STUDIES ON PHILOMETRID AND ANISAKID NEMATODES INFECTING MARINE FISHES IN JAPANESE WATERS

Karl Marx Andaya Quiazon

The University of Tokyo

Department of Aquatic Bioscience

Laboratory of Fish Diseases

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水　圏　生　物　科　学　専　攻
平成１８年度博士課程　進学
氏名　キアゾン　カール　マルクス　アンダヤ
指導教員名　小川和夫

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SUMMARY

Chapter 1. General Introduction

Marine fishes are frequently infected with nematodes. However, information on them, such as taxonomy and biology, are generally limited. In this study, I focused on two nematode families, namely Philometridae and Anisakidae considering their importance. Philometrid nematodes are known for their economic impact on fish production, especially those species infecting the gonads and body muscles of their respective host fishes. Proper species identification is important, not only in the re-evaluation of the taxonomical classification of these poorly studied group, but also in understanding the biology and pathology on their host fishes.

Taxonomical and biological studies were carried out on six different gonad-infecting *Philometra* species and one muscle-infecting *Philometroides* species, thus adding some basic but very relevant information on this group of nematode.

On the other hand, anisakid nematodes are known for their zoonotic contribution to human anisakiasis and as source of allergens. With this, studying this group of nematode is very important from the viewpoint of food safety. Previous identification of *Anisakis* larvae are based on two types, *Anisakis* Type I and *Anisakis* Type II, wherein *A. simplex*¸ *A. ziphidarum*, *A. pegreffii* and *A. typica* were on the former type, whereas *A. paggiae*, *A. brevispiculata* and *A. physeteris* were on the latter type. At present, little is known about the taxonomy and infection, especially those of the two widely reported sibling species of *Anisakis simplex* complex, namely *A. simplex* (sensu stricto) (s.s.) (Rudolphi, 1809) and *A. pegreffii* Campana-Rouget et Biocca, 1955. Taxonomical studies and experimental infection studies were carried out on *Anisakis* species in Japanese waters, with emphasis on these two sibling species.

Chapter 2. Studies on philometrid nematodes

1. *Taxonomical studies*

In philometrid nematodes, males are generally variant in morphology and

crucial to their taxonomy. Due to the difficulty in finding tiny males, description of many philometrids were based mainly on large females. In this study, males of six philometrid species were successfully obtained and used for their taxonomy. Six gonad-infecting *Philometra* species and one muscle-infecting *Philometroides* species in Japanese waters were taxonomically examined by morphological and molecular analyses. Males of *Philometra lateolabracis* (Yamaguti, 1935), females of which have been reported from various fish species, were discovered for the first time in the gonads of its type host, Japanese seaperch, *Lateolabrax japonicus*. Morphological comparisons were carried out by light microscopy and scanning electron microscopy between *P. lateolabracis* collected from Japanese seaperch and other philometrid nematodes previously reported as *P. lateolabracis* from chicken grunt, *Parapristipoma trilineatum*, and red sea bream, *Pagrus major*. Results revealed that the latter two represent new species, *Philometra isaki* Quiazon, Yoshinaga et Ogawa, 2008 and *Philometra madai* Quiazon, Yoshinaga et Ogawa, 2008, respectively. Also, new *Philometra* species, *P. sawara* Quiazon, Yoshinaga et Ogawa, 2008, was described based on male and female specimens collected from the gonads of Japanese Spanish mackerel, *Scomberomorus niphonius*. Three additional species, *Philometra nemipteri* Luo, 2001, *Philometra sciaenae* Yamaguti, 1941 and

Philometroides seriolae (Ishii, 1931) were confirmed as valid species and were redescribed based on specimens collected from the gonads of golden threadfin bream, *Nemipterus virgatus* and silver croaker, *Pennahia argentata*, and from the body muscle of Japanese amberjack, *Seriola quinqueradiata*, respectively. Male *P. nemipteri* were first reported and described in this study, whereas male *Philometroides seriolae* were unsuccessfully collected. Molecular results of the ITS2 sequences supported the morphological findings that all seven philometrid species examined are independent from each other and from other species, the sequence of which were already reported. These findings indicate that philometrids have strong host specificity and the identity of a species reported from several host species should be re-evaluated.

2. *Biological studies*

In this study, the infection of six gonad-infecting *Philometra* species (*P. sciaenae*, *P. sawara*, *P. isaki*, *P. madai*, *P. lateolabracis* and *P. nemipteri*) and one muscle-infecting *Philometroides* species were examined. Results of examination revealed that both live and dead *Philometra* species were mainly found in the ovarian lumen and some were also found in the oviduct. In male host fishes, only *P. nemipteri* were found mainly in the spermatic duct, whereas majority of the other

five *Philometra* species, live and dead, were found in the seminiferous tubules. The developmental stages and prevalence of gonad-infecting *Philometra* species were found synchronized with the spawning season of their host fishes. Hence, it is clear that presence of fully gravid female *Philometra* in the gonads of their hosts possibly facilitate the release of first-stage larvae, as host gametes were released during host spawning. On the other hand, *Philometroides seriolae* were mainly found in the body muscle and only very few of them were situated under the skin of the fish. For those found under the skin, only few (8.7 % of fully gravid females) were found protruding their anterior end outside the fish skin, possibly to release first-stage larvae into the environment as suggested in a previous report. As all developmental stages, particularly fully gravid females, were observed throughout the year, no seasonal fluctuation in the prevalence of *Philometroides seriolae* was observed. Up to date, its mode of releasing hatched larvae remains largely unknown. However, the fully gravid females under the skin possibly contribute to the continuous existence of this parasite in the marine environment. In the infection with *Philometroides seriolae*, a layer of inflammatory tissue was formed in the muscle surrounding worms, where numerous leukocytes infiltrated. In gonad-infecting *Philometra* species, in general, leukocyte infiltrations were mostly observed in live worms and fibrous tissue layers

were frequently observed around remnant dead worms.

Chapter 3. Studies on anisakid nematodes

1. *Taxonomical studies*

Studies on anisakid nematodes were carried out in aspects mainly focusing on *A. simplex* (s.s.) and *A. pegreffii*: (1) morphological differences between *A. simplex* (s.s.) and *A. pegrefii*, (2) First report of two *Anisakis* species belonging to *Anisakis* Type II in Japan, (3) comparisons of *A. pegreffii* from the Far East and Mediterranean, and (4) *Anisakis* distribution in Japanese waters.

Morphological differences in larvae and adults between A. simplex (s.s.) and A. pegreffii. Proper identification of *Anisakis* species infecting host fishes is very important to both human health and fish disease diagnosis. The foremost problem in the identification of *Anisakis* larvae in fishes is that L3 larvae cannot be easily differentiated morphologically, especially between *A. simplex* (s.s.) and *A. pegreffii*. Instead, molecular means such as allozyme, mitochondrial DNA (*mt*DNA) *cox*2 gene and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses had been successfully used. However, morphological distinction is important in facilitating studies especially on these two species. In this study, morphological differences of L3 larvae collected from fishes and *in*

vitro-cultured L4 larvae and adults between *A. simplex* (s.s.) and *A. pegreffii* were evaluated. L3 larvae were collected from seven different host fishes within Japan. L4 larvae and adults were obtained by culturing L3 larvae *in vitro*. After morphological examination, each parasite was subjected to PCR-RFLP analysis of the ITS region (ITS1-5.8S-ITS2) for species identification. The identification was confirmed by *mt*DNA *cox*2 gene sequencing of representative specimens. Results revealed that L3, L4 and adult stages of *A. simplex* (s.s.) and *A. pegreffii* are morphologically distinguishable based on ventriculus length, wherein the former has longer ventriculus (0.90–1.50 mm) than the latter (0.50–0.78 mm). Also, adult male *A. simplex* (s.s.) and *A. pegreffii* were found to be distinguishable by differences in the distribution pattern of the caudal papillae, particularly the 3rd pair of distal papillae.

First report of two Anisakis species belonging to Anisakis Type II in Japan. Morphological and molecular approaches were conducted in order to precisely identify different *Anisakis* species infecting Alaska pollock, *T. chalcogramma*, in northern Japan. Morphologically, both *Anisakis* Type I and *Anisakis* Type II larvae were found. *Anisakis* identification using PCR-RFLP analysis generated four different fragment patterns. Analysis of the nucleotide and amino acid sequences of the ITS region and *mt*DNA *cox*2 gene, respectively, indicated that the four fragment

patterns corresponded to *A. simplex* (s.s.), *A. pegreffii*, *A. brevispiculata* and *A. paggiae*. Among these four species, the predominant species infecting *T. chalcogramma* was *A. simplex* (s.s.) (91.0%), followed by *A. pegreffii* (5.2%), *A. paggiae* (2.4%), and *A. brevispiculata* (1.4%). This is the first evidence of the occurrence of *A. brevispiculata* and *A. paggiae* in the north-west Pacific region.

Comparison of A. pegreffii from the Far East and Mediterranean. *Anisakis pegreffii* has been widely reported, not only in the Mediterranean and Atlantic but also in the Far East region. Recently, two base difference was reported in the sequence of 5.8S rDNA between *A. pegreffii* from the Mediterranean and that from the Far East region. Based on this difference, the latter, which was originated from China, was tentatively designated as *Anisakis pegreffii* JP (Japan) in a previous report. In this study, *A. pegreffii* from the Mediterranean and from the Far East were morphologically and molecularly compared to confirm the validity of *A. pegreffii* JP and to confirm the two base difference in the 5.8S rDNA region. Morphologically, little difference was detected among the specimens. In PCR-RFLP and DNA sequences of ITS region, identical results were obtained from all the present and previously reported specimens of *A. pegreffii* except that the two base difference was detected previously in only one sequence reported from the Mediterranean (GenBank

Acc. No. AY826720), in which an unclear base was sandwiched between the different base positions, suggesting possible error in sequencing. In *mt*DNA *cox*2 region, no geographical difference was detected. These results indicate that *A. pegreffii* JP is identical morphologically and molecularly with *A. pegreffii* from Europe.

Anisakis distribution in Japanese waters. Combining data obtained in the present studies (1) and (2), other host fishes presently examined and data obtained from available literature, distributions of *Anisakis* species in Japanese waters were described. It was revealed that *A. simplex* (s.s.) are mainly present in all host fishes examined in northern part of Japan in Hokkaido and in the Pacific side, whereas *A. pegreffii* are mainly present in all host fishes examined in the Sea of Japan and the East China Sea. In addition, low prevalence and intensity of three *Anisakis* species belonging to *Anisakis* Type II (*A. physeteris*, *A. brevispiculata* and *A. paggiae*) and hybrid genotype were found. The former species was reported from Kyushu, whereas the latter two species were found in north-west Pacific side near Iwate Prefecture, whereas hybrid genotypes were found in northern and southern part of Japan. Interestingly, the current data indicate difference in distribution pattern between *A. simplex* (s.s.) and *A. pegreffii*. It is also very interesting to know the exact boundaries

of such distribution between the Pacific, the Sea of Japan and East China Sea sides, from the viewpoint of the distribution of host populations including fishes and cetaceans.

2. *Experimental infection studies*

Anisakis Type I larvae are commonly found in many marine fish species. In spite of its presence in the body muscles of some host fishes, little information is available regarding the tissue specificity of *A. simplex* (s.s.) and *A. pegreffii*. In this study, experimental infection of rainbow trout, *Oncorhynchus mykiss*, and Japanese flounder, *Paralichthys olivaceus* with L3 larvae of *A. simplex* (s.s.) and *A. pegreffii* was conducted independently on both parasites. Fishes were orally challenged with L3 larvae. Sites of infection of the parasites were monitored 3, 7, 14, 21, 28 and 35 days postinfection (dpi). In rainbow trout, all *A. simplex* (s.s.) and *A. pegreffii* were recovered at 3dpi in the gastrointestinal lumen and body cavity. Migration of *A. simplex* (s.s.) in the body muscle was observed 7 dpi onwards, whereas *A. pegreffii* remained freely moving in the body cavity and finally disappeared at 35 dpi. In Japanese flounder, only 78% and 53% of *A. simplex* (s.s.) and *A. pegreffii*, respectively, were recovered at 3 dpi in the body cavity only. Migration in the body muscles was only obserevd in *A. simplex* (s.s.) from 7 dpi onwards, whereas *A.*

pegreffii was found encycted in the body cavity in lumps. The results demonstrate that the migration and survival of both parasites differ depending on host species.

Chapter 4. General Discussion

The implication of both parasites to wild fisheries, aquaculture and food safety cannot be underestimated. This study was able to answer some taxonomical and biological information which could partly contribute in fully understanding these groups of nematodes. Taxonomically, the finding of males and usage of SEM and molecular approaches had shown its importance for precise identification of philometrid nematodes. Biologically, further researches are needed particularly in carrying out studies in understanding mechanisms involved for host specificity and its relation to the evolutionary context of these nematodes, relationship fo infection with host spawning, host response to infection and their life cycle. With regards to the anisakid nematodes, finding of morphological keys for distinguishing between the two sibling species, not only in adult stage but also in L3 stage, will be very significant during immediate on-site investigation. Current data obtained in both taxonomical and experimental infection studies would be helpful in further preventing anisakiasis. The findings that only *A. simplex* (s.s.) only migrates in the body muscle of two host fishes used in this study somehow supports our questions

why only this sibling species are mainly present in human patients. In view of this, more works has to be carried out for wide fish species to confirm our hypothesis that only *A. simplex* (s.s.) infects the body muscle while *A. pegreffii* only remains in the body cavity.

CHAPTER 1. GENERAL INTRODUCTION

The roundworms or nematodes are one of the most diverse of all animals and the most diverse phylum of pseudocoelomates, where over 80,000 species have been described, of which over 15,000 species are parasitic. Nematodes have successfully to nearly adapted to every ecological niche, including those from brackishwater and freshwater environment. The parasitic nematodes are found in most plants, animals and humans. Among these parasitic nematodes are the philometrid and anisakid nematodes, the former are known to infect fishes only while the latter are found in fishes and has zoonotic features.

Both philometrid and anisakid nematodes significantly affects fish and human lives. Considering the significant impact and possible contribution on studying the philometrid nematodes, particularly those known to infect fish gonads and body muscles, this group has been chosen as one aspect in my research work. On the other hand, the zoonosis of anisakid nematodes to human health had made the standards in quality control of fish consumption very strict, particularly to countries like Japan where raw seafood dishes are popularly consumed. Although anisakid nematodes are only present from wild catch, the first report of anisakid infection in aquaculture resulted in the initiative of the Japanese government to investigate the reported incidence of anisakid infection in fish (Yoshinaga et al., 2006). In a media report, one case of *Anisakis* type I larva in the examined body muscle in fish is very alarming in view of human food safety. In this regard, in addition to philometrid nematodes, I had thought that chosing also the anisakid nematode as another aspect in my research work can also generate information that can somehow contribute in understanding fully this group, thus preventing or reducing future risks in human health.

PHILOMETRID NEMATODES

Nematodes of the superfamily Dracunculoidea Stiles, 1907 represent a widely distributed and very diverse group of species, which infect different freshwater, brackish-water and marine fishes. Most species of these large histozoic parasites are poorly known, both biologically and taxonomically. Especially males have not been discovered in most dracunculoid species. Given this, identification of some fish dracunculoids are mainly based on morphology of females such as the variations on the structures and arrangement patterns of cephalic papillae, a sensory structures located at the anterior end and variation on the structure of oesophagus (Fig. 1). For male dracunculoids, identification can be based on the variation in the structures on caudal ends (Moravec 2004).

In many dracunculoids, the life cycle is as follows. The female grows markedly after insemination and fills with huge numbers of first-stage larvae, which must be dispersed into the aquatic environment where they will be available to copepod intermediate hosts. In some forms, the gravid female must be in contact with freshwater, where its body burst due to osmotic pressure to release the larvae. The female (e.g., in *Dracunculus*) may elicit a skin lesion to gain access to freshwater, initially in the form of blister containing huge numbers of larvae which breaks when immersed in water; therefore, larvae can be released from the female worm repeatedly when the ulcer over the worm is bathed in water (Anderson 2000). In some fish parasites (e.g., philometrids), the gravid dracunculoid female penetrate through the host's skin and pushes a part of its body into the water to achieve the same result. In some species, gravid female pass out with the reproductive products of the host during spawning (Moravec 2006)

Nematodes belonging to the family Philometridae Baylis et Daubney, 1926 are frequently found in various organs, tissues and body cavities of a wide variety of fishes. Identification of philometrid species has often been done solely using females, as males

(TL <5 mm) are generally much smaller than females (TL usually >100 mm) and cannot be collected easily. Most philometrids remain poorly known, and their identification is usually very difficult and problematic because of difficulties associated with their morphological and biological peculiarities (Moravec et al. 2003, Moravec and Genc 2004). The use of light microscopy (LM) alone is insufficient for the identification of these species. 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 such suggestion of Moravec (2004) that a fundamental re-evaluation is necessary with respect to the taxonomy and classification of dracunculoids. However, to date, only few molecular studies have been carried out on these group of nematodes, particularly those included in the family Philometridae (Wu et al. 2005, Wijová et al. 2006).

The genus *Philometra* Costa, 1845 currently contains 112 reported species (Moravec 2006). These infect various organs, tissues and body cavities of a wide range of host fishes worldwide (Moravec 2006). To date, including the newly raised three *Philometra* species from this study and the validation of one species previously placed under the status *species inquirenda* (Quiazon et al. 2008a, 2008b), 18 *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. managatuwo* 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. nemipteri* Luo, 2001, *P. madai* Quiazon, Yoshinaga et Ogawa, 2008, *P. isaki*

Quiazon, Yoshinaga et Ogawa, 2008 and *P. sawara* Quiazon, Yoshinaga et Ogawa, 2008. Of these species, seven (*P. lateolabracis*, *P. scomberomori*, *P. managatuwo*, *P. sciaenae*, *P. sebastisc*i, *P. nemipteri*, *P. madai*, *P. isaki* and *P. sawara*) 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. 2008a, 2008b). 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, wherein even males of *P. lateolabracis*, a frequently reported gonad-infecting species, were not described from the type host until I found them in this study.

On the other hand, *Philometroides seriolae* (Ishii, 1931) is the recognized type species of the genus *Philometroides* Yamaguti, 1935, which is known to infect body muscle of its type host Japanese amberjack, *Seriola quinqueradiata* Temminck et Schlegel. To date, there are total of 31 recognized *Philometroides* species worldwide, among which 19 species were known to infect the body cavity and subcutaneous tissue of different body organs. *Philometroides seriolae* infects the body muscle only, whereas three other species (i.e., *Philometroides cyprini* (Ishii, 1931), *Philometroides hydrocyoni* Fahmy, Mandour et El-Nafar, 1976 and *Philometroides strelkovi* Vismanis et Yunchis, 1994) were known to infect mainly the subcutaneous tissue from other organs of their host fishes and only some parts of the body muscle (Moravec 2006).

In this area of study, I examined six different marine fish species infected with gonad-infecting philometrids, wherein tiny males from all six fish species examined were successfully collected. Also, muscle-infecting philometrids were collected from the body muscle of one marine fish species. All collected philometrids were taxonomically

identified using morphological approach, which was supported by molecular data. Aside from the taxonomical studies, biological studies were also carried out focusing on the infection of these gonad and muscle-infecting philometrids to their respective host fishes.

Fig. 1 (cephalic end among dracunculoids)

Fig. 2 (Oesophagus among dracunculoids)

ANISAKID NEMATODES

Anisakids are group of nematodes under the family Anisakidae Railliet et Henry, 1912 that are well known as the causative agents of human anisakiasis. This disease can be acquired by ingestion of live larval anisakids, which were commonly reported from those included in the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* in raw seafood dishes such as sashimi, sushi, ceviche and pickled herring. Morphological differentiation among these anisakid groups is based on the presence or absence of intestinal caecum and ventricular appendix (Fig. 3) (Koyama et al 1969). Symptoms of anisakiasis include abdominal pain, nausea, vomiting and diarrhea. This disease is often misdiagnosed as appendicitis, acute abdomen, stomach ulcers or ileitis. Endoscopic examination with biopsy forceps has facilitated the diagnosis of gastric aniaskiasis. The anisakids can be invasive and migrate beyond the stomach, penetrating the intestine, omentum, liver, pancreas and probably lungs of human (Ishikura and Namiki 1989, Sakanari and McKerrow 1989, Umehara et al. 2008).

Kagei (1969) described the life cycle of anisakids as follows. After the eggs contained in the feces of dolphins and other animals, which are the host of the adult worms, are released into the sea and become first-stage larvae. Subsequently, the eggs are consumed by tiny crustaceans, such as *Euphausia* or *Thysanoessa* (the first intermediate host), and then become the second- and third-stage larvae. In fish (the second intermediate host) that consume the crustaceans, the larvae developed to third and fourth stage. The larvae are then consumed by humans resulting to anisakiasis. At present, it has been known that the hatched larvae from the released eggs are not on their first-satge but rather on their third-stage, which then infects tiny crustaceans/euphasiids that are consumed by the fish.

Anisakis Dujardin, 1845 are parasitic nematodes that have a global distribution. The third-stage larvae (L3 larvae) of *Anisakis* are found not only in the body cavities, but also in the body muscle of selected host species (Templeman et al. 1957, Scott and Martin 1959, Novotny and Uzmann 1960, Wootten and Waddell 1977, Smith 1984, Strømnes and Andersen 1998). Conventionally, morphological diagnosis using L3 larvae can only determine its type groupings, *Anisakis* Type I or *Anisakis* Type II; the former has a longer ventriculus and has a mucro at the posterior tip, whereas the latter has a shorter ventriculus and no mucro (Berland 1961, Koyama et al. 1969).

Until early 1980s when allozyme analysis was used in identifying sibling species, only three valid species had been recognized, namely *A. simplex* (Rudolphi, 1809, det. Krabbe, 1878), *A. typica* and *A. physeteris* (Nascetti et al. 1983, 1986, Davey 1971). Recent molecular studies on *Anisakis*, which launched from a series of new studies using allozyme, ribosomal DNA and mitochondrial DNA (*mt*DNA) *cox*2 gene analyses, revealed that the *Anisakis* species consisted of eight valid species, five species (*A. ziphidarum*, *A. typica* and three sibling species of *A. simplex* complex (or *A. simplex* sensu lato), namely *A. simplex* [in a strict sense or sensu stricto] (s.s.), A. *pegreffii* Campana-Rouget et Biocca, 1955 and *A. simplex* C) and three species (*A. paggiae*, *A. brevispiculata* and *A. physeteris*) of which are in the *Anisakis* Type I and *Anisakis* Type II groupings, respectively (Mattiucci et al. 1986, Nascetti et al. 1986, Mattiucci et al. 1997, 1998, 2001, 2002, 2005, Paggi et al. 1998, Mattiucci and Nascetti 2006, Valentini et al. 2006).

In previous reports, the sibling species of the reported *A. simplex* have not been specified. In this regard, the *A. simplex* referred in these previous reports should be referred to as *A. simplex* complex, which is either *A. simplex* (s.s.), *A. pegreffii*, *A. simplex* C or a combination of these three sibling species. Hence, in this manuscript, all *Anisakis* species referred only as "*A. simplex*" are assumed to be referred to *A. simplex* complex.

Anisakis pegreffii, which was the dominant species in the genus *Anisakis* in the Mediterranean (Mattiucci and Nascetti 2006), was first described from the digestive tract of a Mediterranean monk seal, *Monachus monachus* (Hermann), captured on eastern Sardinia (Campana-Rouget and Biocca 1955). In Japan, *Anisakis* species identified through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Abe et al. 2005, Umehara et al. 2006) and multiplex PCR (Umehara et al. 2008) analyses of the ITS region (ITS1-5.8S rDNA-ITS2) were *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris*. The *A. simplex* (s.s.), which is abundantly distributed worldwide, can be found mostly in northern Japan, while *A. pegreffii*, a dominant species in Mediterranean, can be found mostly in southern Japan (Abe et al. 2005, Umehara et al. 2006). Aside from causing human anisakiasis, potential allergens have been reported in *A. simplex* and *A. pegreffii*, which may cause allergic reactions in humans when consumed together with fish as fresh, frozen, cooked, or processed food (Armentia et al. 1998, del Pozo et al. 1999, Daschner et al. 2000, Alonso-Gómez et al. 2004, Caballero and Moneo 2004, Nieuwenhuizen et al. 2006, Kobayashi et al. 2008).

There may have been a compilation of taxonomical, biological and pathological research works dealing on *A. simplex*. Unfortunately, morphological distinction between its two sibling species, *A. simplex* (s.s.) and *A. pegreffii* were not known. This created some questions which among these two sibling species has been dealt with, especially if the parasites studied were only identified as *A. simplex.* In this area of study, I focused on the two sibling species *A. simplex* (s.s.) and *A. pegreffii.* This study is divided into two major aspects; the taxonomical studies and infection challenges. In the taxonomical studies, L3 and *in vitro*-cultured adults of *A. simplex* (s.s.) and *A. pegreffii* were morphologically examined. Also, the distribution of *Anisakis* species in Japanese waters were surveyed, wherein the present results obtained in this study were combined with previously reported

results to see the overview of *Anisakis* distribution in Japan. Lastly, experimental challenges in the infection and migration of the two sibling species were examined *in vivo* using two different host fishes.

Fig. 3 Morphological key among anisakid groups (Koyama et al 1969)

CHAPTER 2. STUDIES ON PHILOMETRID NEMATODES

SUBCHAPTER 1. TAXONOMICAL STUDIES

Morphological and molecular taxonomy of philometrid nematodes in Japan

Introduction

Philometra lateolabracis has been repeatedly reported in the gonads not only from its type host Japanese seaperch, *Lateolabrax japonicus* (Cuvier) from family Percichthyidae, but also in members of other perciform families (Ariidae, Carangidae, Centropomidae, Glaucosomatidae, Haemulidae, Hemiramphidae, Lutjanidae, Mullidae, Muraenesocidae, Paralichthyidae, Polynemidae, Pomadasyidae, Psettodidae, Sciaenidae, Serranidae and Sparidae) from tropical and subtropical regions of the Mediterranean, Pacific, South Pacific, Indian and Atlantic Oceans. Reported hosts of *P. lateolabracis* are as follows: chicken grunt (*Parapristipoma trilineatum* (Thunberg)), Hong Kong grouper (*Epinephelus akaara* (Temminck et Schlegel)), dusky grouper (*Epinephelus marginatus* (Lowe)), speckled blue grouper (*Epinephelus cyanopodus* (Richardson)), blacktip grouper (*Epinephelus fasciatus* (Forsskål)), grunt (*Haemulon plumieri* (Lacépède)), red sea bream (*Pagrus major* (Temminck et Schlegel)), snapper (*Pagrus auratus* (Forster)), westralian jewfish (*Glaucosoma hebraicum* Richardson), greater amberjack (*Seriola dumerili* (Risso)), mottled grouper (*Mycteroperca rubra* (Bloch)), daggertooth pike conger (*Muraenesox cinereus* (Forsskål)), giant sea catfish (*Arius thalassinus* (Rüppell)), long billed halfbeak (*Rhynchorhamphus georgii* (Valenciennes)), barramundi (*Lates calcarifer* (Bloch)), lane snapper (*Lutjanus synagris* (Linnaeus)), Indian goatfish (*Parupeneus indicus* (Shaw)), fourfinger threadfin (*Eleutheronema tetradactylum* (Shaw)), Belanger's croaker (*Johnius belangerii* (Cuvier)), coitor croaker (*Johnius coitor* (Hamilton)), sin croaker (*Johnius dussumieri* (Cuvier)), blotched croaker (*Nibea maculata* (Bloch et Schneider)), tiger-toothed croaker (*Otolithes rubber* (Bloch et Schneider)), bronze croaker

(*Otolithoides biauritus* (Cantor)), greyfin croaker (*Pennahia anea* (Bloch)), blackspotted croaker (*Protonibea diacanthus* (Lacépède)), tomato hind (*Cephalopholis sonnerati* (Valenciennes)), black grouper (*Mycteroperca bonaci* (Poey)), Javan flounder (*Pseudorhombus javanicus* (Bleeker)) and Indian spiny turbot (*Psettodes erumei* (Bloch et Schneider)) (Yamaguti 1935, 1941, Crisp and Klein 1973, Sakaguchi et al. 1987a, 1987b, Sharples and Evans 1995a, 1995b, Hesp et al. 2002, Moravec et al. 2003, Moravec and Genc 2004, Merella et al. 2004, Moravec and Justine 2005, Moravec 2006). Since morphologically more variable males had not mostly been discovered for nematodes reported as *P. lateolabracis*, even from its type host Japanese seaperch, researchers used less variable females for species identification. In this regard, there is a high possibility that these nematodes reported as *P. lateolabracis* are different, yet unidentified *Philometra* species.

On the other hand, little is known about the morphological taxonomy of *Philometroides seriolae*, not only its males but also in the full description of its females, in spite of the economic implication of this parasite in the aquaculture industry of Japanese amberjack in Japan. In this subchapter, redescription of female *Philometroides seriolae* were found necessary because of the following: (1) Yamaguti (1935) redescribed only one female specimen; (2) Moravec et al. (1998) redescribed only one fragment female specimen; (3) no SEM examination, which is an important tool in taxonomical studies of these group, has been carried out by both Yamaguti (1935) and Moravec et al. (1998) and; (4) the unavailability of SEM examination made the description of the distribution pattern of the cephalic papillae doubtful. Molecular studies on the 18S and ITS2 rDNA were also carried out to strengthen and support results obtained from the morphological studies.

Finally, since new species have already been raised and reported by Quiazon et al.

2008a, 2008b in this subchapter, the proper rule in zoological nomenclature in naming species was followed. Also, throughout the manuscript, the fishbase name is used.

Materials and methods

A. Morphological studies

Sources of philometrid nematodes. Philometrid nematodes were collected from the gonads of the Japanese seaperch, chicken grunt, red seabream, Japanese Spanish mackerel, golden threadfin bream and silver croaker caught in off Futtsu, off Nomaike, off Shimonada, near Awaji Island, off Ichiki and off Shimabara in Japan, respectively. On the other hand, *Philometroides seriolae* were isolated from the body muscle of cage-cultured Japanese amberjack from Saga, Miyazaki, Kochi and Oita Prefectures in Japan (Figs. 4,5). Females were collected macroscopically. For detection and collection of male philometrids in male fish, the testes were pressed between two glass plates and examined under a stereomicroscope, whereas collection of male philometrids in fish ovaries were carried out by dissecting and washing the ovaries with physiological saline solution and collection was aided with stereomicroscope. Collected male and female philometrids were fixed in 70% ethanol. *Philometra* species were cleared in glycerin, whereas female *Philometroides* species were cleared in lacto-phenol solution. 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. The general features of males, females and first-stage larvae in the uterus of a fully gravid female philometrid were drawn using a compound LM with a Nikon drawing tube attached. Females were categorized as gravid (i.e., designated larvigerous specimen), subgravid (i.e., those containing no larvae but only eggs or developing embryos) and nongravid (i.e., those without larvae or eggs). All measurements were in millimetres. Lengths of each morphological features examined

were expressed in proportional values (enclosed in parentheses) in body length. Identification up to species level was initially carried out using the morphological keys reported (Appendix 1,2) (Moravec and Genc 2004, Moravec 2006). With the continuous discovery and redescription of gonad-infecting philometrids, a revised morphological keys for the identification of these parasites created with the addition of new data obtained from the present study (Appendix 3). All *Philometra* species were deposited at the Meguro Parasitological Museum, Tokyo (M.P.M. Coll. Nos. 18858, 18859, 18860, 18861, 18862, and 18863) and the Institute of Parasitology, České Budějovice (Coll. Nos. N-252, N-888, N-889, N-916, N-917 and N-703). Measurements of some morphological structures difficult to examine under LM were carried out using SEM. Specimens to be examined using SEM were fixed with 70% ethanol, post-fixed in 1.25–1.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and dehydrated through series of ascending ethanol concentrations. Samples were subjected to three changes of absolute butyl alcohol and freeze-dried. Freeze-dried samples were subsequently sputter-coated with gold and observed under a scanning electron microscope (SEM S-4000, Hitachi).

B. Molecular studies

DNA extraction and PCR program. The entire genome of one male and three females of the 100% ethanol-fixed philometrids from each host fish were extracted separately using a DNeasy™ Tissue Kit from Qiagen Inc. (protocol for animal tissues). The designed forward primer 35f (5'-TATAATGGTGAAACCGCGAACGGC-3') and the universal reverse primer 18gM (5'-GGAAACCTTGTTACGACTTTTGCC-3') were used for the amplification of 18S ribosomal DNA (rDNA) region of the six philometrids. The forward primer NC5f (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and the reverse primer NC2r (5'-TTTAGTTTCTTTTCCTCCGCT) reported by Zhu et al. (1998) were used for sequencing the ITS region. In between these forward and reverse primers at

terminal ends, sets of primers were designed in both 18S rDNA and ITS region (Appendix 4,5). Similar PCR program was used for both 18S and ITS rDNA regions. The PCR assay was performed with 1 µL sample DNA as template in a total volume of 20 µL which contained 0.6 µL forward and reverse primer, 14.1 µL DDW 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). After the DNA had been initially denaturized at 94 °C for 4 min, 30 cycles were carried out using iCycler™ (BIO-RAD). Each cycle consisted of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s with final extension at 72 °C for 7 min.

DNA sequencing and analyses. Nucleotide bases were sequenced using a DNA automatic sequencer (ABI Prism® 310 Genetic Analyzer, Applied Biosystems) after purification of the selected amplified DNA. The sequenced DNA of philometrids was checked for contamination by performing a BLAST search in the NCBI (http://www.ncbi.nlm.nih.gov/). The boundaries between ITS1, 5.8S, ITS2 and 28s were determined manually by comparison with other reported nematodes in the NCBI. Only the sequences encompassing the ITS2 region were used for analysis. The obtained sequences were aligned with the aid of BioEdit version 7.0.4.1. The analysis of percentage similarity (i.e., gaps included) was calculated using Clustal_W (Thompson et al. 1994) and BioEdit (Ibis Biosciences, Carlsbad, CA, USA). 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 database under the accession numbers EF203081, EF203082, EU443201, EU443202, FJ155812 and EU443203. Also, the 18S rDNA sequences were deposited under the accession numbers FJ161971, FJ161972, FJ161973, FJ161974, FJ161975, and FJ155811. Neighbour-joining (NJ) (maximum composite likelihood) and maximum parsimony (MP) trees inferred based

on the *p-distance* values in 18S rDNA and ITS2 regions were constructed with the aid of MEGA 4.0 program (Saitou and Nei 1987, Tamura et al. 2007). Using non-parametric bootstrap analysis (Felsenstein 1985) with 1000 replicates, the reliability of phylogenetic relationships was evaluated. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). The reliability was considered to be high if bootstrap values exceeded 70% of the replicates (Hills and Bull 1993).

Molecular analyses. The molecular examinations were subdivided into three studies: (1) molecular comparison of *Philometra* previously identified as *P. lateolabracis*; (2) molecular comparison with other *Philometra* species in Japan; and (3) molecular comparison of *Philometroides seriolae* with other dracunculoids. For phylogenetic analyses on the 3rd aspect (3), molecular comparison of 18S rDNA was carried out between the currently examined philometrid species with other available dracunculoid sequences in GenBank database, four genera from the family *Philometridae* (i.e., *Philometroides*, *Philometra*, *Clavinema* Yamaguti, 1935 and *Dentiphilometra* Moravec et Wang, 2002) and one genus from the family *Micropleuridae* Baylis et Daubney, 1922 (i.e., *Philonema*). On the other hand, molecular comparison on ITS2 region was carried out with the available sequences of three genera only under the family *Philometridae* (i.e., *Philometroides*, *Philometra* and *Clavinema*). Since *Philometra fujimotoi* Furuyama, 1932 has been transferred by Margolis and Moravec (1987) to the genus *Clavinema*, a change that was confirmed by Moravec (2006), the reported *P. fujimotoi* in GenBank database was treated in this paper as *Clavinema fujimotoi* (Furuyama, 1932). *Philometroides pseudaspii* Moravec et Ergens, 1970 was among the valid species in the monograph of Moravec (2006), which was synonymized with *Philometroides ganzhounensis* Yu, 1998 whose ITS sequence was deposited by Wu et al. (2005) in GenBank database. Also, *Philometroides carassii* (Ishii, 1931) was synonymized with the reported valid species *Philometroides sanguineus* (Rudolphi, 1819).

Fig.4 Host fishes

Fig.5 host locality

Results

A. Morphological studies

A.1. Descriptions

1. Philometra lateolabracis (Yamaguti, 1935) Figs. 6,7

(proportional values in body length of each morphological characters are enclosed in parentheses)

Male. Body length of twenty specimens collected in January 2005 ranging from 2.07 to 2.73; filiform body; transparent to whitish-cream body colouration when alive; maximum width at mid-portion of body 0.040–0.052 (1.8–2.4%); gradually tapering towards anterior portion, then gradually broadening forming a bulbous anterior extremity; cuticle smooth; rounded anterior end; cephalic end dome-shaped, with four submedian pairs of cephalic papillae forming outer ring and four single papillae present as inner ring; small rounded mouth opening, 0.0008–0.0010 (0.04%) in diameter, with pair of small round amphids situated laterally; overall length of oesophagus 0.255–0.363 (10.9–16.3%), with distinct inflation (bulb formation) at its anterior end; oesophageal gland very distinct with large rounded nucleus, 0.006–0.012 in diameter (0.3–0.5%), visible at its mid-portion; length of anterior part of oesophagus (i.e., not overlapped by oesophageal gland) 0.072–0.138 (2.9–6.3%), that of posterior part overlapped by oesophageal gland 0.12–0.25 (5.4–10.3%); distance of oesophageal gland nucleus and nerve ring 0.180–0.249 (8–11.3%) and 0.030–0.116 (1.2–5%) from anterior extremity, respectively; ventriculus present but barely visible; testis extending posteriorly up to base of spicules; spicules narrow, needle-like and unequally long; length of longer and shorter spicules 0.071–0.130 (2.8–5.9%) and 0.065–0.124 (2.5–5.1%), respectively; length ratio of spicules 1:1.03–1.12; gubernaculum narrow, 0.050–0.093 (2–4.1%) long, with proximal end part bent dorsally and with lamellate-like structures on its dorsal portion; length ratio

of gubernaculum to spicules 1:1.30–2.02; posterior end of body rounded, with two large lobes at both sides of spicules and gubernaculum; each large lobe subdivided into two smaller equal lobes; caudal papillae observed on posterior extremity on each pair of large lobe; outlets of phasmids not observed.

Gravid female. Body length of ten specimens collected in January 2005 ranging from 112 to 206; filiform body, with slightly yellowish brown to reddish body colouration and with light to dark brown intestine when alive; maximum width at mid-portion of body 0.95–1.12 (0.6–1.0%); width in anterior portion of body broader, then tapering gradually to posterior portion; cuticle smooth; anterior end of body rounded; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring and four round single papillae present as inner ring; paired cephalic papillae with slightly rounded and elongated papillae; width of small mouth opening 0.010, triangular in shape, with a pair of amphids present on both sides; overall length of oesophagus 0.785–1.030 (0.5–0.8%), broad and slightly swollen near mouth, forming slightly distinct bulb [0.065–0.105 long (0.03–0.06%) and 0.099–0.130 wide (0.06–0.07)]; narrowest width at nerve ring level, 0.068–0.110 (0.04–0.06%); oesophageal gland prominent, extending anteriorly to level of nerve ring and posteriorly to ventriculus, with large rounded nucleus 0.028–0.038 in diameter (0.01–0.02), located at its mid-portion; length of anterior part of oesophagus 0.212–0.230 (0.16–0.18%); posterior part overlapped by oesophageal gland 0.572–0.805 long (0.4–0.5); distance of oesophageal gland nucleus and nerve ring 0.388–0.598 (0.2–0.5%) and 0.170–0.295 (0.1–0.3%), respectively from anterior extremity; well-developed ventriculus with length and width of 0.080–0.092 (0.05%) and 0.090–0.095 (0.05–0.07%), respectively; two long ovaries situated near anterior and posterior ends of body; anterior ovary sometimes reaching up to level of nerve ring, while posterior ovary nearly to body end; uterus occupying most space in body, filled with

different stages of developing embryos and some first-stage larvae; posterior end of body rounded with two lateral papilla-like projections; intestine relatively narrow, with distinctly thick intestinal wall; almost black, straight and with its posterior end atrophied, forming ligament attached ventrally to body wall, far anterior to posterior extremity; vagina and vulva not observed.

Subgravid female. Body length of eight specimens collected in January 2005 ranging from 35.7 to 66.8.

Nongravid female. Body length of ten specimens collected in January 2005 ranging from 12.65 to 16.06.

First-stage larva. Total length and maximum width of ten first-stage larvae from uterus of gravid females collected in January 2005 ranging from 0.385 to 0.453 and from 0.015 to 0.020, respectively; proportion of oesophageal, intestinal and tail length in relation to total body length 21–26%, 46–49% and 27–30%, respectively.

Taxonomic summary

Type host: *Lateolabrax japonicus* (Cuvier) (Perciformes: Lateolabracidae); FishBase name: Japanese seaperch; Japanese name: suzuki.

Site of infection: Gonads.

Prevalence: 89% (24 fish infected out of 27 fish examined).

Mean intensity: Male parasites, 153 per fish; female parasites, 10 per fish.

Locality: Off Futtsu, Chiba Prefecture, Tokyo Bay, Japan (35°45’N, 139°48’E).

Deposition of specimens: Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18862) and Institute of Parasitology, České Budějovice (Coll. No. N-252).

Fig.6

Fig. 7

2. Philometra isaki Quiazon, Yoshinaga et Ogawa, 2008 Figs. 8,10

Male. Body length of eleven specimens (holotype and paratypes) collected from September 2001 to March 2003 ranging from 2.62 to 3.26; body filiform; transparent to whitish-cream body colouration when alive; maximum width at mid-portion of body 0.068–0.090 (2.6–2.9%); broad body rapidly tapering towards anterior end, and gradually and slightly broadening forming small highly distinct bulbous inflation at anterior extremity; cuticle smooth; anterior end rounded; overall length of oesophagus 0.246–0.308 (7.5–10.4%), with very small distinct inflation (bulb formation) at its anterior end; oesophageal gland very distinct with large round nucleus, 0.007–0.010 in diameter (0.2–0.3%), visible at its mid-portion; length of anterior part of oesophagus 0.081–0.168 (2.8–5.5%), that of posterior part overlapped by oesophageal gland 0.107–0.220 (3.3–6.8%); distance of oesophageal gland nucleus and nerve ring 0.178–0.259 (6.2–8.8%) and 0.100–0.157 (3.1–5.3%) from anterior extremity, respectively; testis extending posteriorly up to base of spicules; spicules narrow, needle-like and unequally long; length of longer and shorter spicules 0.107–0.127 (3.7–4.0%) and 0.105–0.122 (3.6–3.9%), respectively; length ratio of spicules 1:1.01–1.09; gubernaculum narrow, 0.07–0.09 long (2.3–2.9%), with proximal part bent dorsally; length ratio of gubernaculum to spicules 1:1.33–1.74; posterior end of body rounded with two large lobes at both sides of spicules and gubernaculum; each large lobe subdivided into two smaller equal lobes; outlets of phasmids not observed.

Gravid female. Body length of ten specimens (allotype and paratypes) collected in July 2002 ranging from 167 to 420; body filiform, with yellowish-brown to reddish body colouration and with dark brown to black intestine when alive; maximum width at mid-portion of body 0.69–1.30 (0.4–0.5%), anterior end of body rounded, tapering gradually to posterior portion; cuticle smooth; cephalic end dome-shaped containing four

submedian pairs of cephalic papillae arranged in outer ring and four single round papillae present as inner ring; width of small mouth opening, 0.010, somewhat triangular in shape with a pair of amphids present on both sides; overall length of oesophagus 0.79–1.10 (0.2–0.6%), broad and slightly swollen near mouth forming slightly distinct bulb; narrowest width in nerve ring portion, 0.058–0.100 (0.03%); bulb length, 0.058–0.115 (0.02–0.04%), generally shorter than width, 0.078–0.120 (0.02–0.06%); oesophageal gland prominent, extending anteriorly to level of nerve ring and posteriorly to ventriculus, with large round nucleus, 0.02–0.03 in diameter (0.02%), located at its mid-portion; length of anterior part of oesophagus 0.175–0.245 (0.05–0.11%), that of posterior part overlapped by oesophageal gland 0.60–0.86 (0.15–0.50%); distance of oesophageal gland nucleus and nerve ring 0.418–0.545 (0.2–0.3%) and 0.175–0.225 (0.05–0.14%) from anterior extremity, respectively; two long ovaries situated near anterior and posterior end of body; anterior ovary extending up to level of nerve ring, extending downward up to anterior portion of intestine just after ventriculus; posterior ovary reaching far posterior to intestinal ligament, sometimes almost extending to posterior end of body; uterus occupying most space in body, filled with different stages of developing embryos, ready-to-hatch larvae and first-stage larvae; well-developed ventriculus with length and width of 0.075–0.095 (0.02–0.05%) and 0.080–0.112 (0.02–0.07%), respectively; posterior end of body rounded, with two lateral papilla-like projections; intestine relatively narrow, straight and with its posterior end atrophied, forming ligament attached ventrally to body wall, 0.95–1.12 (0.8%) from posterior extremity; vagina and vulva not observed.

Subgravid female. Body length of one specimen collected in July 2002 was 131.

First-stage larva. Total length and maximum width of ten first-stage larvae from uterus of gravid females collected in July 2002 ranging from 0.355 to 0.450 and from 0.015 to 0.018, respectively; proportion of oesophageal, intestinal and tail length in

relation to total body length 27–31%, 37–41%, and 28–36%, respectively; mouth opening 0.0015 in width; two small round papillae 0.00075 in diameter, observed below the mouth; pointed boring tooth present in upper portion near mouth; cylindrical amphids protruding at both sides of mouth; width of anus, 0.002, present in posterior portion of body besides a smaller-sized unidentified opening, 0.0005 in diameter.

Taxonomic summary

Type host: *Parapristipoma trilineatum* (Thunberg) (Perciformes: Haemulidae); FishBase name: chicken grunt; Japanese name: isaki.

Site of infection: Gonads.

Prevalence: 74% (216 fish infected out of 291 fish examined).

Intensity: Male parasites, 1–2 per fish; female parasites, 2–3 per fish.

Type locality: Off Nomaike, Kagoshima Prefecture, East China Sea, Japan (31°35'N, 130°08'E).

Deposition of specimens: Male holotype, allotype and paratypes deposited in the Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18860); paratypes also in the Institute of Parasitology, České Budějovice (Coll. No. N-888).

Etymology: This scientific name relates to the Japanese name of the fish host, i.e., isaki.

Fig.8

3. Philometra madai Quiazon, Yoshinaga et Ogawa, 2008 Figs. 9,10

Male. Body length of eight specimens (holotype and paratypes) collected from September 2004 to October 2005 ranging from 3.92 to 5.94; filiform body; transparent to whitish-cream body colouration when alive; maximum width at mid-portion of body 0.096–0.132 (1.9–2.2); gradually tapering towards anterior end without formation of bulbous anterior extremity; cuticle smooth; rounded anterior end; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring and four single papillae present as inner ring; small round mouth, 0.003 in diameter, with a pair of amphids on its opposite sides; overall length of oesophagus 0.43–0.49 (7.5–11.5%), with inflation (bulb formation) at anterior end; oesophageal gland very distinct, with large round nucleus, 0.007 (0.2%) in diameter, at its mid-portion; length of anterior part of oesophagus 0.12–0.14 (2.4–3.6%), that of posterior part overlapped by oesophageal gland 0.31–0.35 (5.9–8.0%); distance of oesophageal gland nucleus and nerve ring 0.317–0.354 (7.0–8.1%) and 0.04–0.08 (0.7–1.9%) from anterior extremity, respectively; length and width of ventriculus 0.028–0.032 (0.5%) and 0.025 (0.4–0.5%), respectively; testis very broad occupying most spaces within body during its spawning season from June to July 2005, extending posteriorly up to base of spicules; spicules narrow, needle-like and slightly unequally long; length of longer and shorter spicules 0.084–0.100 (1.4–1.9%) and 0.077–0.100 (1.3–1.9%), respectively; length ratio of spicules 1:1.00–1.16; gubernaculum narrow, 0.064–0.084 long (1.2–1.6%), with proximal tip bent and with lamellate-like structures on its dorsal portion; length ratio of gubernaculum to spicules 1:1.01–1.40; posterior end of body rounded, with two large lobes at both sides of spicules and gubernaculum; each posterior lobe subdivided into two smaller unequal lobes, 0.012 and 0.005 in length; two tail papillae observed in posterior extremity in smaller-sized subdivided lobe; outlets of phasmids not observed.

Gravid female. Body length of four specimens (allotype and paratypes) collected from May to July 2005 ranging from 103.8 to 394.4; filiform body with yellowish brown to reddish body colouration and with light- to dark brown intestine when alive; maximum width at mid-portion of body 0.62–1.60 (0.6–0.7%); body at anterior portion broader, tapering gradually to posterior portion; cuticle smooth; anterior end of body rounded; cephalic end dome-shaped containing four submedian pairs of cephalic papillae as outer ring and four single papillae as inner ring; wide rounded mouth opening, 0.025 in diameter, with pair of amphids on its opposite sides; overall length of relatively narrow oesophagus 1.06–1.12 (0.4–1.0%); its anterior portion highly swollen forming distinct bulb; narrowest width in nerve ring portion, 0.050–0.115 (0.04–0.05%); bulb length, 0.090–0.102 (0.04–0.10%), generally shorter than width [0.110–0.158 (0.05–0.11%)]; oesophageal gland prominent, extending anteriorly to level of nerve ring and posteriorly to ventriculus, with large round nucleus, 0.032 in diameter (0.03%), located at its mid-portion; length of anterior part of oesophagus 0.265–0.312 (0.1–0.3%), that of posterior part overlapped by oesophageal gland 0.748–0.835 (0.4–0.7%); distance of oesophageal gland nucleus and nerve ring 0.67–0.71 (0.3–0.6%) and 0.24–0.34 (0.2%) from anterior extremity, respectively; well-developed ventriculus with length and width of 0.095–0.140 (0.06–0.09%) and 0.078–0.100 (0.04–0.07%), respectively; with two long and broad ovaries, situated near anterior and posterior end of body; anterior ovary usually starting at about mid-length of oesophagus, extending downward up to anterior portion of intestine just after ventriculus; posterior ovary reaching far posterior to intestinal ligament, sometimes almost extending to posterior end of body; uterus very broad, occupying most space in body, filled with different stages of developing embryos and first-stage larvae; posterior end of body rounded; lateral papilla-like projections not observed; intestine relatively narrow with moderately thick intestinal wall, straight and with posterior end

atrophied, forming ligament attached ventrally to body wall; vagina and vulva not observed.

Subgravid female. Body length of three specimens collected in June 2005 ranging from 31.66 to 39.0.

Nongravid female. Body length of a single specimen collected in June 2005 was 20.47.

First-stage larva. Total length and maximum width of ten first-stage larvae from uterus of gravid females collected in July 2005 ranging from 0.450 to 0.522 and from 0.012 to 0.018, respectively; proportion of oesophageal, intestinal and tail length in relation to total body length was 28–34%, 36–43% and 25–31%, respectively.

Taxonomic summary

Type host: *Pagrus major* (Temminck et Schlegel) (Perciformes: Sparidae); FishBase name: red seabream; Japanese name: madai.

Site of infection: Gonads.

Prevalence: 11% (75 fish infected out of 650 fish examined).

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

Type locality: Off Shimonada, Ehime Prefecture, Seto Inland Sea, Japan (33°41'N, 132°36'E).

Deposition of specimens: Male holotype, allotype and paratypes deposited in the Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18858); paratypes also in the Institute of Parasitology, České Budějovice (N-889).

Etymology: This scientific name relates to the Japanese name of the fish host, i.e., madai.

Fig.9

Fig. 10

4. Philometra sawara Quiazon, Yoshinaga et Ogawa (2008) Figs. 11,12

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 (1.7–2.2%)], tapering gradually towards anterior section before broadening leading to bulbous terminal structure near round anterior end; overall oesophagus length 0.252–0.460 (8.5–15.6%), enlarged (bulb formation) near anterior end; distinct oesophageal gland with large rounded nucleus, 0.008–0.016 in diameter (0.3–0.5%), visible near mid–section; anterior section of oesophagus 0.065–0.220 in length (2.6–7.5%); posterior section overlapped by oesophageal gland 0.16–0.24 in length (5.9–9.5%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.169–0.283 (5.6–9.6%) and 0.084 (2.8%), respectively; ventriculus length and width 0.02 (0.7%) and 0.03 (1.0%), respectively; testis extended posteriorly to base of spicules, with white spots visible along each testis in 8 out of 18 specimens examined; spicules narrow, needle-like, and of unequal lengths; longer spicule 0.074–0.135 in length (2.9–4.3%); shorter spicule 0.071–0.131 in length (2.8–4.2%); length ratio of spicules 1:1.03–1.05; gubernaculum narrow, 0.040–0.076 long (1.5–2.2%), 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 (0.9–1.0%)], 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 (0.4–1.5%), broad and highly enlarged near mouth forming very distinct bulb [0.110–0.165 long (0.2%) and 0.116–0.180 wide (0.2%)]; narrowest width of oesophagus around nerve ring, 0.058–0.100 in diameter (0.1%); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.019–0.048 in diameter (0.02–0.03%), located near mid-section of oesophageal gland; anterior section of oesophagus 0.202–0.325 in length (0.1–0.4%); posterior section partially overlapped by oesophageal gland 0.545–0.772 in length (0.5–0.6%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.468–0.705 (0.02–0.44) and 0.170–0.265 (0.1–0.2%), respectively; ventriculus well developed, 0.095–0.150 in length (0.1%) and 0.082–0.130 in width (0.1%); 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 occupied 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 comprised 26-31%,

40–45%, and 28–31% of the total length, respectively.

Taxonomic summary

Type host: *Scomberomorus niphonius* (Cuvier) (Perciformes: Scombridae); FishBase name: Japanese Spanish mackerel; Japanese name: sawara.

Host's body size: fork length, 400–520 mm; body weight, 475–975 g.

Site of infection: Ovary.

Date of collection: April, May and August 2003; January and February 2004; January 2005.

Prevalence: 71% (43 fish infected out of 61 fish examined).

Intensity: Male parasites, 1–6 per fish; female parasites, 1–11 per fish.

Type locality: northern (134˚N, 35˚E) and southern (136˚N, 34˚E) part of Awaji Island, Hyogo Prefecture, Seto Inland Sea, Japan.

Deposition of specimens: 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, BCASCR, České Budějovice (N-916).

Etymology: The species name relates to the Japanese name of the fish host, i.e., sawara.

Fig.11

Fig.12

5. Philometra nemipteri Luo, 2001 Figs. 12,13

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 (2.1–3.1%)], 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 (11.1–14.5%), enlarged (bulb formation) near anterior end; distinct oesophageal gland with large rounded nucleus, 0.007–0.012 in diameter (0.2–0.3%), visible near mid-section; anterior section of oesophagus 0.131–0.205 in length (3.4–5.9%); posterior section overlapped by oesophageal gland 0.225–0.330 in length (6.5–8.2%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.308–0.340 (7.6–10.1%) and 0.15–0.19 (4.7–5.5%), respectively; ventriculus length and width 0.015–0.023 (0.4–0.7) and 0.018–0.027 (0.5–0.8%), respectively; testis extended posteriorly to base of spicules; spicules narrow, needle-like, and of unequal lengths with passage canal for sperm cells located in central section of spicules; longer spicule 0.093–0.126 in length (2.9–3.7%); shorter spicule 0.085–0.113 in length (2.6–3.4%); length ratio of spicules 1:1.02–1.21; gubernaculum narrow, 0.073–0.101 in length (2.2–2.8%), 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 (0.9–1.2%)], 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 (1.0–2.9%), highly enlarged near mouth forming distinct bulb, 0.085–0.120 long (0.2%) and 0.078–0.110 wide (0.1–0.3%), with protruding lobular structures on anterior tip of each oesophageal lobe; narrowest width of oesophagus around nerve ring, 0.035–0.060 in diameter (0.1–0.2%); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.018–0.028 in diameter (0.04–0.05%), located near mid-section of oesophageal gland; anterior section of oesophagus 0.175–0.250 in length (0.3-0.4%); posterior section partially overlapped by oesophageal gland 0.477–0.800 in length (1.4–2.1%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.418–0.627 (0.9–1.1%) and 0.175–0.245 (0.3–0.4%), respectively; ventriculus well developed, 0.040–0.085 in length (0.1–0.2%) and 0.060–0.088 in width (0.1%); 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 occupied 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 comprised 26-34%, 37–45%, and 27–32% of total length, respectively.

Taxonomic summary

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, BCASCR, České Budějovice (N-917).

Fig.13

6. Philometra sciaenae Yamaguti, 1941 Figs. 14,15

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 (2.5–2.9%)], 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 opening relatively small (0.001 in diameter) with pair of amphids situated laterally; overall oesophagus length 0.245–0.390 (10.9–17.4%), enlarged (bulb formation) near anterior end; oesophageal gland very broad with large rounded nucleus, 0.008–0.011 in diameter (0.4–0.5%), visible near mid-section; anterior section of oesophagus 0.033–0.140 in length (1.6–5.3%); posterior section overlapped by oesophageal gland 0.16–0.30 in length (7.4–12.5%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.140–0.255 (6.6–11.8) and 0.032–0.070 (1.2–3.7%), respectively; ventriculus present but barely visible; testis extended posteriorly to base of spicules; spicules narrow, needle-like, and of unequal lengths with passage canal for sperm cells located in central section of spicules; longer spicule 0.098–0.138 in length (4.6–5.3%); shorter spicule 0.096–0.135 in length (4.2–5.2%); length ratio of spicules 1:1.02–1.06; gubernaculum narrow, 0.045–0.074 in length (2.1–3.2%), with proximal end bent dorsally and has 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 in 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 (0.6–0.9%)], 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 (0.9–1.7%), enlarged near mouth forming a distinct bulb, 0.088–0.115 long (0.1–0.2) and 0.072–0.088 wide (0.1–0.2); narrowest width of oesophagus around nerve ring, 0.038–0.055 in diameter (0.1%); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.035 in diameter (0.1%), located near mid-section of oesophageal gland; anterior section of oesophagus 0.210–0.228 in length (0.2–0.5%); posterior section partially overlapped by oesophageal gland, 0.532–0.735 in length (0.7–1.2%); distance from anterior end to oesophageal gland nucleus and nerve ring 0.480 (1.1%) and 0.220–0.270 (0.3–0.5%), respectively; ventriculus well developed, 0.070–0.080 in length (0.1–0.2%) and 0.075–0.080 in width (0.1–0.2); 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 occupied 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 and 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 comprised 30-33%, 37–42%, and 27–31% of total body length, respectively.

Taxonomic summary

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, BCASCR, České Budějovice (N-703).

Fig.14

Fig.15

7. Philometroides seriolae (Ishii, 1931) Figs. 16,17

Male. Unknown.

Gravid female. (28 specimens collected from July and August 2006 and February and May 2007) length 236–530; filiform body; milky white and red in body colour; cuticle with numerous papilla-like protuberance (cuticular bosses) individually scattered on body surface, 0.002–0.005 in high, giving rise to conspicuous bossy appearance; slight yellowish brown to reddish body colouration; body widest near mid-section (1.85–2.25) (0.68–0.78%), almost uniformly broad, tapering gradually towards posterior end; anterior end of body broad and rounded; cephalic end dome-shaped with 8 pieces of very small cephalic papillae (1.5µm) arranged as ring around the mouth; paired cephalic papillae very small; rounded mouth opening, 25 µm in diameter, with no amphids observed on both sides; overall oesophagus length 4.1–6.0 (1.48–1.72%), broad and highly enlarged near mouth forming a highly distinct bulb, 0.105–0.140 long (0.03–0.04%) and 0.105–0.150 wide (0.03–0.04%); narrowest width of oesophagus around nerve ring, 0.085–0.100 in diameter (0.02–0.04%); oesophageal gland well developed, prominent and extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.100–0.200 in diameter (0.04%), located near mid-section of oesophageal gland; anterior section of oesophagus 0.080–0.155 in length (0.03–0.05%); posterior section partially overlapped by oesophageal gland 3.92–5.20 in length (1.58–1.66%); distance from anterior end to oesophageal gland nucleus and nerve ring 1.22–3.56 (0.5–0.7%) and 0.30–0.41 (0.1%), respectively; ventriculus well-developed, 0.180 in length (0.06%) and 0.135–0.148 in width (0.03–0.06%); 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; both ovaries filled with unfertilized eggs; uterus occupied majority of body space and filled

with developing embryos and first-stage larvae; posterior end of body rounded; intestine light to dark-brown in colour when alive; intestine relatively narrow, 0.135–0.500 in width, straight and atrophied near posterior end, forming a solid cord attached ventrally to body wall, anterior to posterior end; no vagina or vulva observed. In a cross-sectional view, muscle cells in dorsal and ventral sides well developed; peripheral contractile muscles laminated; two granular median lines present in the dorsal and ventral sides separating dorsal and ventral muscle portions into two portions; very broad and flat lateral fields with finely fibrous substances containing numerous nuclei.

Nongravid female. Body length of 2 specimens collected in August and October 2006 is 10.

First-stage larva. Total length and maximum width of 10 first-stage larvae isolated from uterus of gravid females collected in August 2004 ranging from 0.540 to 0.588 and 0.016 to 0.019, respectively; oesophagus, intestine, and tail comprised 19–28%, 48–56%, and 23–26% of the total length, respectively.

Taxonomic summary

Type host: *Seriola quinqueradiata* Temminck et Schlegel (Perciformes: Carangidae); FishBase name: Japanese amberjack; Japanese name: buri.

Type locality: Tokyo and Izu Peninsula, Honshu Island, Pacific Ocean off Japan.

Other localities: Infected juveniles were caught from Saga, Miyazaki, Kochi and Oita Prefectures, Japan.

Host's body size: 5.2–9.1 kgs.

Site of infection: body muscle.

Materials studied: Paratypes - 30 whole specimens (28 gravid; 2 nongravid) collected from cultured *Seriola quinqueradiata* from Saga and Oita Prefectures, Japan.

Date of collection: July 2004 to July 2007.

P r e v a l e n c e : 85% (47 fish infected out of 55 fish examined).

I n t e n s i t y : Male parasites, unknown; female parasites, 1–20 per fish.

D e p o s i t i o n o f s p e c i m e n s : Paratypes will be deposited in the Meguro Parasitological Museum, Tokyo and in the Institute of Parasitology, České Budějovice.

Fig.16

Fig.17

A.2. Comments

The major distinguishing morphological features among the six *Philometra* species examined are summarized in Table 1.

Philometra lateolabracis. This species was originally reported and described by Yamaguti (1935) as *Sanguinofilaria lateolabracis* from fishes belonging to three different families: Japanese seaperch (Lateolabracidae), chicken grunt (Haemulidae) and Hong Kong grouper (Serranidae). Among these three fish species, Yamaguti did not mention the type host. He only reported that *Sanguinofilaria lateolabracis* occurred in the ovary or oviduct of various marine fishes from the Pacific or the Inland Sea such as Japanese seaperch, chicken grunt, Hong Kong grouper and many others. Later, Yamaguti (1941) synonymized *Sanguinofilaria* with *Philometra* and transferred this species to the latter genus. Since female *P. lateolabracis* have subsequently been reported by many authors from a wide range of host fishes, it cannot be excluded that several species may have been confused under this name (Moravec et al. 1998).

Japanese seaperch, as the first host stated by Yamaguti (1935) to be infected by *P. lateolabracis*, can now be considered as the type host. Therefore, there was no doubt that the collected philometrids in this study, from the same host fish species, was *P. lateolabracis.* This was confirmed by morphological comparisons with the female *P. lateolabracis* briefly described by Yamaguti (1935). After Yamaguti's work, no other researchers have examined *P. lateolabracis* from Japanese seaperch in Japan in detail. Therefore, a full redescription of *P. lateolabracis* is provided in the present study. Moravec and Genc (2004) reported female *P. lateolabracis* from different host fishes (i.e., dusky grouper and mottled grouper) with characteristics fitting the general description of the female *P. lateolabracis* observed in this study. However, detailed morphological comparisons revealed that female *P. lateolabracis* reported by Moravec and Genc (2004)

were in fact not *P. lateolabracis*, but rather a different species. The morphological differences of specimens reported by Moravec and Genc (2004) from dusky grouper, against those observed in the Japanese seaperch in this study, include the following: big rounded mouth opening (0.020–0.040 in diameter) as compared with the observed relatively small and triangular (0.010 maximum width) mouth opening; wider maximum body width; longer overall length of the oesophagus; bigger inflated anterior portion of the oesophagus; longer distance of the nerve ring and oesophageal gland nucleus from the anterior extremity; and longer first-stage larvae inside the uterus. Yamaguti (1935, 1961), Moravec et al. (2003), and other authors did not observe any male *P. lateolabracis*, except for Crisp and Klein (1973), Moravec and Genc (2004), and Moravec and Justine (2005), who reported that their male philometrids obtained from fish species other than Japanese seaperch were *P. lateolabracis.* Since morphologically variable and tiny males normally occur together with females, a more thorough search to actually locate these males is required. A comparison between the reported male *P. lateolabracis* as described by Crisp and Klein (1973) from grunt with that observed in this study from its type host, Japanese seaperch, was impossible since most organs of the poorly preserved male specimen of Crisp and Klein (1973) were not visible. The only character which was similar is the presence of unequally long spicules. On the other hand, comparison between the male *P. lateolabracis* reported by Moravec and Genc (2004) from mottled grouper with the male *P. lateolabracis* from Japanese seaperch observed in this study revealed that these two philometrids were entirely different species; the former has longer proportion of the oesophagus in relation to the total body length, longer distance of the nerve ring and oesophageal gland nucleus to the anterior extremity and longer equal-sized spicules. Also, comparison with the male *P. lateolabracis* reported by Moravec and Justine (2005) from speckled blue grouper and blacktip grouper did not coincide with the observed

morphological features of the male *P. lateolabracis* examined in this study; the former has longer body length, broad U-shaped lobular-mound at the posterior end, longer distance of the nerve ring to the anterior extremity, longer equal-sized spicules, and lacks distinct inflation at the anterior portion of the wider oesophagus.

I cannot ignore the possibility that these previously reported records of *P. lateolabracis* from other host fishes are not actually *P. lateolabracis*, but rather new and different species. This study has shown the importance of assessing males from other host fishes reported as infected with *P. lateolabracis* to clarify if these nematodes are really *P. lateolabracis* or new *Philometra* species. Moravec et al. (2006) reported initially that the female philometrids examined from spotted weakfish (*Cyanoscion nebulosus* (Cuvier)) on the Atlantic coast of South Carolina were mostly similar to *P. lateolabracis*, but later declared it a new species (*P. carolinensis* Moravec, de Buron et Roumillat, 2006) due to the discovery of males with differently shaped and relatively shorter spicules, and with gubernaculum possessing a dorsal barb on the distal end compared to those reported in *P. lateolabracis* males by Crisp and Klein (1973), Moravec and Genc (2004), and Moravec and Justine (2005). *Philometra carolinensis* females are distinctly different from *P. lateolabracis* examined in this study, particularly regarding the shape of the mouth, but the metric and meristic features in the spicules and gubernaculum are within the range of male *P. lateolabracis.*

Philometra isaki. Chicken grunt was one of the three host fish species in which Yamaguti (1935) reported the presence of *P. lateolabracis* females. Crisp and Klein (1973) also reported the presence of a single “assumed” male and five female *P. lateolabracis* in the gonads of grunt, a fish belonging to the same family Haemulidae. However, some morphological features used to distinguish *P. lateolabracis* from the male reported by Crisp and Klein (1973) appear doubtful wherein some differences may be due

to a certain amount of intraspecific variability and some inaccuracies in observations. Recently, Merella et al. (2004), Moravec and Genc (2004), and Moravec and Justine (2005), reported *P. lateolabracis* males which they found in other host fishes. A morphological comparison between the observed male philometrids from chicken grunt with the observed male *P. lateolabracis* from its type host Japanese seaperch in this study revealed that the philometrids from chicken grunt were clearly distinguishable from *P. lateolabracis*; the former have longer body length, broader body width, shorter proportion of oesophageal length in relation to total body length, longer distance of nerve ring to anterior extremity and longer unequal-sized spicules. The discovery of male *P. lateolabracis* in this study will serve as a basis in the re-identification of *P. lateolabracis* reported from fishes other than Japanese seaperch. The female *P. isaki* from chicken grunt was compared with the *P. lateolabracis* reported by Yamaguti (1935), and with the fully described female of *P. lateolabracis* from Japanese seaperch observed in this study. Generally, similar morphological features were observed except for the nearly double maximum length of gravid females and relatively lower oesophageal length ratio in relation to the total body length in *P. isaki*. With these morphological differences observed, it is necessary to classify *P. isaki* from its type host fish chicken grunt as a new species. Additionally, some morphological differences were also observed in the first-stage larvae found inside the uterus of gravid females. These differences between the hatched larvae of *P. lateolabracis* and those of *P. isaki* include a shorter proportion of oesophageal length and longer proportion of intestinal length in relation to total body length in *P. lateolabracis*.

Philometra madai. Yamaguti (1961) found and identified for the first time single, not fully mature female nematodes in the swimbladder of the black porgy [*Acanthopagrus schlegeli* (Bleeker)] as *P. spari*. The nematode found by Sakaguchi and Matsusato (1978)

in the body cavity of red seabream caught in Kariya Bay, Saga Prefecture was also identified as *P. spari* due to the morphological similarities observed. Nakajima and Egusa (1979) examined cultured red seabream from the coast of Amami Island, Kagoshima Prefecture, and found philometrid nematodes in its gonads. They reported it as an unknown species (*Philometra* sp.), and not *P. spari* because of the difference in habitat of the worm in the host and the location of the uterus in the gravid female worm. Sakaguchi et al. (1987a,b) re-identified the philometrid nematodes obtained from red seabream as *P. lateolabracis*, which was previously reported as *P. spari.* Sharples and Evans (1995a,b) also reported the presence of female *P. lateolabracis* in New Zealand from snapper, *Pagrus auratus*, a host fish belonging to the same genus (*Pagrus* Cuvier). Sakaguchi et al. (1987b) and Sharples and Evans (1995a,b) did not describe morphological characteristics of their nematodes in detail. Their morphological examinations, being closest to *P. lateolabracis*, which was always reported to infect diverse marine fishes, might have made them identify their specimens as *P. lateolabracis.* Since all of these researchers were not successful in finding the morphologically more variable males, it was not clear if these nematodes were *P. spari*, *P. lateolabracis*, or other *Philometra* species.

The majority of morphological structures of the *P. lateolabracis* females described in this study have some similarities with *P. madai.* However, there were several features that differentiate *P. madai* from *P. lateolabracis.* The major difference observed in the *P. madai* female is the highly inflated anterior portion of the narrow oesophagus, which is distinctly different from that of the female *P. lateolabracis* (i.e., slightly inflated and wide oesophagus). In males, the major differences observed in *P. madai*, compared to *P. lateolabracis*, include the total body length (nearly two times longer), narrower body, presence of paired large posterior lobes on both sides of the spicule and gubernaculum which are subdivided into two unequal-sized smaller lobes, and a different length ratio of

spicules and gubernaculum. In this regard, the previously reported *P. lateolabracis* from red seabream was a misidentification. Hence, with these various morphological differences observed, it is therefore necessary to suggest *P. madai* as a new species.

Philometra sawara. It is possible that the parasite described here, collected from the ovary of Japanese Spanish mackerel, is identical with the originally described *Sanguinofilaria scomberomori* Yamaguti, 1935, collected from Chinese seerfish (*Scomberomorus 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 Spanish mackerel (*Scomberomorus maculatus* (Mitchill)), but this was questioned by Moravec (2006). Morphologically, *P. globiceps* from its type host Atlantic stargazer (*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 skipjack tuna (*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 Albacore (*Thunnus alalunga* (Bonnaterre)) (type host) and yellowfin tuna (*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* in the present study 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 in examined 20 male *P. lateolabracis* in Japanese seaperch. On the other hand, comparisons carried out with other *Philometra* species identified in Japan 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, I am confident that the specimens isolated from Japanese Spanish mackerel represent a new species, unless sufficient morphological information (as well as molecular data), from both male and female *P. scomberomori* from Chinese seerfish, become available.

Philometra nemipteri. Luo (2001) first reported and described only female *P. nemipteri* from golden threadfin bream 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* examined from Japanese seaperch 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 with the examined male *P. lateolabracis.* 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.

Philometra sciaenae. Yamaguti (1941) originally described *P. sciaenae* from the ovary of silver croaker (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 silver croaker, 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 presence of 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* in the present study 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.

Philometroides seriolae. This species was originally described in brief by Ishii (1931) and identified it as *Filaria seriolae*, which was later transferred and redescribed by Yamaguti (1935) into a new genus, the *Philometroides*. Sixty-three years after its transfer to the genus *Philometroides*, Moravec et al. (1998) again redescribed this species based only on a single fragment of anterior region collected from the same host fish (Japanese amberjack) in Japan. Morphological comparison of the nematode specimens from Japanese amberjack in the current study with the only one specimen redescribed by Yamaguti (1935) and another one specimen redescribed by Moravec et al. (1998) confirmed that the current specimens were *Philometroides seriolae.* General morphological features were found similar except for the number and distribution pattern of the cephalic papillae, a structure that Yamaguti (1935) listed in the generic diagnosis as absent, but was first described by Moravec et al. (1998). The observed number and distribution pattern of cephalic papillae contradicted with the one described by Moravec et al. (1998) using LM. SEM results in the present study revealed only four pairs (i.e., eight pieces) of very small cephalic papillae located only in a single circular pattern around the mouth portion. These cephalic papillae would be hardly visible using LM. Based on the drawings by Moravec et al. (1998), many large cephalic papillae were

arranged in two circular patterns similar with those of the genus *Philometra*. Given that these observed cephalic papillae by Moravec et al. (1998) were very big and its distribution in the cephalic end is somewhat uncommon, it was likely possibly that these were cuticular bosses, which were randomly distributed in the cuticle of this parasite. With the additional new morphological information observed on several whole specimens collected, I feel that redescription is necessary.

Table 1

Table 1, cont

B. Molecular studies

The results in the molecular examinations were mainly subdivided into the following studies:

(1) Molecular comparison of Philometra species previously identified as P. lateolabracis. *Philometra madai* (429 bases) had a higher number of nucleotide bases in the ITS2 region than *P. lateolabracis* (418 bases). The percentage similarity of sequences (gaps included) between the two species was 70% while intraspecific similarities were above 99.7% in both species (Table 2). These molecular data support the finding that *P. lateolabracis* and *P. madai* are different species, which is in contrast to the previous reports that philometrids from red seabream were *P. lateolabracis*. Even though *P. isaki* was not sequenced due to unavailable samples, its description as a new species could still be strongly supported by the morphological data obtained. Further molecular comparison with the ITS2 sequences reported under *Philometra* in the GenBank showed that the *Philometra* species examined in this study were obviously different (Table 3). The similarity of 70% between *P. lateolabracis* and *P. madai* is in contrast to the interspecific similarity of 21.2–43.7% among previously studied species. This suggests a close phylogenetic relationship between *P. lateolabracis* and *P. madai* compared to previously studied species.

(2) Molecular comparison on other Philometra species in Japan. The 18S rDNA of all presently examined *Philometra* species were found to be conserved region. As shown in Table 4, out of a total of 1499-1701 bases comprising partial sequence of the 18S rDNA and partial sequence of the ITS1 region of five *Philometra* species examined in this study, interspecies similarities and number of different bases ranged from 88-97.8 % and 0-2 bases, respectively (Appendix 6,7). On the other hand, ITS2 region was found to be more variable region compared to the 18S rDNA. The total bases of *Philometra sawara, P.*

nemipteri and P. sciaenae in ITS2 region were 479, 425 and 497 nucleotide bases, respectively. No intraspecies variations were observed among four representative specimens for each species. As shown in Table 5, the interspecies similarities among examined *Philometra* species ranged from 63.7–85.9%, while the number of different bases ranged from 29-83 bases (Appendix 8,9). The results indicate that the five species of *Philometra*, molecularly examined in Japan, were independent.

(3) Molecular comparison of Philometroides seriolae with other dracunculoids. The number of partially sequenced nucleotide bases of 18S rDNA and ITS2 regions of *Philometroides seriolae* were 1,672 and 797 bases, respectively. No intraspecies variations were observed between the three subsamples in each of the six philometrid species sequenced in both regions.

Molecular comparison of *Philometroides seriolae* with other reported sequences of *Philometroides* species, including *Philometra clavaeceps* which was observed to be genetically close with the genus *Philometroides* in molecular study (2), revealed very low interspecies similarities of 45.0–46.5% and 16.4–28.3% on 18S rDNA and ITS2 region, respectively (Table 6,7). The number of base difference and *p-distance* value for 18S rDNA were 64–74 bases and 0.080–0.092, and for ITS2 were 156–180 bases and 0.529–0.610, respectively. Generated phylogenetic tree among dracunculoinds reported in the GenBank, using NJ (maximum composite likelihood) and MP methods inferred from 18S rDNA, revealed two major clades separating the genus *Philometra* and *Philometroides* (Fig. 18). The first 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 six *Philometroides* species (i.e., *Philometroides sanguineus* (=*Philometroides carassii*), *Philometroides pseudorasbori*, *Philometroides cyprini* (Ishii, 1931), *Philometroides seriolae*, *Philometroides fulvidraconi* Yu, Wu et Wang, 1993, and

Philometroides pseudaspii (=*Philometroides ganzhounensis*)), two *Clavinema* species (i.e., *Clavinema parasiluri* Yamaguti, 1935 and *Clavinema fujimotoi* (Furuyama, 1932) (=*Philometra fujimotoi*) and one *Philometra* species (i.e., *Philometra clavaeceps* Dogiel et Akhmerov, 1959). Both clades branched out from *Dentiphilometra monopteri* Moravec et Wang, 2002 with *Philonema* sp. as an outgroup. Although percentage similarities among *Philometroides* species were very low, the tree somehow shows close genetic relationship of *Philometroides seriolae* with *Philometroides sanguineus* (=*Philometroides carassii*), *Philometroides pseudorasbori* and *Philometroides cyprini*. The inclusion of the *C. parasiluri, C. fujimotoi* (=*P. fujimotoi*) and *P. clavaeceps* created some possible misidentification of the sequenced specimen. On the other hand, generated NJ and MP trees inferred from ITS2 region also created the same two major clades separating the genus *Philometra* and genus *Philometroides* (Fig. 19).

Table 2

Table 3

Table 4

Table 5

Table 6

Table 7

Fig. 18

Fig. 19

Discussion

Morphological and molecular analyses were used to describe and compare all male and female specimens of philometrid nematodes, revealing that all presently examined seven philometrid nematodes are independent species. Given that males from some previously reported philometrid species have not been discovered, the report on male characteristics in this present study will be very useful for future taxonomical studies.

The discovery of male *P. lateolabracis* from its type host, Japanese seaperch, clarified the taxonomical identity of previously reported *P. lateolabracis* observed in chicken grunt and red seabream. This study has shown the importance of searching for male philometrids from other reported *P. lateolabracis*-infected hosts. Such results would confirm if these philometrids are indeed *P. lateolabracis* or new *Philometra* species. Up to present, the morphology of male *Philometroides seriolae* still remains unknown, whereas the morphological redescription of females, particularly on the arrangement pattern and sizes of the cephalic papillae, adds important morphological information to this species.

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 region compared to 18S rDNA, concluding that 18S rDNA was more suitable for phylogenetic studies. In contrast with their results, the 18S rDNA examined in the present study was highly conserved, than the ITS2 rDNA region, in the genus *Philometra* and cannot be used to clearly distinguish species. Molecular comparisons with other ITS2 sequences of *Philometra* and *Philometroides* from the GenBank database supported the morphological data that the five *Philometra* species (i.e., *P. lateolabracis*, *P. madai*, *P. sawara*, *P. nemipteri* and *P. sciaenae*) examined in the present study were all independent to each other and to other reported species. Although no molecular data was obtained for *P. isaki*,

morphological data still clearly showed that it was a new species. 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. With the combined morphological and molecular data showing that *P. nemipteri* and *P. lateolabracis* are entirely different species, it is suggested that *P. nemipteri* should now be removed from its current status of *Species inquirenda* as reported by Moravec (2006).

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 parasitising a freshwater fish, *Chanodichthys erythropterus* Basilewsky, whereas 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. Also, the genus *Clavinema*, particularly *C. parasiluri*, was also included in the *Philometroides* clade, whereas the other *C. fujimotoi* (=*Philometra fujimotoi*) were separated from both clades of *Philometra* and *Philometroides*. However, due to low bootstrap values, we cannot make a concrete conclusion on the phylogenetic position of this genus. It is possible that 18S rDNA is not a good region to differentiate the phylogenetic position of the genus *Clavinema* with those of the genus *Philometra* and *Philometroides.* In contrast, the ITS2 region generated a phylogenetic tree wherein the genus *Clavinema* becomes an outgroup indicating that this group is not closely related to the genus *Philometroides.* It is also possible that ITS2 is not as good region compared with other regions. Hence, it is

suggested that other variable regions, aside from 18S and ITS2, should also be explored. In conclusion, the morphological and molecular results that were generated from the present study will be very useful for future taxonomical studies with established or newly discovered species under this group.

SUBCHAPTER 2. BIOLOGICAL STUDIES

Infection dynamics of philometrid nematodes in the body muscles and gonads of marine fishes

Introduction

The genera *Philometroides* and *Philometra* are members of the family Philometridae in which recognized species are continuously increasing with the discovery of new species. The use of SEM for morphological taxonomy, supported by molecular data, made it possible to raise new species, redescribed valid species, re-evaluate the taxonomical positions of some mistakenly identified species and validate species under the status *species inquirenda* (see Subchapter 1, Chapter 2). In spite of the numerous reported taxonomical studies on philometrid nematodes, few works have been reported about their infection dynamics and pathology (Hespt et al. 2002, Wang 2002, Oliva et al. 1992, Uhazy 1977, 1978).

Philometroides seriolae has very high economic impact especially to the Japanese amberjack industry in Japan. The body muscle of this fish is relished in Japan in raw form as sashimi and sushi. Although this worm has no zoonotic features, their presence in the body muscle greatly reduces the market value of the fish. To date, only *Philometroides seriolae* is known to infect mainly body muscle of marine fish, whereas three species, *Philometroides hydrocyoni*, *Philometroides cyprini* and *Philometroides strelkovi*, have been reported in some body muscle of freshwater fishes *Hydrocynus forskhlii* (Alestiidae), *Cyprinus carpio* (Cyprinidae) and *Gnathopogon strigatus* (Cyprinidae), respectively (Moravec 2006, see Chapter 1).

The 18 *Philometra* species reported in Japan include nine species which were known to infect fish gonads (see Chapter 1). There were some taxonomical studies wherein the possible effects of these gonad-infecting philometrids to fisheries industry,

such as significant decrease in fish production and full parasitic castration, have been stated (Sakaguchi et al. 1987, Moravec and Genc 2004). Despite of the economic implication of *Philometroides seriolae* and *Philometra* species in aquaculture and wild fisheries, little is known about their biology, thereby justifying the need for more biological studies. This study was carried out by focusing mainly in the understanding of the fundamental infection dynamics and pathology of the muscle and gonad-infecting philometrid nematodes. In this study, seasonal monitoring of the infection of muscle-infecting philometrids in Japanese amberjack was carried out to determine not only the seasonality and sites of infection, but mainly to determine until when the worms will die and be completely removed in the body muscle of the fish making it suitable for selling to the public. Also, the gonad-infecting philometrids were studied to understand the seasonality of infection, relation of the prevalence and intensity of gravid female worms to fish spawning season, and their possible effects in fish reproduction.

Materials and methods

Sample collection and fixation. The marine fishes used in the the taxonomical studies in Subchapter 1 (Chapter 2) were also used for the biological studies in this subchapter. The muscle-infecting *Philometroides* species were collected from three batches of a total of 55 cage-cultured Japanese amberjack, weighing 5.2 to 9.1 kg. Japanese amberjack were originally caught during the spring of 2004 (batch 1), 2005 (batch 2) and 2006-2007 (batch 3) in off Ashizuri Peninsula, Kochi Prefecture, Japan, where they were beleived to have acquired infection, prior to raising in floating net cages in the aquaculture facilities located in Oita, Saga and Miyazaki Prefectures. The whole fish were dissected by slicing the muscles thinly to collect worms. The number of worms and sites of infection were plotted in lateral and cross sections, both of which were divided into two separate sections; section 1 (from head to anus portion) and section 2

(from anus to tail portion) (Fig. 20). Collected worms were fixed in 70% ethanol and cleared in lacto-phenol solution prior to morphological identification using light microscope. A portion of the worm tissue was fixed in 100% ethanol for molecular confirmation of the species level. Species identification was based on the morphological and molecular information previously reported (Moravec et al. 1998, Moravec 2006, see Subchapter 1 (Chapter 2)).

On the other hand, the examined 6 marine fishes infected by gonad-infecting *Philometra* species were silver croaker, Japanese Spanish mackerel, chicken grunt, red seabream, Japanese seaperch and golden threadfin bream. Among these host fishes, only the silver croaker, chicken grunt and red seabream were examined for seasonality of infection, due to availability of samples, whereas the remaining fishes were examined depending on the availability of samples. Large female worms were collected macroscopically from both testes and ovaries, whereas tiny males were collected only from the ovaries following procedures done in Subchapter 1 (Chapter 2). Fixation and identification procedures carried out for muscle-infecting *Philometroides* species were also followed for gonad-infecting *Philometra* species, except for the use of glycerin as clearing solution prior to morphological examination (see Subchapter 1, Chapter 2).

Infection dynamics examination. The infection dynamics of both muscle and gonad-infecting philometrid nematodes were monitored, particularly on the prevalence of infection (i.e., percentage of infected fish in all fish examined), intensity of infection (i.e., number of worms per infected fish), mean abundance of worms (i.e., mean number of worms in all fish examined), and sites of infection. Apart from the developmental stages (nongravid, semigravid and gravid) as categorized in Subchapter 1 (Chapter 2), newly dead and remnants (old dead worms) of lysed worms were also monitored, especially for muscle-infecting *Philometroides* species. A worm was categorized as

newly dead with the presence of disintegrating body, whereas remnants of lysed worms were easily identified as dark, worm-like to irregular-shaped materials within the infected tissues.

For the examination of the gonad-infecting philometrids, both fresh (for silver croaker, red seabream, Japanese seaperch and golden threadfin bream) and fixed fish gonads (for Japanese Spanish mackerel and chicken grunt) were examined. Since monitoring and collection of male worms in the host testes were difficult, distinction between male and female worms was carried out only in host ovaries. To determine the relation of gonad-infecting philometrids with the host spawning season, monitoring were carried out only in silver croaker, chicken grunt and red sea bream. The fish spawning seasons in the fishbase (www.fishbase.org) served as a guide in the examination of the maturity of fish ovaries. The spawning season of the currently examined fishes varied slightly from that in the fishbase due possibly to the difference from localities when the spawning seasons were recorded. Given that the developmental stages of fish ovaries were not examined in detail in this study, the ovaries were only categorized to immature (i.e., eggs are at pre-ovulation stage), mature (i.e., eggs are at post-ovulation stage) and spent stages (i.e., large eggs, milky or white in coloration inside the egg).

Finally, the host responses against philometrids and the pathological effects to hosts were monitored histologically. For this purpose, a total of 20 infected muscle tissues of Japanese amberjack and 52, 10, 5 and 24 gonads of silver croaker, chicken grunt, red sea bream and golden threadfin bream, respectively, were fixed in 10% buffered formalin (3.7% formaldehyde) and processed in standard histological sections.

Results

Morphological and molecular analyses confirmed that the muscle-infecting *Philometroides* species was *Philometroides seriolae*, and the six gonad-infecting *Philometra* species were *P. sciaenae*, *P. sawara*, *P. isaki*, *P. madai*, *P. lateolabracis* and *P. nemipteri* as shown in Subchapter 1 (Chapter 2).

Muscle-infecting Philometroides seriolae. Only female worms were collected individually in the cavities formed within the body muscle of Japanese amberjack, whereas males still remained undiscovered. Table 8 shows the number of *Philometroides seriolae* at different developmental stages, prevalence and intensity of infection, and mean abundance. The prevalence, intensity and mean abundance varied among examined batches and seasonality was not detected in them. A total of 519 worms were examined, 39.1% of which were alive and 60.9% were dead. Among the live worms examined, 82.2%, 15.3% and 2.5% were gravid, semigravid and nongravid, respectively, without clear seasonality in the composition of the developmental stages of worms. Although both live and dead *Philometroides seriolae* were observed in every sampling period, prevalence of live worms were lower (50–100%) compared to dead worms (100%). Intensity of both live and dead worms ranged from 1 to 20 worms per infected fish, whereas mean abundance were 0.5–10.0 and 1.0–14.4 for the former and the latter, respectively. Table 9 shows the number of *Philometroides seriolae* in the different locations within the body muscle of Japanese amberjack. Data revealed that majority of worms (91.3%) infected the body muscle portion (Figs. 21 C,D), whereas only 8.7% were found under the skin (Figs. 21 E). Among those worms observed under the skin, only very few worms were found extruding their anterior end outside the fish skin (Fig. 21 F). Table 10 and Fig. 22 show the number of *Philometroides seriolae* in lateral and cross sections. The total worms found in the epaxial muscle was 56.9%, whereas 43.1% were found in the hypaxial muscle. Also,

51.5% and 48.5% of the worms were found in the left and right sides of the fish body, respectively, whereas 52.4% and 47.6% were found in the portion from the head to anus (section 1) and that from the anus to tail (section 2), respectively.

For the host response of Japanese amberjack against *Philometroides seriolae*, a white-coloured tissue was seen lining all formed cavities (Fig. 21 D; Table 14). Histologically, inflammatory damaged tissues surrounding each cavity were infiltrated with inflammatory cells. No inflammatory cells were seen attached in the cuticle of any live worms (Fig. 21 A). As a result of no inflammatory cells attaching directly to the worms, development up to gravid stage seemed unaffected by host response. In other previously infected portions of the body muscle, remnants of old dead worms with complete healing (tissue regeneration) were observed (Fig. 21 B). Hatched larvae were also found within the formed cavities where fully gravid female worms were found.

Gonad-infecting Philometra species. The prevalence, intensity and mean abundance of male and female gonad-infecting *Philometra* species were recorded only in the host ovaries since collection of tiny worms in the fish testis was difficult. As shown in Table 11, the prevalence, intensity and mean abundance of female *Philometra* species were higher than males. However, as male worms were considerably smaller and difficult to find than females, such difference was possibly due to the difficulty in detection. In female hosts, both live and dead *Philometra* species were mainly found in the ovarian lumen and some were also found in the oviduct. In male host fishes, only *P. nemipteri* were found mainly in the spermatic duct, whereas majority of the other five *Philometra* species, live or dead, were found in the seminiferous tubules.

In the majority of silver croaker, chicken grunt and red seabream examined for the seasonal prevalence and intensity of worms, majority of the collected female worms were gravid during host's spawning season (Table 12). The reported spawning season of silver

croaker in Ariake sound, Japan collected from September 2001-August 2002 was from April to August. In this study, however, matured ovaries were observed, not only until August but until September. Spent ovaries were observed from September until November, whereas immature ovaries were observed from January to April. As shown in Table 12, gravid female *P. sciaenae* were collected from July to September, when most of the silver croaker had matured eggs. Dead worms were recorded mostly in November after the spawning season of the fish. For chicken grunt, the reported spawning period in Kumano-nada, central Japan collected from 1978-1979 was from June to August (Suzuki and Kimura 1980). In the examined specimens, no gravid *P. isaki* was collected except during the examination of three fish in July 2002. This result is not enough to conclude the relation of fish spawning with the presence of gravid worms, but the number of dead *P. isaki* was lowest from July to August, the period when mostly gravid worms are expected. For red seabream, the reported spawning season in Tokyo Bay was from May to June (www.fishbase.org). In this study, red seabream were obtained from southern Japan and were observed to have matured eggs from June to July, which coincides with the season when majority of gravid *P. madai* were collected. Also, dead worms were mostly observed after the spawning season of the fish. Although worms were also observed during off-breeding season of the fish, collected worms were either nongravid or dead.

On the other hand, Table 13 shows the prevalence, intensity and mean abundance of all six *Philometra* species in the testes and ovaries of their respective host fishes. Presented data in Table 13 include all developmental stages of *Philometra* species from nongravid to dead worms. In silver croaker, prevalence, intensity and mean abundance were high from June to November, whereas no clear trend was found in chicken grunt. High intensity was observed in July and September, and in January for the red seabream and Japanese seaperch, respectively, where most of the examined fishes have matured

eggs.

Regardless of the prevalence, intensity and sites of infection, leukocyte infiltrations were mostly observed in live and dead worms (Table 14). Presence and absence of leukocyte infiltrations around *Philometra* species depend on the host gonads, developmental stages of the worms and sites of infection. In silver croaker, host response or accummulation of inflammatory cells around the *P. sciaenae* were observed in both dead and live worms in testes and ovaries (Fig. 23 A-C). There were also some live worms observed to have no inflammatory cells accumulating around their cuticle especially those present in the ovaries. In chicken grunt, all available specimens histologically examined were all old dead *P. isaki* with minimal inflammatory cells observed. In some specimens, no inflammatory cells were observed around these old dead worms. In red seabream, both live and dead *P. madai* were observed to be surrounded with intensive inflammatory cell accummulation (Fig. 23 D). Lastly, live *P. nemipteri* from the spermatic duct of golden threadfin bream were not surrounded by inflammatory cells (Fig. 23 E). However, majority of the released first-stage hatched larvae observed both in the spermatic duct and seminiferous tubules were surrounded by inflammatory cells (Fig. 23 F).

Discussion

Oliva et al. (1992) reported the relationship between first maturity of Chilean sea bass (*Paralabrax humeralis* (Valenciennes)) and the infection process of *Philometra* sp., wherein infection can occur, by expelling viviparous nematode larvae in the environment together with the host's gametes, and the larvae actively penetrate the new host without any reported intermediate hosts. However, no concrete data was presented by Oliva et al. (1992) on the absence of any intermediate hosts to establish re-infection. There was evidence reported that *Philometra* sp. infects Chilean sea bass only after first maturity (Oliva et al. 1992), but in the present study, infection by nongravid and semigravid

female *Philometra* species were not only observed in gravid hosts but as well in immature hosts. However, the incidence of *Philometra* infection in gravid hosts was relatively higher than in immature hosts.

Host response against philometrid infection was reported to be different among host fishes. The absence of inflammatory cell accumulation around live worms in some ovaries of silver croaker coincides with the result obtained by Hesp et al. (2002) that no histological evidence in the oocytes of westralian jewfish were becoming necrotic near the philometrids identified as *P. lateolabracis*. Instead, as also observed in this study, the dead and remnant parasites become encapsulated in a fibrous sheath produced in the host. This observation was also similar to that observed by Oliva et al. (1992), who found no evidence of histological damage caused by live *Philometra* sp. in the gonads of Chilean sea bass, although a moderate leucocytic infiltration was observed suggesting some type of immune response by the host to the infection. Hesp et al. (2002) partly attributed the absence of host response to the relatively low intensity of infection, although the philometrid found in westralian jewfish are large. Furthermore, few inflammatory cells were observed around the old dead *P. isaki* in the present study, possibly indicating that either no leukocyte infiltration occurred, or since the worms were already lyzed, the accumulated inflammatory cells might have subsided. On the other hand, the presence of intensive inflammatory cell accumulation commonly observed around live fully gravid *P. madai* in ovaries of red seabream, indicates an alarming negative effect on the host reproduction. The fresh and histological evidences obtained in this study revealed large portion or space of the fish gonads, instead of being alloted to gamete production, were occuppied by the worms in which some are intensively surrounded by inflammatory tissues occupying also large space as observed in the ovaries of red seabream. For those infecting the male golden threadfin bream, the presence of large number of worms in the

main passageway of the gametes in the fish testis (i.e., spermatic duct) during fish spawning season could completely block the release of gametes making the male fish useless during their breeding season, Host response was also reported in other philometrids in the body cavity, which completes their development in the subcutaneous tissues. Uhazy (1978) noted little or no tissue damage associated with *Philometroides nodulosa* in the body cavity of the white sucker (*Catastomus commersoni*). Wierzbicki (1960) and Crites (1975) reported encapsulated developmental stages of *Philometroides sanguineus* in crucian carp (*Carassius carassius* (Linnaeus)) and *Philometra* sp. in freshwater drum (*Aplodinotus grunniens* Rafinesque).

Reports concerning the mode of releasing hatched larvae of philometrid nematodes were very few (Uhazy 1978, Oliva et al. 1992). According to the study conducted by Uhazy (1978), *Philometroides huronensis,* a philometrid infecting the subepidermal tissue of the white sucker, were reported to release their first-stage larvae through the cutaneous opening of the host resulting in the disruption of the fibrous capsule and rupture of its body, whereas Oliva et al. (1992) reported that the release of *Philometra* sp. larvae was in conjunction with the release of the sexual product of the Chilean sea bass. Both of these modes of releasing hatched larvae were also possible for the presently examined muscle and gonad-infecting philometrids. The observation of *Philometroides seriolae* extruding outside the host skin had suggested that this is the mode for releasing first-stage hatched larvae (Nakajima et al. 1970). However, only 8.7% of the examined worms were found under the skin, whereas the remaining 91.3% were found in the body. The released larvae that remained inside the cavities formed within the body muscle and gonads of the host fishes seemed unable to go out of the host to beome a source of new infection, since leukocyte infiltrations around the larvae were commonly observed. This possibly indicates that the few *Philometroides seriolae* that successfully settled beneath the skin

and extruded their anterior end outside the fish skin can only contribute as the source of infection, and the re-infection for gonad-infecting *Philometra* species were from those released during host copulation.

Given that Japanese amberjack is highly relished food in Japan as sashimi and sushi, complete healing process in the infected areas will significantly contributes to the economic benefit of the fish growers. However, it is still unclear how long it will take until the infected areas become completely healed or until when the worms will be completely removed. On the other hand, low infection of philometrid nematodes infecting the gonads of some host has minimal pathological effect on host reproduction, whereas high infections may affect gamete production and even blocks the release of gametes during spawning as shown in fresh and histological evidences. However, there might be other host fishes having similar severe pathological effects observed in red seabream in spite of low intensity of infection. Generally, although no histological damages or moderate leukocyte infiltrations were detected in the gonads, heavy infection, as well as strong tissue response initiated by dead worms, may reduce the volume of the gonads and may lead to lower fecundity. In conclusion, although the data obtained in this study were limited and have not obtained some major objective of this study, these can somehow contribute to the few biological information on these group of philometrid nematodes.

Table 8

Table 9

Table 10

Table 11

Table 11, cont

Table 11, cont

Table 12

Table 12, cont

Table 13

Table 13, cont

Table 13, cont

Table 14

Fig. 20

Fig. 21

Fig. 22

Fig. 23

CHAPTER 3. STUDIES ON ANISAKID NEMATODES

SUBCHAPTER 1. TAXONOMICAL STUDIES

Section 1. *Morphological differences between larvae and in vitro-cultured adults of Anisakis simplex (sensu stricto) and Anisakis pegreffii*

Introduction

Anisakis simplex, which continuously attracts human health concerns relating to anisakiasis, is the most frequently reported species in the body muscle of some host fishes. Proper species identification of L3 larvae taken from patients with anisakiasis and from wild hosts is very important in implementing guidelines for future reduction and prevention of this disease. Since morphological identification in L3 larvae is only determined by type grouping (see Chapter 1), precise identification up to species level is difficult with the current unavailability of distinguishing morphological keys in L3 larvae, especially in distinguishing *A. simplex* from *A. pegreffii*.

Current use of molecular approaches led to the identification of 8 recognized valid species (see Chapter 1). Even with the success in the development of different molecular protocols, costs, time duration needed for examination and availability of technical expertise limit its widespread application. This is also impractical and impossible during field surveys needing rapid on-site identification of *Anisakis* species.

In contrast to larvae, species identification of adults is morphologically possible in some species. However, no obvious morphological differences have been reported between *A. simplex* (s.s.) and *A. pegreffii*. Among the morphological keys used in species identification by systematists as valid criteria is the number and arrangement of caudal papillae in the males of adult nematodes (Davey 1971, Fagerholm 1988). The caudal papillae of *A. pegreffii* have not been examined. Those of *A. simplex*, which was described when the presence of sibling species has not yet been recognized, was reported (Davey

1971) while those of *A. simplex* (s.s.) are not available. In addition, the spicule length, which is also frequently used in distinguishing one species from another overlapped between *A. simplex* and *A. pegreffii* (Davey 1971, Campana-Rouget and Biocca 1955).

This study was conducted due to the current difficulties with the morphological differentiation, not only on L3 larvae but also in adults of *A. simplex* (s.s.) and *A. pegreffii*. The aim of the present study was to closely examine any major morphological differences to find keys for identification of L3 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii*. Such identification keys would be useful for immediate identification of *Anisakis* recovered from human patients and from infected fishes and other hosts, thereby providing precautionary measures in preventing and reducing anisakiasis. In this study, I applied an *in vitro*-culture technique (Iglesias et al. 1997, 2001) to obtain adult worms as collection of adults from infected cetacean hosts is difficult.

Materials and methods

Parasite collection and fixation. L3 larvae were collected from the body muscle of chum salmon (*Oncorhynchus keta* (Walbaum)) caught in the Chitose River in Hokkaido and from the visceral surface of 5 different fish species, namely Pacific cod (*Gadus macrocephalus* Tilesius), Alaska pollock (*Theragra chalcogramma* (Pallas)), blue mackerel (*Scomber australasicus* Cuvier), chub mackerel (*Scomber japonicus* Houttuyn) and Japanese Spanish mackerel (*Scomberomorus niphonius* (Cuvier)) caught in Japanese waters. Also, L3 larvae were collected from the body cavity of juvenile greater amberjack (*Seriola dumerili* (Risso)) imported from China (Table 15, Fig. 24). L3 larvae collected from fishes and L4 larvae and adults obtained by *in vitro*-culture were fixed in 70% ethanol. After measurement of total length, they were cut into three pieces: the anterior, middle and posterior parts. Anterior and posterior parts were used for light microscopic and scanning electron microscopic observations. The middle part was stored in 100%

ethanol to extract genomic DNA for PCR-RFLP analysis of rDNA and for *mt*DNA *cox*2 gene sequencing.

In vitro-culture. L3 larvae collected from chum salmon caught in Chitose River and chub mackerel caught in off Kumamoto Prefecture were used. *In vitro*-culture from L3 larvae to adult was performed following a previous report (Iglesias et al. 2001). Briefly, undamaged worms were washed and immersed in an antibiotic–antimycotic solution (Iglesias et al. 1997) for 30 min for sterilization before *in vitro*-culture. Subsequently, worms were placed individually in a single well of 24-well plates containing culture media (i.e., RPMI-1640 supplemented with 20% heat-inactivated fetal bovine serum and 1% commercial pepsin and adjusted to pH 4.0). *In vitro*-culture was performed at 37 °C in an air atmosphere with 5% CO2. Medium was replaced twice a week during L3 to L4 stage, and was replaced more frequently during the adult stage, according to the amount of debris and color change observed in the medium. Worms were transferred to bigger 12-well plates after 2 weeks and finally, to 6-well plates after moulting from L4 to adult stage. L4 larvae and adults were sampled during 3 to 26 days and 27 to 60 days of culture, respectively.

DNA extraction and data analyses. The genomic DNA was extracted from 100% ethanol-fixed worms using DNeasy™ Tissue Kit from Qiagen Inc. (protocol for animal tissues). The ITS region (ITS1-5.8S-ITS2) of rDNA was amplified using primers A (5′-GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA-3′) and B (5′-GCCGGAT CCGAATCCTGGTTAGTTTCTTTTCCT-3′) (Umehara et al. 2006). The PCR assay was performed with 1 μL sample DNA as template in a total volume of 20 μL containing 0.6 μL forward and reverse primer, 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). Amplification was carried out using iCycler™ (BIO-RAD, Japan). PCR mixture

was denaturized at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 49 °C for 30 s, 72 °C for 75 s, followed by post-amplification at 72 °C for 7 min following previous reports (Umehara et al. 2006, D'Amelio et al. 2000).

Digestion of PCR products with restriction enzymes. PCR-RFLP analysis was carried out in all specimens for species identification according to the reported genetic keys (D'Amelio et al. 2000, Pontes et al. 2005). The restriction enzymes used in the RFLP analysis of ITS rDNA were *Taq* I, *Hinf* I and *Hha* I (Takara Bio Inc., Japan). Manufacturer's recommendations were followed with regard to the digestion of PCR products. Finally, digested products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide and visualized by illumination with shortwave ultraviolet light.

DNA amplification and sequencing. For further confirmation of the identified *A. simplex* (s.s.) and *A. pegreffii* using PCR-RFLP analysis, the *mt*DNA *cox*2 region of three specimens representing each species was individually sequenced. The *mt*DNA *cox*2 gene was amplified using the primers 210 (5′-CACCAACTCTTAAAATTATC-3′) and 211 (5′-TTTCTAGTTATATAGATTGRTTYAT-3′) (Nadler and Hudspeth 2000). The PCR mixture was denatured at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 46 °C for 1 min, 72 °C for 1 min and 30 s, followed by post-amplification at 72 °C for 10 min following a previous report (Valentini et al. 2006). Nucleotide bases were sequenced using a DNA automatic sequencer (ABI Prism® 310 Genetic Analyzer, Applied Biosystems) after purification of the *mt*DNA *cox*2 gene. Nucleotide sequences were translated into amino acid sequences, compared and aligned with the reported *mt*DNA *cox*2 sequences of all previously studied *Anisakis* species (Valentini et al. 2006) using the blastn and blastp programs (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) for nucleotide and translated amino acid sequences, respectively. Nucleotide sequences were deposited and

made available in the GenBank database under accession numbers EU413958 and EU413959.

Morphological examination. For LM observation, excised anterior and posterior parts were cleared in glycerin and individually mounted on slides. The following morphological characters were measured and examined: total body length, maximum body width, distance of nerve ring to anterior end, oesophagus length, ventriculus length, ventriculus width, ratio between oesophagus and ventriculus length, tail length and mucro length. Relations between the morphological measurements and total body length were plotted on graphs for detailed comparisons between different species detected by PCR-RFLP analysis. All measurements were in millimetres.

SEM examination was performed in order to study other morphological structures of the anterior and posterior portion in detail, which were difficult to examine using LM. For SEM, 57 specimens (i.e., 36 *A. simplex* (s.s.) and 21 *A. pegreffii*) used for LM observation were processed following procedures in Subchapter 1 (Chapter 2).

Results

PCR-RFLP of rDNA. A total of 274 individual worms were examined. Species of all examined L3 larvae, L4 larvae and adults were identified using PCR-RFLP analysis. Based on the genetic keys (D'Amelio et al. 2000, Pontes et al. 2005), 76.5% of the examined worms from different host fishes caught from the Pacific side of Japan, which were represented by locality nos. 1, 2, 4, 6 and 7 in Fig. 24, were *A. simplex* (s.s.), while 98.6% of the examined worms from different host fishes caught from the Sea of Japan and East China Sea regions, which were represented by locality nos. 5, 8 and 9, were *A. pegreffii* (Table 15). Currently, since the number of examined fishes and worms in this study was relatively small, the species distribution between these two major *Anisakis* species is still not conclusive. Aside from these two major *Anisakis* species, there were

some specimens observed to have different PCR-RFLP fragment pattern after *Hinf* I digestion that was not included in the reported genetic keys (D'Amelio et al. 2000, Pontes et al. 2005). This fragment pattern was found similar to that of the previously reported hybrid genotype between *A. simplex* (s.s.) and *A. pegreffii* (Umehara et al. 2006, Abollo et al. 2003, Martín-Sánchez et al. 2005). As shown in Fig. 25, combinations of fragment patterns in both sibling species were found in hybrid genotype. ***mtDNA cox2 analysis.*** The translated amino acid sequences of *mt*DNA *cox*2 gene showed 100% similarities among the three sibling species of *A. simplex* complex (*A. simplex* (s.s.), *A. pegreffii* and *A. simplex* C), while using nucleotide sequences showed 99.0% and 98.2% similarities with the previously reported sequences of *A. simplex* (s.s.) (DQ116426) and *A. pegreffii* (DQ116428) (Valentini et al. 2006), respectively (Table 15). Comparison among other reported *mt*DNA *cox*2 sequences of *Anisakis* species showed 4.9–14.8% interspecies variations, which thereby confirms the species level of the examined worms. In the present study, no intra-species variation was found among the 3 specimens of *A. simplex* (s.s.) and *A. pegreffii*, while interspecies variation observed was 4.3%. The small sequence difference of the species examined in this study from those previously reported (Valentini et al. 2006) might be due to differences on geographical sources of the host fishes.

In vitro-culture. *Anisakis simplex* (s.s.) and *A. pegreffii* successfully reached the adult stage *in vitro*. Both parasites moulted from L3 to L4 stage between 3 and 4 days of culture. Moulting from L4 to adult stage was observed between 27 and 41 days for both species. Moulting from L3 to L4 and from L4 to adult stage occurred not only on the outer cuticle but also on the oesophageal linings. During the *in vitro*-culture period of 60 days, spicules of adult males were not observed.

Morphological differences. One distinguishing morphological character that can differentiate *A. simplex* (s.s.) from *A. pegreffii* was found during the examination of the

different morphological characters plotted in a graph in relation to the total body length of the worms. Among the morphological measurements examined from L3 to adult stages, clear difference was found in the ventriculus length (Fig. 26). As shown in Table 17 and Fig. 26, the ventriculus length did not increase as the worms grew in length and developed from L3 larvae to adults. In any life-history stages examined, the ventriculus length was shorter in *A. pegreffii* than in *A. simplex* (s.s), which ranged from 0.50 to 0.78 mm and 0.90 to 1.50 mm, respectively (Fig. 27). The shorter ventriculus in *A. pegreffii* reflected the ratio between oesophagus and ventriculus length, wherein *A. pegreffii* has higher oesophagus/ventriculus ratio (1:3.08–1:4.7) than those of *A. simplex* (s.s.) (1:1.9–1:2.8), with no overlapping ratios observed particularly during L4 and adult stages.

SEM examination revealed no morphological differences between *A. simplex* (s.s.) and *A. pegreffii* in L3 larvae collected from fishes and *in vitro*-cultured L4 larvae. Difference was only observed in adult males. Out of 36 specimens of *A. simplex* (s.s.) and 21 specimens of *A. pegreffii* examined under SEM, eight and 11 specimens of the former and the latter were males, wherein out of these males, only five and six specimens of the former and the latter were clearly analyzed. Following the nomenclature of papillae from previous reports (Mattiucci et al. 2005, Fagerholm 1988), the observed caudal papillae in all adult males examined are a median papilla, a pair of single proximal papilla, a pair of double procloacal papillae and four pairs of single distal papillae from the anus to the posterior end (Fig. 28 B). No caudal papillae were observed in females, but a pair of phasmids was observed on both male and female at the posterior most ends. Difference was observed in the distribution pattern of the caudal papillae, particularly in the location of the 3rd pair of distal papillae. In *A. simplex* (s.s.), the 3rd pair of distal papillae were located inside the 4th pair (Fig. 28 A, B), while in *A. pegreffii*, the 3rd pair were located outside the 4th pair (Fig. 28 C, D).

Discussion

The ventriculus length, oesophagus/ventriculus ratio and distribution pattern of caudal papillae, together with the body length and spicule length, are among the morphological characters in adults that were considered important in the diagnosis of anisakid nematodes and had been used specifically for species of *Anisakis* (Davey 1971, Mattiucci et al. 2005, Fagerholm 1988). Based on the morphological examination of *A. simplex* (s.s.) and *A. pegreffii*, which were molecularly identified using PCR-RFLP and *mt*DNA *cox*2 analyses, a clear difference was observed in the ventriculus length. As the difference was constant from L3 larvae to adults, the difference could be used as a major identification key for these two species. The ventriculus length had also been used as one criterion in differentiating *Anisakis* Type I larvae from *Anisakis* Type II larvae wherein the former has longer ventriculus length (0.65–1.5 mm) than the latter (0.52–0.75 mm) (Berland 1961, Koyama et al. 1969). Based on these ranges of ventriculus length in *Anisakis* Type I larvae, it is expected that these are mixed specimens of *A. simplex* (s.s.) and *A. pegreffii*, especially if examined closely based on the observed ventriculus length of the former (0.90–1.50 mm) and the latter (0.50–0.78 mm) in the present study. However, such ranges of ventriculus length in the reported *Anisakis* Type II larvae also falls within the range of ventriculus length in *A. pegreffii*, but both could be differentiated on the absence or presence of mucro. Apart from ventriculus length, morphological difference was also observed in the oesophagus/ventriculus ratio. Since no increase in ventriculus length was observed as the total body length increased slightly during *in vitro*-culture, major difference on the oesophagus/ventriculus ratio, without any overlapping ratios, was observed only from L4 to adult stages. The ventriculus length and oesophagus/ventriculus ratio has been reported to differentiate *A. brevispiculata* from *A. paggiae* (Mattiucci et al. 2005) and the present study shows that such morphological characters also applies in

differentiating *A. simplex* (s.s.) from *A. pegreffii*.

In addition to ventriculus length and oesophagus/ventriculus ratio, morphological difference was also found in the distribution pattern of the caudal papillae. SEM examination revealed a remarkable difference between adult male *A. simplex* (s.s.) and *A. pegreffii*, particularly in the position of the 3rd pair of distal papillae. The position of the 3rd pair of distal papillae in *A. simplex* (s.s.) was similar to that of *A. ziphidarum* (Paggi et al. 1998), while that of *A. pegreffii* was similar to that of *A. paggiae* (Mattiucci et al. 2005). However, *A. simplex* (s.s.) and *A. pegreffii* can be differentiated from *A. ziphidarum* and *A. paggiae* based on the difference on the locations of the 1st and 2nd pairs of distal papillae. There has been no reported distribution pattern of the caudal papillae in *A. pegreffii*. Even the original description also failed to describe it, since the specimen sent by Professor G. Pegreffi for examination was preserved in lacto-phenol, which resulted in a deterioration of the entire cuticle and complete destruction of the papillae (Campana-Rouget and Biocca 1955). The distribution pattern of the caudal papillae in *A. simplex* was previously reported, in which the number and origin of the specimens examined were not described though (Davey 1971) (Fig. 28 E, F). This previously reported distribution pattern in *A. simplex* was almost identical to that of *A. pegreffii* observed in the present study. The current findings created confusion as to whether the reported distribution pattern of the distal papillae of *A. simplex* (Davey 1971) was really that of *A. simplex* (s.s.) or rather that of *A. pegreffii* which was mistakenly identified as *A. simplex*.

In contrast to the above morphological characters that can be used to differentiate *A. simplex* (s.s.) from *A. pegreffii*, the spicules were not observed in adult males in the present study, which were obtained by *in vitro*-culture. In this regard, the reported overlapping spicule length between *A. simplex* and *A. pegreffii* (Davey 1971) was not confirmed. Matured adult females of *A. simplex*, which had begun ovoposition 97–143

days after *in vitro*-culture, have been previously reported (Iglesias et al. 2001), but any presence of spicules in adult males were not mentioned. It is possible that the worms in the present study were still young adults and required more time for their spicules to develop.

Based on the present results, specimens of *A. pegreffii* from fish and cetaceans in the Mediterranean should be re-examined. By doing so, *A. pegreffii* can be redescribed and characterized.

In conclusion, morphological characteristics distinguishing L3 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii* were found for the first time in this study. The finding makes the distinction between the two species much easier and will contribute in the epidemiological studies on human and fish anisakiasis.

Table 15

Table 16

Table 17

Fig. 24

Fig. 25

Fig. 26

Fig. 27

Fig. 28

Section 2. First report of two *Anisakis* species belonging to *Anisakis* type II larvae in Japan

Introduction

Conventional morphological studies of *Anisakis* species in the north-west Pacific region reported occurrence of both *Anisakis* Type I and *Anisakis* Type II larvae (Koyama et al. 1969, Moravec et al. 1985, Moravec and Nagasawa 1989), among which the specific species level have been determined molecularly as *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris* (Mattiucci et al. 1998, Abe et al. 2005, Mattiucci and Nascetti 2006, Umehara et al. 2006, 2008, Yoshinaga et al. 2006). *Anisakis simplex*, *Anisakis* sp. and *Anisakis* Type I larvae have been reported both in the body cavity and body muscle of Alaska pollock, which is a major raw material for surimi or fish paste from which various surimi products, such as imitation crab meat, fish balls and fish cakes, are produced for human consumption (Koyama et al. 1969, Oishi and Hiraoki 1971, Arthur et al. 1982). Given that potential allergens have been reported from *A. simplex* and *A. pegreffii* (Armentia et al. 1998, del Pozo et al. 1999, Daschner et al. 2000, Alonso-Gómez et al. 2004, Caballero and Moneo 2004, Nieuwenhuizen et al. 2006, Kobayashi et al. 2008), precise identification of *Anisakis* species is important from the viewpoint of food safety. On the basis of this background, we collected *Anisakis* L3 larvae from Alaska pollock and identified these morphologically and molecularly.

Materials and methods

Fifty Alaska pollock caught in northern Japan (Fig. 29) were examined for *Anisakis* infection. Briefly, a total of 210 L3 larvae were collected from the body cavity of the fish, removed from their sheaths, cleaned in phosphate-buffered saline, and fixed in 70% ethanol. After measurement of total body length, the worms were cut into three pieces: the anterior, middle and posterior parts. The anterior and posterior parts were

cleared in glycerol and morphologically identified based on type groupings (*Anisakis* Type I larvae or *Anisakis* Type II larvae) using LM. The middle part was stored in 100% ethanol for the extraction of genomic DNA. For preliminary species identification, PCR-RFLP analysis was performed on the ITS region (ITS1-5.8S rDNA-ITS2) in all collected L3 larvae following procedures in Section 1 (Subchapter1, Chapter 3). Species identification was carried out based on the resulting fragment patterns according to the genetic keys presented by D'Amelio et al. (2000) and Pontes et al. (2005). Species identity was finally confirmed by sequencing the ITS region and *mt*DNA *cox*2 gene of four representatives for each RFLP pattern obtained following previously described procedures (Nadler and Hudspeth 2000, Abe et al. 2005, Umehara et al. 2006, Valentini et al. 2006, see Section 1 (Subchapter 1, Chapter 3)). The percentage similarities of the nucleotide and amino acid sequences of the ITS region and *mt*DNA *cox*2 gene, respectively, were aligned and compared with the reported *Anisakis* species in the GenBank database using the blastn and blastp programs (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Only published sequences for each given species were selected from GenBank if various sequences were available, whereas even unpublished sequences were selected if limited sequences were available. Based on *p-distance* values for both DNA sequences, a phylogenic tree was inferred using the NJ method (Saitou and Nei 1987) of the MEGA 4.0 program (Tamura et al. 2007). The *mt*DNA *cox*2 and ITS rDNA sequences were deposited and made available in GenBank under the accession numbers EU560907–EU560911 and EU624342–EU624345, respectively.

Results

Anisakis Type I and *Anisakis* Type II larvae were morphologically identified based on the reported morphological features (Berland 1961, Koyama et al. 1969). In *Anisakis* Type I larvae, two of the three sibling species of *A. simplex* complex were distinguished morphologically as *A. simplex* (s.s.) and *A. pegreffii* through the diferrence on their ventriculus length as observed in Chapter 3 (Subchapter 1, section 1), In contrast, no morphological differences were found in species identification of those under the *Anisakis* Type II larvae. In this regard, molecular identification using PCR-RFLP was initially carried out. PCR-RFLP results revealed four different fragment patterns as shown in Fig. 30 and Table 18. Fragment patterns 1 and 2 were similar with the reported fragment patterns of *A. simplex* (s.s.) and *A. pegreffii*, whereas fragment pattern 3 and 4 have not been reported (D'Amelio et al. 2000, Pontes et al. 2005). Sequencing the ITS region of the four fragment patterns confirmed the PCR-RFLP results obtained from fragment patterns 1 and 2, wherein both nucleotide sequences showed 100% siilarities with *A. simplex* (s.s.) and *A. pegreffii*, respectively, whereas those with fragment pattern 3 showed 99.5% similarity with *A. brevispiculata* (Table 19). The sequence corresponding to fragment pattern 4 showed 99.9% similarity with one sequence reported in the GenBank as *A. physeteris* (AB201789) but showed considerably lower similarities (91.2%–91.8%) with five other sequences also reported for this species (GenBank Acc. Nos. EU327690, AY826721, EU327691, AY603530, and AB277821). On the other hand, in the *mt*DNA *cox*2 sequences, two close but different sequences were obtained from specimens with fragment pattern 1, whereas one identical sequence was obtained from specimens with fragment patterns 2 to 4. As shown in Table 20, the nucleotide sequences of *mt*DNA *cox*2 gene in fragment patterns 1 to 3 showed high similarities of 98–99%, 99.7% and 98.8% with those of *A. simplex* (s.s.), *A. pegreffii* and *A. brevispiculata*, respectively (Valentini et

al. 2006). The *mt*DNA *cox*2 nucleotide sequence of fragment pattern 4 only showed 95.8% with that of *A. paggiae* and similarities lower than 90% with other species including *A. physeteris*. The amino acid sequences of *mt*DNA *cox*2 showed considerably high similarities (96–100%), even between different species, and therefore was not used for species identification. Based on the molecular examination on the *mt*DNA *cox*2 gene and ITS region, the L3 larvae showing fragment patterns 1 to 3 were identified as *A. simplex* (s.s.), *A. pegreffii* and *A. brevispiculata*. Given that no control specimens belonging to identified *A. paggiae* can be used for both morphological and molecular comparison those specimens showing fragment pattern 4 were still unidentified *Anisakis* species that belong to *Anisakis* Type II groupings. The constructed NJ and MP trees, based on ITS (Fig. 31) and *mt*DNA *cox*2 (Fig. 32), support and confirm the above identification because the present sequences formed clear clades with those of previously reported species. Among these four different *Anisakis* species, the dominant species infecting Alaska pollock was *A. simplex* (s.s.) (91.0%), followed by *A. pegreffii* (5.2%), *Anisakis* sp. belonging to *Anisakis* Type II groupings (2.4%) and *A. brevispiculata* (1.4%). Both *A. simplex* (s.s.) and *A. pegreffii* were present in the Alaska pollock caught in Hokkaido and Iwate Prefectures, whereas *Anisakis* sp. and *A. brevispiculata* were only observed in the latter location (Fig. 29; Table 18).

Discussion

Current works on anisakid allergens, particularly those in the genus *Anisakis*, have been mainly reported in *A. simplex* complex, which is now molecularly confirmed to be comprised of three sibling species, namely *A. simplex* (s.s.), *A. pegreffii* and *A. simplex* C. Although reports on the presence of allergens is only available for *A. pegreffii*, the possibilities of presence of allergens in *A. simplex* (s.s.) cannot be disregarded. Precise species identification of *Anisakis* species, expecially in mixed infection, is very important

prior to consumption or processing of the infected fishes. In this study, the finding of dominant infection by *A. simplex* (s.s.) and *A. pegreffii* in Alaska pollock have to serve as a warning in possible production of surimi paste products unsuitable for public consumption, particularly to consumers who may be sensitive to such allergens.

Previously, *A. brevispiculata* have only been reported in the Atlantic region, which was reported in black scabbardfish, *Aphanopus carbo* (Trichiuridae) and European hake, *Merluccius merluccius* (Merluccidae) from the Azores Island, Iberian Atlantic coast and Mauritanian coast (Mattiucci and Nascetti 2006). The present study provides the first evidence on the presence of *A. brevispiculata* in the Pacific region. At present, an unknown *Anisakis* sp. belonging to *Anisakis* Type II grouping has been found. Although it is molecularly close to *A. paggiae* in its *mt*DNA *cox*2 gene, it is not 100% similar. Likewise, no ITS sequence of the *A. paggiae* is available for comparison. In this regard, the only way to verify its identity is through morphological and molecular comparison with fully identified *A. Paggaie.*

Alaska pollock was found to be infected with four different *Anisakis* species, two of which could be identified morphologically based on the ventriculus length. No overlapping of ventriculus length was observed in *A. simplex* (s.s.) (0.90–1.50 mm) and *A. pegreffii* (0.52–0.70 mm) (Fig. 33) which coincides with that observed in Section 1 (Subchapter 1, Chapter 3). In contrast, the ventriculus lengths in the L3 larvae of *A. brevispiculata* and *Anisakis* sp. overlapped with each other, making it impossible to morphologically distinguish these two species, although adults of *A. brevispiculata* and *A. paggiae* can be differentiated by their ventriculus length (Mattiucci et al. 2005), the former having a longer ventriculus (0.56–0.60 mm) than the latter (0.35–0.45 mm). Such mixed infections of L3 larvae have also been reported from different host fishes in the Atlantic Ocean and the Mediterranean Sea (Mattiucci et al. 2002, 2004, 2007, Pontes et al. 2005,

Farajallah et al. 2007, 2008), and mixed infections of adult worms have been found in the beaked whales *Ziphius cavirostris* and *Mesoplodon layardii* (Paggi et al. 1998).

The ITS sequence of AB201789 in GenBank was reported to have been taken from *A. physeteris*; however, it appears to be similar with the *Anisakis* sp. The similarity (95.8%) between the present *Anisakis* sp. and the *A. paggiae* reported from the West Atlantic Ocean (Florida coast) in *mt*DNA *cox*2 sequences was not high as expected compared with that of *A. simplex* (s.s.), *A. pegreffii* and *A. brevispiculata.* This may possibly indicate that this is either not that of *A. paggiae,* but the possibility of being same species cannot be disregarded, in which the differences may possibly be due to intraspecies variation which requires further examination using large sample size. Identification of the morphologically similar L3 larvae of *Anisakis* species is only possible through the use of molecular approaches, such as allozyme analysis, PCR-RFLP and sequencing of the ITS region and *mt*DNA *cox*2 gene (Mattiucci et al. 1998, Paggi et al. 1998, Mattiucci et al. 2002, Mattiucci et al. 2005, Mattiucci and Nascetti 2006, Valentini et al. 2006).

Table 18

Table 19

Table 20

Table 20, cont

Fig. 29

Fig. 30

Fig. 31

Fig. 32

Fig. 33

Section 3. *Molecular and morphological comparisons between Far East and Mediterranean Anisakis pegreffii*

Introduction

A case of *Anisakis* infection was reported in cultured greater amberjack, *Seriola dumerili*, in Japan, which had been infected in China and imported to Japan for aquaculture production (Yoshinaga et al. 2006). Considering the two base difference found in the 5.8S rDNA, when compared with that of *A. pegreffii* (Genbank Acc. No. AY826720) from the Mediterranean, the species collected from greater amberjack was tentatively referred to as *A. pegreffii* JP (Yoshinaga et al. 2006) in order to distinguish it from the Mediterranean *A. pegreffii*. Furthermore, other *A. pegreffii* collected in the Far East (EU624343, AB277823, AB196670, AB196671) had the same sequence with *A. pegreffii* JP with the same difference from the Mediterranean specimen (AY826720) in 5.8S rDNA (Abe et al. 2005, Umehara et al. 2008, see Section 1 and 2 (Subchapter 1, Chapter 3)), except one *A. pegreffii* reported from China (AM706346) (Zhu et al. 2007) which has three base difference in the ITS1 and one base difference in the ITS2 region. To date, as detailed comparisons have not been carried out for *A. pegreffii* in the Far East and in Europe, the taxonomy of *A. pegreffii* JP still remains unconfirmed. In order to clarify the taxonomical relations of *A. pegreffii* from the two localities, morphological and molecular examinations, using specimens from the Mediterranean and specimens collected from greater amberjack imported from China, were carried out. Additionally, they were also compared morphologically and molecularly with *A. pegreffii* and other *Anisakis* species previously reported from Mediterranean, Far East and Atlantic regions.

Materials and methods

Specimens used were ethanol-fixed L3 larvae of *A. pegreffii* (n=60) and *A. pegreffii* JP (n=40) collected from Mediterranean and in greater amberjack imported from

China to Japan, respectively (Fig. 34). The latter were subsamples of *A. pegreffii*, referred to *A. pegreffii* JP by Yoshinaga et al. (2006). For morphological and molecular examinations, the procedures described in Section 1 (Subchapter 1, Chapter 3) was followed. Molecular analyses were carried out using PCR-RFLP of the ITS region (ITS1-5.8S rDNA-ITS2) for all samples and sequencing of the ITS region and *mt*DNA *cox*2 gene of three specimens each from Mediterranean and China following previous studies (D'Amelio et al. 2000, Pontes et al. 2005, Valentini et al. 2006, Umehara et al. 2006). Morphologically, the relation between total length and ventriculus length was compared using data obtained from the present samples from Mediterranean and China and from previous data of other *Anisakis* species (Yoshinaga et al. 2006, see Section 1 and 2 (Subchapter 1, Chapter 3)). As only the ITS sequence was available for the reported *A. pegreffii* JP (Yoshinaga et al. 2006), both ITS region and *mt*DNA *cox*2 gene of the present samples were sequenced. The percentage sequence similarities in *mt*DNA *cox*2 gene were aligned and compared among present samples and previously reported *Anisakis* species (Kim et al. 2006, Valentini et al. 2006) in GenBank database using blastn and blastp programs (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Based on *p-distance* values in *mt*DNA *cox*2 gene, analysis of the phylogenic tree inferred using NJ method (Saitou and Nei 1987) of MEGA 4.0 program (Tamura et al. 2007) was carried out following procedures in Section 1 and 2 (Subchapter 1, Chapter 3). The *mt*DNA *cox*2 and ITS rDNA sequences were deposited and made available in GenBank under the accession numbers EU933994-EU933996 and EU933997-EU933998, respectively.

Results

Morphologically, the currently examined specimens of *A. pegreffii* from Mediterranean and *A. pegreffii* JP from China had ventriculus length of 0.50–0.78 mm (mean±SD) (0.61±0.07 mm) and 0.55-0.80 mm (0.69±0.06 mm), respectively, which

corresponded with *A. pegreffii* (0.50–0.78 mm; mean 0.64±0.07 mm) obtained from Alaska pollock, Japanese Spanish mackerel, chub mackerel, greater amberjack and Pacific cod in Japan (see Section 1 and 2 (Subchapter 1, Chapter 3) and *A. pegreffii* JP from China (0.57–0.83 mm; mean 0.67 mm) (Yoshinaga et al. 2006) (Fig. 35). Although total body length overlapped among those from China, Japan and Mediterranean, *A. pegreffii* from Japan were generally shorter and *A. pegreffii* JP from China were longer, whereas *A. pegreffii* from Mediterranean measured in-between lengths.

In a molecular level, the yielded PCR-RFLP fragment pattern of the present samples from Mediterranean and China corresponded with the reported pattern of *A. pegreffii* (D'Amelio et al. 2000, Pontes et al. 2005) and with that obtained in Section 1 and 2 (Subchapter 1, Chapter 3). Sequence data showed only two base difference in the ITS 1 region between *A. simplex* (s.s.) and *A. pegreffii* as previously reported (Umehara et al. 2008). Similarity of 100% was obtained in the ITS and 5.8S rDNA sequences among the present samples from Mediterranean and China and from previously reported *A. pegreffii* from the Far East (EU624343, AB277823, AB196670, AB196671), whereas two base difference in the 5.8S rDNA gene were found when these samples were compared with the *A. pegreffii* accession AY826720 previously reported from the Mediterranean.

The nucleotide sequences of *mt*DNA *cox*2 gene were used in the analysis as the translated amino acid sequences of the gene yielded nearly 100% similarities among the three sibling species of *A. simplex* complex. Sequences in *mt*DNA *cox*2 gene were more variable than the ITS region between *A. simplex* (s.s.) and *A. pegreffii*, having 95.7–96.1% similarities and 21–23 bases difference (Table 21). Two different *A. pegreffii* sequences were obtained from three specimens sequenced from Mediterranean samples, showing three nucleotide base difference. After molecular comparison, 97.4–99.8% similarities were obtained among *A. pegreffii* JP from China (EU933994) and *A. pegreffii* from

Mediterranean (EU933995 and EU933996), Japan (EU560908 and EU413958) (see Section 1 and 2 (Subchapter 1, Chapter 3)) and Atlantic (DQ116428) (Valentini et al. 2006). In addition, the sequence of *A. simplex* from South Korea (AY994157) (Kim et al. 2006) was found to be 97.8–99.8% similar to the above sequences of *A. pegreffii*, suggesting that this is probably *A. pegreffii* rather than its co-sibling species *A. simplex* (s.s.). Although intraspecific variations of 0.2–2.6% were found among sequences of *A. pegrefii* from the Far East regions, including that of *A. simplex* reported by Kim et al. (2006), and Atlantic and Mediterranean regions, no geographical differences were detected (Fig. 36).

Discussion

L3 larvae of *Anisakis* could not be identified morphologically to species level until the morphological difference has been clarified in the ventriculus length between larvae of *A. simplex* (s.s.) and *A. pegreffii* (see Section 1 (Subchapter 1, Chapter 3)). In the present study, little difference was found in the ventriculus length among present specimens of *A. pegreffii* from Mediterranean and China, from the examined *A. pegreffii* from Japan (see Section 1 and 2 (Subchapter 1, Chapter 3)) and from previously reported *A. pegreffii* JP from China (Yoshinaga et al. 2006). The small differences observed in the total body length of *Anisakis* from China, Japan and Mediterranean could possibly be due to different factors such as host species and host age, in which the current study was unable to attend. Molecular results support the morphological findings. All 5.8S rDNA sequences of *A. pegreffii* from present specimens collected in the Mediterranean and China, and previous specimens collected in the Far East, were identical except that the previously reported sequence of *A. pegreffii* from Mediterranean (AY826720) only showed two base difference from the other specimens. Considering that the 5.8S rDNA is generally well preserved and an unknown base separated the two different bases in AY826720, there

could be some error in that position of the sequence. The absence of substantial difference between *A. pegreffii* in the Mediterranean and *A. pegreffii* JP in the Far East clarified that *A. pegreffii* JP is morphologically and molecularly indistinguishable from other *A. pegreffii*. As *A. pegreffii* from Japan (EU560908) shared the same clade in *mt*DNA *cox*2 with *A. pegreffii* from the Atlantic and Mediterranean in the phylogenetic tree, no geographical difference was detected in the present study. However, the geographical difference should be fully verified by examining large number of samples and examining more variant sequences.

Table21

Table21, cont

Fig. 34

Fig. 35

Fig. 36

Section 4. *Anisakis distribution in Japanese waters*

Introduction

Anisakis species had been reported widely in Japanese waters as stated in Section 1 and 2 (see Subchapter 1, Chapter 3). It was revealed that *A. simplex* (s.s.) and *A. pegreffii* were mostly distributed in northern and southern waters in Japan, respectively (Abe et al. 2005, Umehara et al. 2006, 2008). However, previous identifification of *Anisakis* species in Japanese waters were carried out mainly through morphological examination. Moreover, with the distinguishing morphological differences found in L3 larvae in this study (see Section 1, Subchapter 1, Chapter 3), the identification of *Anisakis* species became faster, reliable and accurate. With the zoonotic implication of anisakid nematodes, including those from this genus *Anisakis*, it is worthwhile to know the distribution of *Anisakis* species in Japanese waters. Such information will be very useful for government agencies responsible for human food safety in setting up future guidelines in preventing further incidence of anisakiasis. In this regard, all available information from other researchers on the distribution of examined *Anisakis* species in Japan, as well as those obtained in this study, were combined to have a bird's eyeview on their distribution.

Materials and methods

The data used in this section came from the present results of the taxonomical studies (see Section 1 and 2, Subchapter 1, Chapter 3) and data gathered from available literatures (Abe et al. 2005, Umehara et al. 2006, 2008). Also, other host fishes were examined for this study (Table 22); the flathead flounder (*Hippoglossoides dubius* Schmidt) and sailfin sandfish (*Arctoscopus japonicus* (Steindachner)) from off Fukui Prefecture, a puffer (*Takifugu poecilonotus* (Temminck et Schlegel)) from off Shimanoseki, Yamaguchi Prefecture; *Theragra chalcogramma* from off Rausu, Hokkaido Prefecture, *Gadus macrocephalus* from off Iwate Prefecture, *Scomber japonicus* from off

Nagasaki Prefecture and *Scomber australascicus* from off Nishinoomote, Kagoshima Prefecture . The former two fish species were collected in off Fukui Prefecture and the latter one fish species was collected off Yamaguchi Prefecture, Japan. A total of 19 flathead flounders, 18 sailfin sandfishes and six puffer were examined. Identification of the collected L3 larvae up to species level was carried out morphologically, through ventriculus length examination, and molecularly, through PCR-RFLP, as previously described in Section 1, 2 and 3 (see Subchapter1, Chapter 3).

Results

Four different *Anisakis* species have been found in Japanese waters, namely *A. simplex* (s.s.), *A. pegreffii*, *A. brevispiculata* and *A. physeteris*. *Anisakis* sp. belonging to *Anisakis* Type II grouping has been found. Molecular examination on the *mt*DNA *cox*2 gene showed only 95.8% similar with *A. paggiae*. Given that no ITS sequence of *A. paggiae* can be used for comparison and no adult specimens of these *Anisakis* sp. is available for morphological comparison, the identity of these *Anisakis* sp. remains unknown. *Anisakis* sp. cannot be concluded to be similar with *A. paggiae* since 100% similarities were not obtained in the *mt*DNA *cox*2 gene (see Chapter 3, Subchapter 1, Section 2). Hybrid genotypes of *A. simplex* (s.s.) and *A. pegreffii*, which were also reported in Europe, were also found in Japanese waters. Based on the data examined, *A. simplex* (s.s.) was the most dominant species (68.9%), followed by *A. pegreffii* (28.4%), hybrid genotype (1.6%), *Anisakis* sp. (0.59%), *A. brevispiculata* (0.35%) and *A. physeteris* (0.12%) (Table 22). Plotting the distribution of these *Anisakis* species revealed interesting result. There is a clear distribution on the localities between the two sibling species of *A. simplex* complex, regardless of host fishes. *Anisakis simplex* (s.s.) are mainly found in fishes examined in northern Japan in Hokkaido, as well as in all fishes examined in the Pacific side, whereas *A. pegreffii* are mainly found in fishes examined in the Sea of Japan

and East China Sea sides. The hybrid genotype were found in the northern and southern part of Japan. It seemed that the distribution of *Anisakis* species depend on the localities, not on fish species. *Anisakis* species included in the *Anisakis* Type II groupings (*A. brevispiculata*, *A. physeteris* and *Anisakis* sp.) were found in off Iwate and Kyushu area at low ratios (Fig. 37).

Discussion

The combined data from this present study, and with those previously reported, provided us with an idea on the distribution of different *Anisakis* species in Japanese waters, particularly between the two dominant species, the *A. simplex* (s.s.) and *A. pegreffii*. Species distribution revealed to be site specific and not host specific as *A. simplex* (s.s.) was mostly located in Hokkaido and Pacific sides, whereas *A. pegreffii* was mostly found in host located in the Sea of Japan and East China Sea. Majority of fishes in Hokkaido were infected with *A. simplex* (s.s.), the species that are commonly recovered from patients with anisakiasis. This might possibly explain the high incidence of anisakiasis in this region. Based on our result on the *Anisakis* distribution, fishes examined in Kyushu area were mostly infected with *A. pegreffii*. However, patients with anisakiasis (80 persons) in Kyushu area were found infected with *A. simplex* (s.s.) while only 1 patient was infected by *A. pegreffii* (Umehara et al. 2007). One possibility for this is that *A. pegreffii* might be less pathogenic to human than *A. simplex* (s.s.). Other possibility is that *A. pegreffii* could not reach to body muscle of fishes as shown in our experimental challenge (see Subchapter 2, Chapter 3). In this regard, chances of ingestion of *A. pegreffii* by human may be much lower compared to *A. simplex* (s.s.). Since the location of the source of the consumed fish infected with *A. simplex* (s.s.) were unknown, it was possible that these fishes came from Hokkaido and/or Pacific side. However, this possibility cannot still be disregarded based on the result of the distribution of *A. simplex* (s.s.) and *A. pegreffii* in Japan. Given that

Japanese people relish most seafood dishes uncooked such as sushi and sashimi, continuous accummulation of data on *Anisakis* distribution in Japanese waters among different research works would be very helpful in tracking how this relates directly or indirectly to the incidence of human anisakiasis in Japan. This information can be of help in setting guidelines for the government offices responsible for human food safety.

Table 22

Table 22, cont.

Table 22, cont.

Fig. 37

SUBCHAPTER 2. EXPERIMENTAL INFECTION STUDIES

Experimental infection of rainbow trout, Oncorhynchus mykiss, and Japanese flounder, Paralichthys olivaceus, with Anisakis simplex (s.s.) and Anisakis pegreffii

Introduction

Anisakis simplex is the only *Anisakis* species known to infect body cavities and body muscle of fish belonging to the Gadidae, Salmonidae and Clupeidae families (Arthur et al. 1982, Cattan and Carvajal 1984, Smith 1984, Strømnes and Andersen 1998). Currently, the host specificity and site of infection of *A. simplex* (s.s.) and *A. pegreffii* are largely unknown. The presence of larval anisakids in the body muscle of the host fishes presents potential zoonotic problems (Margolis 1977). Despite this, little is known about how and when *Anisakis* larvae enter the body cavity and migrate into the body muscle. Past studies have evaluated the migration of *A. simplex* and unidentified *Anisakis* larvae into the body muscle of both live and dead fishes (Smith and Wootten 1975, Wootten and Smith 1975, Arthur et al. 1982, Cattan and Carvajal 1984, Smith 1984, Santamarina et al. 1994, Køie 2001). However, these studies did not identify the larvae to the level of the sibling species. Only recently, Abollo et al. (2001) reported the presence of *A. simplex* (s.s.) and *A. pegreffii* in the body cavity and body muscle of European hake (*Merluccius merluccius* (Linnaeus)), blue whiting (*Micromesistius poutassou* (Risso)), angler (*Lophius piscatorius* Linnaeus), Atlantic mackerel (*Scomber scomber* Linnaeus) and Atlantic horse mackerel (*Trachurus trachurus* Linnaeus). However, it was not specified whether *A. simplex* (s.s.), *A. pegreffii*, or both were present in the body muscle. Considering the zoonotic implication of *Anisakis* infection, it would be interesting to determine whether there is any difference in the site specificity of *A. simplex* (s.s.) and *A. pegreffii*, particularly with regard to the body muscle of live fish. In this study, rainbow trout, *Oncorhynchus mykiss* (Walbaum), and Japanese flounder, *Paralichthys olivaceus*

(Temminck et Schlegel), were orally infected with L3 larvae of *A. simplex* (s.s.) and *A. pegreffii* to evaluate the differences in the degree of infection, the site of infection and the host response between the two *Anisakis* species.

Materials and methods

Rainbow trout (average body weight [ABW] 472 g) and Japanese flounder (ABW 352 g) were individually confined and acclimatized in net cages and placed in re-circulating freshwater and saltwater tanks at 12 ˚C and 20 ˚C, respectively. L3 larvae of *A. simplex* (s.s.) were collected from Pacific cod caught off Hokkaido in the Pacific. *Anisakis pegreffii* larvae were collected from chub mackerel caught off Kumamoto Prefecture in the East China Sea. Different batches of the two *Anisakis* species collected from different batches were used in the experiment. The L3 larvae were removed from their sheath and cleaned in phosphate buffered saline. A preliminary identification of *A. simplex* (s.s.) and *A. pegreffii* was made based on the differences in ventriculus length (see Section 1 (Subchapter 1, Chapter 3). Since the *in vitro*-cultured L3 larvae used in Section 1 (Subchapter 1, Chapter 3) were found viable from five to six days storage in PBS at 4°C prior to *in vitro*-culture and reached adult stage, the collected worms that were maintained only for three days in the same condition were assured to be viable prior to oral infection. Undamaged *A. simplex* (s.s.) and *A. pegreffii* individuals were then inserted into a hole in the middle of a commercial feed pellet (Fig. 38 A). The larvae were secured in place by covering with softened pellets. A total of 36 rainbow trout were orally challenged with either *A. simplex* (s.s.) (n=18, experiment 1) or *A. pegreffii* (n=18, experiment 2), whereas a total of 48 Japanese flounder were orally challenged with either *A. simplex* (s.s.) (n=18, experiment 1) or *A. pegreffii* (n=30, experiment 2). Immediately after the pellets were prepared, the fish were fed, one pellet at a time, until a total of 20 *A. simplex* (s.s.) or 40 *A. pegreffii*, and 15 *A. simplex* (s.s.) or 50 *A. pegreffii* were ingested by each individual

rainbow trout and Japanese flounder, respectively, in each experimental group. For rainbow trout, three fish from each experimental group were sampled at 3, 7, 14, 21, 28 and 35 days postinfection (dpi) and the number of larvae recovered, condition of the larvae (i.e., dead or alive), and sites of infection were recorded. For Japanese flounder, three and five fish, respectively, were sampled in the experiments for *A. simplex* (s.s.) and *A. pegreffii* on a similar sampling intervals at the same time. Similar parameters observed in rainbow trout experiment were also observed in Japanese flounder. Both fishes were dissected and larvae were collected and counted by site of infection, in which the sites of infection were categorized to the lumen of the stomach, pyloric caeca, intestine, in the walls of the stomach and intestine, the mesentery and serosa surrounding visceral organs and tissues, and empty space in the body cavity. The body muscle was thinly sliced, pressed between two flat glass plates, and observed under a stereomicroscope using a bright light box to locate larvae. All larvae were removed and fixed in 70% ethanol. All recovered specimens from each experimental group in both fishes were identified to species level using PCR-RFLP, and three representative specimens in each experimental group were further analyzed by sequencing the ITS region (ITS1-5.8S rDNA-ITS2) and *mt*DNA *cox*2 gene following the methods of D'Amelio et al. (2000) and the procedures used in Section 1, 2 and 3 (Subchapter 1, Chapter 3). In addition, we conducted a histological examination of the infected organs from fish challenged with *A. simplex* (s.s.) to determine any host reactions.

Results

The fragment patterns, analyzed by PCR-RFLP, of all the larvae recovered from the two experimental groups in both fishes were similar to those reported from *A. simplex* (s.s.) and *A. pegreffii* (D'Amelio et al. 2000, see Section 1, 2 and 3 (Subchapter 1, Chapter 3)). Sequencing of the ITS rDNA and *mt*DNA *cox*2 regions of the three representative

specimens confirmed that the species used in the two experimental groups were *A. simplex* (s.s.) and *A. pegreffii*, respectively.

Rainbow trout. The recovery of *A. simplex* (s.s.) is summarized in Table 23. All L3 larvae of *A. simplex* (s.s.) were alive at the time they were collected. L3 larvae were found in the lumen of the stomach, the intestine and the pyloric caeca between 3 and 14 dpi, although the total number decreased over time. Similarly, larvae in the body cavity were found between 3 and 35 dpi, and in the body muscle between 7 and 35 dpi. Many of the larvae found in the body cavity were recovered from the mesentery surrounding the pyloric caeca. Infection in the body cavity was greatest between 7 and 28 dpi. None of the larvae found in the body cavity were encapsulated. The ratio of number of larvae in the body muscle to those larvae in the body cavity increased steadily, suggesting that *A. simplex* (s.s.) larvae were migrating from the body cavity into the body muscle. Some haemorrhaging and accumulation of inflammatory cells in the infected mucosal and submucosal layers of the stomach were observed (Fig. 38 B). In addition, we observed one incidence of melanization in a histological sample taken from an open wound in the stomach wall 7 dpi, after the larvae had migrated into the body cavity (Fig. 38 C). Inflammatory cells were observed amongst the lysed and broken-down muscle fibres with the region of body muscle that was infected by *A. simplex* (s.s.) (Fig. 38 D). No evidence of encapsulation was found around the larvae.

On the other hand both live and dead larvae of *A. pegreffii* were recovered from rainbow trout (Table 24). All the larvae recovered from the gastro-intestinal lumen were alive 3 dpi. In contrast, the majority were dead between 7 and 21 dpi. All recovered larvae from the body cavity between 3 and 28 dpi were alive and unencapsulated, but few in number. The total number of recovered larvae decreased over time, with no larvae being found 35 dpi. Evidence of *A. pegreffii* larvae in the body muscle or remaining organs of the

host fish was not found.

Japanese flounder. The recovery of *A. simplex* (s.s.) and *A. pegreffii* is summarized in Table 25 and 26, respectively. L3 larvae of *A. simplex* (s.s.) were recovered from the body cavity and body muscles from 3 dpi onward, whereas no larvae were recovered from the gastro-intestinal tract. Migration of larvae into the body muscle was observed as early as 3 dpi (22%), wherein the recovered larvae was highest at 35 dpi (51%). Recovered larvae in the body cavity were not encapsulated. In the infected muscles, leukocycte infiltration was also observed surrounding the broken muscle fibres and the migrated larvae.

On the other hand, similar results of the *A. pegreffii* infection obtained in the rainbow trout experiment were also obtained in Japanese flounder experiment for *A. pegreffii* infection wherein L3 larvae did not migrated into the body muscle. Instead, the *A. pegreffii* larvae were recovered in the body cavity, encycted in lumps of 4-15 worms per lump. All recovered larvae from 3 dpi onwards were all alive.

Discussion

Previous studies have experimentally infected fish with *A. simplex* and unidentified *Anisakis* species larvae using a variety of methods, including direct feeding, intraperitoneal insertion and intubation. The postinfection recovery of larvae was variable in each of these studies (75%, 74% and 27%, respectively) (Wootten and Smith 1975, Santamarina et al. 1994, Køie 2001). In the present study, 100% and 78% of *A. simplex* (s.s.) were recovered within the first 7 dpi following the oral administration in rainbow trout and Japanese flounder, respectively, using food pellets as a carrier. Our results suggest that this is an effective method for exposure to the larvae.

Undamaged *A. simplex* (s.s.) and *A. pegreffii* placed in PBS at 4°C for five to six days prior to *in vitro* culture (Iglesias et al 2001, see Section 1 (Subchapter1, Chapter3))

developed from L3 to adult stage indicating that prolonged storage of L3 in PBS at this temperature does not affect the viability of the L3 worms. In this regard, the viability of *A. simplex* (s.s.) and *A. pegreffii* stored for 3 days prior to oral infection was not affected as previously discussed. Also, since some *A. pegreffii* successfully migrated from the gatrointestinal lumen to the body cavity, this is already a good indication that the *A. pegreffii* used in this study were viable.

In this study, the migration of *A. simplex* (s.s.) into the body muscle of rainbow trout and Japanese flounder was observed. In contrast, *A. pegreffii* did not migrate to the body muscle. Since viability of *A. pegreffii* used cannot be questioned as discussed, the migration difference between *A. simplex* (s.s.) and *A. pegreffii* in the body muscle of both fishes is possibly be due to host specificity or the nature of the individual *Anisakis* species.

The high recovery of live *A. simplex* (s.s.) from the lumen, body cavity and body muscle suggests that both rainbow trout and Japanese flounder are suitable hosts for *A. simplex* (s.s.) infection. This conclusion is supported by previous reports that *A. simplex* (s.s.) are commonly observed in salmonid fishes (Bouree et al. 1995, Inoue et al. 2000), as well as in flat fishes American plaice, *Hippoglossoides platessoides* (Fabricius), and witch flounder, *Glyptocephalus cynoglossus* (Linnaeus) (Templeman et al. 1957). It is not clear whether the larvae will persist in the body muscle for more than 35 days. However, larval nematodes are known to reside in the body muscle for at least a year, and probably longer (Bishop and Margolis 1955). Based on the presence of larvae in the stomach, intestinal wall, pyloric caeca and the surrounding mesentery, we hypothesize that both *Anisakis* species migrate through these organs into the body cavity. However, the non-observance of *A. simplex* (s.s.) and *A. pegreffii* in the gastro-intestinal tracts at 3 dpi in the Japanese flounder experiment was possibly due to high rearing temperature (20 ^{o}C) compared to that in rainbow trout experiment (12 ^{o}C). It is well known that the site specificity of *A.*

simplex L3 larvae differs among host fish species. For example, salmonid fishes are primarily infected in the body muscle, herring (*Clupea harrengus harrengus* Linnaeus), Japanese anchovy (*Engrauluris japonicus* Temminck et Schlegel) and Alaska pollock are mainly infected both in the body cavity and body muscle, and most other fishes are only infected within the body cavity (Smith and Wootten 1975, Novotny and Uzmann 1960, Arthur et al. 1982, Abollo et al. 2001). The mechanism for this site specificity is poorly understood.

The experimental infections carried out by Køie (2001) in Pacific cod challenged with L3 larvae of *A. simplex* collected from the viscera of herring indicated that about one-third of the ingested larvae passed through the Pacific cod's alimentary tract and were extruded through the anus. Similarly, we recorded very low recovery on *A. pegreffii* postinfection in both rainbow trout and Japanese flounder. Many of the larvae may have passed through the alimentary tract. This may also explain the missing *A. simplex* (s.s.) that should be recovered after the oral infection experiment. The few *A. pegreffii* that successfully migrated into the body cavity were unable to migrate further into the body muscle of rainbow trout and Japanese flounder. This may be related to the water temperature as *A. pegreffii* typically infect fish that are found in warmer waters such as east/southeast China, the Sea of Japan and the Mediterranean (Abe et al. 2005, Mattiucci and Nascetti 2006, Umehara et al. 2006, Zhu et al. 2007, Umehara et al. 2008, see Section 1 and 2 (Subchapter 1, Chapter 3)). Alternatively, rainbow trout and Japanese flounder may be an unfavorable host for *A. pegreffii*. To our knowledge, *A. pegreffii* is rarely recovered from fish body muscle, which may be one characteristic of this species.

The haemorrhaging and accumulation of inflammatory cells in the submucosal and mucosal layers of the stomach found in rainbow trout infected by *A. simplex* (s.s.) is similar to other host responses observed following infection by other parasites. The lysis

and degradation of muscle fibres within the infected body muscle is likely due to mechanical damage, caused by migration of the larvae into the muscle and/or substances excreted by the larvae. The excretory secretory products might also influence the pathology associated with the infection (Raybourne et al. 1986). Audicana et al. (1995) reported no evidence of inflammation in the body muscle of European hake infected with *A. simplex* (s.s.) differently from our result. In our study, the presence of inflammatory cells around the larvae may have been induced by the presence of lysed and degenerative muscle fibres. Following the removal of this tissue, the immune system response may have been down-regulated thus explaining the absence of a response as observed by Audicana et al. (1995).

Table 23

Table 24

Table 25

Table 26

Figure 38

CHAPTER 4. GENERAL DISCUSSION

STUDIES ON PHILOMETRIDS

Current taxonomical and biological information of muscle-infecting *Philometroides seriolae* and gonad-infecting *Philometra* species remains limited. With the economic significance of these nematodes to fish production, understanding these basic information will help in future predictions of parasite infection and in formulating possible preventive measures againsts such infection. Although the results obtained in this study is not comprehensive, the data obtained can contribute to the existing biological information of this group of philometrids and can be served as a foundation of possible future studies that can be explored. This study was focused on two major aspects, the taxonomical and biological studies, wherein the former employed both morphological and molecular approaches for species identification, whereas the latter focused specifically in the infection of the worms to their respective host fishes.

We found the importance of finding males, usage of SEM and application of molecular approaches for precise species identification. Through this, I was able to identify 3 new *Philometra* species, redescribed 3 species and confirmed the validity of 1 species, in which removal from its current status *species inquirenda* has been suggested. In this regard, males of other *Philometra* species, particularly those same species reported from different hosts, should be discovered and females should be re-examined. SEM and molecular approaches should also be employed in many other species in order to re-evaluate the taxonomical classification of this group as suggested by Moravec (2004). At present, the discovery of male *P. lateolabracis* and re-description of its female counterpart from its type host served as a guide in raising new species, validating species and redescribing species that had previously identified as *P. lateolabracis* (Quiazon et al. 2008b, Moravec 2008, Moravec and Justine 2008, Moravec et al.

2008b,c,d). Further molecular studies should also be carried out using other genetic regions. In doing so, the obtained phylogenic relationships in this study, based on 18S and ITS2 regions, can be confirmed. Molecular examination also of other reported species worldwide would be helpful in constructing the phylogenic relationship of this group, thereby providing possible links on how this group had evolved and how it relates to host specificity or host evolution.

Host specificity was observed among the philometrid nematodes examined. With this, different questions aroused for further studies such as; a) what is the mechanisms behind host specificity?; b) is the host specificity due to host evolution long time ago during the evolution period or is it just due to accidental or random infection to a given species and evolves as time passes by?. As host specificity was found, further examination should be carried out in wide varieties of fish species. If host specificity is also found, this can possibly be employed or used for fish species identification and/or fish stock assessment studies.

Although seasonality of infection of gonad-infecting *Philometra* species was only observed in detail in *P. sciaenae* and *P. madai*, it seems that there is a clear relationship between the prevalence and intensity of gonad-infecting gravid female *Philometra* species with their host's spawning season. With this, the factors or mechanisms influencing such relationship between host and worm's maturity can also be a good topic for future studies. Bashirullah and Adams (1983) reported the effect of hormone in the maturation of sockeye salmon, *Oncorhynchus nerka* (Walbaum), and the parasitic nematode *Philonema oncorhynchi* infecting the host coelomic cavity. It is also possible that some released hormones responsible for the maturity of host's gametes can influence the maturity of the currently examined gonad-infecting *Philometra* species. In Japan, high intensity of *Philometra* has been reported in the gonads of red seabream reared in an

aquaculture facility (Sakaguchi et al. 1987). Given that the maturity of this fish can be easily manipulated under laboratory condition, this fish can be used as a possible candidate fish to confirm such connection between the host and worm's maturation under laboratory condition. In the present study, the effect of these gonad-infecting *Philometra* species on the fecundity and quality of gamete production was not examined. It is also interesting to find out if these worms affect fish reproduction by using red seabream as a model fish.

Only host response was examined in the infection of the *Philometra* species. Although host response was observed in some live worms in the host's ovaries, there was a great difference on the intensity of host response in silver croaker and red seabream against *P. sciaenae* and *P. madai*, respectively. In the former host, no or minimal host response was observed, whereas in the latter host, very intense host response was observed. There are other questions left unclear that could be an interesting topics for future studies such as: a) why such differences in the host response occurred?; b) is such difference in host response due to the species of the host or worms?; and c) is *P. madai* more pathogenic than *P. sciaenae*?. With the accumulation of leukocytes around the site of infection, it is also interesting to study the immunological aspects of such infection. Among the questions that currently arises are: a) what types of leukocytes are present?; and b) does the fish develop immunity after infection?.

For *Philometroides seriolae* infection in Japanese amberjack, no seasonality was found. Based on the gathered data, more work has to be done especially in understanding the infection dynamics and life-cycle of the parasite. It is also worth studying when and how infection commences.

Natural healing of affected muscle tissues was found. Such occurrence is good news to fish growers who tend to sell their product for consumption in raw form.

However, there were many major questions that still need to be answered such as: a) how long does it take to have complete healing after the worms die?; b) how long is the life-span or until how long does this worm stay alive in the body muscle?; and c) how long does it take for the dead worms to be completely removed from the host body muscle?. Same with *Philometra* species, it is also interesting to know the immunological aspect of Japanese amberjack against *Philometroides seriolae*. Among the things that can be studied is the possible development of immunity against *Philometroides seriolae.* Also, another very important topic that would greatly and directly benefit final stakeholders is on the aspect of prevention and control, which up to present remains unknown. It would be good to explore the economic feasibility and viability of currently employed preventive and treatment measures in other aquatic organisms such as the use of vaccine, immunostimulant, drugs or manipulation of environmental parameters.

Presence of worms under the skin were observed in small number than those present in the body muscle. Further studies on this aspect may clarify the following: a) the mode of releasing hatched larvae; b) the fate of released larvae within the body muscles; and c) the factors influencing the presence of worms in the muscle and under the skin.

STUDIES ON ANISAKIDS

It is well known that anisakids are harmful to human. The zoonotic implication of these worms resulted in numerous research works on these group of nematodes, particularly on the genus *Anisakis.* Before the conduct of this study, there is no way of easily differentiating morphologically the 2 sibling species of *A. simplex* complex recovered from patients. Proper identification of causative agents of anisakiasis in patients is a basic but very important step needed in the diagnosis and prevention of any potential risks for human consumption. In this study, I fosued mainly on the 2 sibling species, *A. simplex* (s.s.) and *A. pegreffii*, particularly on their taxonomy, distribution in Japanese waters and difference on sites of infection in fish host.

I found many differences between *A. simplex* (s.s.) and *A. pegreffii*. In the taxonomy level, there were great differences in the morphology in the examined L3 and *in vitro*-cultured adults. Molecular examination of the ITS and *mt*DNA *cox*2 regions revealed that these 2 sibling species were also different. From this result, it is also necessary to examine the morphological features and look for any morphological differences in L3 and adult stage, not only of the other remaining sibling species of *A. simplex* complex, the *A. simplex* C, but also of other species belonging from *Anisakis* Type I and *Anisakis* Type II groupings.

Given that the used and examined adult specimens were obtained through *in vitro*-culture, it is worth comparing and studying any morphological difference, which include the arrangement pattern of the caudal papillae and size difference of the spicule length, with adult specimens collected from hosts in the wild.

Two additional *Anisakis* species belonging to *Anisakis* Type II have been found in Japanese waters, making the total number of *Anisakis* species belonging to Type II groupings to 3. Hence, it seems that there are still other *Anisakis* species that needs to be

reported.

Different hybrid genotypes of *A. simplex* (s.s.) and *A. pegreffii* were also found. Up to present, such occurrence of hybrid genotypes is not well studied. With the possibility of culturing *Anisakis* under laboratory condition, the hypothesis that *A. simplex* (s.s.) and *A. pegreffii* could produce such hybrid genotypes can be another interesting work to explore.

No differences were found morphologically and molecularly between Mediterranean and Far East *A. pegreffii.* Given that the samples examined in the Mediterranean and Far East regions are limited, increasing the number of samples and finding any differences of *A. pegreffii* around the world, as well as carrying out population dynamic studies, would be a good topic in the future. Such result may provide information of host-parasite interaction or this can be used as an indicator in fish stock assessments.

Based on the combined results of *Anisakis* distribution in Japan, it seems that clear separation on the distribution between these 2 sibling species were present (i.e., *A. simplex* (s.s.) from the Pacific side and *A. pegreffii* from the Sea of Japan and East China Sea sides). Given that the examined host fishes did not cover all or most locations in Japan, it is good to carry out further field surveys in order to answer remaining unanswered questions such as: a) where is the boundery on the separation between these two sibling species?; and b) what factors (i.e., water temperature, water current, presence of other intermediate and final hosts, species of fishes, others) affect such major distribution?. Present stock assessment of chub mackerel and spotted mackerel (*Scomber australasicus*) is conducted by tentatively distinguishing two stocks namely, the Pacific stock and the Tsushima-current stock for chub mackerel and the Pacific stock and the East China Sea stock for spotted mackerel. Thus, many fish stocks are believed to be

divided into different stocks by the border located around Kagoshima Prefecture, although there are no clear evidence except for indirect information on their distributional and migration pattern. Hence, the use of the two sibling species of *Anisakis* would be helpful indicator for such fish stocks assessments after further verification have been carried out.

Only *A. simplex* (s.s.) migrated into the fish muscle but not *A. pegreffii*. This difference on the behavior of the two sibling species was shown in 2 different host fishes. Since raw fish is a traditional way in preparing food in Japan, it is also very important to determine whether other host fishes are suitable for *A. simplex* (s.s.) or *A. pegreffii* for their migration to the fish body muscle. It seems that *A. simpelx* (s.s.) is more pathogenic or preferred the muscle as the final site of infection. This possibly explains the reason in the report of Umehara et al. (2007) that majority of recovered worms in patients were *A. simplex* (s.s.) and only 1 person (out of 80 patients examined) was infected by *A. pegreffii*. With this difference, it seemed that *A. simplex* (s.s.) is more harmful than *A. pegreffii*. I think it is necessary to confirm the above hypothesis regarding the pathological differences between these 2 sibling species in the future. One aspect that can also be looked into is on the differences on the pathogenecity of the different allergens present in them. Allergens have been reported only in *A. simplex* and *A. pegreffii* (see SubChapter 1, Chapter 3), but have not been examined specifically in the sibling species *A. simplex* (s.s.). Any difference on the allergenic properties will be a good information in determining which of these two sibling species should be strictly be monitored for safe human consumption of infected fish. If *A. simplex* (s.s.) is confirmed as more pathogenic and *A. pegreffii* is less pathogenic, it would be safe to eat raw chub mackerel (saba) caught off western Kyushu and in the Japan Sea, which is commonly infected with *A. pegreffii* and which is commonly eaten raw in Kyushu area.

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2. Parasitic in Scombridae …….....…….3
   - Parasitic in fishes of other families ……...5

3. Parasitic in *Scomberomorus sinensis* in North Pacific (Japan)…… ……...…….….……*P. scomberomori*
4. Spicules sub-equal, 55-78 µm and 50-62 µm long; length of gubernaculums 22-40 µm. male 11-19 mm long. In *Thunnus* spp. ….....……..*P. macroandri*
   - Spicules very unequal, 1,750 – 2,080 µm and 65 – 95 µm long; length of gubernaculums 130-145µm. Male 9.5 – 12 mm long. In *Katsuwonus pelamis* ……....……...……*P. katsuwoni*

5. Body of gravid female 445 – 460 mm long. Male unknown. In *Pampus argenteus* (Stromateidae) and *Epinephelus septemfasciatus* (Serranidae); North Pacific (Japan) …….……...….. *P. managatuwo*
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6. Parasitic in Mugilidae (*Mugil cephalus*). Gravid female up to 250 mm long. Spicules subequal, 74 – 116 µm and 76 – 81 µm long. Indian Ocean (India)…….……...……...……*P. cephalus*
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9. Gravid female 65 – 85 mm long, length of its esophagus 1.07 – 1.18 mm. Length of equal spicules 432 – 468 µm; gubernaculums 84 – 93 µm long. In *Epinephelus morio* (Serranidae); Gulf of Mexico ……...…. *P. margolisi*
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10. Gravid female up to 64 mm long, length of its esophagus 0.62 – 0.86 mm. Male unknown. In *Nemipterus virgatus* (Nemipteridae); Taiwan Strait…….……..…… *P. nemipteri*

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11. Body length of larvae from uterus 350 µm. Esophagus of gravid female 1.3 mm long. Male unknown. In *Serranus cabrilla* (Serranidae); Adriatic Sea …….. *P. serranellicambrillae*
- Body length of larvae from uterus 610 µm. Esophagus of gravid female 0.9 mm long. Length of equal spicules 137 – 156 µm; gubernaculums 44 – 78 µm long. In *Uranoscopus scaber* (Uranoscopidae); reported also from some members of Citharidae, Phycidae, Scophthalmidae and Trachinidae; Mediterranean, Adriatic and Black Seas ……..…… *P. globiceps*

12. Parasitic in scorpaeniform fishes of family Sebastidae. Gravid female 100 – 135 mm long, length of its esophagus 1.25 – 1.59 mm. Larvae from uterus 260 – 350 µm long. Male unknown. In *Sebastiscus marmoratus*; East China Sea and North Pacific Ocean (Japan)….. …….……...…. *P. sebastisci*
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- Gravid female 132 – 230 mm long. Length of equal spicules 114 – 189 µm; gubernaculums 78 – 84 µm long. In *Lateolabrax japonicus* (Percichthyidae); reported also from many fishes of other perciform families (Carangidae, Centropomidae, Glaucosomidae, Mullidae, Pomadasyidae, Sciaenidae, Serranidae, Sparidae); tropical and sub-tropical regions of Pacific, Indian and Atlantic Oceans and Mediterranean Sea …….……. *P. lateolabracis*

Appendix 2. Key to gonad-infecting species of *Philometra* from marine and brackish-water fishes (Moravec et al. 2008b).

1. Gravid females unknown. Males longer than 9 mm. Parasitic in Scombridae in Atlantic ……2
   - Gravid females described. Males less than 6 mm long. Mostly parasitic in fishes belonging to other families ……... 3
2. Spicules subequal, 55-78 µm and 50-62 µm long; length of gubernaculums 22-40 µm. Male 11-19 mm long. In *Thunnus* spp. …….. *P. macroandri*
   - Spicules very unequal, 1,750-2,080 µm and 65-95 µm long; length of gubernaculums 130-145 µm. Male 9.5-12 mm long. In *Katsuwonus pelamis* …..….. ……. *P. katsuwoni*
3. Oesophagus of gravid female without anterior bulbous inflation. Intestine of larvae black-coloured due to presence of large black corpuscles. Male unknown. In *Pagellus erythrinus* (Sparidae); Mediterranean Sea …… *P. filiformis*
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   - Parasitic in fishes of other families ……... 5
5. Gravid female 445-460 mm long. Male unknown. In *Pampus argenteus* (Stromateidae); North Pacific (Japan) ……..…. *P. managatuwo*
   -Gravid female at most 250 mm long ……. 6
6. Parasitic in Mugilidae (*Mugil cephalus*). Gravid female up to 250 mm long. Spicules subequal, 74-116 µm and 76-81 µm long. Indian Ocean (India) ……... *P. cephalus*
   -Parasitic in fishes of other families ……... 7
7. Parasitic in Pomatomidae (*Pomatomus saltatrix*). Spicules equal, 60-70 µm long. Atlantic Ocean (USA) and Mediterranean Sea …….…….. *P. saltatrix*
   -Parasitic in fishes of other families ……...… 8
8. Gravid female at most 85 mm …… 9
   -Gravid female 100 mm or longer (that of *P. inimici* may be shorter) ……. 12
9. Gravid female 65-85 mm long. Spicules equal, 432-468 µm long; gubernaculums 84-93 µm long. In *Epinephelus morio* (Serranidae); Gulf of Mexico …….. *P. margolisi*
   -Gravid female at most 64 mm long …….… 10
10. Gravid female up to 64 mm long, length of oesophagus 0.62-0.86 mm. Male unknown. In *Nemipterus virgatus* (Nemipteridae); Taiwan Strait ……... *P. nemipteri*
    -Gravid female at most 60 mm long, length of oesophagus 0.9-1.3 mm. Parasitic in fishes of other families …….. 11

11. Larvae from uterus 350 µm long. Oesophagus of gravid female 1.3 mm long. Male unknown. In *Serranus cabrilla* (Serranidae); Adriatic Sea …… *P. serranellicabrillae*
-Larva from uterus 610 µm long. Oesophagus of gravid female 0.9 mm long. Spicules equal, 137-156 µm long; gubernaculums 44-78 µm long. In *Uranoscopus scaber* (Uranoscopidae); reported also from some members of other families; Mediterranean, Adriatic and Black Seas ……..……. *P. globiceps*
12. Parasitic in Scorpaeniform fishes ……. 13
-Parasitic in perciform fishes ……. 14
13. Gravid female 100-135 mm long, length of oesophagus 1.25-1.59 mm. Larvae from uterus 260-350 µm long. Male unknown. In *Sebastiscus marmoratus* (Sebastidae); East China Sea and North Pacific Ocean (Japan)…….. *P. sebastisci*
-Gravid female up to 220 mm long, length of oesophagus 0.75-1.48 mm. Larvae from uterus 306-424 µm long. Male unknown. In *Inimicus japonicus* (Synanceiidae) and *Platycephalus indicus* (Platycephalidae); North Pacific Ocean (Japan)…….. …… *P. inimici*
14. Gravid female at most 100 mm long. Spicules equal, 108-126 µm long; length of gubernaculums 48-56 µm. In *Argyrosomus argentatus* (Sciaenidae); South China Sea and North Pacific Ocean (Japan) …… *P. sciaenae*
-Gravid female longer than 100 mm ……. 15
15. Gravid female 150-480 mm long. Larvae from uterus 500-600 µm long. Male unknown. In *Seriolella violacea* (Centrolophidae); South Pacific Ocean (Peru) …….. ……. *P. neptomeni*
-Gravid female less 400 mm long ……. 16
16. Spicules equal, 260-265 µm long; length of gubernaculums 84 µm. Gravid female up to 300 mm long. Larvae from uterus 410 µm long. In *Epinephelus marginatus* (Serranidae); Mediterranean Sea ……. *P. jordanoi*
-Spicules less than 150 µm long ……...…… 17
17. Oral aperture of gravid female triangular, small. Distal end of gubernaculums with dorsal protuberance covered with transverse lamella-like structure …….…… 18
-Oral aperture of gravid female circular or slightly triangular. Neither dorsal protuberance nor lamella-like structures on distal end of gubernaculums ……... 19
18. Anterior end of oesophagus in gravid female slightly inflated. Male 2.07-2.73 mm long, gravid female 112-206 mm long. Spicules 71-130 µm and 65-124 µm long; gubernaculums 50-93 µm long. In *Lateolabrax japonicus* (Lateolabracidae); North Pacific (Japan) ……..... *P. lateolabracis*
-Anterior end of oesophagus in gravid female conspicuously inflated. Male 3.92-5.94 mm long, gravid female 104-394 mm long. Spicules 84-100 µm and 77-100 µm long; gubernaculums 64-84 µm long. In *Pagrus major* (Sparidae); North Pacific (Japan) …….. *P. madai*

19. Oral aperture somewhat triangular, small. Oesophageal inflation slightly outlined. Male 2.62-3.26 mm long, gravid female 167-420 mm long. Spicules 107-127 µm and 105-122 µm long, gubernaculums 70-90 µm long. In *Parapristipoma trilineatum* (Haemulidae); East China Sea (Japan) ……...…….. *P. isaki*
    -Oral aperture circular, large. Oesophageal inflation well developed, bulbous ...… 20
20. Spicules 81-87 µm long. Gubernaculum 57-69 µm long, distal end with distinct dorsal barb. Male 1.59-1.85 mm long, gravid female 115-157 mm long. In *Cynoscion nebulosus* (Sciaenidae); West Atlantic Ocean (South Carolina, USA) ……...….. ……... *P. carolinensis*
    -Spicules 90 µm or longer. Gubernaculum without dorsal barb ……..……. 21
21. Spicules 90-120 µm long, representing 5-7% of male body length. Male 1.63-1.86 mm long. Larvae from uterus 420-435 µm long. In *Epinephelus adscensionis* (Serranidae); Gulf of Mexico ……. *P. mexicana*
    -Spicules 132-141 µm long, representing 4-5% of male body length. Male 2.65-3.14 mm long. Larvae from uterus 582-597 µm long. In *Mycteroperca phenax* (Serranidae); West Atlantic Ocean (South Carolina, USA) ……. *P. charlestonensis*

Appendix 3. Key to gonad-infecting species of *Philometra* from marine and brackish-water fishes (Present study).

1. Gravid females unknown. Males longer than 9 mm. Parasitic in Scombridae in Atlantic ……2
   - Gravid females described. Males less than 6 mm long. Mostly parasitic in fishes belonging to other families ……... 3
2. Spicules subequal, 55-78 µm and 50-62 µm long; length of gubernaculums 22-40 µm. Male 11-19 mm long. In *Thunnus* spp. …….. *P. macroandri*
   - Spicules very unequal, 1,750-2,080 µm and 65-95 µm long; length of gubernaculums 130-145 µm. Male 9.5-12 mm long. In *Katsuwonus pelamis* …..….. …….. *P. katsuwoni*
3. Oesophagus of gravid female without anterior bulbous inflation. Intestine of larvae black-coloured due to presence of large black corpuscles. Male unknown. In *Pagellus erythrinus* (Sparidae); Mediterranean Sea …… *P. filiformis*
   -Oesophagus of gravid female with anterior bulbous inflation. Intestine of larvae from uterus light-coloured …….…. 4
4. Parasitic in Scombridae (*Scomberomorus sinensis*) in North Pacific (Japan). Gravid female up to 150 mm long. Male unknown …… *P. scomberomori*
   - **Male 2.44-3.38 mm long; spicules slightly unequal 74-135 and 71-131 µm long; length of gubernaculum40-76 µm long. Females up to 193 mm long; no U-shaped lobular mound at posterior end present. In *Scomberomorus niphonius* ……..……...….. *P. sawara***
   - Parasitic in fishes of other families ……... 5
5. Gravid female 445-460 mm long. Male unknown. In *Pampus argenteus* (Stromateidae); North Pacific (Japan) ……..…. *P. managatuwo*
   -Gravid female at most 250 mm long …… 6
6. Parasitic in Mugilidae (*Mugil cephalus*). Gravid female up to 250 mm long. Spicules subequal, 74-116 µm and 76-81 µm long. Indian Ocean (India) ……... *P. cephalus*
   -Parasitic in fishes of other families ……... 7
7. Parasitic in Pomatomidae (*Pomatomus saltatrix*). Spicules equal, 60-70 µm long. Atlantic Ocean (USA) and Mediterranean Sea …….…….. *P. saltatrix*
   -Parasitic in fishes of other families ……...… 8
8. Gravid female at most 85 mm …… 9
   -Gravid female 100 mm or longer (that of *P. inimici* may be shorter) ……. 12
9. Gravid female 65-85 mm long. Spicules equal, 432-468 µm long; gubernaculums 84-93 µm long. In *Epinephelus morio* (Serranidae); Gulf of Mexico …….. *P. margolisi*
   -Gravid female at most 64 mm long …….… 10

10. Gravid female up to **85 mm** long, length of oesophagus **0.62-1.025** mm. **Male 2.94-4.02 mm long; unequal spicules 93-126 and 85-113 µm long; gubernaculums 73-101 µm long; U-shaped lobular mound at posterior end present**. In *Nemipterus virgatus* (Nemipteridae); Taiwan Strait **and East China Sea (Japan)** …….……...……... *P. nemipteri*
-Gravid female at most 60 mm long, length of oesophagus 0.9-1.3 mm. Parasitic in fishes of other families …….. 11
11. Larvae from uterus 350 µm long. Oesophagus of gravid female 1.3 mm long. Male unknown. In *Serranus cabrilla* (Serranidae); Adriatic Sea …… *P. serranellicabrillae*
-Larva from uterus 610 µm long. Oesophagus of gravid female 0.9 mm long. Spicules equal, 137-156 µm long; gubernaculums 44-78 µm long. In *Uranoscopus scaber* (Uranoscopidae); reported also from some members of other families; Mediterranean, Adriatic and Black Seas ……..……. *P. globiceps*
12. Parasitic in Scorpaeniform fishes ……. 13
-Parasitic in perciform fishes ……. 14
13. Gravid female 100-135 mm long, length of oesophagus 1.25-1.59 mm. Larvae from uterus 260-350 µm long. Male unknown. In *Sebastiscus marmoratus* (Sebastidae); East China Sea and North Pacific Ocean (Japan)…….. *P. sebastisci*
-Gravid female up to 220 mm long, length of oesophagus 0.75-1.48 mm. Larvae from uterus 306-424 µm long. Male unknown. In *Inimicus japonicus* (Synanceiidae) and *Platycephalus indicus* (Platycephalidae); North Pacific Ocean (Japan)…….. …… *P. inimici*
14. Gravid female at most **104** mm long. **Males 1.46-2.62 mm long; spicules slightly unequal, 98-138 and 96-135 µm long**; length of gubernaculums **45-74 µm; no U-shaped lobular mound at posterior end present**. In *Argyrosomus argentatus* (Sciaenidae); South China Sea and North Pacific Ocean (Japan) …….…… *P. sciaenae*
-Gravid female longer than 100 mm ……. 15
15. Gravid female 150-480 mm long. Larvae from uterus 500-600 µm long. Male unknown. In *Seriolella violacea* (Centrolophidae); South Pacific Ocean (Peru) …….. ……. *P. neptomeni*
-Gravid female less 400 mm long ……. 16
16. Spicules equal, 260-265 µm long; length of gubernaculums 84 µm. Gravid female up to 300 mm long. Larvae from uterus 410 µm long. In *Epinephelus marginatus* (Serranidae); Mediterranean Sea ……. *P. jordanoi*
-Spicules less than 150 µm long ……...…… 17
17. Oral aperture of gravid female triangular, small. Distal end of gubernaculums with dorsal protuberance covered with transverse lamella-like structure …….…… 18

-Oral aperture of gravid female circular or slightly triangular. Neither dorsal protuberance nor lamella-like structures on distal end of gubernaculums ……... 19

18. Anterior end of oesophagus in gravid female slightly inflated. Male 2.07-2.73 mm long, gravid female 112-206 mm long. Spicules 71-130 µm and 65-124 µm long; gubernaculums 50-93 µm long. In *Lateolabrax japonicus* (Lateolabracidae); North Pacific (Japan) ……..... *P. lateolabracis*
    -Anterior end of oesophagus in gravid female conspicuously inflated. Male 3.92-5.94 mm long, gravid female 104-394 mm long. Spicules 84-100 µm and 77-100 µm long; gubernaculums 64-84 µm long; **U-shaped lobular mound at posterior end present**. In *Pagrus major* (Sparidae); North Pacific (Japan) …… …….…….. *P. madai*
19. Oral aperture somewhat triangular, small. Oesophageal inflation slightly outlined. Male 2.62-3.26 mm long, gravid female 167-420 mm long. Spicules 107-127 µm and 105-122 µm long, gubernaculums 70-90 µm long; **no U-shaped lobular mound at posterior end present**. In *Parapristipoma trilineatum* (Haemulidae); East China Sea (Japan) ……...…….. *P. isaki*
    -Oral aperture circular, large. Oesophageal inflation well developed, bulbous ...… 20
20. Spicules 81-87 µm long. Gubernaculum 57-69 µm long, distal end with distinct dorsal barb. Male 1.59-1.85 mm long, gravid female 115-157 mm long. In *Cynoscion nebulosus* (Sciaenidae); West Atlantic Ocean (South Carolina, USA) ……...….. ……... *P. carolinensis*
    -Spicules 90 µm or longer. Gubernaculum without dorsal barb ……..……. 21
21. Spicules 90-120 µm long, representing 5-7% of male body length. Male 1.63-1.86 mm long. Larvae from uterus 420-435 µm long. In *Epinephelus adscensionis* (Serranidae); Gulf of Mexico ……. *P. mexicana*
    -Spicules 132-141 µm long, representing 4-5% of male body length. Male 2.65-3.14 mm long. Larvae from uterus 582-597 µm long. In *Mycteroperca phenax* (Serranidae); West Atlantic Ocean (South Carolina, USA) ……. *P. charlestonensis*

Appendix 4. Designed primers for sequencing 18S rDNA and ITS (ITS1-5.8S rDNA-ITS2) region of the *Philometra* species examined.

Primer code	**Forward / reverse**	**Target rDNA region**	**Nucleotide location (apprx.)**	**Primer sequences (5' – 3')**
35f	Forward primer	18S	1-20	TATAATGGTGAAACCGCGAACGGC
248f	Forward primer	18S	130-150	TTGGTGACTCTGAATAGCT
490f	Forward primer	18S	390-410	CTATGAGAGGGCAAGTCTGG
600f	Forward primer	18S	575-595	CGGCTGCGTAAGGTGGCTAA
970f	Forward primer	18S	863-883	CCTAGTTCTGACCGTAAACG
1652f	Forward primer	18S	1478-1498	TGCCCTTTGTACACACCGCC
490r	Reverse primer	18S	390-410	CCAGACTTGCCCTCTCATAG
970r	Reverse primer	18S	863-883	CGTTTACGGTCAGAACTAGG
18gM	Reverse primer	18S		GGCAAAAGTCGTAACAAGGTTTCC
NC5f	Forward primer	ITS	1 - 26	GTAGGTGAACCTGCGGAAGGATCATT
300f	Forward primer	ITS	290-310	GTCCACGTTGGCGTCTAAACC
600f	Forward primer	ITS	575-603	TTATACTCTTAGCGGTGGATCACTCGGC
990f	Forward primer	ITS	970-990	GAGATACCCGATTTTGATTTGAC
1,040f	Forward primer	ITS	1,020-1,040	GCGATACACATATATGCTGC
NC2r	Reverse primer	ITS	1,493–1,515	TTTAGTTTCTTTTCCTCCGCT
990r	Reverse primer	ITS	970-990	GTCAAATCAAAATCGGGTATCTC
890r	Reverse primer	ITS	870-890	GCCGATTGCTAGTCGTACCACAA
680r	Reverse primer	ITS	660-680	GCGTTCAAAGTCTTAGTGTTC
600r	Reverse primer	ITS	575-603	GCCGAGTGATCCACCGCTAAGAGTATAA
300r	Reverse primer	ITS	290-310	GGTTTAGACGCCAACGTGGAC

Appendix 5. Designed primers for sequencing 18S rDNA and ITS (ITS1-5.8S rDNA-ITS2) region of *Philometroides seriolae*.

Primer code	**Forward / reverse**	**Target rDNA region**	**Nucleotide location (approx.)**	**Primer sequences (5' – 3')**
35f	Forward primer	18S	1-20	TATAATGGTGAAACCGCGAACGGC
248f	Forward primer	18S	130-150	TTGGTGACTCTGAATAGCT
490f	Forward primer	18S	390-410	CTATGAGAGGGCAAGTCTGG
970f	Forward primer	18S	863-883	CCTAGTTCTGACCGTAAACG
1652f	Forward primer	18S	1478-1498	TGCCCTTTGTACACACCGCC
490r	Reverse primer	18S	390-410	CCAGACTTGCCCTCTCATAG
970r	Reverse primer	18S	863-883	CGTTTACGGTCAGAACTAGG
18gM	Reverse primer	18S		GGCAAAAGTCGTAACAAGGTTTCC
NC5f	Forward primer	ITS	1 - 26	GTAGGTGAACCTGCGGAAGGATCATT
600f	Forward primer	ITS	566-589	ACTCTTAGCGGTGGATCACTCGGC
1,300f	Forward primer	ITS	1,319-1,338	CAAAAACACATACGTACACG
800r	Reverse primer	ITS	754-773	AACAGCAACAGCAACACCTG
600r	Reverse primer	ITS	465-484	TTGCTTGCTTGCTGCTACTC
NC2r	Reverse primer	ITS	1,493–1,515	TTTAGTTTCTTTTCCTCCGCT

Appendix 6. Alignment of nucleotide bases in the 18S rDNA among examined *Philometra* species

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            10         20         30         40         50
P.sawara            ---------- ---------- ---------- ---------- ----------
P.nemipteri         ---------- ---------- ---------- ---------- ---------G
P.sciaenae          ACCGCGAACG GCTCATTACA ACAGCTATTA TTTACTTGAT TTAGATTCCA
P.lateolabracis     ---------- ---------- ---------- ---------- ----------
P.madai             ---------- ---------- --------TW TTT-CTTGAT TTAGATTCCA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            60         70         80         90        100
P.sawara            ---------- ---------- ---------- ---------- ----------
P.nemipteri         TCCTCGTGGA TACGTGG-AA TTCTAGAGCT AATACATGCA CCAAAGCTCC
P.sciaenae          TACGTGGATA ACTGTGGCAA TTCTAGAGCT AATACATGCA CCAAAGCTCC
P.lateolabracis     ---------- ---------- TTCTAGAGCT AATACATGCA CCAAAGCTCC
P.madai             TACGTGGATA ACTGTGGCAA TTCTAGAGCT AATACATGCA CCAAAGCTCC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           110        120        130        140        150
P.sawara            ---------- ---------- ---------- ---------- ----------
P.nemipteri         GACTCGTTGA CGAGCGCATC TATTAGAATA AAACCAATCG AGACGCGCAA
P.sciaenae          GACTCGTTGA CGAGCGCATC TATTAGAATA AAACCAATCG AGACA-----
P.lateolabracis     GACTCGTTGA CGAGCGCATC TATTAGAATA AAACCAATCG AGACA-----
P.madai             GACTCCTTGA CGAGCGCATC TATTAGAATA AAACCAATCG AGACT-----

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           160        170        180        190        200
P.sawara            ---------- ---------- ---------- ---------- ----------
P.nemipteri         TGCGTCTCGT GCATTTGGTG ACTCTGAATA GCTTAGCTGA TCGCATGGTC
P.sciaenae          -GTGTCTCGT CCATTTGGTG ACTCTGAATA GCTTAGCTGA TCGCATGGTC
P.lateolabracis     -GTGTCTCGT CCATTTGGTG ACTCTGAATA GCTTAGCTGA TCGCATGGTC
P.madai             -GTGTCTCGT CCATTTGGTG ACTCTGAATA GCTTAGCTGA TCGCATGGTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           210        220        230        240        250
P.sawara            --------GC GACGTATCTA TCAAGTATCT GCCTTATCAA CTTTCGATGG
P.nemipteri         ACGCACCGGC GACGTATCTA TCAAGTATCT GCCTTATCAA CTTTCGATGG
P.sciaenae          ACGCACCGGC GACGTATCTA TCAAGTATCT GCCTTATCAA CTTTCGATGG
P.lateolabracis     ACGCACCGGC GACGTATCTA TCAAGTATCT GCCTTATCAA CTTTCGATGG
P.madai             ACGCACCGGC GACGTATCTA TCAAGTATCT GCCTTATCAA CTTTCGATGG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           260        270        280        290        300
P.sawara            TAGTTTATAT GACTACCATG GTTGTAACGG GTAACGGAGA ATAAGGGTTC
P.nemipteri         TAGTTTATAT GACTACCATG GTTGTAACGG GTAACGGAGA ATAAGGGTTC
P.sciaenae          TAGTTTATAT GACTACCATG GTTGTAACGG GTAACGGAGA ATAAGGGTTC
P.lateolabracis     TAGTTTATAT GACTACCATG GTTGTAACGG GTAACGGAGA ATAAGGGTTC
P.madai             TAGTTTATAT GACTACCATG GTTGTAACGG GTAACGGAGA ATAAGGGTTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           310        320        330        340        350
P.sawara            GACTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACTTCCA AGGAAGGCAG
P.nemipteri         GACTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACTTCCA AGGAAGGCAG
P.sciaenae          GACTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACTTCCA AGGAAGGCAG
P.lateolabracis     GACTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACTTCCA AGGAAGGCAG
P.madai             GACTCCGGAG AGGGAYCATG AGAAACGGCC ACCACTTCCA AGGAAGGCAG
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
P.sawara            CAGGCGCGCA AATTACCCAC TCTCAGCTCA GAGGAGGTAG TGACGAAAAA
P.nemipteri         CAGGCGCGCA AATTACCCAC TCTCAGCTCA GAGGAGGTAG TGACGAAAAA
P.sciaenae          CAGGCGCGCA AATTACCCAC TCTCAGCTCA GAGGAGGTAG TGACGAAAAA
P.lateolabracis     CAGGCGCGCA AATTACCCAC TCTCAGCTCA GAGGAGGTAG TGACGAAAAA
P.madai             CAGGCGCGCA AATTACCCAC TCTCAGCTCA GAGGAGGTAG TGACGAAAAA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
P.sawara            TAACGAGACC GTACTCGATG AGGCCGGTTA TCGGAATGGG TACAATTTAA
P.nemipteri         TAACGAGACC GTACTCGATG AGGCCGGTTA TCGGAATGGG TACAATTTAA
P.sciaenae          TAACGAGACC GTACTCGATG AGGCCGGTTA TCGGAATGGG TACAATTTAA
P.lateolabracis     TAACGAGACC GTACTCGATG AGGCCGGTTA TCGGAATGGG TACAATTTAA
P.madai             TAACGAGACC GTACTCGATG AGGCCGGTTA TCGGAATGGG TACAATTTAA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
P.sawara            ACCCGTTAAC GAGGATCTAT GAGAGGGCAA GTCTGGTGCC AGCAGCCGCG
P.nemipteri         ACCCGTTAAC GAGGATCTAT GAGAGGGCAA GTCTGGTGCC AGCAGCCGCG
P.sciaenae          ACCCGTTAAC GAGGATCTAT GAGAGGGCAA GTCTGGTGCC AGCAGCCGCG
P.lateolabracis     ACCCGTTAAC GAGGATCTAT GAGAGGGCAA GTCTGGTGCC AGCAGCCGCG
P.madai             ACCCGTTAAC GAGGATCTAT GAGAGGGCAA GTCTGGTGCC AGCAGCCGCG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
P.sawara            GTAATTCCAG CTCTCAAAGT GTATATCGGC ATTGCTGCGG TTAAAAAGCT
P.nemipteri         GTAATTCCAG CTCTCAAAGT GTATATCGGC ATTGCTGCGG TTAAAAAGCT
P.sciaenae          GTAATTCCAG CTCTCAAAGT GTATATCGGC ATTGCTGCGG TTAAAAAGCT
P.lateolabracis     GTAATTCCAG CTCTCAAAGT GTATATCGGC ATTGCTGCGG TTAAAAAGCT
P.madai             GTAATTCCAG CTCTCAAAGT GTATATCGGC ATTGCTGCGG TTAAAAAGCT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
P.sawara            CGTAGTTGGA TTTCAGCCTT ACGACGTGGT CCATTCATTG AATGCGAACT
P.nemipteri         CGTAGTTGGA TTTCAGCCTT ACGACGTGGT CCATTCATTG AATGCGAACT
P.sciaenae          CGTAGTTGGA TTTCAGCCTT ACGACGTGGT CCATTCATTG AATGCGAACT
P.lateolabracis     CGTAGTTGGA TTTCAGCCTT ACGACGTGGT CCATTCATTG AATGCGAACT
P.madai             CGTAGTTGGA TTTCAGCCTT ACGACGTGGT CCATTCATTG AATGCGAACT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
P.sawara            ACGCCTCGTA GGCTATATTG ATGTTGGTTT TTCCTTACGT TGCTCTAATC
P.nemipteri         ACGCCTCGTA GGCTATATTG ATGTTGGTTT TTCCTTACGT TGCTCTAACC
P.sciaenae          ACGCCTCGTA GGCTATATTG ATGTTGGTTT TTCCTTACGT TGCTCTAATC
P.lateolabracis     ACGCCTCGTA GGCTATATTG ATGTTGGTTT TTCCTTACGT TGCTCTAATC
P.madai             ACGCCTCGTA GGCTATATTG ATGTTGGTTT TTCCTTACGT TGCTCTAATC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
P.sawara            GGCTGCGTAA GGTGGCTAAC GAGTTTACCT TGAAAAAATT AGAGTGCTCA
P.nemipteri         GGCTGCGTAA GGTGGCTGAC GAGTTTACCT TGAAAAAATT AGAGTGCTCA
P.sciaenae          GGCTGCGTAA GGTGGCTAAC GAGTTTACCT TGAAAAAATT AGAGTGCTCA
P.lateolabracis     GGCTGCGTAA GGTGGCTAAC GAGTTTACCT TGAAAAAATT AGAGTGCTCA
P.madai             GGCTGCGTAA GGTGGCTAAC GAGTTTACCT TGAAAAAATT AGAGTGCTCA
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
P.sawara            ACGCGGGCTA ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT
P.nemipteri         ACGCGGGCTA ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT
P.sciaenae          ACGCGGGCTA ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT
P.lateolabracis     ACGCGGGCTA ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT
P.madai             ACGCGGGCTA ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
P.sawara            CGGTTCTATT TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA
P.nemipteri         CGGTTCTATT TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA
P.sciaenae          CGGTTCTATT TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA
P.lateolabracis     CGGTTCTATT TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA
P.madai             CGGTTCTATT TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            810        820        830        840        850
P.sawara            GACGGGGGCA TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG
P.nemipteri         GACGGGGGCA TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG
P.sciaenae          GACGGGGGCA TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG
P.lateolabracis     GACGGGGGCA TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG
P.madai             GACGGGGGCA TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            860        870        880        890        900
P.sawara            CGAGACGTCC CACTGCGAAA GCATTTGCCA AGAATGTCTT CATTAATCAA
P.nemipteri         CGAGACGTCC CACTGCGAAA GCATTTGCCA AGAATGTCTT CATTAATCAA
P.sciaenae          CGAGACGTCC CACTGCGAAA GCATTTGCCA AGAATGTCTT CATTAATCAA
P.lateolabracis     CGAGACGTCC CACTGCGAAA GCATTTGCCA AGAATGTCTT CATTAATCAA
P.madai             CGAGACGTCC CACTGCGAAA GCATTTGCCA AGAATGTCTT CATTAATCAA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950
P.sawara            GAACGAAAGT CAGAGGTTCG AAGGCGATCA GATACCGCCC TAGTTCTGAC
P.nemipteri         GAACGAAAGT CAGAGGTTCG AAGGCGATCA GATACCGCCC TAGTTCTGAC
P.sciaenae          GAACGAAAGT CAGAGGTTCG AAGGCGATCA GATACCGCCC TAGTTCTGAC
P.lateolabracis     GAACGAAAGT CAGAGGTTCG AAGGCGATCA GATACCGCCC TAGTTCTGAC
P.madai             GAACGAAAGT CAGAGGTTCG AAGGCGATCA GATACCGCCC TAGTTCTGAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            960        970        980        990       1000
P.sawara            CGTAAACGAT ACCAACTAGC GTTCCGTCAG CAGTAATTAC GCCTTGACGG
P.nemipteri         CGTAAACGAT ACCAACTAGC GTTCCGTCAG CAGTAATTAC GCCTTGACGG
P.sciaenae          CGTAAACGAT ACCAACTAGC GTTCCGTCAG CAGTAATTAC GCCTTGACGG
P.lateolabracis     CGTAAACGAT ACCAACTAGC GTTCCGTCAG CAGTAATTAC CCCTTGACGG
P.madai             CGTAAACGAT ACCAACTAGC GTTCCGTCAG CAGTAATTAC GCCTTGACGG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           1010       1020       1030       1040       1050
P.sawara            GCAGCTTTCC GGAAACGAAA GTTTTTCGGT TCCGGGGGAA GTATGGTTGC
P.nemipteri         GCAGCTTTCC GGAAACGAAA GTTTTTCGGT TCCGGGGGAA GTATGGTTGC
P.sciaenae          GCAGCTTTCC GGAAACGAAA GTTTTTCGGT TCCGGGGGAA GTATGGTTGC
P.lateolabracis     GCAGCTTTCC GGAAACGAAA GTTTTTCGGT TCCGGGGGAA GTATGGTTGC
P.madai             GCAGCTTTCC GGAAACGAAA GTTTTTCGGT TCCGGGGGAA GTATGGTTGC
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1060       1070       1080       1090       1100
P.sawara            AAAGCTGAAA CTTAAAGAAA TTGACGGAAG GGCACCACCA GGAGTGGAGC
P.nemipteri         AAAGCTGAAA CTTAAAGAAA TTGACGGAAG GGCACCACCA GGAGTGGAGC
P.sciaenae          AAAGCTGAAA CTTAAAGAAA TTGACGGAAG GGCACCACCA GGAGTGGAGC
P.lateolabracis     AAAGCTGAAA CTTAAAGAAA TTGACGGAAG GGCACCACCA GGAGTGGAGC
P.madai             AAAGCTGAAA CTTAAAGAAA TTGACGGAAG GGCACCACCA GGAGTGGAGC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1110       1120       1130       1140       1150
P.sawara            CTGCGGCTTA ATTTGACTCA ACACGGGAAA ACTCACCTGG CCCGGACACC
P.nemipteri         CTGCGGCTTA ATTTGACTCA ACACGGGAAA ACTCACCTGG CCCGGACACC
P.sciaenae          CTGCGGCTTA ATTTGACTCA ACACGGGAAA ACTCACCTGG CCCGGACACC
P.lateolabracis     CTGCGGCTTA ATTTGACTCA ACACGGGAAA ACTCACCTGG CCCGGACACC
P.madai             CTGCGGCTTA ATTTGACTCA ACACGGGAAA ACTCACCTGG CCCGGACACC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1160       1170       1180       1190       1200
P.sawara            GTGAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCGGTG GTTGGTGGTG
P.nemipteri         GTGAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCGGTG GTTGGTGGTG
P.sciaenae          GTGAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCGGTG GTTGGTGGTG
P.lateolabracis     GTGAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCGGTG GTTGGTGGTG
P.madai             GTGAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCGGTG GTTGGTGGTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1210       1220       1230       1240       1250
P.sawara            CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTTA TTCCGATAAC
P.nemipteri         CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTTA TTCCGATAAC
P.sciaenae          CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTTA TTCCGATAAC
P.lateolabracis     CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTTA TTCCGATAAC
P.madai             CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTTA TTCCGATAAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1260       1270       1280       1290       1300
P.sawara            GAACGAGACT CTAGCCTGCT AAATAGTCGG CG-AATAATT TTTGAACGTT
P.nemipteri         GAACGAGACT CTAGCCTGCT AAATAGTCGG CGTAATAATT TTTGAACGTT
P.sciaenae          GAACGAGACT CTAGCCTGCT AAATAGTCGG CG-AATAATT TTTGAACGTT
P.lateolabracis     GAACGAGACT CTAGCCTGCT AAATAGTCGG CG-AATAATT TTTGAACGTT
P.madai             GAACGAGACT CTAGCCTGCT AAATAGTCGG CG-AATAATT TTTGAACGTT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1310       1320       1330       1340       1350
P.sawara            CGTACGACTT CTTAGAGGGA CAAGCGGAAC TTAAGCCGCA TGAAGTTGAG
P.nemipteri         CGCACGACTT CTTAKAGGGA CAAGCGGAAC TTAAGCCGCA TGAAGTTGAG
P.sciaenae          CGTACGACTT CTTAGAGGGA CAAGCGGAAC TTAAGCCGCA TGAAGTTGAG
P.lateolabracis     CGTACGACTT CTTAGAGGGA CAAGCGGAAC TTAAGCCGCA TGAAGTTGAG
P.madai             CGTACGACTT CTTAGAGGGA CAAGCGGAAC TTAAGCCGCA TGAAGTTGAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1360       1370       1380       1390       1400
P.sawara            CAATAACAGG TCTGTGATGC CCTTAGACGT CCAGGGCTGC ACGCGCGCTA
P.nemipteri         CAATAACAGG TCTGTGATGC CCTTAGACGT CCAGGGCTGC ACGCGCGCTA
P.sciaenae          CAATAACAGG TCTGTGATGC CCTTAGACGT CCAGGGCTGC ACGCGCGCTA
P.lateolabracis     CAATAACAGG TCTGTGATGC CCTTAGACGT CCAGGGCTGC ACGCGCGCTA
P.madai             CAATAACAGG TCTGTGATGC CCTTAGACGT CCAGGGCTGC ACGCGCGCTA
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1410       1420       1430       1440       1450
P.sawara            CACTGGAGGA ATCAACGTGC TATATCCGTT GCCGAAAGGC AGTGGCAACC
P.nemipteri         CACTGGAGGA ATCAACGTGC TATATCCGCT GTCGAAAGGC AGTGGCAACC
P.sciaenae          CACTGGAGGA ATCAACGTGC TATATCCGCT GTCGAAAGGC AGTGGCAACC
P.lateolabracis     CACTGGAGGA ATCAACGTGC TATATCCGTT GTCGAAAGGC AATGGCAACC
P.madai             CACTGGAGGA ATCAACGTGC TATATCCGCT GTCGAAAGGC AGTGGCAACC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1460       1470       1480       1490       1500
P.sawara            CATTGAAAAT CCTCCGTGCT CGGAATCGGG AATTGCAATT ATTTCCCTTG
P.nemipteri         CATTGAAAAT CCTCCGTGCT CGGAATCGGG AATTGCAATT ATTTCCCTTG
P.sciaenae          CATTGAAAAT CCTCCGTGCT CGGAATCGGG AATTGCAATT ATTTCCCTTG
P.lateolabracis     CATTGAAAAT CCTCCGTGCT CGGAATCGGG AATTGCAATT ATTTCCCTTG
P.madai             CATTGAAAAT CCTCCGTGCT CGGAATCGGG AATTGCAATT ATTTCCCTTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1510       1520       1530       1540       1550
P.sawara            AACGAGGAAT TCCTAGTAAG TGTGAGTCAT CAGCTCACGC TGATTACGTC
P.nemipteri         AACGAGGAAT TCCTAGTAAG TGTGAGTCAT CAGCTCACGC TGATTACGTC
P.sciaenae          AACGAGGAAT TCCTAGTAAG TGTGAGTCAT CAGCTCACGC TGATTACGTC
P.lateolabracis     AACGAGGAAT TCCTAGTAAG TGTGAGTCAT CAGCTCACGC TGATTACGTC
P.madai             AACGAGGAAT TCCTAGTAAG TGTGAGTCAT CAGCTCACGC TGATTACGTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1560       1570       1580       1590       1600
P.sawara            CCTGCCCTTT GTACACACCG CCCGTCGCTG CCCGGGACTG AGCCGTTTCG
P.nemipteri         CCTGCCCTTT GTACACACCG CCCGTCGCTG CCCSGKACTG AACCKTTTCK
P.sciaenae          CCTGCCCTTT GTACACACCG CCCGTCGCTG CCCGGGACTG AGCCGTTTCG
P.lateolabracis     CCTGCCCTTT GTACACACCG CCCGTCGCTG CCCGGGACTG AGCCGTTTCG
P.madai             CCTGCCCTTT GTACACACCG CCCGTCGCTG CCCGGGACTG AGCCGTTTCG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1610       1620       1630       1640       1650
P.sawara            AGAAAAGCGG GGACCGCTGT TATTGGGTTA CATTGTGAAT CTGATAAACG
P.nemipteri         A-AAAAACGG GKACCGCTGC TATTGGGTTA CGTTGTGAAT CTGATAACCG
P.sciaenae          AGAAAAGCGG GGACCGCTGT TATTGGGTTA CATTGTGAAT CTGATAAACG
P.lateolabracis     AGAAAAGCGG GGACCGCTGT TATTGGGTTA CATTGTGAAT CTGATAAACG
P.madai             AGAAAAGCGG GGACCGCTGT TATTGGGTTA CATTGTGAAT CTGATAAACG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1660       1670       1680       1690       1700
P.sawara            GTAGAAACCG CCTTAATCGC AGTGGCTTGA ACCGGGCAAA AGTCGTAACA
P.nemipteri         GTAGAAACCG CCTTAATCGC AGTGGCTTGA ACCGGGCAAA AGTCGTAACA
P.sciaenae          GTAGAAACCG CCTTAATCGC AGTGGCTTGA ACCGGGCAAA AGTCGTAACA
P.lateolabracis     GTAGAAACCG CCTTAATCGC AGTGGCTTGA ACCGGGCAAA AGTCGTAACA
P.madai             GTAGAAACCG CCTTAATCGC AGTGGCTTGA ACCGGGCAAA AGTCGTAACA

                    ....|...

P.sawara            AGGTTTCC
P.nemipteri         AGGTTTCC
P.sciaenae          AGGTTTCC
P.lateolabracis     AGGTTTCC
P.madai             AGGTTTCC
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Appendix 7. Alignment of nucleotide bases in the 18S rDNA among *Philometroides* species with the inclusion of *Philometra clavaeceps*.

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
P.seriolae          ---------- ---------- ---------- ---------- ----------
P.fulvidraconi      ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC
P.pseudaspii        ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC
P.cyprini           ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC
P.sanguineus        ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC
P.pseudorasbori     ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC
P.clavaeceps        ATGCATGTCT AAGTTCATAT TGCCTATAAT GGTGAAACCG CGAACGGCTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
P.seriolae          ---------- ---------- ---------T RTTCCATACG TGGATA-CTG
P.fulvidraconi      ATTACAACAG CTATAATTTA CTTGATTTTG ATTCCCTACG TGGATAACTG
P.pseudaspii        ATTACAACAG CTATAATTTA CTTGATTTTG ATTCCATACG TGGATAACTG
P.cyprini           ATTACAACAG CTATTATTTA CTTGATTTTG ATTCCATACG TGGATAACTG
P.sanguineus        ATTACAACAG CTATTATTTA CTTGATTTTG ATTCCATACG TGGATAACTG
P.pseudorasbori     ATTACAACAG CTATTATTTA CTTGATTTTG ATTCCATACG TGGATAACTG
P.clavaeceps        ATTACAACAG CTATAATTTA CTTGATTTTG ATTCCATACG TGGATAACTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
P.seriolae          TSGCAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT -----TTTTG
P.fulvidraconi      TGGTAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ACAAATTTTG
P.pseudaspii        TGGTAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ACAAATTTTG
P.cyprini           TGGCAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ATGA-TTTTG
P.sanguineus        TGGCAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ATCA-TTTTG
P.pseudorasbori     TGGCAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ATCA-TTTTG
P.clavaeceps        TGGTAATTCT AGAGCTAATA CATGCACCAA AGCTCCGACT ACAAATTTTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            160        170        180        190        200
P.seriolae          ACGAGCGCAT CTATTAGAAT -AAAACCAAT CGAGTGTAC- ----------
P.fulvidraconi      ACGAGCGCAT CTATTAGAAT CAAAACCAAT CGAGATCGTG TCGTGGTCGT
P.pseudaspii        ACGAGCGCAT CTATTAGAAT CAAAACCAAT CGAGATCGTG TCGTGGTCGT
P.cyprini           ACGAGCGCAT CTATTAGAAT -AAAACCAAT CGAGACTTT- ----------
P.sanguineus        ACGAGCGCAT CTATTAGAAT -AAAACCAAT CGAGACTTT- ----------
P.pseudorasbori     ACGAGCGCAT CTATTAGAAT -AAAACCAAT CGAGACTTT- ----------
P.clavaeceps        ACGAGCGCAT CTATTAGAAT CAAAACCAAT CGAGATCGTG TCGTGGTCGT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            210        220        230        240        250
P.seriolae          --A----GTC TT-------- ---------- -------GT- GCACTCGTAG
P.fulvidraconi      GGCGTGATTT CTGATTGC-G CGTACGCACA CGCACACGTC AATCTCGTCG
P.pseudaspii        --CGTGATTT TTTG------ CGTACGCACA CGCACACGTC AATCTCGTCG
P.cyprini           --T----TTC TTTATT---- ---------- CGAGAAAGT- GGTCTCGTCG
P.sanguineus        --T----TTC TTCAAT---- ---------- CGAGAAGAT- CGCCTCGTCC
P.pseudorasbori     --T----TTC TTCAAT---- ---------- CGAGAAGAT- CGCCTCGTCC
P.clavaeceps        --CGTGATTT TTTGTGATTG CGTACGCACA CGCACACGTC AATCTCGTCG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
P.seriolae          ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTC GTACCGGCGA
P.fulvidraconi      ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTT GTACCGGCGA
P.pseudaspii        ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTT GTACCGGCGA
P.cyprini           ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTC GTACCGGCGA
P.sanguineus        ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTC GTACCGGCGA
P.pseudorasbori     ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTC GTACCGGCGA
P.clavaeceps        ATTTGGTGAC TCTGAATAGC TTAGCTGATC GCATGGTCTT GTACCGGCGA
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
P.seriolae          CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATATGA
P.fulvidraconi      CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA
P.pseudaspii        CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA
P.cyprini           CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA
P.sanguineus        CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA
P.pseudorasbori     CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA
P.clavaeceps        CGTATCTATC AAGTATCTGC CTTATCAACT TTCGATGGTA GTTTATGTGA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
P.seriolae          CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.fulvidraconi      CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.pseudaspii        CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.cyprini           CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.sanguineus        CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.pseudorasbori     CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG
P.clavaeceps        CTACCATGGT TGTAACGGGT AACGGAGAAT AAGGGTTCGA CTCCGGAGAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
P.seriolae          GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.fulvidraconi      GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.pseudaspii        GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.cyprini           GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.sanguineus        GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.pseudorasbori     GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA
P.clavaeceps        GGAGCCTGAG AAACGGCTAC CACTTCCAAG GAAGGCAGCA GGCGCGCAAA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
P.seriolae          TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.fulvidraconi      TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.pseudaspii        TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.cyprini           TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.sanguineus        TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.pseudorasbori     TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT
P.clavaeceps        TTACCCACTC TCAGCACAGA GGAGGTAGTG ACGAAAAATA ACGAGACCGT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
P.seriolae          ACTCGATGAG GCCGGTTATC GGAATGGGTA CAATTTAAAC CCGTTAACGA
P.fulvidraconi      ACTCGATGAG GCCGGTTATC GGAATGGGCA CAATTTAAAC CCGTTAACGA
P.pseudaspii        ACTCGATGAG GCCGGTTATC GGAATGGGCA CAATTTAAAC CCGTTAACGA
P.cyprini           ACTCGATGAG GCCGGTTATC GGAATGGGTA AAATTTAAAC CCGTTAACGA
P.sanguineus        ACTCGATGAG GCCGGTTATC GGAATGGGTA CAATTTAAAC CCGTTAACGA
P.pseudorasbori     ACTCGATGAG GCCGGTTATC GGAATGGGTA CAATTTAAAC CCGTTAACGA
P.clavaeceps        ACTCGATGAG GCCGGTTATC GGAATGGGCA CAATTTAAAC CCGTTAACGA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
P.seriolae          GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.fulvidraconi      GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.pseudaspii        GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.cyprini           GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.sanguineus        GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.pseudorasbori     GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
P.clavaeceps        GGATCTATGA GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
P.seriolae          CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.fulvidraconi      CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.pseudaspii        CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.cyprini           CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.sanguineus        CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.pseudorasbori     CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT
P.clavaeceps        CTCAAAGTGT ATATCGGCAT TGCTGCGGTT AAAAAGCTCG TAGTTGGATT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
P.seriolae          TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.fulvidraconi      TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.pseudaspii        TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.cyprini           TGAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.sanguineus        TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.pseudorasbori     TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG
P.clavaeceps        TCAGCCTTAC GACGTGGTCC ATTCATTGAA TGCGAACTAC GCCTCGTAGG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
P.seriolae          CTCGTTTGAT GTTGGTTTTT CCTTACGTTG CTCTCAACGG CTGCGTAAGG
P.fulvidraconi      CTATTTGAAT GTTGGTTTTT CCTTACTTGG CTCTCAACGG CTGAGTAAGG
P.pseudaspii        CTATTTGAAT GTTGGTTTTT CCTTGCTTGG CTCTCAACGG CTGAGTAAGG
P.cyprini           CTATTTTGAT GTTGGTTTTT CCTTATGTCG CTCTCATCGG CAGCGTAAGG
P.sanguineus        CTATTTTAAT GTTGGTTTTT CCTTACGTCG CTCTCATCGG CAGCGTAAGG
P.pseudorasbori     CTATTTTAAT GTTGGTTTTT CCTTACGTCG CTCTCATCGG CAGCGTAAGG
P.clavaeceps        CTATTTGAAT GTTGGTTTTT CCTTGCTTGG CTCTCAACGG CTGAGTAAGG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
P.seriolae          TGGCTGACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTA--
P.fulvidraconi      TGGCTGACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTCAT
P.pseudaspii        TGGCTGACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTATT
P.cyprini           TGGCTAACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTA--
P.sanguineus        TGGCTAACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTA--
P.pseudorasbori     TGGCTAACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTA--
P.clavaeceps        TGGCTGACGA GTTTACCTTG AAAAAATTAG AGTGCTCAAC GCGGGCTATT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            810        820        830        840        850
P.seriolae          ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT CGGTTCTATT
P.fulvidraconi      ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATACGACTT CTGGTCTATT
P.pseudaspii        ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATACGACTT CTGGTCTATT
P.cyprini           ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT CGGTTCTATT
P.sanguineus        ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT CGGTTCTATT
P.pseudorasbori     ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATAGGATCT CGGTTCTATT
P.clavaeceps        ACGCCTGAAT ACTCGTGCAT GGAATAATGG AATACGACTT CTGGTCTATT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            860        870        880        890        900
P.seriolae          TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.fulvidraconi      TTGTTGGTTT TCTGATCGGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.pseudaspii        TTGTTGGTTT TCTGATCGGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.cyprini           TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.sanguineus        TTGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.pseudorasbori     TCGTTGGTTT TCTGATCTGA GATAATGGTT AAGAGGGACA GACGGGGGCA
P.clavaeceps        TTGTTGGTTT TCTGATCGGA GATAATGGTT AAGAGGGACA GACGGGGGCA
```

```
                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950
P.seriolae          TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.fulvidraconi      TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.pseudaspii        TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.cyprini           TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.sanguineus        TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.pseudorasbori     TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC
P.clavaeceps        TTCGTATCGC TGCGTGAGAG GTGAAATTCT TGGACCGTAG CGAGACGTCC

                    ....|....| ....|....| ....|.
                            960        970
P.seriolae          CACTGCGAAA GCATTTGCCA AGAATG
P.fulvidraconi      CACTGCGAAA GCATTTGCCA AGAATG
P.pseudaspii        CACTGCGAAA GCATTTGCCA AGAATG
P.cyprini           CACTGCGAAA GCATTTGCCA AGAATG
P.sanguineus        CACTGCGAAA GCATTTGCCA AGAATG
P.pseudorasbori     CACTGCGAAA GCATTTGCCA AGAATG
P.clavaeceps        CACTGCGAAA GCATTTGCCA AGAATG
```

Appendix 8. Alignment of nucleotide bases in the ITS2 region among examined *Philometra* species.

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
P.sawara            ATAATACACA AAAACTCGTG TACT--A--- -------CAT CATCATCATC
P.nemipteri         ATAATACACA AAAACTCGTG TACC------ ---------- -----ACATC
P.sciaenae          ATAATAAACA AAAACTCGTG TACCACATCC TCATGATCAT CATCATCATC
P.lateolabracis     ATAATACACA AAAACTCGTG TACCACAT-- -------CCT CATCATCATC
P.madai             ATAATACACA AAAACTCGTG TACTCTA--- -------CAT CCTCGTCATC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
P.sawara            ATCATCATCA -TCA-TCACG CACATGTGT- --GTGTGTGT -------GTG
P.nemipteri         ATCATCATCA TTCA------ ---ATGTC-- ---------- -------ATG
P.sciaenae          ATCATCAGCA TTCACTTACT CCCATGTGTT GTATGTGTAT ATGGGTTTGG
P.lateolabracis     ACCATCATCA TTCACTTACT CCCATGTGTT GTATGTGTAT ATGG---GTG
P.madai             ATCATCGTCA CTCACTCACC CCCATGTGT- --ATGTGTAT ATGGATGGTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
P.sawara            TGTG-TGTGT GTATAGATG- --TGATTGCG TGA------- --TGGTTGTG
P.nemipteri         TGTG-TGTGT GTATAGATG- --TGATTGTG TGTG------ -ATGGTTGTG
P.sciaenae          TGTGCTGCGT GTATCGATGA TGTGATTGTG TGTGTGTATG TGTGGTTGTG
P.lateolabracis     TGGTGTGCGT GTGTAGATGA TGTGATTGTA GATGT--ATG TGTGCTTGTG
P.madai             TGTG-TGTGT GTATAGATGA TGTGATTGTG TG-------- --TGGTTGTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            160        170        180        190        200
P.sawara            GTACG----A CTAGCAATCG GCTATGTGT- -GATCGCG-- TACATCAGCA
P.nemipteri         GTACG----A CTAGCAATCG GCT------- --ATCGCG-- TACATCAGCA
P.sciaenae          GTACG----A CTAGCAATCG GCTATGTAT- -GATCAGTGT TGTGTGTGTG
P.lateolabracis     GTACG----A CTAGCAATCG GCTATGTGTA TGATCGAGGG TAGATCAGTG
P.madai             GTACGTACGA CTAGCAATCG GCTATGTGT- -GATCGCGCG TACATCAGCA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            210        220        230        240        250
P.sawara            AATTTGTTGT TGCTGCTG-G TGTGTGC-TG TACTCGATCG AGATACCCGA
P.nemipteri         AATTCGTT-- ---------- --TGTGC-TG TACTCGCGCG AGATACCCGA
P.sciaenae          TGTGTGCA-- ---------- --TGTGC-TG CACTCGATCG AGATACCCGA
P.lateolabracis     T-TGTGTG-- ------TGCT GCTGTACTCA CTCGATCGTG AGATACCCGA
P.madai             AATTTGTTGC TGTTGGTGTG TGTGTGTGCG TGCTGTACT- AGATACCCGA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
P.sawara            TTTTGATTTG ACATGTAATG CATATATATG TGTGTACGTG TGCGCGATAC
P.nemipteri         TTTTGATTTG ACATTTAA-G TGCATATATA TGTGTGTACG TGTGCGATAC
P.sciaenae          TTTTGATTTG ACATGTAA-- TGCATATATA TGTGTGTACG TGTGCGATAC
P.lateolabracis     TTTTGATTTG ACATATA--- ---------- ---------- TATGCGATAC
P.madai             TTTRGATTTG ACATGTAATG CGCATATATA TGTGTGTACG TGTGCGATAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
P.sawara            ACATATATGC TGCTGTGAAT TGTCATGGTG TGTATGTG-A TGTGCAG---
P.nemipteri         ACATATATGC TGCTGTGAAT TGTCATGATG TATATGTG-C TGCGTCGTCG
P.sciaenae          ACATATATGC TGCTGTGAAT TCTCATGGTG TATATGTGTG TATGTCGT--
P.lateolabracis     ACATATATGC TGCTGTGAAT A--TATG-TG TGTATGTG-C TGCGTCG---
P.madai             ACATATATGC TGCTGTGAAT TGTCATGGTG TATATGTGTA TGTGCTG---
```

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
P.sawara            -CGTCGTCGT TTGAATGACA C--AATAACA AGGTTGTTTT GTGTATG---
P.nemipteri         TCGTCGTCGT TTGAATGACG CGCAATAACA AGGTTGTTTT GTGTGTG---
P.sciaenae          -TGTCG-CGT TTGAATGACA C--AATAACA AGGTTGTTTT GTGTGCG---
P.lateolabracis     ---TTGTCGT TTGAATGACA C--AATAACA AGGTTGTTTT TTGTGTGTGA
P.madai             -CGTCGTCGT TTGAATGACA C--AATAACA AGGTTGTTTT GTGTGTG---

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
P.sawara            --ACGACGAA AACACATAC- ACATA--CAT ---CATCAT- ----CTACAT
P.nemipteri         --ACGACAAA AAGACATGC- ACATG--CAC ATGCACCAC- ----ATACAT
P.sciaenae          --ACGACGAC AAAA---AC- ACATA--CAT CATCATCAT- ----ATCAAC
P.lateolabracis     CGACGACAGC AACACATACT ACATCGTCAT CGTCATCATG ATCAACATAT
P.madai             --ACGACAAA AACACATAC- ACAAA--CAC ATACATCAT- ----ATACAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
P.sawara            AC-ACTATAA ACTGAAAACA A-TCATTATT TTGACCTCAA CTCAGTCGAG
P.nemipteri         A---CCACA- ---------- ------TATT TTGACCTCAA CTCAGTCGAG
P.sciaenae          A----TATGT ACAAAAAT-- --TCATTATT TTGACCTCAA CTCAGTCGAG
P.lateolabracis     ACTACTATAT ATGTACACAA ATTCATTATT TTGACCTCAA CTCAGTCGAG
P.madai             AT-ACTATAT ACGCAAAT-- --TCATTATT TTGACCTCAA CTCAGTCGAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
P.sawara            ATTACCCGCT GAATTTAAGC ATATAAACTA AGCGGAGGAA AAGAAACTAA
P.nemipteri         ATTACCCGCT GAATTTAAGC ATATAA-CTA AGCGGAGGAA AAGAAACTAA
P.sciaenae          ATTACCCGCT GAATTTAAGC ATATAA-CTA AGCGGAGGAA AAGAAACTAA
P.lateolabracis     ATTACCCGCT GAATTTAAGC ATATAAACTA AGCGGAGGAA AAGAAACTAA
P.madai             ATTACCCGCT GAATTTAAGC ATATAA-CTA AGCGGAGGAA AAGAAACTAA

                    .
P.sawara            A
P.nemipteri         A
P.sciaenae          A
P.lateolabracis     A
P.madai             A
```

Appendix 9. Alignment of nucleotide bases in the ITS2 region among *Philometroides* species with the inclusion of *Philometra clavaeceps*.

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
P.seriolae          GTATTAGAAA AAACAATTCG TGTATGCGAC TACTACAGGT GTTGCTGTTG
P.fulvidraconi      ---------- ---------- ---------- ---------- ----------
P.pseudaspii        ---------- ---------- ---------- ---------- ----------
P.cyprini           ---------- ---------- ---------- ---------- ----------
P.sanguineus        ---------- ---------- ---------- ---------- ----------
P.clavaeceps        ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
P.seriolae          CTGTTGCTAT TGCTGCTACT ACTGTTGTTG TTGTTGTTTG CTTGTATGAA
P.fulvidraconi      ---------- ---------- ---------- ---------- ----------
P.pseudaspii        ---------- ---------- ---------- ---------- ----------
P.cyprini           ---------- ---------- ---------- ---------- ----------
P.sanguineus        ---------- ---------- ---------- ---------- ----------
P.clavaeceps        ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
P.seriolae          TGAATGGCAG ACAGAGTAGT AGTAGTAGTA GTAGTAGTAG TAGTAGTAGT
P.fulvidraconi      ---------- ---------- ---------- ---------- ----------
P.pseudaspii        ---------- ---------- ---------- ---------- ----------
P.cyprini           ---------- ---------- ---------- ---------- ----------
P.sanguineus        ---------- ---------- ---------- ---------- ----------
P.clavaeceps        ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            160        170        180        190        200
P.seriolae          AGTAGTAGTA GTAGTACSTA CSATCAACAA TTAGCTATAC TMATAGYAAT
P.fulvidraconi      ---------- -------GTA TTAAAGAACA AGATTCGTGT ACGTGACTGT
P.pseudaspii        ---------- -------GTA TTAAAGAACA AGATTCGTGT ACGTGACTGT
P.cyprini           ---------- -------GTA TTATAAAACA --ACTCGTGT ACCCGCT---
P.sanguineus        ---------- -------GTA TTATAAAACA --ACTCGTGT ACGCGCTC--
P.clavaeceps        ---------- -------GTA TTAAAGAACA AGATTCGTGT ACGTGACTGT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            210        220        230        240        250
P.seriolae          ACTTTCATTT ATTACCATCT ATCTATCTAT CTATCTATCT ATCYATCAGC
P.fulvidraconi      GACTCTCTTG TCGGCTTTA- -------CTC CTCCTCCTGA TCA-------
P.pseudaspii        GTGACTCTTG TTGGCTTTA- ----TACTCC GTCTTCCTCC TCT----CAT
P.cyprini           ---------G TTTGCTAGC- -------TTA ATTAA----- ----------
P.sanguineus        -CAACTCGAA TTCACTCAC- -------TCA CTCGGCTAGT GATGA-----
P.clavaeceps        GTGACTCTTG TTGGCTTTAC TCCTTCTTCC TTCCTCCTCT TCTTGATCAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
P.seriolae          AGCTTGTGAT AGTAGTAGTA GTAGTGATGA TGATGATGAT AGTAGTAGTA
P.fulvidraconi      ------TGGT GGTGGTG--- GTGGTGGTGG TAAACAACAA ----------
P.pseudaspii        CCTCTCTGCT TGTTGTGT-G ATGATGGTGA TGAAGAAAGA AGGAAAGATG
P.cyprini           ---------- ---------- ATGATGATGA TAAAGCTGCT ----------
P.sanguineus        ------TGCT GGTGATG--- ATGATGATGA TGATGATGAT ----------
P.clavaeceps        CCTCTCTGCT TGTTGTG--- ATGATGGTGA TGAAGAAAGA ----------
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
P.seriolae          GTAGTAGTAT GCGTATACCT GATTTGATAA TATATTCATG TGTGTATGTA
P.fulvidraconi      --------GG ACAAGA---- GATGCG-TAC GATATACAAT CAGCTATCAT
P.pseudaspii        GTGGTGGTAA ACAACA-AGA GAGACG-TAC GATATACAAT CAGCTATCAT
P.cyprini           ---------G ACCGGC---- GAT-CG-TAC GACTAACAAT TGGCTATATG
P.sanguineus        ---------G ATGAGG---- AACGTG-TAC GACTAACAAT CGGCTATATG
P.clavaeceps        -TGGTGGTAG ACAAGAGAGA GAGACG-TAC GATATACAAT CAGCTATCAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
P.seriolae          TGACACACAC ACATATACAC ATTGATTTTG ACCGCGAAGT GTTGTGTATG
P.fulvidraconi      CGTCGT-CAT CATCATCAT- ---CATC-GT CCATTAGTGC AGCG-TGATG
P.pseudaspii        CGTCGT-CGT CGTCATCATC ATCCATTAGT GCAGT-GTGT GGTGCTGATG
P.cyprini           TTTTGCACAG CGCTCTACTA ATACTCTACA ACTAACGGGT ACCA-TTGTG
P.sanguineus        TGTGGT-TAC TGCTATATTA ----TATATA TTTA-TGTGT GC-G-TCTTG
P.clavaeceps        CAACGT-CAT CGTCATC--- ---CATTAGT GCAGT-GTGT GGTGCTGATG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
P.seriolae          TATGTTGATT GATTTGGTGT TTCTTGTGCA TACGCATTCG TCGTTTCGGC
P.fulvidraconi      -GTGTGTGTA TGTATG---- ---------- TGTGTGATAC CTGATTAGGC
P.pseudaspii        -GTGTGATTG -ATACGCGCG CGCGCGTG-- TGTGTGATAC CTGATTAGGC
P.cyprini           -TGCTTATTG GGTGTGTA-- ---------- -TTTGAGCGC CGACCTGTGC
P.sanguineus        -GTATTAGCA CATTGATA-- ---------- -ATTTAGCAT ATATATGTGC
P.clavaeceps        -GTGTGATTG -ATACG---- ---------- --TGTGATAC CTGATTAGGC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
P.seriolae          AACAAGAACG GTATGGTTGA CCGAATATGA TTATGGACGA GCATGTGCAT
P.fulvidraconi      -------AC- ---TAAAATG TT---TGTGG ACACGAAAAG ATGTGT-GAT
P.pseudaspii        GACGACTAC- ---TAAAATG TT---TGTGG ACGCGAAAAG ATGTGT-GAT
P.cyprini           ATATA---TA -TATACCTGA TT---TGATT ATTCGACATC ATGCAT-CGT
P.sanguineus        ACACCACCCG -TATACCTGT TT---TGGTA TTTTGACAT- GCGCAT-CAT
P.clavaeceps        GACGACGACG -ACTGAAATG TT---TGTGG ACGCGAAAAG ATGTGT-GAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
P.seriolae          ATTTGTGTGT GCGTTTGTGT TTAT--GATG ATGAAGAATT ATTATGTGTG
P.fulvidraconi      GCGCATGT-C ACGCACATTC TTTG---AGA ATTGTTGACT TGCTGGT---
P.pseudaspii        GCGCATGT-C ACGCACATTC TTTG---ATA ATTGTTGACT TGCTGGT---
P.cyprini           ----TCACAC TTGTACATTT TTGG-CAACA ACAATGGATA TGTTTGT---
P.sanguineus        GCGATTGTGT TTGTACGCGT TTGGACAACA ACAATGGATA TGTTTGT---
P.clavaeceps        GCGCATGT-C ACGCACATTC TTTG---ATA ATTGTTGACT TGCTGGTTGG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
P.seriolae          CTGCACTCAT ACATATTAAT TCGTTTCGTT TCGTTTCGTT TCATGAACGA
P.fulvidraconi      ---------- -GATTGTGAA C--------- ------AAAC ACACACACAA
P.pseudaspii        -----GATTG TGGTTGTGGA CAACACCATA CAAAACAAAC AAACACACAA
P.cyprini           ---------- -TGACG-AAA TG------AA CAACG-GAAT ACACACAAGA
P.sanguineus        ---------- -TGAGACGAA TGCTGCTTGT CGTTT-GGGC AAACACAACC
P.clavaeceps        TTGGTGATTG TGGTTGTGGA CAACACCATA CAAAA-AAAC AAACACACAA
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
P.seriolae          AACAAAAACA CATACGTA-C ACGAAAATAA GAGAGAACAC TATACAAGAT
P.fulvidraconi      CAACGGAATG GTTGTGTCTT GTTTGATTGT GGATGAATGT TGATTGGAAC
P.pseudaspii        CAACGGAATG GTTGTGG--T GTT--GTTG- G-ATGGATGT TGATTGAAAC
P.cyprini           TGTGGAGATG AGGA------ GAC--ATGT- GAGAGAAATC GAAAAGAAAG
P.sanguineus        AACAATGATT ATTATTT--T GAT--ATTTT GACCTCAACT CAGTCGAGAT
P.clavaeceps        CAACGGAATG GTTGTG---T GTT--GTTG- GGATTGATGT TGATTGAAAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
P.seriolae          GAATGCTGAA AAATGCGTAT CATCGTCGTC GTCGTCGCGC GCGCATACAT
P.fulvidraconi      AAACATACGC AAGCAG--GC AGCAGCAGCA GCA------A ACGATGAAAT
P.pseudaspii        AAACGCAAGC AAGAAGCAGC AGCAGCAGCA GCA------A ACGATGAAAT
P.cyprini           TGAC------ ---------- ---------- ---------- ----------
P.sanguineus        TACCCGCTGA ACTTAAGCAT AT-------- ---------- ----------
P.clavaeceps        AAACGCAAGC AAGCAGCAGC AGCAGCAGCA GCAGCAACAA ACGATGAAAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
P.seriolae          ATACAATGAA TGTACG-AAA AAATACATTT TTGACCTCAA CTCAGTCGAG
P.fulvidraconi      GAAGAATTAT TT-------- ---CATAATT TTGACCTCAA CTCAGTCGAG
P.pseudaspii        GT-GAATTGT TT-----CAT CATCATAATT TTGACCTCAA CTCAGTCGAG
P.cyprini           ---------- ---------- ---------- ---------- ----------
P.sanguineus        ---------- ---------- ---------- ---------- ----------
P.clavaeceps        GT-GAATTGT GTTTCATCAT CATCATAATT TTGACCTCAA CTCAGTCGAG
                      .
                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
P.seriolae          ATTACCCGCT GAATTTAAGC ATATAACTAA GCGGAGGAAA AGAAACTAAA
P.fulvidraconi      ATTACCCGCT GAACTTAAGC ATAT------ ---------- ----------
P.pseudaspii        ATTACCCGCT GAACTTAAGC ATAT------ ---------- ----------
P.cyprini           ---------- ---------- ---------- ---------- ----------
P.sanguineus        ---------- ---------- ---------- ---------- ----------
P.clavaeceps        ATTACCCGCT GAACTTAAGC ATAT------ ---------- ----------

                    .

P.seriolae          A
P.fulvidraconi      -
P.pseudaspii        -
P.cyprini           -
P.sanguineus        -
P.clavaeceps        -
```

Table 1. Major morphological characters observed among six *Philometra* species examined in the present study

Morphological keys	*Philometra* species					
	P. lateolabracis	*P. isaki*	*P. madai*	*P. sawara*	*P. nemipteri*	*P. sciaenae*
I. Host						
Order	Perciformes	Perciformes	Perciformes	Perciformes	Perciformes	Perciformes
Family	Lateolabracidae	Haemulidae	Sparidae	Scombridae	Nemipteridae	Sciaenidae
Genus	*Lateolabrax*	*Parapristipoma*	*Pagrus*	*Scomberomorus*	*Nemipterus*	*Pennahia*
Species	*japonicus*	*trilineatum*	*major*	*niphonius*	*virgatus*	*argentata*
II. Male						
Total length	2.07–2.73	2.62–3.26	3.92–5.94	2.44–3.38	2.94–4.02	1.46–2.62
Body width	0.040–0.052	0.068–0.090	0.096–0.132	0.045–0.070	0.068–0.092	0.040–0.076
Oesophagus length	0.255–0.363	0.246–0.308	0.43–0.49	0.252–0.460	0.423–0.478	0.245–0.390
Length of longer spicule	0.071–0.130	0.107–0.127	0.084–0.100	0.074–0.135	0.093–0.126	0.098–0.138
Length of shorter spicule	0.065–0.124	0.105–0.122	0.077–0.100	0.071–0.131	0.085–0.113	0.096–0.135
Length ratio of spicules	1 : 1.03–1.12	1 : 1.01–1.09	1 : 1.00–1.16	1 : 1.03–1.05	1 : 1.02–1.21	1 : 1.02–1.06
Gubernaculum length	0.050–0.093	0.07–0.09	0.064–0.084	0.040–0.076	0.073–0.101	0.045–0.074
Length ratio of gubernaculums to spicules	1 : 1.30–2.02	1 : 1.33–1.74	1 : 1.01–1.40	1 : 1.34–1.88	1 : 1.08–1.66	1 : 1.23–2.08
Shape of anterior end	Fig. 6 E (narrowest at nerve ring portion)	Fig. 8 E (narrowest at nerve ring portion)	Fig. 9 E (narrowest at anterior most end)	Fig. 11 E (narrowest at nerve ring portion)	Fig. 13 E (narrowest from nerve ring to anterior end portion)	Fig. 14 E (narrowest at anterior most end)
Size differences on posterior lobes	Fig. 6 I, Fig. 7 B (equal size)	Fig. 8 F (equal size)	Fig. 9 H , Fig. 10 E (unequal size)	Fig. 11 F (equal size)	Fig. 12 D, Fig. 13 F (unequal size)	Fig. 14 F, Fig. 15 B (equal size)
Others				With white spots along each testis		

Morphological keys	*Philometra* species					
	P. lateolabracis	*P. isaki*	*P. madai*	*P. sawara*	*P. nemipteri*	*P. sciaenae*
III. Female						
Total length	112–206	167–420	104–394	68–193	23–85	44–104
Body width	0.95–1.12	0.69–1.3	0.62–1.60	0.71–1.70	0.28–0.74	0.40–0.65
Oesophagus length	0.785–1.030	0.79–1.10	1.06–1.12	0.76–1.145	0.655–1.025	0.760–0.945
Bulb length x bulb width	0.065–0.105 x 0.099–0.130	0.058–0.115 x 0.078–0.120	0.090–0.102 x 0.110–0.158	0.110–0.165 x 0.116–0.180	0.085–0.120 x 0.078–0.110	0.088–0.115 x 0.072–0.088
Mouth shape	Triangular	Somewhat triangular	Round	Rounded	Round	Round
Mouth width	0.010	0.010	0.025	0.025	0.020–0.025	0.025
Shape of bulb formation of the anterior oesophagus	Fig. 6 D (slight inflation)	Fig. 8 D (slight inflation)	Fig. 9 D (highly inflated)	Fig. 11 D (highly inflated)	Fig. 13 D (highly inflated)	Fig. 14 D (moderately inflated)

Note: proportional values for each morphological characters, in relation to body length, were enclosed in parentheses

Table 2. Percentage similarities on ITS2 region between four individual specimens of *Philometra lateolabracis* and *Philometra madai* (gaps included)

Species	Individual specimens	No. bp	A	B	C	D	E	F	G	H
Philometra lateolabracis	A	418 bp	-	100	100	100	69.7	69.7	70.0	70.0
(from Japanese seaperch)	B	418 bp	100	-	100	100	69.7	69.7	70.0	70.0
	C	418 bp	100	100	-	100	69.7	69.7	70.0	70.0
	D	418 bp	100	100	100	-	69.7	69.7	70.0	70.0
Philometra madai	E	429 bp	69.7	69.7	69.7	69.7	-	100	99.7	99.7
(from red seabream)	F	429 bp	69.7	69.7	69.7	69.7	100	-	99.7	99.7
	G	429 bp	70.0	70.0	70.0	70.0	99.7	99.7	-	100
	H	429 bp	70.0	70.0	70.0	70.0	99.7	99.7	100	-

Legend: A – male *P. lateolabracis*; B – female *P. lateolabracis* No. 1; C – female *P. lateolabracis* No. 2; D – female *P. lateolabracis* No. 3; E – male *P. madai*; F – female *P. madai* No. 1; G – female *P. madai* No. 2; H – female *P. madai* No. 3.

Table 3. Percentage similarities on ITS2 region between *Philometra lateolabracis*, *Philometra madai* and other reported sequences of *Philometra* in GeneBank database (gaps included)

Species	Host	Location	No. bp	*P. lat*	*P. mad*	*P. fuj*	*P. cla*	*P.* sp.	GeneBank Acc. No.	Author
P. lateolabracis	Japanese seaperch	Tokyo Bay, Japan	418 bp	-					EF203081	Present study
P. madai	Red seabream	Seto Inland Sea, Japan	429 bp	69.7	-				EF203082	Present study
P. fujimotoi (Furuyama, 1932)	*Channa argus* (Cantor)	Upper Yangtze River, China	517 bp	34.8	35.5	-			DQ076690	Wu et al. 2005
P. clavaeceps Dogiel et Akhmerov, 1959	*Culter erythropterus* Basilewsky	Liangzi lake, Hubei, China	505 bp	41.8	43.7	34.3	-		DQ076696	Wu et al. 2005*
Philometra sp.	*Siniperca chuatsi* Basilewsky	China	731 bp	24.3	23.6	27.9	21.2	-	EF127904	Wu and Wang (unpublished)

* The reported host (*Cultrichthys erythropterus*) is a synonym of *Culter erythropterus.*

Table 4. Percentage similarities[a], number of base differences[b], [in square brackets], and *p-distance* values[b] (in parentheses) on 18S rDNA between presently and previously examined *Philometra* species

Species	No. bp	1	2	3	4	5	6	7	8	GeneBank Acc. No.	Author
1. P. lateolabracis	1631	-								FJ161972	Present study
2. P. madai	1672	97.0 [2] (0.003)	-							FJ161974	Present study
3. P. sawara	1499	91.7 [0] (0)	89.3 [2] (0.003)	-						FJ161973	Present study
4. P. nemipteri	1657	97.1 [2] (0.003)	96.4 [4] (0.006)	89.3 [2] (0.003)	-					FJ161975	Present study
5. P sciaenae	1701	95.7 [0] (0)	97.8 [2] (0.003)	88.0 [0] (0)	95.0 [2] (0.003)	-				FJ161971	Present study
6. P. fujimotoi	924	43.5 [5] (0.007)	45.3 [7] (0.010)	36.7 [5] (0.007)	44.3 [7] (0.010)	472 [5] (0.007)	-			DQ076680	Wu et al. 2005
7. P. clavaeceps	974	45.2 [17] (0.025)	47.1 [19] (0.028)	38.4 [17] (0.025)	454 [16] (0.024)	491 [17] (0.025)	91.6 [16] (0.024)	-		DQ076686	Wu et al. 2005
8. Philometra sp.	1749	85.9 [40] (0.060)	87.6 [42] (0.063)	79.5 [40] (0.060)	858 [42] (0.063)	895 [40] (0.060)	46.0 [41] (0.061)	47.7 [53] (0.079)	-	EF127905	Wu and Wang unpublished

[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).

Table 5. Percentage similarities[a], number of base differences[b] [in square brackets] and *p-distance* values[b] (in parentheses) on ITS2 region between presently and previously examined *Philometra* species

Species	No. of 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]	418	71.5 [68] (0.154)	63.7 [53] (0.137)	73.4 [49] (0.109)	ID							EF203081	present study
5. *Philometra madai* [g]	429	85.9 [36] (0.076)	74.1 [42] (0.101)	76.9 [66] (0.140)	70.5 [83] (0.181)	ID						EF203082	present study
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

[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).

Table 6. Percentage similarities, *p-distance* values (in parentheses) and number of base difference [in square brackets] on 18S rDNA between the current *Philometroides seriolae* specimen and reported sequences of *Philometroides* species, including *Philometra clavaeceps*, in GenBank database

Species	1	2	3	4	5	6	7
1. *Philometroides seriolae*	ID						
2. *Philometroides fulvidraconi*	45 (0.092) [74]	ID					
3. *Philometroides pseudaspii* (=*Philometroides ganzhounensis*)	45.3 (0.091) [73]	98.5 (0.007) [6]	ID				
4. *Philometroides cyprini*	46.4 (0.086) [69]	92.5 (0.046) [37]	93.2 (0.046) [37]	ID			
5. *Philometroides sanguineus* (=*Philometroides carassii*)	46.5 (0.080) [64]	92.7 (0.042) [34]	93.4 (0.042) [34]	98.7 (0.015) [12]	ID		
6. *Philometroides pseudorasbori*	46.4 (0.081) [65]	92.6 (0.044) [35]	93.3 (0.044) [35]	98.6 (0.016) [13]	99.8 (0.001) [1]	ID	
7. *Philometra clavaeceps*	45.1 (0.091) [73]	98.6 (0.007) [6]	99.3 (0) [0]	92.7 (0.046) [37]	92.9 (0.042) [34]	92.8 (0.044) [35]	ID

Table 7. Percentage similarities, *p-distance* values (in parentheses) and number of base difference [in square brackets] on ITS2 region between the current *Philometroides seriolae* specimen and reported sequences of *Philometroides* species, including *Philometra clavaeceps*, in GenBank database

Species	1	2	3	4	5	6
1. *Philometroides seriolae*	ID					
2. *Philometroides fulvidraconi*	24.8 (0.559) [165]	ID				
3. *Philometroides pseudaspii* (=*Philometroides ganzhounensis*)	28.3 (0.566) [167]	72.8 (0.122) [36]	ID			
4. *Philometroides cyprini*	16.4 (0.610) [180]	31.5 (0.519) [153]	29.4 (0.505) [149]	ID		
5. *Philometroides sanguineus* (=*Philometroides carassii*)	20.2 (0.529) [156]	34.7 (0.417) [123]	32.6 (0.410) [121]	52.2 (0.359) [106]	ID	
6. *Philometra clavaeceps*	27.8 (0.576) [170]	72.0 (0.125) [37]	85.7 (0.027) [8]	28.7 (0.502) [148]	33.0 (0.414) [122]	ID

Table 8. Number at developmental stages, prevalence, intensity and mean abundance of *Philometroides seriolae* in the body muscle of Japanese amberjack

Date of collection		ABW (kg)	No. of fish	Developmental stages of worms								Prevalence [f] (%)		Intensity [g] (Range)		Mean abundance [h]	
				Live			Sub total	Dead		Sub total	Total						
Year	Month			NG	SG	G		ND	R			Live	Dead	Live	Dead	Live	Dead
2004	July [a]	7.9	3	0	5	16	21	(-) [e]	(-) [e]	(-) [e]	21	100	100	2-9	(-) [c]	7	(-) [c]
	Aug [a]	6.7	1	0	0	9	9	(-) [e]	(-) [e]	(-) [e]	9	100	100	9	(-) [c]	9	(-) [c]
	Nov [a]	9.0	3	0	6	7	13	8	4	12	25	67	100	1-12	2-8	4.3	4
	Nov [b]	9.1	2	0	0	1	1	0	5	5	6	50	100	1	1-4	0.5	2.25
2005	Mar*	-	1	0	0	6	6	(-) [e]	(-) [e]	(-) [e]	6	100	100	6	(-) [c]	6	(-) [c]
	Apr [b,c]	6.3	5	1	0	5	6	5	3	8	14	60	100	1-5	3	1.2	1.6
	Nov [b]	-	1	0	7	0	7	0	4	4	11	100	100	7	4	7	4
2006	Jul [d]	6.4	5	1	2	20	23	9	8	17	40	80	100	3-8	1-7	4.6	3.4
	Aug [d]	7.5	5	1	1	48	50	6	21	27	77	100	100	2-20	4-9	10	5.4
	Sept [d]	5.2	1	0	0	6	6	0	1	1	7	100	100	6	1	6	1
	Oct [b,d]	7.3	8	1	5	5	11	14	37	51	62	75	100	1-5	1-11	1.4	6.4
	Nov [d]	8.5	5	1	3	7	11	7	45	52	63	80	100	1-6	3-15	2.2	10.4
2007	Feb [d]	8.0	5	0	0	10	10	8	64	72	82	60	100	1-7	10-20	2.0	14.4
	May [d]	8.6	5	0	2	16	18	3	36	39	57	100	100	2-7	4-11	3.6	7.8
	Jul [d]	7.0	5	0	0	11	11	1	27	28	39	80	100	1-4	2-11	2.2	5.6
Total			55	5	31	167	203	61	255	316	519						

a, Examined fish came from Oita Prefecture, Japan; b, Examined fish came from Saga Prefecture, Japan; c, Examined fish came from Kochi Prefecture, Japan; d, Examined fish came from Miyazaki Prefecture, Japan; * location unknown; e, Observed but not counted; f, Number of fish infected out of total fish examined; g, Number of *Philometroides seriolae* per fish infected; h, Number of *Philometroides seriolae* in all fish examined out of number of fish examined (= mean no. of *Philometroides seriolae* per fish examined); NG, nongravid; SG, semigravid; G, gravid; ND, newly dead; R, remnants.
Note: Batch 1 (2004), Batch 2 (2005), Batch 3 (2006 – 2007)

Table 9. Number of *Philometroides seriolae* at different locations in the body muscle of Japanese amberjack

Date of collection		No. of fish	Sites of infection																					
			Near the vertebral column							In the body muscle							Under the skin							
			Live			Subtotal	Dead		Subtotal	Live			Subtotal	Dead		Subtotal	Live			Subtotal	Dead		Subtotal	Total
Yr	Mo		NG	SG	G		ND	R		NG	SG	G		ND	R		NG	SG	G		ND	R		
2004	Nov	3	0	0	5	5	4	2	6	0	6	1	7	1	5	6	0	0	1	1	0	0	0	25
	Nov	2	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	4	4	6
2006	Jul	5	1	0	7	8	1	2	3	0	1	12	13	7	5	12	0	1	1	2	1	1	2	40
	Aug	5	0	0	18	18	1	8	9	0	1	27	28	5	12	17	1	0	3	4	0	1	1	77
	Sept	1	0	0	2	2	0	0	0	0	0	2	2	0	1	1	0	0	2	2	0	0	0	7
	Oct	8	1	1	2	4	3	4	7	0	3	3	6	11	29	40	0	1	0	1	0	4	4	62
	Nov	5	0	1	0	1	0	9	9	1	2	7	10	7	35	42	0	0	0	0	0	1	1	63
2007	Feb	5	0	0	2	2	2	17	19	0	0	8	8	6	38	44	0	0	0	0	0	9	9	82
	May	5	0	0	2	2	0	7	7	0	2	14	16	2	26	28	0	0	0	0	1	3	4	57
	Jul	5	0	0	4	4	0	6	6	0	0	7	7	1	16	17	0	0	0	0	0	5	5	39
Total		55	2	2	43	47	11	55	66	1	15	81	97	40	168	208	1	2	7	10	2	28	30	458

Locations of worms in the body musculature of Japanese amberjack were not examined in samples obtained in July and August of 2004, and March, April and November of 2005.

Table 10. Number of *Philometroides seriolae* in the lateral and cross sections

Date of collection		No. of fish examined	Cross-section							Longitudinal section						
			Epaxial muscle		Sub total	Hypaxial muscle		Sub total	Total	Left side		Sub total	Right side		Sub total	Total
Yr	Mo		S1	S2		S1	S2			S1	S2		S1	S2		
2006	Jul	5	13	7	20	12	8	20	40	11	9	20	14	6	20	40
	Aug	5	27	19	46	8	23	31	77	13	20	33	22	22	44	77
	Sept	1	4	1	5	2	0	2	7	3	0	3	3	1	4	7
	Oct	8	17	13	30	18	14	32	62	19	14	33	16	13	29	62
	Nov	5	26	13	39	10	14	24	63	22	13	35	14	14	28	63
2007	Feb	5	25	25	50	17	17	34	84	23	26	49	19	16	35	84
	May	5	15	20	35	9	13	22	57	11	15	26	13	18	31	57
	Jul	5	12	7	19	10	10	20	39	13	9	22	9	8	17	39
Total		39	139	105	244	86	99	185	429	115	106	221	110	98	208	429

Table 11. Prevalence, intensity and mean abundance of male and female *Philometra* species in the ovaries of their respective host fishes

Date of collection		No. of female fish examined	Host fishes	*Philometra* species	Prevalence (%)			Intensity (range)			Mean abundance		
Yr	Mo				Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex
2004	Jun	27	Silver croaker	*P. sciaenae*	22.2	59.3	70.4	1-12	1-19	1-19	1.0	4.4	5.4
	Jul	17			64.7	58.8	82.4	1-13	1-13	1-13	3.4	1.9	5.2
	Sept	51			35.3	35.3	52.9	1-12	1-9	1-12	1.3	1.2	2.5
	Oct	ND			5.0	25.0	30.0	1-5	1-4	1-5	0.2	0.4	0.6
	Nov	14			14.3	71.4	71.4	1-8	1-6	1-8	0.6	2.1	2.7
2005	Jan	19			5.3	5.3	10.5	1.0	1.0	1.0	0.05	0.05	0.11
2003	Apr	4	Japanese Spanish mackerel	*P. sawara*	25.0	100	100	2.0	1-3	1-3	0.5	2.2	2.8
	May	5			40.0	100	100	2-3	1-5	1-5	1.0	2.6	3.6
	Aug	5			40.0	100	100	1-4	2-5	1-5	1.0	2.8	3.8
2004	Jan	4			75.0	50	100	1-6	1-3	1-6	2.2	1.5	3.8
	Feb	13			53.8	100	100	1-2	1-3	1-3	0.7	1.8	2.5
2005	Jan	13			53.8	69.2	92.3	1-4	1.0	1-4	1.2	0.7	1.9

(Table 11, continued)

Date of collection		No. of female fish examined	Host fishes	*Philometra* species	Prevalence (%)			Intensity (range)			Mean abundance		
Yr	Mo				Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex
2001	Sept	32	Chicken grunt	*P.isaki*	6.2	56.2	56.2	1.0	1-2	1-2	0.1	1.1	1.1
	Oct	19			31.6	73.7	73.7	1-2	1-3	1-3	0.4	1.2	1.5
	Nov	19			15.8	84.2	84.2	1-3	1-4	1-4	0.3	1.5	1.8
	Dec	5			80.0	80.0	80.0	1-2	2.0	1-2	1.2	1.6	2.8
2002	Jan	11			0.0	81.8	81.8	0.0	1-5	1-5	0.0	1.7	1.7
	Jul	3			0.0	100	100	0.0	1-2	1-2	0.0	1.3	1.3
	Aug	1			0.0	100	100	0.0	1.0	1.0	0.0	1.0	1.0
	Sept	7			0.0	100	100	0.0	1-3	1-3	0.0	1.7	1.7
	Oct	17			17.6	64.7	64.7	1-2	1-5	1-5	0.2	0.9	1.2
	Nov	3			0.0	100	100	0.0	1-2	1-2	0.0	1.7	1.7
	Dec	5			0.0	100	100	0.0	1-3	1-3	0.0	2.0	2.0
2003	Jan	6			16.7	83.3	83.3	1.0	1-2	1-2	0.2	1.2	1.3
	Feb	7			14.3	85.7	85.7	1.0	1-4	1-4	0.1	1.7	1.9
	Mar	14			0.0	78.6	78.6	0.0	1-4	1-4	0.0	1.3	1.3

(Table 11, continued)

Date of collection		No. of female fish examined	Host fishes	*Philometra* species	Prevalence (%)			Intensity (range)			Mean abundance		
Yr	Mo				Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex	Male worm	Female worm	Regardless of sex
2004	Sept	42	Red seabream	*P. major*	2.4	16.7	19.0	1.0	1-2	1-2	0.02	0.19	0.21
2005	Apr	41			7.3	14.6	19.5	1.0	1-2	1-2	0.07	0.17	0.24
	May	38			7.9	13.2	18.4	1.0	1-2	1-2	0.08	0.18	0.26
	Jun	56			1.8	7.1	8.9	2.0	1.0	1-2	0.04	0.07	0.11
	Jul	55			9.1	10.9	18.2	1.0	1-3	1-3	0.09	0.16	0.25
	Aug	47			2.1	8.5	8.5	1.0	1-2	1-2	0.02	0.11	0.13
	Sept	22			0.0	13.6	13.6	0.0	1.0	1.0	0.0	0.14	0.14
	Oct	48			0.0	16.7	16.7	0.0	1.0	1.0	0.0	0.17	0.17
2003	Sept	10	Japanese seaperch	*P.lateola bracis*	30.0	10.0	30.0	1-20	1.0	1-20	2.0	0.1	2.1
	Oct	1			100	100	100	24.0	7.0	7-24	24.0	7.0	31.0
2004	Jan	16			100	81.2	100	15-870	1-20	1-870	152.6	8.1	160.7
2005	Sept	2	Golden threadfin bream	*P. nemipteri*	50	100	100	2.0	1-9	1-9	1	6	7

Table 12. Number of male and female *Philometra* species in the ovaries of seasonally examined silver croaker, chicken grunt and red seabream

Date of collection		No. of female fish examined	Host fishes	*Philometra* species	Total number of worms			
					Females			
Year	Month				Nongravid	Gravid	Dead	Male
2004	Jun	27	Silver croaker	*P. sciaenae*	118	0	4	27
	Jul	17			0	32	1	57
	Sept	51			13	40	11	68
	Oct	ND			0	6	3	3
	Nov	14			0	0	29	9
2005	Jan	19			1	0	0	2
2001	Sept	32	Chicken grunt	*P. isaki*	1	0	33	2
	Oct	19			0	0	22	7
	Nov	19			0	0	29	5
	Dec	5			0	0	8	6
2002	Jan	11			1	0	18	0
	Jul	3			0	1	3	0
	Aug	1			0	0	1	0
	Sept	7			0	0	12	0
	Oct	17			0	0	16	4
	Nov	3			0	0	5	0
	Dec	5			0	0	10	0
2003	Jan	6			0	0	7	1
	Feb	7			0	0	12	1
	Mar	14			0	0	18	0

(Table 12, continued)

Date of collection		No. of female fish examined	Host fishes	*Philometra* species	Total number of worms			
					Females			
Year	Month				Nongravid	Gravid	Dead	Male
2004	Sept	42	Red seabream	*P. madai*	0	0	8	1
2005	Apr	41			5	0	2	3
	May	38			5	1	1	3
	Jun	56			0	5	0	2
	Jul	55			0	10	1	5
	Aug	47			0	0	5	1
	Sept	22			0	0	3	0
	Oct	48			0	1	7	0

Note: Reported spawning season from fishbase (www.fishbase.org)
Silver croaker (April to August in Ariake Sound from September 2001 – August 2002)
Chicken grunt (June to August in Kimono-nada, Central Japan from 1978-1979)
Red seabream (May to June in Tokyo Bay [date unspecifie])

Table 13. Prevalence, intensity, and mean abundance of *Philometra* species infection in the testes and ovaries of their respective host fishes

Date of collection Yr	Mo	No. of male and female fish examined	Host fishes (*Philometra* sp.)	Prevalence (%)			Intensity (range)			Mean abundance		
				Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex
2004	April	37	Silver	60	52.9	56.8	1-7	1-4	1-7	1.9	1.0	1.7
	May	51	craoker	27.8	46.7	52.9	1-5	1-5	1-5	0.47	1.47	0.76
	Jun	36	(*P. sciaenae*)	91.8	78.9	83.4	3-56	1-40	1-56	6.4	6.3	6.4
	Jul	35		82.5	89.5	85.9	1-16	1-13	1-16	3.5	4.8	4.1
	Sept	36		60.1	44.8	54.2	1-13	1-12	1-13	2.9	2.7	2.8
	Oct	20		ND	ND	30.0	ND	ND	1-3	ND	ND	0.6
	Nov	32		100	71.4	87.5	1-63	1-8	1-63	18.0	2.7	11.3
2005	Jan	35		6.2	10.5	8.6	1.0	1.0	1	0.06	0.11	0.09
2003	Apr	4	Japanese	ND	ND		ND	ND		ND	ND	
	May	5	Spanish	ND	ND		ND	ND		ND	ND	
	Aug	5	mackerel	ND	ND		ND	ND		ND	ND	
2004	Jan	4	(*P. sawara*)	ND	ND		ND	ND		ND	ND	
	Feb	13		ND	ND		ND	ND		ND	ND	
2005	Jan	13		ND	ND		ND	ND		ND	ND	

ND - not determined

(Table 13, continued)

Date of collection Yr	Mo	No. of male and female fish examined	Host fishes (*Philometra* sp.)	Prevalence (%)			Intensity (range)			Mean abundance		
				Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex
2001	Sept	69	Chicken grunt (*P. isaki*)	89.2	56.2	73.9	1-2	1-2	1-2	1.8	1.1	1.5
	Oct	25		50.0	73.7	68.0	1	1-2	1-2	0.5	1.5	1.3
	Nov	23		50.0	84.2	78.3	1-2	1-3	1-3	0.8	1.7	1.6
	Dec	12		57.1	80.0	66.7	1-4	1-4	1-4	1.1	2.8	1.9
2002	Jan	33		95.4	81.8	90.9	1-4	1-5	1-5	1.9	1.7	1.8
	Jul	14		81.8	100	85.7	1-5	1-2	1-5	1.7	1.3	1.6
	Aug	5		75.0	100	80.0	1	1	1	0.8	1.0	0.8
	Sept	17		90.0	100	94.1	1-3	1-3	1-3	1.3	1.7	1.5
	Oct	22		40.0	64.7	59.1	1	1-7	1-7	0.4	1.2	1.0
	Nov	5		0.0	100	60.0	0	1-2	1-2	0.0	1.7	1.0
	Dec	13		87.5	100	92.3	1-3	1-3	1-3	1.5	2.0	1.7
2003	Jan	20		14.3	83.3	35.0	1	1-3	1-3	0.1	1.3	0.5
	Feb	12		60.0	85.7	75.0	1-2	1-4	1-4	0.8	1.9	1.4
	Mar	21		14.3	78.6	57.1	1	1-4	1-4	0.1	1.2	0.9

(Table 13, continued)

Date of collection Yr	Mo	No. of male and female fish examined	Host fishes (*Philometra* sp.)	Prevalence (%)			Intensity (range)			Mean abundance		
				Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex	Testes	Ovaries	Regardless of sex
2004	Sept	96	Red seabream (*P. madai*)	14.8	19.0	16.7	1-3	1-2	1-3	0.22	0.21	0.22
2005	Apr	81		5.0	22.0	13.6	1	1-2	1-2	0.05	0.24	0.15
	May	72		8.8	18.4	13.9	1	1-2	1-2	0.09	0.26	0.18
	Jun	88		3.1	8.9	6.8	1	1-2	1-2	0.03	0.11	0.08
	Jul	106		7.8	18.2	13.2	1	1-3	1-3	0.08	0.25	0.17
	Aug	76		3.4	8.5	6.6	1	1-2	1-2	0.03	0.13	0.09
	Sept	49		0.0	13.6	6.1	0	1	1	0.0	0.13	0.06
	Oct	82		8.8	16.7	13.4	1	1	1	0.09	0.17	0.13
2003	Sept	10	Japanese seaperch (*P. lateolabracis*)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Oct	1		ND	ND	ND	ND	ND	ND	ND	ND	ND
2004	Jan	16		ND	81.2	ND	ND	1-870	ND	ND	152	ND
2005	Sept	30	Golden threadfin bream (*P. nemipteri*)	85.7	100	86.7	1-23	5-9	1-23	7.5	7.0	7.0

Table 14. Host response against muscle-infecting *Philometroides seriolae* and gonad-infecting *Philometra* species

Philometrids	n	Body muscle		Gonads											
				Testis								Ovary			
				Spermatic duct				Seminiferous tubules							
				+		-		+		-		+		-	
		+	-	LW	DW	LW	DW	LW	DW	LW	DW	LW	DW	LW	DW
Philometroides seriolae	20	20/20	20/20*												
Philometra sciaenae	63			5/63	8/63	8/63	0/63	9/63	16/63	3/63	1/63	1/63	10/63	2/63	0/63
Philometra isaki	10				1/10				5/10		4/10				
Philometra madai	5											2/5	3/5		
Philometra nemipteri	24			6/24[a]	9/24	6/24		3/24[a]							

Note: +, with host response (accumulated inflammatory cells); -, no host response (no accumulation of inflammatory cells); LW, live worms; DW, dead worms; n, total number of specimens examined; *, around worms cuticle; **, within inflamed tissues only; a, these are hatched larvae;

Table 15. Identification of *Anisakis simplex* (s.s.) and *Anisakis pegreffii* from different host fishes in Japanese waters

Fish species	Locality[a]	Date of collection	No. of worms collected	PCR-RFLP identified *Anisakis* species		Hybrid genotype[b]
				A. simplex (s.s.)	*A. pegreffii*	
Oncorhynchus keta[c]	Chitose river, Hokkaido Prefecture (1)	October 2005 and October 2006	101	99	0	2
Theragra chalcogramma	Hokkaido Prefecture (2)	March 2007	10	10	0	0
Theragra chalcogramma	Between Aomori-Hokkaido (3)[d]	September 2006	20	18	0	2
Theragra chalcogramma	Off Iwate Prefecture (4)	April 2007	22	10	7	5
Scomberomorus niphonius	Off Kyoto Prefecture (5)	January 2005	6	0	6	0
Scomber australasicus	Off Kanagawa Prefecture (6)	June 2006	8	8	0	0
Scomber australasicus	Off Miyazaki Prefecture (7)	June 2006	5	5	0	0
Scomber japonicus	Off Kumamoto Prefecture (8)	October – November 2006	67	0	66	1
Seriola dumerili[e]	Off Kagoshima Prefecture (9)[d]	June 2005	10	0	9	1
Gadus macrocephalus	Between Aomori-Hokkaido (3)[d]	April 2007	19	8	8	3
Gadus macrocephalus	Off Iwate Prefecture (4)	May 2006	6	6	0	0

[a] Number coding (in parenthesis) after each locality is shown in Fig. 24; [b] Hybrid genotype showed same fragment pattern after *Hinf* I digestion as previously reported (Umehara et al. 2006, Abollo et al. 2003, Martín-Sánchez et al. 2005); [c] Wild salmons migrating upstream to spawn were examined; [d] A detailed locality was not specified; [e] *Anisakis*-infected juveniles imported from China and reared in net cages were examined.

Table 16. Percentage similarities on the nucleotide sequences[a] and translated amino acid sequences[b] (in parenthesis) of *mt*DNA *cox*2 gene between presently and previously examined *Anisakis* species

Species	1	2	3	4	5	6	7	8	9	10	Authors	GenBank Acc. No.
1. *A. simplex* (s.s.)	-										present study	EU413959
2. *A. pegreffii*	95.7 (100)	-									present study	EU413958
3. *A. simplex* (s.s.)	99.0 (100)	95.7 (100)	-								Valentini et al. (2006)	DQ116426
4. *A. pegreffii*	96.1 (100)	98.2 (100)	96.5 (100)	-							Valentini et al. (2006)	DQ116428
5. *A. simplex* C	95.1 (100)	93.9 (100)	94.2 (100)	93.2 (100)	-						Valentini et al. (2006)	DQ116429
6. *A. ziphidarum*	89.6 (97.6)	87.4 (97.6)	89.2 (98.1)	89.3 (98.1)	88.3 (98.1)	-					Valentini et al. (2006)	DQ116430
7. *A. typica*	87.6 (97.1)	87.6 (97.1)	88.0 (97.6)	88.3 (97.6)	88.3 (97.6)	87.2 (99.5)	-				Valentini et al. (2006)	DQ116427
8. *A. brevispiculata*	85.9 (95.9)	85.2 (95.9)	86.6 (94.7)	86.3 (94.7)	86.1 (94.7)	87.8 (96.6)	84.9 (96.2)	-			Valentini et al. (2006)	DQ116433
9. *A. paggiae*	88.3 (96.5)	87.6 (96.5)	88.2 (96.2)	88.2 (96.2)	88.0 (96.2)	89.8 (98.1)	86.4 (97.6)	88.8 (97.1)	-		Valentini et al. (2006)	DQ116434
10. *A. physeteris*	88.7 (95.9)	88.1 (95.9)	88.6 (96.2)	88.5 (96.2)	88.5 (96.2)	87.6 (98.1)	86.9 (97.6)	90.3 (98.6)	88.9 (98.1)	-	Valentini et al. (2006)	DQ116432

[a] percentage similarities computed using blastn program; [b] percentage similarities computed using blastp program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 17. Measurements of some morphological characters in *Anisakis simplex* (s.s.) and *Anisakis pegreffii* at different developmental stages

Morphological characters	Developmental stages							
	L3 larvae		L4 larvae		Adult			
	A. simplex (s.s.)	*A. pegreffii*	*A. simplex* (s.s.)	*A. pegreffii*	*A. simplex* (s.s.)		*A. pegreffii*	
					Male	Female	Male	Female
No. of specimens examined	112	71	16	4	8	28	11	10
Total body length	12.75-29.94	11.10-26.78	12.07-33.72	11.20-17.76	17.68-29.65	14.95-32.65	14.30-20.55	15.90-29.01
Maximum body width	0.45-0.75	0.38-0.60	0.56-1.00	0.38-0.79	0.74-1.10	0.80-1.33	0.68-1.03	0.72-1.15
Distance of nerve ring to anterior end	0.21-0.35	0.20-0.31	0.29-0.48	0.27-0.29	0.40-0.42	0.37-0.47	0.27-0.34	0.34-0.36
Length of oesophagus	1.18-2.58	1.04-2.11	2.23-3.18	1.55-1.85	2.33-2.95	2.75-3.40	2.00-2.95	2.27-3.07
Ventriculus length	0.90-1.50	0.50-0.78	0.95-1.33	0.50-0.60	1.10-1.40	0.90-1.42	0.50-0.70	0.60-0.70
Ventriculus width	0.13-0.31	0.12-0.27	0.18-0.32	0.18-0.22	0.24-0.32	0.20-0.34	0.21-0.24	0.25-0.27
Ratio between oesophagus and ventriculus length	1:0.9-1:2.3	1:1.5-1:3.1	1:2.0-1:2.5	1:3.08-1:3.1	1:2.1-1:2.6	1:1.9-1:2.8	1:3.1-1:4.2	1:3.5-1:4.7
Tail length	0.04-0.14	0.05-0.12	0.10-0.25	0.12-0.14	0.18-0.22	0.18-0.28	0.14	0.17-0.21
Mucro length	0.02-0.03	0.02-0.03	Absent	Absent	Absent	Absent	Absent	Absent

Table 18. PCR-RFLP fragment patterns in the ITS region (ITS1-5.8S rDNA-ITS2) of *Anisakis* species infecting Alaska pollock

Fragment patterns (*Anisakis* species)	No. of specimens examined				Fragment lengths		
	Off Rausu, Hokkaido Pref.	Off Miyako, Iwate Pref.	Total	Percentage	*Taq*I	*Hinf*I	*Hha*I
Pattern 1 (*A.simplex* s.s.)	104	87	191	91.0%	430–400	620–240	550–440
Pattern 2 (*A.pegreffi*)	6	5	11	5.2%	390–310–140	370–320–260	550–440
Pattern 3 (*A.brevispi-culata*)	0	3	3	1.4%	[280–260]–150–(60~90)	900	400–330–190
Pattern 4 (*Anisakis* sp.)	0	5	5	2.4%	[290–270]–135–(50~90)	900	530–430
Total	110	100	210	100%			

Note: values in square brackets represent two very close bands visible on gels as one thick band, whereas values in parentheses are approximate fragment sizes of minor and barely visible bands.

Table 19. Percentage similarities[a] on ITS region (ITS1-5.8S rDNA-ITS2) between presently and previously examined *Anisakis* species

Species/fragment pattern (No. of specimens examined	1	2	3	4	5	6	7	8	9	10	11	Authors	GenBank Acc. No.	Locality
1. Fragment pattern 1 (4 specimens)	ID											Present study	EU624342	Hokkaido and Iwate, Japan (Pacific)
2. Fragment pattern 2 (4 specimens)	99.8	ID										Present study	EU624343	Hokkaido and Iwate, Japan (Pacific)
3. Fragment pattern 3 (4 specimens)	87.9	88.1	ID									Present study	EU624344	Iwate, Japan (Pacific)
4. Fragment pattern 4 (4 specimens)	88.4	88.5	92.6	ID								Present study	EU624345	Iwate, Japan (Pacific)
5. *A. simplex* (s.s.)	100	99.8	87.9	88.4	ID							Umehara et al. (2008)	AB277822	Hokkaido, Japan (Pacific)
6. *A. pegreffii*	99.8	100	88.1	88.5	99.8	ID						Umehara et al. (2008)	AB277823	Kyushu, Japan (Pacific)
7. *A. brevispiculata*	88.0	88.2	99.5	91.5	88.0	88.2	ID					D'Amelio et al. [b]	AY826719	Florida (Atlantic)
8. *A. physeteris*	89.2	89.4	95.3	91.8	89.2	89.4	95.2	ID				Umehara et al. (2008)	AB277821	Kyushu, Japan (Pacific)
9. *A. physeteris*	89.1	89.2	95.2	91.7	89.1	89.2	94.9	99.8	ID			D'Amelio et al. [b]	AY826721	Italy
10. *A. physeteris*	87.3	87.5	94.9	91.2	87.3	87.5	94.8	99.5	99.3	ID		Kijewska et al. [b]	AY603530	Azores (Atlantic)
11. *A. physeteris*	88.4	88.5	92.2	99.9	88.4	88.5	91.5	91.7	91.5	91.1	ID	Abe and Yagi [b]	AB201789	Japan (Pacific)

[a] percentage similarities computed using the blastn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi); [b] unpublished

Table 20. Percentage similarities on the nucleotide sequences[a] and translated amino acid sequences[b] (in parentheses) of *mt*DNA *cox*2 gene between presently and previously examined *Anisakis* species

Species/fragment patterns (No. of specimens examined)	1	2	3	4	5	6	7	8	9	10	11	12	13	Authors	GenBank Acc. No.	Locality
1. Fragment pattern 1[c] (3 specimens)	ID													Present study	EU560907	NW Pacific Ocean (Hokkaido & Iwate, Japan)
2. Fragment pattern 1[c] (1 specimen)	97.6 (100)	ID												Present study	EU560911	NW Pacific Ocean (Hokkaido & Iwate, Japan)
3. Fragment pattern 2 (4 specimens)	94.9 (99.4)	95.7 (99.4)	ID											Present study	EU560908	NW Pacific Ocean (Hokkaido & Iwate, Japan)
4. Fragment pattern 3 (4 specimens)	85.5 (95.9)	86.6 (95.9)	85.8 (95.3)	ID										Present study	EU560909	NW Pacific Ocean (Iwate, Japan)
5. Fragment pattern 4 (4 specimens)	85.9 (96.5)	86.7 (96.5)	86.6 (95.9)	88.8 (98.4)	ID									Present study	EU560910	NW Pacific Ocean (Iwate, Japan)
6. *A. simplex* (s.s.)	98.0 (100)	99.0 (100)	95.9 (99.5)	86.6 (95.9)	87.0 (96.0)	ID								Valentini et al. (2006)	DQ116426	NE Pacific and Atlantic Ocean
7. *A. pegreffii*	95.3 (100)	96.1 (100)	99.7 (99.5)	86.3 (95.9)	87.4 (96.0)	96.5 (100)	ID							Valentini et al. (2006)	DQ116428	NE and SE Atlantic Ocean
8. *A. simplex* C	93.1 (100)	95.1 (100)	93.4 (99.5)	86.1 (95.9)	86.8 (96.0)	94.8 (100)	94.0 (100)	ID						Valentini et al. (2006)	DQ116429	NE Pacific coast and NE Pacific Ocean

(Table 20, continued)

Species/fragment patterns (No. of specimens examined)	1	2	3	4	5	6	7	8	9	10	11	12	13	Authors	GenBank Acc. No.	Locality
9. *A. ziphidarum*	89.1 (97.6)	89.7 (97.6)	88.5 (97.4)	87.1 (98.0)	88.2 (98.0)	89.8 (98.1)	89.2 (98.1)	88.5 (98.1)	ID					Valentini et al. (2006)	DQ116430	SE Atlantic Ocean
10. *A. typica*	87.5 (97.1)	87.6 (97.1)	87.5 (96.9)	84.1 (97.5)	86.2 (97.5)	88.7 (97.6)	88.5 (97.6)	88.5 (97.6)	88.3 (99.5)	ID				Valentini et al. (2006)	DQ116427	NW Atlantic Ocean, W Atlantic Ocean and Caribbean Sea
11. *A. brevispiculata*	85.0 (95.9)	85.9 (95.9)	86.2 (93.9)	98.8 (100)	88.6 (96.5)	86.2 (94.7)	85.8 (94.7)	85.6 (94.7)	87.9 (96.6)	84.6 (96.2)	ID			Valentini et al. (2006)	DQ116433	SE and W Atlantic Ocean
12. *A. paggiae*	87.1 (96.5)	88.4 (96.5)	88.0 (95.4)	89.2 (98.5)	95.8 (99.0)	87.9 (96.2)	87.6 (96.2)	87.6 (96.2)	90.0 (98.1)	86.7 (97.6)	89.4 (97.1)	ID		Valentini et al. (2006)	DQ116434	W Atlantic Ocean
13. *A. physeteris*	87.5 (95.9)	88.9 (95.9)	86.7 (95.4)	90.5 (100)	88.6 (98)	88.4 (96.2)	87.8 (96.2)	87.8 (96.2)	88.6 (98.1)	87.3 (97.6)	90.7 (98.6)	90.1 (98.1)	ID	Valentini et al. (2006)	DQ116432	Mediterranean Sea

[a] percentage similarities computed using the blastn program.
[b] percentage similarities computed using the blastp program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
[c] two different sequences were obtained from specimens with fragment pattern 1, whereas sequences of all four specimens examined in fragment pattern 2, 3 and 4 are similar.

Table 21. Percentage similarities on the nucleotide[a] and translated amino acid[b] sequences in parentheses (on the left lower side) and number of base differences[c] (on the right upper side) of the *mt*DNA *cox*2 gene between presently and previously examined *Anisakis* species

Species	Locality	GenBank Acc. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *A. peg**	China	EU933994	ID	11	3	11	12	10	4	22	30	64	64	77	70	62
2. *A. peg*	Japan1	EU560908	97.4 (99.4)	ID	10	0	3	1	11	22	35	62	65	77	66	65
3. *A. peg*	Japan2	EU413958	99.2 (100)	97.8 (99.4)	ID	10	11	9	1	23	33	65	64	78	69	63
4. *A. peg**	Mediterranean1	EU933995	97.6 (100)	99.8 (99.4)	98.0 (100)	ID	3	1	11	22	35	62	65	77	66	65
5. *A. peg**	Mediterranean2	EU933996	97.4 (100)	99.1 (99.4)	97.8 (100)	99.4 (100)	ID	2	10	21	35	61	62	74	67	62
6. *A. peg*	Atlantic	DQ116428	97.8 (100)	99.6 (99.5)	98.2 (100)	99.8 (100)	99.6 (100)	ID	10	21	35	61	64	76	67	64
7. *A. sim*	South Korea[d]	AY994157	99.0 (100)	97.8 (99.5)	99.8 (100)	97.8 (100)	98.0 (100)	98.2 (99.5)	ID	22	32	64	63	77	70	62
8. *A. sim* (ss)	Japan	EU413959	95.7 (100)	95.7 (99.4)	95.7 (100)	95.9 (100)	96.1 (100)	96.1 (100)	95.7 (100)	ID	26	53	64	73	69	59
9. *A. sim* C	Pacific	DQ116429	94.3 (100)	92.8 (99.5)	93.9 (100)	93.5 (100)	93.5 (100)	93.2 (100)	94.0 (99.5)	95.1 (100)	ID	66	67	79	70	66

(Table 21, continued)

Species	Locality	GenBank Acc. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
10. A. zip	Atlantic	DQ116430	87.4 (97.6)	88.5 (97.4)	87.4 (97.6)	88.0 (97.6)	88.2 (97.6)	89.2 (98.1)	88.5 (97.6)	89.6 (97.6)	88.3 (98.1)	ID	70	68	60	61
11. A. typ	Atlantic & Caribbean	DQ116427	87.4 (97.0)	87.5 (96.9)	87.6 (97.1)	87.4 (97.0)	88.0 (97.0)	88.3 (97.6)	88.5 (97.1)	87.6 (97.1)	88.3 (97.6)	87.2 (99.5)	ID	87	77	72
12. *A. bre*	Japan	EU560909	85.2 (95.8)	85.8 (95.3)	85.1 (95.9)	85.4 (95.8)	86.0 (95.8)	86.3(95.9)	85.9 (95.9)	85.9 (95.9)	86.1 (95.9)	87.2 (98.0)	84.9 (97.5)	ID	60	49
13. *A. pag*	Atlantic	DQ116434	87.6 (96.4)	88.0(95.4)	87.6 (96.5)	87.8(96.4)	88.4(96.4)	88.2(96.2)	87.8(95.7)	88.2 (96.5)	88.0 (96.2)	89.8 (98.1)	86.4 (97.6)	88.8 (98.5)	ID	61
14. *A. phy*	Mediterranean	DQ116432	88.1 (95.8)	88.3 (95.4)	88.1 (95.9)	87.7 (95.8)	88.3 (95.8)	88.5 (96.2)	88.7 (95.7)	88.7 (95.9)	88.5 (96.2)	87.6 (98.1)	86.9 (97.6)	90.5 (100)	88.9 (98.1)	ID

a, Similarity computed using blastn; b, Similarity computed using blastp (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi); c, computed using pairwise analysis in MEGA4. All positions containing gaps and missing data were eliminated from the dataset (pairwise deletion); d, Origin of host fish not reported; *, present study. *Abbreviations*: *A. peg* - *A. pegreffii*; *A. sim* (ss) – *A. simplex* (s.s.); *A. sim*C – *A. simplex* C; *A. zip* – *A. ziphidarum*; *A. typ* – *A. typica*; *A. bre* – *A. brevispiculata*; *A. pag* – *A. paggiae*; *A. phy* – *A. physteris*.

Table 22. Distribution of presently and previously examined *Anisakis* species in Japanese waters

References (Code in Fig. 34)	Host fishes	Locality	No. of worms examined	Number of worms (percentage %)					
				A. simplex (s.s.)	*A. pegreffii*	*Anisakis* sp. (*Anisakis Type II)*	*A.brevispiculata*	*A. physeteris*	Hybrid genotypes
Umehara et al. 2008 (A)	*Pleurogrammus azonus*	Hokkaido*	10	10 (100%)	0	0	0	0	0
	Scomber japonicus	Kyushu *	11	0	10 (90.9%)	0	0	1 (9.1%)	0
Umehara et al. 2006 (B)	*Pleurogrammus azonus*	Hokkaido*	20	20 (100%)	0	0	0	0	0
	Theragra chalcogramma		19	19 (100%)	0	0	0	0	0
	Scomber japonicus		16	16 (100%)	0	0	0	0	0
	Hypomesus pretiosus japonicus		10	10 (100%)	0	0	0	0	0
	Scomber japonicus	Fukuoka	38	0	37 (97.4%)	0	0	0	1 (2.6%)
Abe et al. 2005 (C)	*Gadus macrocephalus*	Hokkaido*	26	24 (92.3%)	2 (7.7%)	0	0	0	0

*exact location not specified

(Table 22, continued)

References (Code in Fig. 34)	Host fishes	Locality	No. of worms examined	Number of worms (percentage %)					
				A.simplex (s.s.)	*A.pegreffii*	*Anisakis* sp. (*Anisakis Type II*)	*A.brevispiculata*	*A. physeteris*	Hybrid genotypes
This study See Section 1, Subchapter 1, Chapter 3 (D)	*Oncorhynchus keta*	Hokkaido	101	99 (98%)	0	0	0	0	2 (2%)
	Theragra chalcogramma	Hokkaido	10	10 (100%)	0	0	0	0	0
	Theragra chalcogramma	Between Aomori-Hokkaido	20	18 (90%)	0	0	0	0	2 (10%)
	Gadus macrocephalus	Off Iwate	22	10 (45.3%)	7 (31.8%)	0	0	0	5 (22.7%)
	Scomberomorus niphonius	Off Kyoto	6	0	6 (100%)	0	0	0	0
	Scomber australasicus	Off Kanagawa	8	8 (100%)	0	0	0	0	0
	Scomber australasicus	Miyazaki	5	5 (100%)	0	0	0	0	0
	Scomber japonicus	Off Kumamoto	67	0	66 (98.5%)	0	0	0	1 (1.5%)
	Gadus macrocephalus	Between Aomori-Hokkaido	19	8 (42.1%)	8 (42.1%)	0	0	0	3 (15.8%)
	Gadus macrocephalus	Off Iwate	6	6 (100%)	0	0	0	0	0
This study See Section 2, Subchapter1, Chapter 3 (E)	*Theragra chalcogramma*	Off Rausu, Hokkaido	110	104 (94.5%)	6 (5.5%)	0	0	0	0
	Theragra chalcogramma	Off Miyako, Iwate	100	87 (87%)	5 (5%)	5 (5%)	3 (3%)	0	0

(Table 22, continued)

References (Code in Fig. 34)	Host fishes	Locality	No. of worms examined	Number of worms (percentage %)					
				A.simplex (s.s.)	*A.pegreffii*	*Anisakis* sp. (*Anisakis Type II)*	*A.brevispiculata*	*A. physeteris*	Hybrid genotypes
Other examined host fishes (present study) (F)	*Takifugu poecilonotus*	Off Shimanoseki, Yamaguchi	1	0	1 (100%)	0	0	0	0
	Arctoscopus japonicus	Fukui	1	0	1 (100%)	0	0	0	0
	Hippoglossoides dubius	Fukui	28	0	28 (100%)	0	0	0	0
	Scomber australasicus	Off Nishinomote, Kagoshima	47	32 (68.1%)	15 (31.9%)				
	Scomber japonicus	Nagasaki	50		50 (100%)				
	Theragra chalcogramma	Off Rausu, Hokkaido	50	50 (100%)					
	Gadus macrocephalus	Off Iwate prefecture	50	50 (100%)					
Total number			851	586	242	5	3	1	14
Percentage				68.9%	28.4%	0.59%	0.35%	0.12%	1.6%

Table 23. Recovery of *Anisakis simplex* (s.s.) from the organs of rainbow trout following oral administration of 20 larvae (day 0)

Days post-infection (dpi)	No. of infected fish/No of fish examined	Mean number of worms recovered (range of worms recovered) [percentage (%) of worms recovered relative to the initial infection]													
		Lumen				Wall			Body cavity			Body muscle			Total recovery
		Sto	Int	PyC	Sub-total	Sto	Int	Sub-total	PyC	Oth	Sub-total	Ent	Ins	Sub-total	
3	3/3	11.3 (10-12) [56.7]	6.0 (5-7) [30]	1.3 (1-2) [6.7]	18.7 (18-19) [93.3]	0	0	0	1.3 (1-2) [6.7]	0	1.3 (1-2) [6.7]	0	0	0	20.0 (20) [100]
7	3/3	4.0 (3-5) [20]	4.0 (3-5) [20]	1.7 (1-2) [8.3]	9.7 (9-10) [48.3]	0	0	0	5.3 (5-6) [26.7]	3.7 (3-4) [18.3]	9.0 (8-10) [45]	1.3 (1-2) [6.7]	0	1.3 (1-2) [6.7]	20.0 (20) [100]
14	3/3	1.3 (0-3) [6.7]	1.7 (0-4) [8.3]	0.3 (0-1) [1.7]	3.3 (1-6) [16.7]	2.3 (1-4) [11.7]	0.3 (0-1) [1.7]	2.7 (1-5) [13.3]	5.0 (3-6) [25]	2.7 (1-4) [13.3]	7.7 (4-10) [38.3]	0.7 (0-2) [3.3]	1.3 (0-2) [6.7]	2.0 (2) [10]	15.7 (14-17) [78]
21	3/3	0	0	0	0	0	0	0	4.3 (2-6) [21.7]	2.3 (0-4) [11.7]	6.7 (2-10) [33.3]	1.3 (0-3) [6.7]	3.7 (2-5) [18.3]	5.0 (3-7) [25]	11.7 (7-15) [58]
28	3/3	0	0	0	0	0	0	0	3.7 (3-4) [18.3]	2.0 (1-3) [10]	5.7 (5-6) [28.3]	1.3 (0-3) [6.7]	3.3 (3-4) [16.7]	4.7 (4-6) [23.3]	10.3 (9-12) [52]
35	3/3	0	0	0	0	0	0	0	2.0 (1-4) [10]	0.3 (0-1) [1.7]	2.3 (1-4) [11.7]	1.3 (1-2) [6.7]	3.3 (3-4) [16.7]	4.7 (4-5) [23.3]	7.0 (6-8) [35]

Legend: Sto: stomach; Int: intestine; PyC: pyloric caeca; Oth: other visceral organs and mesenteries; Ent: entering the muscles wherein the posterior end is still in the body cavity area; Ins: entire larvae is inside the muscle

Table 24. Recovery of *Anisakis pegreffii* from the organs of rainbow trout following oral administration of 40 larvae (day 0)

Days post-infection (dpi)	No. of infected fish/No of fish examined	Mean number of worms recovered (range of worms recovered) [percentage (%) of worms recovered relative to the initial infection]															
		Lumen						Wall			Body cavity			Body muscle			Total recovery
		Sto		Int		PyC	Sub-total	Sto	Int	Sub-total	PyC	Oth	Sub-total	Ent	Ins	Sub-total	
		Alive	Dead	Alive	Dead												
3	3/3	13.0 (9-21) [32.5]	0	5.3 (2-8) [13.3]	0	0	18.3 (11-27) [45.8]	0.3 (0-1) [0.8]	0	0.3 (0-1) [0.8]	0.3 (0-1) [0.8]	1.0 (0-2) [2.5]	1.3 (1-2) [3.3]	0	0	0	20.0 (13-28) [50]
7	3/3	0	29.0 (12-38) [72.5]	1.7 (1-2) [4.2]	1.7 (0-4) [4.2]	0	32.3 (17-40) [80.8]	0	0	0	0	0	0	0	0	0	32.3 (17-40) [80.8]
14	3/3	0	3.3 (1-6) [8.3]	0	0	0	3.3 (1-6) [8.3]	0	0	0	0.3 (0-1) [0.8]	0	0.3 (0-1) [0.8]	0	0	0	3.7 (1-7) [9.2]
21	3/3	0	0.3 (0-1) [0.8]	0	0.7 (0-2) [1.7]	0	1.0 (0-2) [2.5]	0	0	0	0.3 (0-1) [0.8]	1.0 (0-2) [2.5]	1.3 (0-2) [3.3]	0	0	0	2.3 (2-3) [5.8]
28	3/3	0	0	0	0	0	0	0	0	0	0.3 (0-1) [0.8]	0	0.3 (0-1) [0.8]	0	0	0	0.3 (0-1) [0.8]
35	3/3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Legend: Sto: stomach; Int: intestine; PyC: pyloric caeca; Oth: other visceral organs and mesenteries; Ent: entering the muscles wherein the posterior end is still in the body cavity area; Ins: entire larvae is inside the muscle

Table 25. Recovery of *Anisakis simplex* (s.s.) from the organs of Japanese flounder following oral administration of 15 larvae (day 0)

Days post-infection (dpi)	No. of infected fish/No of fish examined	Mean number of worms recovered (range of worms recovered) [percentage (%) of worms recovered relative to the initial infection]								
		Body cavity				Body muscle			Vascular cavity	Total recovery
		Sto	Int	Oth	Sub-total	Ent	Ins	Sub-total		
3	3/3	2.3 (2-3) [15.6]	3 (2-4) [20]	3 (1-5) [20]	8.3 (1-5) [55.6]	3 (2-4) [20]	0	3 (2-4) [20]	1 (1) [2.2]	11.7 (1-5) [77.8]
7	3/3	0	3 (3) [20]	1.7 (0-3) [11.1]	4.7 (0-3) [31.1]	1 (1) [6.7]	1.3 (1-2) [8.9]	2.3 (1-2) [15.6]	0	7.0 (1-3) [46.7]
14	3/3	0.6 (0-2) [4.4]	5 (3-7) [33.3]	1.7 (1-3) [11.1]	7.3 (0-7) [48.9]	1.7 (1-2) [11.1]	2 (1-3) [13.3]	3.7 (1-3) [24.4]	0	11.0 (1-7) [73.3]
21	3/3	0.3 (0-1) [2.2]	3 (2-4) [20]	1 (0-2) [6.7]	4.4 (0-4) [28.9]	1.3 (1-2) [8.9]	0.7 (0-1) [4.4]	2 (0-2) [13.3]	0	6.3 (1-4) [42.2]
28	3/3	0	2.7 (2-3) [17.8]	0	2.7 (2-3) [17.8]	3 (2-4) [20]	3 (2-4) [20]	6 (2-4) [40]	0	8.7 (2-4) [57.8]
35	3/3	0	3.7 (2-6) [24.4]	0	3.7 (2-6) [24.4]	0.3 (0-1) [2.2]	7.3 (5-10) [48.9]	7.6 (1-10) [51.1]	0	11.3 (1-10) [75.6]

Legend: Sto: surrounding stomach; Int: surrounding intestine; Oth: other visceral organs and mesenteries; Ent: entering the muscles wherein the posterior end is still in the body cavity area; Ins: entire larvae is inside the muscle

Table 26. Recovery of *Anisakis pegreffii* from the organs of Japanese flounder following oral administration of 50 larvae (day 0)

Days post-infection (dpi)	No. of infected fish/No of fish examined	Mean number of worms recovered (range of worms recovered) [percentage (%) of worms recovered relative to the initial infection]								
		Body cavity				Body muscle			Vascular cavity	Total recovery
		Sto	Int	Oth	Sub-total	Ent	Ins	Sub-total		
3	5/5	4.8 (0-9) [9.6]	13 (0-22) [26]	8.6 (6-11) [17.2]	26.4 (0-22) [52.8]	0	0	0	0	26.4 (0-22) [52.8]
7	5/5	0	13.6 (9-20) [27.2]	3.8 (0-7) [7.6]	17.4 (0-20) [34.8]	0	0	0	0	17.4 (0-20) [34.8]
14	5/5	0	12 (9-16) [24]	1.2 (0-3) [2.4]	13.2 (0-16) [26.4]	0	0	0	0	13.2 (0-16) [26.4]
21	5/5	1.2 (0-3) [2.4]	22.6 (14-30) [45.2]	1 (0-2) [2]	24.8 (0-30) [49.6]	0	0	0	0	24.8 (0-30) [49.6]
28	5/5	0.8 (0-4) [1.6]	18.8 (12-26) [37.6]	0.2 (0-1) [0.4]	19.8 (0-26) [39.6]	0	0	0	0	19.8 (0-26) [39.6]
35	5/5	0.6 (0-3) [1.2]	19.8 (17-22) [39.6]	1.4 (0-2) [2.8]	21.8 (0-22) [43.6]	0	0	0	0	21.8 (0-22) [43.6]

Legend: Sto: surrounding stomach; Int: surrounding intestine; Oth: other visceral organs and mesenteries; Ent: entering the muscles wherein the posterior end is still in the body cavity area; Ins: entire larvae is inside the muscle

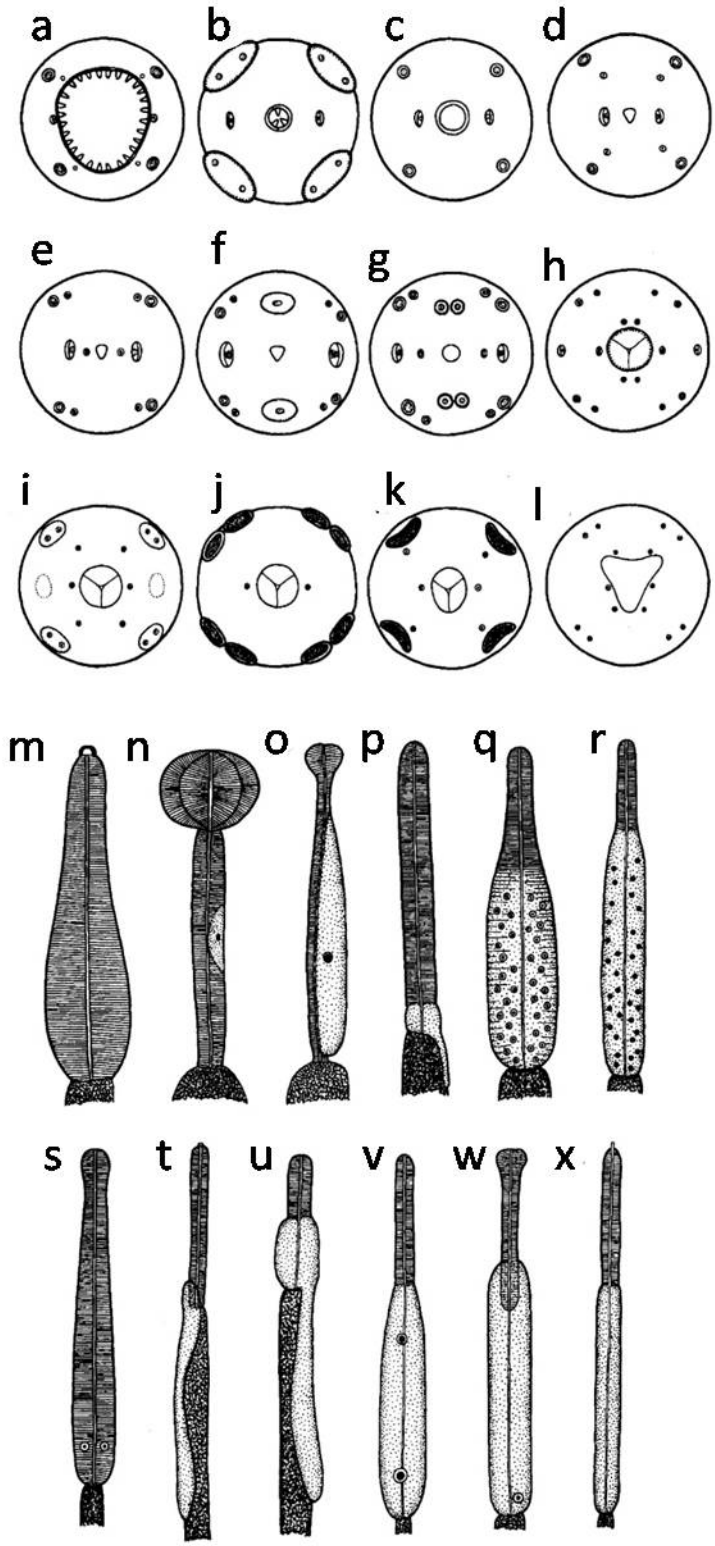


Figure 1. Variations in the arrangement of cephalic papillae (apical view, schematized) (a-l) and variability in the structure of oesophagus (schematized) (m-x) in fish dracunculoids. a – *Anguillicola*; b – *Molnaria*; c – *Skrjabillanus*; d – *Lucionema*; e – *Granulinema*; f – *Daniconema*; g – *Histodytes*; h – *Dentiphilometra*; i – *Philometra* (*P. ovata*); j – *Philometra* (*P. salgadoi*); k – *Philometra* (*P. ocularis*); l – *Clavinema*; m – *Anguillicola*; n – *Clavinema*; o – *Philometra*; p – *Ichthyofilaria*; q – *Philonema*; r – *Histodytes*; s – *Lucionema*; t – *Skrjabillanus*; u – *Travassosnema*; v – *Mexiconema*; w – *Syngnathinema*; x – *Esocinema* (Moravec 2004).

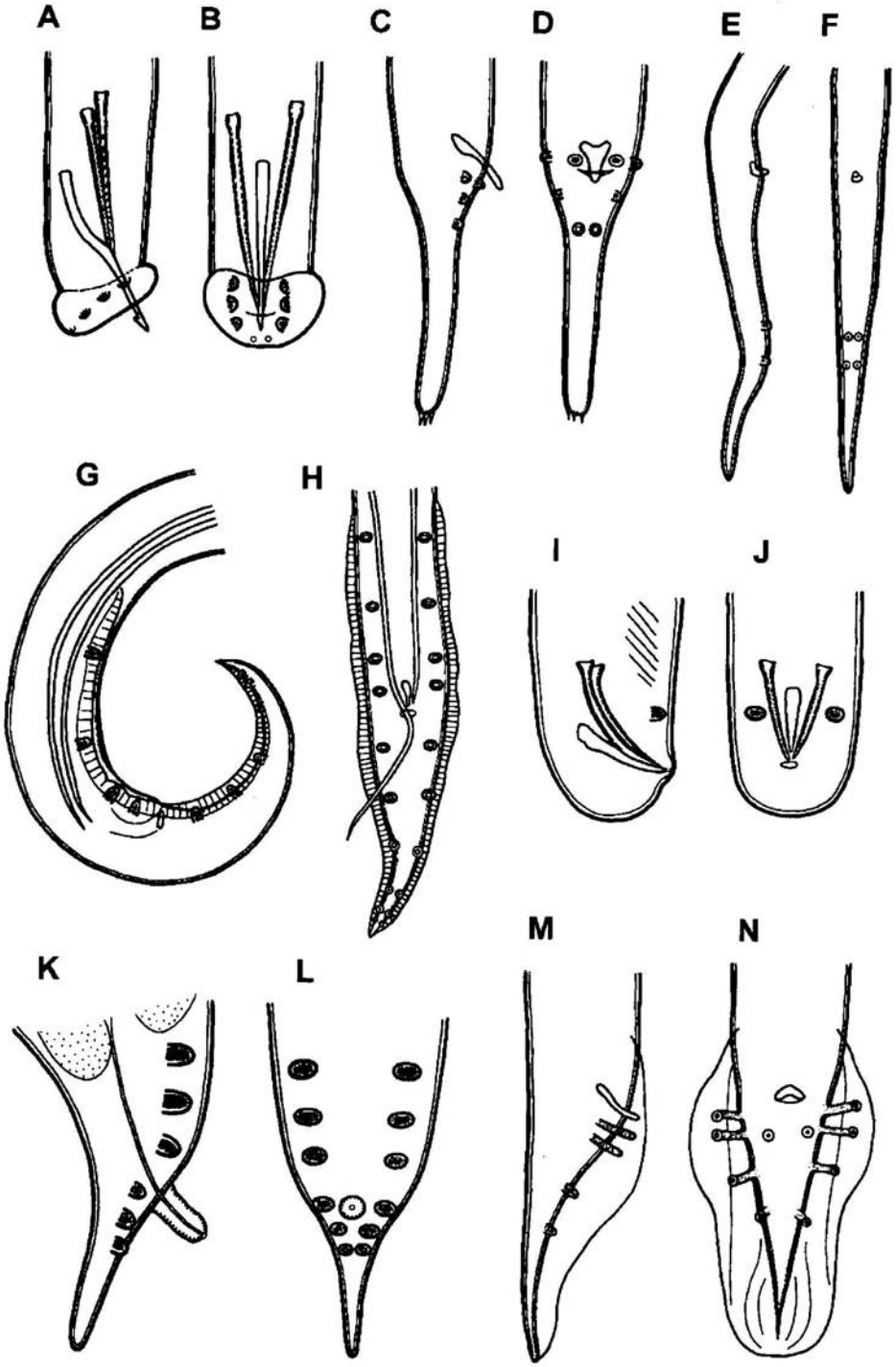


Figure 2. Variations in the structure of the male caudal end in some fish dracunculoinds (lateral and ventral views, schematized) . A,B – *Philometra*; C,D – *Mexiconema*; E,F – *Lucionema*; G,H – *Guyanema*; I,J – *Neophilometroides*; K,L – *Anguillicola*; M,N – *Skrjabillanus* (Moravec 2004).

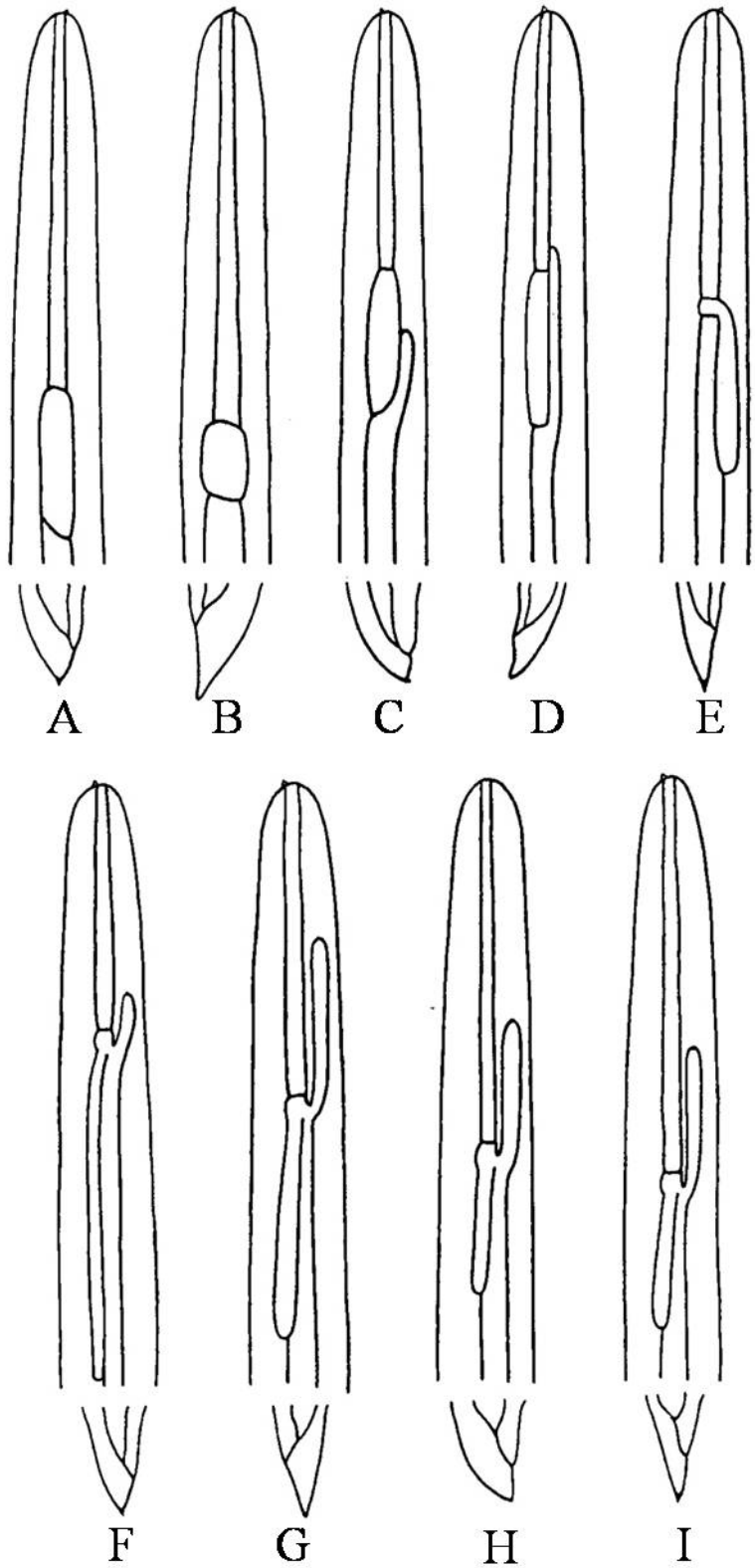


Figure 3. Schema of head and tail in distinguishing larval nematodes under anisakid groups. A – *Anisakis* (Type I); B – *Anisakis* (Type II); C – *Terranova* (Type A); D – *Terranova* (Type B); E – *Raphidascaris* or *Raphidascaroides* sp.; F – *Contracaecum* (Type A); G – *Contracaecum* (Type B); H – *Contracaecum* (Type C); I – *Contracaecum* (Type D) (Koyama et al. 1969).

Family: Lateolabracidae (Asian seaperches)
Genus: *Lateolabrax*
Species: *japonicus* (Cuvier, 1828)
Fishbase name: Japanese seaperch
Japanese name: Suzuki

Family: Sparidae (Porgies)
Genus: *Pagrus*
Species: *major* (Temminck et Schlegel, 1843)
Fishbase name: Red seabream
Japanese name: Madai

Family: Haemulidae (grunts)
Genus: *Parapristipoma*
Species: *trilineatum* (Thunberg, 1793)
Fishbase name: Chicken grunt
Japanese name: Isaki

Family: Scombridae (mackerels, tunas, bonitos)
Genus: *Scomberomorus*
Species: *niphonius* (Cuvier, 1832)
Fishbase name: Japanese Spanish mackerel
Japanese name: Sawara

Family: Nemipteridae (threadfin breams, whiptail breams)
Genus: *Nemipterus*
Species: *virgatus* (Houttuyn, 1782)
Fishbase name: Golden threadfin bream
Japanese name: Itoyoridai

Family: Sciaenidae (Drums or croaker)
Genus: *Pennahia*
Species: *argentatus* (Houttuyn, 1782)
Fishbase name: Silver croaker
Japanese name: Shiroguchi

Family: Carangidae (Jacks and pompanos)
Genus: *Seriola*
Species: *quinqueradiata* Temminck et Schlegel, 1845
Fishbase name: Japanese amberjack
Japanese name: Buri

Figure 4. Host fishes of examined philometrid nematodes.

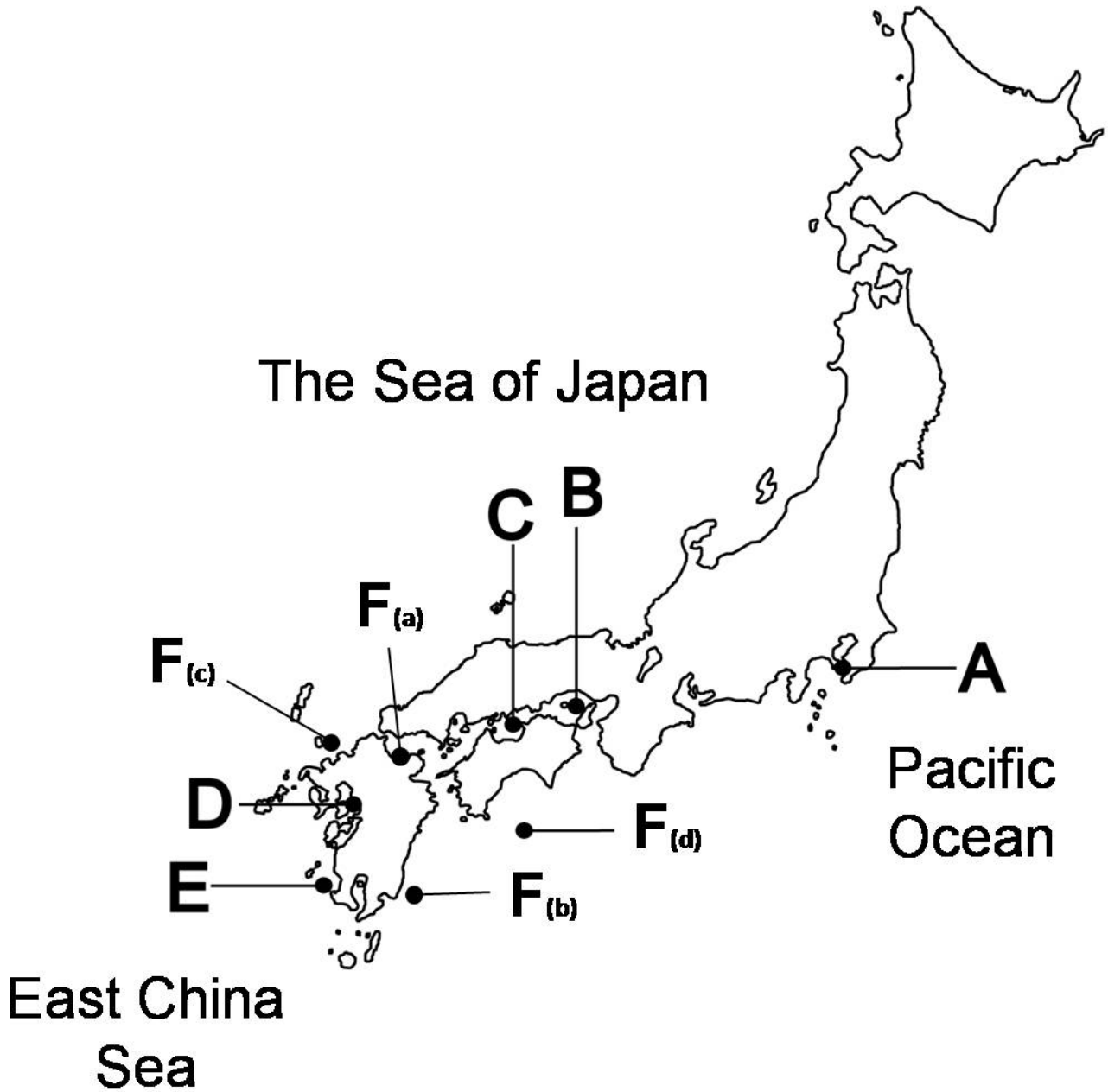


Figure 5. Geographical locations of examined host fish infected with philometrid nematodes in Japan. (**A** – Chiba Prefecture (Japanese seaperch), **B** – Hyogo Prefecture (Japanese Spanish mackerel); **C** – Ehime Prefecture (red seabream); **D** – Nagasaki Prefecture (silver croaker); **E** – Kagoshima Prefecture (golden threadfin bream); **F** – Oita (a), Miyazaki (b), Saga (c) and Kochi (d) Prefectures (Japanese amberjack).

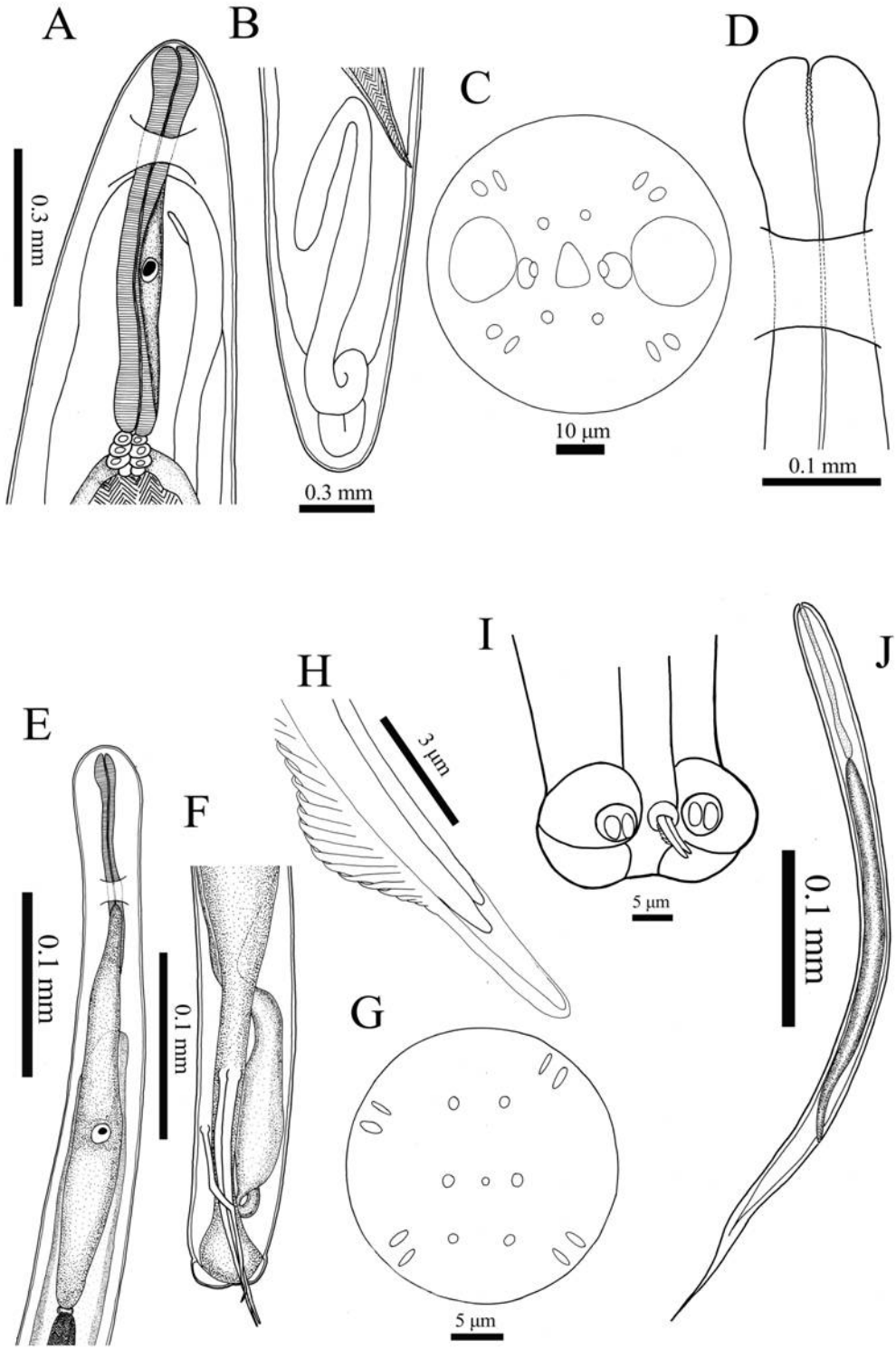


Figure 6. *Philometra lateolabracis* (Yamaguti, 1935). **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – slightly inflated anterior end of the oesophagus in female; **E, F** – anterior and posterior end of male; **G** – cephalic end of male, apical view; **H** – distal ends of the spicules and gubernaculum; **I** – posterior end of male showing equal-sized subdivided smaller lobes, tail papillae, spicules and gubernaculum; **J** – first-stage larvae.

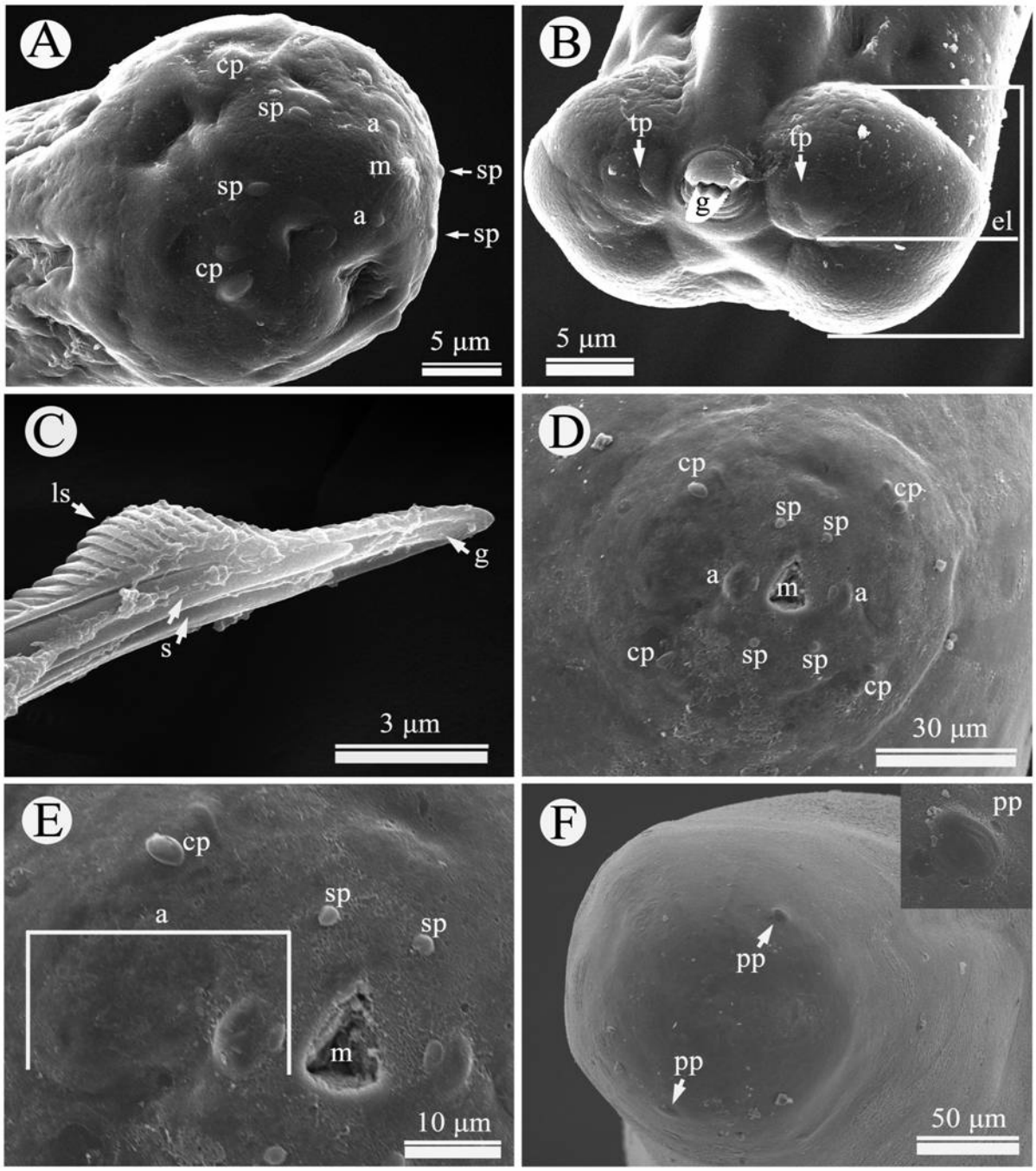


Figure 7. Scanning electron micrograph of *Philometra lateolabracis* (Yamaguti, 1935). **A** – cephalic end of male; **B** – posterior end of male; **C** – distal ends of spicules and gubernaculum; note the lamellate-like structures on the gubernaculum; **D** – cephalic end of female; **E** – closer view of the amphids; **F** – closer view of the lateral papilla-like projections. *Abbreviations*: a – amphid; cp – paired cephalic papillae of outer ring; el – equal-sized lobes; g – gubernaculum; sp – single papilla of inner circle; ls – lamellate-like structures; m – mouth; pp – lateral papilla-like projection; s – spicules; tp – tail papillae.

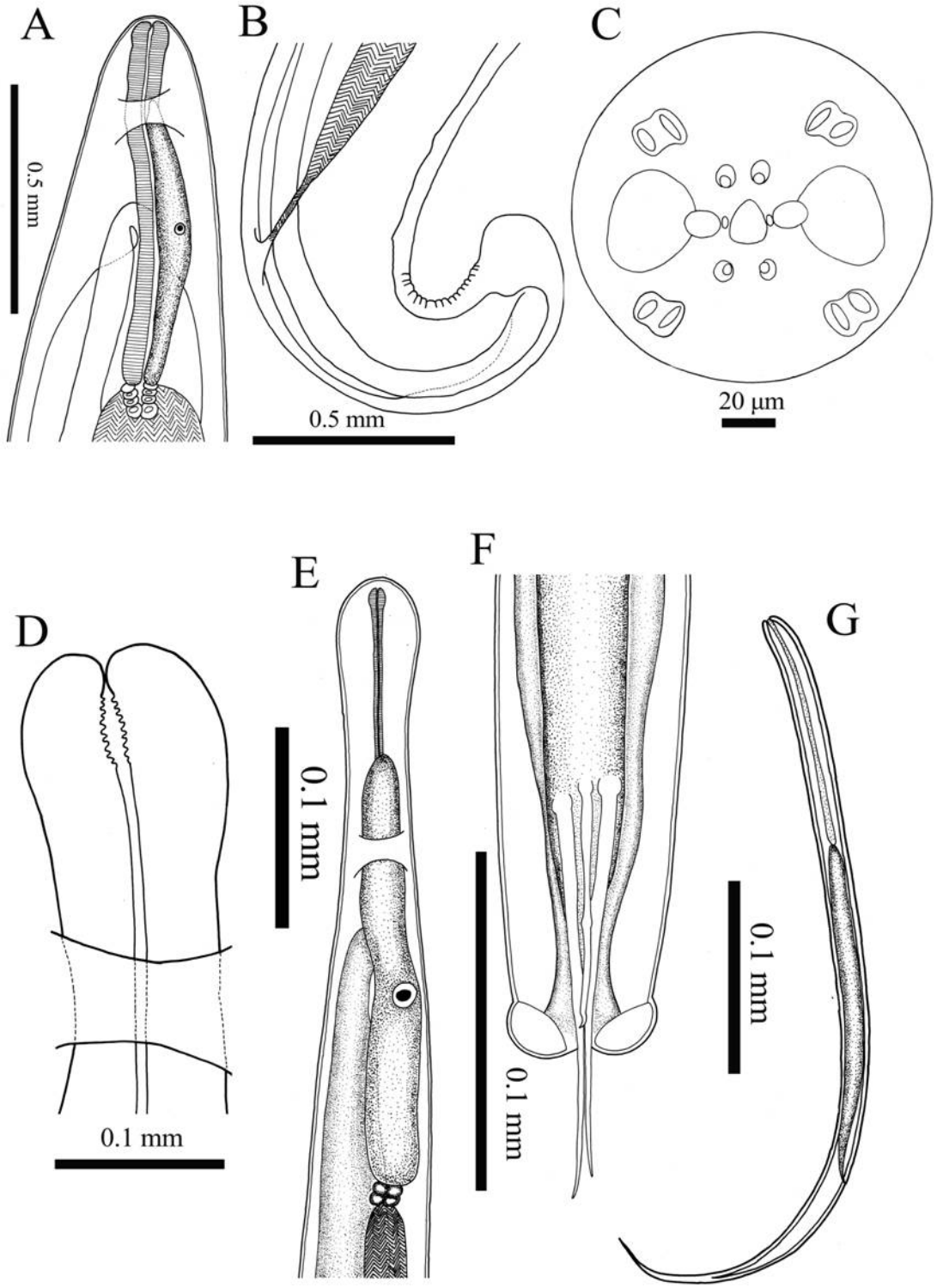


Figure 8. *Philometra isaki* Quiazon, Yoshinaga et Ogawa, 2008. **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – slightly inflated anterior end of the oesophagus in female; **E, F** – anterior and posterior end of male; **G** – first-stage larva.

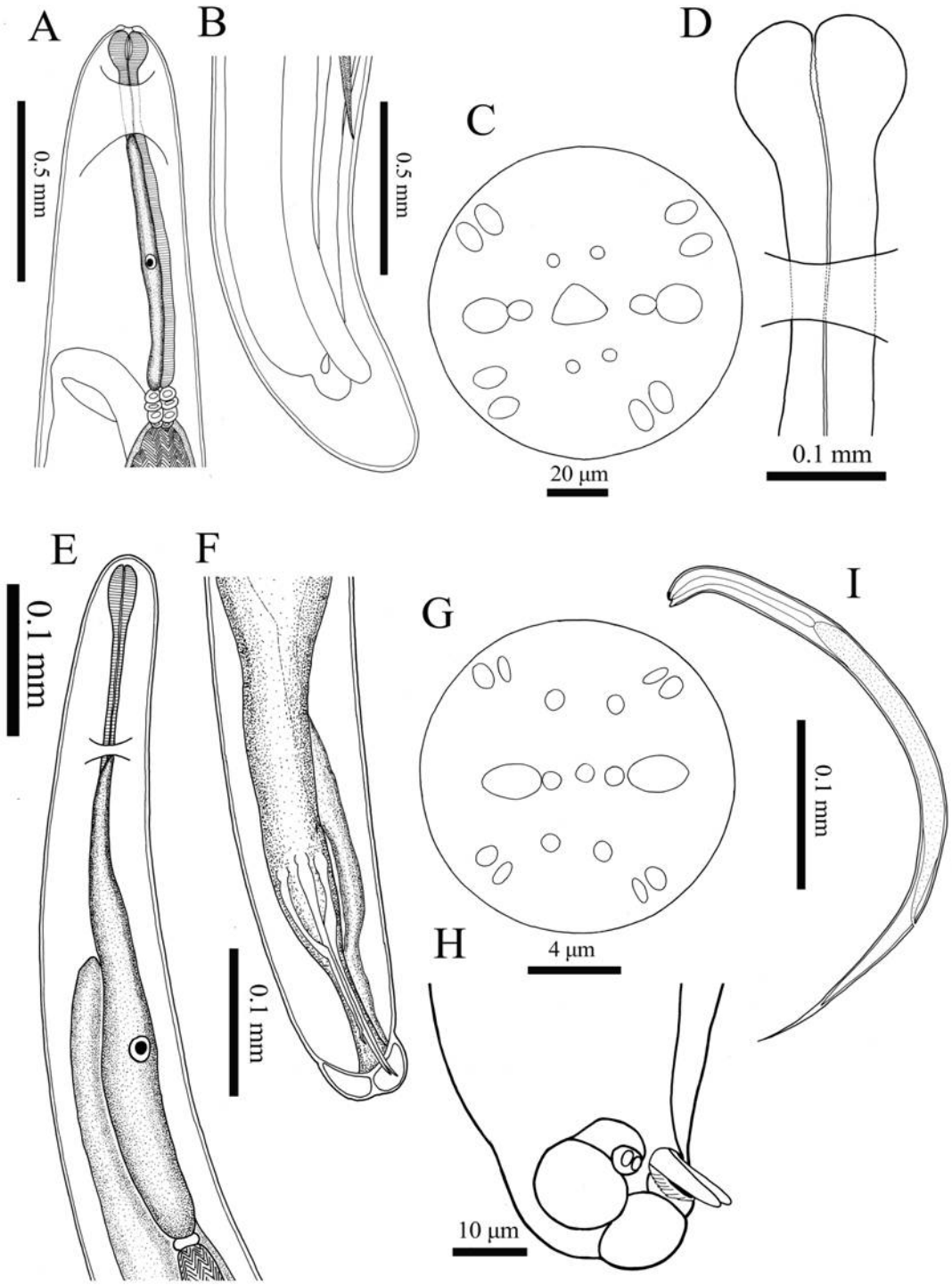


Figure 9. *Philometra madai* Quiazon, Yoshinaga et Ogawa, 2008. **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – highly inflated anterior end of the oesophagus in female; **E, F** – anterior and posterior end of male; **G** – cephalic end of male, apical view; **H** – closer view of posterior end of male showing the unequal-sized subdivided smaller lobes, tail papillae, spicules and gubernaculum; **I** – first-stage larva.

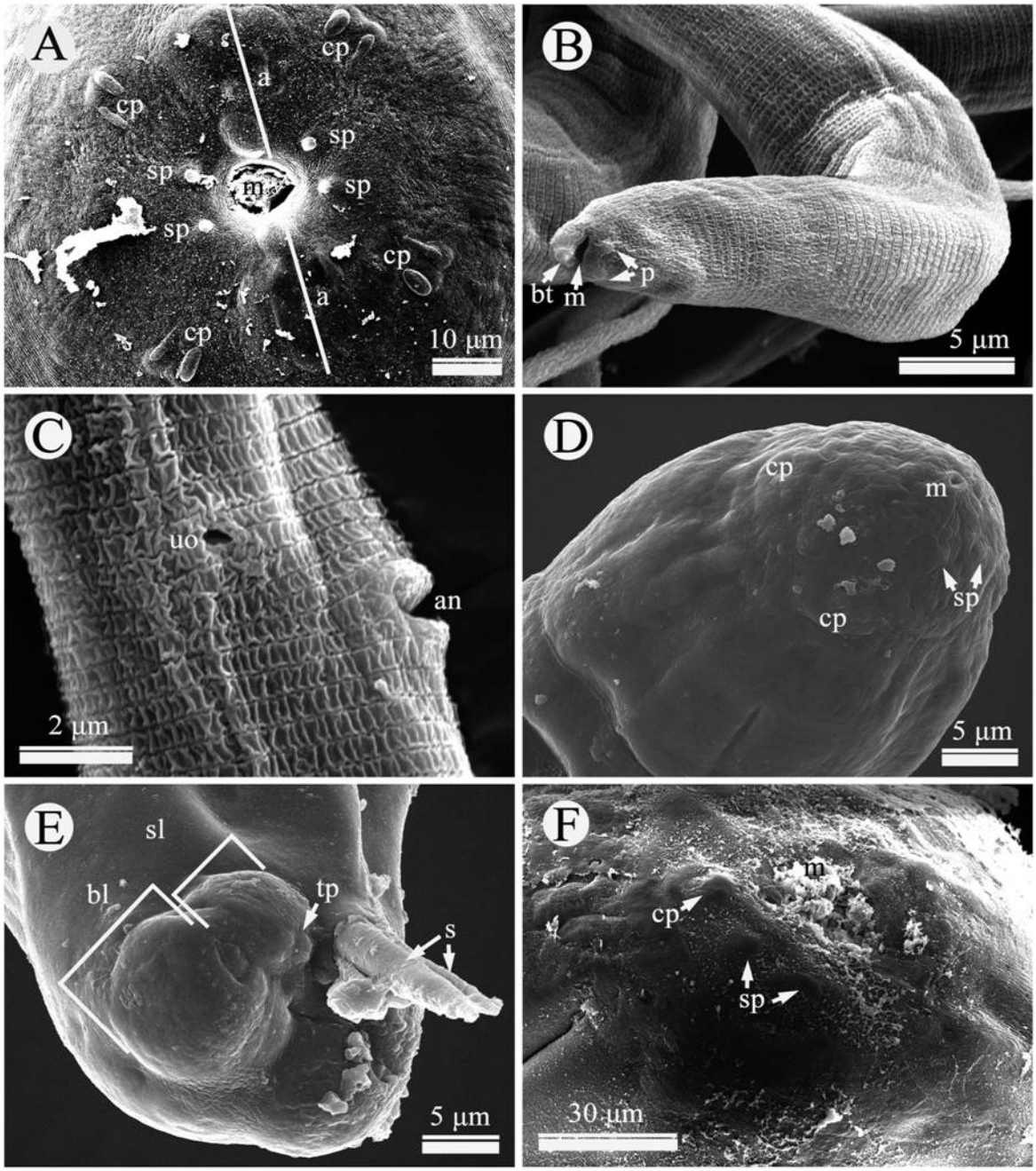


Figure 10. Scanning electron micrographs of *Philometra isaki* Quiazon, Yoshinaga et Ogawa, 2008 (A–C) and *Philometra madai* Quiazon, Yoshinaga et Ogawa, 2008 (D–F). **A** – cephalic end of female, apical view; **B** – anterior end of first-stage larva showing the boring tooth, papillae and amphids near the mouth opening; **C** – posterior end of first-stage larva showing the bigger-sized anus besides an unidentified smaller-sized opening; **D** – cephalic end of male, sub-apical view; **E** – posterior end of male showing the unequal-sized subdivided smaller lobes; **F** – cephalic end of female. *Abbreviations*: a – amphid; an – anus; bl – bigger-sized lobe; bt – boring tooth; cp – paired cephalic papillae of outer circle; g – gubernaculum; sp – single papillae of inner circle; m – mouth; p – papillae; sl – smaller-sized lobe; s – spicules; tp – tail papillae; uo – unidentified opening.

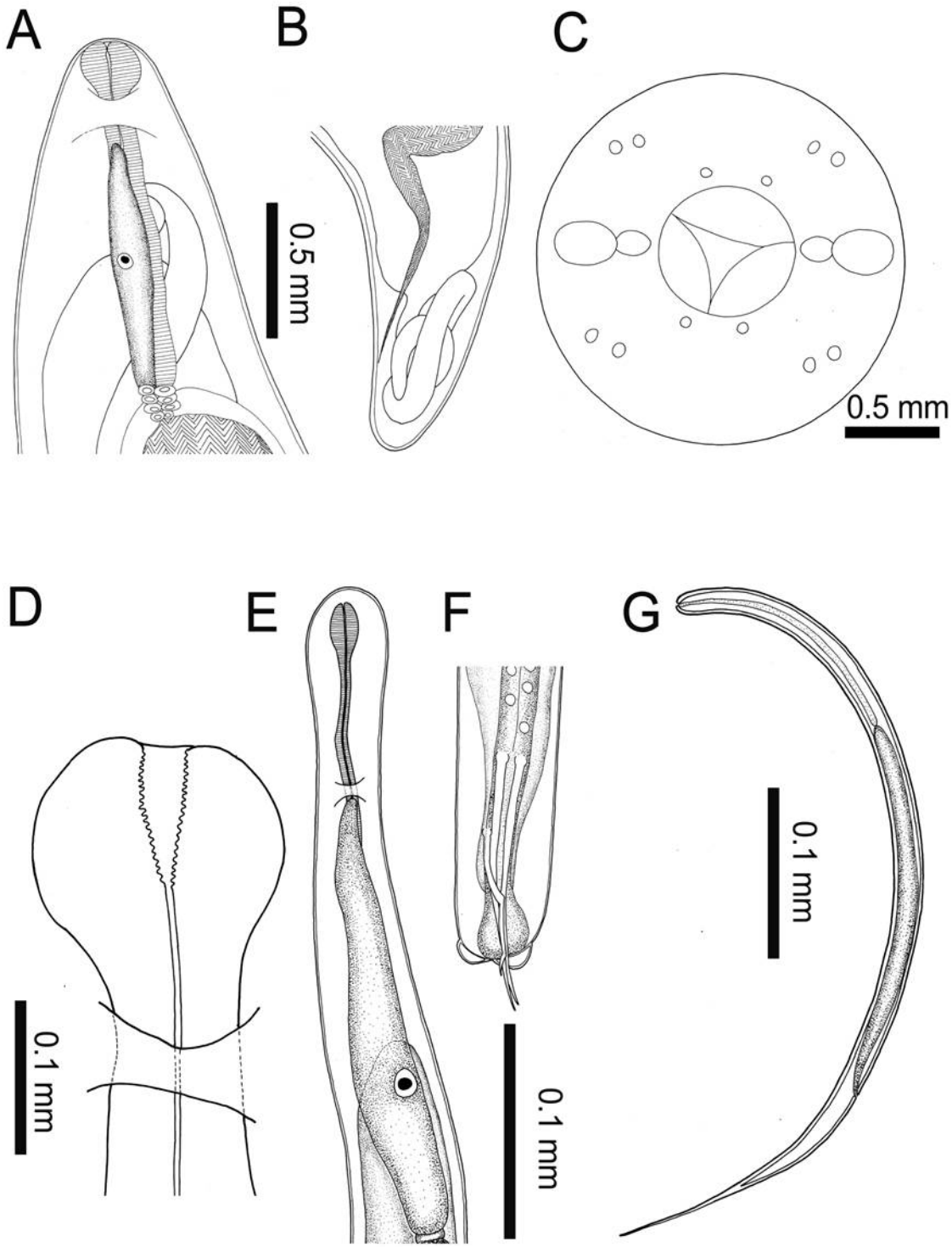


Figure 11. *Philometra sawara* Quiazon, Yoshinaga et Ogawa, 2008. **A, B** - anterior and posterior end of female; **C** - cephalic end of female, apical view; **D** - highly inflated anterior end of the oesophagus in female; **E, F** - anterior and posterior ends of male; **G** - first-stage larva.

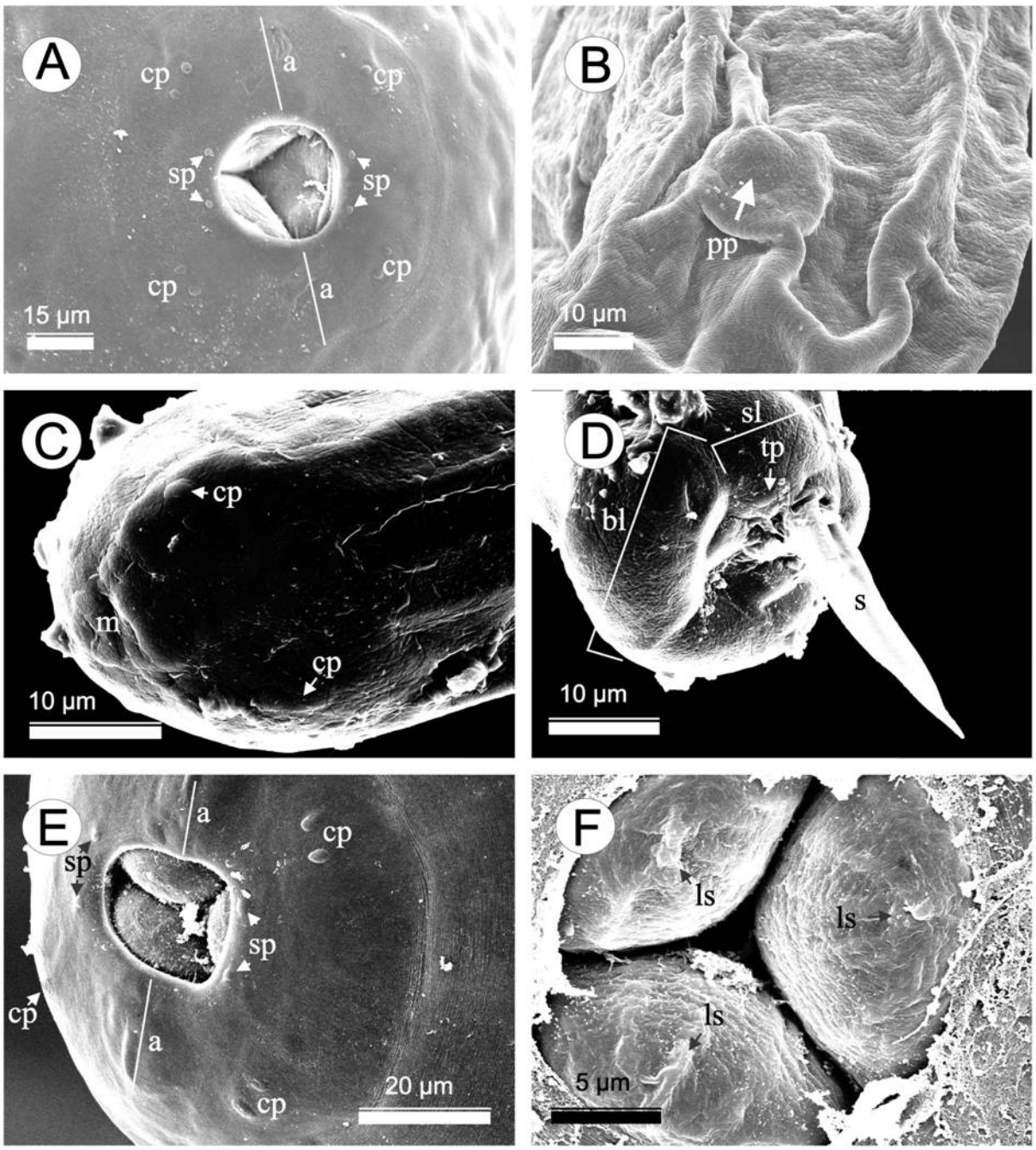


Figure 12. Scanning electron micrographs of *Philometra sawara* Quiazon, Yoshinaga et Ogawa, 2008 (A-B) and *Philometra nemipteri* Luo, 2001 (C-F). **A** - cephalic end of female *P. sawara*, apical view; **B** – closer view of the lateral papilla-like caudal projection at the caudal end; **C** - cephalic end of male *P. nemipteri*; **D** - posterior end of male *P. nemipteri*; **E** - cephalic end of female *P. nemipteri*, sub-apical 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; s - spicules; pp – papilla-like caudal projections; sl - smaller-sized lobe; sp - single papillae of inner ring; tp - tail papillae.

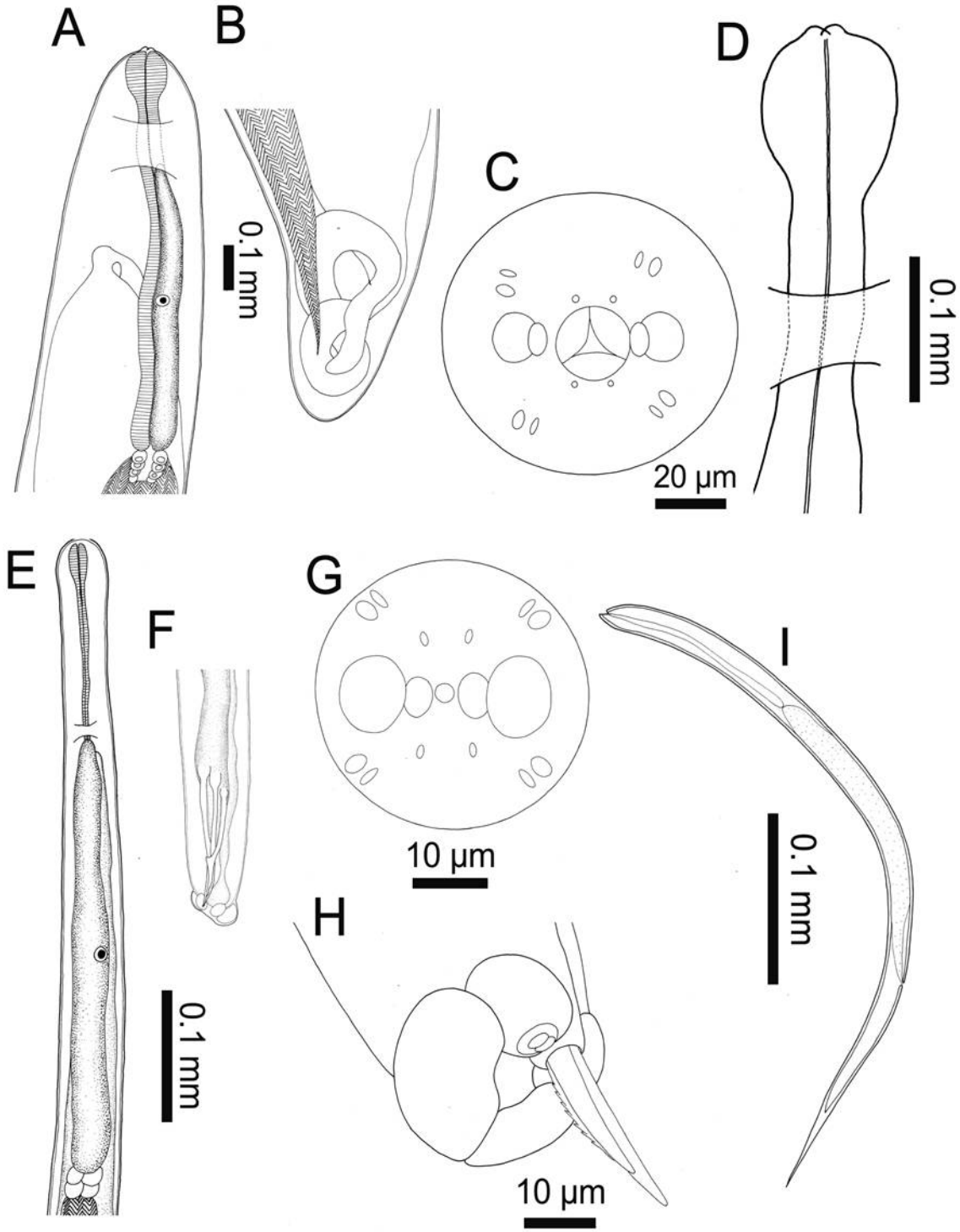


Figure 13. *Philometra nemipteri* Luo, 2001. **A, B** - anterior and posterior end of female; **C** -cephalic end of female, apical view; **D** - moderately inflated anterior end of oesophagus with protruding small lobular structure at the anterior tip in each oesophageal lobe in female; **E, F** - anterior 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.

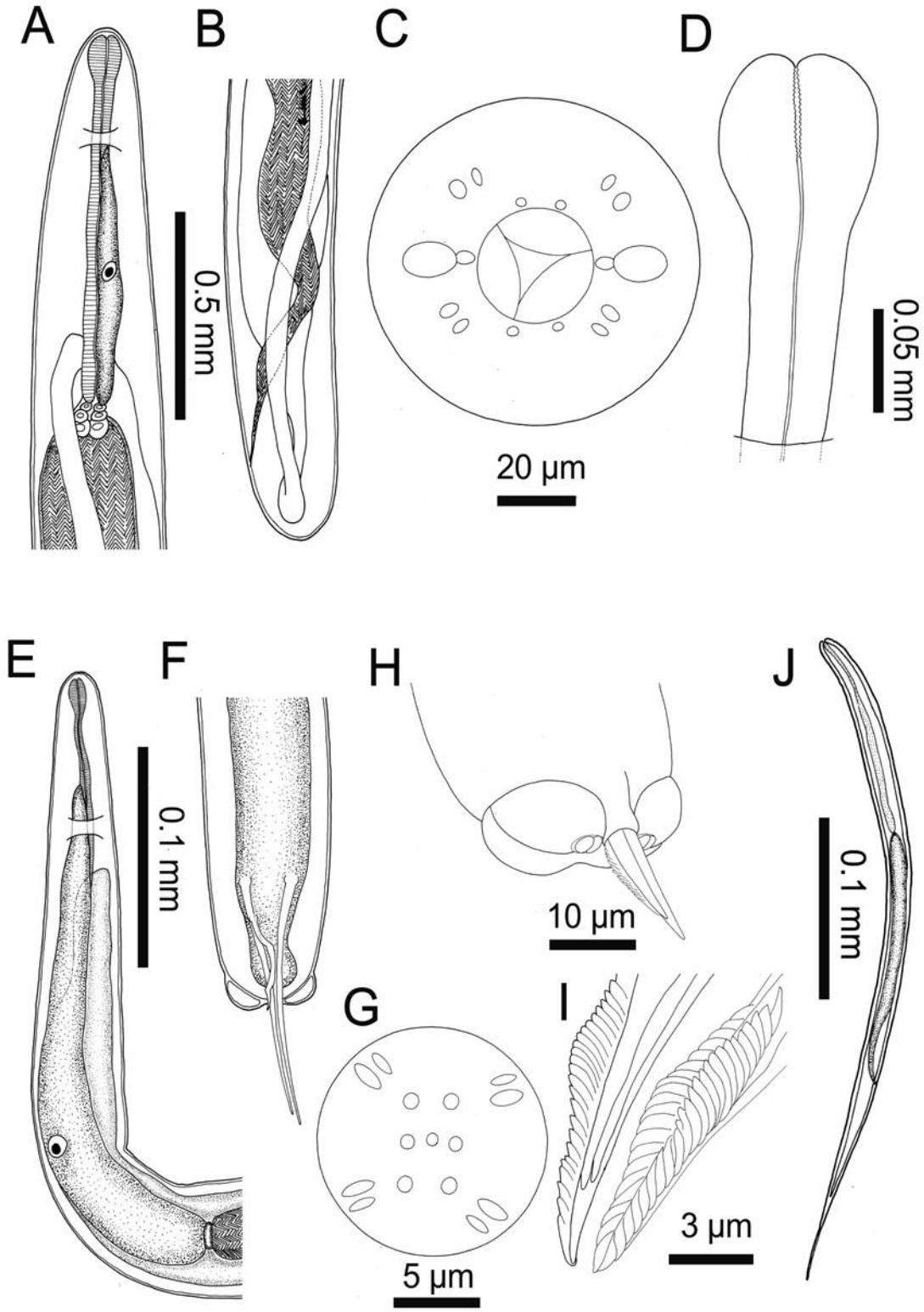


Figure 14. *Philometra sciaenae* Yamaguti, 1941. **A, B** - anterior and posterior end of female; **C** - cephalic end of female, apical view; **D** - moderately inflated anterior end of oesophagus in females; **E, F** - anterior and posterior end of male; **G** - cephalic end of male, apical view; **H** - 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.

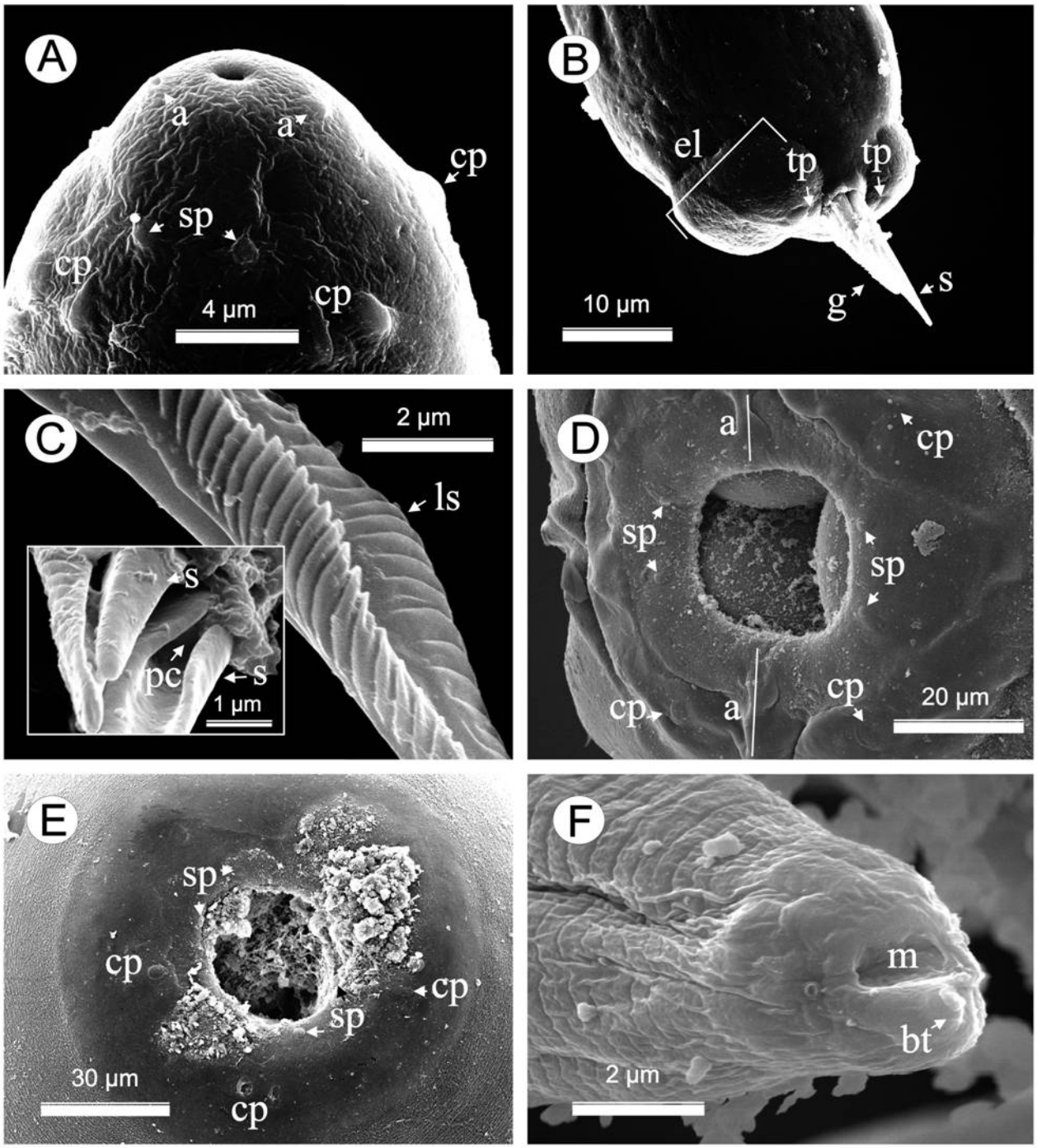


Figure 15. Scanning electron micrographs of *Philometra sciaenae* Yamaguti, 1941. **A** - cephalic end of male; **B** - posterior end of male; **C** - closer view of spicules and lamellate-like structures of the gubernaculum (inset-closer view of spicules showing the passage canal for releasing of sperm 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.

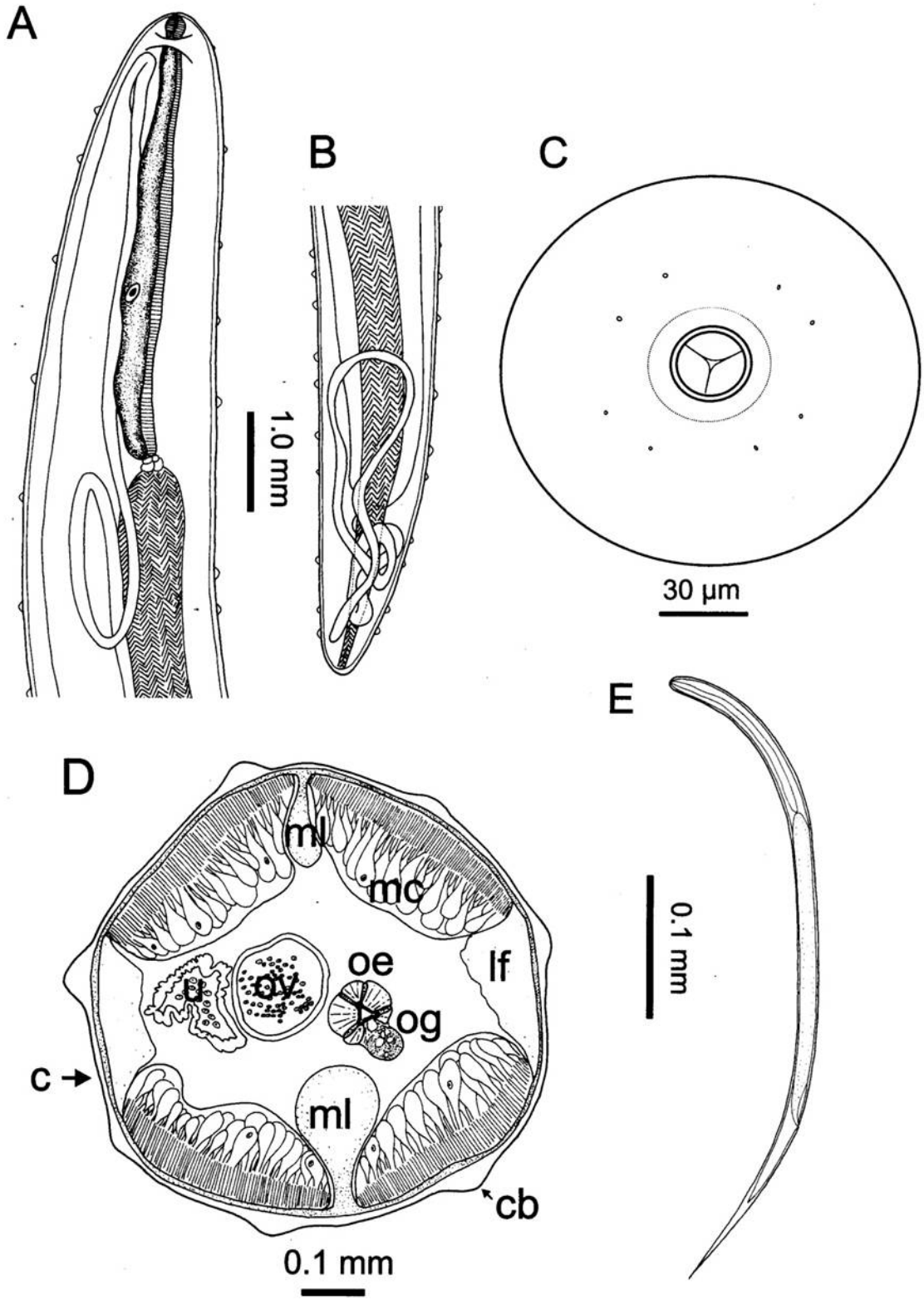


Figure 16. *Philometroides seriolae* (Ishii, 1931). **A, B** – anterior and posterior end of gravid females; **C** – cephalic end of female, apical view; **D** – cross-sectional view of gravid female; **E** - first-stage larva. *Abbreviations*: cb – cuticular bosses; c – cuticle; ml – median line; mc – muscle cells; lf – lateral fields; ov – ovary; u – uterus; oe – oesophagus; og – oesophageal gland.

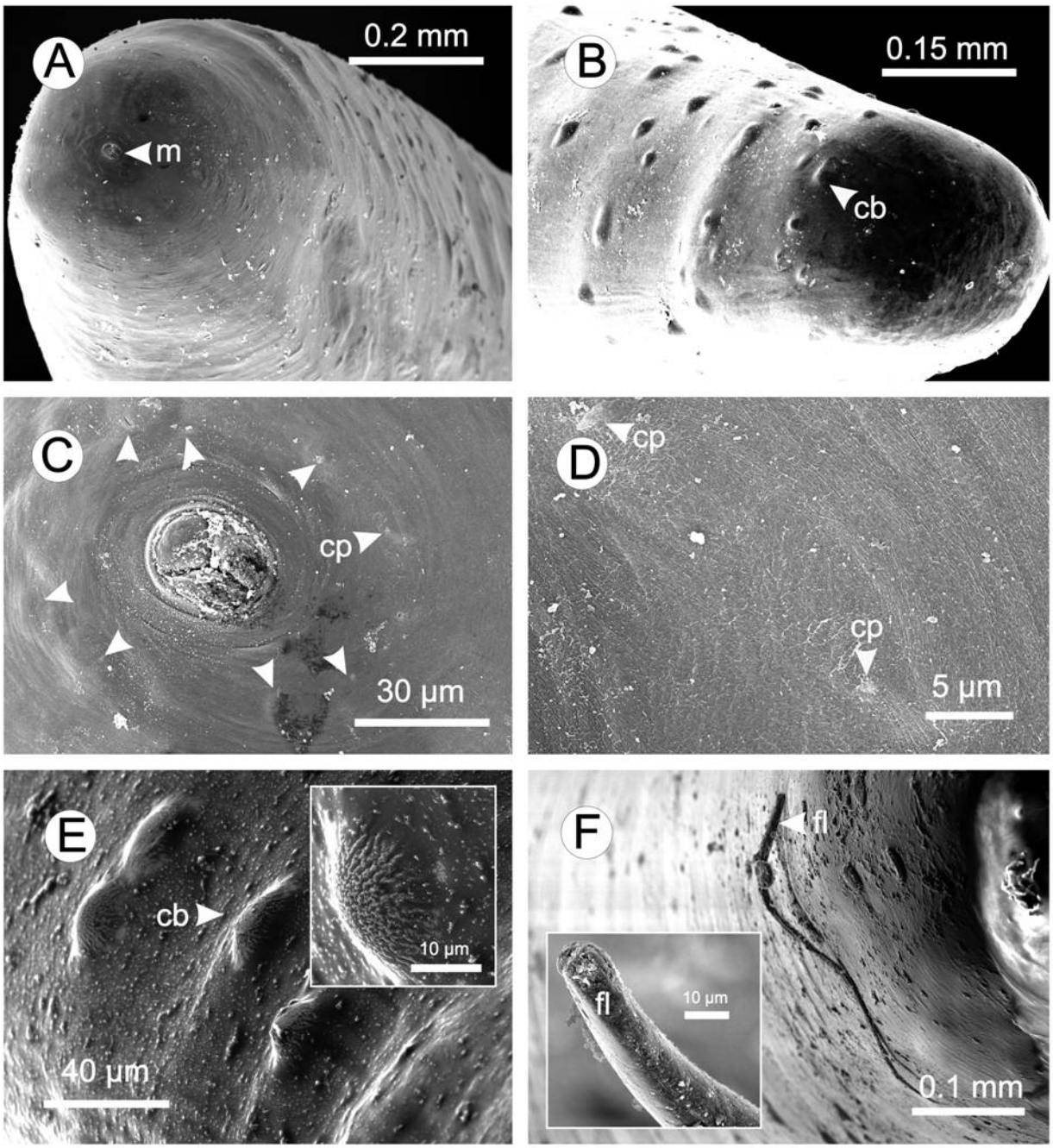


Figure 17. Scanning electron micrographs of gravid female *Philometroides seriolae* (Ishii, 1931). **A** – anterior end; **B** – posterior end; **C** – cephalic end showing 8 cephalic papillae arranged in a single circular pattern; **D** – closer view of the tiny cephalic papillae; **E** – cuticular bosses scattered randomly in the cuticle of the parasite (inset-closer view of cuticular bosses); **F** – first-stage larva on the surface of the cuticle of gravid female (inset-closer view of the anterior end of first-stage larva). *Abbreviations*: cb – cuticular bosses; cp – cephalic papillae; fl – first-stage larva; m – mouth/oral aperture.

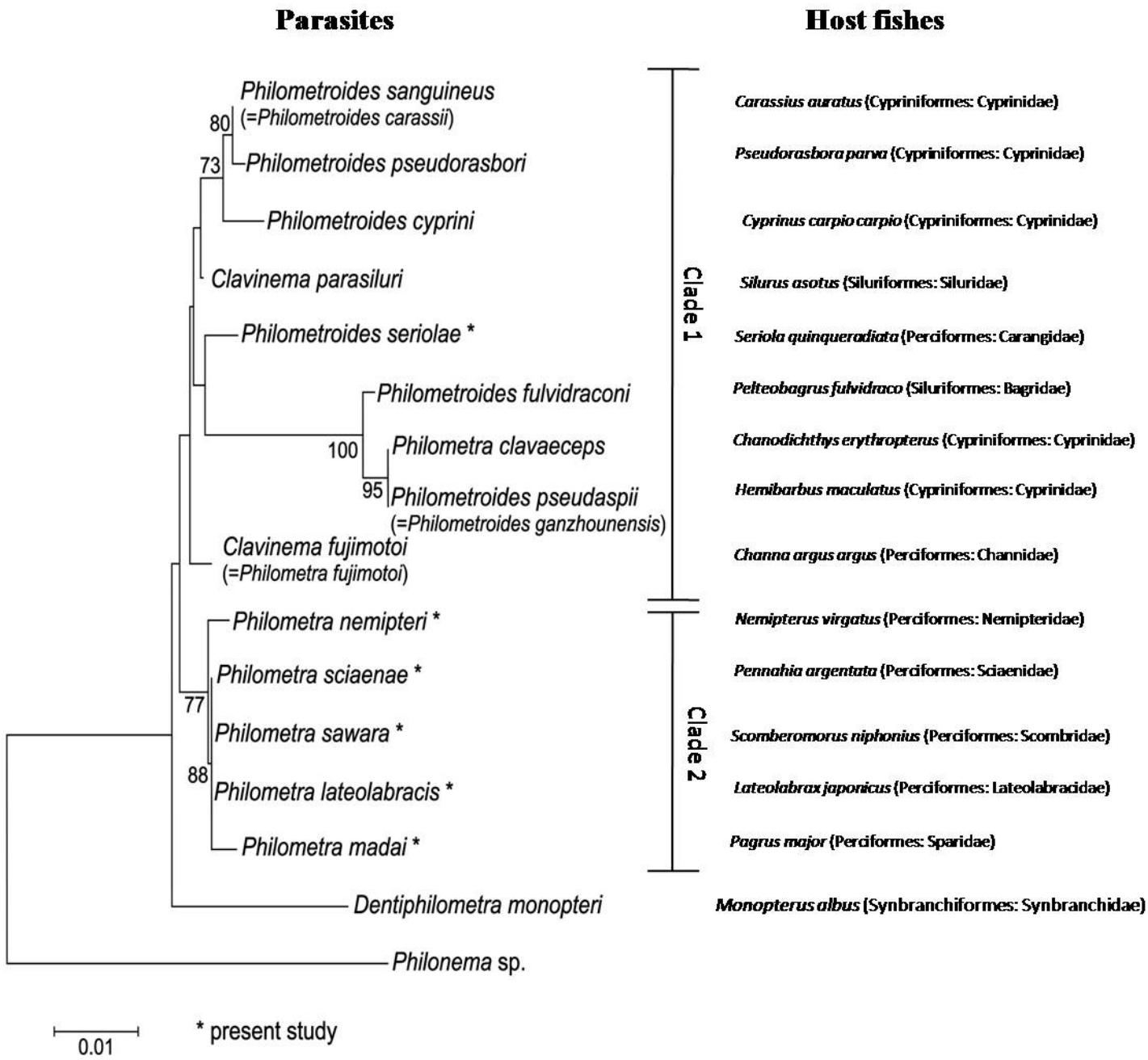


Figure 18. NJ tree derived from 18S rDNA of all reported sequences of dracunculoids in GenBank database.

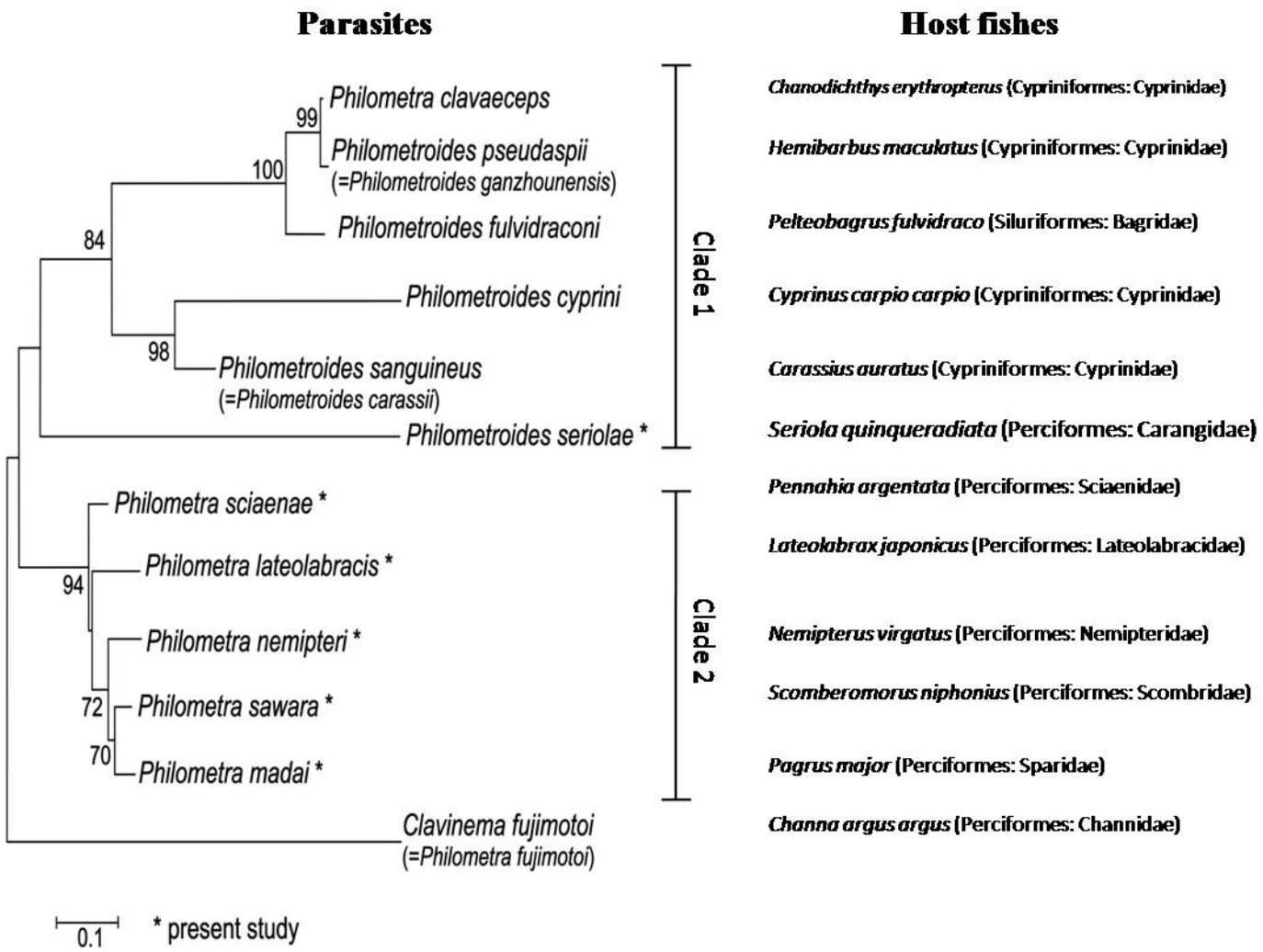


Figure 19. NJ tree derived from ITS2 region of all reported sequences of philometrids in GenBank database.

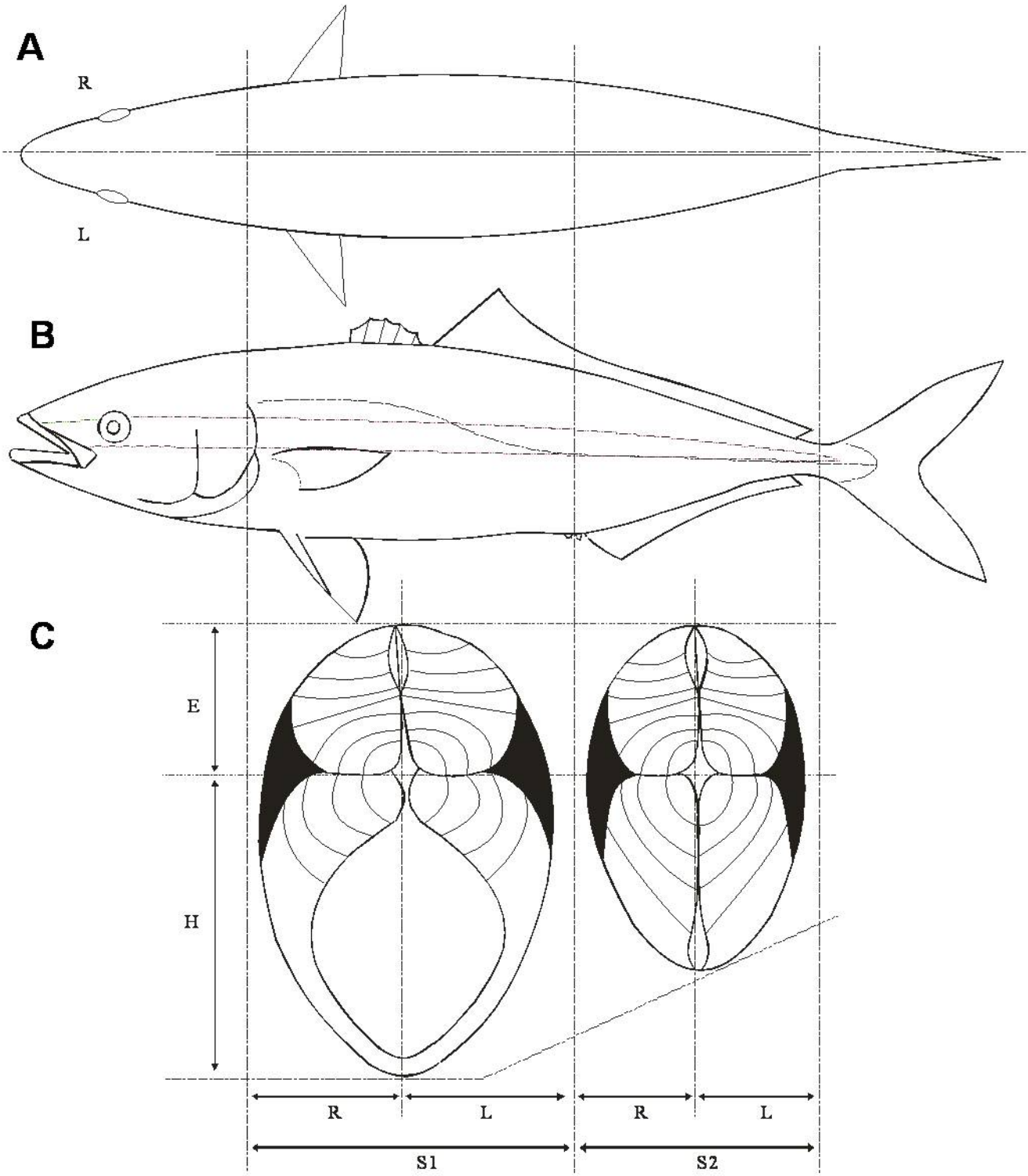


Figure 20. Lateral (top view, A; side view, B) and cross sections (C) of Japanese amberjack. *Abbreviations*: E – epaxial muscle; H – hypaxial muscle; L – left side; R – right side; S1 – section 1; S2 – section 2.

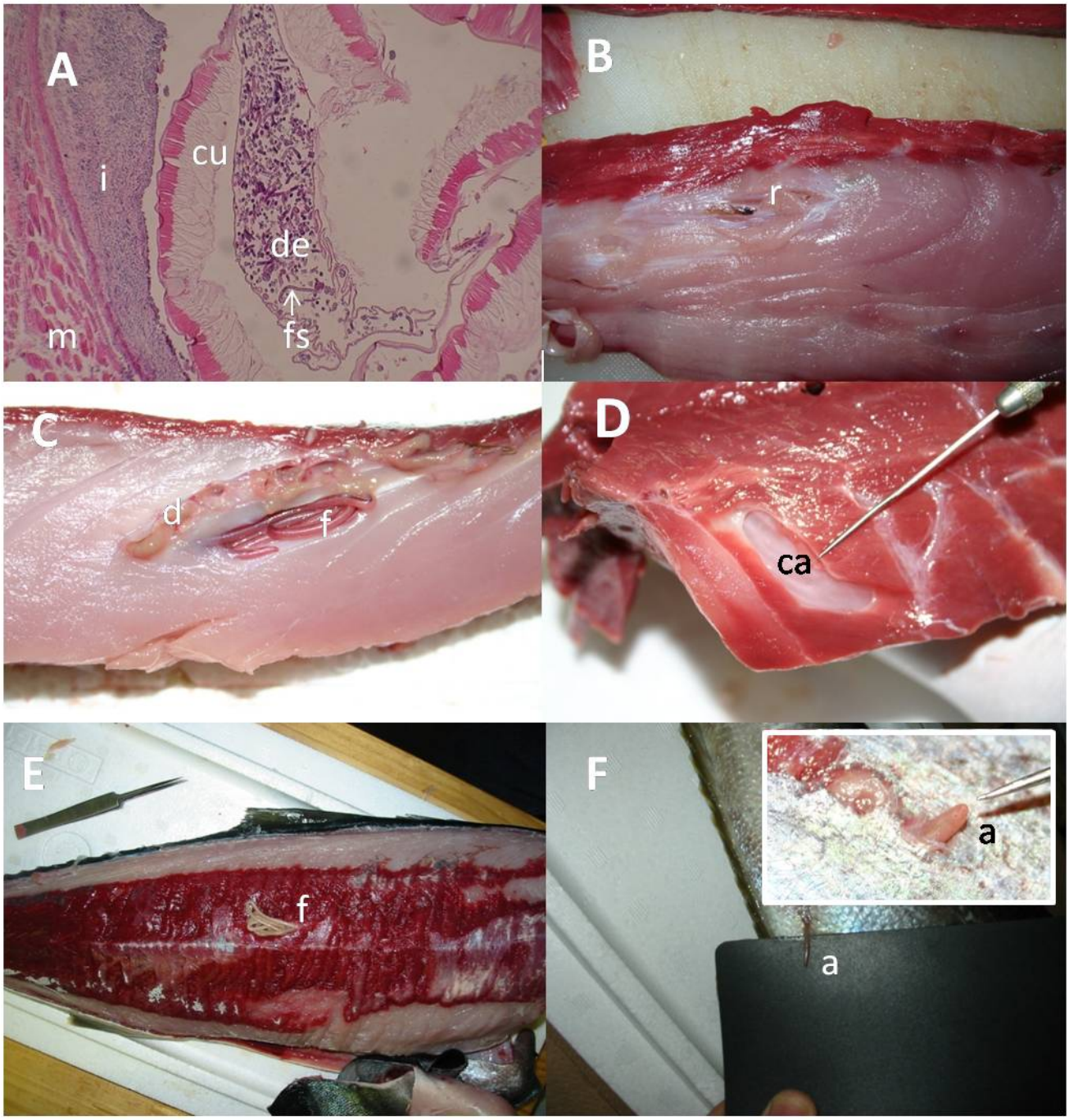


Figure 21. Responses of Japanese amberjack (A-B) against *Philometroides seriolae* infection in the body muscle (C-D) and under the skin (E-F). A – histological section of infected body muscle showing accumulation of inflammatory cells in the inflamed tissues surrounding each formed cavity; B – completely healed portion of the body muscle showing small remnants of lysed worm; C – gravid female worms besides dead worm which were situated individually in each formed cavity in the body muscle; D – cavity where folded female worms are located; E – milky white-coloured fully gravid female worm located under the host skin; F – anterior end of reddish-coloured gravid female worm protruding outside the host skin with exposed anterior end about 4 cm in length; inset, anterior end of gravid female worm starting to protrude outside the host skin (other specimen with exposed anterior end about 1 cm in length); *Abbreviation*: a – anterior end of the worm; ca- formed cavity; cu – thick cuticle of the worm; d – dead worms; de – developing embryos; f – fully gravid worms; fs – first-stage larvae; i – inflammatory cells; m – muscle fibres; r – remnants of lysed worm.

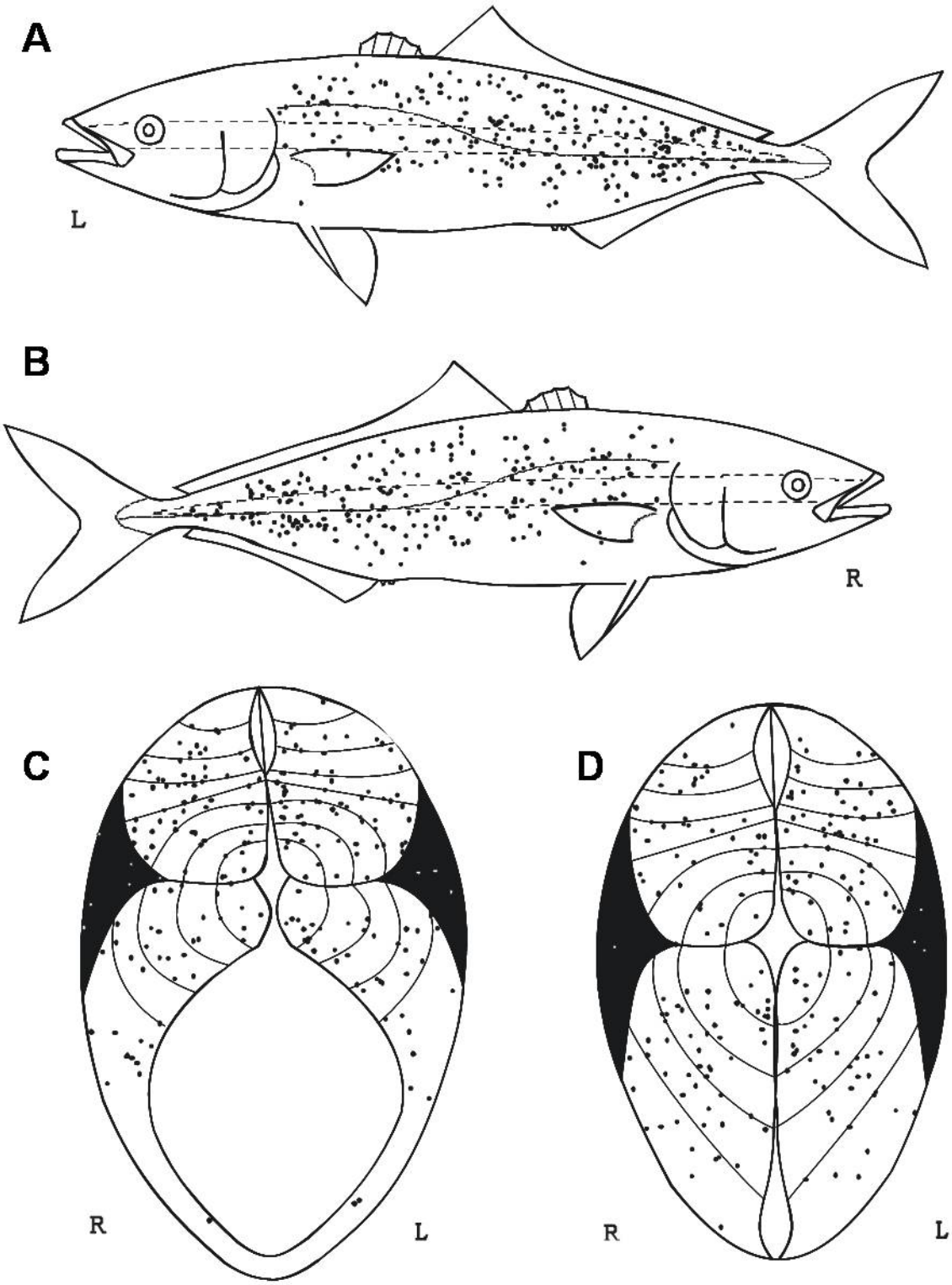


Figure 22. Sites of infection, regardless of developmental stages (nongravid, semigravid, gravid and dead) of *Philometroides seriolae*, in the lateral (A–B) and cross sections (C–D) of the body muscle of Japanese amberjacks examined from 2006 to 2007. A–left side (L); B–right side (R); C–cross sections in section 1 (from head to anus portion); D–cross sections in section 2 (from anus to tail end portion).

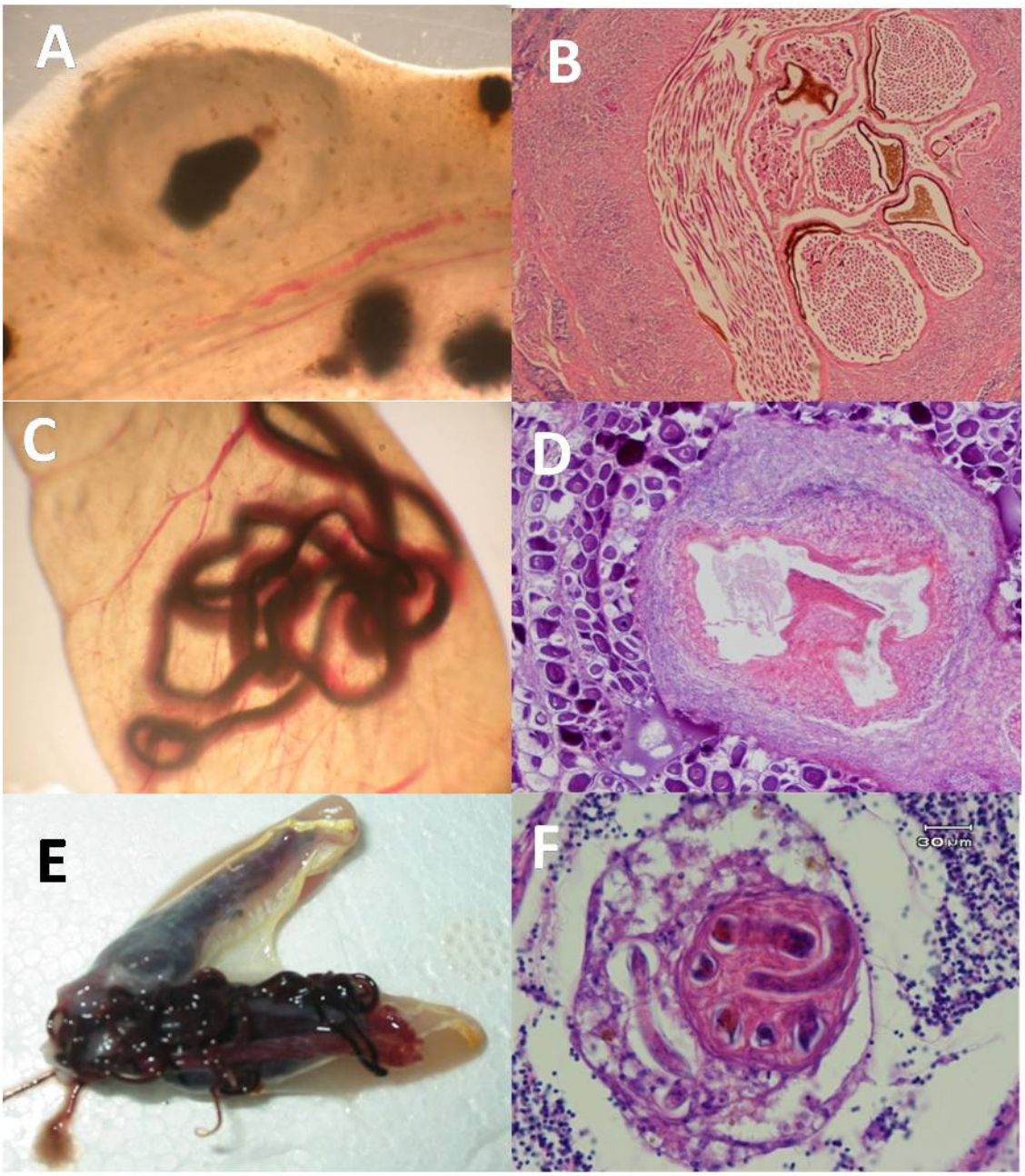


Figure 23. Responses of different host fishes against the gonad-infecting *Philometra* species. A – gravid female (live) *P. sciaenae* in the testes of silver croaker showing evident inflammation; B – gravid female *P. sciaenae* in the testis of silver croaker (histological section of (A)); C – gravid female *P. sciaenae* in the ovary of silver croaker showing absence of host response; D – dead female *P. madai* in the ovary of red seabream surrounded by leukocyte infiltrations; E – a group of < 20 gravid female *P. nemipteri* in the spermatic duct of golden threadfin bream showing no accumulation of inflammatory cells; F – a group of released first-stage larvae in the seminiferous tubule in the host testis surrounded by inflammatory cells.

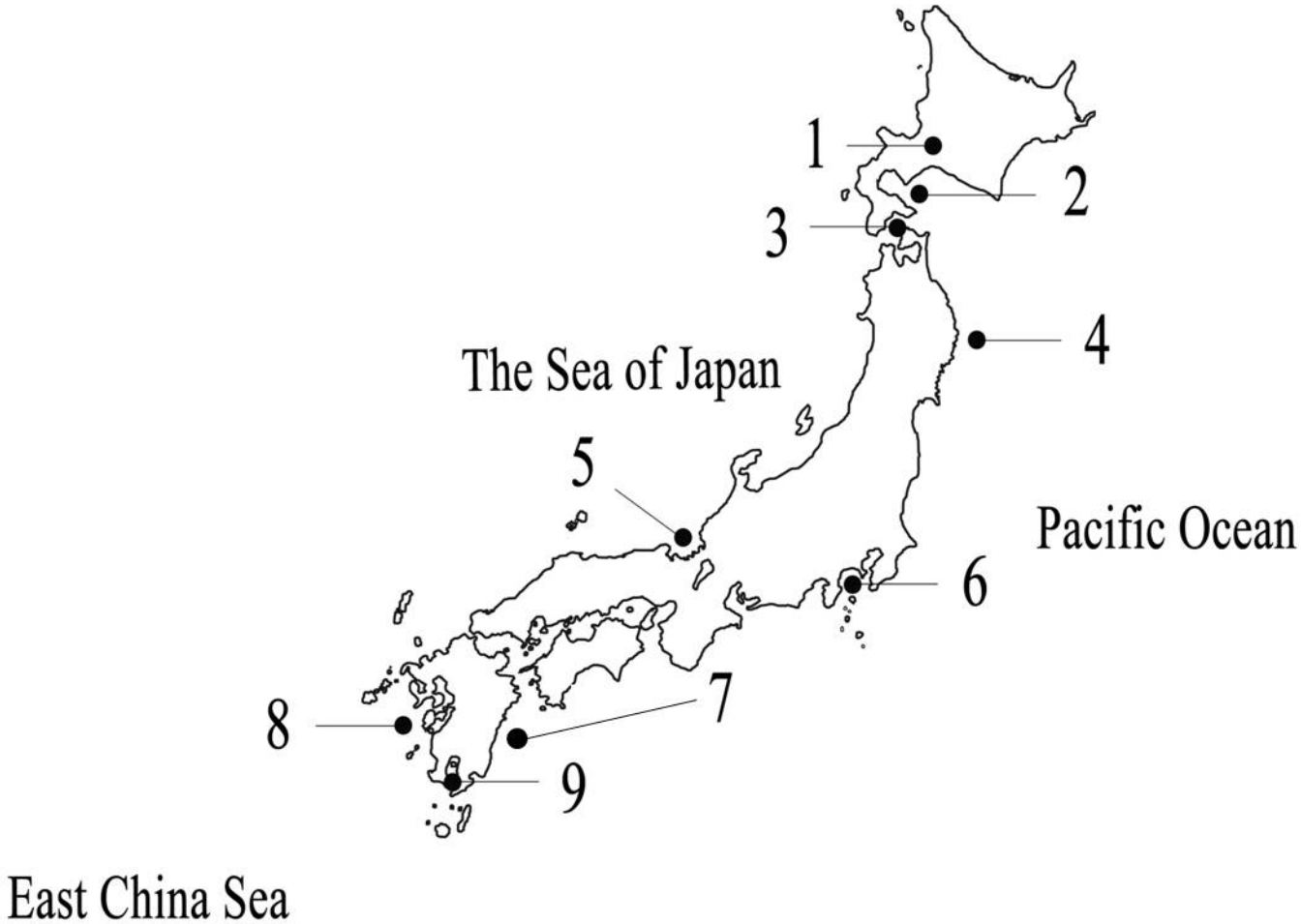
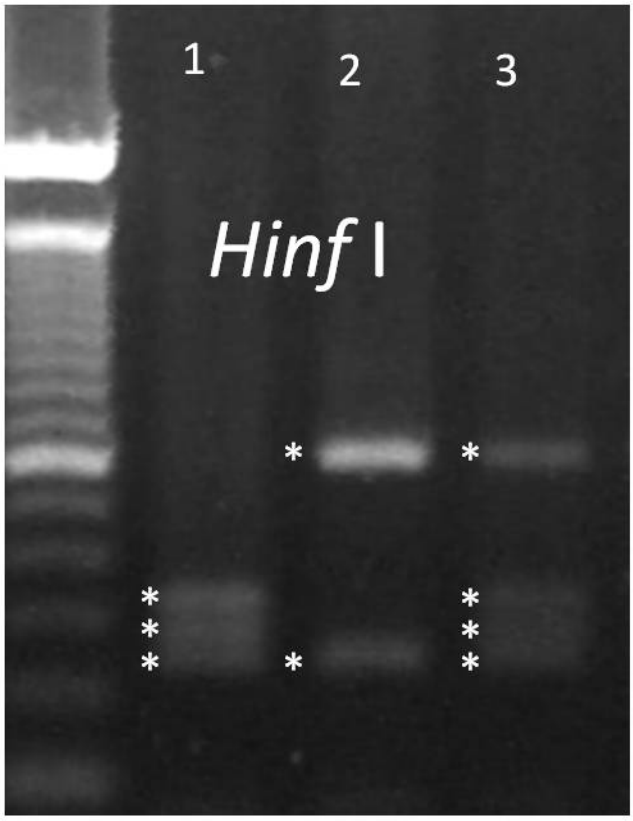


Figure 24. Geographical locations of examined host fishes infected with *Anisakis* species in Japanese waters. The numbers correspond to the locality numbers in Table 15.

A



B

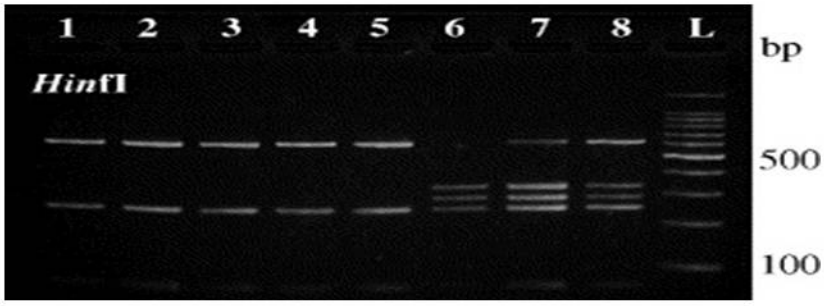


Figure 25. Fragment patterns generated after *Hinf* I digestion in PCR-RFLP. A – present study (lane 1, *A. pegreffii*; lane 2, *A. simplex* (s.s.); lane 3, hybrid genotype ; B – data from Umehara et al. 2006 (lanes 1-5, *A. simplex*(s.s.); lane 6, *A. pegreffii*; lanes 7-8, hybrid genotype.

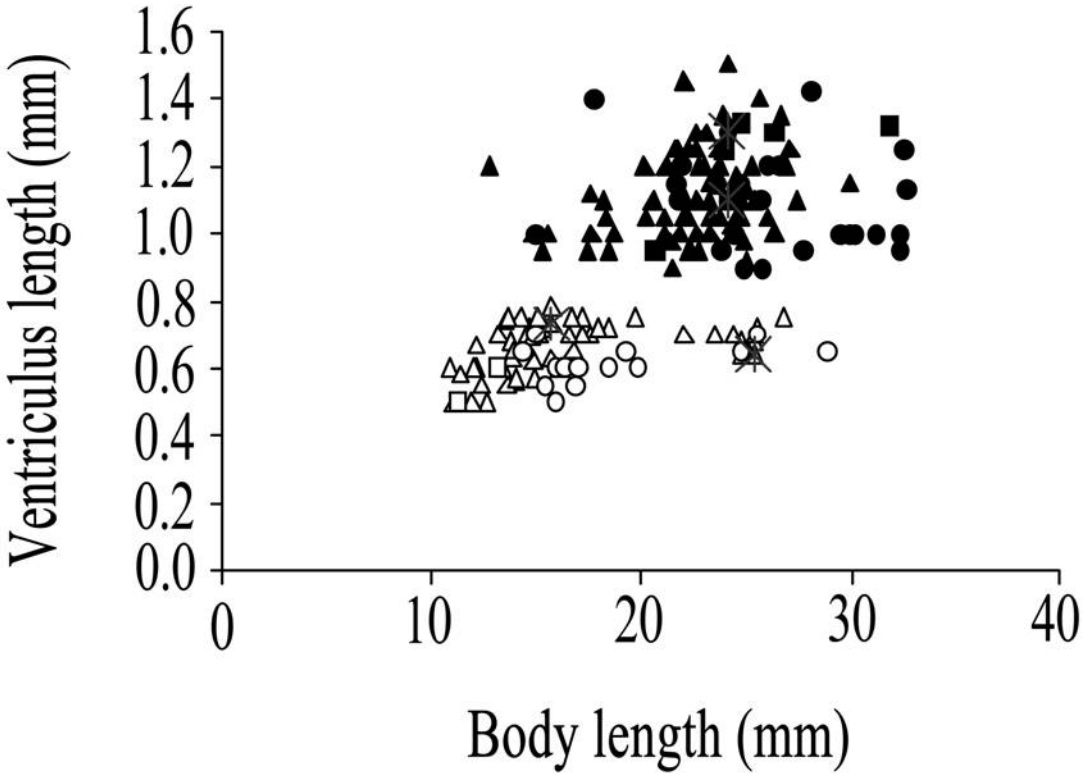


Figure 26. Relationship between the ventriculus and total body length in L3 larvae and *in vitro*-cultured L4 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii* infecting fishes in Japanese waters (n=274). Legend: △: *A. pegreffii* (L3 larvae); □: *A. pegreffii* (L4 larvae); ○: *A. pegreffii* (adult); ▲: *A. simplex* (s.s.) (L3 larvae); ■: *A. simplex* (s.s.) (L4 larvae); ●: *A. simplex* (s.s.) (adult); ✳: Hybrid genotype.

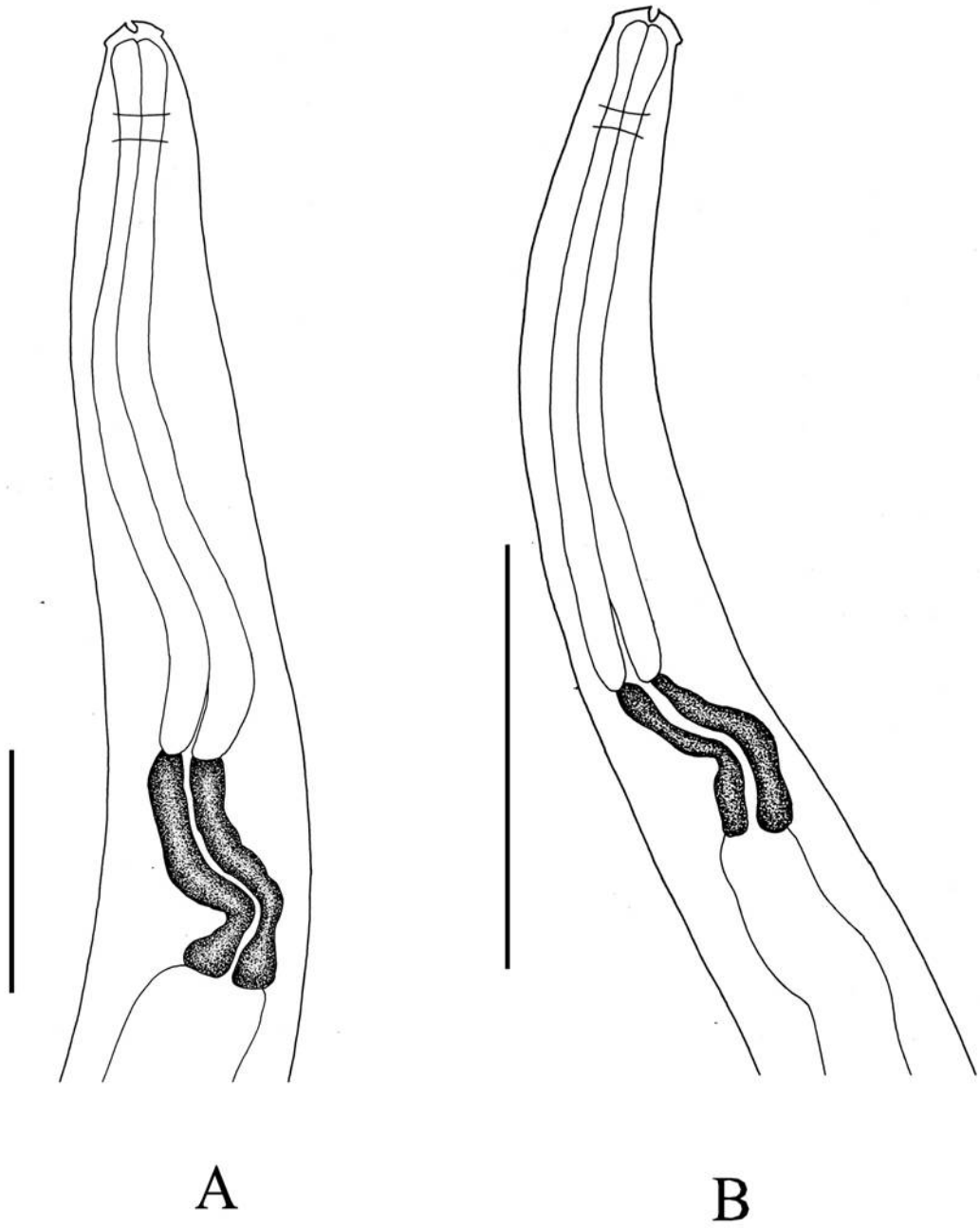


Figure 27. Morphological key found in distinguishing larval (L3-L4) and adult stage *Anisakis simplex* (s.s.) (A) from *Anisakis pegreffii* (B) through differences on the ventriculus length. Ventriculus length at different life-history stages of *A. simplex* (s.s.) ranges from 0.90 to 1.50 mm while that of *A. pegreffii* ranges from 0.50 to 0.78 mm. Scale bar=1 mm.

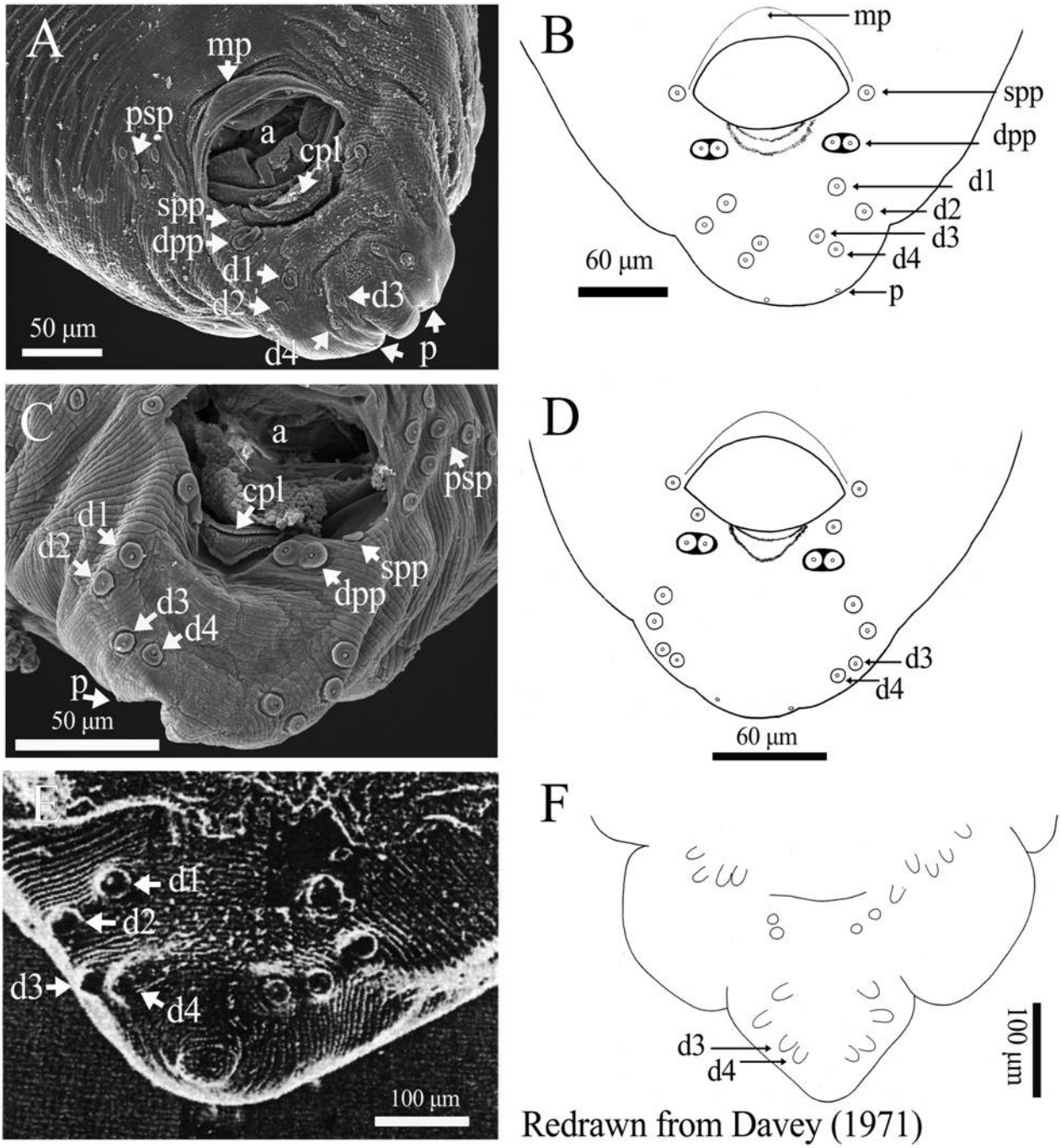


Figure 28. Distribution patterns of caudal papillae in the *in vitro*-cultured adult males of *A. simplex* (s.s.) (A and B) and *A. pegreffii* (C and D) and that of *A. simplex* (E and F) reported by Davey (1971). Left and right rows are SEM micrographs and drawings based on SEM micrographs, respectively. *Abbreviations*: a, anus; cpl, caudal plates; d1, distal papilla no.1; d2, distal papilla no.2; d3, distal papilla no.3; d4, distal papilla no.4; dpp, double procloacal papillae; mp, median papilla; p, phasmid; psp, proximal subventral papillae; spp, single proximal papilla.

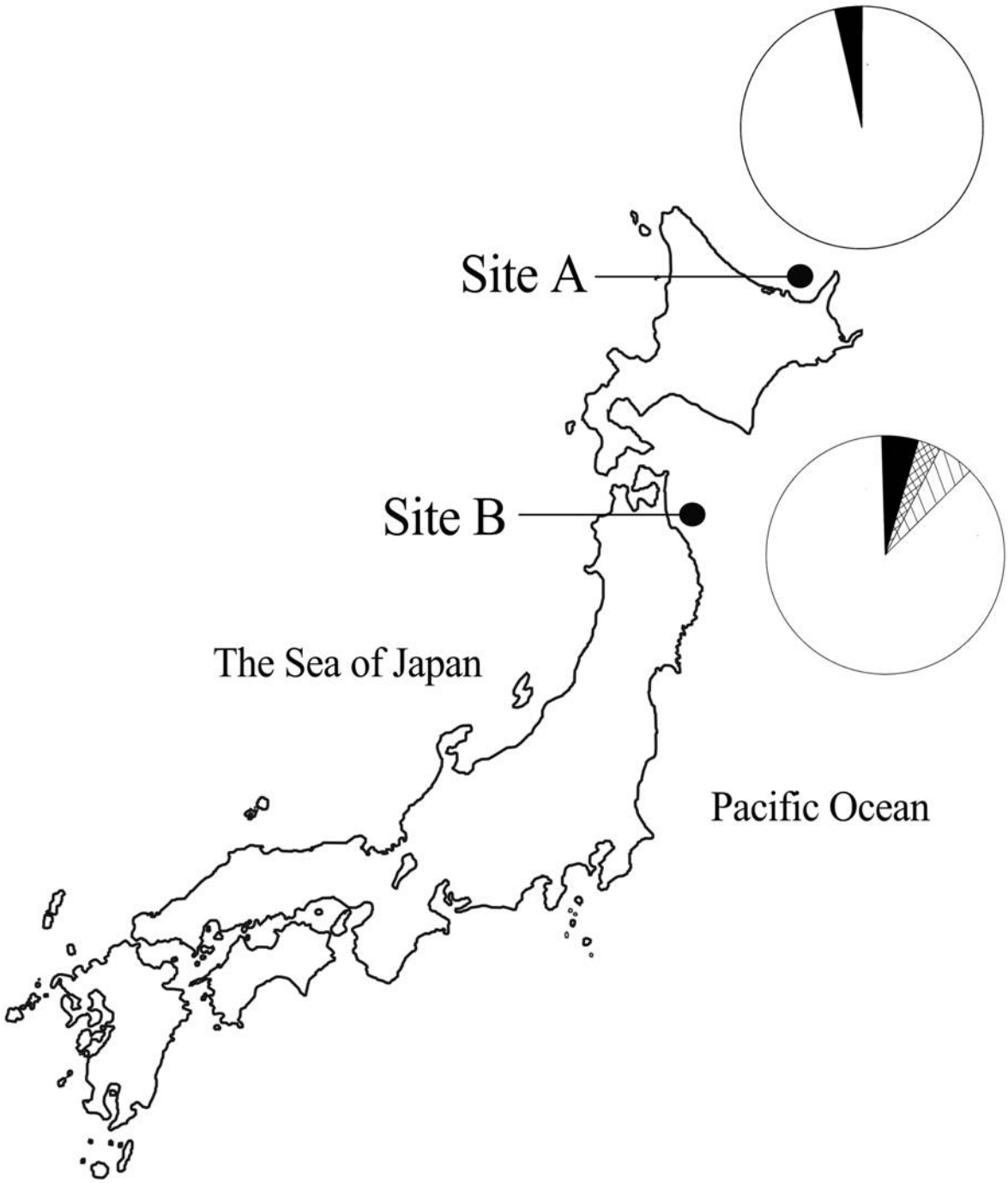


Figure 29. Geographical locations and relative proportions of *Anisakis* species in the examined Alaska pollock in northern Japan. Site A: off Rausu, Hokkaido Prefecture; Site B: off Miyako, Iwate Prefecture; ○: *A. simplex* (s.s.); ●: *A. pegreffii*; ⊘ : *Anisakis* sp. (*Anisakis* Type II); ⊗ : *A. brevispiculata*.

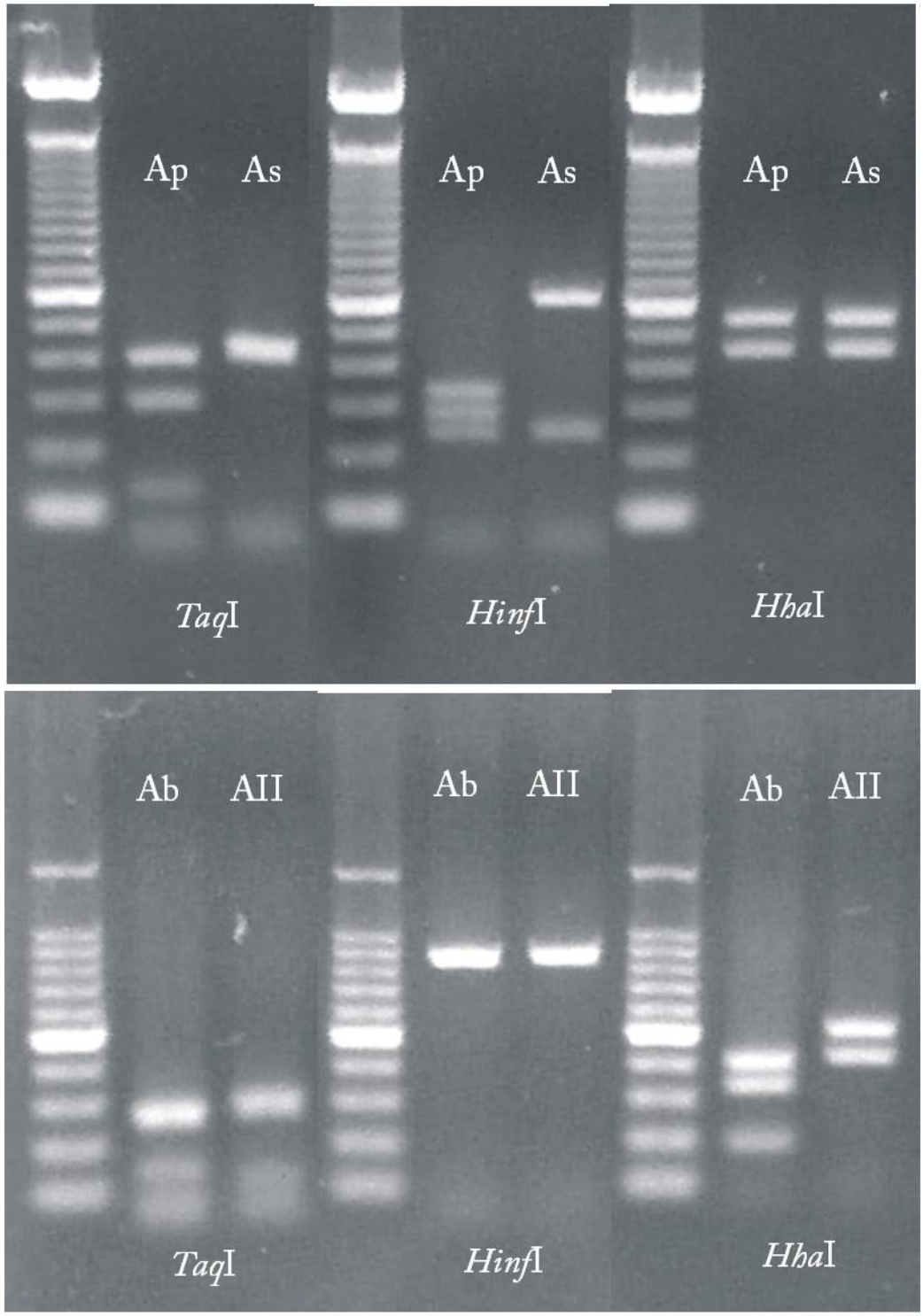


Figure 30. PCR-RFLP fragment patterns of two *Anisakis* species belonging to *Anisakis* Type I (A) and Type II (B) groupings after digestion with restriction enzymes. Ap – *Anisakis pegreffii*; As – *Anisakis simplex* (s.s.); Ab – *Anisakis brevispiculata*; AII – *Anisakis* sp. belonging to *Anisakis* Type II groupings; M – 100 bp ladder.

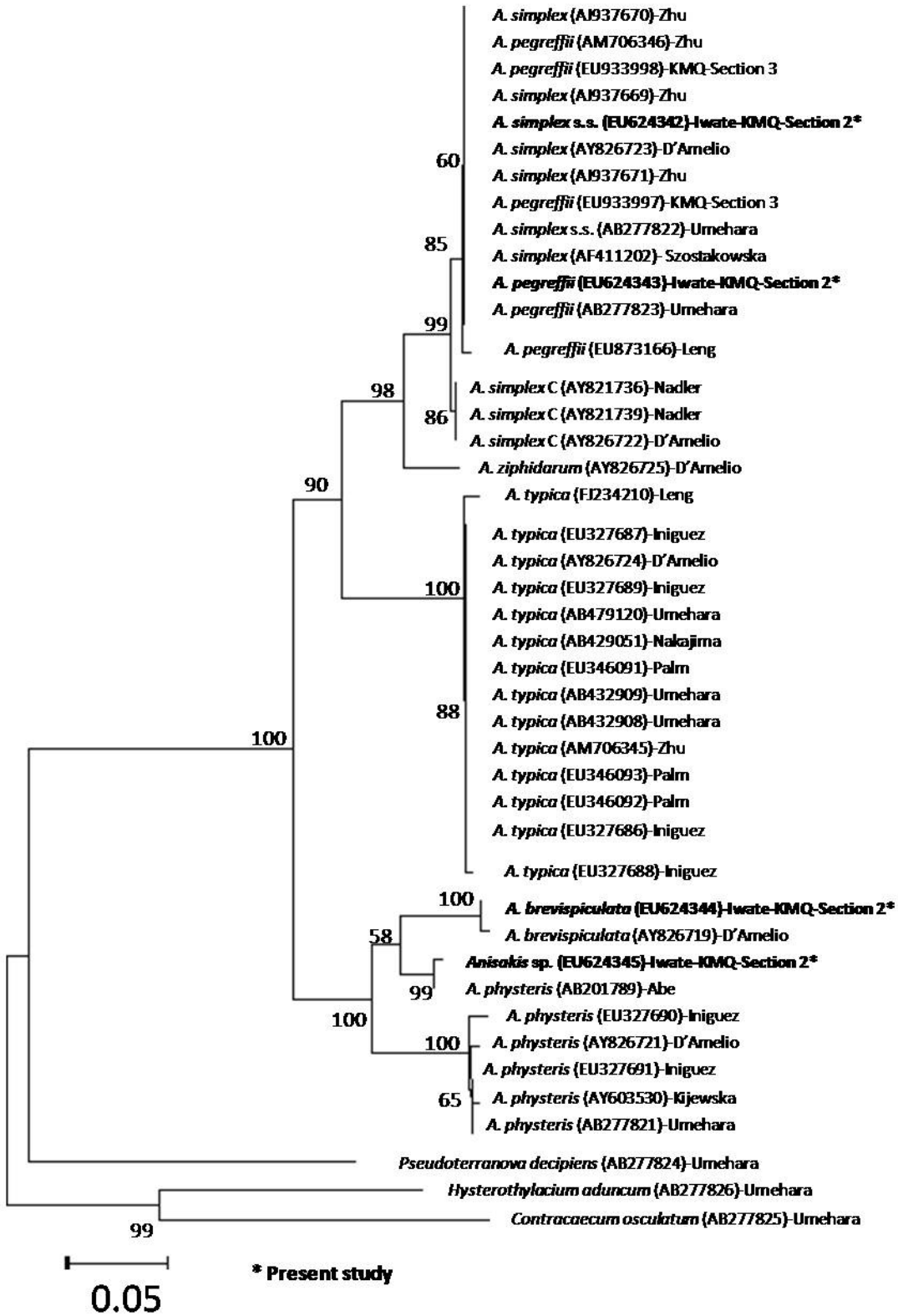


Figure 31. NJ tree inferred from *p-distance* values of the ITS region.

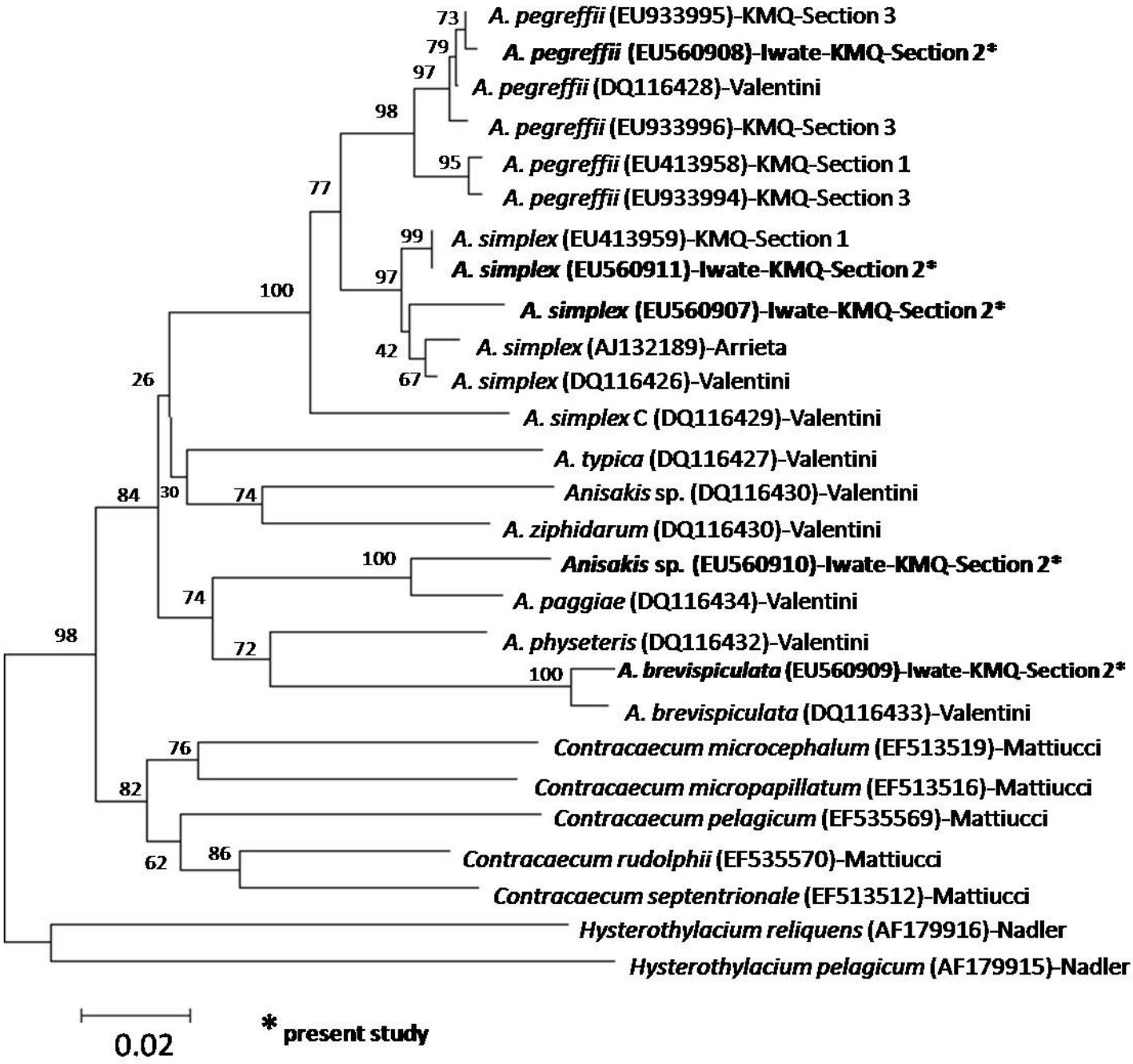


Figure 32. NJ tree inferred from *p-distance* values of the *mt*DNA *cox*2 gene showing the evolutionary relationships among the presently and previously studied *Anisakis* species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) 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. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

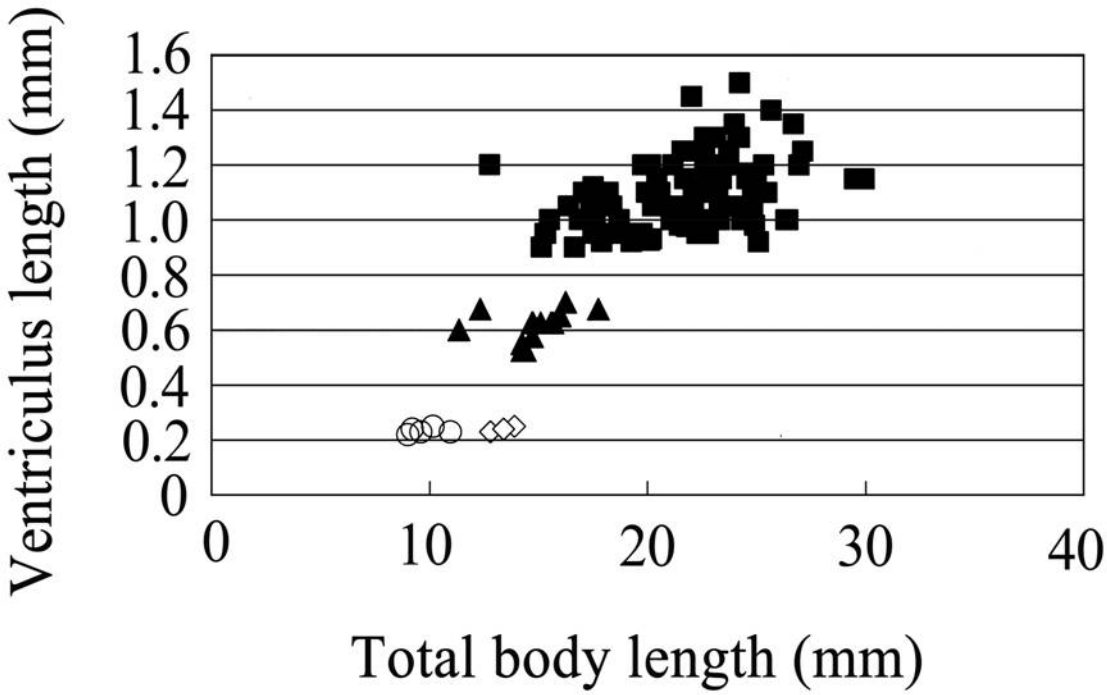


Figure 33. Relationship between the ventriculus length and total body length in the L3 larvae of *A. simplex* (s.s.), *A. pegreffii*, *A. brevispiculata* and *Anisakis* sp. *i*nfecting Alaska pollock caught in northern Japan (n = 210). Closed symbols indicate *Anisakis* Type I, whereas open symbols indicate *Anisakis* Type II larvae. ■: *A. simplex* (s.s.); ▲: *A. pegreffii*; ◊: *A. brevispiculata*; ○: *Anisakis* sp.

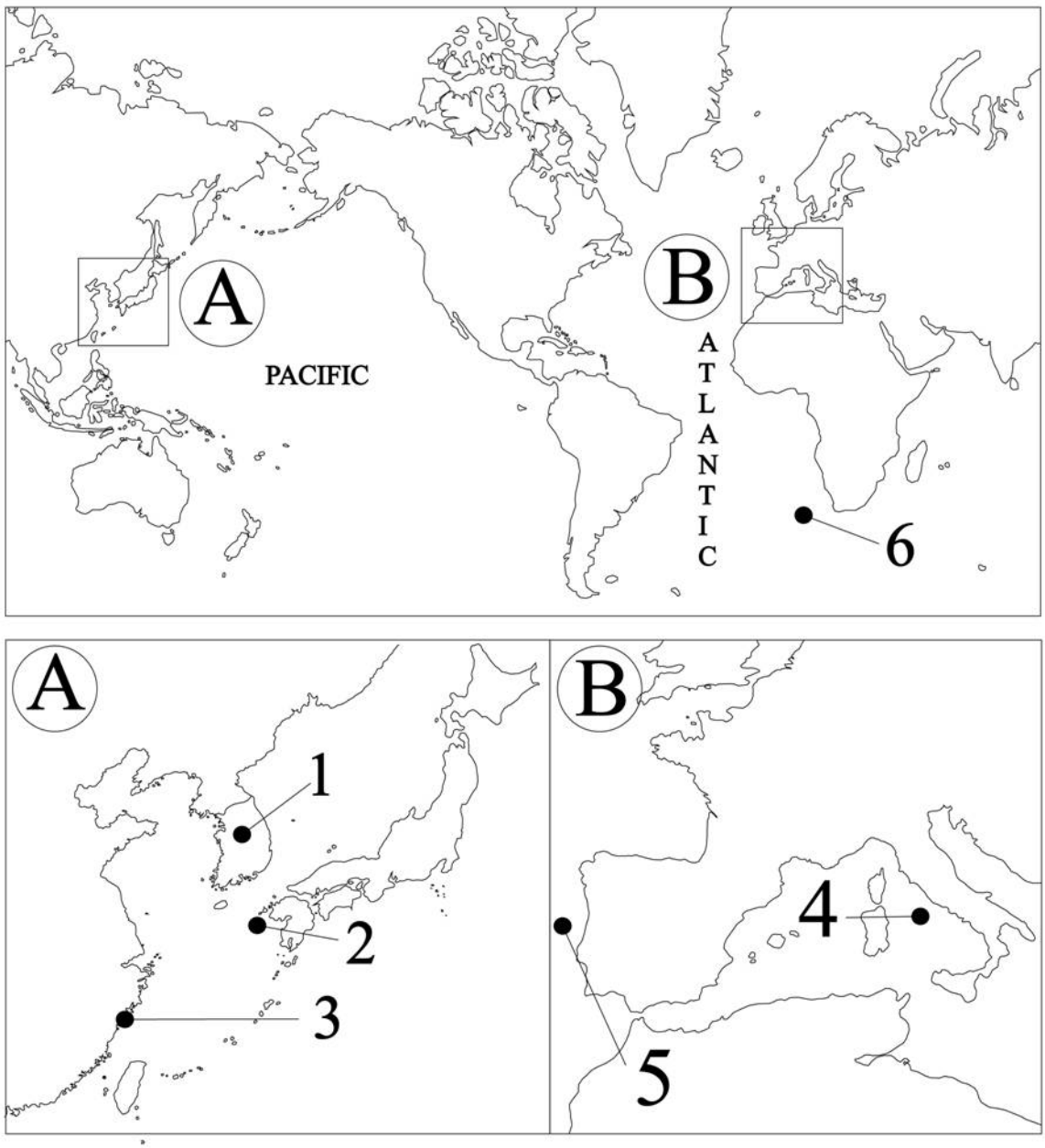
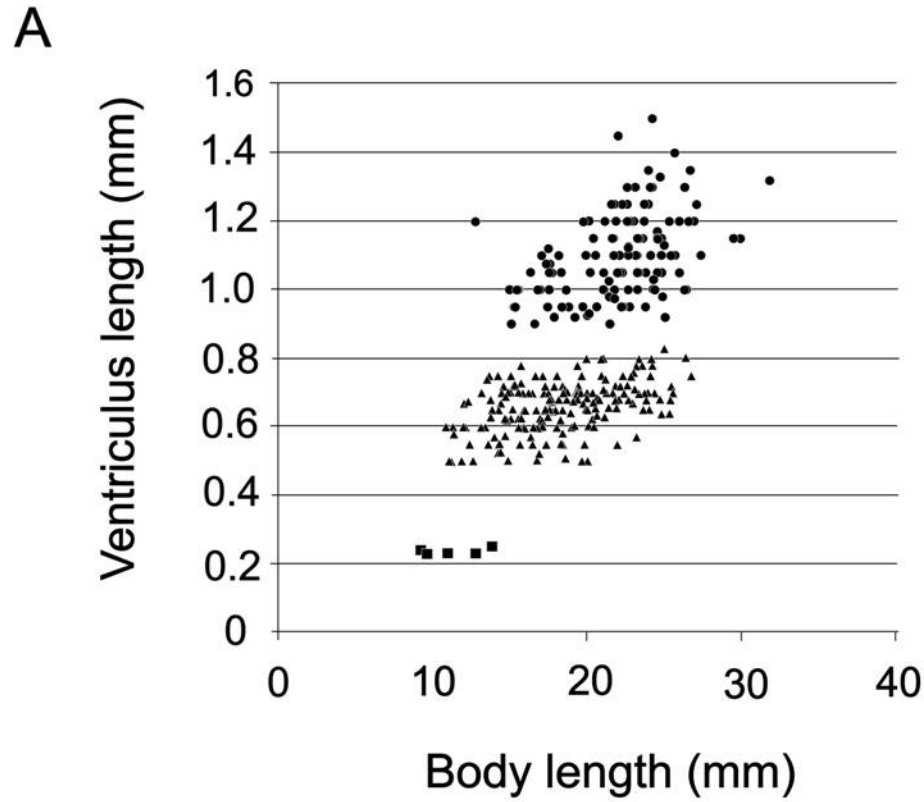


Figure 34. Geographical locations of the examined *A. pegreffii* from the Far East and Mediterranean regions.



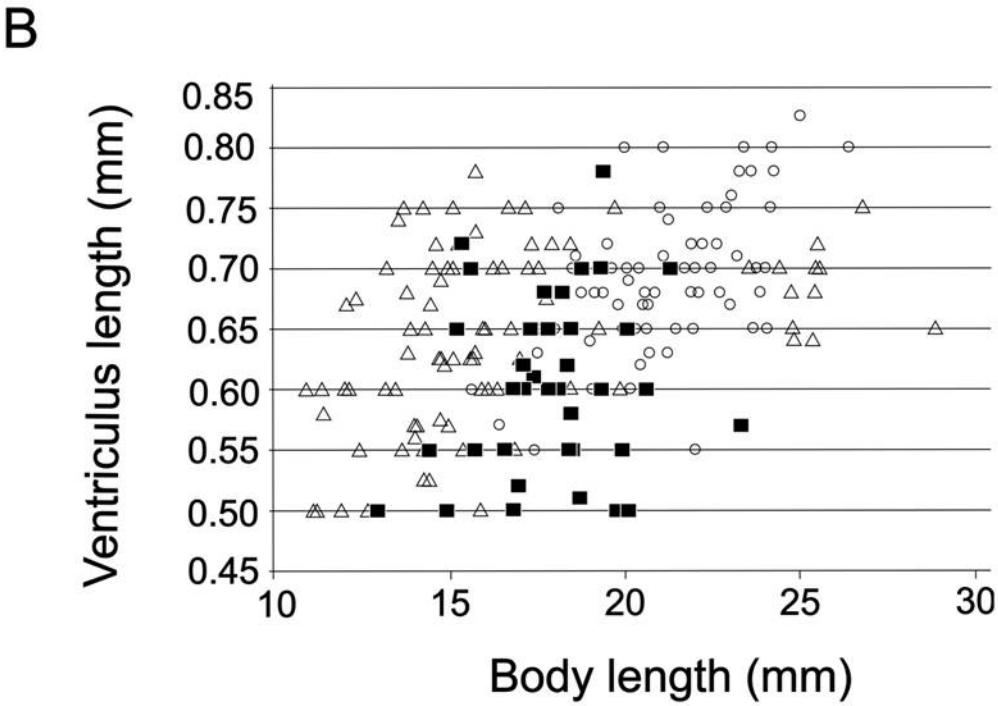


Figure 35. Ventriculus length of L3 larvae of *A. pegreffii* from Mediterranean and *A. pegreffii* JP from China, together with other previously reported *Anisakis* species. A – ventriculus length of *A. simplex* (s.s.) (●), *A. pegreffii* (▲) and *Anisakis* Type II larvae (■); B – ventriculus length of *A. pegreffii* from Japan (△), Mediterranean (■) and China (○).

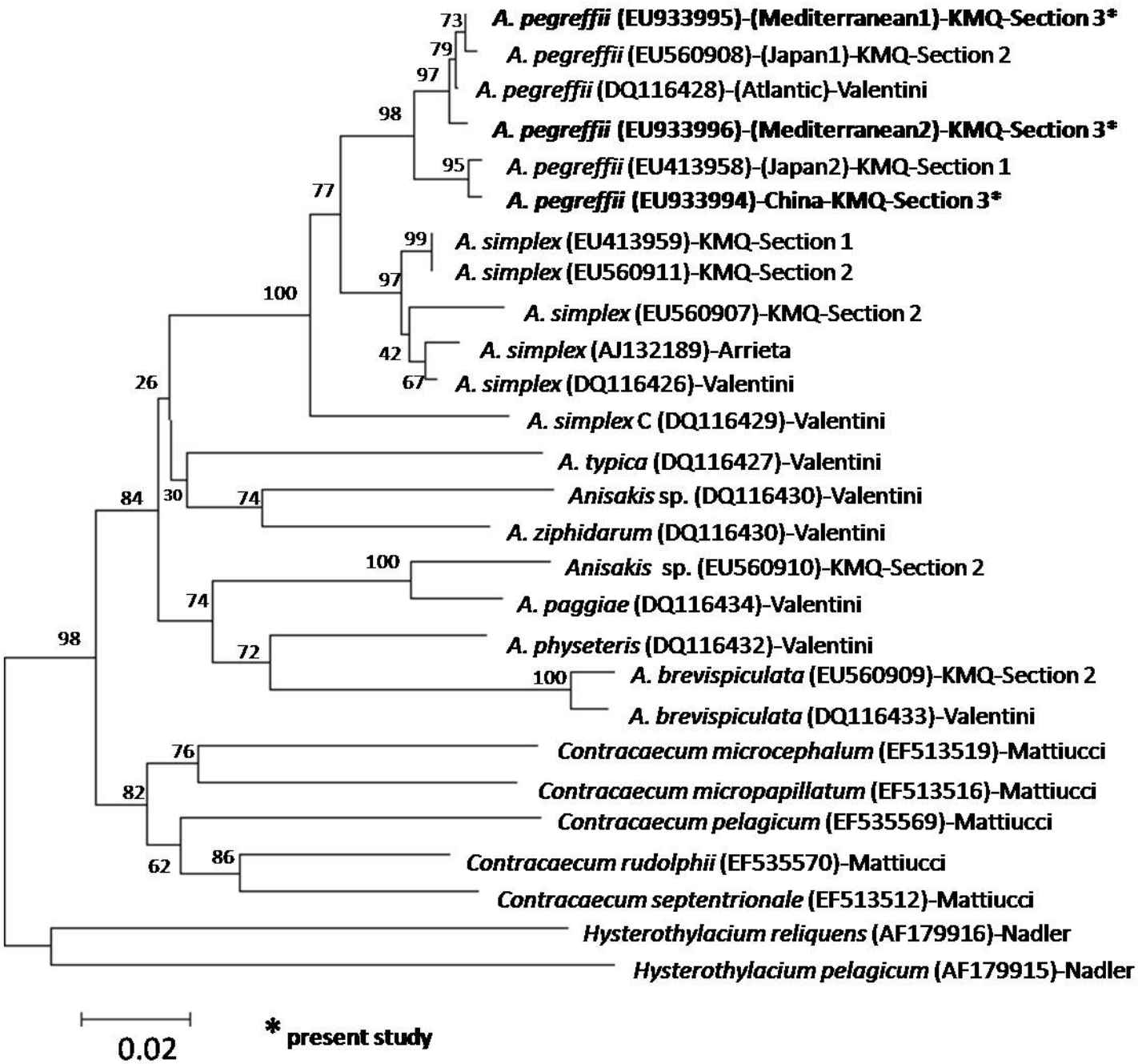


Figure 36. NJ tree inferred from *p-distance* values based on the nucleotide bases of *mt*DNA *cox*2 gene showing the evolutionary relationships among presently and previously studied *Anisakis* species.

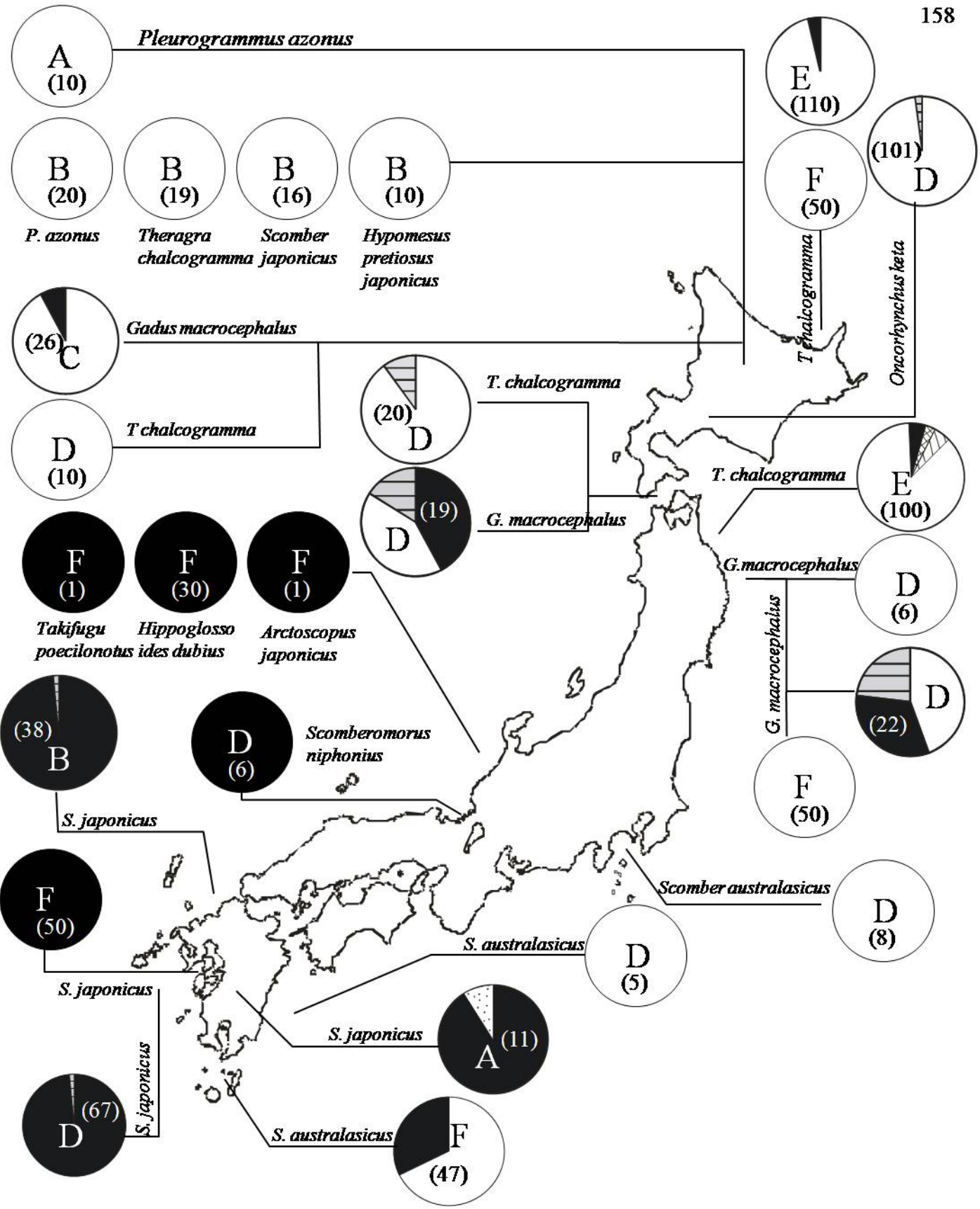


Figure 37. Distribution and relative proportions of different *Anisakis* species. ○: *A. simplex* (s.s.); ●: *A. pegreffii*; ⊘ : *Anisakis* sp.; ⊗: *A. brevispiculata*; ⚽ : *A. physteris*; ⊜ : hybrid genotypes . A-Umehara et al. 2008; B-Umehara et al. 2006; C-Abe et al. 2005; D-present study from Section 1 (Subchapter 1, Chapter 3); E-present study from Section 2 (Subchapter 1, Chapter 3); F-other examined host fishes (present study). Number of examined worms are in parentheses.

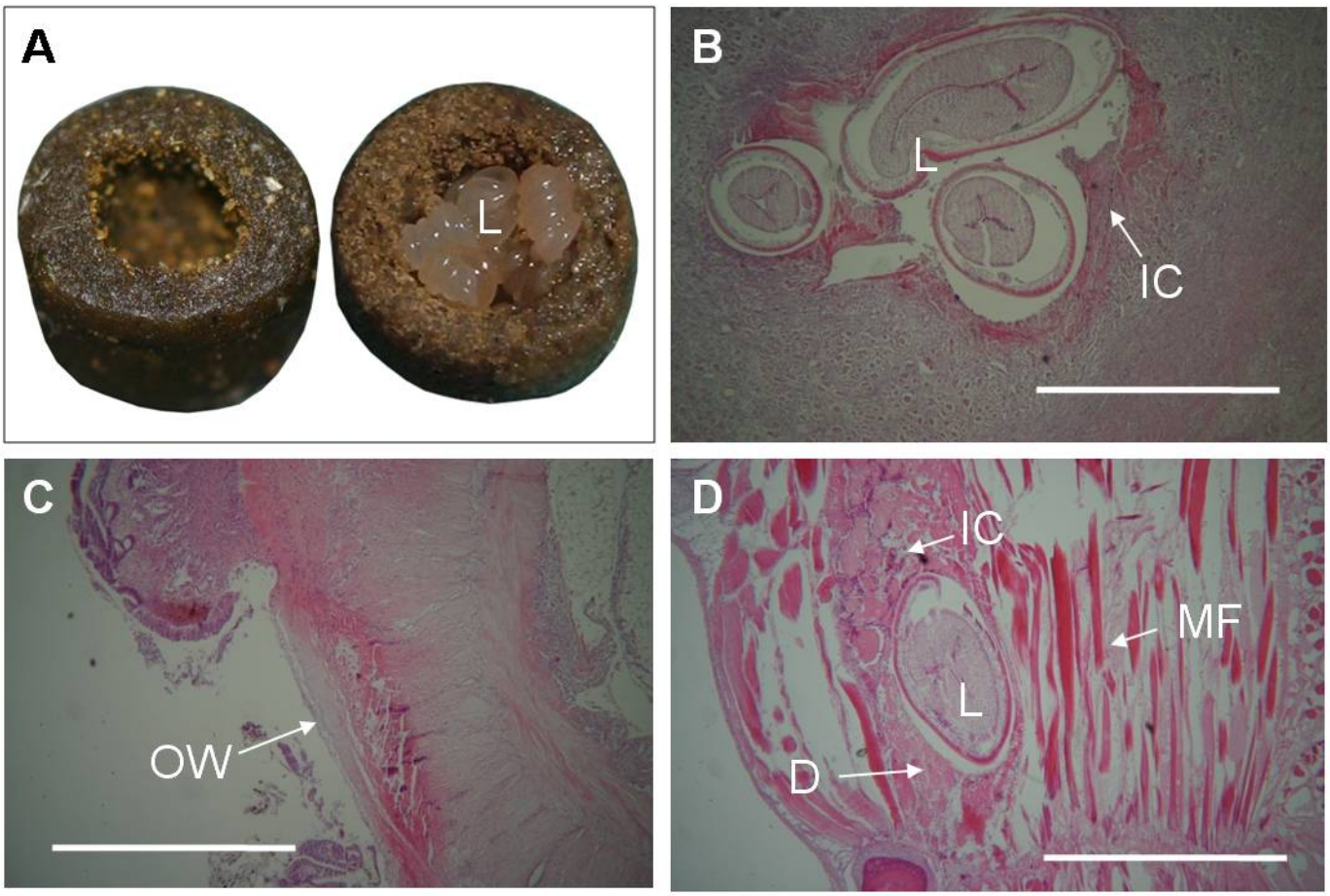


Figure 38. Migration and pathological effect of *Anisakis simplex* (s.s.) in rainbow trout. A – pellet bored with hole in the middle (left) where L3 larvae (L) were inserted (right); B – L3 larvae within the sub mucosal layer showing haemorrhages and accumulation of inflammatory cells (IC) around the larva (L); C – open wound (OW) in the stomach wall created by migrated L3 larvae (L); D – L3 larvae (L) inside the infected musculature surrounded by degenerated muscle fibres (D) where inflammatory cells (IC) can be seen in between. Intact and normal muscle fibres (MF) can be seen in the vicinities of the worm in the musculature. Scale bar = 1 mm.