論 文 の 内 容 の 要 旨

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論文題目　Studies on philometrid and anisakid nematodes infecting marine fishes in Japanese waters
（日本の海産魚に寄生するフィロメトラ科およびアニサキス科線虫に関する研究）

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 rDNA 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) morphological and molecular approaches for multi-species identification of *Anisakis* species in *Theragra chalcogramma*, (3) comparisons of *A. pegreffii* from the Far East and Mediterranean, and (4) distribution of different *Anisakis* species 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 region 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) of rDNA 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.

Multi-species identification of Anisakis in Theragra chalcogramma 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 and *mt*DNA *cox*2 regions, 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 rDNA, 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 genotypes 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 bounderies 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.