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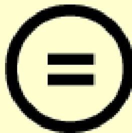

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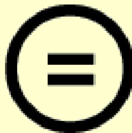

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의학석사 학위논문

Molecular Identification of *Anisakis pegreffii* (Nematoda: Anisakidae) in the Sea Eels (*Astroconger myriaster*) from a Southern Coastal Area of Korea

남해안에 서식하는 붕장어에 기생하는 *Anisakis pegreffii* (Nematoda: Anisakidae) 의 유전학적 동정

2013년 08월

서울대학교 대학원

의학과 기생충학전공

임 혜 미

Molecular Identification of *Anisakis pegreffii* (Nematoda: Anisakidae) in the Sea Eels (*Astroconger myriaster*) from the South Coast of Korea

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이 논문을 의학석사 학위논문으로 제출함

2013년 04월

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임 혜 미

임혜미의 의학석사 학위논문을 인준함

2013년 07월

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Molecular Identification of *Anisakis pegreffii* (Nematoda: Anisakidae) in the Sea Eels (*Astroconger myriaster*) from a Southern Coastal Area of Korea

by

Hyemi Lim

(Directed by Prof. Jong-Yil Chai)

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Parasitology) at Seoul National University College of Medicine

July 2013

Approved by Thesis Committee:

Professor ____________________Chairman

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Abstract

Human anisakiasis is an important fish-borne parasitic zoonosis. It is caused by anisakid larvae that include the species of *Anisakis* and *Pseudoterranova*. In Korea, *Anisakis* type I larvae (mixture of *Anisakis simplex* and *Anisakis pegreffii*) were reported from various species of marine fish, including the sea eel and yellow corvina. However, the presence of *A. pegreffii* has seldom been documented. In this study, *Anisakis* larvae were collected from the sea eel (*Astroconger myriaster*) collected from Tongyeong City, a southern coastal area of Korea in March 2013, and molecular analysis was performed. All sea eels examined (20/20, 100%) were found infected with *Anisakis* larvae. In total, 160 *Anisakis* type I larvae were recovered from 20 sea eels (average 8 per fish). They were morphologically *Anisakis* type I larvae, but may be either *A. simplex* or *A. pegreffii*. These two species can be differentiated only through molecular analysis of PCR-RFLP and sequencing based on PCR-RFLP patterns using sequences of internal transcribed spacer (ITS1, 5.8 subunit gene and ITS2) of nuclear ribosomal DNA and sequencing analysis of mitochondrial cytochrome *c* oxidase 2 (*cox*2). The results showed

that 87% of the *Anisakis* type I larvae (113/129 larvae) were identified as third-stage larvae of *A. pegreffii*, and 8% (10/129 larvae) were *A. simplex* larvae. The species of the remaining 5% (6/129) were unknown. This is the first report of *A. pegreffii* from the sea eels of the south coast in the Republic of Korea.

Key words: *Anisakis pegreffii*, sea eel, Republic of Korea, PCR-RFLP, ITS1, *cox*2

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different patterns (370, 300 and 250 bp) C. *A. simplex* has 2 different patterns (370 and 700 bp) D. Infection rate of anisakis larvae from sea eels in this study (n=129).

Figure 4. Phylogenetic tree based on mtDNA *cox*2 (629 bp) gene sequences exploring the relationships among *A. simplex*, *A. pegreffii,* and sample A,B,C.

INTRODUCTION

Human anisakiasis is an important fish-borne parasitic zoonosis caused by ingestion of raw or undercooked fish infected by the larvae of the genera *Anisakis*, *Pseudoterranova*, *Contracaecum,* or *Hysterothylacium* [1]. Humans acquire the infection by two species of *Anisakis, A. simplex* sensu stricto and *A. pegreffii* [2]. These two species have been found to cause human infections [2-6], and identification of parasites has been confirmed by molecular techniques [3-6].

Anisakid larvae can penetrate into the gastrointestinal tract of humans and invade adjacent organs, which give rise to edema, hyperemia, and bleeding in the surrounding gastric mucosa, normally within 6 hr after the ingestion of the infected fish host. Some larvae may remain in the gastrointestinal tract, without penetrating tissues, causing an asymptomatic infection [7].

Human anisakiasis was reported for the first time in the Netherlands [8], and then reports have been made particularly in Japan and some European countries where there is some eating habit of raw and/or undercooked fish. In Italy, in recent years, several cases have been reported [5,9-16]; most of these were

based on histopathological findings, and only in three cases molecular diagnoses have been made on larval nematodes extracted by gastroduodenoscopy [4,5,16]. So far, human *A. pegreffii* infection has not been reported in countries other than Japan and Italy [3-6].

Several studies have been performed on the infection status of anisakid larvae in fish intermediate hosts in Korea. However, most studies were performed on the morphological basis targetting various fish species, such as the yellow croaker (*Pseudosciaena polyactis),* white-spotted conger, salmon (*Onchorhynchus keta),* sea trout (*Oncorhynchus masou),* or anchovy (*Engraulis japonicus)* [17-23], Recently, there were two reports on molecular identification of *Anisakis pegreffi* and *Anisakis simplex* from several species of fish or squids in Korea [23,24]; one reported *A. simplex* from the chum salmon [23], and the other reported a predominance of *A. pegreffii* in several species of fish and squids [24].

In this study, we determined the status of anisakid larval infections in the sea eels caught in the Republic of Korea. The larvae were morphologically *Anisakis* type I which may include *A. simplex* and *A. pegreffii*. Molecular characterization was

performed by PCR, PCR-RFLP, and DNA sequencing of nuclear (ITS1) and mitochondrial (*cox*2) genes to identify the *Anisakis* species occurring in the sea eels.

MATERIALS AND METHODS

1. *Anisakis* larvae from fish

Twenty refrigerated sea eels (*Astroconger myriaster*) were purchased on March 2013 at Guri Agricultural and Marine Products Market, Gyeonggi-do, Korea. The sea eels were told to have been caught around Tong-yeong city in the south coast of Korea. In total, 160 third stage larvae (L3) were collected.

2. Genomic DNA extraction

The total genomic DNA (gDNA) from individual worms was extracted by DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After DNA extraction, the samples were checked nucleic acid concentration with Nanodrop 2000 spectrometer (Thermo Scientific, Wilmington, Delaware, USA).

3. PCR-RFLP analysis

Identification to the species level was carried out using a 629 bp fragment of the mitochondrial cytochrome *c* oxidase 2 (*cox2*)

gene. The *cox2* gene from *Anisakis* spp. was amplified using the primers 211F 5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3' and 210R 5'-CAC CAA CTC TTA AAA TTA TC-3'. Polymerase chain reaction (PCR) was carried out using the Smart 2x PCR premix Taq (Solgent co., Ltd, Daejeon, Korea), containing 10 pmol of each primer and 30 ng of total DNA. The mixture was denatured at 94°c for 3 min, followed by 34 cycles at 94°c for 30 sec, 46°c for 1 min and 72°c for 1.5 min, followed by post-amplification at 72°c for 10 min. The PCR product automated DNA sequencing was performed by Solgent co., Ltd. (Daejeon, Korea) using *cox2* 211F and 210R primers.

A region of nuclear ribosomal DNA (rDNA) was amplified using internal transcribed spacers (ITS1, 5.8 subunit rRNA gene, and ITS2) A (5'-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3') and B (5'-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3' with Smart 2x PCR premix Taq (Solgent co., Ltd, Daejeon, Korea), containing 10 pmol of each primer and 30 ng of the total DNA. The mixture was undergone initial denaturation at 95°c for 10 min, followed by 30 cycles of denaturation at 95°c for 30 sec, annealing at 55°c for 30 sec, extension at 72°c for 1.15 min with a final extension step at 72°c

for 7 min.

Individual ITS1 5.8 subunit rRNA gene and ITS2 PCR products (17 ul) were digested with 10 unit of the restriction endonuclease *Hin*f1 (1 μl) (Enzynomics, Daejeon, Korea) and 10x EZ-one buffer (2 μl) (Enzynomics, Daejeon, Korea) in a final volume of 20 μl at 37°c for 1 hr. Digestion products were then separated by electrophoresis on a 3% agarose gels, containing 1 μg/ml ethidium bromide and visualized under ultraviolet light. The comparison of the fragments profile generated by enzymatic digestion of the 900 bp rDNA region amplified by PCR from gDNA of the isolated worm unambiguously identified it as *A. pegreffii*.

4. DNA sequencing and phylogenetic analysis

Nucleotide sequences obtained from each larval specimen that randomly selected were aligned using the program Geneious v.6.0.3.

RESULTS

1. Collection of anisakid larvae from fish

A total of 160 *Anisakis* third stage larvae (8.0 infected per sea eel) were collected in 20 sea eels caught around Tong-yeong city in the south coast of Korea. All of the sea eels (100%) examined were founf to be infected with *Anisakis* larvae (Fig. 1)

2. Morphological examination

These larvae were identified as *Anisakis* type I morphologically. They had a boring tooth, a ventriculus, and a mucron. The anterior portion showed a prominent boring tooth anteriorly in the cephalic reion (Fig. 2-A). In the digestive tract, the ventriculus level showed the simply connected esophagus, ventriculus, and intestine (Fig. 2-B). The posterior portion showed a mucron in the caudal region (Fig. 2-C).

3. PCR-RFLP

Out of the 129 *Anisakis* larvae identified by PCR-RFLP analysis, approximately 900 bp fragment was produced after

amplification of the rDNA region (ITS-1, 5.8 subunit, and ITS-2). The PCR products were processed in order to identify the species with restriction enzyme *Hin*f1. The *Hin*f1 was the most appropriate and the best known enzyme for anisakid larvae molecular identification. RFLP produced two patterns (Fig. 3A), one of which was three different fragments of approximately between 250 and 500 bp (*A. pegreffii*) and the other was two different fragments between 500 bp and 1,000 bp (*A. simplex*). According to this tecjnique, 8.0% (10/129) of *Anisakis* larvae were *A. simplex* and 87% (113/129) were *A. pegreffii*. The remaining 5% (6/129) included blank or unknown bands (Fig. 3B).

4. DNA sequencing and phylogenetic analysis

The final confirmation of the species identification was made after sequencing of the mtDNA *cox*2 gene and aligned using the program Geneious v.6.0.3. Randomly selected samples were undergone sequence analysis of the mtDNA *cox*2 gene of 629 bp, and the results indicated that more closely the sequences were obtained for *A. pegreffii* compared to *A. simplex* (Table 1). The sequencing results were identical with *A. pegreffii* in sample A (97.8%) and sample B (99.6%), with only minor differences, and

were completely identical in sample C (100%). On the other hand, their homology with *A. simplex* was lower than 94.0%.

Table 1. Estimation of the evolutionary divergence between *A. pegreffii* and *A. simplex* based on mtDNA *cox*2 region of 3 randomly sampled larval specimens

Sample	*A. pegreffii* (%)	*A. simplex* (%)
A	97.8	93.4
B	99.6	93.7
C	100	94.0

Table 2. History of *Anisakis pegreffii* studies in Japan, Korea, and China

Authors (year)	Subjects	Area	Method	Reference No.
Abe et al. (2005)	Fish	Japan	PCR-RFLP	25
Umehara et al. (2006)	Fish	Japan	PCR-RFLP	37
Zhang et al. (2007)	Fish	China	PCR-coupled mutation scanning sequence analysis	60
Lee et al. (2009)	Fish, Squid*	Korea	PCR-RFLP	
Fang et al. (2010)	Fish	Taiwan strait	Multiple primer PCR	38
Du et al. (2010)	Fish	China	PCR-RFLP	59
Shih et al. (2010)	Fish	Taiwan	PCR-RFLP	58
Umehara et al. (2010)	Fish	Taiwan and Japan	PCR-RFLP	57
Fang et al. (2011)	Fish	Taiwan strait	Real-time PCR	55
Chou et al. (2011)	Fish	Taiwanese coast of the NWP**	PCR-RFLP	54
Murata et al. (2011)	Fish	Japan	Sequence analysis	38
Quiazon et al. (2011)	Fish	Japan	PCR-RFLP	51

Arizono et al. (2012)	Human	Japan	Real-time PCR	29
Zhang et al. (2013)	Fish	China	PCR-RFLP	44

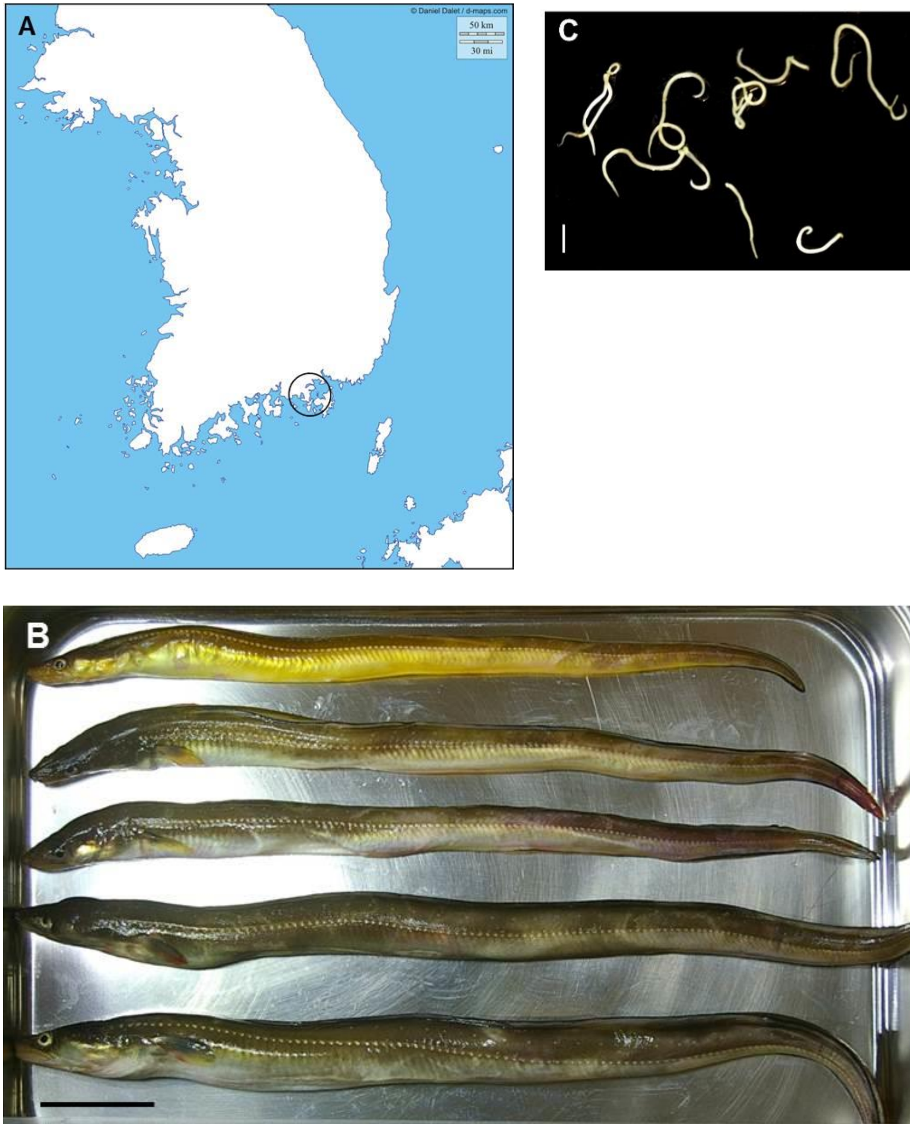
*Chub mackerel (*Scomber japonicas*), Ribbon fish (*Trichiurus lepturus*), Pacific squid (*Todarodes pacificus*)
**Northwestern Pacific

Table 3. History of *Anisakis pegreffii** studies in Europe, Australia, and America

Authors (year)	Subjects	Area	Method	Reference No.
Nascetti et al. (1986)	Fish	Mediterranean and Atlantic	Electrophoretic analysis	65
Mattiucci et al. (1997)	Fish and squid	Italy	Genetic and ecological data	33
D'Amelio et al. (1999)	Human	Italy	PCR-RFLP	4
D'Amelio et al. (2000)	Fish	Thyrrhenian sea	PCR-RFLP	34
Abollo et al. (2001, 2003)	Fish	Spain	Morphology, PCR-RFLP	62, 63
Martin et al. (2005)	Fish	Spain	PCR-RFLP, RAPD**-PCR	64
Pontes et al. (2005)	Fish	Portugal	PCR-RFLP	61
Fumarola et al. (2009)	Human	Italy	PCR-RFLP	5
Santoro et al. (2010)	Marine mammal	Italy	Sequence analysis	56
Cavallero et al. (2011)	Fish	Southeastern Atlantic	PCR-RFLP	53
Petric et al. (2011)	Squid	Eastern Adriatic	Sequence analysis	52
Mattiucci et al. (2011)	Human	Italy	Sequence analysis	6

Meloni et al. (2011)	Fish	Western Mediterranean	PCR-RFLP	36
Baldwin et al. (2011)	Fish	California	Sequence analysis	50
Abattouy et al. (2011)	Fish	North of Morocco	PCR-RFLP	49
Chaligiannis et al. (2012)	Fish	Aegean sea	PCR-RFLP	27
Hermida et al. (2012)	Fish	Portugal	PCR-RFLP	48
Jabbar et al. (2012)	Fish	Australia	PCR-coupled mutation scanning sequence analysis	47
Cavallero et al. (2012)	Fish	Tyrrhenian sea	PCR-RFLP	46
Mladineo et al. (2012)	Fish	Adriatic sea	Sequence analysis	45
Jabbar et al. (2013)	Fish	Western Australia	PCR-coupled mutation scanning sequence analysis	43
Abattouy et al. (2013)	Fish	Northern Morocco	PCR-RFLP	42
Serracca et al. (2013)	Squid and Fish	Ligurian sea	PCR-RFLP	41
Mattiucci et al. (2013)	Human	Italy	Sequence analysis	26

*The original description of *Anisakis pegreffii* was reported in 1955 by Campana-Rouget & Biocca.
**Random amplified polymorphic DNA



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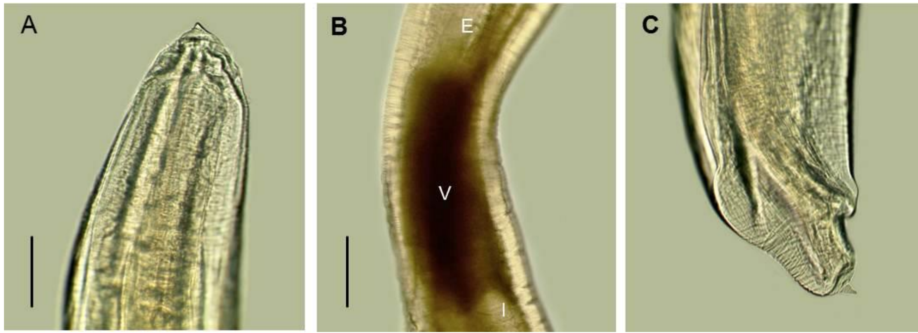

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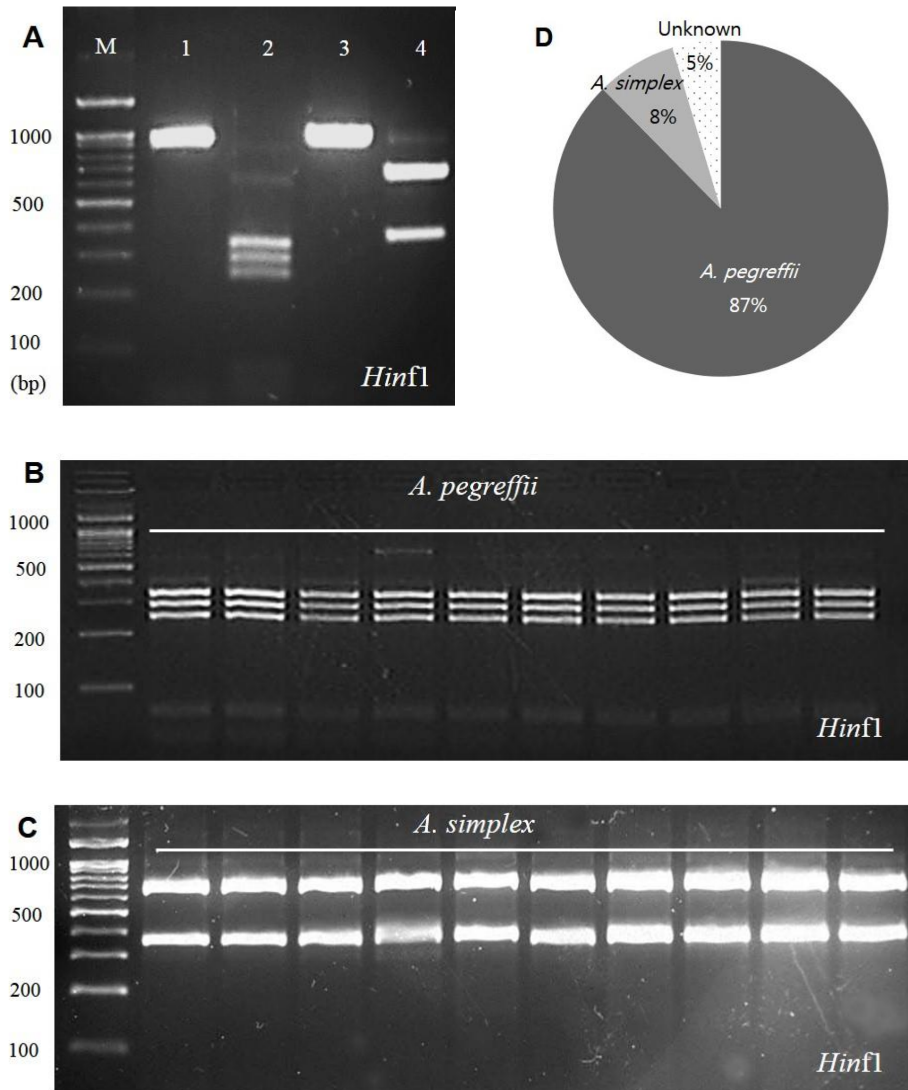

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A. simplex (PCR-RFLP from lane 2 gDNA). B. *A. pegreffii* has 3 different patterns (370, 300 and 250 bp) C. *A. simplex* has 2 different patterns (370 and 700 bp) D. Infection rate of anisakis larvae from sea eels in this study (n=129).

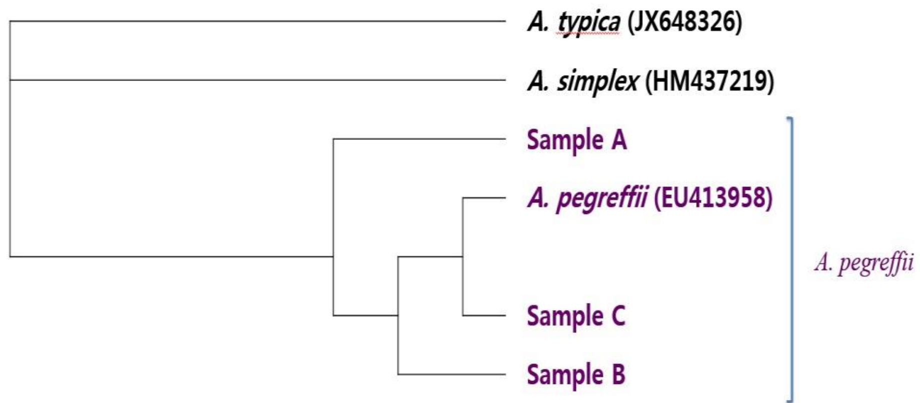

Figure 4. Phylogenetic tree based on mtDNA *cox*2 (629 bp) gene sequences exploring the relationships among *A. simplex*, *A. pegreffii,* and sample A,B,C.

DISCUSSION

Human anisakiasis is an important fish-borne parasitic zoonosis and caused by the ingestion of raw or improperly cooked fish infected by the larvae [1]. The overall infection rate of *Anisakis* larvae in sea eels was 100%, with the infection density of 8.0 larvae per sea eel. As seen from its infection status, the sea eel is suspected as one of the most important paratenic fish hosts that may be related with human anisakiasis in Korea. Also, *Anisakis* larval infection has seasonal variations, as Stømnes and Andersen [25] reported that *Anisakis* larvae significant increase in number during the spring time of March and April.

Anisakis pegreffii was first reported in 1955 by Campana-Rouget & Biocca. After the report, in 1986, *A. pegreffii* was found in the Mediterranean and Atlantic Ocean using the electrophoretic technique [66]. Since then, several studies have reported on *A. pegreffii* in various localities and using various methods. In Europe, Australia, and America, *A. pegreffii* was reported repeatedly in marine fish, squids, and humans (Table 3).

Based on the larval morphological features, genus *Anisakis* can be identified as *Anisakis* type I or type II, which can be

mainly differentiated by the length of ventriculus [6,23]. However, the morphological characteristics are not always consistent. In East Asia, studies on molecular analysis of anisakid larvae began in Japan in 2005 [26] and then in 2007 in China [61], and in 2009 in Korea [24]. Molecular studies became more active in East Asia after 2010 (Table 2). The ribosomal DNA gene is an important multigene family. One unit of ribosomal DNA consists of three genes encoding ribosomal RNA separated by internal transcribed spacers (ITS). The ITS region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions. ITS provides a useful approach for the specific identification of both distantly and closely related anisakis species, the level of variation in ITS region makes it suitable for detecting genetic variation within species. D'Amelio et al. (2010) suggested that mtDNA region is highly reliable for species discrimination [23, 35, 38, 67].

The first report of human *A. pegreffi* infection was by D'Amelio et al. [4] in 1999 in Italy using a molecular technique, PCR-RFLP. The second was from Japan in 2007, in which one of the 85 anisakid larvae extracted from human anisakiasis patients in Kyushu was identified as *A. pegreffii* by PCR-RFLP of ITS

regions including 5.8S rRNA [3]. Thereafter, in 2009, two Italian women were diagnosed as gastric anisakiasis due to *A. pegreffii* through PCR-RFLP of the ITS1, 5.8S gene, and ITS2, plus about 70 nucleotides of the 28S gene [5]. Subsequently, in 2011, DNA was extracted from a paraffin-embedded granuloma taken from an Italian man which was followed by a molecular confirmion as *A. pegreffii* [6]. It has been also documented in 2013 that human *A. pegreffii* infection is associated with gastroallegic reactions of the patients in several Italian cases [27].

In Korea, morphological studies of *Anisakis* spp. larvae have been reported. One of the reports was on *A. simplex* and *P. decipiens* larvae obtained from 107 human cases through gastrofiberscopy in Cheju-do [33]. A recent report was on the infection of marine fish and cephalopods with *Anisakis* spp. in Busan, in which a total of 2,537 specimens were collected [29]. The overall infection rate of the seafoods was 34.3% and *A. simplex* L3 showed the highest abundance in the spring season in most of the fish species [29]. In 2012, marine fish from three sea areas of the Republic of Korea were examined on anisakid larval infection; the result was 52.3% (45 of 86) of fish infected with *Anisakis* spp. from the East Sea, 76.6% (131 of 171) of fish in the

South Sea, and 40.2% (37 of 92) of fish from Yellow Sea were infected [36]. Species and type of larvae were determined by their morphological characteristics and measurement data [36]. The prevalence of infection and the identification of anisakid larvae in chum salmon from the Namdae River, the east coast of Korea, were investigated in 2008. All the chum salmon (120/120; 100%) were infected with anisakid larvae. Based on the morphological and the molecular analysis of PCR-RFLP and sequencing of mitochondrial DNA cox2 gene, these *Anisakis* larvae were identified as *A. simplex* (s.s) [23]. So far, *A. pegreffii* has seldom been studied in Republic of Korea through morphological and molecular approaches.

Anisakis larvae, particularly *A. simplex* sensu stricto and *A. pegreffii,* are difficult in morphological identification and can only be defined by molecular examination [23,24]. The rDNA gene is an important multigene family consisting of tandem repeats of genes interspersed with transcribed and non-transcribed spacers. One unit of rDNA consists of three genes encoding rRNA separated by ITS or mitochondrial cytochrome *c* oxidase 2 (*cox2*) gene. These are the most frequent targets used to identify *Anisakis* spp. The ITS region does not encode any

product, permitting it to evolve at a faster rate than the ribosomal coding regions. The level of variation in ITS region makes it suitable for detecting genetic variation within species [6,23,31,37-39]. In our study, rDNA RFLP analysis results support the identification of *A. simplex* and *A. pegreffii*. In total, 113 (87.0%) of *A. pegreffii* were identified 129 of anisakis larvae and 10 (8.0%) *A. simplex*, 6 (5%) unknown anisakis larvae were detected.

A. pegreffii is widely distributed in the south Atlantic and north Pacific, as well as Japanese water. *A. pegreffii* more detected from Fukuoka prefecture in Japen where near the Korea site than *A. simplex* [26,38]. *A. pegreffii* is irrefutably pathogenic to humans but not much study with *A. pegreffii* as invasive and resistant to the human gastrointestinal tract as *A. simplex*.

In this study, we report infection of anisakid larvae in paratenic host as sea eel and identification of *A. simplex* and *A. pegreffii* in sea eels in Reublic of Korea. This is the first report on the molecular identification of *Anisakis pegreffii* from sea eels in the area of southern coastal area of Korea. The larvae of *Anisakis* spp. have similar morphologic features that could be used to identify them. Molecular characterization by PCR-RFLP

was used to identify the species of Anisakis, and DNA sequencing of nuclear (ITS1) and mitochondrial (*cox*2) genes to identify the *Anisakis* spp.

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국문 초록

인체 고래회충유충증은 중요한 어류매개성 인수공통감염증으로 고래회충(*Anisakis)*종과 물개회충(*Pseudoterranova*)종을 포함하는 고래회충 유충에 의해 일어난다. 한국에서는 고래회충 제 1유형(*Anisakis simplex* 와 *Anisakis pegreffii* 혼합)이 붕장어와 조기와 같은 바다 물고기에서 발견된 보고가 있었다. 하지만 현재까지 한국에서의 *A. pegreffii*의 존재의 보고는 극히 드물고 특히나 남해안의 *A. pegreffii*의 존재 유무에 대한 보고는 없었다. 이 연구에서는 한국 남해안에 위치한 통영시에서 잡은 붕장어에서 고래회충유충을 수집하여 유전자적 확인을 하였다. 2013년 3월에 20마리의 붕장어로부터 총 160마리의 고래회충유충을 수집하였다. 확인한 붕장어들은 모두 고래회충유충에 감염되어 있었다(20/20, 100%). 이 유충들은 형태학적으로 고래회충유충의 제1유형과 동일하였으나, *A. simplex* 이거나 *A. pegreffii* 일 수 있다. 이 두 종들은 PCR-RFLP 법의 유전자적 분석과 핵의 리보솜 DNA의 internal transcribed spacer(ITS1, 5.8subunit rRNA gene and ITS2)의 염기서열을 사용한 PCR-RFLP 유형을 기반으로 한 염기서열 분석법 그리고 mitochondrial cytochrome *c* oxidase 2(*cox2*)의 염기

서열 분석을 통해서만 구별할 수 있으며, 이 선충들 중 129중 113 마리 (87%)가 *A. pegreffii*의 3기 유충들로 확인되었다. 129중 10 마리 (8%)가 *A. simplex*로 확인 할 수 있었다. 나머지 129중 6 마리 (5%)는 알 수 없었다. 이 연구는 한국 남해안의 붕장어에서 확인된 *A. pegreffii*의 첫번째 보고이다.

주제어 : *Anisakis pegreffii*, 붕장어, 한국, PCR-RFLP, ITS1, *cox2*

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