저작자표시-비영리-변경금지 2.0 대한민국

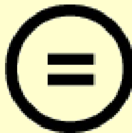
이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

연세대학교
YONSEI UNIVERSITY
[UCI]I804:11046-000000521925

Molecular Identification of *Anisakis* larvae (Nematode: Anisakidae) from marine fishes collected from three Korean sea waters

Marck António Augusto Magno Neves

Graduate School of Public Health
Yonsei University
Department of Global Health Security
Division of Global Health Security Detection Program

연세대학교
YONSEI UNIVERSITY

Molecular Identification of *Anisakis* larvae (Nematode: Anisakidae) from marine fishes collected from three Korean sea waters

Directed by Professor Tai-Soon Yong

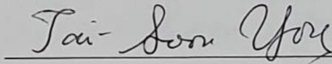
A Master's Thesis
Submitted to the Department of Global Health Security
Division of Global Health Security Detection Program
And the Graduate School Public Health of Yonsei University
In partial fulfilment of the
requirements for the degree of
Master of Public Health

Marck António Augusto Magno Neves

December 2019

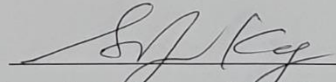
연세대학교
YONSEI UNIVERSITY

This certifies that the Master's Thesis
of Marck António Augusto Magno Neves is approved.



Thesis Committee Chairman : Tai-Soon Yong

Committee Member : Myeong Heon Shin



Committee Member : Sunjoo Kang

Graduate School of Public Health
Yonsei University
December 2019

연세대학교
YONSEI UNIVERSITY

Acknowledgements

First, I would like to thank almighty GOD and my Lord Jesus Christ for this beautiful life as His blessing on me and I want to thank also my mother Virgin Mary that always with me. I want to thank my beloved country Timor-Leste that build or established a relation and cooperation between this two country. I want to thank Korean people through the Government as the representative of the people (KOICA) for this golden opportunity to study here in Korea. I also thank to YONSEI University for receiving me and give me a chance to study Public Health at this wonderful University. And to my supervisor also as thesis committee, Prof. Tai-Soon Yong, for the guidance, encouragement, and advice he has provided throughout my time as his student. And my Thanks to all Thesis committee Prof. Myeong Heon Shin and Prof. Sunjoo Kang for their efforts on making my thesis better. I would also like to thank all the members of staff at Yonsei University College of Medicine. In particular, I would like to thank Prof. Myungken Lee for his continued support and encouragement. I was continually amazed by his willingness to encourage and motivated me and all other students fulfilling our duties and completing our theses on time. I also acknowledge that completing this thesis would have been more difficult if I wasn't helped, guided and supported by the other members of the Institute of Tropical Medicine of Yonsei University. Especially for all my lab mate, thanks to my lab supervisor Ju Yeong Kim that always helps and guides me on how to do the experiment on the lab, and Myung-hee Yi and team. I am indebted and grateful to them for their help. Thank you very much.

Last, I would like to express my gratitude to my family that always with me and give me continuous support, guidance, trust, encouragement and endless love. Father and Mother, thank you for never forget to say my name in your prayers.

Marck António Augusto Magno Neves

연세대학교
YONSEI UNIVERSITY

Table of Contents

Table of Contents i
Abbreviations iii
List of Tables iv
List of Figures v
ABSTRACT vi
I. INTRODUCTION 1
II. LITERATURE REVIEW 3
2.1. Preface. 3
2.2. *Anisakis* (parasitic zoonoses) 3
2.3. Phylogenies of *Anisakis* 5
2.4. Life cycle of *Anisakis spp.* 6
III. HYPOTHESIS, QUESTIONS and PURPOSE 8
3.1. Study Hypothesis 8
3.2. Study Questions 8
3.3. Purpose 8
IV. MATERIALS AND METHODS 9
4.1. Materials 9
4.1.1. Specimens: Fish species 9
4.1.2. Specimens: Locations 10
4.1.3. Dissection 13
4.2. Methods 16
4.2.1. DNA extraction 16

연세대학교
YONSEI UNIVERSITY

4.2.2. PCR for identification of pathogens 17
4.2.3. Species identification strategies 19
4.2.4. Sequence analysis 19
4.2.5. Nucleotide Identity Percentage (%) analysis 19
V. RESULT 21
5.1. PCR analysis 21
5.2. Validation of *Anisakis* species collected from Korean waters 21
5.2.1. Sequencing result 22
5.2.2. Nucleotide Identity Percentage (%) 23
VI. DISCUSSION 26
VII. CONCLUSION 28
VIII. REFERENCES 29
IX. Appendix 34

연세대학교
YONSEI UNIVERSITY

Abbreviations

A. pegreffi	*Anisakis pegreffi*
A. simplex	*Anisakis simplex*
DNA	Deoxyribonucleic Acid
EMBOSS	European Molecular Biology Open Software Suite
Fig.	Figure
H. aduncum	*Histerothylacium aduncum*
INFOSAN	International Food Safety Authorities Network
ITS	International Transcribed Spacer
L (1,2,3,4,5)	Larvae Stage 1,2,3,4,5
NIP	Nucleotide Identity Percentage
PCR	Polymerase Chain Reaction
P. decipiens	*Pseudoterranova decipiens*
PSA	Pairwise Sequence Alignment
UV	Ultraviolet
WHO	World Health Organizations

연세대학교
YONSEI UNIVERSITY

List of Tables

Table 1. Fish species, number of samples, body measurement (weight), total parasites found on each samples and location area that from three different Korea sea waters. 12
Table 2. PCR Master Mix ingredients and total the amount for master mix (μL) 17
Table 3. Result total of DNA sequence positive as *Anisakis pegreffi* species........................23
Table 4. Nucleotide identity (%) of anisakid larvae stage 3 from mackerel, yellow croaker fish, and sea eel with *Anisakis pegreffi, A. simplex, Pseudoterranova decipiens and Hysterothylacium aduncum* 24

연세대학교
YONSEI UNIVERSITY

List of Figures

Figure 1. The life cycle of *Anisakis* spp with each stages and different hosts (heteroxenuous) including human as an accidental host. 6
Figure 2. The five different sampling collected locations from three Korean sea waters. 10
Figure 3. Total amount of three different types of fish that collected from different location in three Korean sea waters 11
Figure 4. Longitudinal dissection from the anus until the gills (branchiopores) of the sea eel (*Conger myriaster)* to expose the internal organs. 14
Figure 5. *Anisakid* larvae L3 presence in different part of Sea eel organs 14
Figure 6. The *Anisakis spp.* larvae L3 stage at below the skin (subcutaneous) 15
Figure 7. PCR product (DNA fragments band) in 2% agarose gel that electrophoresed for molecular identification of anisakid larvae, here showed the result all were belongs to species of *A. pegreffi*..22

연세대학교
YONSEI UNIVERSITY

ABSTRACT

Anisakis infects humans who eat raw seafood such as squid, mackerel, and conger, which is considered as one of the parasitic diseases in Korea. Molecular study for *Anisakis* L3 larvae was performed to identify the species of *Anisakis*.

Molecular analysis for *Anisakis* L3 larvae was performed to identify the species of *Anisakis* and by using the DNA alignment tool to identify the DNA sequence relation. Total 98 *Anisakis* type I L3 larvae were collected from 63 fishes among three kinds of fish species (mackerel (*Scomberomorus japonicus*), anago (*Conger myriaster*), and yellow croaker (*Larimichthys polyactis*)) that came from different location near sea areas (Namhae-gun, Uljin-gun, Daecheon-si, Pohang-si and Seoul).

DNA were extracted from *Anisakis* and ITS gene (forward primer: Nc5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3'); reverse primer: Nc2 (5'-TTAGTTTCTTTTCCTCCGCT-3')) was amplified using PCR and sequenced.

By using some method that considered for the species identification strategies, such as: Sequence analysing (*Anisakis pegreffi* (GGCAGAGT**C**) => C/C allele and *Anisakis simplex* (GGCAGAGT**T**) => T/T allele) and the strategy to find the rest total of unidentified in the first strategy by using nucleotide identity percentage: *A. pegreffi* (AB277823), *A. simplex* (AB277822), *P. decipiens* (AB277824) *and H. aduncum* (AB277826).

It was found that *Anisakis pegreffi* was the most predominant *Anisakis* species from all fish species and in all Korean locations with 67 as a result of the total. And in the result of nucleotide identity percentage for analyzed the rest 31 samples that not identified during the sequence

연세대학교
YONSEI UNIVERSITY

procedure, based on matched result, 1 were more matched to *A. simplex*, 25 to *A. pegreffi* and 5 to *H. aduncum.*

Keyword: *Anisakis*. PCR. DNA sequence.

연세대학교
YONSEI UNIVERSITY

I. INTRODUCTION

Anisakiasis is considered as a fish-borne parasitic infection and this parasite can be associated with human diseases. This parasitic zoonoses can be occurred with people that live in some specific area where presented some food behavior by eating raw marine animal (different type of fish) like in Korea and Japan. (11)

The World Health Organization (WHO) 2012, estimates approximately 1.5 billion people are infected with soil-transmitted by helminth worldwide and approximately 56 million cases of parasite infections associated with the consumption of fish product. Among all categories of parasites are also implicated to cause harm on human health and even can have as a result some severe pathologies in human. This parasite also wide distributed in different part of globe. (1, 16)

Since the case of Anisakiasis being reported for the first time in Korea by (Kim et.al)(29) the occurrence of this infection consistently occurred here in Korea. In some data source we can observed that the incidence of this infection are growing in number each time. (12) With this condition considered as one of the reason that motivated the Author to conducted this study.

Anisakis can caused some clinical issues, such as nauseas, vomiting, abdominal pain and even allergic reaction. (28)

There is relatively some study realized on *Anisakis* here in Korea related to this diseases, some of them even targeting all three Korean sea water (2), only the sample target and some location is different but in result presented some similarities with more predominantly *A. pegreffi*. Here the author tried to focused more in three specific fish and mostly the mackerel. Actually even some of Korean media such as The Korea Bizwire even published that mackerel fish and

연세대학교
YONSEI UNIVERSITY

together with yellow croaker or yellow corvine is being considered as the most popularly consumed fish in Korea in 2018. (31)

The aims of this study is to provide the information about the presence of some Anisakid nematode species that can be occurred in marine fish by using molecular study for identification. And knowing the distribution of the *Anisakis* spp. that found in three types of fish collected and the distribution in different location here in Korea.

The study covered the species of Anisakid nematodes larvae 3 that collected from different types of fish that brought to the lab from different are that this part will more specific in material and method and other body part of this thesis.

연세대학교
YONSEI UNIVERSITY

II. LITERATURE REVIEW

2.1. Preface.

We human being, in our daily life we always consumed different food product with variety of origins, it can be from vegetables or animal, and by consumed different animal product, including marine animal (fish, crustaceans and others) made us always being susceptible to variety of foodborne diseases including the Parasitic foodborne zoonosis

Actually marine animal is wild animal, and they can be parasitized by different parasites, including nematodes (round worm), one of the types of round worm is Anisakid worm.

Human get zoonotic diseases because of their living habits. In most cases related to human food behaviour like the traditional or modern habits for consumptions of raw or half cooked fish, and sometimes this are the reason that how we acquired this infections (zoonotic (parasitic) infection). (18)

During the third quarter of 2018, the INFOSAN (International Food Safety Authorities Network), (is a global network of national food safety authorities, managed jointly by FAO and WHO. Aims to: Promote the rapid exchange of information during food safety related events) 141 WHO member states has been involved in 32 food safety events. There were 19 events involving a biological hazard and *Anisakis*. (15)

2.2. *Anisakis* (parasitic zoonoses)

The association between human diseases and a number of nematode (genus *Anisakis*) are frequently associated. Anisakid worm (larvae) can provoke gastrointestinal tract penetration and do harm to adjacent human organs, which can cause hyperemia, edema and bleeding in the

연세대학교
YONSEI UNIVERSITY

surrounding gastric mucosa. This parasitic zoonosis considerable as a global concern, include South Korea, meaning that by the occurrence is not only occurred in Korea, but the occurrence also can be found in many countries in different part of the world, e.g. in some north-eastern Asia country like Japan, U.S.A. in America continent and some European countries; (Spain, Netherland, France, Italy and Germany). (13, 14)

Is also considered important to know what is Anisakid worm, how its classified as a parasite and maybe what is a parasite;

A parasite is an organism that lives on or in a host organism and gets its food from or at the expense of its host. There are three main classes of parasites that can cause disease in human: protozoa, helminths, and ectoparasites. (6) There are different types of parasites, and of them recognised as Helminth.

As its name origin, it was derivate from Greek "Helmins" mean parasitic worm (7) is a multicellular organism, and the helminth can be classified in; platyhelminth (flatworm), acanthocephalins (thorny-headed worm), cestodes (tape worms), trematodes (flukes), and the Phylum of genus *Anisakis* "nematodes (roundworm)". The adult form of these worm can reside in the gastrointestinal tract, blood, lymphatic system subcutaneous tissue. (9,10) In this thesis, we will have focused more in one of the nematode member, the "*Anisakis*".

Genus *Anisakis*, Order Ascaridida, Familly Anisakidae are belongs to the Nematode phylum, commonly known as the whale or herring worm. Occur at their third larval stage in numerous marine teleost fish species around the world. (3)

연세대학교
YONSEI UNIVERSITY

2.3. Phylogenies of *Anisakis*

Genus *Anisakis* belongs to Kingdom: Animalia, Phylum: Nematoda, Class: Secernentea, Order: Ascaridida, Family: Anisakidae, Genus: *Anisakis*. (19, 20)

Recently in molecular phylogenies (20, 25, 21) recognized five major clades in nematode (phylum Nematoda) and seven order that before is free leaving and then independently start the transition and move towards the parasitism that occurred in vertebrates. And the order of Ascaridida (Superfamily: Ascaridoidea (round worm)) is belongs to the Clade III i.e. Spirurina. (20-26)

Historically the species in the genus *Anisakis* were recognised very few (Davey 1971), however, nowadays there are nine nominal *Anisakis* species classified in two main phylogenetic clades. Currently in first clade contains six species including *A. simplex* complex whose members share the larval morphology known as *Anisakis* Type I (Berland, 1961). (3)

Until this date, there are nine total of *Anisakis* have been confirmed using and/or biochemical methods, i.e. *A. simplex sensu stricto, A. pegreffi, A. berlandi, A. ziphidarum, A. nascettii, A. paggiae, A. physeteris, A. brevispiculata* and *A. typica*.) (4)

During last three decades, with advanced of technology and science until this modern era human were capable to developed new and better genetic tools that allowed the identification of the nine *Anisakis* species by using multilocus allozyme electrophoresis (MAE) and DNA based methods such as PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). (4)

연세대학교
YONSEI UNIVERSITY

2.4.Life cycle of *Anisakis spp.*

Anisakid nematode have free leaving life in marine water, the way they evolved they need a host organism and its capable of infecting its definitive host, meanwhile before they reaching the final host, the worm also passed through the intermediate and the paratenic host during its life cycle.

The Anisakid nematodes have complex and indirect (heteroxenous) life cycle that passed through one or more intermediate hosts, that needed for completion of their larval stage that comprising five larval stages and four moults. (4)

The adult *Anisakis* spp., can be found inside the body (e.g. lumen of the stomach, alone or clusters) of the definitive host; cetaceans. Inside the body of the definitive hosts the adult *Anisakis* spp., released the eggs to the sea together with the hosts faeces. (4, 19)

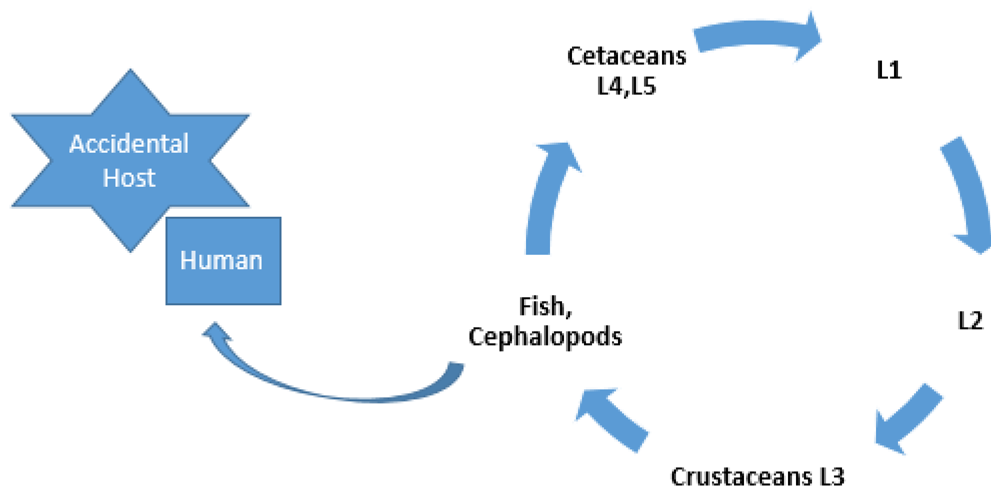


Figure 1. The life cycle of *Anisakis* spp. with each stages and different hosts (heterogeneous) including human as an accidental host.

연세대학교
YONSEI UNIVERSITY

The adult worm mostly in the lumen of the stomach of the final or the definitive host, and they released eggs with host faeces. There are five stages and four moults, after larva was excreted there started with L1 and L2 occurred during those stages. And those larvae ere ingested by small crustaceans (euphauciaceans(krill) and cephalopods) and then start to grow into L3(still with L2 cuticle), here already 3 stages and two moults happened, this small crustacean was eaten by another crustaceans and small fish as a paratenic host, and this crustaceans and small fish was eaten by bigger fish were the larva reach stage 4 and become adult in the final host (cetaceans). (4,5)

Human is being considered as accidental host, because of the food behaviour that consumed the raw or undercooked fish, that's how human ingested the larvae and start to develop the infection that known as anisakiasis.

연세대학교
YONSEI UNIVERSITY

III. HYPOTHESIS, QUESTIONS and PURPOSE

3.1. Study Hypothesis

1. Several species of anisakid worms will be identified from the sample that collected from fishes obtained from different Korean sea waters.

3.2. Study Questions

1. What kind of method should we use for identification of the anisakid worms?

2. What is the reason by collecting the fish from different location in Korean sea?

3.3. Purpose

To study about different types of anisakid worms that presented in the different fishes that collected from the different location in Korean seas water.

The detailed objectives:

a. Analyze the anisakid worms collected by using the DNA extraction and PCR study and later continue with the sequence analysis to identify the types of *anisakis* based on the result from the sample studied.
b. To know the distribution of *Anisakis* spp. in different types of marine fish species that brought to lab from different part of Korean sea-water.

연세대학교
YONSEI UNIVERSITY

IV. MATERIALS AND METHODS

4.1. Materials

4.1.1. Specimens: Fish species

The *Anisakis* worm will be collected from different types of fish, with more focused at three main species such as mackerel (*S. japonicus*), sea eel (*C. myriaster*) and yellow croaker (*L. polyactis*), that brought to our laboratory from different location that located in three different Korean sea waters area.

The process of obtaining the sample is divided into different stages according to the date of obtaining the samples that are brought from different locations to our laboratory to start with the necropsy procedure for the samples obtained.

On March 09, 2019, the specimens (4 mackerels, 6 yellow croaker and 2 sea eels) was bought in (Mapo Agricultural and Fish market) at Mapo-gu, Seoul. After arrived at laboratory, the specimens were dissected immediately. On May 14, 2019, fishes (4 mackerels, 3 yellow croakers and 2 sea eels) from Namhae-gun (Gyeongsangnam-do) was brought to our lab and immediately being examined. Fish was brought (2 sea eels) to our lab on July 17, 2019 from Uljin-gun, Gyeongsangbuk-do province. Two days later the fish was bought to the lab from two different locations. From Uljin-gun there were three mackerels, and the rest were from Daecheon, Boryeong-si, Chungcheongnam-do with two different species of fishes (three mackerels and two sea eels). Finally, a total of 32 fishes were sent to the laboratory from Pohang-si, Gyeongsangbuk-do with three different species, 29 mackerels, 2 sea eels and 1 yellow

연세대학교
YONSEI UNIVERSITY

croaker fish. After arrived at our laboratory these samples were immediately dissected, the parasite collection was performed.

4.1.2. Specimens: Locations

A total of 63 fishes were purchased from different sites in Korea, such as; Seoul, Namhae, Uljin, Daecheon and Pohang, that located in three different sea water (east, west and south). (Fig.2) and in the next figure (Fig.3) we observed the total number from different types of fish that collected from different location in different Korean sea waters.

Figure 2. The five different sampling locations from three Korean sea waters.

연세대학교
YONSEI UNIVERSITY

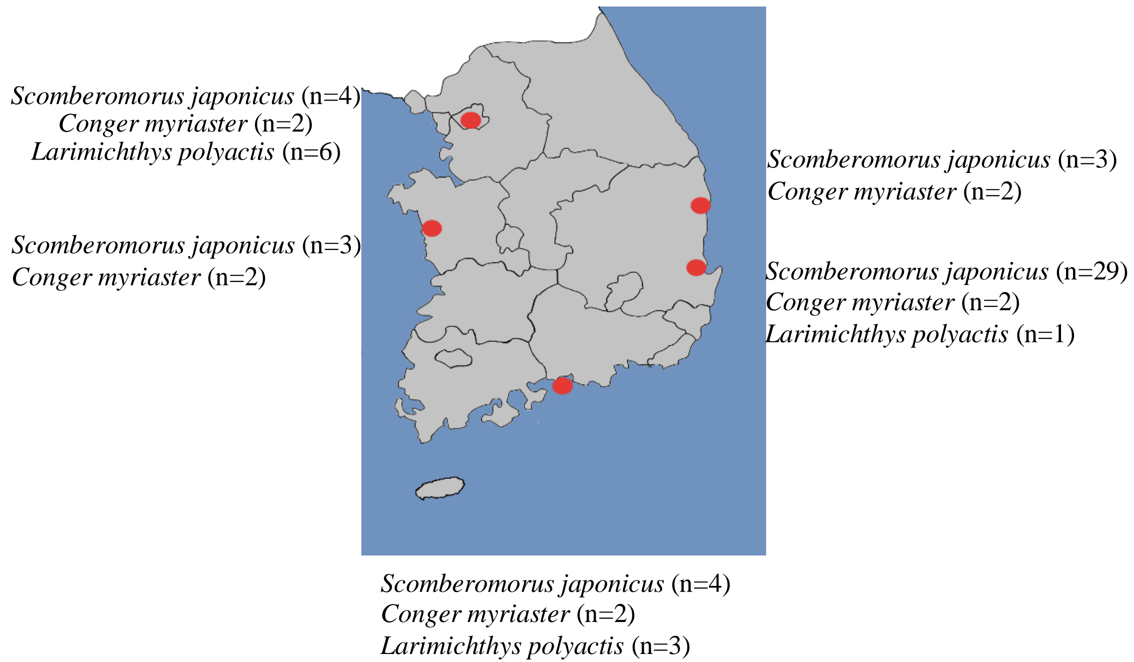


Figure 3. Total number of three different kinds of fish collected from different locations in three Korean sea waters

The samples were transported to our laboratory and immediately dissected and in the next step those samples will be examined for anisakid larvae. The anisakid larvae were collected from the body of the fish (collected from the different organs, cavities, flesh even from the skin). After being washed with 0.9% NaCl, the anisakid worm were counted and fixed in 70% ethanol and some in PBS (Phosphate-buffered saline) solution then stored in the tube collector following by stored at the refrigerator. The information related to the fish samples is being summarized as we can observe the summarized in the Table 1.

연세대학교
YONSEI UNIVERSITY

Table 1. Fish species, number of samples, body measurement (weight), total parasites found on each samples and location area that from three different Korea sea waters.

Sea water	Location area	Fish Species	Total samples	Body weight (g)	No. of larvae detected
West	Seoul	*S. japonicus*	4	-	254
	Seoul	*C. myriaster*	2	-	87
	Seoul	*L. polyactis*	6	-	51
	Daecheon	*S. japonicus*	3	544.1 ± 491.2	1,044
	Daecheon	*C. myriaster*	2	459.3 ± 442.2	20
South	Namhae	*S. japonicus*	4	328.3 ± 293.1	162
	Namhae	*C. myriaster*	2	243.0 ± 224.9	42
	Namhae	*L. polyactis*	3	171.8 ± 161.2	11
East	Uljin	*S. japonicus*	3	599.2 ± 526.1	>1,214
	Uljin	*C. myriaster*	2	428.0 ± 324.1	17
	Pohang	*S. japonicus*	29	454.0 ± 72.1	2,385
	Pohang	*C. myriaster*	2	823.4 ± 410.0	6
	Pohang	*L. polyactis*	1	61.0	2
	Total	-	63	-	-

연세대학교
YONSEI UNIVERSITY

4.1.3. Dissection

Data total on number of larvae detected and weight were recorded for each specimen. Even including the locations that brought from (Tab.1).

Following that, by carrying out an incision on fish in longitudinal direction until reach the last pair of fish gills or the branchiopores and began to open and to expose the internal organs (Fig. 5). First, conducted the macroscopic observation on *Anisakid* larvae without removed the internal organs from the fish visceral cavity. Finally, the head region being separated from body and start with dissection and extraction of all internal organs to started the inspections on presence of anisakid larvae in different part of the organs (Fig.6, 7).

The extraction of the anisakid larvae L3 is being extracted with the fresh sample that immediately dissected after brought to our laboratory. During this procedure we can observed macroscopically the worm in form of larvae stage 3 inside the fish body (Fig.5, 6). As one of the life cycle process, sometimes when the host died the larvae started to migrated in to the host muscle as what found and here some even migrate until the skin (subcutaneous) (Fig.7).

연세대학교
YONSEI UNIVERSITY

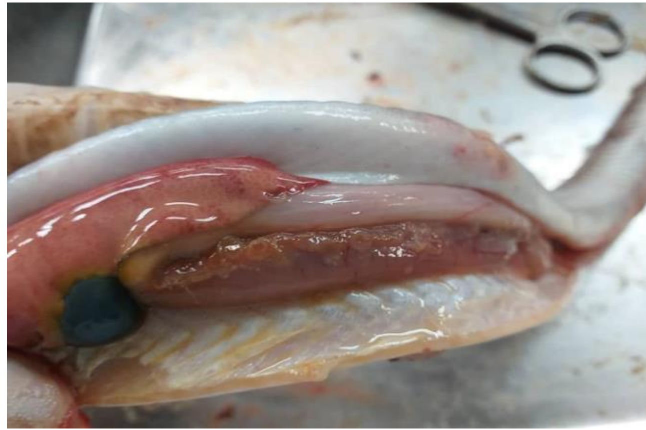


Figure 4. Longitudinal dissection from the anus until the gills (branchiopores) of the sea eel (*Conger myriaster)* to expose the internal organs.

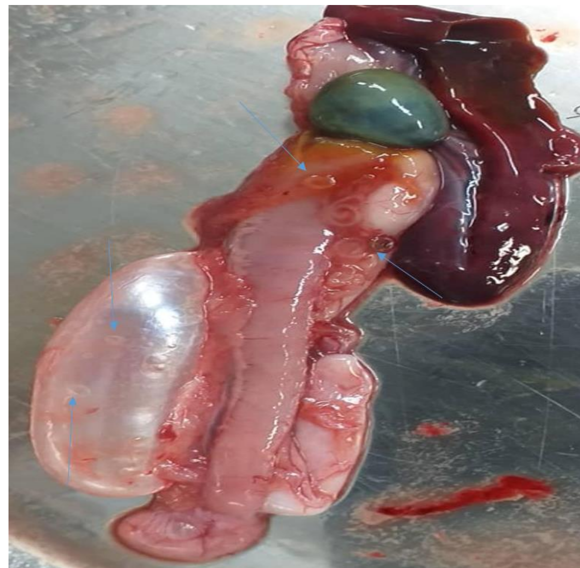


Figure 5. *Anisakid* larvae L3 presence in different part of Sea eel organs

연세대학교
YONSEI UNIVERSITY

So, as the result of this study that conducted in different locations in three Korean sea waters with total 63 different fish samples and 98 larvae L3 that were extracted and studied.

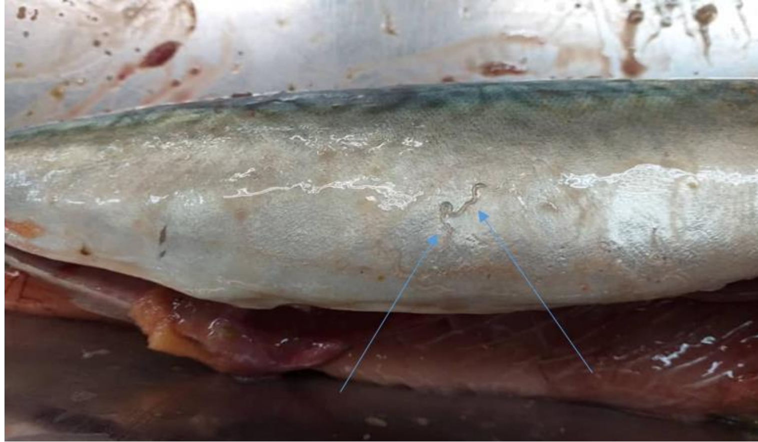


Figure 6. The *Anisakis* spp. larvae L3 stage at below the skin (subcutaneous)

The mackerel fish (*S. japonicus*) were more predominated with total 43 fishes from 63 fishes or more than a half of the fish samples that brought to our laboratory. Then followed by the Yellow corvina or yellow croaker (*L. polyactis*) and the sea eel (*Conger myriaster*) where both of them presented the same amount with 10 samples each one.

연세대학교
YONSEI UNIVERSITY

4.2. Methods

4.2.1. DNA extraction

In this step, by using the Germany kits product, (*Macherey-Nagel, Genomic DNA extraction from insects (NucleoSpin® DNA Insect)*) (www.mn-net.com) DNA extraction was performed. The extraction of the DNA from the sample were done according to the processes registered as shown in the following steps: The sample was inserted into NucleoSpin®Bead bead tubes type D. First and second step is to prepare and lyse the sample; collect <40 mg insect material putted into the bead tubes type D together with the 100 µL Elution Buffer (BE), 40 µL MG buffer and 10 µL Liquid Proteinase K and then agitate for 15 to 20 minutes after that continue with the centrifuge (speed: 11,000 x g durations: 30 seconds) to clean the lid. Third: to adjust DNA binding conditions in order to clean the lid, sediment glass and cell debris with 600 µL MG buffer and then continued to centrifuge with same speed and duration. Fourth: to bind the DNA; transferred the loading sample (600 µL) into the DNA insect column that placed together with 2 mL collection tube and centrifuged then discarded before tube and putted a new collection tube. Fifth: Wash silica membrane; divided into two steps, first with 500 µL BW buffer, centrifuged and discarded the flow-through and placed back the column into the collection tube then continue with 500 µL B5 buffer, centrifuged, discarded flow-through and placed back onto the collection tube. Sixth: Dry silica membrane; this step is just a continuous procedure from recent procedure only here not loading of any buffer or any substance, but as its name is to dry by continued to being centrifuged without any loading material, then before continued to the next step the collection tube was discarded. Seventh: Elute highly pure DNA; here the insect column was placed into 1.5 mL nuclease-free tube, then added 100 µL BE buffer onto the

연세대학교
YONSEI UNIVERSITY

column then incubated at room temperature for 1 minute before continued to being centrifuged with the same speed and duration (11,000 x g during 30 seconds).

4.2.2. PCR for identification of pathogens

PCRs were applied for rapid screening of *Anisakis* worm for detection of selected pathogens following by specific identification of the pathogens using species-specific primers using a conventional PCR.

For realization of PCR procedure, the PCR master mix was prepared by using the last product from the DNA extraction and put into the PCR tube. The calculation for the master mix based on the total number of the PCR product targeted and then tried to multiplied with the total of master mix with formula as shown below;

(Total number of PCR product targeted x Total master mix (μL))

Table 2. PCR Master Mix ingredients and total the amount for master mix (μL)

Master mix substances	Total master mix (μL)
Forward primer Nc5 (GTAGGTGAACCTGCGGAAGGATCATT)	1 μL
Reverse primer Nc2 (TTAGTTTCTTTTCCTCCGCT)	1 μL
10x Buffer	2.5 μL
dNTPs (Deoxynucleoside triphosphates)	2.5 μL
Taq DNA polymerase	0.1 μL
DW (Distilled Water)	16.9 μL
+ Template	4 μL
Total	28 μL

연세대학교
YONSEI UNIVERSITY

By finished the calculation the total amount was injected into the template before continued to put onto the PCR machine.

The PCR steps was set up as bellow;

Step	Temperature	Time	
Step 1 (Denaturing)	95 ºC	5 minutes	
Step 2 (Annealing)	95 ºC	30 Seconds	X 34 cycles
	55 ºC	30 Seconds	
	72 ºC	45 Seconds	
Step 3 (Extending)	72 ºC	5 minutes	

After finishing the PCR procedure that ran during 1 hour and 31 minutes, electrophoresis was carried out; the agarose gel was prepared.

Began with by pouring the 40 mL TAE (Tris-acetate) buffer in measuring tube then continued to pouring onto lab glass ware (Erlenmeyer flask) and put 0.4mg of agarose powder. Then stored in microwave for 2 minutes continued with by added 2 μL of midori green the pour into the template then waited for 15 to 30 minutes to let the agarose cool down before started adding the PCR products into the agarose gel. After cooled down it become solidified, then placed into the gel box (electrophoresis unit) then started to adding the PCR product and the loading marker (100 bp) into the additional wells gel and after finished, started to run the electrophoresis unit during the 30 minutes. The last step was by used the UV light, that can help to visualize the DNA fragments as bands depends on their appearance in the gel. The PCR products were sequenced using primers (Nc5 and Nc2) and analyzed.

연세대학교
YONSEI UNIVERSITY

4.2.3. Species identification strategies

Being electrophoresed, the PCR product together with the forward primer was sent to the company (Bionics, Seoul, Korea) for sequencing analysis.

After the result obtained from the company, identification of the *Anisakis* species was done using the Sequence analysis and the Nucleotide Identity Percentage analysis (used for those unidentified DNA sequencing during the Sequence analyzing procedure).

4.2.4. Sequence analysis

During this procedure the DNA sequence result obtained are identified by using two DNA sequence as our target to find the matchings result. And those are;

Anisakis pegreffi (GGCAGAGT**C**) => C/C allele

Anisakis simplex (GGCAGAGT**T**) => T/T allele

As a result 67 revealed as *A. pegreffi* (Table 3) and 31 unidentified that will continue to conduct other strategies to find the matching relation of the sequences and no one were matched as *A. simplex* the one with the T/T allele.

4.2.5. Nucleotide Identity Percentage (%) analysis

The strategy for species identification of the rest unidentified ones in the first strategy is by using the nucleotide identity percentage analysis: *A. pegreffi* (AB277823), *A. simplex* (AB277822), *P. decipiens* (AB277824) *and H. aduncum* (AB277826). (30)

Pairwise Sequence Alignment (PSA), a sequence alignment tool, was used for nucleotide identity percentage analysis. The functional, structural and/or evolutionary indication that shows relationship between two biological sequences (protein or nucleic acid) for the

연세대학교
YONSEI UNIVERSITY

identification of similarity regions can be identified by using this sequence alignment tool (PSA). (32)

Local alignment tool with the Water (EMBOSS) was used to identify the matchings between two sequences using a rigorous algorithm based on the LALINGN application (Table 4). (32)

연세대학교
YONSEI UNIVERSITY

V. RESULT

5.1. PCR analysis

The PCR products were analysed after finished the PCR procedures and next being examined with electrophoresis we can observed the presence of the banned and it have revealed different patterns depending on the *Anisakis* species. The banding patterns observed in this study was corresponded to *A. pegreffi* (Fig. 4) and some other species that will need the DNA sequencing for species types identification.

5.2. Validation of *Anisakis* species collected from Korean waters

In the result we found that the genotype of the 98 anisakid larvae more predominantly revealed 67 homozygous C/C allele (GGCAGAGT**C**) as *A. pegreffi*. Before sending the PCR product to company for DNA sequencing process, those PCR products were being electrophoresed first and by observed and identified the positive sample of the DNA fragments that appeared as bands (Fig. 7).

연세대학교
YONSEI UNIVERSITY

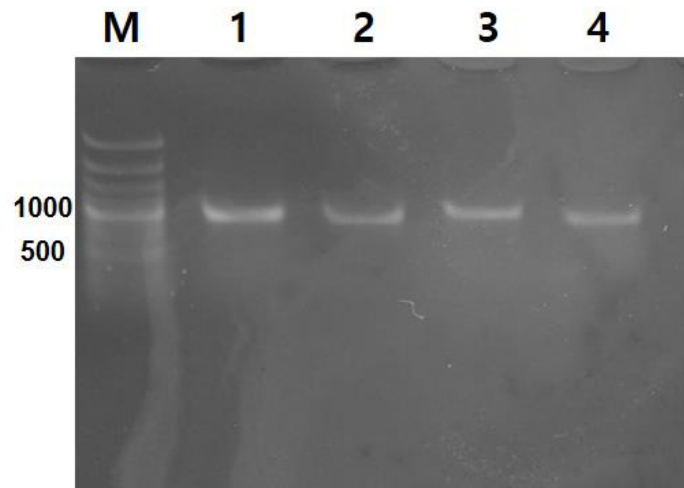


Figure 7. PCR product (DNA fragments band) in 1% agarose gel that electrophoresed for molecular identification of anisakid larvae.

5.2.1. Sequencing result

After received the sequence result from company, then tried to analysed the sequence data by matching the DNA code with some of anisakid species.

Only 67 samples among 98 PCR product positives were identified as *A. pegreffi*, having C/C allele (Table 3). By using other strategy for the rest 31 DNA sequences for matching the DNA was used the Nucleotide Identity Percentage.

연세대학교
YONSEI UNIVERSITY

Table 3. Result total of DNA sequence positive as *Anisakis pegreffi* species

Location	Type of fish	Total positive *A. pegreffi*
Seoul	*S. japonicus*	10
	C. myriaster	11
	L. polyactis	8
Namhae	*S. japonicus*	4
	C. myriaster	4
	L. polyactis	1
Uljin	*S. japonicus*	6
	C. myriaster	12
	L. polyactis	0
Daecheon	*S. japonicus*	4
	C. myriaster	2
	L. polyactis	0
Pohang	*S. japonicus*	4
	C. myriaster	1
	L. polyactis	0
Total		**67**

5.2.2. Nucleotide Identity Percentage (%)

After the 31 DNA sequences result obtained, 25 specimens were identified as *A. pegreffi* since the nucleotide identity percentage showed more similar to *A. pegreffi* sequence. One sequence was most closely related with *A. simplex*, and the remaining 5 specimens were identified to be *Histerothylacium aduncum*.

So through this strategy 31 unidentified specimens by using PCR and gel electrophoresis were identified to be 3 species: *A. simplex* (AB277822), *A. pegreffi*, and *H. aduncum* (AB277826). (30)

연세대학교
YONSEI UNIVERSITY

Particularly one specimen showed 100% matching to *H. aduncum* as shown in the bellow (Table 4).

Table 4. Nucleotide identity (%) of anisakid larvae stage 3 from mackerel, yellow croaker fish, and sea eel with *Anisakis pegreffi, A. simplex, Pseudoterranova decipiens and Hysterothylacium aduncum*

Sample no.	**ITS2 region**				***Fish type***	***Location***	***Sea***
	Anisakis simplex	*Anisakis pegreffi*	*Pseudo-terranova decipiens*	*Hysterot-hylacium aduncum*			
L12-2	**84.0**	83.5	67.9	54.3	*C. myriaster*	Seoul	West
L12-3	85.5	**85.9**	68.9	57.2	*C. myriaster*	Seoul	West
L12-4	88.6	**88.8**	68.2	56.2	*C. myriaster*	Seoul	West
L12-7	90.1	**90.3**	71.7	58.9	*C. myriaster*	Namhae	South
L12-10	84.8	**85.1**	67.4	52.9	*C. myriaster*	Namhae	South
L13-2	82.8	**83.0**	65.2	53.9	*L. polyactis*	Namhae	South
L13-6	80.1	**80.2**	65.3	54.4	*L. polyactis*	Namhae	South
L14-3	58.8	58.8	54.6	**90.9**	*L. polyactis*	Seoul	West
L14-4	84.3	**84.7**	67.6	54.1	*L. polyactis*	Seoul	West
L14-10	57.9	58.0	54.4	**88.9**	*L. polyactis*	Seoul	West
L15-14	61.6	61.7	57.6	**96.3**	*C. myriaster*	Uljin	East
L18-1	83.0	**83.2**	67.3	56.6	*S. japonicus*	Daecheon	West
L18-2	90.8	**91.0**	72.1	60.6	*S. japonicus*	Daecheon	West
L18-3	88.1	**88.4**	70.6	56.3	*S. japonicus*	Daecheon	West
L18-4	84.9	**85.1**	68.5	57.3	*S. japonicus*	Daecheon	West

연세대학교
YONSEI UNIVERSITY

L18-5	82.0	**82.2**	66.6	56.1	*C. myriaster*	Daecheon	West
L18-6	90.9	**91.2**	71.7	58.7	*C. myriaster*	Daecheon	West
L18-7	84.4	**84.6**	68.4	56.8	*C. myriaster*	Daecheon	West
L18-8	85.0	**85.3**	68.4	58.0	*C. myriaster*	Daecheon	West
L19-2	91.5	**91.7**	72.6	59.8	*S. japonicus*	Daecheon	West
L20-2	79.1	**79.4**	65.6	51.7	*C. myriaster*	Daecheon	West
L20-3	92.4	**92.6**	73.9	59.9	*C. myriaster*	Daecheon	West
L20-4	91.2	**91.4**	73.2	60.4	*C. myriaster*	Daecheon	West
L20-5	92.2	**92.4**	72.8	58.7	*C. myriaster*	Daecheon	West
L20-6	91.7	**91.9**	71.5	60.1	*C. myriaster*	Pohang	East
L20-7	90.5	**90.7**	71.9	60.6	*C. myriaster*	Pohang	East
L20-8	90.6	**90.8**	71.7	59.3	*C. myriaster*	Pohang	East
L20-9	83.0	**83.2**	67.3	54.4	*C. myriaster*	Pohang	East
L20-10	87.4	**87.6**	70.3	58.9	*C. myriaster*	Pohang	East
L21-2	64.8	65.0	55.5	**100.0**	*L. polyactis*	Namhae	South
L21-3	73.3	73.5	60.7	**80.7**	*L. polyactis*	Namhae	South

연세대학교
YONSEI UNIVERSITY

VI. DISCUSSION

By the present study, 98 anisakid worm were being analized from 63 fishes that focused at 3 main species; mackerel (*Scomberomorus japonicus*), sea eel *(Conger myriaster*) and yellow croaker or yellow corvine (*Larimichthys polyactis*). The study that realized by Chun et al (33) showed that South sea reported more infection density of anisakid larvae is higher than the Yellow sea.

In this study we found that, from the 63 fishes collected from different sea areas, the larvae were being counted (Table 1.) and it revealed that the fishes from the East sea show higher infection rate of anisakid larvae than the other two seas (yellow sea and south sea).

Several species of anisakid worm presented in samples of fresh fish that brought to our lab, and the identification methods used; PCR and the DNA sequence analysis. In this study, after identified 98 anisakid worms, we found 67 positives samples belongs to *Anisakis pegreffi*. With the total number of 67 *A. pegreffi* represent 68.37% from all the study. And from this result obtained, revealed that the Yellow sea presented the higher infection rate of anisakid larvae than the South sea and the East sea. Comparing the West sea (Yellow sea) and the East sea, the presence of this *Anisakis* species (*A. pegreffi*) are higher in yellow sea (52.24%) than the East sea (34.33%).

The *Anisakis* spp. also can be found in human being, after ingestion of the infected fish in raw condition, and by this food behaviour have possibility to get the infection (Anisakiasis). There are also some studies here in Korea about the presence of anisakid worm inside human body. Some study shown that from the anisakiasis cases, was predominantly by *A. pegreeffi* rather than the *A. simplex*. (11,34,35)

연세대학교
YONSEI UNIVERSITY

And in the other hand, between 1989 and 1992, there are some study that reported that *A. simplex* was the major cause of human anisakiasis in Korea. (30, 36, 370)

In this study what we found were predominated by *A. pegreffi* with 67 total cases, and the rest were analysed by Nucleotide Identity Percentage (%).

There was some study realized here in Korea in the same three different Korean sea waters, and by using more samples in bigger total number and also in more different species of fishes with total 174 (n=174) in 10 different species of fishes. Around 17 conger myriaster that also included as one of the species that they used for the study. And based on the result they obtained mostly from the result shown *A. pegreffi* is the most predominant. Related to my study it is also share some similarities as the *A. pegreffi* is the most predominant species from genus *Anisakis* found. (2)

In Spain is conducted some study about the Anisakid larval by using 663 total specimens from nine different species, and mostly from mackerel family. And as a result shown that 40.33% is related to *Anisakis* type I. And there also shown that the prevalence (%) for fishes with higher risk of *Anisakis* type I larvae are from mackerel families with horse mackerel as the highest one. Related to my study mostly of my result are *A. pegreffi* that belongs to genus *Anisakis* amd mostly found in mackerel (*Scomberomorus japonicas*). (38)

연세대학교
YONSEI UNIVERSITY

VII. CONCLUSION

- Anisakid larvae were obtained from 63 fishes with 3 species from different location in three different Korean sea waters.
- Totally 97 *Anisakis* larvae were examined for identification of the anisakid species by a molecular technique (PCR).
- *Anisakis pegreffi* was predominantly found with 67 positive cases.
- The rest 30 samples were analyzed by DNA alignment tool (Water (EMBOSS)) and by using available DNA sequences of four different anisakid worms; *Anisakis pegreffi, Anisakis Simplex, Pseudoterranova decipiens* and *Histerothylacium aduncum.* From this result obtained, 25 specimens were identified as *A. pegreffi,* one specimen was identified as *A. simplex*, and the remaining 5 specimens were identified as *Histerothylacium aduncum*.

연세대학교
YONSEI UNIVERSITY

VIII. REFERENCES

1. Aibinu IE, Smooker PM, Lopata AL. IJP : Parasites and Wildlife Anisakis Nematodes in Fish and Shellfish- from infection to allergies. IJP Parasites Wildl [Internet]. 2019;9(April):384–93. Available from: https://doi.org/10.1016/j.ijppaw.2019.04.007
2. Sohn W, Kang J, Na B. Molecular Analysis of Anisakis Type I Larvae in Marine Fish from Three Different Sea Areas in Korea. 2014;52(4):383–9.
3. Wichmann K. Pathobiology and Protection.
4. Domínguez MB. A multidisciplinary approach to tackle the problem of the zoonotic parasite Anisakis in fish. 2017;(April).
5. Mattiucci S, Nascetti G. Chapter 2. 2008;66(08).
6. Parasites. https://www.cdc.gov/parasites/about.html
7. Helminth. https://www.etymonline.com/word/helminth
8. Clasification and Identification of Helminth. https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Boundless)/8%3A_Microbial_Evolution%2C_Phylogeny%2C_and_Diversity/8.20%3A_Helminths/8.20B%3A_Classification_and_Identification_of_Helminths
9. Mattiuci, S.,D'Amelio, S.,2014. Anisakiasis, in: Bruschi, F. (Ed), Helmith Infection and Their Impact on Global Public Health. Springer Vienna, Vienna, pp.325-365. doi: 10.1007/978-3-7091-1782-8
10. Mattiuci, S., Fazii, P., De Rosa, A., Paoletti, M., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C.,Calvaruso, V., Sorentini, I., Palma, G., Bruschi,

연세대학교
YONSEI UNIVERSITY

F., Nascetti, G.,2013. Anisakiasis and gastroalllergic reactions associated with *Anisakis pegreffi* infection, Italy. Emerg. Infect. Dis. 19, 496-499. doi: 10.3201/eid1903.121017

11. Sohn WM, Na BK, Kim TH, Park TJ. *Anisakiasis: Report of 15 gastric Cases Caused by Type I Larvae and a Brief Review of Korean Anisakiasis Cases*. Korean J Parasitol Vol. 53, No.4: 465-470, August 2015. http://dx.doi.org/10.3347/kjp.2015.53.4.465
12. HIRA data. https://opendata.hira.or.kr/
13. Kim, C.H., Chung, B.S., Moon, Y.I., Chun, S.H., 1971. A case report on human infection with Anisakis sp. In Korea. Korean J. Parasitol. 9, 39-43.
14. Hyunsu Kim, Kyung-Wan Baek, Mi-Kyung Park, Kyung-Yoon Jeon, Eun-Ji Ko, Hee-Jae Cha, Mee Sun Ock. Establishment and validation of of ARMS (amplification-refractory mutation system) for identification of *Anisakis* species collected from Korean waters. *a.Department of Parasitology and Genetics, Kosin University College of Medicine, Busan 49267, Republic of Korea.b.Department of Parasitology and Tropical Medicine, Pusan National University, Busan 50612, Republic of Korea.*https://doi.org/10.1016/j-gene.2018.12.045
15. INFOSAN, food safety. https://www.who.int/foodsafety/areas_work/infosan/INFOSAN-QS3/en/
16. World Health Organization, 2012. Soil-transmitted Helminth,WHO.http://www.who.int/intestinal_ worms/en/
17. FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization].2014. Multicriteria-based ranking for risk management of food-borne parasites. Microbiological Risk Assessment Series No. 23. Rome. 302pp.(https://www.who.int/foodsafety/publications/mra_23/en/)

연세대학교
YONSEI UNIVERSITY

18. Natalie E. Nieuwenhuizen. Anisakis-immunology of a foodborne parasitosis. Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany. doi: 10.1111/pim.12349
19. Anisakis. https://en.wikipedia.org/wiki/Anisakis
20. Serge Morand, Salah Bouamer, Jean-Pierre Hugot. 2015/01/01.63.80. Chapter 4: Nematodes. Micromammals and Macroparasites: From Evolutionary Ecology to Management. doi:10.1007/978-4-431-36025-4_4
21. (KOSIS; Korean Statistical Information Service). http://kosis.kr/search/search.do
22. Roy C. Anderson, Alain G. Chabaud, Sheila Willmott (eds) (1974-1983) CIH keys to the nematode parasites of vertebrates. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England
23. Roy C. Anderson. Nematode parasites of vertebrates: their development and transmission. CABI Publish, Wallingford, Oxon, UK. doi:10.79/9780851994215.0000
24. De Ley, P. A quick tour of nematode diversity and the backbone of nematode phylogeny. (January 25,2006), WormBook, ed. The C. *elegans* Research Community, WormBook, doi/10.1895/wormbook.1.41.1, http://www.wormbook.org
25. Blaxter ML, de Ley P, Garey JR, Liu LX, Scheldeman P, Vierstrate A, Van-fleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas Wk (1998) A molecular evolutionary framework for the phylum Nematoda. Nature 392:71-75
26. De Ley P, Blaxter ML (2002) Systematic position and phylogeny. In: Lee D (ed). The biology of nematodes. Hardwood Acad Publ, Reading, pp 1-30
27. **Creative Commons Attribution Share Alike (CC BY-SA).** https://creativecommons.org/licenses/by-sa/3.0/?
28. Chai JY, Murrell KD, Lymbery AJ. Fish-borne parasitic zones: status and issues. Int J Parasitol 2005; 35: 1233-1254

연세대학교
YONSEI UNIVERSITY

29. Kim, C.H., Chung, B.S., Moon, Y.I., Chun, S.H., 1971. A case report on human infection with *Anisakis sp.* In Korea. Korean J Parasitol. 1971;9 (1): 39-43. https://doi.org/10.3347/kjp.1971.9.1.39
30. Song H, Jung BK, Cho J, Chang T, Huh S, Chai JY. *Molecular Identification of Anisakis Larvae Extracted by Gastrointestinal Endoscopy from Health Check-up Patients in Korea.* April 2019. Korean J Parasito Vol. 57, No. 2: 207-211, April 2019. DOI: https://doi.org/10.3347/kjp.2019.57.2.207
31. Mackerel most popular fish in Korea. (http://koreabizwire.com/mackerel-most-popular-fish-in-south-korea/112480)
32. Sequence Alignment tool. https://www.ebi.ac.uk/Tools/psa/
33. Chung SK, Chung BK, Ryu BS. Studies on Ansakis sp. On the infectionstate of anisakis-like larvae isolated from various marine fishes. Korean J Fiss Aquact Sci 1968; 1: 1-6 (in Korean).
34. Lee EJ, Kim YC, Jeong HG, lee OJ. The mucosal changes and influencing factors in upper gastrointestinal anisakiasis: analysis of 141 cases. Korean J Gastroenterol 2009; 53(2): 90-97 (in Korean).
35. Lim H, Jung BK, Cho J, Yooyen T, Shin EH, Chai JY. Molecular diagnosis of cause of anisakiasis in humans, South Korea. Emerg Infect Dis. 2015; 21: 342-344.
36. Im KI, Shin HJ, Kim BH, Moon SI. Gastric anisakiasis cases in Cheju-do, Korea. Korean J Parasitol 1995; 33: 179-186.
37. Choi SC, Lee SY, Song HO, Ryu JS, Ahn MH. Parasitic infections based on 320 clinical samples submitted to Hanyang Univesrsity, Korea (2004-2011). Korean J Parasitol 2014; 42: 215-220.
38. Debenedetti AI, Madrid E, Trelis M, Codes FJ,Gil-Gómez F, Sáez-Durán S, and Fuentes MV. Prevalence and Risk of Anisakid Larvae in Fresh Fish Frequntly

연세대학교
YONSEI UNIVERSITY

Consumed in Spain: An Overview. Fishes 2019, 4, 13; doi: 10.3390/fishes4010013. www.mdpi.com/journal/fishes.

IX. Appendix

1. Poster presentation in 61st Annual Meeting and 60th Anniversary of the Korean Society for Parasitology and Tropical Medicine at Seoul National Hospital.