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SYSTEMATIC AND DISTRIBUTION STUDIES OF ANISAKIS NEMATODES INFECTING YELLOW FIN TUNA (*Thunnus albacares)*, MACKEREL (*Rastrelliger* sp.), GROUPER (*Epinephelus* sp.) and ROUNDSCAD (*Decapterus* sp.) MARKETED IN THE PHILIPPINES

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ABSTRACT

This study morphologically and molecularly identifies Anisakis using PCR-RFLP and sequenced rDNA ITS region. It also determines *Anisakis* presence and distribution in the country. The results of the morphological examination, PCR-RFLP and sequencing of the ITS region has already proven the correct identity of the *Anisakis* species collected in the current study in which*A. typica* is the dominant species which do not cause human anisakiasis and allergies. With the absence of *A. simplex* s.s. and *A. pegreffi* from our exportable tunas, we can assure the international community of the safeness of our exported tuna for human consumption.

Keywords: ***Anisakis, Thunnus albacares, Rastrelliger sp., Epinephelus sp., Decapterus sp.***

INTRODUCTION

Philippines with 36, 289 kilometer coastline is considered as one of the most diverse habitat of marine aquatic flora and fauna in the [1]. Along with the country's long shoreline lies a teeming and diverse aquatic flora and fauna which is identical to a rich coastal area and it is widely distinguished as a worldwide priority for marine conservation [2].

Tuna is considered as one of the high valued and commercially important marine fish species in the Philippines. Tuna fishery contributed to almost 56,708 tons in terms of volume of exports [3]. In our country ranked second as the largest producer of tuna and tuna-like species in the world, behind Indonesia and ahead of China [4].

Anisakis, a parasitic nematode of the family Anisakidae has essential used as water pollution indicator[5], global climate changes [6,7], anthropogenic impacts and environmental stresses [8], fish stock assessment [9,10] and general ecosystem health [8]. Anisakid nematodes in the Pacific region of the Philippine archipelago still remain unexplored.

The risk of raw tuna meat consumption due to Anisakiasis, a disease caused by ingesting *Anisakis* larvae gained approximately 20,000 cases of Anisakiasis reported globally [11]. *A. simplex* sensu strictoand *A. pegreffii* which is considered as the most pathogenic species known to cause human anisakiasis. Anisakiasis infection on humansmanifest long-lasting sharp epigastric pain, nausea and vomiting without diarrhea [12]. The increasing threat of human anisakiasis worldwide is now posing human health risk for tuna and mackerel consumers.This study carries out systematic and distribution studies on the presence of zoonotic anisakid nematodes marketed in the Philippines using morphological and molecular approaches.

MATERIALS AND METHODS

Sample Collection

Samples were collected from five different sites including Palawan, Quezon, Zambales, Davao, General Santos and Nueva Ecija. Sites were selected basically to cover major fishing areas in the country.

The anisakid nematodes were collected from the tissue outside the stomach and visceral organ of yellowfin tuna (*Thunnus albacares*), mackerel (*Rastrelliger* sp.), grouper (*Epinephelus* sp.), slipmouth (*Eubleekeria splendens*), amberjack (*Seriola* sp.) and roundscad (*Decapterus* sp.). Samples were placed immediately in a cylindrical tubes containing Natural Saline Solution and then transferred into another tube containing 70% ethanol. Each worm was cut into three portions: the anterior end, middle portion, and posterior end. The anterior and posterior ends of the isolated worms were cleared in glycerin prior to mounting in slides for the initial parasite identification using morphological keys [13-17]. Middle portion of worms were used for further molecular identification. Anisakid nematodes were identified up to genus level

based on morphological characteristics as previously reported [18, 19].

Specimen Examination

A total of 1,546 worms were examined (anterior and posterior portion) by the aide of light microscope. 62 worms (16 from Palawan mackerel, 20 from Palawan yellowfin tuna, 6 from Quezon grouper and 20 from Nueva Ecija roundscad and mackerel) were morphologically identified as *Anisakis* species.

The parasites were examined at Fish Pathology Laboratory, College of Fisheries, Central Luzon State University. Characterization of different *Anisakis* species using morphological cues were assessed through ventriculus length (i.e., *Anisakis* type 1 group has 0.5-1.5 mm ventricular length while *Anisakis* type 2 group has ventricular length of below 0.5 mm.). A schematic drawing was made using Corel Draw Version 12 software on the anterior and posterior ends of the worms containing the vital keys for morphological examination. On the other hand, ImageJ software was used in measuring the length of each worm. Measurements taken were on the following: a) TL: total length, b) VL: ventriculus length c) TLe: tail length, d) BW: body width (maximum) and e) VW: ventriculus width.

DNA Extraction

The genomic DNA was extracted from middle portion of every worm using Wizard® Genomic DNA Purification Kit (Promega corporation) following manufacturer's protocol.

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism

Among the morphologically identified anisakid samples, digestion of PCR products with restriction enzyme *Hinf* I(Takara Bio Inc., Otsu, Japan) werecarried out on the ITS region to initially determine species differences using the reported molecular keys [16, 20-23]. The samples were incubated at 37 ºC for one hour. Then stainedwith GR Green Loading Buffer (Bio-craft, Tokyo, Japan) and were electrophoresed in 2.0% agarose gel and visualized by illumination with shortwave ultraviolet light.

Polymerase Chain Reaction

PCR was performed to amplify the largest gene region. Initial species identification was done using PCR-RFLP of the Internal Transcribe Spacer (ITS) rDNA region, whereas final species identification was done by DNA sequencing of the ITS gene region. The ITS region was amplified using forward primer NC5F (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and reverse primer NC2R (5'-

TTAGTTTCTTTTCCTCCGCT-3'). The PCR assays were performed using thermal cycler (Flex Cycler, Analytic Jena AG, Germany) with the following PCR profile: initial denaturation at 94°C for 4 min, followed by 30 cycles consisting of denaturation at 94°C for 30 secs, annealing at 55°C for 30 secs, and extension at 72°C for 30 secs, with final extension at 72°C for 7 min [24].

Gel Electrophoresis

Five (5) µL of the PCR product with loading dye were loaded into 1% agarose gel and have undergone size separation using electrophoresis machine at 100 volts for 45 minutes. The size of the amplicons was determined using 250 base pair molecular weight ladder (Hoffman-La Roche Ltd., Switzerland). The gel was stained using GelRed™ Nucleic Acid Gel Stain (Life Technologies, India) for 30 minutes and was visualized using Alphadigidoc Pro Imaging System (Alphainnotech Corp.,USA).

DNA Purification, Quantification and Sequencing

Amplicons were incised from the agarose gel for purification using QIAquick Gel Extraction Kit (QIAGEN™ Group, Germany) following the manufacturer's protocol and the concentration of DNA was quantified using Thermo Scientific Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., USA). The PCR products were sent to the Philippine Genome Center, UP-Diliman for bidirectional sequencing.

Sequence Assembly, Alignment and Phylogenetic Analyses

Bidirectional sequence trace files were assembled using BioEdit 7.2.5 [25] and homologous sequences were aligned with the program ClustalX 2 [26].Species identities wasconfirmed at GenBank using BLAST program. A Neighbor-Joining (NJ) Tree with 1000 pseudo replications and model testing were implemented in Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 [27]. Pairwise comparison analysis was also accomplished using MEGA v.6.

RESULTS AND DISCUSSION

Morphological Examination

Anisakis were initially identified based on the reported keyssuch as: pronounced ventriculus, no intestinal or ventricular caeca, excretory pore at head and mucron at tail tip [28,29]. The vital parts of collected *Anisakis* worms have the following morphometric data: total length of 5.198-21.875mm; maximum body width of 0.049-0.561mm; tail length of 0.019-0.261mm; and ventriculus length of 0.559-1.512mm.

A total of 1,500 worms were collected from the host fishes and 74 out of

1,500 worms collected were identified under genus *Anisakis*based on morphological examinations. The samples were initially identified to be included in the *Anisakis*type 1 groupcomposed of *A. simplex* s.s., *A. pegreffii, A. berlandi, A. typica*and *A. ziphidarum.*

PCR-RFLP

PCR fragments size were analyzed for comparable length by enzymatic digestion with endonucleases, enzymes able to cut DNA by recognition of short and specific oligonucleotide sequences. Three different species were revealed consisting of *A. typica*, *A. pegeffii* and a hybrid genotype of *A. simplex* s.s. and *A. pegreffii*.

DNA Analyses

The DNA sequence conforms to the results of the morphological examination. The phylogenetic tree showed that two samples(PYT13 and PM15)were grouped with *A. typica*, while five samples(URs3, URs72, UM1, UM4 and UM5) were located within *A. simplex* s.s., *A. pegreffii*,*A. berlandi* and *A. ziphidarum*group. Based on pairwise comparison analyses, PYT13 and PM15 have the lowest p-distance (0.000-0.025) and number of base differences (0-4 base differences) when compared to *A. typica*;while higher values when compared with other *Anisakis* species within clade 1.

Furthermore, high p-distance values (0.299-0.350) and number of base differences (47-55 base differences) were found when compared with *Anisakis* species belonging to clade 2 group. On the other hand, URs3, URs72, UM1, UM4 and UM5 have lowest p-distance values of 0.000-0.102 and 0-16 base differences when compared to *A. simplex*s.s., *A. berlandi*, *A. pegreffii* and *A. ziphidarum*. Furthermore, higher p-distance values (0.236-0.261) and number of base differences (36-41 base differences) were found when compared with other *Anisakis* species.

Distribution of Anisakis in the Philippines

Based on the present samples isolated from the marine fishes marketed in the Philippines, six out of 20 samples turned out to be *A. pegreffi*. This six samples were all acquired from the unknown source. Record shows that this is the first time that *A. pegreffii* has been found infecting marine waters in the country. Since the origin of the fish are unknown, there is a possibility that these sample fishes where these *A. pegreffi* isolated were imported.

Though there are no records yet stating that there is *A. pegreffii* isolated in the country, the presence of its hosts along the waters enclosing Philippines is giving the possibility that it is really present at the fish stocks in our marine waters. Another possible

cause is due to the climate change that might have affected the migration pattern of its intermediate hosts forcing them to enter the Philippine archipelago. There is also a possibility that the fish collected and used in this study were collected from countries where *A. pegreffii* and hybrid genotypes of *A. simplex* s.s. and *A. pegreffii* are present. Though this hybrid genotype has been reported in Japanese waters [30, 31] findings of such hybrid genotype in the Philippines could be the first record.

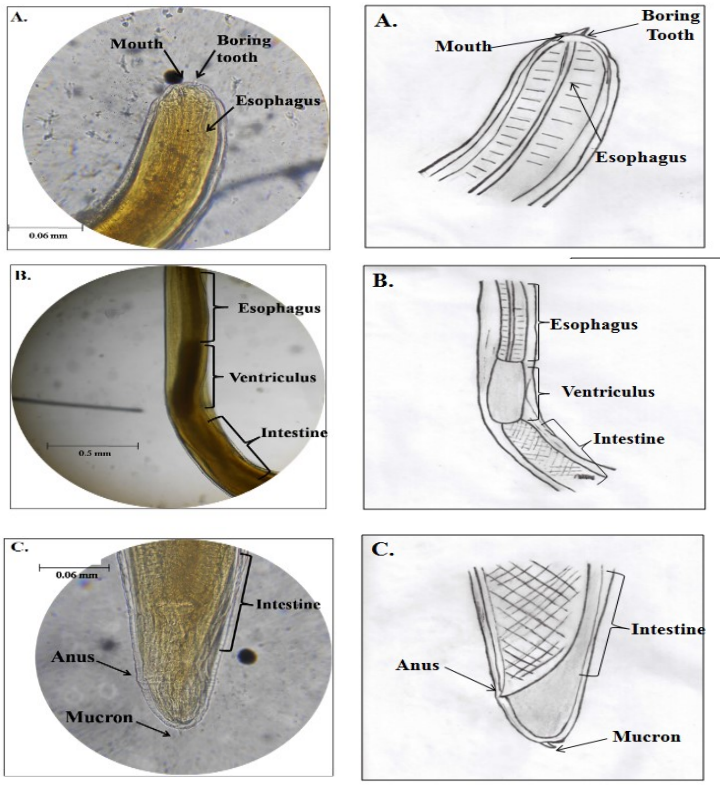
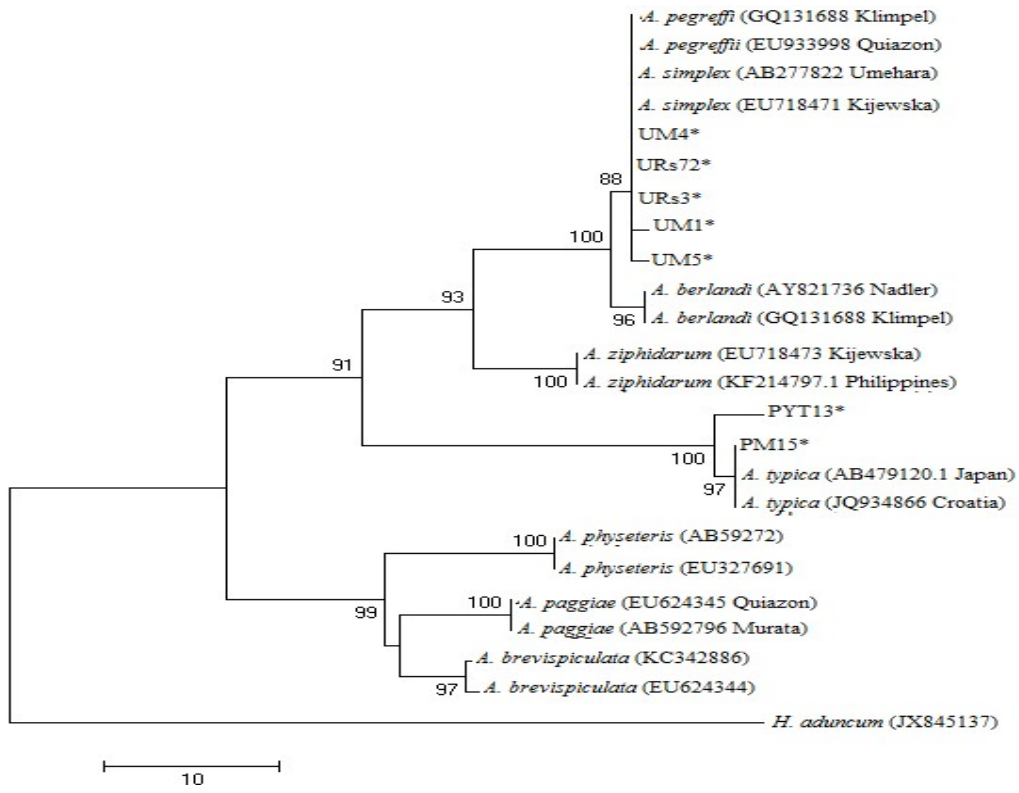


Figure 1: Actual photo (left) and schematic drawing (right) of three different sections of *Anisakis*: A. anterior portion; B. middle portion; C. posterior portion



Note: Code for the samples of the present study are as follows: P= Palawan; U= Unknown; M= Mackerels; Roundscad; YT= Yellowfin tuna

Figure 2: Phylogeny of *Anisakis* species identified from yellowfin tuna and mackerel based on the ITS region (NJ tree, p-distance, complete deletion, bootstrap method, MEGA6).

CONCLUSION

The results of the morphological examination, PCR-RFLP and sequencing of the ITS region has already proven the correct identity of the *Anisakis* species collected in the current study in which*A. typica* isthe dominant species which do not cause human anisakiasis and allergies. This could be the reason why incidence of human anisakiasis is not known in the country. With the absence of *A. simplex* s.s. and *A. pegreffi* from our exportable tunas, we can assure the international community of the safeness of our exported tuna for human consumption. On the other hand, importation of marine products especially tuna, may pose threat like dissemination of *Anisakis* infection in Philippine waters.

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