The Philippine Journal of Fisheries 27(2): 183-192 July-December 2020
DOI: 10.31398/tpjf/27.2.2020-0007

FULL PAPER

Parasitic Anisakid Nematode Isolated from Stranded Fraser's Dolphin (*Lagenodelphis hosei* Fraser, 1956) from Central Philippine Waters

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ABSTRACT

Cetaceans, including dolphins, serve as definitive hosts of zoonotic anisakid nematodes, which are important etiological agents for human anisakiasis and allergy-associated health risks. With limited knowledge of these zoonotic parasites from the marine environment in the Philippine waters, the stranding of a Fraser's dolphin (*Lagenodelphis hosei* Fraser, 1956) off the central Philippines made it possible to identify the worm species isolated from its gut. Parasitological examinations were carried out using morphological and molecular tools. Morphologically, the SEM and LM data revealed that the specimens belong to the genus *Anisakis* of the Type 1 group. Molecularly, PCR-RFLP results of the ITS region generated only a single fragment pattern on all worm samples corresponding to the reported molecular keys for *A. typica.* Further sequence and phylogenetic analyses of both ITS rDNA and mtDNA COX2 genes confirmed the anisakid nematodes' identity as *A. typica.* The molecular data obtained in this study support previous findings on the possible existence of local variants of *A. typica* in this region.

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Received: *April 5, 2020*
Accepted: *July 9, 2020*

Keywords: *Lagenodelphis hosei, Fraser's dolphin, Anisakis typica, Philippines, PCR-RFLP, ITS rDNA, mtDNA COX2*

Abbreviations: ITS–Internal Transcribed Spacer, LM–light microscope, mtDNA *COX2*–mitochondrial DNA cytochrome oxidase 2, PCR-RFLP–polymerase chain reaction-restriction fragment length polymorphism, SEM–scanning electron microscope

1. INTRODUCTION

Dolphins are among the members of the cetacean group where anisakid infections have been reported. Cetaceans served as definitive hosts of these parasitic nematodes, particularly of the genus *Anisakis* Dujardin, 1845. The life cycle of these parasites involves marine fishes and cephalopods as paratenic hosts, which can cause human infections such as human anisakiasis and allergies if improperly-handled infected fishery products are consumed. Among the nine known *Anisakis* species, the *A. typica* Diesing, 1860 has been reported from various dolphin species belonging to the families Delphinidae, Phocoenidae, and Pontoporidae from warmer temperate, tropical, and subtropical waters, as well from the South West (Brazil) and North West (Florida) Atlantic, and Mediterranean waters (North Africa) (Mattiucci et al. 2002, 2005; Nadler et al. 2005; Palm et al. 2008; Colón-Llavina et al. 2009; Kuhn et al. 2011; Kleinertz et al. 2014). Among the reported *A. typica*-infected dolphin species within the family Delphinidae include the following: short-finned pilot whale (*Globicephala macrorhynchus* Gray, 1846) from Florida coast; striped dolphin (*Stenella coeruleoalba* Meyen, 1833) from eastern Mediterranean Sea; common bottlenose dolphin (*Tursiops truncatus* Montagu, 1821) and pantropical spotted dolphin (*Stenella attenuata* Gray, 1846) from Florida coast and Carribean Sea; tucuxi (*Sotalia fluviatilis* (Gervais and Deville 1853)) and spinner dolphin (*Stenella longirostris* Gray, 1828) from Brazil Atlantic coast; rough-toothed dolphin (*Steno bredanensis*) from Carribean Sea and Indo-Pacific bottlenose dolphin (*Tursiops aduncus* (Ehrenberg, 1833)) from northern Red Sea.

Moreover, other dolphin species of the same family that were also reported to be infected with other *Anisakis* species include the following: short-beaked common dolphin (*Delphinus delphis* Linnaeus, 1758) (*A. simplex* sensu stricto (AS) from Iberian Atlantic coast; *A. pegreffii* (AP) from Iberian coast and western Mediterranean Sea); long-finned pilot whale (*Globicephala melaena* Traill, 1809) (AS from Iberian coast and South African coast; *A. berlandii* (AB) from South African coast); white-beaked dolphin (*Lagenorhynchus albirostris* Gray, 1846) (AS from North East Atlantic); northern right whale dolphin (*Lissodelphis borealis* Peale, 1848) (AB from North East Pacific); killer whale (*Orcinus orca* Linnaeus, 1758) (AS from North East Pacific); false killer whale (*Pseudorca crassidens*) (AS and AB from North East Pacific); striped dolphin (AS from Iberian Coast and AP from Western Mediterranean Sea); and common bottlenose dolphin (AP from central Mediterranean Sea and South African coast) (Mattiucci et al. 2002, 2005; Mattiucci and Nascetti 2008; Kleinertz et al. 2014).

The initial record of *Anisakis* infection in Fraser's dolphin (*Lagenodelphis hosei* Fraser, 1956) was from Brazil and Caribbean Sea; however, species level was not determined (Colón-Llavina et al. 2009; Carvalho et al. 2010). It was only recently that *A. typica* and *A. pegreffii* have been identified in this dolphin species from the Gulf of Mexico (Florida coast) (Cavallero et al. 2011). To date, no other reports on anisakid infection in Fraser's dolphin from different parts of the world, including the Philippines. Hence, the stranding of this dolphin species in the Pacific region is a rare opportunity to conduct such parasitological work, particularly looking at what species of anisakid nematodes present in the gut of this dolphin inhabiting central Philippine waters, which is part of the Western Pacific Ocean. Such information on the identity of anisakid species in Fraser's dolphin would help the local understanding of the possible *Anisakis* species that may be possibly infecting the marine fishes in the locality, which may pose human health concerns.

2. MATERIALS AND METHODS

Fragments of 45 individual worm specimens collected from the gut of Fraser's dolphin stranded in the West Pacific off central Philippine waters of Bantaya, Dumaguete City, Negros Oriental (9° 19' 43 "N; 123° 18' 45 "E) on February 2009 were fixed in 100% ethanol. The availability of solely undamaged anterior portions of the fragmented worms was used to identify the worms' genus level using SEM and LM. All available worms were then molecularly examined through PCR-RFLP of the ITS rDNA region (ITS1-5.8S-ITS2) using three restriction enzymes (*Taq* I, *Hinf* I, and *Hha* I). After initial species identification using the reported molecular keys (D'Amelio et al. 2000), the ITS rDNA and mtDNA *COX2* gene regions of representative samples were sequenced and analyzed following previous reports (Valentini et al. 2006; Quiazon et al. 2013; Quiazon 2016). In addition to the specimens from the current study, the DNA templates of other *Anisakis* species from previous studies (Quiazon et al. 2013) were used. Sequence alignments, construction of Neighbor-Joining tree (NJ), and phylogenetic analysis using Maximum Parsimony (MP) were carried out using the BioEdit 7.2.5, ClustalX 2.0.10, MEGA6 (bootstrap, 1,000 replicates, complete deletion), and PAUP 4.0. Some nucleotide sequences of *Anisakis* species examined in this study were deposited in the GenBank database with the following accession numbers: KF356673–KF356675 (ITS) and KF356648–KF356653 (mtDNA *COX2*).

3. RESULTS AND DISCUSSION

Morphologically, the presence of distinct ventriculus and the absence of ventricular appendix and intestinal caecum reveals that the worm samples belong to the genus *Anisakis* (Figs. 1A, 1B). Based on the presence of a long ventriculus with an oblique ventricular-intestinal junction, the *Anisakis* worms belong to the Type 1 group (Umehara et al. 2010; Murata et al. 2011).

Molecularly, the PCR-RFLP results of all worm specimens generated only one fragment pattern similar to the reported pattern for *A. typica* from each of the three enzymes used (D'Amelio et al. 2000; Lee et al. 2009; Quiazon et al. 2013; Zhang et al. 2013) (Figs. 1C–E). The PCR-RFLP results supported the sequence data of the two gene regions examined that the worms isolated from the gut of Fraser's dolphin are all 100% *A. typica*. The phylogenetic analysis

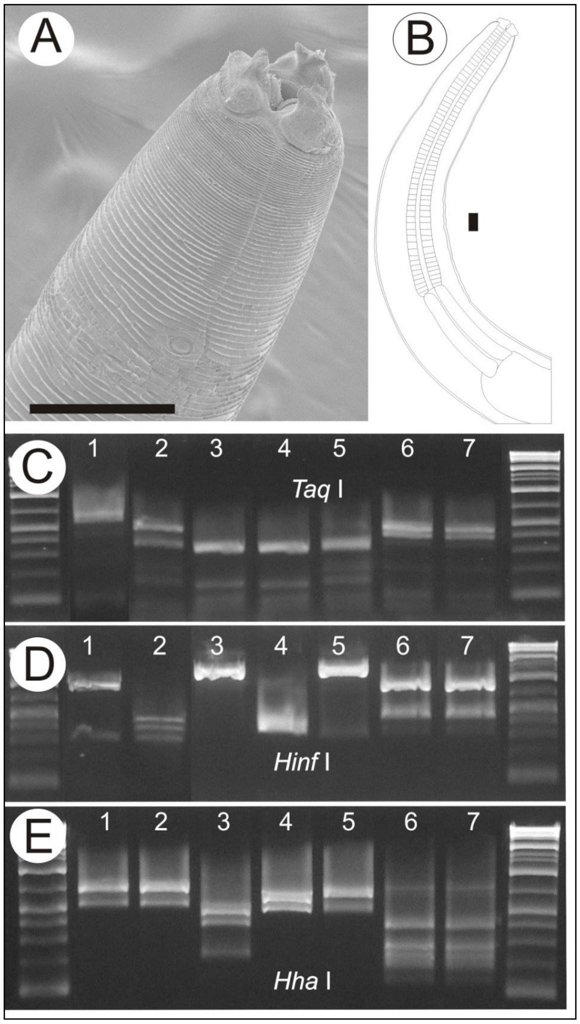

Fig. 1. Morphological and molecular results on the *Anisakis* infecting Fraser's dolphin (A, SEM micrograph of the anterior end showing the mouth opening; B, LM result showing the distinct ventriculus as characteristics of the genus *Anisakis*; C, PCR-RFLP results after digestion with different restriction enzymes [Lane 1, *A. simplex* s.s.; lane 2, *A. pegreffii*; lane 3, *A. brevispiculata*; lane 4, *A. ziphidarum*; lane 5, *A. paggiae*; lane 6, *A. typica*; lane 7, Samplesfrom Fraser's dolphin]). Scale bar: 150µm.

produced tree topology similar to the NJ and MP results wherein worms from the Fraser's dolphin were all clustering in the *A. typica* branch (Fig. 2). The ITS region revealed the closest similarity with the reported *A. typica* (Palm et al. 2008; Umehara et al. 2010; Kuhn et al. 2011; Koinari et al. 2013; Quiazon et al. 2013; Zhang et al. 2013) compared to other congeners. Final species confirmation using the mtDNA *COX2* region revealed that the current samples were indeed *A. typica* compared with other reported congeners (Table 1). Percent similarities within the reported *A. typica* showed slightly lower similarities (96.2%) to those *A. typica* reported from the Atlantic (DQ116427), Brazil (JQ798968), Japan (AB517571), Philippines (KC821728), and Croatia (JQ934884), but higher similarities with specimens reported from Papua New Guinea (JX648323) (99.1-99.7%) and Indonesia (KC928263) (97.7–98%) (Table 2).

There have been reports on *A. typica* infection from several marine fishes and cetaceans in the Western Pacific side. Worldwide zoogeographical modeling of the zoonotic parasite *Anisakis* from cetaceans and fishes had been done wherein the species found to inhabit the tropical regions in the South China Sea is the *A. typica* (Kuhn et al. 2011). *Anisakis typica* was reported from different marine fish species in Korea (Lee et al. 2009), South China Sea (Zhang et al. 2013), Taiwan, Japan (Umehara et al. 2010), Papua New Guinea (Koinari et al. 2013), and Indonesian waters (Palm et al. 2008; Anshary et al. 2014). In the Philippines, a multi-infection of *A. typica*, *A. brevispiculata*, and two unknown *Anisakis* species genetically close to *A. paggiae* and *A. ziphidarum* have been reported from Dwarf sperm whale (Quiazon et al. 2013). Although other parasites have been reported in Fraser's dolphin such as *Phyllobotrium delphini* (cysrs and larval stage in blubber) (Cestoda, Tetraphyllidea), *Monorygma grimaldii* (in the abdominal cavity and urinary bladder) (Cestoda: Phyllobothriidae), and *Tetrabothrius forsteri* (Cestoda: Tetrabothriidae) (Carvalho et al. 2010, Colón-Llavina et al. 2009, Failla Siquier and Le Bas 2003, Mignucci-Giannoni et al. 1999, Moreno et al. 2003), the available parasite samples sent to the researchers for identification were mainly nematode fragments of *Anisakis* samples. Despite the identification of only *Anisakis* species in this study, it is likely possible that other endo-parasites are present but were not collected.

Since dolphins are one of the final hosts of *Anisakis* species, the primary potential source of infection is through their diet, which serves as the parasite's intermediate host during their third larval stage. Mesopelagic fishes, particularly myctophids (mainly *Ceratoscopelus warmingi, Diaphus* spp. and *Myctophum asperum*), were equally important diet with mesopelagic cephalopods (*Abraliopsis, Onychoteuthis, Histioteuthis,* and *Chiroteuthis*), and crustaceans (*Notostomos elegans, Acanthephyra quadrispinosa,* and *Acanthephyra carinata*) for Fraser's dolphin (Dolar et al. 2003). Among these food items, *Anisakis* infections have been reported in *Diaphus* (Cabrera-Gil et al. 2018, Gaglio et al. 2018), *Myctophum* (Cabrera-Gil et al. 2018; Gaglio et al. 2018; Klimpel et al. 2008, 2010a, 2010b; Mateu et al.

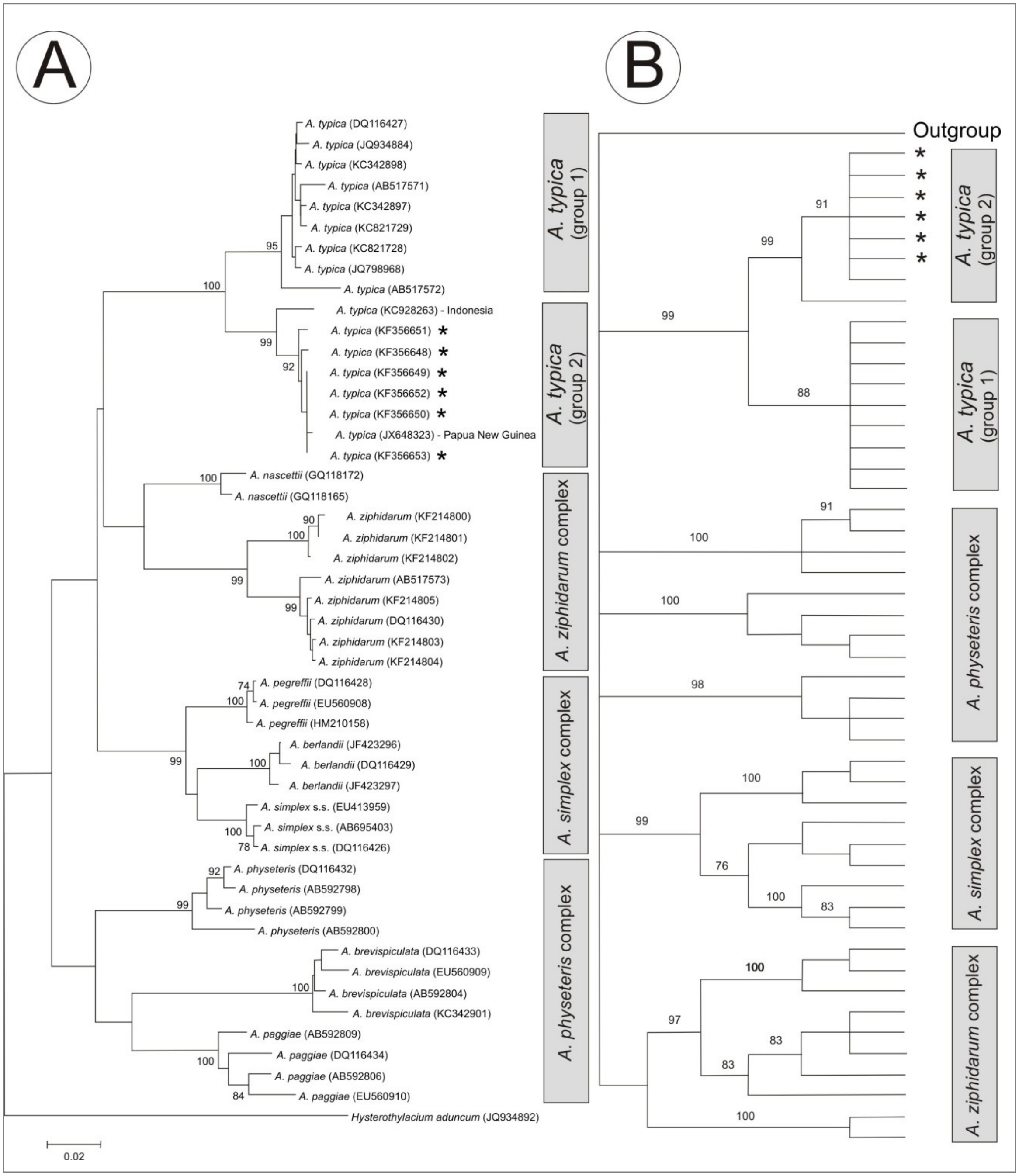

Fig. 2. Evolutionary relationship of *Anisakis* species from Fraser's dolphin (in *) based on the mtDNA *COX2* region (A - NJ tree [K2P, complete deletion, bootstrap, 1,000 replicates, MEGA6]; B - MP tree [bootstrap, PAUP4.0]).

Table 1. Pairwise comparison on the genetic distances (nucleotide, K2P, complete deletion, MEGA 5) and amino acid differences (in parentheses, complete deletion) in the mtDNA *COX2* region between the current specimenswith other congeneric species deposited in the GenBank

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**GenBank Acc. No.**
1 Fraser's dolphin	0 – 0. 009 (0 – 4)										This study
2 *A. typica* grp2	0.002 – 0. 007 (0 – 2)	-									JX648323
3 *A. typica* grp1	0.051 – 0. 053 (21 – 23)	0.053 (22)	-								DQ116427
4 *A. ziphidarum*s.s.	0.148 – 0. 151 (57 – 58)	0.151 (58)	0.144 (57)	-							DQ116430
5 *A. nascettii*	0.135 – 0. 137 (55 – 57)	0.132 (56)	0.121 (51)	0.088 (34)	-						GQ118172
6 *A. simplex* s.s.	0.132 – 0. 134 (50 – 52)	0.134 (51)	0.130 (54)	0.113 (47)	0.130 (54)	-					DQ116426
7 *A. pegreffii*	0.132 – 0. 134 (50 – 52)	0.134 (51)	0.134 (55)	0.123 (51)	0.132 (53)	0.037 (17)	-				DQ116428
8 *A. berlandii*	0.132 – 0. 138 (47 – 49)	0.138 (48)	0.136 (51)	0.137 (51)	0.141 (55)	0.056 (22)	0.068 (30)	-			DQ116429
9 *A. paggiae*	0.161 – 0. 166 (58 – 59)	0.166 (59)	0.161 (61)	0.115 (43)	0.136 (50)	0.134 (47)	0.136 (46)	0.140 (45)	-		DQ116434
10 *A. brevispiculata*	0.189 – 0. 192 (72 – 73)	0.194 (73)	0.197 (74)	0.143 (55)	0.177 (66)	0.167 (65)	0.174 (65)	0.176 (64)	0.123 (48)	-	DQ116433
11 *A. physeteris*	0.149 – 0. 158 (56 – 59)	0.154 (57)	0.151 (60)	0.134 (53)	0.160 (61)	0.133 (52)	0.142 (54)	0.144 (52)	0.117 (48)	0.107 (44)	DQ116432

Table 2. Pairwise comparison on the percentage similarities on the mtDNA *COX2* region between *A. typica* specimens from Fraser's dolphin with other deposited *A. typica* from the GenBank

Group	Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	GenBank Acc No.
A.typica group 2	1 Fraser's dolphin1	-													KF356648
	2 Fraser's dolphin2	99.5	-												KF356649
	3 Fraser's dolphin3	99.7	99.8	-											KF356650
	4 Fraser's dolphin4	99.2	99.7	99.5	-										KF356651
	5 Fraser's dolphin5	99.5	100	99.8	99.7	-									KF356652
	6 Fraser's dolphin6	99.7	99.8	100	99.5	99.8	-								KF356653
	7 Indonesia	97.8	98	97.8	97.7	98	97.8	-							KC928263
	8 Papua New Guinea	99.1	99.7	99.5	99.3	99.7	99.5	97.9	-						JX648323
A.typica group 1	9 Atlantic	94.9	95.1	95	95.1	95.2	95	95	95	-					DQ116427
	10 Brazil	95.1	95.3	95.1	95.3	95.3	95.1	94.8	95	99.3	-				JQ798968
	11 Japan	94.4	94.7	94.5	94.6	94.7	94.5	94.9	94.5	98.9	98.4	-			AB517571
	12 Philippines	94.5	94.7	94.5	94.7	94.7	94.5	94.5	94.4	99.3	99.4	98.4	-		KC821728
	13 Croatia	94.5	94.8	94.7	94.7	94.8	94.7	94.7	94.6	99.4	98.9	98.6	99	-	JQ934884

Note: Percentage similarities were determined using emboss matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/nucleotide.html).

2015), *Onychoteuthi* (Nagasawa and Moravec 1995, 2002; Sun et al. 1991), and *Histioteuthis* (Culurgioni et al. 2010).

The identification of *Anisakis* species through a morphological examination of the posterior end of adult male specimens is a conventional approach in the taxonomical studies of anisakid worms. Unfortunately, identification to genus level was only made possible using SEM and LM with the availability of some good specimens of fragmented anterior end only. Thus, a molecular tool was the only possible way to identify anisakid worms species from the Fraser's dolphin in this West Pacific region. Apart from the PCR-RFLP on the ITS region, the use of mtDNA *COX2* region for final species confirmation on anisakid nematodes, including the genus *Anisakis*, has been widely used and adopted (Valentini et al. 2006; Colón-Llavina et al. 2009; Umehara et al. 2010; Koinari et al. 2013; Quiazon et al. 2013, 2016). The similarity of the fragment patterns generated from PCR-RFLP with the reported molecular keys (D'Amelio et al. 2000; Lee et al. 2009; Quiazon et al. 2013; Zhang et al. 2013) has already provided the initial identity of the anisakid worms from the Fraser's dolphin as *A. typica*. Despite the clustering of the present samples in the phylogenetic trees with several reported *A. typica* sequences, we also found similar results from previous studies from Indonesia (Balinese waters, Javanese waters, and Southern Makassar Strait) (Palm et al. 2008; Anshary et al. 2014) and Papua New Guinea (Koinari et al. 2013) on the intra-variable differences on the nucleotide bases within the *A. typica* groups forming further two sub-groups. Such sub-grouping has been observed in both gene regions examined.

Given that no available specimens of whole adult male *A. typica* of these possible local variants have been ever examined morphologically and compared to the reference specimen of *A. typica*, local variance will remain a possibility. To this point, our data supports such possible existence of local variants or sibling species of *A. typica* not only from the Indonesian waters (Palm et al. 2008; Anshary et al. 2014) and Papua New Guinea (Koinari et al. 2013) but as well in the Philippine waters. Moreover, despite the several global reports of *A. typica*, this study is the first to report the *Anisakis* infection from Fraser's dolphin off central Philippine waters within the West Pacific region, second only to that reported from the Florida coast (Cavallero et al. 2011).

4. CONCLUSION

The possible health risk brought about by these zoonotic parasites, and the limited parasitological study in the Philippine waters warrants the investigation of *Anisakis* infection on their definitive hosts as these are mainly responsible for the release and spread of worm larvae to several economically important marine fishes and cephalopods. Hence, stranding of Fraser's dolphin made it possible to examine the species of *Anisakis* present in this marine mammal, which may lead to knowledge of the potential *Anisakis* species infecting its paratenic hosts within the region. The documentation of *Anisakis* infection in Philippine waters is of scientific importance in view of food safety, particularly in this archipelagic country where vast marine fish food products are abundant for its local and international consumers.

5. ACKNOWLEDGMENT

The authors wish to thank Janet Estarion and Leslie Callanta of Siliman University - Institute of Environmental and Marine Sciences for collecting the parasites from the Fraser's dolphin. Japan Society partly supported this study for the Promotion of Science (JSPS) Postdoctoral Fellowship for Foreign Researchers and Grant-in-Aid for JSPS fellows (23-01405).

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