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Food and Waterborne Parasitology

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Anisakid nematodes in *Trichiurus lepturus* and *Saurida undosquamis* (Teleostea) from the South-West Indian Ocean: Genetic evidence for the existence of sister species within *Anisakis typica* (s.l.), and food-safety considerations

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ARTICLE INFO

Keywords:

Anisakis typica

Sister taxa

South-West Indian Ocean

Trichiurus lepturus

Saurida undosquamis

Intra-vitam larval migration

Food safety

ABSTRACT

Nematode parasites of the genus *Anisakis* (Nematoda, Anisakidae) are considered among the most important biological hazards in seafood products worldwide. In temperate and tropical waters, the most common species appears to be *Anisakis typica*, generally found around the viscera and sporadically in the flesh of various fish host species. This study investigated the infection sites and genetic diversity of *A. typica* infecting commercial fishes from the South-West Indian Ocean. Largehead hairtail ($N = 20$) and brushtooth lizardfish ($N = 72$) fished off Tanzania were inspected for anisakid nematodes by UV-press. A subsample of 168 nematodes were identified by sequence analyses of the *cox2* mtDNA gene and ITS region of rDNA. The species *A. typica* (s.l.) ($N = 166$), *Pseudoterranova ceticola* ($N = 1$) and *Anisakis paggiae* ($N = 1$) were molecularly identified. Phylogenetic analysis of *A. typica* (s.l.) sequences based on both genes, indicated the existence of two distinct phylogenetic lineages forming two well-supported clades. The first clade comprised 12 *A. typica* specimens including individuals from its type locality (central Atlantic Ocean). The second clade comprising 154 specimens, clustered with reference sequences retrieved from GenBank including one apparently undescribed taxon, i.e., *Anisakis* sp. 1, and *A. typica* var. *indonesiensis*. The two reciprocally monophyletic clades are closely related and correspond to two distinct sister species within *A. typica* (s.l.), presently indicated as *A. typica* sp. A and *A. typica* sp. B. Two and four fixed alternative nucleotide substitutions (SNPs), i.e., diagnostic positions, between the two taxa, respectively, were found at the mtDNA *cox2* and the ITS region of rDNA. The genetic data, as well as their occurrence in sympatry, strengthens the hypothesis that the actual specimens represent two distinct gene pools. The occurrence of both *A. typica* sp. A and *A. typica* sp. B in the musculature of freshly examined *T. lepturus* and *S. undosquamis*, suggests that both species can migrate *intra-vitam* into the flesh. Although the zoonotic potential of *A. typica* s.l. is still unclear, the presence of these parasites in the musculature, edible part of the fish, raises health concerns for consumers.

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<https://doi.org/10.1016/j.fawpar.2022.e00177>

Received 27 June 2022; Received in revised form 2 August 2022; Accepted 18 August 2022

Available online 23 August 2022

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1. Introduction

Nematode parasites of the genus *Anisakis* (Anisakidae, Nematoda) are considered among the most important biological hazards in seafood products (Ishikura et al., 1993). Larvae of *Anisakis* spp. may cause human anisakiasis, a seafood-borne parasitic zoonosis provoked by accidental ingestion of viable larvae of certain *Anisakis* species which infect the edible parts of fish or squid (Audicana and Kennedy, 2008). The potential human health hazards associated with the presence of these parasites in fish, combined with their repellent appearance if present in large numbers, represent a serious concern for consumers, food safety authorities and the fishing industries (Bao et al., 2017; Bao et al., 2019; D'Amico et al., 2014; Karl and Levsen, 2011; Levsen and Karl, 2014; Llarena-Reino et al., 2013).

Anisakis species have heteroxenous life cycles, that involve various hosts at different levels across the marine food web. Crustaceans act as first intermediate hosts, while fish and squids serve as intermediate and/or paratenic hosts, transferring the larvae to their respective cetacean definitive hosts (Mattiucci et al., 2018; Mattiucci and Nascetti, 2006). In fish, most *Anisakis* larvae reside encapsulated in the viscera (Levsen et al., 2018; Mattiucci et al., 2018; Mattiucci and Nascetti, 2008), while some species may migrate into the muscle, thus, posing a potential zoonotic risk (Cipriani et al., 2018; Karl and Levsen, 2011; Levsen et al., 2018; Llarena-Reino et al., 2013; Mattiucci et al., 2018). To date, out of the nine characterized *Anisakis* species (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009), *A. simplex* (s. s.) and *A. pegreffii* seem to be the only species capable of migrating *intra-vitam* and *post-mortem* into the fish flesh (reviewed by Mattiucci et al., 2018). Moreover, they are the only genetically recognized species that may induce human anisakiasis (Arai et al., 2014; D'Amelio et al., 1999; Fumarola et al., 2009; Guardone et al., 2018; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2017; Mladineo et al., 2016; Umehara et al., 2007). Due to their zoonotic potential and their ability to migrate into the flesh of many commercially important fishes, *Anisakis simplex* (s. s.) and *A. pegreffii* are currently the most intensively studied species. Thus, extensive and systematic epidemiological surveys have been conducted on these species using internationally recognized and validated detection methods, i.e. the UV-press method (ISO 23036-1:2021) (Gómez-Morales et al., 2018; Karl and Leinemann, 1993; Karl and Levsen, 2011; Levsen et al., 2018; Pippy, 1970) and artificial digestion (ISO 23036-2:2021) (Llarena-Reino et al., 2013; Lunestad, 2003).

In the warmer temperate and tropical waters of the world's oceans from 35° N to 39° S in a circum-equatorial distribution, *A. typica* (Diesing, 1860) appears to be one of the most common *Anisakis* species (Irigoitia et al., 2021; Lanfranchi et al., 2018; Mattiucci et al., 2018). Adult individuals of this species were originally described from *Delphinus delphis* in the Central Atlantic Ocean (Diesing, 1860). Since then, *A. typica* has been frequently recorded in temperate waters of the Atlantic and Indo-Pacific oceans, both at the adult stage from several dolphin species (Colón-Llavina et al., 2009; Gomes et al., 2021; Iñiguez et al., 2009, 2011; Mattiucci et al., 2002; Mattiucci and Nascetti, 2008; Quiazon et al., 2020), and at the larval stage in numerous fishes (Mattiucci et al., 2018; Shamsi, 2021). In recent years, a new taxon genetically distinct from all known species of *Anisakis* but closely related to *A. typica*, has been found at the larval stage in several fish hosts from tropical temperate waters of the Indo-Pacific area (Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). This taxon was detected for the first time in the visceral cavity of *Nemipterus japonicus* caught off the Malaysian coast, identified as a distinct gene pool by multiple genetic markers (allozymes and mtDNA analysis), and provisionally named *Anisakis* sp. 1 (Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). Palm et al. (2008, 2017) identified several different "genotypes" of *A. typica* larvae from fish hosts caught in Balinese and Javanese waters (Indonesia) by ITS rDNA sequence analysis, and designated them as a variant of *A. typica*, namely *Anisakis typica* var. *indonesiensis*.

The commonly reported site of infection in fish with *A. typica* larvae is limited to the visceral body cavity; the larvae are generally free or coiled on mesentery, liver and gonads (Borges et al., 2012; Palm et al., 2017). However, some occasional findings of *A. typica* larvae in the muscle tissue have been reported in fishes from the Western Pacific Ocean (Anshary et al., 2014; Koinari et al., 2013; Palm et al., 2008, 2017). Only scanty data exist on the occurrence of human anisakiasis from the geographic areas where *A. typica* has been found. Rare cases of positive seroprevalence for anisakiasis in humans have been reported in Indonesia (Anshary et al., 2014; Uga et al., 1996), but the etiological agents, possibly responsible for that positive serodiagnosis, were not identified.

Anisakis epidemiological surveys in commercial fish from the Western Indian Ocean region (WIO) are scarce or incomplete. Indeed, fish consumption per capita in this area has always been low, and there was little commercial interest in investigating zoonotic and quality reducing fish parasites (FAO, 2018; Giulietti et al., 2020). In recent years, local governments of WIO region have shown a growing interest in exploring the stock status of new local seafood resources (FAO, 2018). Among them, the largehead hairtail, *Trichiurus lepturus* Linnaeus, 1758, may represent a new potentially important fishery. The largehead hairtail is mainly a coastal benthopelagic fish, heavily targeted in small scale and commercial fisheries worldwide (Kang et al., 2015), being among the 25 commercial fish species with highest landings (FAO, 2018; Al-Nahdi et al., 2009; Kim et al., 2005; Kwok and Ni, 1999). Despite its global commercial importance, this fish species is still under-utilised in most of the WIO region, particularly in artisanal and commercial fisheries in Tanzania, where this species is considered of low commercial value and often discarded (Fennessy, 2009; Oduor-Odote and Kazungu, 2009; Van der Elst et al., 2005).

Another abundant fish species in the South-West Indian Ocean is the brushtooth lizardfish, *Saurida undosquamis* (Richardson, 1848) (Johannessen et al., 2018). The species is a demersal fish, mostly found above 100 m depth over sand or mud bottoms of coastal waters (Golani, 1993; Heemstra, 1995). Although not commercially attractive for international markets, brushtooth lizardfish is targeted by artisanal local fisheries in WIO region, being a fish species mostly consumed by populations along the coastal areas. The potential economic yield from largehead hairtail and brushtooth lizardfish emerging fisheries raises the need for deepening the knowledge of their parasitic fauna, with special emphasis on zoonotic and quality reducing species.

The present study aimed to investigate anisakid species diversity in largehead hairtail and brushtooth lizardfish fished off the coast

of Tanzania, South-West Indian Ocean, and to provide data on the infection levels and spatial distribution of these parasites in the fish hosts.

2. Materials and methods

2.1. Fish sampling and parasitological examination

A total of 20 *T. lepturus* and 72 *S. undosquamis* were sampled in April 2018 off Tanzania, in the South-West Indian Ocean (Fig. 1), during an ecosystem survey with the research vessel Dr. Fridtjof Nansen (NORAD-FAO project GCP/INT/730/NOR; survey number 2018404), operating within the EAF-Nansen Project. Fish were caught using bottom trawls at 100–600 m depth off the coast of Tanzania (7°16' S 39°41' E; FAO Subarea 51.5). Fishes were identified to species level by local taxonomists, measured (total body length, TL, 5 mm accuracy), and weighed (total body weight, TW, in g) (Table 1). Parasite inspection was conducted onboard right after catch using the UV-press method for anisakid nematodes (ISO 23036-1:2021) (Karl and Leinemann, 1993; Levsen et al., 2018). Fish were dissected, each flesh side (i.e., fillets including belly flaps) and the visceral organs of each fish, were placed in individual plastic bags and then pressed using a hydraulic pressing device to a 1–2 mm thick layer. The bags were then frozen for 24 h, thawed, and subsequently examined for anisakid nematodes under UV-light (366 nm). Anisakid nematodes were localized in the fish host, counted, washed in saline solution, and stored at –20 °C for further morphological and molecular identification.

2.2. Identification of anisakid nematodes

A subsample of 168 out of 1634 (around 10%) anisakid nematodes, randomly selected from the viscera and flesh, were subjected to a morphological examination using light microscopy (Olympus BX51 microscope) at 200× magnification. The nematodes were assigned to genus level according to diagnostic morphological characters (Berland, 1961).

Subsequently, these morphologically examined nematodes ($N = 168$) were subjected to genetic identification by sequence analysis of the mitochondrial cytochrome *c* oxidase II (*cox2*) gene. In addition, a subsample of 80 specimens randomly selected among those previously sequenced at the *cox2* mtDNA, were genetically identified by sequence analysis of the ITS region of rDNA. DNA was extracted from 2 mg of tissue from each specimen, using the DNeasy® Blood and Tissue Kit (QIAGEN® GmbH, Hilden, Germany) and following the manufacturer's instructions.

The mtDNA *cox2* gene was amplified by polymerase chain reaction (PCR) using the primers 211F (5'-TTT TCT AGT TAT ATA GAT TGR TTT YAT-3') and 210R (5'-CAC CAA CTC TTA AAA TTA TC-3') (Nadler and Hudspeth, 2000), following the procedures provided by Mattiucci et al. (2014). The ITS region rDNA, including the first internal transcribed spacer (ITS-1), the 5.8S and the second transcribed spacer (ITS-2), was sequenced using the primers NC5 (forward; 5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and NC2 (reverse; 5'-TTA GTT TCT TTT CCT CCG CT-3') (Zhu et al., 2000). PCR was carried out according to the procedures reported in Zhu

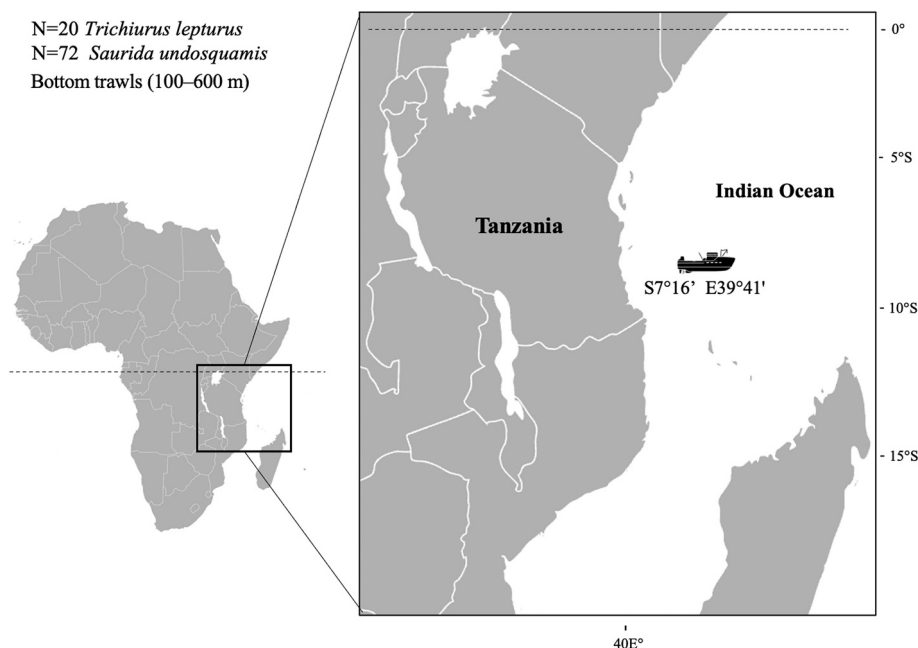


Fig. 1. Sampling localities of specimens of *Trichiurus lepturus* and *Saurida undosquamis* examined in the present study (for detailed information see Table 1).

Table 1

Infection parameters of *Anisakis typica* (s.l.) in *Trichiurus lepturus* and *Saurida undosquamis* collected off the coast of Tanzania (9°54' S 39°52' E, 7°16' S 39°41' E), in South-West Indian Ocean: number of infected fish (N), prevalence (P, %), mean abundance (A), mean intensity (mI) with standard deviation (\pm SD) and range (min-max).

		Musculature		Viscera	Overall	
		Ventral	Dorsal			
<i>Trichiurus lepturus</i>	Infected	17	0	20	20	
	N = 20			100.0	100.0	
	TL mean (\pm SD) = 111.8 \pm 18.6	A	4.5	0	53.1	57.6
	TW mean (\pm SD) = 842.8 \pm 594.1	mI (\pm SD) (min-max)	5.29 \pm 7.37 (1–31)	0 \pm 0	53.05 \pm 53.86 (9–238)	57.55 \pm 59.90 (9–269)
		$N_{I,Tot}$ (%)	90 (7.8%)	0 (0%)	1061 (92.2%)	1151 (100%)
<i>Saurida undosquamis</i>	Infected	11	2	44	45	
	N = 72			61.1	62.5	
	TL mean (\pm SD) = 29.2 \pm 5.4	P (%)	15.3	0.08–0.26	2.8	0.003–0.013
	TW mean (\pm SD) = 190.4 \pm 122.7	A	0.19	0.06	6.46	6.71
		mI (\pm SD) (min-max)	1.27 \pm 0.47 (1–2)	2.00 \pm 1.41 (1–3)	10.57 \pm 11.04 (1–50)	10.73 \pm 11.25 (1–50)
	$N_{I,Tot}$ (%)	14 (2.9%)	4 (0.8%)	465 (96.3%)	483 (100%)	

Number of total larvae ($N_{I,Tot}$) and their relative proportions (%) in different sites of infection are also given.

et al. (2000). Purification and sequencing of PCR products were carried out by Eurofins (Cologne, Germany), using the same primers as the ones used for the amplification. Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST) (Morgulis et al., 2008).

2.3. Phylogenetic analysis and genetic distance estimation

The sequences obtained of the mtDNA *cox2* and the ITS region of rDNA were aligned with sequences of other anisakid species previously identified and deposited in GenBank, as well as with other reference sequences obtained in our previous studies, using ClustalX v2.0 (Larkin et al., 2007) (see Tables 2 and 3). The mtDNA *cox2* and ITS region of rDNA alignments were manually edited and trimmed by using BioEdit v7.0.5.3 (Hall, 1999).

Phylogenetic trees based on mtDNA *cox2* and ITS rDNA were made using Bayesian Markov Chain Monte Carlo (MCMC) analysis in BEAST v1.10.4 (Suchard et al., 2018). Best-fit substitution model was selected using the Bayesian information criterion (BIC) and Akaike information criterion (AIC) as implemented in jModeltest v2.1.7. (Darriba et al., 2012). TN93 + G + I (*cox2*) and GTR + G (ITS)

Table 2

Details of all mtDNA *cox2* Ascaridoid nematode sequences included in the BI phylogenetic analyses (Fig. 2): species, stage (A: adult, L3: third larval stage), host, geographic location, and accession number of sequences deposited in Genbank.

Species (genotype)	New classification	Stage	Host	Geographical location	Accession number	References
<i>Anisakis typica</i>	<i>A. typica</i> A	L3	–	–	DQ116427	Valentini et al., 2006
<i>A. typica</i>	<i>A. typica</i> A	A	<i>Stenella clymene</i>	Brazil	JQ859923	Di Zevedo et al., 2017
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Auxis thazard</i>	Indonesia	KC928272	Anshary et al., 2014
<i>A. typica</i> *	<i>A. typica</i> B	L3	<i>Auxis thazard</i>	Indonesia	KC928269	Anshary et al., 2014
<i>A. typica</i>	<i>A. typica</i> B	L3	<i>Priacanthus tayenus</i>	Thailand	MF399482	Eamsobhana et al., 2018
<i>A. typica</i>	<i>A. typica</i> B	A	<i>Tursiops aduncum</i>	Red Sea	KF701409	Kleinertz et al., 2014
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Katsuwonus pelamis</i>	Japan	LC543820	Takano et al., 2021
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>T. lepturus</i>	Bangladesh	ON109754	Bao et al., 2022
<i>A. typica</i>	<i>A. typica</i> A?	L3	<i>Selar crumenophthalmus</i>	Bangladesh	ON109756	Bao et al., 2022
<i>A. typica</i>	<i>A. typica</i> B	L3	<i>Pinjalo lewisi</i>	Papua New Guinea	JX648323	Koinari et al., 2013
<i>A. sp.1</i>	<i>A. typica</i> B	L3	<i>Nemipterus japonicus</i>	Malaysian coast	OL456210	Mattiucci and Nascetti, 2008
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Trichiurus lepturus</i>	Brazil	JQ798968	Borges et al., 2012
<i>A. typica</i>	<i>A. typica</i> A	A	<i>Stenella longirostris</i>	Caribbean	AY826724	Cavallero et al., 2011
<i>A. typica</i>	<i>A. typica</i> A	A	<i>Pontoporia blainvillei</i>	Argentina	MT534169	Irigoitia et al., 2021
<i>A. typica</i>	<i>A. typica</i> B	A	<i>Lagenodelphis hosei</i>	Philippines	KF356648	Quiazon et al., 2020
<i>A. simplex</i> (s.s.)	<i>A. simplex</i> (s.s.)	–	–	–	DQ116426	Valentini et al., 2006
<i>A. pegreffii</i>	<i>A. pegreffii</i>	A	<i>Delphinus delphis</i>	–	DQ116428	Valentini et al., 2006
<i>A. berlandi</i>	<i>A. berlandi</i>	–	–	–	DQ116429	Valentini et al., 2006
<i>A. ziphidarum</i>	<i>A. ziphidarum</i>	–	–	–	DQ116430	Valentini et al., 2006
<i>A. nascettii</i>	<i>A. nascettii</i>	–	–	–	DQ116431	Valentini et al., 2006
<i>A. physeteris</i>	<i>A. physeteris</i>	–	–	–	DQ116432	Valentini et al., 2006
<i>A. brevispiculata</i>	<i>A. brevispiculata</i>	–	–	–	DQ116433	Valentini et al., 2006
<i>A. paggiae</i>	<i>A. paggiae</i>	–	–	–	DQ116434	Valentini et al., 2006
<i>Pseudoterranova ceticola</i>	<i>Pseudoterranova ceticola</i>	–	–	–	DQ116435	Valentini et al., 2006

(–: data not reported). The new classification is provided according to the novel results obtained.

Table 3

Details of all ITS rDNA anisakid nematode sequences included in the BI phylogenetic analyses (Fig. 2): species, stage (A: adult, L3: third larval stage), host, geographic location, and accession number of sequences deposited in Genbank.

Species (genotype)	New classification	Stage	Host	Geographical location	Accession number	References
<i>A. typica</i>	<i>A. typica</i> A	–	<i>Stenella longirostris</i>	Brazil	AY826724	D'Amelio et al., 2004 (Unpublished)
<i>A. typica</i>	<i>A. typica</i> A	A	<i>Sotalia fluviatilis</i>	Brazil	JQ912690	Mattiucci et al., 2014
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Trichiurus lepturus</i>	Indonesia	JN968936	Kuhn et al., 2013
<i>A. typica</i>	<i>A. typica</i> B	L3	<i>Trichiurus lepturus</i>	Indonesia	JN968940	Kuhn et al., 2013
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Selar crumenophthalmus</i>	Hawaii	JN968907	Kuhn et al., 2013
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Nemipterus japonicus</i>	China	MT020144	(Guo et al., 2020)
<i>A. typica</i> *	<i>A. typica</i> B	L3	<i>Nemipterus japonicus</i>	China	MT020143	Guo et al., 2020
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>T. lepturus</i>	Bangladesh	ON065559	Bao et al., 2022
<i>A. typica</i> *	<i>A. typica</i> B	L3	<i>Decapterus macarellus</i>	Vietnam	LC592876	Van Hien et al., 2021
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Trichiurus</i> sp.	Taiwan	AB551660	Umehara et al., 2010
<i>A. typica</i>	<i>A. typica</i> A	A	<i>Steno bredanensis</i>	Brazil	FJ161066	Iniguez et al., 2011 (Unpublished)
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Auxis thazard</i>	Indonesia	KC928261	Anshary et al., 2014
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Stegastes apicalis</i>	Australia	JX848665	Jabbar et al., 2012
<i>A. typica</i> *	<i>A. typica</i> B	L3	<i>Grammatorcynus bicarinatus</i>	Australia	JX848663	Jabbar et al., 2012
<i>A. typica</i>	<i>A. typica</i> A	–	<i>Nemipterus hexodon</i>	Thailand	MN420660	Tunya et al., 2020
<i>A. typica</i>	<i>A. typica</i> A	L3	<i>Auxis rochei rochei</i>	Indonesia	EU346091	Palm et al., 2008
Anisakis cf. <i>typica</i>	<i>A. typica</i> A	L3	<i>A. rochei rochei</i>	Indonesia	EU346092	Palm et al., 2008
<i>A. typica</i> var. <i>indonesiensis</i>	<i>A. typica</i> B	L3	<i>A. rochei rochei</i>	Indonesia	EU346093	Palm et al., 2008
<i>A. typica</i> var. <i>indonesiensis</i>	<i>A. typica</i> B	L3	<i>Brama</i> cf. <i>orcini</i>	Indonesia	KY524198	Palm et al., 2017
<i>A. typica</i>	<i>A. typica</i> B	A	<i>Lagenodelphis hosei</i>	Phillippines	KF356673	Quiazon et al., 2020
<i>A. typica</i>	<i>A. typica</i> B	L3	<i>Scomberomorus niphonius</i>	China: South China Sea	KP326555	Zhao et al., 2016
<i>A. typica</i> *	<i>A. typica</i> B	A	<i>Tursiops aduncus</i>	Northern Red Sea	HF911524	Kleinertz et al., 2014
<i>Anisakis</i> sp.	<i>P. ceticola</i>	A	<i>Kogia sima</i>	Phillippines	KC342892	Quiazon et al., 2013
<i>A. paggiae</i>	<i>A. paggiae</i>	L3	–	–	JQ912695	Mattiucci et al., 2014
<i>A. brevispiculata</i>	<i>A. brevispiculata</i>	–	–	–	JQ912694	Mattiucci et al., 2014
<i>A. physeteris</i>	<i>A. physeteris</i>	–	–	–	JQ912693	Mattiucci et al., 2014
<i>A. berlandi</i>	<i>A. berlandi</i>	–	–	–	JX535519	Mattiucci et al., 2014
<i>A. simplex</i> (s.s.)	<i>A. simplex</i> (s.s.)	–	–	–	JX535521	Mattiucci et al., 2014
<i>A. pegreffii</i>	<i>A. pegreffii</i>	–	–	–	JX535520	Mattiucci et al., 2014
<i>A. ziphidarum</i>	<i>A. ziphidarum</i>	–	–	–	JQ912691	Mattiucci et al., 2014
<i>A. nascettii</i>	<i>A. nascettii</i>	–	–	–	JQ912692	Mattiucci et al., 2014

(–: data not reported). The new classification is provided according to the novel results obtained.

substitution model, strict molecular clock (Zuckerandl and Pauling, 1965) and Yule speciation process were used as the tree priors (Gernhard, 2008), as well as other default parameters. For each marker, a run of 10 million iterations was completed, logging parameters every 10,000 iterations, and checked for stationarity and effective sample sizes (ESS) of parameters >200 with Tracer v1.7. (Rambaut et al., 2018). The first 10% of the trees were discarded as burn in, and the remaining 9000 were analyzed and visualized by TreeAnnotator v.1.10.4 (Suchard et al., 2018), and FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>), respectively. *Caenorhabditis elegans* and *Ascaris lumbricoides*, and *A. suum* and *Toxocara canis*, were set as outgroups in the mtDNA *cox2* and ITS rDNA phylogenetic analyses, respectively.

Estimates of nucleotide genetic distance were calculated on both mtDNA *cox2* and ITS rDNA sequences datasets by using the Kimura-2-Parameters (K2P) model (Kimura, 1980), as implemented by MEGAX (Kumar et al., 2018).

2.4. Statistical analysis of the epidemiological data

Quantitative infection assessment of nematode prevalence and abundance, separately for viscera and flesh of the fish, was performed. Quantitative descriptors of anisakid infection, such as prevalence (P, %), mean abundance (A), mean intensity (mI) with standard deviation (\pm SD) and range of infection (min – max) were calculated as defined in Bush et al. (1997).

Spearman rank tests were run to analyse the relationship between fish total length (TL) and parasite abundance, using Statistica® 13.5.0.17 (TIBCO Software Inc., CA, USA).

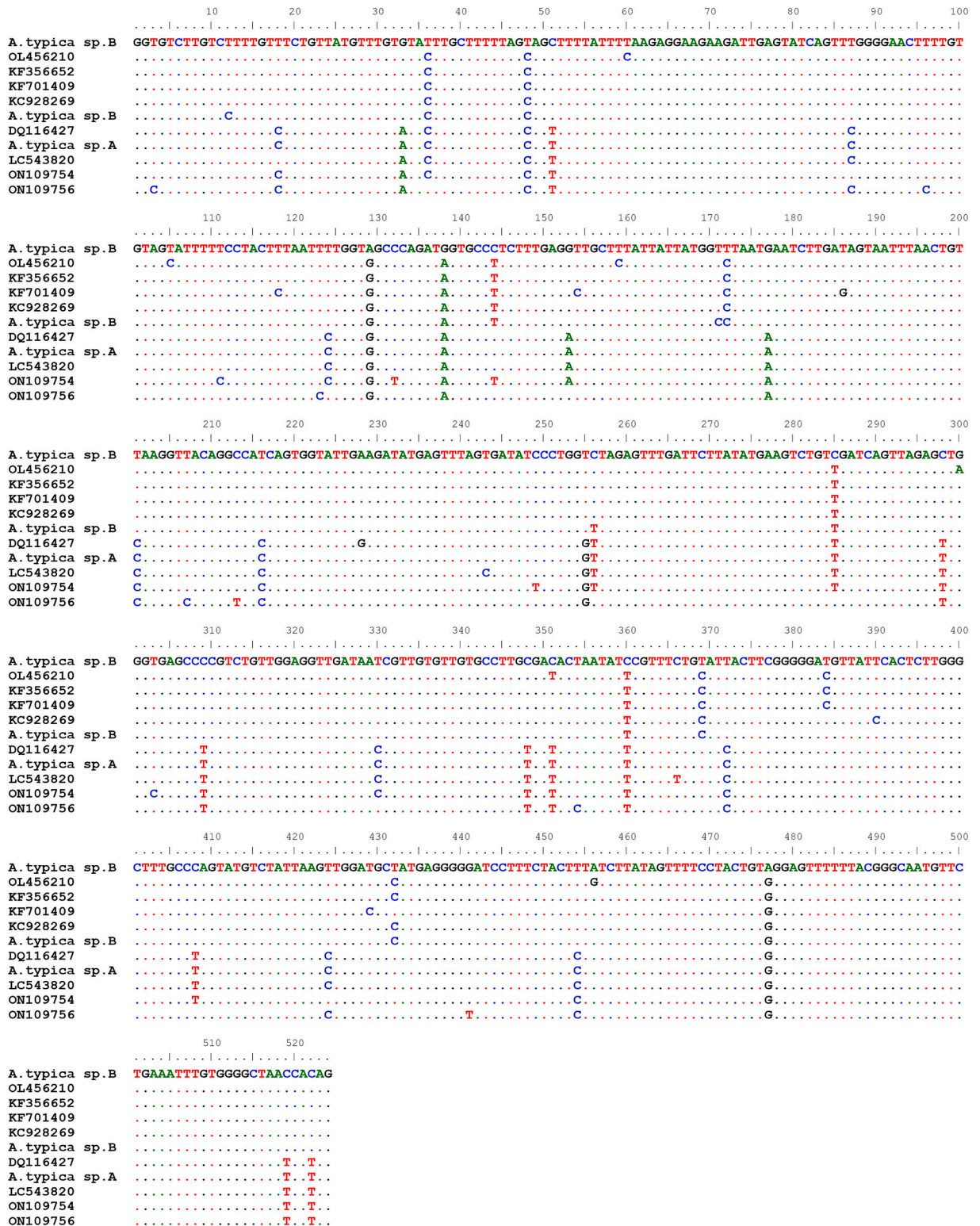
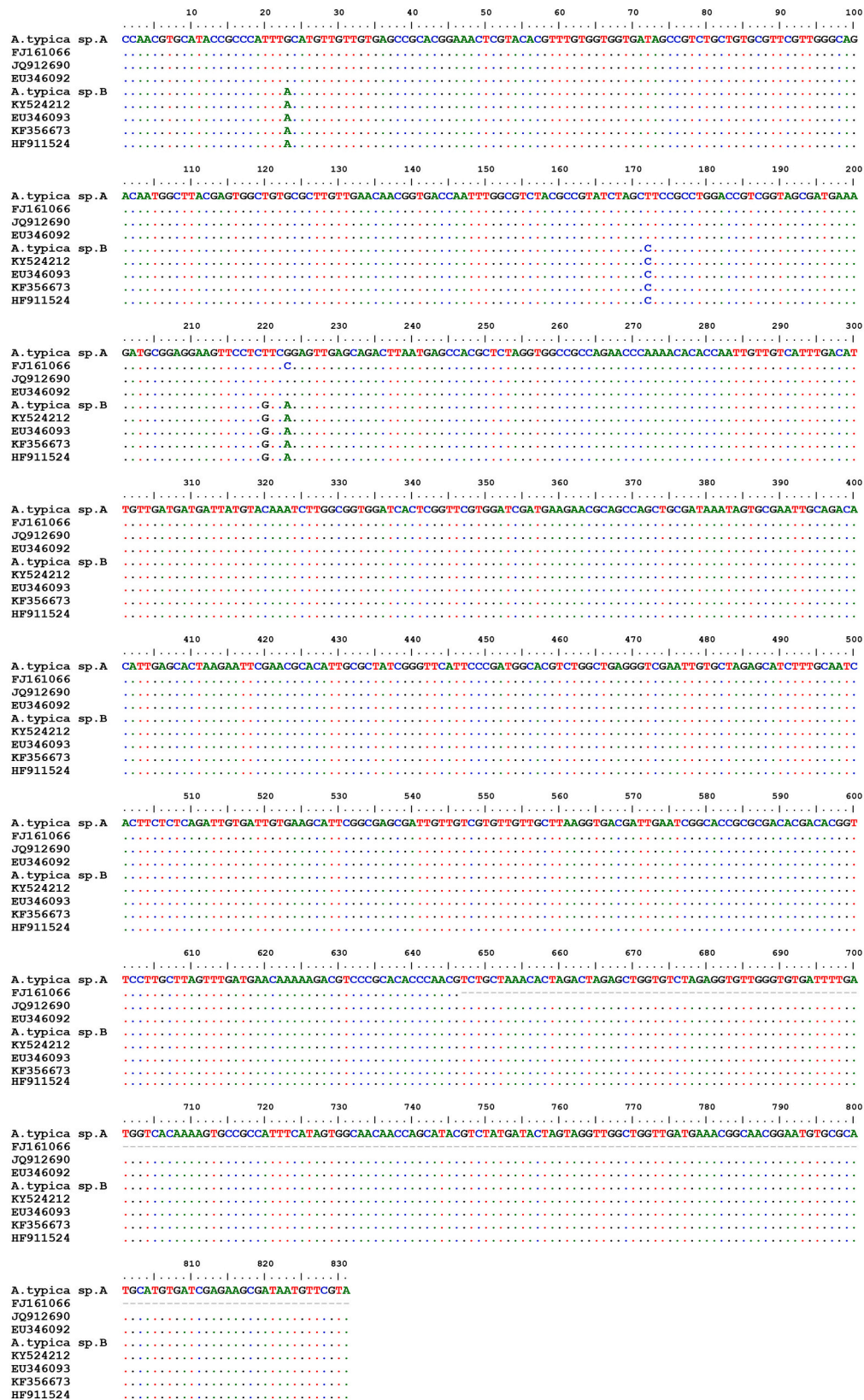


Fig. 2. Alignment performed by Bioedit software (Hall, 1999) for the mtDNA *cox2* gene sequences of *A. typica* sp. A (OP094645), *A. typica* sp. B (OP094650 and OP094651) from the present study, and reference sequences of both taxa retrieved from GenBank (Table 2). Dots indicate identity and dashes indicate gaps.



(caption on next page)

Fig. 3. Alignment performed by Bioedit software (Hall, 1999) for the ITS rDNA gene sequences of *Anisakis typica* sp. A (OP101841), *A. typica* sp. B (OP101846) from the present study, and reference sequences of both taxa retrieved from GenBank (Table 3). Dots indicate identity and dashes indicate gaps.

3. Results

3.1. Parasite species identification

In total, 1151 and 483 anisakid nematodes were detected in 20 *T. lepturus* and 72 *S. undosquamis*, respectively. Out of 168 larval specimens morphologically examined, 166 and 1 were recognized as *Anisakis* third-stage larvae (L3), Type I and Type II, respectively (sensu Berland, 1961). In addition, one single larva infecting *S. undosquamis* presented an intestinal caecum aside the ventriculum, thus resembling *Pseudoterranova* sp.. However, the morphological characterization of this specimen was not sufficiently exhaustive to attribute the specimen to any described larval type.

A total of 168 mtDNA *cox2* (524 bp) sequences and 80 rDNA ITS (830 bp) sequences were successfully obtained from the larval specimens recovered from *T. lepturus* and *S. undosquamis*. Among the 166 mtDNA *cox2* sequences obtained from the *Anisakis* larvae Type I (sensu Berland, 1961), $N = 12$ matched >99% with *A. typica* recorded from *Sotalia fluviatilis* from the Atlantic South American waters (Genbank accession number DQ116427; Valentini et al., 2006) and *Stenella clymene* from Brazil (JQ859923; Di Azevedo et al., 2017), whereas $N = 154$ larvae matched >99% with *A. typica* (s.l.) detected in *Nemipterus japonicus* off the Malaysian coast (OL456210) provisionally indicated as *Anisakis* sp. 1 by Mattiucci and Nascetti (2008), and with those from *Auxis thazard* in Indonesia (KC928269; Anshary et al., 2014) and *Pingjalo lewisi* in Papua New Guinea (JX648323; Koinari et al., 2013). Sequences alignment of the 12 aforementioned mtDNA *cox2* sequences showed 2 fixed alternative nucleotide substitutions (SNPs) in position 124 and 216 with respect to the other 154 sequences (Fig. 2). Out of the $N = 80$ specimens sequenced at the ITS region rDNA, 66 matched >99% with sequences of *A. typica* var. *indonesiensis* from *Priacanthus tayenus* in Indonesia (KY524212; Palm et al., 2017), whereas 12 specimens matched >99% with *A. typica* from *Sotalia fluviatilis* in Brazil (JQ912690; Mattiucci et al., 2014). The first 66 ITS sequences and the other 12 aforementioned sequences differed by 4 fixed alternative nucleotide substitutions (SNPs) in positions 23, 172, 220, 223 (Fig. 3).

In addition, the mtDNA *cox2* sequence of the single *Anisakis* Type II larva isolated from *S. undosquamis* matched >99% with *Anisakis* cf. *paggiae* from *Kogia sima* in the waters off Northeast Brazil (KF693770; Di Azevedo et al., 2017). The rDNA ITS sequence of this specimen matched >99% with *A. paggiae* identified from *Anoplogaster cornuta* in Greenland (GU295973; Klimpel et al., 2011). Finally, a single anisakid larva resembling morphologically a *Pseudoterranova* sp. third-stage larva, matched 98.4% with samples identified as *Pseudoterranova ceticola* from *Kogia breviceps* in the Caribbean Sea (DQ116435; Valentini et al., 2006). The rDNA ITS sequence of this specimen matched 100% with an unidentified *Anisakis* sp. from a *K. sima* stranded off the Pacific coast of southern Philippine archipelago (KC852171; Quiazon et al., 2013).

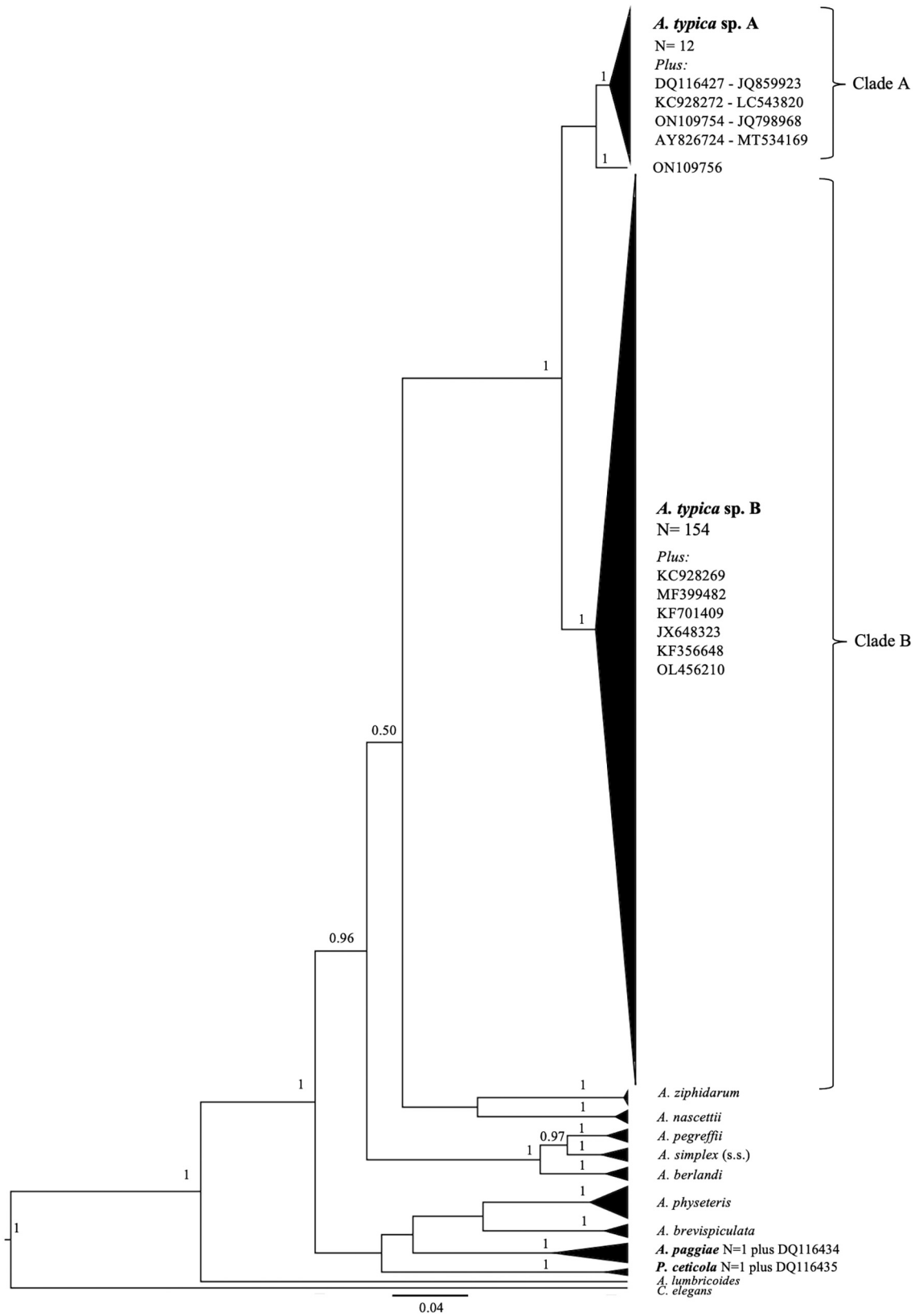
3.2. Phylogenetic analysis and genetic distance

Phylogenetic analysis included 168 *cox2* mtDNA and 80 ITS rDNA sequences generated in this study, plus 24 and 31 reference sequences retrieved from GenBank from each marker, respectively (see Table 2). Phylogenetic trees of similar topology were obtained for both mtDNA *cox2* and ITS rDNA datasets using Bayesian analyses (Figs. 4 and 5). The *cox2* mtDNA phylogenetic analysis indicated that *A. typica* (s.l.) forms a clade comprised of two closely related, but distinct phylogenetic lineages robustly supported by high posterior probability values. The first (clade A) consisted of 12 new nematode sequences obtained in this study from *T. lepturus* and *S. undosquamis* and numerous *A. typica* sequences deposited in GenBank, including *A. typica* adult specimens obtained from type locality (i.e. central West Atlantic Ocean) (Fig. 4). The second phylogenetic lineage (clade B) comprised 154 new nematode sequences obtained from *T. lepturus* and *S. undosquamis*, one sequence of an unidentified *Anisakis* sp. 1 (OL456210), and several sequences of *A. typica* var. *indonesiensis* from different geographic areas of the Indo-Pacific Ocean (see Fig. 4).

The ITS rDNA phylogenetic tree analysis showed a tree topology congruent with the mtDNA *cox2* tree, with the existence of two separated phylogenetic lineages in the clade of *A. typica* (s.l.) (Figs. 4 and 5). The first lineage (clade A) consisted of 12 new nematode sequences obtained in this study from both fish hosts examined and 13 sequences of *A. typica* previously deposited in GenBank, including *A. typica* type locality, such as a sequence from *Sotalia fluviatilis* from SE Atlantic Ocean (JQ912690) (see Fig. 5). The second phylogenetic lineage (clade B) comprised 66 new nematode sequences obtained from *T. lepturus* and *S. undosquamis*, several sequences of *A. typica* var. *indonesiensis* (see Table 3) by Palm et al. (2017), and 7 additional sequences of *A. typica* previously deposited in GenBank (see Table 3 and Fig. 5).

In addition, one sequence obtained at both mtDNA *cox2* and ITS rDNA from the *Anisakis* Type II larva from *S. undosquamis* clustered in the clade including the reference sequences of *A. paggiae* previously deposited in GenBank (respectively DQ116434 and JQ912695) (Figs. 4 and 5, Tables 2 and 3). Finally, the mtDNA *cox2* sequence obtained from the single larva found in *S. undosquamis* resembling a *Pseudoterranova* sp. larva, clustered in another well separate clade with the sequence of *P. ceticola* (DQ116435) (Fig. 4, Table 2). The ITS rDNA sequence of this same specimen clustered instead with a sequence on an unidentified *Anisakis* sp. deposited in GenBank (KC342892; Quiazon et al., 2013) (Fig. 5, Table 3).

Estimates of genetic distance by K2P at mtDNA *cox2* and ITS rDNA between the two *A. typica* lineages and other species so far included in the genus *Anisakis*, are shown in Table 4. In pairwise comparisons, the genetic distance of mtDNA *cox2* and ITS rDNA



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Fig. 4. Bayesian inference (BI) phylogenetic tree based on *cox2* mtDNA sequences dataset including those obtained from specimens analyzed in this study and reference sequences retrieved from GenBank (for detailed information see Table 2). *Ascaris lumbricoides* and *Chaenorhabdites elegans* were set as outgroups.

between *A. typica* and *Anisakis* sp. 1 lineages, were 0.059 and 0.004, respectively. The values were similar or higher than those found between the other sibling species of the *A. simplex* complex (see Table 4). The two distinct phylogenetic lineages, i.e. *A. typica* and *Anisakis* sp. 1 (also corresponding to *A. typica* var. *indonesiensis*), are hereafter referred to as *A. typica* sp. A and *A. typica* sp. B, respectively (Figs. 4 and 5, Tables 2 and 3).

The DNA sequences of the presently identified *Anisakis* species were deposited in GenBank under the following accession numbers: mtDNA *cox2*: OP094645–49 (*A. typica* sp. A); OP094650–54 (*A. typica* sp. B); OP094656 (*A. paggiae*); ON155434 (*P. ceticola*). ITS rDNA: OP101841–45 (*A. typica* sp. A); OP101846–50 (*A. typica* sp. B); OP101852 (*A. paggiae*); ON128286 (*P. ceticola*).

3.3. *Anisakis* infection data

In the two fish species examined, both *A. typica* sp. A and *A. typica* sp. B larvae were detected in viscera, mostly encapsulated outside the internal organs, and in the flesh, mostly in the ventral part of the musculature. *A. typica* sp. A represented 7.2% of larvae genetically identified, while *A. typica* B was 92.8%. *A. typica* sp. A and *A. typica* sp. B larvae were recorded in syntopy, both in viscera and in fish muscle.

In *T. lepturus*, 5.8% of genetically identified *Anisakis* larvae resulted *A. typica* sp. A, while 94.2% were *A. typica* sp. B. The two species were found in syntopy in the visceral organs and in the ventral part of the musculature. In *S. undosquamis*, *A. typica* sp. A represented 14.8% of the molecularly identified larvae, whereas 85.2% were *A. typica* sp. B. However, while *A. typica* sp. A was detected in the visceral organs and in the ventral and dorsal part of the musculature, *A. typica* sp. B was only found in the viscera. The *A. paggiae* and *P. ceticola* larvae were both found in the viscera of a single individual of *S. undosquamis*.

Since only a subsample of the total amount of larvae were identified as *A. typica* sp. A and sp. B, general infection parameters are aggregated and given as *Anisakis typica* (s.l.). Data on infected fish number, prevalence (P), mean abundance (mA), and mean intensity (mI) of *A. typica* (s.l.) larvae at different sites of infection (viscera and flesh) in the two fish species are given in Table 1. The most infected fish species was *T. lepturus* showing 100% overall *A. typica* s.l. prevalence and 57.6 mean abundance. The prevalence of *A. typica* (s.l.), when considering only the musculature of the fish, was $P = 85.0\%$, with a maximum of 31 larvae found in the ventral muscle of a single fish. All *A. typica* (s.l.) larvae were detected in the ventral part of the musculature. Considering the localization of *A. typica* (s.l.) larvae by infection site, 92.2% of the larvae was detected in the viscera, and 7.8% in the ventral section of the musculature.

Saurida undosquamis showed lower infection rates compared to *T. lepturus*. Out of 72 fish examined, 45 were infected with *A. typica* (s.l.), with a prevalence of $P = 62.5\%$. The overall mean abundance was 6.71 (Table 1). The prevalence of *A. typica* (s.l.) larvae, when considering only the musculature of the fish, was $P = 18.0\%$. Larvae were distributed in both the ventral and the dorsal part of the fish musculature, but most of them were found in the ventral part. Mean abundance of *A. typica* (s.l.) in the ventral part of the musculature of *S. undosquamis* was 0.19, with a maximum of 2 larvae found in the ventral fillet of a single fish. In the dorsal part of the muscle mean abundance was 0.06, with a maximum of 3 larvae found in a single fish (Table 1). *A. typica* (s.l.) larvae were detected in the dorsal musculature of 2 (out of 72) specimens of *S. undosquamis*.

Considering *A. typica* (s.l.) larvae distribution by infection site in *S. undosquamis*, 96.3% were located in the viscera, 2.9% in the ventral part of the musculature, and 0.8% in the dorsal part.

A moderate positive and statistically significant correlation was recorded between the abundance of *A. typica* (s.l.) in *T. lepturus* and the fish total length and total weight ($r = 0.46$, $p = 0.04$ and $r = 0.51$, $p = 0.02$, respectively,) whereas for *S. undosquamis* these correlations were statistically significant ($r = 0.51$, $p < 0.001$ and $r = 0.44$, $p < 0.001$, respectively).

4. Discussion

The presence of anisakid nematode parasites in fish and cephalopods is monitored worldwide due to the zoonotic risk associated with certain species within this family, and their repellent appearance which reduces the quality of fish products. Because of the low fish consumption in the Western Indian Ocean region (WIO) (FAO, 2018), epidemiological surveys on parasitic nematodes in fish from this area are scarce, and the anisakid nematode fauna is still poorly known.

The present study presents molecular epidemiological data on anisakid nematodes in two new potential local fisheries in the WIO region, namely large head hairtail (*T. lepturus*) and brushtooth lizardfish (*S. undosquamis*).

4.1. Genetic identification of the anisakid nematodes

The taxonomy of *A. typica* is still largely unresolved. Previous studies that indicated the possible existence of two distinct taxa within *A. typica* (s.l.) from the Indo-Pacific area were based on allozymes (Mattiucci and Nascetti, 2008), on the ITS region rDNA (Palm et al., 2008, 2017), or mtDNA *cox2* sequence analysis (Eamsobhana et al., 2018; Mattiucci et al., 2018). The present study, based on a comparatively higher number of specimens analyzed at both the mitochondrial *cox2* gene locus and nuclear ribosomal ITS region, strongly supports the existence of a genetic heterogeneity within the nominal morphospecies *A. typica* (s.l.), likely representing a

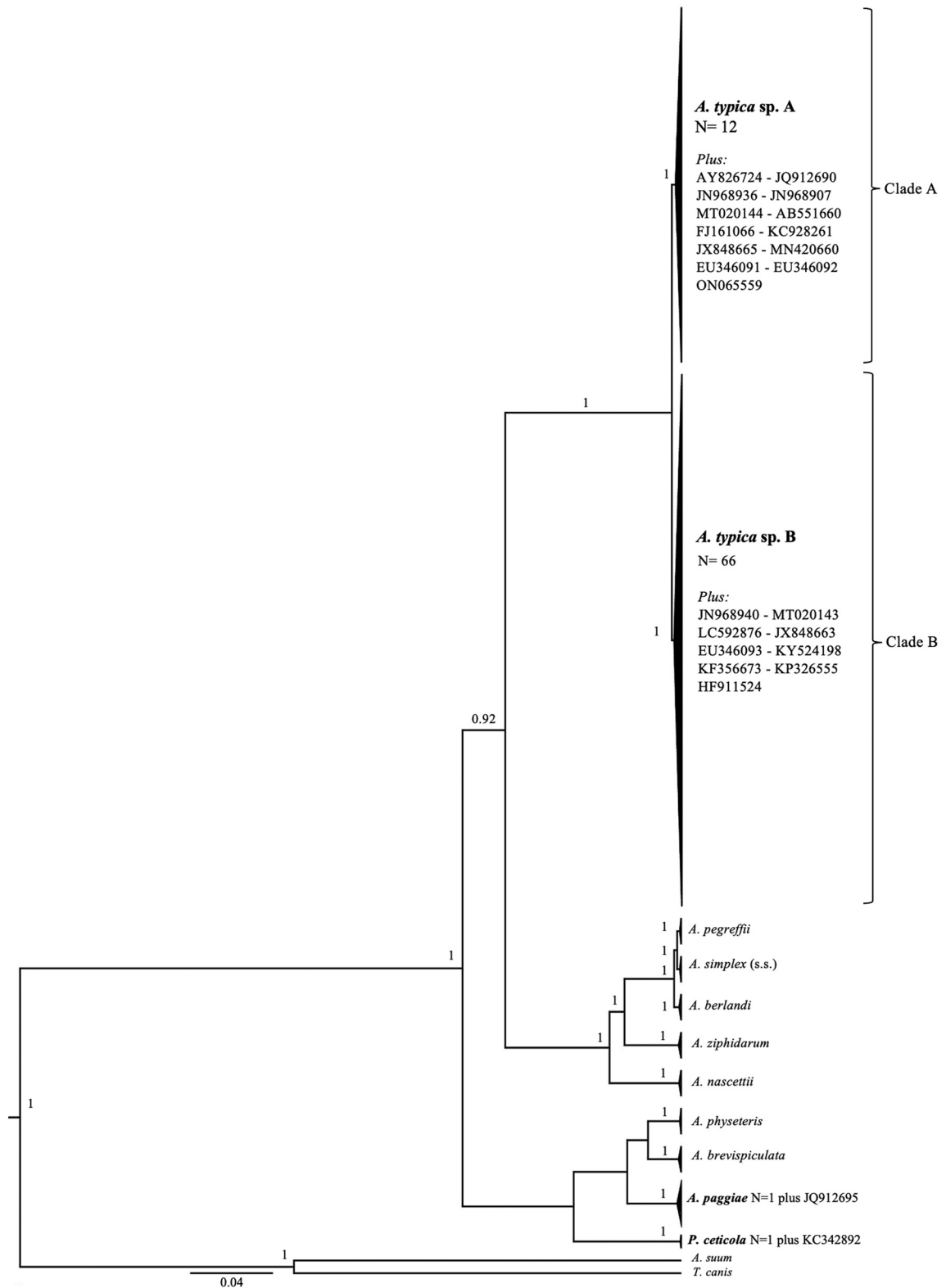


Fig. 5. Bayesian inference (BI) phylogenetic tree based on ITS rDNA sequences dataset including those obtained from specimens analyzed in this study and reference sequences retrieved from GenBank (for detailed information see [Table 3](#)). *Ascaris suum* and *Toxocara canis* were set as outgroups.

Table 4

K2P genetic distance values between *Anisakis typica* sp. A, *A. typica* sp. B, and other *Anisakis* species, based on the mtDNA *cox2* (a) and ITS r DNA (b) sequences.

a)		cox2 mtDNA									
		1	2	3	4	5	6	7	8	9	10
1	<i>A. pegreffii</i>		0.0050	0.0098	0.0172	0.0165	0.0195	0.0171	0.0168	0.0153	0.0168
2	<i>A. simplex</i> (s.s.)	0.0272		0.0101	0.0180	0.0174	0.0202	0.0182	0.0177	0.0158	0.0175
3	<i>A. berlandi</i>	0.0617	0.0591		0.0169	0.0170	0.0194	0.0175	0.0172	0.0165	0.0170
4	<i>A. physeteris</i>	0.1433	0.1424	0.1406		0.0167	0.0154	0.0191	0.0185	0.0173	0.0193
5	<i>A. paggiae</i>	0.1458	0.1487	0.1495	0.1362		0.0157	0.0200	0.0196	0.0153	0.0172
6	<i>A. brevispiculata</i>	0.1711	0.1709	0.1697	0.1153	0.1255		0.0229	0.0216	0.0175	0.0205
7	<i>A. typica</i> sp. A	0.1457	0.1483	0.1432	0.1579	0.1777	0.1969		0.0102	0.0183	0.0168
8	<i>A. typica</i> sp. B	0.1442	0.1461	0.1406	0.1575	0.1798	0.1951	0.0588		0.0176	0.0166
9	<i>A. zypthidarum</i>	0.1276	0.1238	0.1349	0.1423	0.1267	0.1418	0.1572	0.1578		0.0142
10	<i>A. nascettii</i>	0.1459	0.1489	0.1443	0.1678	0.1530	0.1783	0.1385	0.1493	0.1014	

b)		ITS rDNA									
		1	2	3	4	5	6	7	8	9	10
1	<i>A. pegreffii</i>		0.0017	0.0024	0.0166	0.0162	0.0165	0.0153	0.0151	0.0086	0.0105
2	<i>A. simplex</i> (s.s.)	0.0024		0.0025	0.0167	0.0164	0.0162	0.0153	0.0151	0.0086	0.0105
3	<i>A. berlandi</i>	0.0056	0.0056		0.0166	0.0164	0.0164	0.0153	0.0151	0.0086	0.0108
4	<i>A. physeteris</i>	0.1917	0.1933	0.1946		0.0095	0.0079	0.2013	0.2045	0.0156	0.0170
5	<i>A. paggiae</i>	0.1848	0.1864	0.1894	0.0660		0.0088	0.1869	0.1904	0.0151	0.0163
6	<i>A. brevispiculata</i>	0.1844	0.1826	0.1855	0.0458	0.0504		0.1981	0.2012	0.0161	0.0174
7	<i>A. typica</i> sp. A	0.1561	0.1544	0.1534	0.0181	0.0173	0.0186		0.0022	0.0147	0.0162
8	<i>A. typica</i> sp. B	0.1568	0.1551	0.1541	0.0184	0.0176	0.0189	0.0045		0.0149	0.0161
9	<i>A. zypthidarum</i>	0.0539	0.0552	0.0543	0.1758	0.1621	0.1727	0.1355	0.1389		0.0687
10	<i>A. nascettii</i>	0.0838	0.0852	0.0890	0.1893	0.1832	0.1926	0.1572	0.1582	0.0097	

Each taxon included all the *Anisakis* corresponding sequences shown in the trees (see Figs. 2 and 3). The number of base substitutions per site from averaging over all sequence pairs between groups (below the diagonal) are shown with standard error estimate(s) (above the diagonal).

complex of two distinct sister species, indicated as *A. typica* sp. A and *A. typica* sp. B (Figs. 4 and 5). In fact, the presence of the two closely related, but clearly distinct, phylogenetic taxa in sympatry in the same hosts, even at their larval stages, suggests absence of gene flow between them, likely as a result of reproductive isolation in the current geographic area.

Differential nucleotide substitutions in the two taxa *A. typica* sp. A and *A. typica* sp. B seem to support this view. In the 80 ITS rDNA sequences analyzed in this study, the two taxa showed 4 fixed distinctive nucleotide substitutions at four positions (Fig. 3), confirming the findings reported by Palm et al. (2008, 2017) and Anshary et al. (2014). No heterozygote genotypes were presently observed at that locus in this sample. The mtDNA *cox2* sequences alignments, performed on all the specimens presently obtained, showed only 2 fixed nucleotide substitutions (SNPs) diagnostic between *A. typica* sp. A and *A. typica* sp. B (Fig. 2). It is worth to notice that two diagnostic nucleotide substitutions at the mtDNA *cox2* locus are even existing between the two closely related species *A. simplex* (s.s.) and *A. pegreffii* (Mattiucci et al., 2014, 2016).

The nucleotide genetic distance (K2P) calculated in pairwise comparisons between *A. typica* sp. A and *A. typica* sp. B at mtDNA *cox2* and ITS rDNA was 0.059 and 0.004, respectively. Similar K2P values were also obtained between sibling species of the *A. simplex* (s.l.) complex, e.g., between *A. pegreffii* and *A. berlandi* (0.062 ± 0.006) or *A. simplex* (s.s.) and *A. pegreffii* (0.027 ± 0.002) at the mtDNA *cox2* and ITS rDNA loci, respectively (see Table 4). Therefore, it can be concluded that K2P distance values between *A. typica* sp. A (ex *A. typica*) and *A. typica* sp. B (ex *Anisakis* sp.1, or *A. typica* var. *indonesiensis*) is assessed at interspecific level, providing further evidence that these taxa likely represent two distinct sister species, belonging to the *A. typica* (s.l.) species complex.

Anisakis typica was originally described from *Delphinus delphis* in the Central Atlantic Ocean. Since then, numerous records of *A. typica* (s.l.) have been reported in temperate waters of Atlantic and Indo-Pacific oceans, often under different names or as geographic variants. The first detection of the undescribed species related to *A. typica*, initially considered a sister species, and indicated as *Anisakis* sp. 1, was provided by allozyme analysis of several larval specimens collected from *Nemipterus japonicus* caught off the Malaysian coast (Mattiucci and Nascetti, 2008). Although not fully described as a new species, other findings of this taxon were probably reported, but they have never been associated to the first report of this species. Palm et al. (2008, 2017) described a “new genotype” named “*A. typica* var. *indonesiensis*”, as a “genetic variant” of the *A. typica*, obtained in several fish species from Indonesian waters. However, “*A. typica* var. *indonesiensis*” most likely refers to the same taxon previously characterized as *Anisakis* sp. 1 and herein named *A. typica* sp. B. In this study, the match between the two taxa was demonstrated and the name *A. typica* sp. B is retained instead of *Anisakis* sp.1 and *A. typica* var. *indonesiensis*, for taxonomical reason. The term “variant” in particular, as defined by the International Code of Zoological Nomenclature (Ferraris and Eschmeyer, 2000), identifies a “[...] taxa below the rank of subspecies which groups specimen(s) within a species differing from other specimens in consequence of intrapopulation variability [...]” (Art. 45.6.3 “Determination of subspecific or infrasubspecific rank of names following a binomen”). In this study, we showed that the two gene pools included in the clade of *A. typica* likely correspond to distinct species, rather than populations. Considering these aspects, the term “variant” should not be used to

identify this taxon (i.e., *A. typica* var. *indonesiensis*). Therefore, it is recommended that *A. typica* sp. B is henceforth used unambiguously to define the distinct lineage in close sister relationship with *A. typica* sp. A. However, the nomenclature designation based on adult specimens is needed for the taxon indicated as *A. typica* sp. B, with the formal description of this provisional taxon, and its differential diagnosis based on morphological features with respect to *A. typica* sp. A. In this respect, it would be diriment to investigate adult stages of both taxa in their definitive hosts, as was attempted by Quiazon et al. (2020) from a stranded specimen of Fraser's dolphin, *Lagenodelphis hosei*, from central Philippine waters. Unfortunately, the authors could only obtain fragmented or degraded adult stage worms, which did not allow to perform a full morphological description. Hence, based solely on genetic sequence analysis, these worms were identified as *A. typica* (s.l.) (Quiazon et al., 2020). Interestingly, these specimen sequences (*cox2*, KF356648; ITS, KF356673; Quiazon et al., 2020), clustered with *A. typica* sp. B (clade B) in both phylogenetic trees inferred by mtDNA *cox2* and ITS sequences analysis.

The newly recognized *A. typica* sp. B was previously identified as *A. typica* from Red Sea (Kleinertz et al., 2014), Papua New Guinea (Koinari et al., 2013), Indonesia (Kuhn et al., 2013) and South Pacific Ocean (Jabbar et al., 2012; Shamsi et al., 2018). In particular, Jabbar et al. (2012) identified three genotypes (namely A, B and C) of *A. typica* in the visceral organ of various fish hosts in the South Pacific Ocean, off North-eastern Australia. Phylogenetic analyses based on the ITS-1 rDNA sequence data, showed that the genotypes B and C clustered with sequences of *A. typica* previously reported from China and Indonesia (Kuhn et al., 2011; Palm et al., 2008; Zhang et al., 2007), whereas the genotype A clustered with sequences of *A. typica* collected from other geographic areas. Interestingly, we found that the genotypes A and B, and the genotype C, clustered within *A. typica* sp. B and *A. typica* sp. A, respectively (see Figs. 4 and 5), thus indicating that they represent distinct species rather than genotypes. This finding suggests that the sympatric area of the two siblings *A. typica* also includes the South Pacific Ocean, off North-eastern Australia.

In the same broad geographic area, Shamsi et al. (2015) reported the occurrence of *A. typica* larvae in several fish species, including *T. lepturus*. However, these specimens were identified only based on the ITS-2 region of rDNA, which does not include the diagnostic positions distinguishing between the two *A. typica* species. More recently, Shamsi et al. (2018) identified larvae of *A. typica* in fish from New Caledonian waters, using the ITS-1 and the ITS-2 rDNA loci. The alignment of the ITS-1 sequences of these specimens (MH190354–62) with our sequence dataset revealed that they belong to *A. typica* sp. B, thus widening the range of distribution of this species to New Caledonia.

Interestingly, one single *A. typica* retrieved from GenBank (ON109756) found in *S. crumenophthalmus* from the Bay of Bengal (Bao et al., 2022), seemed to cluster separately from the two existing branches, with a strong posterior probability support (Fig. 4). According to Bao et al. (2022), this specimen showed several differences once aligned and compared to other *A. typica* (s.l.) specimens, suggesting that it would represent another genetically distinct taxon belonging to the *A. typica* (s.l.). In fact, the mtDNA *cox2* sequence alignment showed a mismatch in one of the two fixed nucleotide substitutions of this specimen (ON109756) versus *A. typica* sp. A and *A. typica* sp. B (see Fig. 2). Considering the geographic location of the finding, in proximity to the Bangladesh river system estuaries, it could be hypothesized that this genotype may belong to a third distinct taxon related to estuarine/river realm.

Beside the two taxa within the *A. typica* (s.l.) species complex described above, two other *Anisakis* species have been detected in the viscera of *S. undosquamis*: a type II larva, genetically identified as *A. paggiae* (Fig. 4 and Fig. 5), and a specimen morphologically showing a short ventriculus and a small intestinal caecum, belonging to *P. ceticola*. The latter larva matches the recent description of *P. ceticola* L3 from meso/bathypelagic fishes from off Macaronesia waters (NW Africa) (Bao et al., submitted). Indeed, the mtDNA *cox2* sequence of the latter specimen showed 98.4% match with a sequence of *P. ceticola* obtained from a *K. breviceps* stranded in the Caribbean Sea (DQ116435), also clusters together with *P. ceticola* larvae from meso/bathypelagic fish (Bao et al., submitted). On the other hand, the ITS rDNA sequence of the current specimen had 100% identity with unidentified preadult worm, namely *Anisakis* sp. (KC852171) (Quiazon et al., 2013), from a *K. sima* stranded off the Pacific coast of southern Philippine archipelago. The congruence of the clade position at both loci strongly suggests that the specimen belongs to *P. ceticola*, as probably did the sample isolated by Quiazon et al. (2013) named *Anisakis* sp.. The phylogenetic analyses based on mtDNA *cox2* and ITS rDNA (Figs. 4 and 5) indicates that *P. ceticola* clusters in the clade comprising *Anisakis* Type II species (e.g. *A. physeteris*, *A. paggiae* and *A. brevispiculata*). The nesting of *P. ceticola* in this clade was previously reported by Cavallero et al. (2011), and recently by Bao et al. (submitted). Surely *P. ceticola* represents a still poorly studied species, whose phylogenetic position, and adult morphology are yet to be properly described.

The occurrence of *A. paggiae* and *P. ceticola* in *S. undosquamis*, even if at relatively low prevalence, represents a finding of ecological interest. Both parasite species are mostly related to kogiid whales (definitive host) (Di Azevedo et al., 2015; Mattiucci et al., 2018; Shamsi et al., 2019). Probably *S. undosquamis* has more demersal trophic habits compared to *T. lepturus* (where only *A. typica* sp. A and B larvae have been identified), preying upon some deep dwelling organisms that may represent the first intermediate/paratenic hosts (such as meso/bathypelagic, benthopelagic or reef-associated fishes (see Bao et al., submitted)) for these rarely found *Anisakis* and *Pseudoterranova* species. In fact, *S. undosquamis* is predominantly piscivorous (Golani, 1993), but also feeds on shrimps and other bottom dwelling invertebrates (Kadharsha et al., 2013; Rajkumar et al., 2003).

4.2. Geographic distribution of *A. typica* (s. l.)

According to the biogeographical data available in literature for *A. typica* (s.l.), this complex of species is generally distributed in the temperate waters of tropical latitudes. The two newly recognized taxa show different macro-biogeographical distribution. The *A. typica* sp. A taxon has apparently a worldwide distribution, being detected genetically in temperate waters of East Atlantic Ocean (Caribbean area, off the coasts of Florida, Brazil and northern Argentine Sea) (Borges et al., 2012; Cavallero et al., 2011; Iniguez et al., 2011; Irigoitia et al., 2021; Lanfranchi et al., 2018), in the Western Atlantic Ocean (from the North African coasts up to waters off Portugal) (Hermida et al., 2011; Marques et al., 2006; Mattiucci et al., 2002, 2014), in the Mediterranean Sea (Farjallah et al., 2008), in the

Central Pacific Ocean around Hawaii (Kuhn et al., 2013), in the Pacific Ocean waters off Japan (Takano et al., 2021), in the Gulf of Bangladesh (Bao et al., 2022) and, in sympatry with *A. typica* sp. B (ex *Anisakis* sp.1), in several areas of the Indo-Pacific waters (Anshary et al., 2014; Kuhn et al., 2013; Palm et al., 2008, 2017) and off Northeastern Australia (Jabbar et al., 2012). Instead, the *A. typica* sp. B taxon seems to retain a narrower distribution, from the Red Sea and the Eastern African coasts to the Northeast Australian waters, off New Caledonia, in the whole Indo-Pacific temperate waters, always occurring in sympatry with *A. typica* sp. A (Anshary et al., 2014; Eamsobhana et al., 2018; Kleinertz et al., 2014; Kuhn et al., 2013; Jabbar et al., 2012; Palm et al., 2008, 2017; Shamsi et al., 2018).

4.3. Parasites infection data and seafood quality aspects

The results obtained in this study offer unprecedented evidence that both *A. typica* sp. A and *A. typica* sp. B can migrate into the musculature of *T. lepturus* and *S. undosquamis*, and that the migration within the host occurs during the life of fish (i.e., *intra-vitam* migration). In fact, the current fish have been examined immediately after the catch, thus preventing any eventual *post-mortem* migration of *Anisakis* larvae (Cipriani et al., 2016). *Intra-vitam* flesh migration has been described for other *Anisakis* species in several fishes (Cipriani et al., 2014, 2018; Karl, 2008; Karl et al., 2002; Quiazon et al., 2011; Smith, 1984). So far, the presence of *A. typica* sp. B (ex *A. typica* sp.1 or var. *indonesiensis*) in the musculature of certain fish hosts was considered accidental, and only sporadic cases were reported (Palm et al., 2008, 2017; Anshary et al., 2014). For instance, Palm et al. (2008) found a single *A. typica* (s.l.) larva in the musculature of a large batch of *Auxis rochei* and another single specimen in the muscle tissue of *Selar crumenophthalmus* (Palm et al., 2017). Koinari et al., 2013 reported the occurrence of some *A. typica* (s.l.) larvae from the musculature of *Pinjalo pinjalo* from Papua New Guinea, whereas Anshary et al. (2014), found a single larva in the fish musculature and speculated that a potential risk of anisakiasis may occur in connection with the consumption of this fish species. The sporadic and lower infection levels of *A. typica* (s.l.) observed in aforementioned studies compared to our current findings can be explained by the differential detection efficiencies of the inspection methods adopted. The visual inspection and candling method for nematode detection used by Palm et al. (2008, 2017), Koinari et al. (2013) and Anshary et al. (2014), are considered less accurate than UV-press (Levsen et al., 2005). In particular, these methods can significantly underestimate the total burden of anisakid larvae possibly present in the edible part of the fish, i.e. the flesh (Levsen et al., 2005). Thanks to the proven efficiency of the UV-press method (Gómez-Morales et al., 2018), recently approved as international standard method (ISO 23036-1:2021), it is now possible to accurately assess the occurrence of larval anisakid nematodes in the flesh of fish and cephalopods (Gómez-Morales et al., 2018; Levsen et al., 2018), thus the method could be implemented by food businesses in Hazard Analysis and Critical Control Points (HACCP) plans to assess the presence of potentially zoonotic parasites in the edible part of the fish product.

All presently found *A. typica* sp. A and *A. typica* sp. B larvae were residing in the epaxial muscle section of *T. lepturus*, mostly in the anterior part which surrounds the visceral organs and largely comprise the belly flaps. This apparently preferred site within the fish muscle is known from other species as well, e.g., *A. pegreffii* and *A. simplex* (s. s.). Larvae of these parasite species have been systematically reported in the muscle from several fish and squid species (Aco Alburquerque et al., 2020; Bušelić et al., 2018; Cipriani et al., 2018, 2021; Levsen et al., 2018; Pascual et al., 2018; Santos et al., 2022), which so far appear to be the only *Anisakis* species capable to migrate into the muscle or mantle of their hosts, both *intra-vitam* and *post-mortem* (Cipriani et al., 2016; Mattiucci et al., 2018). A similar pattern of distribution of *Anisakis* larvae in muscle was observed in another Trichiuridae, *Lepidopus caudatus*, sharing great ecological affinities with *T. lepturus*. In a batch of heavily infected *L. caudatus* sampled in Mediterranean Sea, most of *A. pegreffii* larvae were located in the viscera, while only 6.4% were detected in the ventral musculature (Levsen et al., 2018; Cipriani pers. obs). Similarly, we reported a prevalence of 7.8% of *A. typica* (s.l.) larvae from the ventral section of the musculature of *T. lepturus*.

In *S. undosquamis*, only a few *A. typica* sp. B larvae were detected in both ventral and dorsal parts of the muscular tissue, while a few *A. typica* sp. A larvae were observed in the viscera. These findings further confirm that *intra-vitam* migration of *A. typica* sp. B occurs systematically in different fish hosts and that some larvae can even reach the dorsal musculature.

The infection levels, when combining the two taxa as *A. typica* (s.l.), showed a clear correlation of prevalence and intensity with fish length and weight. The prevalence of infection in *T. lepturus* was 100%, with all 20 examined fish infected, while 17 fish ($P = 85.0\%$) were carrying larvae in the musculature. In some larger specimens of *T. lepturus* the highest number of larvae per fish was recorded, with a specimen measuring a total length of 168 cm harbouring 269 larvae, of which 31 (11.5%) were located in the epaxial portion of the fish musculature. These data seem to be higher when compared to previous epidemiological studies conducted on the same fish species from several other geographic areas. Considering the Indo-Pacific region, Palm et al. (2017) reported low prevalence of infection (2.9%) of *A. typica* in 35 *T. lepturus*, with a single larva detected over the intestine of a fish. A survey on 22 *T. lepturus* from Northern Taiwan reported 100% prevalence and mean intensity of 49 larvae per fish, mostly represented by *A. pegreffii*, and just 2.4% by *A. typica* (Sonko et al., 2019). In 20 specimens of *T. lepturus* caught in the waters off Southern Vietnam, a prevalence of 30% by *A. typica* was recorded, with a maximum of 5 larvae detected in the viscera of a single fish (Van Hien et al., 2021). Additionally, Umehara et al., 2010 reported the presence of *A. typica* in 6 out of 7 *Trichiurus* spp. from Taiwan, in syntopy with *A. pegreffii*. Recently, Bao et al. (2022) reported low prevalence (10%) of *A. typica* in 38 *T. lepturus* inspected by UV-press from waters off Bangladesh.

In the Atlantic area, Borges et al. (2012) examined by visual inspection 64 specimens of *T. lepturus* caught off the coasts of Rio de Janeiro (Brazil) and found low infection levels of *A. typica* with a prevalence of 20% and a maximum of 10 larvae recorded in the viscera of a single fish. If compared to *T. lepturus*, *S. undosquamis* showed lower infection rates of *A. typica* (s.l.).

The presence of both *A. typica* sp. A and *A. typica* sp. B larvae in the edible part of fish musculature of *T. lepturus* and *S. undosquamis*, raises concern about the zoonotic risk associated with these parasites. Moreover, the repellent appearance of nematodes in fish fillets may also reduce the economic value of the product. So far, anisakiasis cases in humans have only been associated with consumption of

raw or poorly cooked fish infected with *A. simplex* (s. s.) and *A. pegreffii* (Arai et al., 2014; D'Amelio et al., 1999; Fumarola et al., 2009; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2017; Mladineo et al., 2016; Umehara et al., 2007), and the zoonotic risk has been demonstrated only for these two species. Prior to this study, according to the rare findings in fish muscles of *A. typica* (s.l.) larvae, Palm et al. (2008) stated that the risk of human infection by *A. typica* is rather low. However, considering the results obtained here, the apparently low risk associated with *A. typica* cannot be neglected and may depend upon prevention methods (i.e., proper cooking or freezing treatments) applied to the fillets before consumption. Although *A. typica* (s.l.) larvae can actively migrate into the flesh of their fish hosts, there may be numerous reasons explaining the absence of reliable reported cases of anisakiasis caused by fish parasitized with this species. So far, cases of human anisakiasis have been not properly diagnosed and reported in the regions where these species are mostly distributed. Moreover, local fish preparation and food consumption habits could play a crucial role in preventing the health risk to the consumer. According to Daschner and Cuellar (2020), fish preparation and culinary habits represents indeed the main risk factor when dealing with anisakiasis. Palm et al. (2017) suggests that *A. typica* (s.l.) specimens could regularly be ingested by consumers, but they seem to cause no serious disease symptoms. According to Shamsi et al. (2015) no cases of human anisakiasis have been reported in New Caledonia, although the consumption of raw fish is common in the population. However, positive seroprevalence for anisakiasis in humans have been reported in Indonesia (Anshary et al., 2014; Uga et al., 1996), but the etiological agents possibly responsible for these cases were not identified. Recently, using SYBER green-based real-time PCR (targeting the ITS-1 region of rDNA), Najjari et al. (2022) recently investigated the occurrence of *Anisakis* DNA in paraffine embedded biopsies blocks from hospitalized patients with gastritis in Iran. The authors reported the existence of 3 positive Anisakidae DNA matches. Considering the geographic location of these cases (Iran, Persian Gulf area) and that most likely the patients have acquired the parasite by consumption of "local" seafood, it can not be excluded that the agent responsible for the gastritis was *A. typica* (s.l.). Despite these reports, data concerning the occurrence of human anisakiasis from those geographic areas where *A. typica* has been found infecting natural hosts needs further studies to evaluate the zoonotic potential of these neglected parasite species.

It is worth mentioning that infections with *A. typica* in commercial fish species have also been reported from southern Japan and Taiwan (Umehara et al., 2010) where the habit of eating raw or lightly cooked fish is part of the daily diet (Chen and Shih, 2015; Shih et al., 2010) and where cases of human anisakiasis are recorded every year (Suzuki et al., 2021). Most of these cases seem to be linked to *A. simplex* (s. s.) and *A. pegreffii*, and, apparently, no cases of anisakiasis genetically referred to *A. typica* have been reported so far.

5. Conclusion

The present study suggests that the nominal morphospecies *A. typica* (s.l.) likely represents a complex of sister species. Further taxonomic studies based on nuclear markers and morphology, with a direct comparison of adult specimens of the two taxa could eventually lead to a formal description and nomenclature designation of the species provisionally indicated as *A. typica* sp. A and *A. typica* sp. B. The occurrence of these taxa in sympatry corroborates the hypothesis that they are not conspecific.

Further, the results obtained in this study provides first evidence that both *A. typica* sp. A (ex *A. typica*) and *A. typica* sp. B (*Anisakis* sp. 1 and *A. typica* var. *indonesiensis*) can migrate (with subsequent encapsulation) in the musculature of their fish hosts *T. lepturus* and *S. undosquauamis*, and that migration occurs *intra-vitam*. Besides the two taxa herein reported, only two *Anisakis* species (i.e. *A. simplex* (s.s.) and *A. pegreffii*) have been reported from the musculature of various fish and squid hosts.

The ability of both *A. typica* taxa to migrate into the flesh may represent a possible zoonotic risk for consumers when eating raw or undercooked infected fish, although cases of anisakiasis with *A. typica* have not yet been reported worldwide.

More epidemiological data accomplished with updated and efficient detection methods are needed to evaluate the extent of muscle invasion capability by *A. typica* (s.l.) larvae in other commercially important fish species, mostly in areas where direct consumption of fish in raw or marinated preparation is common.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This paper uses data collected through the scientific surveys with the R/V Fridtjof Nansen as part of the collaboration between the EAF-Nansen Programme and Tanzania, where the survey took place. The EAF-Nansen Programme is a partnership between the Food and Agriculture Organization of the United Nations (FAO), the Norwegian Agency for Development Cooperation (NORAD), and the Institute of Marine Research (IMR), Norway, for sustainable management of the fisheries of partner countries. M. Palomba and P. Cipriani (partially) were funded by Italian MUR-PON grants.

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