Genetic differentiation of *Anisakis* species (Nematoda: Anisakidae) in marine fish *Priacanthus tayenus* from Gulf of Thailand

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Abstract. Members of the genus *Anisakis* are parasitic nematodes of the family Anisakidae. They are potential zoonotic parasites, causing anisakiasis in humans who consume raw or undercooked seafood (fish or squid) infected with the larvae of this nematode. In the present study, anisakid nematodes collected from the marine fish *Priacanthus tayenus* (Purple-spotted big-eye) caught from the Gulf of Thailand were examined morphologically and characterized genetically by DNA sequence analysis. Sequence data from the mitochondrial cytochrome *c* oxidase subunit II (mtDNA *cox2*) gene were used to identify these nematodes to species level and to evaluate the phylogenetic relationship among various taxa. All the 15 third-stage larvae of *Anisakis* nematodes investigated in this study belonged to the same genetic lineage as the *A. typica* species complex (named here as *A. typica* sp. T – T for Thailand). Eight mtDNA *cox2* haplotypes were revealed in the 15 isolates of this *Anisakis* from Thailand. The mtDNA *cox2* haplotypes of *A. typica* sp. T from Thailand were genetically distinct from those of the *A. typica* sensu stricto. Taxonomic description of this *A. typica* sp. T as a distinct species however awaits the availability of adult specimens.

INTRODUCTION

Human anisakiasis is an important fish-borne zoonotic disease caused primarily by nematode larvae of the genus *Anisakis* Dujardin, 1845 and less commonly, of the genus *Pseudoterranova* Mozgovoi, 1951 (Hochberg & Hamer, 2010; Mattiucci *et al.*, 2017). Both genera are included in the family Anisakidae. Members of the genus *Anisakis* are characterized by marked differences in their genetic structure and ecological traits (Mattiucci *et al.*, 2009).

The life cycle of anisakid nematodes involves small crustaceans and various marine fish as intermediate/paratenic hosts, marine mammals as final definitive host, whereas humans are the accidental host

(Klimpel & Palm, 2011; Mattiucci & Nascetti, 2008). In humans, the infective anisakid larvae produce severe gastroenteritis and/or allergic reactions following the consumption of raw or undercooked parasitized fish (Sakanari & McKerrow, 1989; Audicana & Kennedy, 2008; Dorny *et al.*, 2009).

The taxonomy of anisakid fish nematodes has been substantially redefined in recent decades using allozyme and DNA-based methods (D'Amelio *et al.*, 2000; Mattiucci & Nascetti, 2006; Mattiucci *et al.*, 2002, 2004, 2005, 2008, 2009, 2014, 2017; Valentini *et al.*, 2006). Based on the combined nuclear ITS rDNA, mitochondrial *cox2* and *rrnS* sequences, *A. simplex* sensu stricto (s.s.) formed a sister group with *A. pegreffii* in the lineage which also contained the *A.*

berlandi (= *A. simplex* sp. C), whereas *A. typica* is basal to the other members of the genus *Anisakis* (Mattiucci *et al.*, 2014).

In recent years, the risk of acquiring anisakid infections through the consumption of raw or undercooked seafood has increased as a result of the growing globalization of food industry as well as the eating habits (Murrell, 2002). To date, it is estimated that more than 20,000 human anisakiasis cases have been recorded worldwide; most of the cases were diagnosed in Japan and followed by Spain (Madrid *et al.*, 2016). There has been increasing awareness of this fish-borne parasitic disease worldwide. Therefore, identifying and differentiating the causative *Anisakis* nematode at genotypic and/or species levels will be important in aiding physicians in diagnosis and managing human anisakiasis, tracing the source of infection, determining the area of origin of the infected food, as well as in developing the most appropriate measures for controlling the infection at all phases of food production (i.e., fishing, breeding, processing and post-processing).

In Thailand, the *Anisakis* morphotypes, type I and *Terranova* larva type B from marine fishes caught in the Gulf of Thailand (Bhaibulaya, 1981), as well as one clinical case of *Anisakis* infection had been documented to date (Hemsrichart, 1993). However, the genetic structure and ecological traits of these anisakid larvae have not been studied. With the increasing popularity of eating raw fish dishes (e.g. sashimi and sushi), infection with *Anisakis* parasite is expected to occur in the Thai population. In addition, fisheries and fish products play an important role in the economic development of Thailand; indeed, fish and fish products are highly valuable export commodity of Thailand. As the species belonging to the genus *Anisakis* are grouped as complexes of morphologically indistinguishable species, and each species has a specific distribution area and range of hosts, identification of these parasites at the species level is crucial in reducing the risk for the consumer, which may include such measures as avoiding particular fishing areas, or particular species of fish (Murrell, 2002).

Given that morphological traits of larvae are not sufficient for the definitive identification of *Anisakis* spp. to the species level, the aim of this study was to identify *Anisakis* specimens isolated from the marine fish *Priacanthus tayenus* off the Gulf of Thailand which were sold for human consumption in the Samut Sakhon province (Thailand).

MATERIALS AND METHODS

Anisakid worms
A total of 59 specimens of *P. tayenus* (Purple-spotted big-eye) caught from the Gulf of Thailand (a seabed area of 304,000 km^2 from 6° N to 13°30' N latitude and 99°E to 104° E longitude) from December 2015 to May 2016 were examined for the larvae of *Anisakis* spp.. The fish were obtained from the fish wholesale markets in the Samut Sakhon province, Thailand and transported on ice to the Parasitology Laboratory, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok. In the laboratory, the body cavity of fish was opened and studied by naked eye. The internal organs were separated and all parasites were removed and washed in physiological saline. The larval parasites were examined micro-scopically and identified based on the morphological characteristics as described for nematodes of the *Anisakis* genus (Smith, 1983; Davey, 1971; Mattiucci *et al.*, 2009). A total of 37 anisakid larvae were detected in 16 fish (27.1%), and the mean larval density was 2.3 per infected fish. They were then fixed, stored in absolute ethanol and kept at –20ºC until DNA extraction.

Isolation of genomic DNA, PCR ampli-fication and sequencing of COII
Fifteen morphologically identified larvae of *Anisakis* were subjected to molecular identification. Genomic DNA was isolated from individual larvae by using the fast technology for analysis of nucleic acid (FTA) classic card method (Whatman BioScience,

Newton Center, Massachusetts, USA) and according to the instructions of the manufacturer (Eamsobhana *et al.*, 2010a,b). Captured nucleic acid on the FTA cards was purified and polymerase chain reaction (PCR) master mix was added directly to the DNA punch in a PCR tube (Eamsobhana *et al.*, 2010a,b).

In polymerase chain reactions (PCR), a partial region of the mtDNA *cox2* gene was amplified using the previously described forward primer 210 (5´-CACCAACTCTT AAAATTATC-3´) and reverse primer 211 (5´-TTTCTAGTTATATAGATTGRTTYAT-3´) (Nadler and Hudspeth, 2000). The PCR mixture was denatured at 94ºC for 3 min, followed by 35-cycles at 94ºC for 30 sec, 46ºC for 1 min, 72ºC for 1 min and 30 sec, followed by post-amplification at 72ºC for 10 min (Eamsobhana *et al.*, 2010a). The PCR products obtained were visualized by electrophoresis in agarose gels and visualized under ultraviolet (UV) light, after ethidium bromide staining. The sequencing procedure follows that previously described by Eamsobhana *et al.* (2010a) using the 210 and 211 primers.

Mitochondrial cytochrome *c* oxidase subunit II nucleotide sequences from GenBank

The mtDNA *cox2* sequences of the present 15 isolates of *A. typica* sp. T from Thailand (T for Thailand) had been deposited in the GenBank – accession number MF399481-MF399495. Representative mtDNA *cox2* sequences of *A. typica* from Indonesia, Japan, Brazil, Papua New Guinea, Western Atlantic, Phillippines, *A. physeteris*, *A. brevispiculata*, *A. paggiae*, *A. ziphidarum*, *A. nascettii*, *A. simplex* complex, and *A. pegreffii* were obtained from the GenBank database (Fig. 1) and used for intrageneric comparison. MtDNA *cox2* sequences of *Pseudoterranova azarasi*, *P. krabbei*, *P. decipiens*, *P. cattani*, *P. bulbosa*, *Contra-caecum osculatum*, *C. ogmorhini* and *C. rudolphii* were also obtained from the GenBank database (Fig. 1) for intergeneric comparison and in the case where the anisakid larval specimens were not members of the genus *Anisakis*. *Ascaris suum* and *A. lumbricoides* were selected as outgroup taxa.

Sequence alignment and phylogenetic analysis

The total length of the aligned sequences of the mtDNA *cox2* gene was 487 bp and the selected models used for maximum likelihood (ML) and Bayesian Inference (BI) analyses were TN+Gamma and TN93+ Gamma, respectively. Alignment of the mtDNA *cox2* sequences, determination of the best-fit nucleotide substitution models and phylogenetic analysis based on nucleotide sequences was performed as described by Yong *et al.* (2015).

Genetic divergence

To assess the level of variation, uncorrected (p) pairwise genetic distances were estimated using the PAUP* 4.0b10 software (Swofford, 2002).

Haplotype network reconstruction

The median joining (MJ) network (Bandelt *et al.*, 1999) was used to estimate the genealogical relationships of the haplotypes. The MJ network was calculated using the NETWORK v5.0.0.1 (http://www.fluxus-engineering.com).

RESULTS

Phylogenetic relationships

The aligned mtDNA *cox2* sequences consisted of 487 characters, of which 417 were invariable (monomorphic), 70 were variable (polymorphic) and 37 were parsimony informative. The phylogenetic trees constructed using the BI and ML methods had similar topology (Fig. 1). The *Anisakis* taxa were grouped into three distinct clades (Fig. 1): (1) *A. typica* species complex – *A. typica* sensu stricto (s.s.), *A. typica* var. *indonesiensis*, and *A. typica* sp. T from Thailand; (2) *A. physeteris*, *A. brevispiculata*, *A. paggiae*, *Pseudoterranova* spp., *A. ziphidarum* and *A. nascettii*; and (3) *A. simplex* (s.s.), *A. berlandi*, *A. simplex* complex and *A. pegreffii*. The 15 sequences
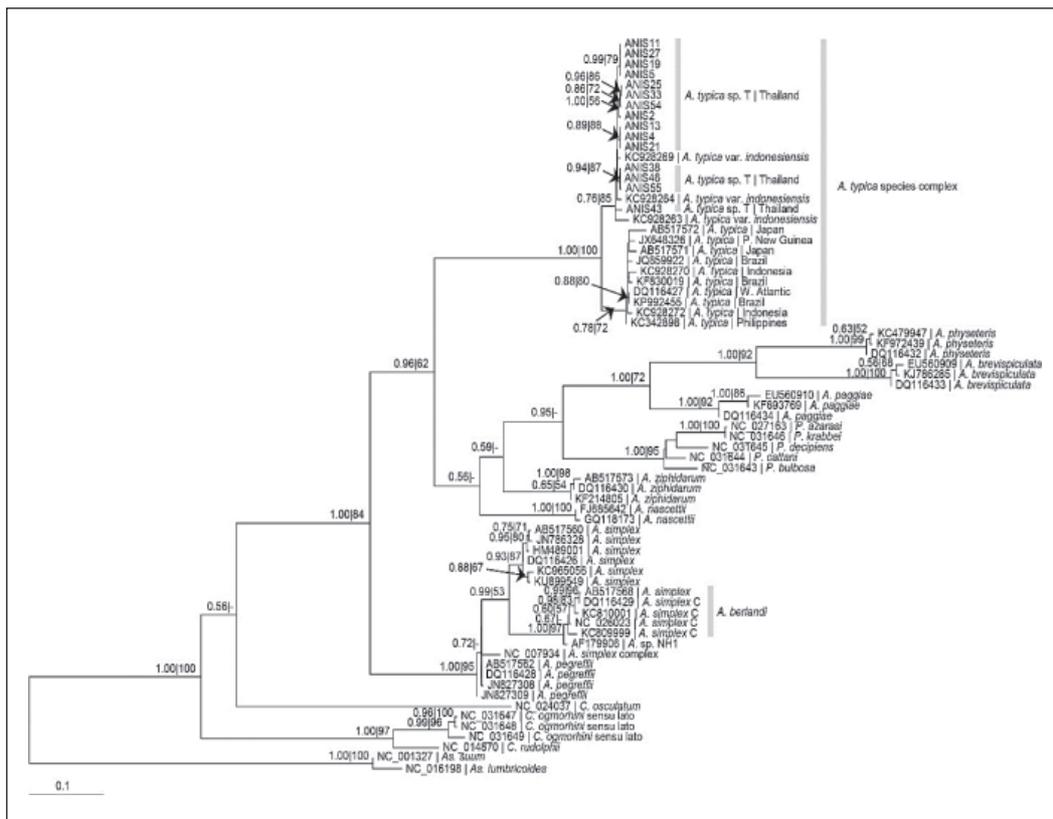
Figure 1. Bayesian inference (BI) and maximum likelihood (ML) phylogenetic tree of *Anisakis* taxa and related anisakid genera based on mitochondrial cytochrome *c* oxidase subunit II nucleotide sequences. *Anisakis typica* sp. T from Thailand and *A. typica* var. *indonesiensis* from Indonesia formed a lineage distinct from *A. typica* sensu stricto. *A.*, *Anisakis*; *As.*, *Ascaris*; *C.*, *Contracaecum*; *P.*, *Pseudoterranova*.

of *A. typica* sp. T from Thailand formed a lineage with *A. typica* var. *indonesiensis* from Indonesia.

The uncorrected 'p' genetic distances for *A. typica* sp. T from Thailand, *A. typica* var. *indonesiensis* and *A. typica* (s.s.) are summarized in Table 1. The Thailand isolates of *A. typica* sp. T had a 'p'-distance of 0-2.1%, while *A. typica* var. *indonesiensis* from Indonesia had a 'p'-distance of 1.2-2.5%. The genetic distances between *A. typica* sp. T and *A. typica* var. *indonesiensis* was 'p' = 0.8-3.1%. Similar genetic distances were found in *A. typica* (s.s.): 'p' = 0-3.3% (Table 1). The genetic distance between *A. typica* (s.s.) and the lineage comprising *A. typica* sp. T and *A. typica* var. *indonesiensis* was p = 4.7-7.6%.

Haplotype diversity and nucleotide diversity

The mtDNA cox^2 haplotype/gene diversity was 0.9683 ± 0.0193 and the nucleotide diversity was 0.033560 ± 0.017161. Eight mtDNA $cox2$ haplotypes were revealed in the isolates of *A. typica* sp. T from Thailand, and three in *A. typica* var. *indonesiensis* from Indonesia (Fig. 2). Most of the haplotypes were represented by singletons, except for one haplotype with 4 isolates (ANIS5, ANIS11, ANIS19, ANIS27) and another with 3 isolates (ANIS4, ANIS13, ANIS21). There were 9 mtDNA $cox2$ haplotypes in the representative sequences of *A. typica* (s.s.) (Fig. 2).

Table 1. Percentage of uncorrected 'p' genetic distance between different pairs of *Anisakis typica* sp. T from Thailand (1-15), *A. typica* var. *indonesiensis* (16-18) and *A. typica* sensu stricto from GenBank (19-28) based on nucleotide sequence of cytochrome *c* oxidase subunit II (*cox2*) gene

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. ANIS25	-																											
2. ANIS33	0.0	-																										
3. ANIS54	0.4	0.4	-																									
4. ANIS2	0.6	0.6	0.6	-																								
5. ANIS11	1.0	1.0	1.0	0.4	-																							
6. ANIS27	1.0	1.0	1.0	0.4	0.0	-																						
7. ANIS19	1.0	1.0	1.0	0.4	0.0	0.0	-																					
8. ANIS5	1.0	1.0	1.0	0.4	0.0	0.0	0.0	-																				
9. ANIS13	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	-																			
10. ANIS21	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.0	-																		
11. ANIS55	1.2	1.2	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-																	
12. ANIS46	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.2	-																
13. ANIS4	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.0	0.0	1.0	0.8	-															
14. ANIS38	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.2	0.0	0.8	-														
15. ANIS43	2.1	2.1	2.1	1.9	1.9	1.9	1.9	1.9	1.9	1.9	2.1	1.9	1.9	1.9	-													
16. KC928264	1.4	1.4	1.4	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.4	1.2	1.2	1.2	1.9	-												
17. KC928269	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.0	0.8	0.8	0.8	1.9	1.2	-											
18. KC928263	2.7	2.7	2.7	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.7	2.5	2.5	2.5	3.1	2.5	2.5	-										
19. JQ859922	5.8	5.8	5.8	5.5	5.5	5.5	5.5	5.5	5.5	5.5	6.2	6.0	5.5	6.0	6.6	5.5	5.5	6.0	-									
20. KC928272	5.8	5.8	5.8	5.5	5.5	5.5	5.5	5.5	5.5	5.5	6.2	6.0	5.5	6.0	6.6	5.5	5.5	6.0	1.2	-								
21. KC342898	4.9	4.9	4.9	4.7	4.7	4.7	4.7	4.7	5.1	5.1	5.3	5.1	5.1	5.1	5.8	4.7	4.7	5.1	0.8	0.8	-							
22. KF830019	5.3	5.3	5.3	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.8	5.5	5.1	5.5	6.2	5.1	5.1	5.5	1.2	1.2	0.8	-						
23. KP992455	5.3	5.3	5.3	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.8	5.5	5.1	5.5	6.2	5.1	5.1	5.5	0.8	0.8	0.4	0.8	-					
24. KC928270	5.3	5.3	5.5	5.3	5.3	5.3	5.3	5.3	5.3	5.3	6.0	5.8	5.3	5.8	6.4	5.3	5.3	5.8	1.9	1.9	1.4	1.4	1.4	-				
25. DQ116427	5.3	5.3	5.3	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.8	5.5	5.1	5.5	6.2	5.1	5.1	5.5	0.8	0.8	0.4	0.8	0.0	1.4	-			
26. JX648326	5.8	5.8	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	6.2	6.0	5.5	6.0	6.2	5.5	5.5	6.0	1.2	1.6	1.2	1.6	1.2	2.3	1.2	-		
27. AB517571	6.0	6.0	6.0	5.8	5.8	5.8	5.8	5.8	5.8	5.8	6.4	6.2	5.8	6.2	6.2	5.8	5.8	5.8	1.9	1.9	1.4	1.9	1.4	2.5	1.4	1.9	-	
28. AB517572	7.2	7.2	7.2	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.6	7.4	7.0	7.4	7.6	7.0	6.6	7.4	3.1	3.1	2.7	3.1	2.7	3.3	2.7	3.1	3.3	-

DISCUSSION

At present, the genus *Anisakis* consists of some 11 recognized species (Gibson, 2017). Genetically identified *Anisakis* has been recorded in 177 teleost species worldwide (Palm *et al.*, 2017). Larvae of the genus *Anisakis* were found in four teleost species (*Muraenesox* sp., *Epinephelus areolatus*, *Rachycentron canadum*, *Trichiurus lepteru*s) out of 78 species from the Gulf of Thailand (Purivirojkul, 2009). However, in Chon Buri Province of Thailand, no anisakid third-stage larvae were found in any of the 16 species of marine fish studied (Nuchjangreed *et al.*, 2006). The present finding of *A. typica* sp. T in *P. tayenus* is the first locality record from Thailand. In an earlier study, *P. tayenus* from the Gulf of Thailand were reported to be infected with *Contracaecum* sp. (Anisakidae) and *Porrocaecum* (Ascarididae) (Purivirojkul, 2009).

A. typica has a worldwide distribution and has been reported in 19 species of definitive hosts – 16 species of Delphinidae, 1 species of Pontoporiidae, and 2 species of Kogiidae (Kuhn *et al.*, 2016). In Indonesia, 28 species of marine fish have been reported to be infected with *A. typica* var. *indonesiensis* compared to 10 species with *A. typica* (s.s.), and one species with a mixture of *A. typica* (s.s.) and *A. typica* var. *indonesiensis* (Palm *et al.*, 2017). All the 10 specimens of *P. tayenus* in Indonesia were infected with *A. typica* var. *indonesiensis* (Palm *et al.*, 2017).

In the present study based on sequences of mtDNA *cox2* gene sequences, all the 15 third-stage larvae of *Anisakis* from Thailand's *P. tayenus* belonged to the same genetic lineage as the *A. typica* var. *indonesiensis* (Fig. 1). It is noteworthy that *P. tayenus* from the Gulf of Thailand and Indonesia does not seem to harbor *A. typica* (s.s.). In contrast, the marine fish *Auxis thazard* and *Katsuwonus pelamis* from the Southern Makassar Strait, Indonesia were infected with *A. typica* (s.s.) and *A. typica* var. *indonesiensis* (Anshary *et al.*, 2014).

The mtDNA *cox2* haplotypes of Thailand isolates of *A. typica* sp. T and *A. typica* var. *indonesiensis* were genetically distinct from those of the *A. typica* (s.s.) (Fig. 2). This
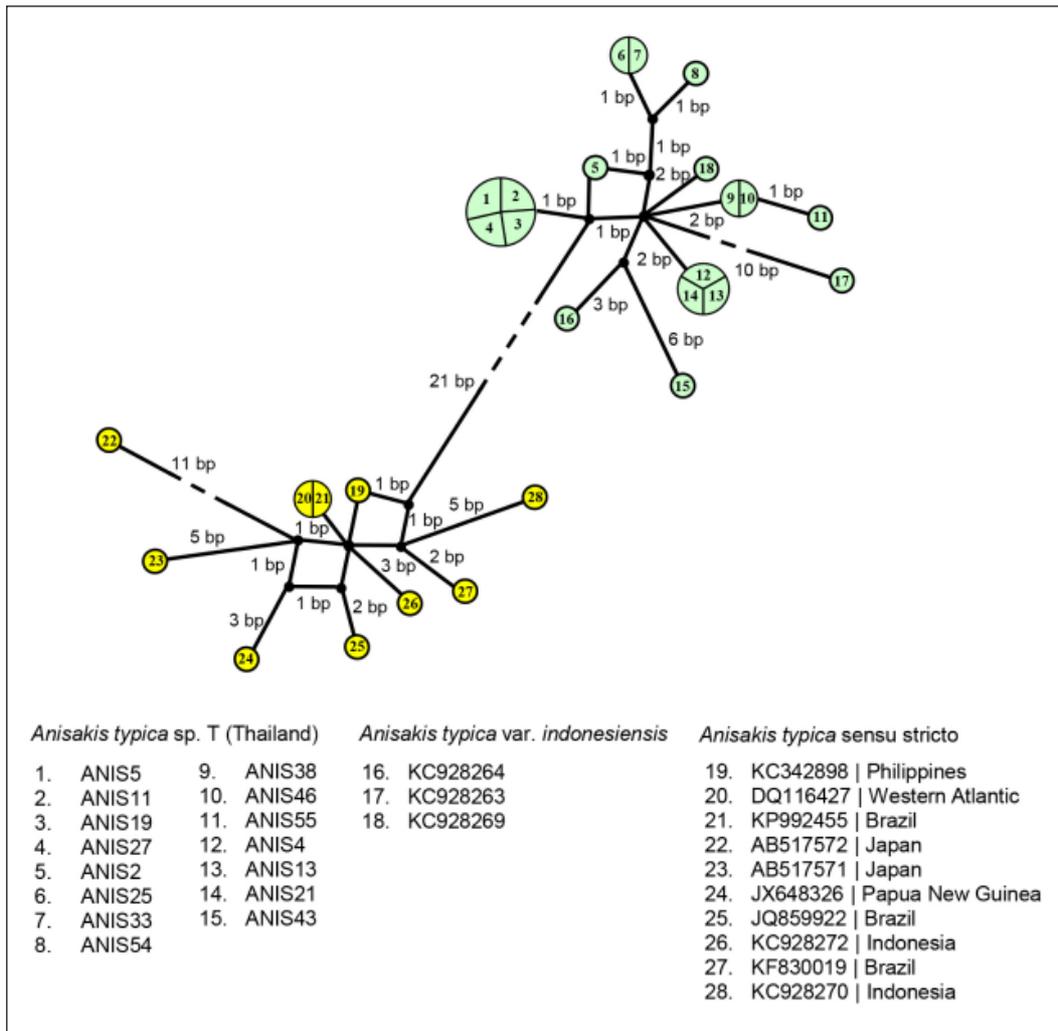
Figure 2. Haplotype networks of *Anisakis typica* sp. T, *A. typica* var. *indonesiensis* and *A. typica* sensu stricto based on mitochondrial cytochrome *c* oxidase subunit II nucleotide sequences generated by NETWORK software. MtDNA *cox2* haplotypes of *A. typica* complex comprising *A. typical* sp. T and *A. typica* var. *indonesiensis* were distinctly separated from those of *A. typica* sensu stricto. Circle represents haplotype and sizes are relative to the number of individuals sharing the specific haplotype.

concurs with the findings on Indonesian isolates of *A. typica* based on the mtDNA *cox2* gene and nuclear ITS1-5.8S-ITS2 marker as described earlier (Anshary *et al.*, 2014; Palm *et al.*, 2017).

The uncorrected genetic distances (p = 4.7-7.6%, Table 1) between *A. typica* (s.s.) and the lineage comprising *A. typica* sp. T of Thailand and *A. typica* var. *indonesiensis* were higher than the intra-taxon genetic distances (p = 0-3.3% for *A. typica* (s.s.) and p = 0-2.5% for the lineage comprising *A. typica* sp. T and *A. typica* var. *indonesiensis*). This, together with distinct mtDNA *cox2* haplotypes, suggests that the larval isolates of *A. typica* sp. T and *A. typica* var. *indonesiensis* warrant species status and not a variety (e.g. var *indonesiensis*) of *A. typica* (s.s.). Taxonomic description of these larval isolates of *A. typica* species complex as a distinct species however awaits the availability of the adult specimens.

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