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Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea

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Abstract

The third-stage larvae of several genera of anisakid nematodes are important etiological agents for zoonotic human anisakiasis. The present study investigated the prevalence of potentially zoonotic anisakid larvae in fish collected on the coastal shelves off Madang and Rabaul in Papua New Guinea (PNG) where fish represents a major component of the diet. Nematodes were found in seven fish species including *Decapterus macarellus, Gerres oblongus, Pinjalo lewisi, Pinjalo pinjalo, Selar crumenophthalmus, Scomberomorus maculatus* and *Thunnus albacares.* They were identified by both light and scanning electron microscopy as *Anisakis* Type I larvae. Sequencing and phylogenetic analysis of the ribosomal internal transcribed spacer (ITS) and the mitochondrial *cytochrome C* oxidase subunit II (*cox2*) gene identified all nematodes as *Anisakis typica.* This study represents the first in-depth characterization of *Anisakis* larvae from seven new fish hosts in PNG. The overall prevalence of larvae was low (7.6%) and no recognised zoonotic *Anisakis* species were identified, suggesting a very low threat of anisakiasis in PNG.

Keywords: Anisakid nematodes, *Anisakis typica*, marine fish, ITS, mt-DNA *cox2*, zoonotic, Papua New Guinea.

1. Introduction

The family Anisakidae includes parasitic nematodes of marine fauna. They have a worldwide distribution and a complex life-cycle which involves invertebrates, fish, cephalopods and mammals (Chai et al., 2005). Anisakid nematodes can accidently infect humans who can suffer from several symptoms including sudden epigastric pain, nausea, vomiting, diarrhoea and allergic reaction (Sakanari and McKerrow, 1989; Audicana and Kennedy, 2008). Most cases of human infection involve anisakid species belonging to the genus *Anisakis* Dujardin, 1845. There are nine described species of *Anisakis*, which are further subdivided into two types. Type I consists of *Anisakis simplex* sensu stricto (s.s), *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum* and *A. nascettii* while Type II consists of *A. paggiae*, *A. physeteris* and *A. brevispiculata* (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009). Of these, only *A. simplex* s.s, *A. pegreffii* and *A. physeteris* have been shown to cause infection in humans (Mattiucci et al., 2011; Arizono et al., 2012).

Anisakid nematodes can be differentiated based on their morphological characteristics and molecular data. According to Berland (1961), larval morphological features including the absence of a ventricular appendage and an intestinal caecum are useful for distinction between several anisakid genera. *Anisakis* Type I or Type II larvae can be identified based on ventriculus length and the presence of a tail spine (or mucron) (Berland, 1961). More recently, polymerase chain reaction (PCR) based tools have been widely used for characterisation of anisakid species at multiple loci, including ribosomal internal transcribed spacer (ITS) regions (Zhu et al., 1998; D'Amelio et al., 2000; Nadler et al., 2005; Pontes et al., 2005; Abe et al., 2006; Umehara et al., 2006; Zhu et al., 2007; Umehara et al., 2008; Kijewska et al., 2009) and the mitochondrial *cytochrome C* oxidase subunit II (*cox2*) gene (Valentini et al., 2006; Mattiucci et al., 2009; Murphy et al., 2010; Cavallero et al., 2011; D'Amelio et al., 2011; Setyobudi et al., 2011).

Anisakid nematodes are a major public health concern. In the last thirty years, there has been a marked increase in the prevalence of anisakiasis throughout the world, due in part to growing consumption of raw or lightly cooked seafood (Audicana and Kennedy, 2008). Over 90%

of cases of anisakiasis are from Japan where consumption of raw fish is popular, with most of the rest from other countries with a tradition of eating raw or marinated fish, such as the Netherlands, France, Spain, Chile and the Philippines (Chai et al., 2005; Choi et al., 2009).

Fish are one of the most important food sources in the coastal areas of Papua New Guinea (PNG). A wide variety of fish species are caught and sold at local markets. Little is known about the prevalence of zoonotic animal parasites including anisakids in fish or of anisakiasis in humans in PNG (Koinari et al., 2012). A review paper mentioned *A. simplex* in skipjack tuna (*Katsuwonus pelamis*) in waters on the south coast of PNG, but did not provide any supporting information (Owen, 2005). The present study was aimed at investigating the distribution of anisakid species in the archipelago off the New Guinean northern coast and specifically to screen for zoonotic species in fish using both morphology and PCR analysis of the ITS region and the mitochondrial *cox2* gene.

2. Materials and methods

2.1. Parasite collection

A total of 276 whole fresh fish were collected from markets in the coastal towns of Madang and Rabaul from March to August 2011 (Fig. 1). The fish were necropsied and nematodes were collected from the body cavities. The muscles of the fish were thinly sliced and investigated under white light to check for nematode larvae. Nematodes were preserved in 70% ethanol and transported to Murdoch University, Australia, for analysis. The prevalence of anisakids in each fish host was expressed as the percentage of positive samples; with 95% confidence intervals calculated assuming a binomial distribution (Rosza et al., 2000).

2.2. Morphological analysis

Whole nematodes were cleared in lactophenol for more than 48 hours and individually mounted onto microscope slides. The body lengths of the nematodes were directly measured. Images were taken with an Olympus BX50 light microscope equipped with Olympus DP70 Camera at 40/100X magnification. The following features were measured: body width, oesophagus length,

ventriculus length and mucron length. Morphological identification was conducted according to keys previously reported (Berland, 1961; Cannon, 1977).

Scanning electron micrographs (SEMs) were taken for representative specimens to study further morphological details. SEMs were obtained on a Phillips XL30 scanning electron microscope at the Centre for Microscopy Characterization and Analysis at the University of Western Australia. Parasite samples were fixed in 2% glutaraldehyde and 1% paraformaldehyde in PBS for 60 min at 4°C and washed twice with PBS (pH = 7.4) in 1.5 mL eppendorf tubes. Samples were dehydrated using a PELCO Biowave microwave processor (TedPella Inc., Redding, CA, USA) by passage through increasing ethanol concentrations in water (33%, 50%, 66% and 100%) followed by two washes in dry acetone. Samples were then dried in a critical point dryer (Emitech 850, Quorum Technologies, Ashford, UK), attached to aluminium sample holders and coated with a 5 nm thick platinum coating to enable surface electrical conduction.

2.3. Genetic characterisation and phylogenetic analysis

DNA from individual nematodes was isolated using a DNeasy® Tissue Kit (Cat. No. 69504, Qiagen, Hilden, Germany). The ITS rDNA region was amplified using primers NC5 5'-GTAGGTGAACCTGCGGAAGGATCAT-3' and NC2 5'-TTAGTTTCTTTTCCCTCCGCT-3' (Zhu et al., 1998) and the mt-DNA *cox2* gene was amplified using primers 210 5'-CACCAACTCTTAAAATTATC-3' and 211 5'-TTTTCTAGTTATATAGATTGRTTYAT-3' (Nadler and Hudspeth, 2000).

Each PCR was performed in a reaction volume of 25 µL using 1 µL of DNA, 1 x PCR buffer (Kapa Biosystems, Cape Town, South Africa), 1.5 mM $MgCl_2$, 200 µM (each) dNTP (Fisher Biotech, Australia), 12.5 pmol of each primer and 0.5 U of *kapa* Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). Negative (no DNA template) and positive (genomic DNA from L3 *Anisakis typica* larvae) controls were included in all PCR reactions. Thermal cycling was performed in a Perkin Elmer Gene Amp PCR 2400 thermal cycler at conditions as previously described (Valentini et al., 2006; Kijewska et al., 2009).

All amplicons were purified using an Ultra Clean® DNA purification kit (MolBio, West Carlsbad, CA, USA). Sequencing was performed using the ABI Prism BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730 DNA Analyser instrument according to manufacturer's instructions except that the annealing temperature was lowered to 46 °C for the *cox2* locus. Sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and compared with published sequences for identification using the National Institute of Health's National Centre for Biotechnology Information Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov). Additional known ITS and *cox2* nucleotide sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) for phylogenetic analysis.

MEGA5 (http://www.megasoftware.net/) was used for all phylogenetic analyses (Tamura et al., 2011). The nucleotide sequences were aligned using MUSCLE (Edgar, 2004), edited manually and tested with MEGA5 model test to find the best DNA model to infer the phylogenetic trees. Phylogenetic analysis with other known anisakid species was conducted using both neighbour-joining (NJ) and maximum-likelihood (ML) analysis for both loci. Evolutionary relationships were calculated using the Kimura two-parameter model for ITS sequences and the Tamura-Nei model for *cox2* sequences with *Contracaecum osculatum* as an outgroup. Reliabilities for of both NJ and ML trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993). The nucleotide sequences were deposited in GenBank under the accession numbers: **JX648312-JX648326.**

3. Results

3.1. Anisakid prevalence

The overall prevalence of anisakids in fish from PNG was 7.6% (21/276, 95% CI=0.05-0.11). Anisakid larvae were found in 7 fish species, at prevalences ranging from 2.9% to 100% (Table 1). The larvae were observed mostly within the body cavities of the fish and their intensity

ranged from 1 to 6 per infected fish host with the exception of *Pinjalo pinjalo,* which had an intensity of 120 larvae per fish, with larvae being found in many other body parts including muscles, pyloric region and liver.

3.2 Morphology of Anisakis *Type I larvae*

Morphological analysis showed that all anisakid nematodes examined were *Anisakis* Type I larvae. The larvae were white and cylindrical in shape. They measured between 20 mm to 36 mm in length and 0.4 to 0.45 mm in width. SEM revealed that the cuticles were irregularly striated transversely at 5.5 µm intervals. The larvae had inconspicuous lips with six papillae, a prominent boring tooth and excretory pore which opened ventrally at the cephalic end (Fig. 2, panels A and E). The mouth opening led to a cylindrical striated oesophagus (length 1.6-2.1 mm), which was followed by a slightly wider ventriculus (length 0.98-1.13 mm). The junction between oesophagus and ventriculus was transverse (Fig. 2 panel B). The ventriculus connected obliquely with the intestine, without a ventricular appendage and intestinal caecum (Fig. 2 panel C). The intestine filled the remaining part of the body. The mucron was distinct and was located at the caudal end (length 17.5-18.0 µm) (Fig. 2 panels D, F and G).

3.3 Sequence and phylogenetic analysis of the ITS region

Amplification of the ITS rDNA generated an approximately 900 bp product. Both neighbour-joining and maximum-likelihood analyses produced trees with similar topology. Neighbour-joining analysis of the ITS nucleotide sequences from the present study with previously reported sequences from GenBank clustered all the *Anisakis* Type I larvae examined with *Anisakis typica* (Fig. 3). The ITS nucleotide sequences of all the *Anisakis* Type I larvae from the present study exhibited 99.1% to 100% similarities to the published sequence of *Anisakis typica* (AB432909) found in Indian mackerel (*Rastrelliger kanagurta*) in Thailand and 96.1% to 97.6% similarities to the published sequence of *Anisakis typica* (JQ798962) found in cutlassfish (*Trichiurus lepturus*) from Brazil. The sequences exhibited 82.7% to 88.7% similarities with other *Anisakis* species (Table 2).

Amplification of the *cox2* gene generated an approximately 629 bp product. As with the ITS locus, neighbour-joining and maximum-likelihood analyses produced trees with similar topology. Neighbour-joining analysis of *cox2* nucleotide sequences showed that all isolates clustered broadly with *A. typica* (DQ116427) but revealed more variation. Two broad groups were produced with subgroup I consisting of 5 isolates and *A. typica* reference sequence (DQ116427), and subgroup II containing 16 isolates (Fig. 4). Based on genetic distance analysis, subgroup I had 98.9% to 99.3% similarity to *A. typica* (DQ116427) while subgroup II had 92.4% to 94.6% similarity to *A. typica* (DQ116427). The *cox2* nucleotide sequences from the present study shared 77.0% to 87.2% similarity with other known *Anisakis* species (Table 2).

4. Discussion

Anisakid larvae were found in 7.6% (21/276) of the 7 fish species examined. The intensity of infection was low (1 to 6) in all fish hosts except for *Pinjalo pinjalo* (120) (Table 1). Previous studies have reported wide variation in prevalence and intensity of infection of anisakids in other fish hosts (Costa et al, 2003; Farjallah et al., 2008a, b; Setyobudi et al., 2011). The relatively low infection level found in the present study could be due to the fact that most of the fish hosts sampled were relatively small in size (range 16-49 cm fork length) compared to previous studies. In general, prevalence and parasite burden tends to increase with the size and the age of the fish host (Setyobudi et al., 2011).

All nematodes in the present study were identified morphologically as *Anisakis* Type I larvae, based on an oblique connection between the ventriculus and the intestine, lack of a ventricular appendage and intestinal caecum, and the presence of a mucron (Berland, 1961; Cannon, 1977). Larvae of *A. typica* found in cutlassfish (*Trichiurus lepturus*) from Brazil shared similar morphological characteristics with the *A. typica* larvae from the present study (Borges et al., 2012).

Phylogenetic analysis of DNA sequences indicated that all examined samples were *Anisakis typica*. At the ITS locus, all isolates examined formed a single clade with *A. typica*. The comparison of the ITS nucleotide sequences from this study with sequences previously deposited in Genbank resulted in 96.1% to 97.6% similarities to *A. typica* found in cutlassfish (accession no. JQ798962) from Brazil and 99.1% to 100% similarities to *A. typica* (accession no. AB432909) from Indian mackerel in Thailand.

At the *cox2* locus, whilst the isolates clustered broadly with the reference *A. typica* genotype, two distinct subgroups (I: 98.9% to 99.3% similarity and II: 92.4% to 94.6% similarity) were identified. Previously reported *cox2* trees by Valentini et al. (2006) also showed similar genetic divergence within the *Anisakis typica* clade. Furthermore, the sequence difference of 5.4% to 7.6% between the subgroup II clade and the reference *A. typica* sequence is still within the range found between conspecifics in other nematode taxa (Blouin et al., 1998).

According to Mattiucci and Nascetti (2006), *Anisakis* species form two sister clades and *A. typica* is grouped within clade I, based on phylogenetic relationships inferred from allozyme and mitochondrial gene markers. In the present study, *A. typica* clustered within clade I at the *cox2* locus, consistent with previously reported phylogenetic trees (Valentini et al., 2006; Mattiucci et al., 2009; Cavallero et al., 2011; Setyobudi et al., 2011). However, at the ITS locus, *A. typica* did not cluster within clade 1 and formed a separate group to the two clades. Other studies have shown similar tree topologies at the ITS locus (Kijewska et al., 2009; Cavallero et al., 2011) and according to Cavallero et al. (2011), *A. typica* could form a distinct lineage (resulting in three clades, rather than two, for the genus *Anisakis*). It should be noted, however, that the position of *A. typica* in both the ITS tree and *cox2* tree was not well supported (<50% bootstrap support) in our study and therefore more sampling of the species from a wider range of hosts and geographical areas is needed to resolve this discrepancy.

The present study identified seven new fish species as hosts for *A. typica*; *Decapterus macarellus, Gerres oblongus, Pinjalo lewisi, Pinjalo pinjalo, Selar crumenophthalmus,*

Scomberomous maculatus and *Thunnus albacares*. Previous studies have identified *A. typica* in more than 15 different fish hosts, which have an epipelagic distribution in the Atlantic Ocean close to the coast lines of Brazil, Mauritius, Morocco, Portugal and Madeira (Mattiucci et al., 2002; Pontes et al., 2005; Marques et al., 2006; Farjallah et al., 2008a; Iniguez et al., 2009; Kijewska et al., 2009, Borges et al., 2012). *Anisakis typica* has also been found in the Mediterranean Sea close to Tunisia, Libya, Cyprus and Crete, and in the Indian ocean off Somalia (Mattiucci et al., 2002; Farjallah et al., 2008b) and Australia (Yann, 2006). Furthermore *A. typica* has been found in Japan, Taiwan, China, Thailand and Indonesia (Chen et al., 2008; Palm et al., 2008; Umehara et al., 2010). Although it has been hypothesized that *A. typica* has a global distribution that extends from a 30°S to a 35°N latitude (Mattiucci and Nascetti, 2006), a previous distribution model for anisakid species has not included PNG (Kuhn et al., 2011).

In conclusion, all anisakids identified from PNG in the present study were *A. typica*, which has not previously been associated with human infections. Further studies are needed to extend the knowledge of anisakid species distribution in larger fish hosts and other seafood hosts in PNG waters, but the present study results suggest that the danger from zoonotic anisakid species in PNG is very low.

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Figure 1: Map of the study sites. Samples were collected on the coastal shelves off Madang and Rabaul in Papua New Guinea.

Figure 2. *Anisakis* Type I larvae from *S. crumenophthalmus*. These images are exemplary for all larvae found in the present study. Light microscopy images show: **A**. Cephalic end of larva showing the boring tooth and the excretory pore; **B**. ventriculus - oesophagus junction; **C**. ventriculus - intestine junction; **D**. claudal end showing the mucron, anal opening and anal glands. Scanning electron microscopy images show: **E**. cephalic end; **F**. rounded tail with a mucron; **G**. mucron. ag = anal glands, ao = anal opening, bt = boring tooth, e = oesophagus, ep=excretory pore, int=intestine, l = lips, mu = mucron, ve = ventriculus.

Figure 3: Phylogenetic relationships between *Anisakis* species from the present study (*) and other *Anisakis* species as inferred by neighbour-joining analysis of ITS rDNA. The evolutionary distances were computed using the Kimura-2 parameter method and the rate variation among sites was modelled with a gamma distribution with *Contracaecum osculatum* as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1, 000 replicates) are shown at the internal nodes (> 50% only). Specimen codes are given in Table 1.

Figure 4: Phylogenetic relationships between *Anisakis* species from the present study (*) and other *Anisakis* species inferred using the neighbour-joining analysis *of* *cox2* genes. The evolutionary distances were computed using Tamura-Nei model and the rate variation among sites was modelled with a gamma distribution with *Contracaecum osculatum* as an outgroup. The percentage of trees in which the associated taxa clustered together in a bootstrap test (1, 000 replicates) are shown next to the branches (> 50% only). Specimen codes are given in Table 1.

1 **Table 1: Fish species from which anisakid larvae were collected in the present study.**

2 N is the number of fish sampled, prevalence is the % of infected fish (95% CI in parentheses) and

3 mean intensity (MI) is the mean number of larvae in the infected fish hosts ±SD (range). Where no

4 SD value was given, there was one or similar observation and SD could not be calculated.

5

Fish Species	N	Prevalence (CI)	MI±SD (min-max)	Specimen Code
Decapterus macarellus (Mackerel Scad)	29	6.9 (-0.03-0.17)	1	DM23, DM24
Gerres oblongus (Slender Silver-biddy)	54	3.7 (-0.02-0.09)	3±0.4 (2-4)	GO14, GO15
Pinjalo lewisi (White-spot Pinjalo Snapper)	14	50 (0.2-0.8)	5±0.92 (1-6)	PL1, PL5, PL8, PL9
Pinjalo pinjalo (Pinjalo)	1	100 (0.2-0.8)	120	PP1
Scomberomous maculatus (Spanish Mackerel)	3	33.3 (-1.1-1.8)	1	SM3
Thunnus albacares (Yellowfin Tuna)	34	2.9 (-0.3-0.09)	3	TA3
Selar crumenophthalmus (Bigeye Scad)	106	6.6 (0.02-0.11)	2.9±0.95 (1-3)	SC76, SC77, SC78, SC88, SC97, SC100, SC102

6

Table 2: Percentage similarity of the *Anisakis* species analysed in the present study and their closest relatives. At the ITS locus, comparison with *A. typica*, accession numbers AB432909 and JQ798962 were presented. *Anisakis* sp.* is conspecific with *A. nascettii* (Mattiucci et al., 2009).

	% similarity at:	
Species compared	ITS rRNA locus	*Cox2* locus
A. typica	96.1 - 100	92.4 - 99.3
A. ziphidarum	87.5 - 88.7	82.1 - 84.3
A. pegreffii	85.4 - 86.2	84.7 - 87.2
A. simplex s. s	85.6 - 86.3	84.7 - 86.7
A. simplex C	85.8 - 86.6	84.2 - 85.8
Anisakis sp.*	86.5 - 87.8	not analysed
A. nascettii	not analysed	83.9 - 86.7
A. physeteris	82.7 - 83.9	82.8 - 84.5
A. brevispiculata	78.6 - 80.1	77.0 - 79.1
A. paggiae	83.8 - 84.7	79.3 - 82.2

Figure 1

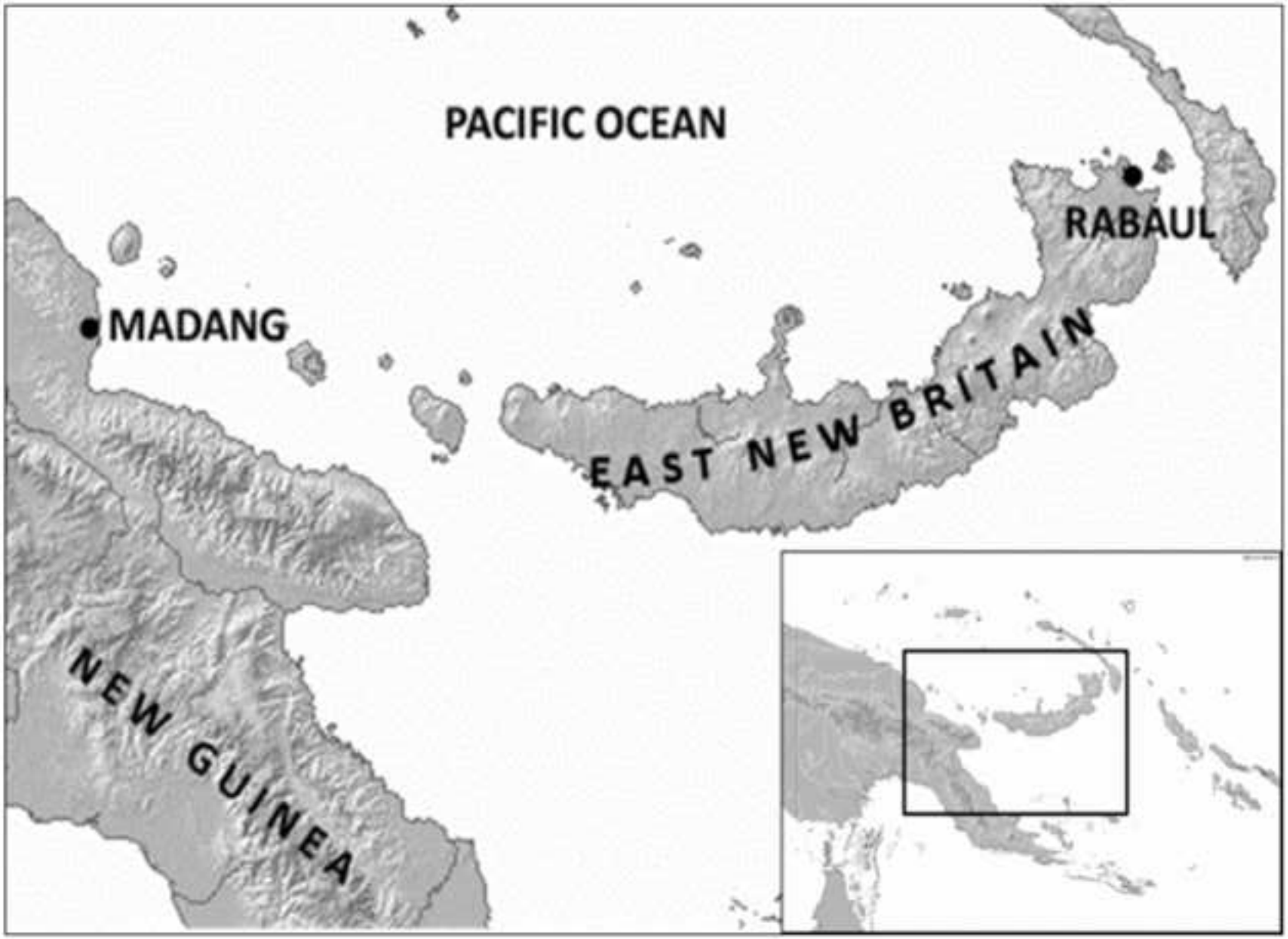
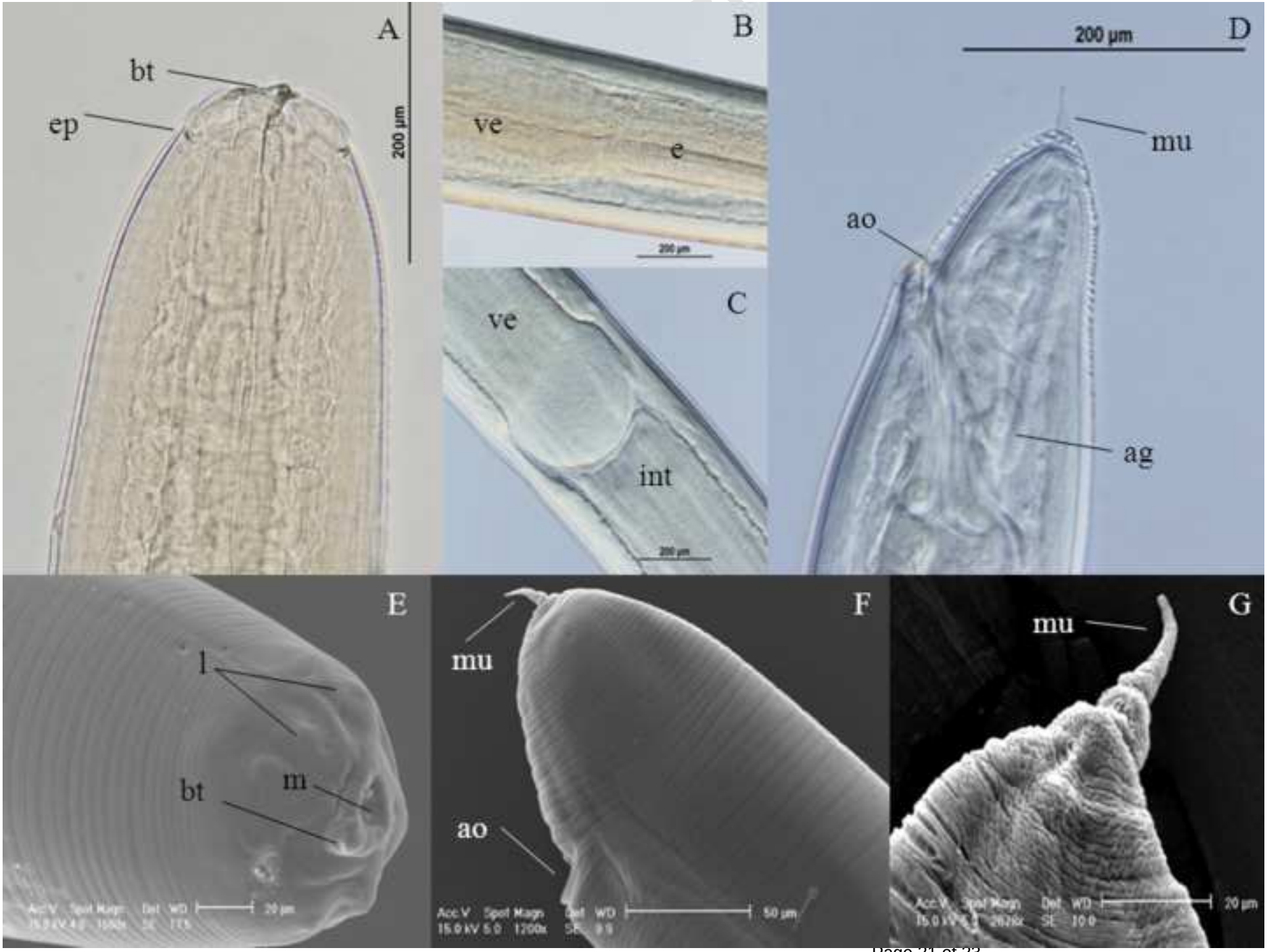


Figure 2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