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Molecular and immunological characterisation of tropomyosin from *Anisakis pegreffii*

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Abstract Tropomyosin (TM) is a major allergen in shellfish, known to cross-react with mite, cockroach and/or some roundworm (nematode) TM. In this study, we aimed to express and purify TM from the parasitic nematode *Anisakis pegreffii* and also to characterise its cross-reactivity with TM from shellfish. *A. pegreffii* was isolated from the flathead tiger fish (*Neoplatycephalus richardsoni*) and characterised using single-strand conformation polymorphism (SSCP)-based sequencing of the first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA. The recombinant tropomyosin (rTM) of *A. pegreffii* was expressed, purified and confirmed by immunohistochemistry, sequencing and LC-MS/MS analyses. Immunohistochemistry showed the muscle and the base layer of the third-stage larvae (L3) of *A. pegreffii* as the location of TM in *A. pegreffii*. The molecular relationship of TM of *A. pegreffii* with homologs from other nematodes and crustaceans was inferred from phylogenetic analysis. Immunogenicity of TM from *A. pegreffii* was tested by immunoblotting, which showed that rTM from *A. pegreffii* binds to IgE from sera of patients with allergy to crustaceans. Immunoblotting also showed that the anti-TM monoclonal antibody (MAb) did not recognise rTM from *A. pegreffii*. The rTM from *A. pegreffii* was, however, recognised by anti-TM polyclonal antibodies (PAbs) as well as anti-crustacean polyclonal antibodies (PAbs). The detection of specific serum IgE antibody against parasite TM has been proposed as a useful approach for the diagnosis of parasite-induced allergy. The findings of this study merit further exploration of the cross-reactive allergenic proteins of *Anisakis* for improved, future diagnosis of allergenic diseases.

Keywords *Anisakis pegreffii* · Tropomyosin · Shellfish · Allergens · Cross-reactivity

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Introduction

The third-stage larvae of the nematode worm *Anisakis* present a major economic problem for the fish-trade industry (Mattiucci et al. 1997; Mattiucci and Nascetti, 2008). They are also identified as potential threats to human health causing anisakiasis and as potential allergenic food-borne pathogens (Bao et al. 2017; Fumarola et al. 2009; Gregori et al. 2015; Kirstein et al. 2010; Umehara et al. 2007). *Anisakis simplex* third-stage larva (L3) is classified as *Anisakis* type I and has been regarded as the primary cause of anisakiasis, mostly acquired through the ingestion of infected, raw fish (Daschner et al. 2002). However, molecular studies have revealed that *A. simplex* represents at least three sibling species,

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namely *A. simplex* sensu stricto (s.s.) (Nascetti et al. 1986), *A. pegreffii* (Campana-Rouget and Biocca, 1955) and *A. simplex* C (Mattiucci et al. 2002; Mattiucci and Nascetti, 2007; Nascetti et al. 1986). It is now established that there is a wide distribution of *A. pegreffii* and *A. simplex* s.s. across the globe. They are characterised on the basis of their genetic and morphological features as well as their ecological traits, such as preference for the definitive host. These parasitic nematodes do have geographical distributions; they, however, do not have a preference for their distribution. Results of various researches worldwide have also shown *A. simplex* s.s. and *A. pegreffii* as the principal etiologic agents of anisakiasis (Abollo et al. 2003; Mattiucci et al. 1997, Mattiucci and Nascetti, 2006; 2008; Mattiucci et al. 2009).

Parasite hosts are constantly being challenged by the large numbers of somatic and excretory/secretory antigens produced by parasites, which in some cases would induce strong allergic reactions in hosts to these antigens (Acevedo and Caraballo 2011). Allergic reactions to *Anisakis* have been reported, and these allergens from *Anisakis* are considered to be of importance (Albrecht et al. 2008; Anibarro et al. 1998; Kamath et al. 2013; Kim et al. 2005).

Tropomyosin (TM), a major allergen in shellfish, is known to be an isoallergen and panallergen, which is cross-reactive with mite, cockroach and some nematode homologs (Lopata and Lehrer, 2009; Lopata et al. 2010). In many species of crustaceans, TM has been identified and found to be implicated in ~ 80% of cases associated with allergic reactions (Faeste et al. 2003). Functionally, shellfish TM shares various highly conserved IgE-binding B cell epitopes. These epitopes are known to induce immune responses and, in addition, also have a high cross-reactivity with TM of invertebrates of diverse species. In *A. simplex*, TM has been described as a major somatic allergen present in third- and fourth-stage larvae (O'Donnell et al. 1989). The proteins of *A. simplex* have been shown to display notable cross-reactivity to proteins of other invertebrates, such as house dust mites and crustaceans as well as related nematodes (Nieuwenhuizen and Lopata, 2013).

This study therefore aimed to characterise the TM of *A. pegreffii* and to determine whether TM of *A. pegreffii* shares epitopes with TM of *A. simplex*, and other organisms, particularly crustaceans. Results from this work will be used to infer the allergenicity of TM from *A. pegreffii*.

Material and methods

Isolation of anisakid larvae and morphological identification

Live larvae (L3) of *A. pegreffii* were recovered from the uppermost layer of the internal cavity and organs such as gut, pyloric caecum, stomach, gonads and liver of parasitised *Neoplatycephalus richardsoni* (flathead fish). The fish were purchased from a local seafood market in Melbourne and transported on ice to the laboratory for immediate processing. Encapsulated larvae were isolated from the fish, and larvae were freed from the host tissue as described by Werner et al. (2011). Briefly, capsules were opened and removed to liberate the larvae. Larvae were thereafter exhaustively washed in phosphate-buffered saline (PBS; pH 7.4) and then fixed in ethanol for subsequent examination. The anterior and posterior parts of each nematode were cut out with a sterile scalpel and preserved in lactophenol for morphological identification. The mid-section (3 mm) of the body was used for subsequent molecular studies. Using the light optical microscope, nematodes were examined morphologically and assigned to a larval 'type' (Cannon, 1977; Shamsi et al., 2011). Briefly, the presence or absence of mucron at the tail end as well as the length of the ventriculus was assessed to assign the larval type.

Molecular identification of anisakid larvae

Sodium dodecyl–sulphate/proteinase K treatment was used to extract genomic DNA from the mid-section of the larvae. This was purified using a mini-column according to the manufacturer's protocol (Wizard DNA Clean-Up System, Promega, Fitchburg, WI, USA). Amplification of ITS-1 and ITS-2 nuclear rDNA, from 10 to 50 ng of genomic DNA, was performed in a PCR using the primer sets SS1/NC13R and SS2/NC2 respectively (Shamsi et al. 2008; Zhang et al., 2007). PCR conditions were as previously described (Jabbar et al. 2012). Known positive controls and negatives (no-DNA) were included for each set of PCRs. Amplicon (5 μl), following PCR, was examined on an ethidium bromide-stained 1.5% *w/v* agarose gel, and thereafter visualised using ultraviolet light.

Single-strand conformation polymorphism (SSCP) and sequencing

In order to determine sequence variation within and among amplicons, ITS-1 and ITS-2 amplified products were subjected to SSCP analysis (Gasser et al. 2006; protocol B). Amplicon representatives for unique SSCP profile for each locus were selected and treated with exonuclease I and shrimp alkaline phosphatase (Fermentas Inc., Glen Burnie, MD, USA). These were subsequently sequenced, using the same PCR primers, in a bi-directional, automated sequencing (BigDye terminator v.3.1, Applied Biosystems, Foster City, CA, USA). Each sequence quality was assessed by evaluating its electropherogram with the Bio-Edit program (Hall, 1999). Polymorphic sites were assigned using International Union of Pure and Applied Chemistry (IUPAC) codes. Reference sequences, AB277823 (representing *A. pegreffii* L3s from *Scomber japonicus* (Pacific chub mackerel)) (Umehara et al.

2008) and JQ900763 from *Engraulis* spp. (anchovy) (Mattiucci et al. 2013), from the GenBank database were compared with selected sequences.

Preparation of protein extracts

An extract was made from *A. pegreffii* by snap-freezing the posterior and anterior portions of 30 L3s (25 mg) in liquid nitrogen, homogenising the extract in 2.5 ml of a solution of 0.1 M Tris (hydroxymethy1) aminomethane and 0.5 M glycine buffer (pH 8.7). This extract was then sonicated using a probe sonicator (Branson digital sonifier, 35% amplitude; pulse on 15 s, pulse off 30 s; three cycles), and thereafter centrifuged for 5 min at 10,000×*g* to remove debris. The supernatant was filtered using a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) and supernatant protein concentration was performed using Millipore concentration tubes (5 kDa cutoff; Amicon Ultra, USA). The total protein concentration of the extract was estimated using the Quick Start Bradford Assay kit (BioRad, USA) and bovine serum albumin (BSA) as a standard. The filtered and concentrated supernatant of the *Anisakis* extract was stored in 1-ml aliquots at − 80 °C prior to use.

Cloning of the tropomyosin coding sequence

Total RNA was extracted from *A. pegreffii* larvae (n = 35) using RNA Mini Kit (Bioline) and cDNA synthesised using a conventional reverse transcription protocol (Bioline) following the manufacturer's instructions. The coding region of TM was PCR-amplified from this cDNA using the oligonucleotide primer pair *A. pegreffii*-TM forward (5′-CGGG ATCCATAGACGCAATCAAGAAGAAGATG-3′)/ *A. pegreffii*-TM reverse (5′-CGGAGCTCTATTAATATCCA GACAGCTCTTG-3′) under the following conditions: an initial denaturation at 94 °C for 2 min; the subsequent denaturation cycle was at 95 °C for 20 s, annealing at 55 °C for 15 s, and elongation at 72 °C for 50 s for 35 cycles, with a final elongation step at 72 °C for 7 min. The amplicon (855 bp) was purified using the Qiagen PCR Purification Kit following the manufacturer's instructions and cloned into the sequencing vector, pCR 2.1, using a TOPO TA cloning kit and cloning reaction protocol (Invitrogen, Carlsbad, CA, USA). Subsequently, the coding region for TM was cloned into the expression vector pRSET A into the *Bam*H1 and *Xho*I sites using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* (BL21 strain) was then transformed with this construct by electroporation and incubated in SOC medium (Hanahan, 1983) at 37 °C for 4 h. Transformed *E. coli* was grown overnight on Luria Bertani (LB) agar with 100 μg/ml of ampicillin at 37 °C. Colonies were screened by PCR using the gene-specific oligonucleotide primers for the presence of inserts. Plasmid DNA was purified from overnight cultures using the QIAprep SPIN reaction kit (Qiagen, Germany) and sequenced to verify its integrity. Cells were stored in glycerol stock at −80 °C.

Recombinant TM was expressed at 37 °C in LB medium (containing 100 μg/ml of ampicillin) and grown until the culture reached middle to late log phase, after which the culture was induced with 0.1 mM IPTG. Recombinant TM was extracted from *E. coli* using lysis buffer (25 mM Tris–HCl, 300 mM NaCl, 1 mM imidazole, 2 mg/ml of lysozyme, pH 8) and purified employing a nickel-charged metal chelate affinity chromatography and ion exchange chromatography columns (GE Healthcare, USA). Purified protein was then stored in 1-ml aliquots at −80 °C prior to use.

SDS-PAGE analysis

Electrophoresis was performed to analyse the protein repertoire in the parasite extract (Abdel Rahman et al. 2010; Abdel Rahman et al. 2013). The extract (12 μg) was briefly heated in Laemmli buffer with dithiothreitol, and ~ 10 μl of protein was loaded onto a 12% bis-acrylamide gel. The protein components were resolved at 170 V until the tracking dye reached the base. The separated proteins were detected by staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA).

Immunoblotting with monoclonal, polyclonal anti-tropomyosin and anti-crustacean antibody

To confirm the presence of native (n-) and recombinant (r-) TM of *A. pegreffii*, poly- and mono-clonal antibodies were used. Four micrograms of *A. pegreffii* protein extract and purified proteins were resolved by SDS-PAGE. The Semi-dry TransBlot Apparatus (BioRad, Hercules, CA, USA) was used to transfer protein onto activated polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked in 5% (w/v) skim milk powder (SMP) in PBS containing 0.05% Tween-20 (PBS-T). The rTM membrane was exposed to (1) monoclonal anti-crustacean TM antibody, MAC 141 (ab50567; Abcam, Cambridge, MA, USA) diluted 1:6000 in 1% SMP in PBS-T and subsequently to rabbit anti-mouse IgG antibody conjugated to HRP (Sigma, St. Louis, MO, USA) diluted 1:8000. (2) An in-house-generated rabbit polyclonal anti-crustacean tropomyosin antibody (Kamath et al., 2014.) diluted 1:10,000 and then to goat anti-rabbit IgG antibody conjugated with HRP (Promega, USA) diluted 1:40,000. The n-TM was exposed to the in-house-generated rabbit polyclonal anti-crustacean tropomyosin antibody as described for rTPM. After the necessary washes (3×) using PBS-T, antibody binding was observed on the membrane using two methods: (a) enhanced chemiluminescent technique, as previously reported (Abdel Rahman et al., 2010, 2011). Briefly, blots were either incubated with chemiluminescent substrate (Sigma, St. Louis, MO, USA) and thereafter exposed to photographic film

(GE Healthcare Biosciences, USA.) or (b) membrane was immersed in TMB (3,3′, 5,5′-tetramethylbenzidine) chromogenic HRP-substrate for 5 min, rinsed in distilled water and antibody-binding protein bands detected thereafter. The rTMs of shellfish were used as positive controls (obtained from Molecular Allergy Laboratory of Andreas Lopata).

Immunoblotting with IgE in human patient sera

To determine the allergenicity of rTM of *A. pegreffii*, IgE antibody reactivity was assessed by immunoblotting using a pool of sera from patients with confirmed shellfish allergy. Serum samples were obtained from patients with a history of allergic reactions to shellfish and/or positive by SPT and/or ImmunoCAP to prawn, crab, or lobster (mean age 32 ± 10.5 years). A serum from a healthy donor was the negative control used. Informed, written consent was obtained from all study subjects, and experimental protocols were approved by The Alfred Hospital Research Ethics Committee (Project number 192/07) and the Monash University Human Ethics Committee (MUHREC CF08/0225).

IgE immunoblotting was conducted as previously described (Abdel Rahman et al. 2013). Briefly, the proteins on the membrane were first incubated with the serum pool (1:10 in 1% SMP, PBS-T) and, thereafter, with rabbit anti-human IgE polyclonal antibody (DAKO Corporation, Carpinteria, CA, USA) and goat anti-rabbit IgG labelled with HRP (Promega, USA). IgE reactivity was then assessed.

Immunofluorescence staining and microscopy

L3s of *A. pegreffii* were placed in a small plastic cassette and embedded in optimal cutting temperature (OCT) compound medium. The OCT-solidified sample was frozen and cut into serial sections (7–10 μm thick) using a microtome cryostat machine. Sections were melted onto super frost slides and labelled. Drying of sections was by placing slides in blocks and leaving at 4 °C overnight. Sections were thereafter fixed by placing slides into cold acetone (100%) for 10 min and allowed to dry. To dissolve the OCT medium, the slides were put into a staining chamber containing PBS for 10 min. For the immunofluorescent labelling step, 200 μl of rabbit polyclonal anti-TM antibody solution (primary antibody) was added directly to slides to cover the whole sections and incubated at 4 °C overnight, followed by a PBS wash for 10 min. Subsequently, 200 μl of HRP mouse anti-rabbit IgG secondary antibodies conjugated to fluorescein isothiocyanate (FITC) was added directly to the slides and incubated at 24 °C for 30 min, followed by a PBS wash for 10 min. Detection was carried out using 20 μl of DAB substrate solution for 10 min, and slides were washed in PBS for 10 min to remove the excess of this solution. The slides were kept in a chamber in PBS overnight and then dried. Glycerol solution (50%; 5–10 μl) was pipetted onto the section and cover-slipped and allowed to dry. Images were then taken using a fluorescent microscope (Nikon) and studied.

Phylogenetic analyses and amino acid alignment

Representative sequences obtained from Genbank for ITS-1 and ITS-2 respectively (*Anisakis simplex* C-AY826722.1; *A. simplex*-EU624342; *Anisakis* spp.-JN005759.1; *Anisakis ziphidarum*-AY826725.1; *Anisakis brevispiculata*-AB592794.1; *Anisakis typica*-JX848663.1, JX848680.1; *Anisakis paggiae*-AB592797.1,GU295975.1; *Anisakis physeteris*-EU718475.1, JX523714.1; *Pseudoterranova decipiens*-AJ413968.1, AJ413967.1 and *Contracaecum rudolphii* A-DQ316967.1, DQ316969.1) and the TM sequences obtained from the National Centre for Biotechnology Information (NCBI) for *A. simplex*, *Ascaris lumbricoides*, *Onchocerca volvulus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Penaeus monodon*, *Habronattus americanus*, *Periplaneta americana*, *Hediste diversicolor*, *Paralithodes camtschaticus*, *Homo sapiens* and *Gallus gallus* (Uniprot accession IDs, JF902160, FJ655903, L41633.1, CELTMY1B, NM_169633, AY827100, AF034954.1, Y14854.1, AF216518.1, AB270632.1, J00910.1 and M19267.1, respectively) were aligned with the inferred amino acid ITS-1, ITS-2 and TM of *A. pegreffii* respectively, employing ClustalW alignment Thompson et al. (1994) in MEGA version. 6.0. Phylogenetic analyses of sequence data for the above-named species were performed using the maximum likelihood statistical method. The following default parameters were used for the phylogenetic tree construction: Poisson model, bootstrap (10,000 replicates; random seed) and complete deletion of gaps (Tamura et al. 2013).

In-gel tryptic digestion and LC-MS/MS analysis

Tryptic digestion was performed by excising a band corresponding to 37 kDa in SDS-PAGE protein analysis of *A. pegreffii* crude extract. The gel band was washed and destained twice in 50% acetonitrile with 50 mM triethylammonium bicarbonate (TEAB) and dehydrated with acetonitrile until the gel plug turned opaque. The gel plug was then rehydrated and incubated in reduction buffer, consisting of 50 mM tris (2-carboxyethyl) phosphine (TCEP) in 50 mM TEAB at 60 °C. Incubation was for 20 min with 20–50 μl of reduction buffer to cover the gel plug. This was followed by alkylation using 100 mM iodoacetamide in 50 mM TEAB at 24 °C in the dark. The sample was washed in 50 mM TEAB twice and dehydrated with acetonitrile. Digestion of the gel plug was with 1 μg of molecular-grade trypsin (Sigma-Aldrich) at 37 °C overnight. Tryptic peptides were extracted from the gel, acidified with formic acid and then analysed. Digested peptides were injected into the column, separated

using a gradient of acetonitrile in water and analysed by liquid chromatography/mass spectroscopy (LC-MS/MS), using a Q-Exactive mass spectrometer (Thermo Scientific). The derived peak list was searched using the Mascot search engine (version 2.4; Matrix Science, London, UK) against the UniProt database. The TM from *A. pegreffii* was identified by peptide sequence identity to TM in this database in two separate experiments.

Results

Verification of the identity of L3s as *A. pegreffii*

The anisakid larvae from *N. richardsoni* (flathead fish) used in this study were individually identified as *Anisakis* larval type 1 (Cannon, 1977; Shamsi et al. 2011), which is consistent with *A. pegreffii.* The sequencing of ITS-1 and ITS-2 from such larvae supported this identification, with these sequences being 99% identical to two reference sequences (Fig. 1a, b). Phylogenetic analysis showed the concatenated ITS-1 and ITS-2 sequences representing *A. pegreffii* (those determined herein) grouped together to the exclusion of other members of the Anisakidae family (Fig. 2a, b).

A. pegreffii tropomyosin (TM)—characterisation, phylogenetic relationship and localisation

The cDNA encoding TM of *A. pegreffii* is 855 bp in length and is 99% similar to that of *A. simplex. A. pegreffii* TM is 284 amino acids in length and has an estimated molecular mass of 33 kDa (Fig. 3a, b). The signature patterns for TM are L-K-E-A-E-x-R-A-E, indicated in red (underlined), conserved in the C-terminal region of *A. pegreffii* TM, and the highly conserved N-terminal motif DAIKKK, which binds actin (underlined in yellow).

Phylogenetic analysis of TM homologues from *A. pegreffii* and other organisms, including *A. simplex*, *A. lumbricoides*, *O. volvulus*, *C. elegans*, *D. melanogaster*, *P. monodon*, *H. americanus*, *P. americana*, *H. diversicolor*, *P. camtschaticus*, humans and chickens, is shown in Fig. 4. Uniprot Accession IDs JF902160, FJ655903, L41633.1,

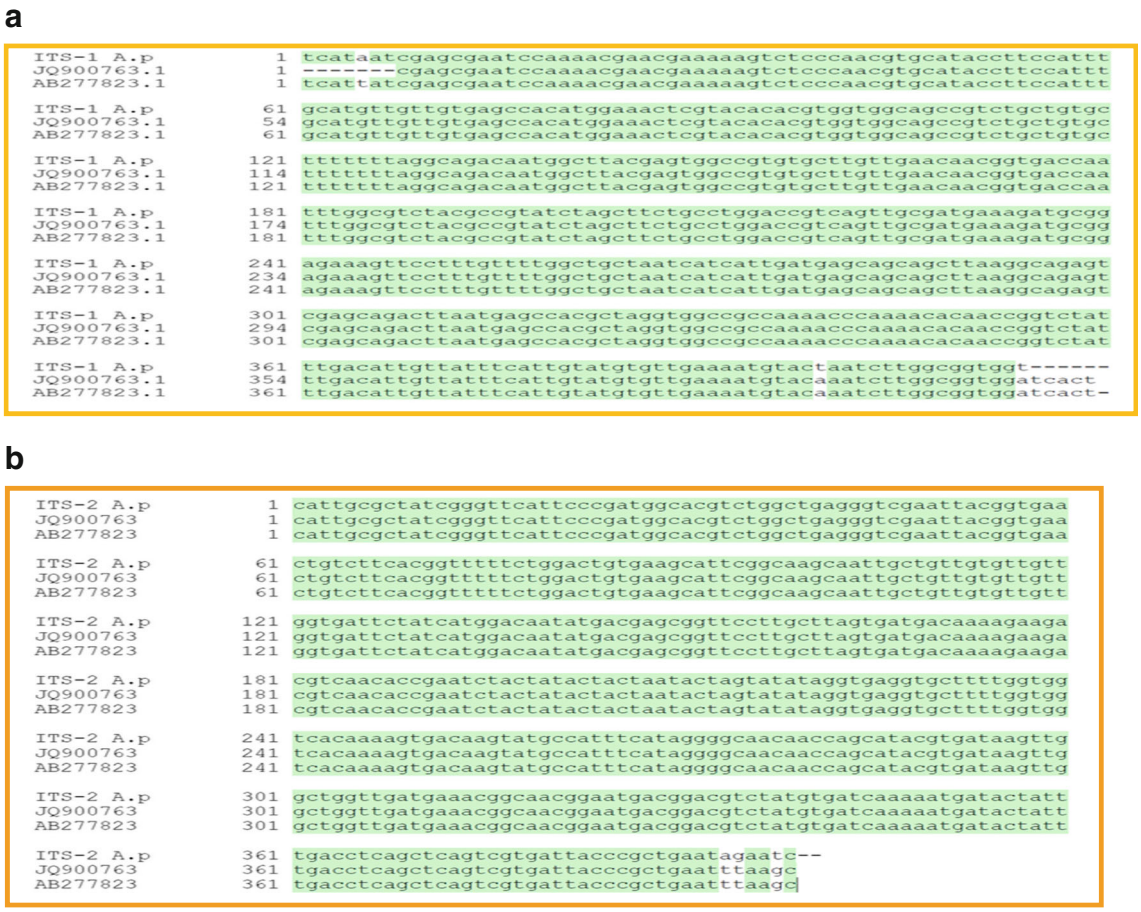

Fig. 1 **a** ITS-1 and **b** ITS-2 sequences of *Anisakis pegreffii* compared with two reference *A. pegreffi* sequences, JQ900763.1 (Mattiucci et al. 2013) and AB277823.1 (Umehara et al. 2008)

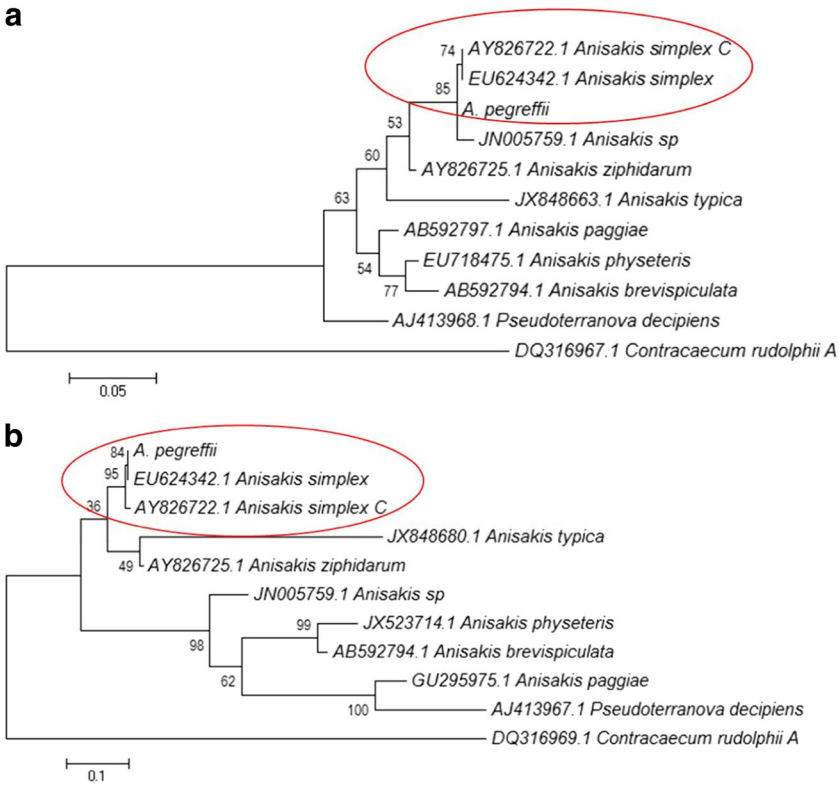

Fig. 2 The phylogenetic analysis of **a** ITS-1 and **b** ITS-2 sequences of *Anisakis pegreffii* and some members of the Anisakidae family

CELTMY1B, NM_169633, AY827100, AF034954.1, Y14854.1, AF216518.1, AB270632.1, J00910.1 and M19267.1, respectively, show that *Anisakis* TM form a unique clade, clustering closely with *A. lumbricoides*. Interestingly, the TM representing shellfish is more closely related to the TM of *Anisakis* spp. than to other nematodes such as *C. elegans* and *O. volvulus*, which may explain their cross-reactivity.

An independent analysis of the somatic extract of *A. pegreffii* by LC-MS/MS combined with a database search confirmed the presence of *A. pegreffii* TM with a match of 14 peptides and sequence coverage of 52% to *A. simplex* TM Ani

Fig. 3 **a** The cDNA and **b** the amino acid sequences encoding tropomyosin of *Anisakis pegreffii*. The signature patterns for TM are L-K-E-A-E-x-R-A-E, indicated in red (underlined), conserved in the C-terminal region of *A. pegreffii* TM, and the highly conserved N-terminal motif DAIKKK, which binds actin (underlined in yellow)

a Nucleic acid sequence

ATGGACGCAATCAAGAAGAAGATGCGCAAGGCAATGGAAATCGAGAAGGACAATGCC
CTCGATCGAGCCGATGCCGCCGAAGAGAAAGTCCGACAGATGACCGACAAGTTGGAG
AGAATCGAAGAGGAACTCCGAGACACACAAAAGAAGATGATGCAAACTGAGAATGAT
TTGGACAAAGCTCAAGAAGACCTTTCAACGGCCAACTCGAATCTGGAAGAGAAGGAA
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TCGAAGAGGAACTCGAAAGGGCTGAAGAACGTCTGAAATTGGCAACTGACAAACTTG
AAGAGGCGACACATACAGCTGATGAATCTGAGCGTGTGCGCAAGGTGATGGAGAACC
GCTCATTCCAAGATGAAGAGCGTGCGAATACGGTAGAATCACAACTCAAGGAAGCGC
AAATGCTTGCCGAAGAGGCTGATCGCAAATACGATGAGGTTGCCCGTAAATTGGCCAT
GGTTGAAGCGGATTTAGAAAGAGCTGAAGAGCGTGCAGAGGCCGGAGAAAATAAGAT
CGTTGAATTGGAAGAGGAATTGCGAGTCGATGGTAACAACTTGAAATCGTTGGAGGTT
TCTGAAGAGAAGGCTTTGCAACGAGAGGATTCATACGAAGAACAGATCCGTACCGTTT
CTGCACGTCTCAAGGAGGCCGAAACTCGTGCCGAATTTGCAGAAAGATCCGTTCAGAA
ACTGCAGAAGGAAGTTGACAGACTCGAAGACGAACTCGTCCACGAGAAAGAGCGATA
CAAGAGCATTTCTGAGGAACTCGATCTACTCCAAGAGCTGTCTGGATATTAATA

b Protein sequence

M D A I K K K M R K A M E I E K D N A L D R A D A A E E K V R Q M T D K L E R I E E E
L R D T Q K K M M Q T E N D L D K A Q E D L S T A N S N L E E K E K K V Q E A E A E V
A A L N R R M T L L E E E L E R A E E R L K L A T D K L E E A T H T A D E S E R V R K V
M E N R S F Q D E E R A N T V E S Q L K E A Q M L A E E A D R K Y D E V A R K L A M V
E A D L E R A E E R A E A G E N K I V E L E E E L R V D G N N L K S L E V S E E K A L Q
R E D S Y E E Q I R T V S A R L K E A E T R A E F A E R S V Q K L Q K E V D R L E D E L
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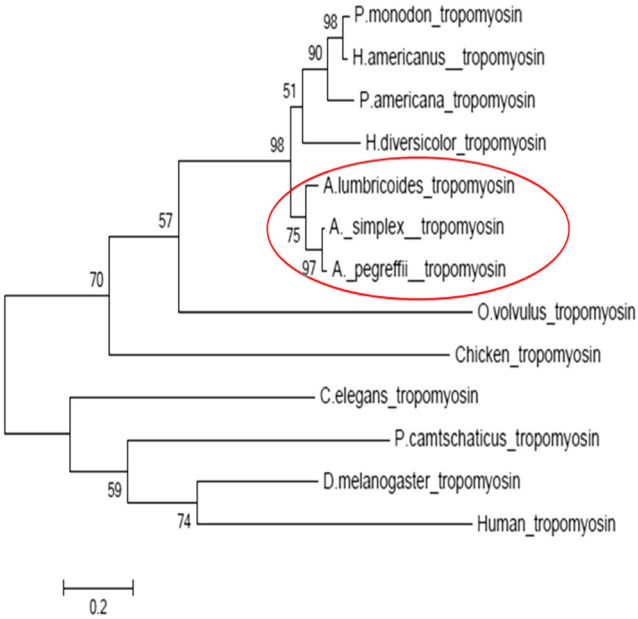

Fig. 4 Phylogenetic analysis of tropomyosin homologues of *Anisakis pegreffii* and other organisms, including *Anisakis simplex*, *Ascaris lumbricoides*, *Onchocerca volvulus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Penaeus monodon* (giant tiger prawn), *Homarus americanus* (American lobster), *Periplaneta americana* (American cockroach), *Haliotis diversicolor* (coloured abalone), *Paralithodes camtschaticus* (red king crab), *Homo sapiens* (human) and *Gallus gallus domesticus* (chicken)

s 3 (accession number: Q9NAS5) and a match of two peptides and sequence coverage of 15% to TM of *O. volvulus* (*Ov-tmy-1*; accession number: Q25632; Fig. 5**)**.

To localise TM in *A. pegreffii*, L3 larvae were subjected to immunofluorescent staining with an anti-TM polyclonal antibody. This staining revealed preferential binding to and intense labelling of native TM in the cuticle of *A. pegreffii* L3 larvae (Fig. 6).

SDS-PAGE and immunoblot analyses of *A. pegreffii* and/or shellfish proteins

The analysis of native extracts from shellfish and *A. pegreffii* showed a variety of proteins ranging from 10 to 250 kDa (Fig. 7a, b). Whole protein extracts from shellfish and *A. pegreffii* showed many intense bands significantly in the size between 30 and 50 kDa on a typical SDS-PAGE gel. Distinct protein bands at 37–39 kDa were observed for the shellfish extracts. The rTM protein of *A. pegreffii* runs slightly slower than the predicted molecular weight (Fig. 7b, c).

In the immunoblot analysis, a distinct band of 37 kDa was detected in crustacean extract using the monoclonal anti-TM antibody, which represented a positive control (Fig. 8a). However, no band was detected in *A. pegreffii* extract, by this antibody, even using a prolonged exposure time. Using anti-TM crustacean polyclonal antibody, a distinct band of 37 kDa was observed in the crustacean TM (Fig. 8a) and a single band of the same size was detected in the crude extract of *A. pegreffii* (lane 6; Fig. 8b). The anti-TM crustacean polyclonal antibody, in addition, also recognised a band of ~ 37 kDa in *A. pegreffii* extract (lane 6; Fig. 8c). A subsequent immunoblot analysis of rTM of *A. pegreffii* probed with sera from patients with shellfish allergy revealed IgE antibody binding (Fig. 9). Most of the sera (60%) showed some or faint reactivity.

Discussion

Consumption of raw or undercooked seafood contaminated with *Anisakis* L3s can result in anisakiasis and/or allergic reaction in humans (Daschner et al. 2002). Such allergic responses is characterised by high-level production of IgE antibodies to both somatic and/or excretory/secretory proteins of *Anisakis* L3s. Diagnostic testing is by detection of specific IgE antibody binding to crude extract of parasite or the recombinant proteins derived from such allergen genes.

TM is the major allergen protein of invertebrates, which has epitopes, featuring high cross-reactivity among invertebrate TMs from other species and which is suspected to elicit hypersensitivity reactions in atopic individuals. Due to its wide distribution, it is considered a pan-allergen (Reese et al. 1999; Acevedo and Caraballo, 2011).

In this study, immunoblotting results revealed that the recombinant *A. pegreffii* TM protein was not recognised by an anti-TM monoclonal antibody. Thus, we demonstrated that the specific epitope, to which the crustacean anti-TM monoclonal antibody reacts, is distinct in sequence from *Anisakis* TM, which is consistent with the findings from

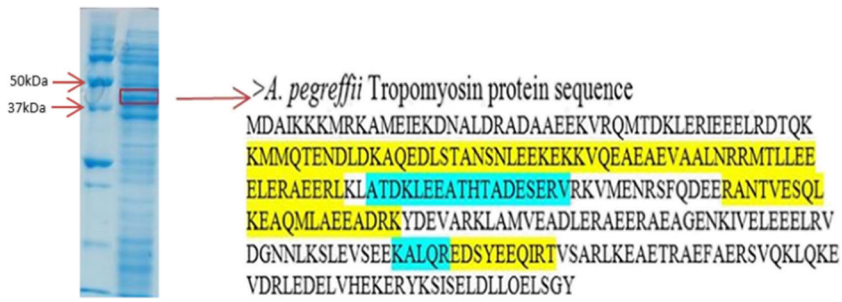

Fig. 5 Peptides of tropomyosin protein identified by LC-MS/MS from somatic extracts of *A. pegreffii*. Yellow, peptides matching *Anisakis simplex* tropomyosin protein; green, peptides matching *Onchocerca volvulus* tropomyosin protein

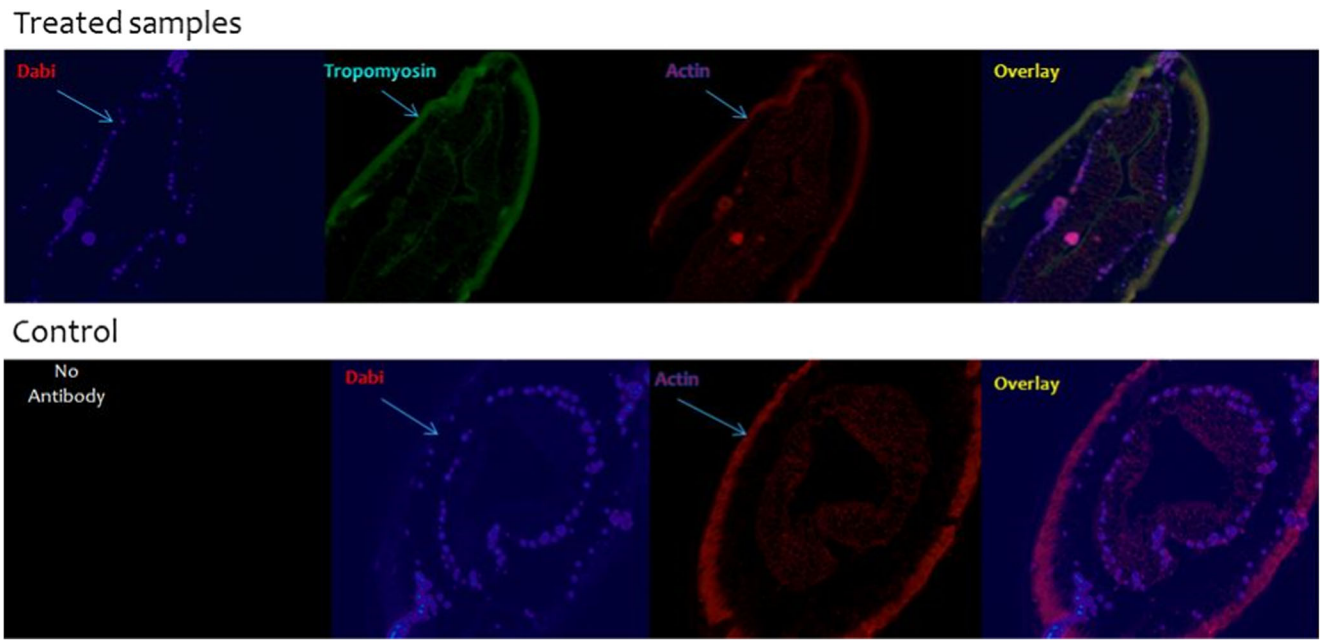

Fig. 6 Localisation of tropomyosin protein in the cuticle of *Anisakis pegreffii* L3 larvae by immunofluorescent staining. Treated samples = sections incubated with anti-tropomyosin antibody

the phylogenetic analysis (Fig. 4). By contrast, immunoblotting using anti-TM and anti-crustacean polyclonal antibodies showed reactivity with *A. pegreffii* TM. Polyclonal antibodies usually recognise multiple epitopes of a protein. Hence, overall reactivity, with the use of a polyclonal antibody, may not be greatly affected by small changes in the structure of single or a small number of epitopes (Lipman et al. 2005). In addition, to confirm that the rTM of *A. pegreffii* retained the allergenic characteristics of natural *A. pegreffii* TM, IgE in sera from shellfish allergic patients reacted with rTM of *A. pegreffii*. This result agrees strongly with other studies that have reported sera reaction from allergic patients with *A. simplex* TM (Acevedo and Caraballo, 2011). Although TM of *A. pegreffii* did not react with all patient sera, this result validates cross-reactivity between TM of *A. pegreffii* and shellfish allergic patient sera. The close relatedness of some of these species can be observed in the phylogenetic tree (Fig. 4). Phylogenetic analysis showed that TM of *A. pegreffii* has closer relationship to TM from other nematodes and crustaceans but

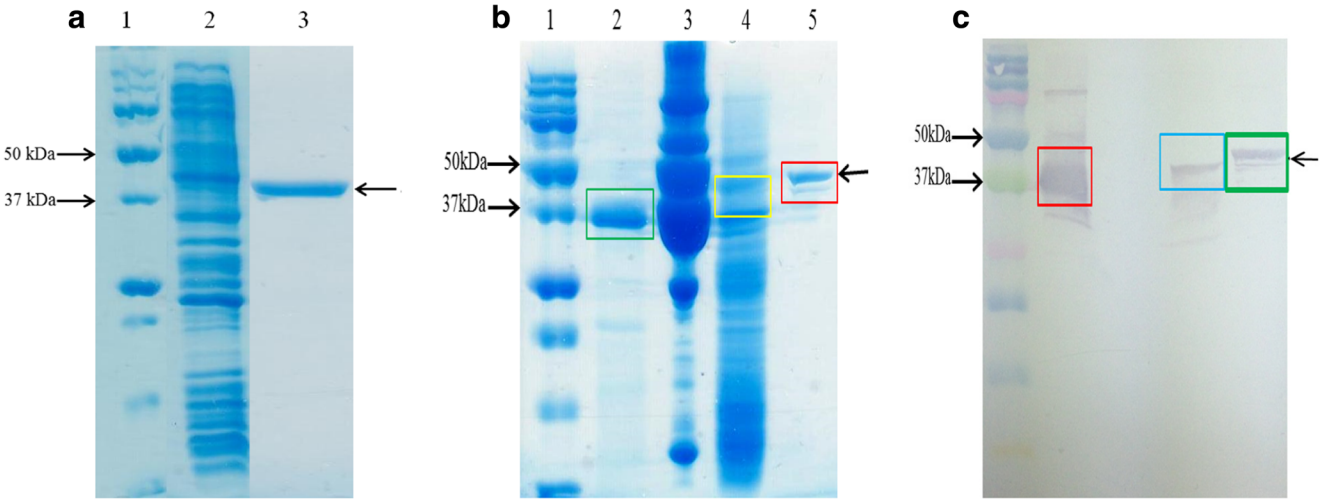

Fig. 7 **a** SDS-PAGE gel of the recombinant tropomyosin protein of *Anisakis pegreffii*. Lane 1 = molecular marker, lane 2 = crude extract of *Anisakis pegreffii*, lane 3 = recombinant tropomyosin of *Anisakis pegreffii*. **b** visualisation of the sizes of recombinant tropomyosin proteins of a crustacean and *Anisakis pegreffii* using SDS-PAGE. **c** Immunoblot analysis using anti-tropomyosin polyclonal antibody. Lane 1 = molecular marker, lane 2 = positive control (recombinant tropomyosin of black tiger 37 kDa), lane 3 = fish extract protein (negative control), lane 4 = crude extract of *Anisakis pegreffii* (41 kDa), lane 5 = recombinant tropomyosin protein of *Anisakis pegreffii* (44 kDa)

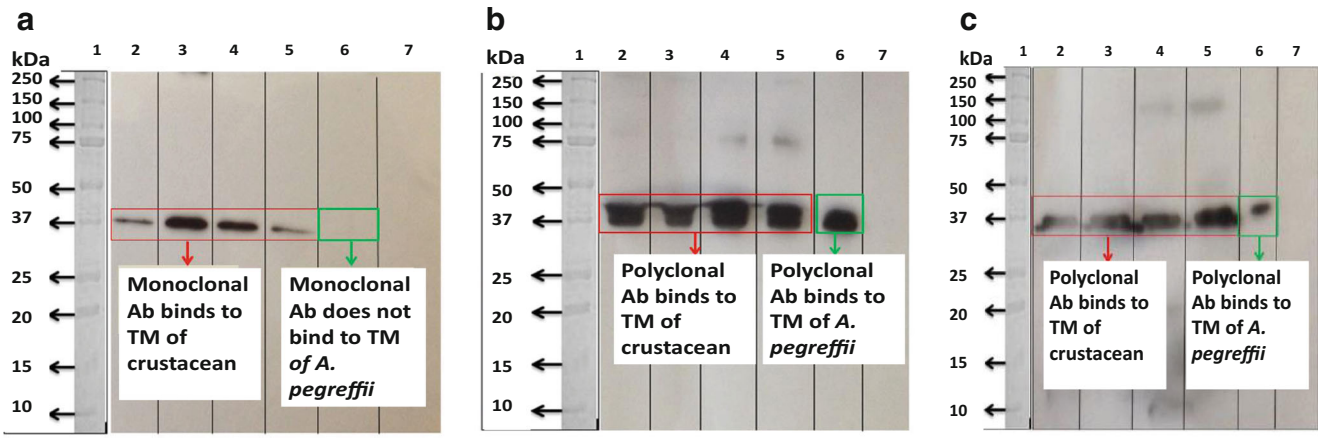

Fig. 8 Immunoblot analysis with (**a**) Monoclonal (**b**) Polyclonal anti–tropomyosin antibodies and (**c**) Polyclonal anti-crustacean antibody against *Anisakis pegreffii* crude extract. L = Lane; L1 = Molecular marker, L2 = Black Tiger (positive control), L3 = King Prawn (positive control), L4 = Green prawn (positive control), L5 = Banana prawn (positive control), L6 = *Anisakis pegreffii*, L7 = Phosphate buffered saline (PBS; negative control)

revealed less similarity to TM from vertebrates (chickens and humans). These results are in agreement with the proposal that TM is a pan-allergen.

We localised TM in *A. pegreffii* for the first time using an immunohistochemistrical approach. TM was detected in the muscles and under the basal layer of the cuticle of *A. pegreffii* L3 larvae using anti-TM polyclonal antibodies. This finding agrees with several studies that have reported the detection of TM in the muscles of parasites (Jenkins et al. 1998; Macgregor and Shore 1990). rTM protein was observed to be larger than the native one and migrated slower on SDS-PAGE than its predicted mass of 37 kDa. The slower run is a consequence of the higher mass of the recombinant protein due to the N-terminal extension encoded by the expression vector.

Sequence similarity searches showed that *A. pegreffii* TM has the highest identity to the alpha-TM isoform from L3s of *A. simplex* (98%) and *A. lumbricoides* (84%), respectively. The deduced amino acid sequence of *A. pegreffii L3* TM has revealed that the TM signature, L-K-E-A-E-x-R-A-E, is conserved in the C-terminal region at position 231–239, as is the highly conserved N-terminal motif D-A-I-K-K-K, which is significant for actin binding. Findings from this study agree with several studies reporting similar results in other organisms (Asturias et al. 2000; Santos et al. 1999; Santos et al. 2008). Furthermore, a potential N-linked glycosylation site N-R-S in TM of *A. pegreffii* was identified from the data obtained from this study.

The protein sequence of TM from *A. pegreffii* L3 showed a sequence of seven heptad-repeating amino acids residues

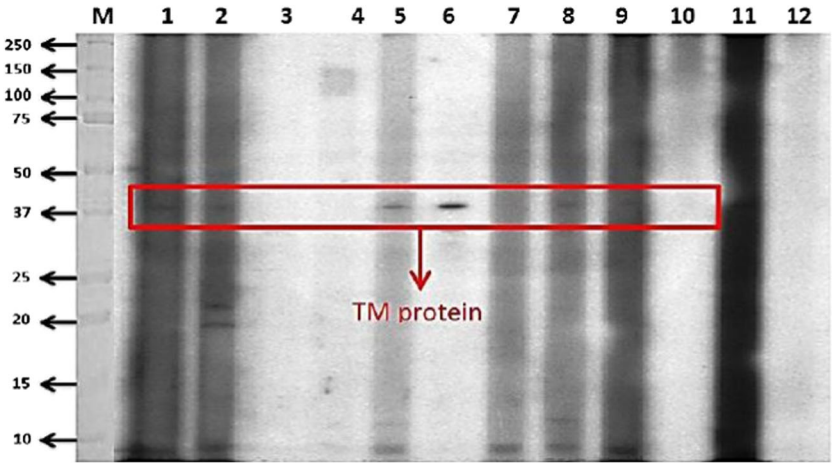

Fig. 9 Immunoblot analysis of recombinant tropomyosin of *Anisakis pegreffii* probed with sera from shellfish-allergic patients. Keys: L = lane, M = molecular marker, L1–10 = patients' sera (DM32, GC10, AN01, CF 31, JP47, CF31, DG46, KF41 and EV09, respectively), L11 = fish extract, L12 = negative control. This figure shows *Anisakis pegreffii* recombinant protein tropomyosin allergen at 44 kDa in L1, 2, 5, 6, 7, 8 and 9. The positive bands are shown in the red box

usually labelled *abcdefg*. Results similar to this, were obtained by Asturias et al. (2000) who investigated the stage 3 larvae of *A. simplex* rTM and showed that in the sequence 'a' and 'd' amino acids, are the hydrophobic positions of each chain. Interactions of these amino acids with each other result in the parallel α-helical coiled coil chain for TM (Mason and Arndt, 2004). This report presents evidence of high indications of α-helical coiled-coil structures of *A. pegreffi*'s rTM. This study confirms cross-reactivity between *A. pegreffii* and shellfish TM. It also demonstrates that the specific epitope to which the crustacean anti-TM monoclonal antibody reacts is not conserved in *Anisakis*; however, the nematode TM tested share one or more epitopes with crustacean TM, as polyclonal antibodies can recognise TM from both species.

In conclusion, we have confirmed the presence of TM protein in *A. pegreffi* with anti-TM crustacean polyclonal antibodies. In contrast, *A. pegreffii* TM was not recognised by immunoblotting with an anti- crustacean TM monoclonal antibody (MAb). This information suggests that *A. pegreffi* TM share one or more epitopes with crustacean TM, as the PAbs is able to recognise multiple epitopes on any one antigen, while they lack specific antigenic determinant, defined by the anti-TM monoclonal antibody present in crustacean TM. The result of this study showed that the size of *A. pegreffii* -TM is similar with previously studied *A. simplex* and *A. lumbricoides* TM, as may be expected by the close relationship between these species. Furthermore, results obtained from this study indicate that the rTM of *A. pegreffii* binds to IgE from different sera samples of crustacean allergic patients confirming cross reactivity between nematodes and different classes of shellfish. Previous studies had shown the function of TM protein to be associated with motility. In the current study, we substantiated the evidence of the association of TM proteins with motility role by determining its localisation in *A. pegreffii* by immunohistochemistry. This showed *A. pegreffii* -TM to be located in both the muscles and under the basal layer of the cuticle in the *A. pegreffii* L3 anatomy. Future studies should explore the cross-reactivity of the allergenic protein repertoire of *Anisakis* in more detail in order to work toward improving diagnosis of allergenic diseases in humans.

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Compliance with ethical standards Informed, written consent was obtained from all study subjects, and experimental protocols were approved by The Alfred Hospital Research Ethics Committee (Project number 192/07) and the Monash University Human Ethics Committee (MUHREC CF08/0225).

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