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Molecular characterization of anisakid nematode larvae from 13 species of fish from Western Australia

Abdul Jabbar [a,*], Rachel W.J. Fong [a], Kia X. Kok [a], Andreas L. Lopata [b,c], Robin B. Gasser [a], Ian Beveridge [a]

[a] *Faculty of Veterinary Science, The University of Melbourne, Werribee, Victoria 3030, Australia*
[b] *School of Pharmacy and Molecular Sciences, James Cook University, Townsville, 4811 Queensland, Australia*
[c] *Centre for Biodiscovery and Molecular Development of Therapeutics, Queensland, Australia*

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ABSTRACT

This study characterized anisakid nematodes in estuarine and near-shore species of fish in southern Western Australia. A total of 108 fish representing 13 species were examined for anisakid larvae. For the molecular characterization of anisakid larvae ($n=218$), we used PCR–coupled mutation scanning–sequencing–phylogenetic analyses of sequence variation in the internal transcribed spacers of nuclear ribosomal DNA. With the exception of *Sillaginoides punctatus* and *Sillago schomburgkii*, all the fish species examined (*Aldrichetta forsteri*, *Arripis georgianus*, *Hyporhamphus regularis*, *Mugil cephalus*, *Platycephalus speculator*, *Pomatomus saltatrix*, *Pseudocaranx dentex*, *Pseudocaranx wrighti*, *Thysanophrys cirronatus*, *Trachurus novaezeelandiae* and *Upeneichthys lineatus*) harboured at least one species of anisakid. Mutation scanning analysis identified 11 different genotypes of anisakid larvae. Phylogenetic analyses of the sequence data, employing reference sequence data for a wide range of anisakids (31 species) from public databases, revealed the presence of *Anisakis pegreffii* ($n=3$), *Contracaecum multipapillatum* (49), *Contracaecum ogmorhini* (1), *Hysterothylacium* larval type IV (82), *Hysterothylacium* larval type Vb (14), *Hysterothylacium* larval type VIII (3), *Hysterothylacium* larval type X (65), and *Terranova* type I (1) in the fish examined. The present study provides valuable information on the diversity of anisakids in southern Western Australia and also a basis for future investigations to assess the public health significance of these parasites.

1. Introduction

Food-borne diseases (FBDs) include a wide spectrum of illnesses and represent a growing public health concern worldwide. FBDs can be caused by microbial pathogens, parasites, chemical contaminants and/or biotoxins (WHO, 2006). Parasitic FBDs are generally under-recognized; however, awareness of these diseases is increasing. Recently, the WHO (2007) formed a task force charged with estimating the disease burden due to infections, particularly parasitic diseases. One of the key groups of parasites contributing towards parasitic FBDs is *Anisakis*. Larval forms of this genus, together with related genera, such as *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*, cause a disease known as anisakidosis. Although members of the genus *Anisakis* are recognized to represent the most common causes of anisakidosis (Van Thiel, 1962; Daschner et al., 2000; Dominguez-Ortega et al., 2001; Audicana et al., 2002; Audicana and Kennedy, 2008), the related genera of anisakid nematodes have also been reported to infect humans (Oshima, 1987; Yagi et al., 1996; Shamsi and Butcher, 2011).

The life cycle of anisakids is indirect and quite complex. Definitive hosts (marine mammals, birds or teleosts) become infected when they ingest either infected intermediate (euphausiids or copepods) or paratenic hosts (fish or cephalopods) harbouring the infective third-stage larvae (L3s), in which two moults occur, before they develop to dioecious adults and produce eggs (Audicana et al., 2002; Mattiucci and Nascetti, 2006, 2008). Anisakidosis occurs when people accidentally ingest L3s in infected raw, undercooked or improperly cured, smoked or processed fish or squid, and can cause abdominal discomfort (colic), nausea, vomiting, diarrhoea, cutaneous swellings, urticaria and even a life-threatening, anaphylactic shock or chronic, debilitating conditions (Van Thiel, 1962; Alonso et al., 1997; Daschner et al., 2000; Dominguez-Ortega et al., 2000, 2001; Audicana et al., 2002; Audicana and Kennedy, 2008).

In addition, allergic sensitization to anisakids is frequently reported due to ingestion or handling of contaminated fish products (Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Lopata and Lehrer, 2009).

In Australia, our knowledge of the prevalence and abundance of different species of anisakid nematodes is very limited. Using morphological characteristics, a few studies have reported larval anisakid nematodes from different states, including New South Wales (Hooper, 1983; Nash, 1998), Queensland (Cannon, 1977a,b; Bruce and Cannon, 1989; Bruce, 1990a,b) and Western Australia (Lymbery et al., 2002; Doupe et al., 2003); however, in these studies, anisakids had been identified only to

* Corresponding author. Tel.: +61 3 9731 2022; fax: +61 3 9731 2366.
E-mail address: jabbara@unimelb.edu.au (A. Jabbar).

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the genus level (Cannon, 1977a,b; Hooper, 1983; Bruce and Cannon, 1989; Bruce, 1990a,b; Nash, 1998). With the advent of molecular approaches, it is now possible not only to identify but also to delimit anisakid nematodes to the species level and also to reveal cryptic species (Mattiucci and Nascetti, 2006, 2008). A number of studies have demonstrated that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA (rDNA) provide suitable genetic markers for the identification of anisakid species, irrespective of their developmental stage (Zhu et al., 1998; Zhu et al., 2001; Zhang et al., 2007; Shamsi et al., 2008, 2009a,b, 2011a,b; Jabbar et al., 2012a,b), and PCR–coupled mutation scanning of the ITS-1 and/or ITS-2, combined with targeted sequencing (Gasser et al., 2006) and phylogenetic analysis (Jabbar et al., 2012a,b), provides a powerful approach for exploring the genetic composition of anisakid populations and for investigating their biology.

In the last five years, using combined morphological and molecular approaches, some studies have described various genera, species and even cryptic species of different anisakid nematodes from various parts of Australia (Shamsi et al., 2008, 2009a,b, 2011a,b; Shamsi and Butcher, 2011; Jabbar et al., 2012a,b). However, these studies were limited mainly to eastern as well as south-eastern parts of the country. Despite these investigations, our knowledge of the diversity, prevalence and genetic composition of anisakid nematodes in other parts of the country, viz. Northern Territory, South Australia and Western Australia, is limited, apart from the studies of Lymbery et al. (2002) and Doupe et al. (2003). Therefore, the aim of the present study was to genetically characterize the larval anisakid nematodes present in estuarine and near-shore fish species in southern Western Australia, employing established integrated PCR–coupled analyses of sequence variation in the internal transcribed spacers (ITS) of nuclear ribosomal DNA.

2. Materials and methods

2.1. Study area and parasite collection

In November 2011, 108 fish representing 13 important commercial and recreational estuarine and near-shore species (*Aldrichetta forsteri* [number of fish examined = 9], *Arripis georgianus* [10], *Hyporhamphus regularis* [10], *Mugil cephalus* [5], *Platycephalus speculator* [5], *Pomatomus saltatrix* [4], *Pseudocaranx dentex* [10], *Pseudocaranx wrighti* [10], *Sillaginoides punctatus* [10], *Sillago schomburgkii* [10], *Thysanophrys cirronatus* [5], *Trachurus novaezeelandiae* [10] and *Upeneichthys lineatus* [10]), caught in the Indian Ocean off the coast of southern Western Australia, were purchased from commercial fish suppliers in Fremantle (32°03′ S 115°44′ E), Western Australia. Each fish was dissected according to an established protocol (Cribb and Bray, 2010) and only the body cavity was examined for the presence of anisakid nematodes. These nematodes were collected and washed extensively in physiological saline (pH 7.4), and stored in 70% ethanol at −20 °C for subsequent identification. Larval nematode prevalence (percentage of fish infected), and mean intensity (mean number of parasites per infected fish) were calculated.

2.2. Morphological identification

Each anisakid larva was dissected and a small portion of the mid-body of each nematode was excised and stored in 70% ethanol at −20 °C for molecular study, while the remainder of each nematode was then cleared in lactophenol for morphological examination. Anisakid larvae were identified to genus according to established morphological characters (Cannon, 1977b; Deardorff and Overstreet, 1981; Shamsi, 2007). Representatives of each morphological nematode type, corresponding to each genotype defined in the present study, have been deposited (under registration numbers AHC46384–AHC46402) in the South Australian Museum, Adelaide, Australia.

2.3. Molecular characterization

2.3.1. Isolation of genomic DNA and PCR amplification

Genomic DNA was isolated from individual larvae by suspending them in DNA extraction buffer (20 mM Tris–HCl [pH 8.0], 100 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl-sulphate) containing 10 mg/ml proteinase K (Amresco LLC, Solon, OH, USA) and isolated from the homogenised suspension using a mini-column (Wizard®DNA Clean-Up System, Promega, Madison, WI, USA) according to the manufacturer's protocol.

Two nuclear ribosomal loci were PCR-amplified using the primers SS1/NC13R (ITS-1) and SS2/NC2 (ITS-2), under the same conditions as described previously (Jabbar et al., 2012a,b). For each set of PCRs, negative (no-DNA) and known positive controls were included. Following PCR, an aliquot of 5 μl of each amplicon was examined on a 1.5% w/v agarose gel stained with ethidium bromide and photographed.

2.3.2. Single-strand conformation polymorphism (SSCP) and sequencing

For both ITS-1 and ITS-2, amplicons were subjected (separately) to SSCP analysis (Gasser et al., 2006; protocol B) to display sequence variation within and among amplicons, as described previously (Jabbar et al., 2012a). For each locus, amplicons representing each unique SSCP profile were selected, treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., Glen Burnie, Maryland, USA), and subjected to bidirectional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, Foster City, California, USA) using (separately) the same primers employed in PCR. The quality of each sequence was assessed by appraising its electropherogram using the programme BioEdit (Hall, 1999). Polymorphic sites were designated using International Union of Pure and Applied Chemistry codes.

2.4. Phylogenetic analyses

Prior to phylogenetic analyses, sequence types defined herein for each locus (ITS-1 and ITS-2) were subjected (separately) to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) to establish the 'top hits' to all nucleotide sequences available in current databases and identities (in %) calculated by pairwise comparisons. Subsequently, the consensus sequence was aligned with a selected subset of closely related reference sequences (*Anisakis pegreffii*, selected species of *Contracaecum*, *Hysterothylacium*, *Pseudoterranova* and *Terranova* as well as *Raphidascaris trichiuri* [outgroup] (Zhu et al., 1998; Damin and Heqing, 2001; Szostakowska et al., 2001; Zhu et al., 2001; Abe et al., 2005; Nadler et al., 2005; Zhang et al., 2007; Zhu et al., 2007; Shamsi et al., 2008; Umehara et al., 2008; Kijewska et al., 2009; Quiazon et al., 2009; Quiazon et al., 2011; Shamsi et al., 2011b; Jabbar et al., 2012a,b)) using the programme Clustal X (Thompson et al., 1997), and alignments were adjusted manually. Phylogenetic analyses were performed on individual (ITS-1 or ITS-2) or concatenated (ITS-1 + ITS-2) sequence datasets. Each concatenated sequence was derived from the same individual nematode. Phylogenetic analysis of nucleotide sequence data was conducted by Bayesian inference (BI), employing the Markov chain Monte Carlo (MCMC) method in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criteria (AIC) test in Modeltest v3.7 (Posada and Crandall, 1998). For all three datasets, AIC revealed the general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR + Γ + I), as the 'best' model. Estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100th tree being saved. At this point, the potential scale reduction factor approached one, and the standard deviation of split frequencies was <0.01. A consensus tree (50% majority rule) was constructed based on the final 75% of trees generated by BI. Sequence data were also analyzed using the Neighbour-Joining (NJ) method