



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

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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

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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 bi-directional, 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

employing PAUP (PAUP 4.0b10) where molecular distances were estimated by the general time-reversible model of evolution and the nodes were tested for robustness by 100,000 bootstrap replicates (Felsenstein, 1985). Phylogenetic trees constructed using the BI and NJ methods were examined for concordance in topology.

3. Results

3.1. Prevalence of larval anisakid nematodes

A total of 108 fish was examined for larval anisakid nematodes. Larvae were found within the liver, visceral organs, mesenteries and peritoneum of 46.3% (50/108) of fishes. Larval anisakid prevalence, and their mean intensity per infected fish varied considerably among different fish species (see Table 1), with no larval nematodes recovered from *S. punctatus* or *S. schomburgkii* and 100% infection in *M. cephalus*, *P. saltatrix* and *T. novaezeelandiae*.

3.2. Morphological identification

Morphological examination showed that the anisakid larvae found in the present study represented *Anisakis* larval type I (L3), *Contracaecum* larval types I and II (L3), *Hysterothylacium* larval type IV (L4) and *Terranova* type I (L3) of Cannon (1977b) as well as *Hysterothylacium* larval types Vb, VIII and X (L3), as described by Shamsi (2007).

3.3. Mutation scanning analysis

Since anisakid larvae cannot be identified morphologically to species, SSCP analysis of ITS-1 and ITS-2 was conducted for 218 anisakid specimens. Eleven genotypes (i.e. nine for ITS-1 and 11 for ITS-2; designated genotypes A–G) were detected by SSCP analysis for all 218 samples (Table 2). Selected amplicons ($n = 1–5$) representing each of these 9–11 SSCP profiles were subjected to sequencing. The consensus lengths of individual ITS-1 and ITS-2 sequence types, mean nucleotide frequencies, polymorphic positions (if any), G + C content and respective accession numbers are listed in Table 2.

3.4. Phylogenetic analyses and host associations

In order to identify the anisakid larvae using ITS sequence data and to verify morphological identities, the concatenated ITS-1 and ITS-2 data for all 11 genotypes detected herein (GenBank accession nos. KC437336–KC437352) were compiled, together with key reference data (comprising concatenated sequences from previous studies representing *A. pegreffii*, selected species of *Contracaecum*, *Hysterothylacium*, *Pseudoterranova* and *Terranova*, using *R. trichiuri* as the outgroup). The concatenated sequences ($n = 65$) were aligned over 831 positions and subjected to phylogenetic analyses. The analyses

grouped three genotypes (B–D) with *Contracaecum multipapillatum*, while one genotype (E) clustered with *Contracaecum ogmorhini* with strong nodal support ($pp = 1.0$; bootstrap value = 100%) (see Fig. 1). Genotype F grouped with *Terranova* type I, and two genotypes (G, H) clustered with *Hysterothylacium* type IV of Shamsi (2007), whereas one genotype (I) grouped with *Hysterothylacium* type V of Shamsi (2007) with strong statistical support (1.00; 100%) (Fig. 1). Genotypes J and K grouped with *Hysterothylacium* types VIII and X of Shamsi (2007), respectively, and genotype A grouped with published sequences representing *A. pegreffii* (see Fig. 1). Separate analyses of ITS-1 and ITS-2 sequence datasets produced trees with identical topologies to that generated for the concatenated ITS-1 and ITS-2 datasets (not shown). In addition, the topology of the trees constructed using the two different algorithms was the same, with some minor variation in nodal/bootstrap support values (see Fig. 1). The associations of the studied larval anisakid nematodes with their different host fish species is presented in Table 3. Host specificity of these nematode was variable as some of them were found exclusively in one fish species (e.g., *A. pegreffii*, *C. ogmorhini*, *Hysterothylacium* type Vb and *Terranova* type I), whereas others in more than one species of fish (*C. multipapillatum*, *Hysterothylacium* types IV, VIII and X) (see Table 3).

4. Discussion

Anisakidosis in humans is a well known disease resulting from accidental infestation with larvae of certain genera of anisakids, causing severe gastrointestinal disorders, allergic reactions and even death (Van Thiel, 1962; Alonso et al., 1997; Daschner et al., 2000; Dominguez-Ortega et al., 2000, 2001; Audicana et al., 2002; Lopata and Lehrer, 2009). The accurate identification of anisakid nematodes is of paramount importance in relation to the diagnosis of infection, epidemiological studies and control. In the present study, we used both molecular-phylogenetic and morphological approaches to characterize larval anisakid nematodes from 13 fish species collected from southern Western Australia, and we found species of *Anisakis*, *Contracaecum*, *Hysterothylacium* and *Terranova*.

In the present study, based on morphological characteristics of larval anisakid nematodes, we found eight different nematodes, whereas PCR-coupled mutation scanning analysis revealed nine (for ITS-1) to eleven (for ITS-2) profiles representing 11 distinct genotypes following sequencing. We identified two *Contracaecum* species in three fishes, including *A. forsteri*, *M. cephalus* (*Contracaecum* type I) and *P. dentex* (*Contracaecum* type II). However, the results of a previous survey for anisakid nematodes, in the same locality, showed that only *Contracaecum* type I was prevalent in four of 17 fishes examined (Lymbery et al., 2002). The prevalence of *Contracaecum* type I in different types of fishes reported by Lymbery et al. (2002) and that found in the present study was similar. In addition, Lymbery et al. (2002) found heavy infections in two species of mullets (*A. forsteri*

Table 1
Prevalence and intensity of infection of fish examined for larval anisakid nematodes.

Fish species	Common name	Prevalence (%)	Intensity of infection (mean)	No of larvae examined
<i>Aldrichetta forsteri</i>	Yellow-eye mullet	88 (8/9) ^a	0–5 (2.75)	21
<i>Arripis georgianus</i>	Tommy rough	10 (1/10)	0–3 (0.30)	3
<i>Hyporhamphus regularis</i>	Western river garfish	50 (5/10)	0–8 (1.80)	17
<i>Mugil cephalus</i>	Flathead grey mullet	100 (5/5)	2–13 (5.60)	28
<i>Platycephalus speculator</i>	Blue spotted flathead	20 (1/5)	0–1 (0.20)	1
<i>Pomatomus saltatrix</i>	Tailor	100 (4/4)	1–4 (2.50)	10
<i>Pseudocaranx dentex</i>	White trevally	50 (5/10)	0–6 (1.60)	17
<i>Pseudocaranx wrighti</i>	Sand trevally, Skipjack trevally	40 (4/10)	0–7 (1.60)	14
<i>Sillaginodes punctatus</i>	King George whiting	0 (0/10)	–	–
<i>Sillago schomburgkii</i>	Yellow finned whiting	0 (0/10)	–	–
<i>Thysanophrys cirronatus</i>	Tasselsnout flathead	40 (2/5)	0–3 (1.00)	5
<i>Trachurus novaezeelandiae</i>	Yellow-tailed scad	100 (10/10)	5–61 (22.60)	72
<i>Upeneichthys lineatus</i>	Red mullet	50 (5/10)	0–28 (6.00)	30

^a Number of infected fish/total number of fish examined.

Table 2

The classification of anisakid nematode specimens based on single-strand conformation polymorphism (SSCP) profiles for two loci (ITS-1 and ITS-2) used in the present study. The sequence linked to each unique SSCP profile is represented by its GenBank accession number, its length, polymorphism and G + C content. Mean nucleotide frequencies for the three main anisakids are also provided.

Genotype	ITS-1										ITS-2									
	SSCP profile (no. of samples with unique profile)	Accession no.	Length (bp)	Polymorphism ^a (alignment position)	GC content (%)	Mean nucleotide frequencies					SSCP profile (no. of samples with unique profile)	Accession no.	Length (bp)	Polymorphism ^a (alignment position)	GC content (%)	Mean nucleotide frequencies				
						A	C	G	T							A	C	G	T	
A	P1-1 (3)	KC437336	392	-	47.19	0.25765	0.22449	0.24745	0.27041	P2-1 (3)	KC437345	308	-	-	42.21	0.29221	0.17208	0.25000	0.28571	
B	P1-2 (48)	KC437337	443	-	44.47	0.26637	0.20137	0.24379	0.28847	P2-2 (47)	KC437346	231	-	-	42.42	0.27638	0.18525	0.23877	0.29960	
C	-do-	-do-	-do-	-	-	-	-	-	-	P2-3 (1)	KC437347	231	R (189)	-	42.42	-	-	-	-	
D	P1-3 (1)	KC437338	443	Y (104)	44.47	-	-	-	-	P2-4 (1)	KC437348	231	Y (180)	-	41.99	-	-	-	-	
E	P1-4 (1)	KC437339	451	-	50.78	0.22395	0.23947	0.26829	0.26829	P2-5 (1)	KC437349	277	-	-	47.29	0.22022	0.20007	0.27646	0.30325	
F	P1-5 (1)	KC437344	437	-	46.68	0.25172	0.21510	0.25172	0.28146	P2-6 (1)	KC437355	264	-	-	50.94	0.24719	0.19618	0.31907	0.23755	
G	P1-7 (82)	KC437341	438	-	51.14	0.21689	0.22374	0.28767	0.27169	P2-8 (80)	KC437351	345	-	-	54.20	0.16667	0.23478	0.30870	0.28986	
H	-do-	-do-	-do-	-	-	-	-	-	-	P2-9 (2)	KC437352	345	G (37)	-	54.49	-	-	-	-	
I	P1-8 (14)	KC437342	437	-	49.66	0.23799	0.22883	0.26773	0.26545	P2-10 (14)	KC437353	272	-	-	50.00	0.19118	0.20588	0.29412	0.30882	
J	P1-9 (3)	KC437343	431	-	49.88	0.22738	0.21810	0.28074	0.27378	P2-11 (3)	KC437354	348	-	-	50.29	0.18966	0.20977	0.29310	0.30747	
K	P1-6 (65)	KC437340	436	-	51.38	0.22018	0.22936	0.28440	0.26606	P2-7 (65)	KC437350	286	-	-	53.50	0.17832	0.23077	0.30420	0.28671	
Total	(larvae)		218									218								

^a Polymorphism for each sequence type was assessed by aligning these sequences with the reference sequences. R = A/G; K = G/T; S = C/G; Y = C/T.

and *M. cephalus*), which is also in concordance with our findings. We also found a high prevalence (88–100%) of *Contracaecum* type I in the specimens examined for these fishes (see Table 1). In addition to *Contracaecum* type I, we also found *Anisakis* larval type I, *Contracaecum* larval type II, *Hysterothylacium* larval type IV and *Terranova* type I of Cannon (1977b) as well as *Hysterothylacium* larval types V, VIII and X of Shamsi (2007) in the fishes studied, and these nematodes are documented for the first time in the southern part of Western Australia.

Molecular-phylogenetic analysis revealed that *Contracaecum* type I of Cannon (1977b) found herein was *C. multipapillatum* (see Fig. 1; genotypes B–D), as described previously by Shamsi et al. (2008, 2011b), the adults of which occur in pelicans. These authors, using a combined morphological and molecular approach, showed that *Contracaecum* type I of Cannon (1977b) represented two species (*C. multipapillatum* and *C. pyripapillatum*). Findings of the present study suggest that *Contracaecum* type I of Cannon (1977b) reported by Lymbery et al. (2002) may represent *C. multipapillatum*. The genetic characterization of *Contracaecum* type II indicated that the ITS-1 and ITS-2 sequences determined herein (genotype E) matched those of *C. ogmorhini*, as described previously (Zhu et al., 2001; Nadler et al., 2005; Shamsi et al., 2011b), the adults of which are found in seals. The ITS sequences determined herein (genotype F) for *Terranova* type I of Cannon (1977b) were identical to those of *Terranova* type I from Lizard Island in the Great Barrier Reef, Queensland, Australia, as reported previously by Jabbar et al. (2012b). Adults of *Terranova* occur as parasites of sharks. Based on morphological characteristics, four types of *Hysterothylacium* were identified; adults of species of this genus are found in large teleosts. However, their molecular characterization allowed the identification of five genotypes (G–K). Sequence data for *Hysterothylacium* larval type IV reported herein (genotypes G, I) matched those of *Hysterothylacium* larval type IVb of Shamsi (2007). Although *Hysterothylacium* larval types Vb reported herein resembled, based on morphological characteristics, *Hysterothylacium* larval type V of Shamsi (2007), the comparison of ITS-1 and ITS-2 sequences of these two morphotypes revealed that the sequences of *Hysterothylacium* larval type V (genotype I) matched those of *Hysterothylacium* larval type VI of Shamsi (2007) (see Fig. 1) and formed a sister clade with *Hysterothylacium* larval type V of Shamsi (2007). This discrepancy between morphological and molecular identification of the same larval type in two different studies cannot be readily explained, as we ensured that the sequence types being reported in this present study corresponded to the correct morphotypes (for more details about morphological descriptions see Jabbar et al., 2012b). The ITS sequences of *Hysterothylacium* larval types VIII and X reported herein (genotypes J and K) were identical to those of the corresponding types of Shamsi (2007). In the present study, ITS sequences of *A. pegreffii* (genotype A) matched those of previously published sequences of this species in Australia (Jabbar et al., 2012a) and other parts of the world (Abe et al., 2005; Zhang et al., 2007; Zhu et al., 2007; Umehara et al., 2008; Quiazon et al., 2009); adults of this species are found in small cetaceans.

The presence of *A. pegreffii* and *Contracaecum* species in the commercial and recreational fishes studied here raises human health concerns, as these anisakid nematodes have been found previously to be associated with human anisakidosis and allergic sensitization (Umehara et al., 2007; Lopata and Lehrer, 2009; Mattiucci et al., 2011; Shamsi and Butcher, 2011). A recent report of anisakidosis, caused by *Contracaecum* sp., from South Australia (Shamsi and Butcher, 2011) suggests an emerging importance of anisakidosis in Australia. Humans suffer from anisakidosis when they consume infected raw, undercooked or improperly processed fishes or squid, and a number of anisakid nematodes, including *A. simplex* (principal cause of anisakidosis), *A. pegreffii*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*, are known to be linked to this disease (Oshima, 1987; Audicana et al., 2002; Umehara et al., 2007; Lopata and Lehrer, 2009; Mattiucci et al., 2011; Shamsi and Butcher, 2011). Anisakidosis has been reported from five

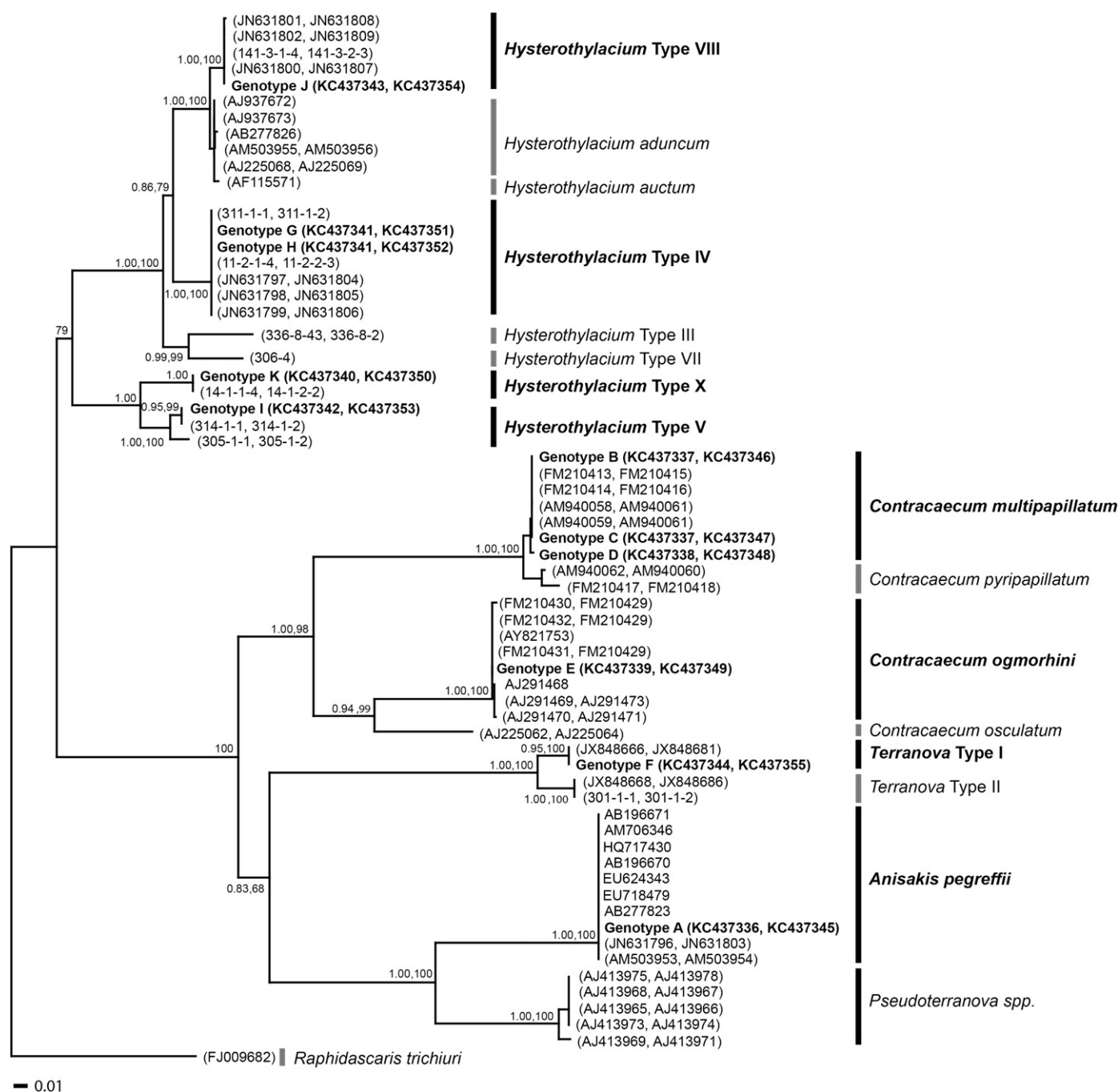


Fig. 1. Phylogenetic relationships of anisakid nematodes representing genotypes A–K, based on concatenated ITS-1 and ITS-2 sequence data determined herein, together with selected reference sequences for anisakid nematodes. Relationships were inferred based on analyses employing Bayesian Inference (BI) and distance-based Neighbour-Joining (NJ) methods. Nodal support is given as a posterior probability for BI (first) and bootstrap value for NJ (second). The scale bar indicates distance.

continents, including Asia (endemic foci mainly in south-east Asia), Africa, the Americas, Australia and Europe (Audicana et al., 2002; Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Shamsi and Butcher, 2011), and it is likely that the incidence of this disease will increase as the consumption of raw and/or under-cooked seafood (e.g., sushi and sashimi) increases in popularity in Australia and other western countries.

Larval anisakid nematodes also pose a threat to fishermen and fish-processing workers in the fishing industries as they may develop occupational seafood allergies/hypersensitivity due to exposure to anisakid antigens in infected fish or fish products (Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008). Previously, a number of studies have shown that a significant number of such workers, in

different parts of the world, were found to be sensitised to anisakid larvae and had high levels of specific immunoglobulin E (IgE) (del Pozo et al., 1999; Nieuwenhuizen et al., 2006). Thus, the findings of the present investigation highlight the need for regular screening of workers routinely handling or processing fish and consumer of fish produce for allergic sensitization against anisakid larvae.

In conclusion, the molecular characterization of larval anisakid nematodes, employing a combined approach of morphological and molecular-phylogenetic analyses, is practical and effective at gaining a better understanding of the biodiversity, systematics and ecology of anisakid nematodes. This approach can also be used to study the life cycles and transmission patterns of anisakid nematodes. From an applied perspective, the molecular tools employed here will be useful for the

Table 3
Distribution of anisakid larvae among their host fish species.

Fish species	<i>Anisakis pegreffii</i>	<i>Contracaecum multipapillatum</i> (Cm)/ <i>Contracaecum ogmorhini</i> (Co)	<i>Hysterothylacium</i> types IV, Vb, VIII, X	<i>Terranova</i> type I
<i>Aldrichetta forsteri</i>	—	Cm (21)	—	—
<i>Arripis georgianus</i>	—	—	IV (3)	—
<i>Hyporhamphus regularis</i>	—	—	X (16)	+ (1)
<i>Mugil cephalus</i>	—	Cm (28)	—	—
<i>Platycephalus speculator</i>	—	—	IV (1)	—
<i>Pomatomus saltatrix</i>	—	—	X (10)	—
<i>Pseudocaranx dentex</i>	+ (3) ^a	Co (1)	IV (4), X (7), VIII (2)	—
<i>Pseudocaranx wrighti</i>	—	—	IV (8), X (6)	—
<i>Thysanophrys cirronatus</i>	—	—	X (5)	—
<i>Trachurus novaezelandiae</i>	—	—	IV (62), X (10)	—
<i>Upeneichthys lineatus</i>	—	—	IV (4), Vb (14), VIII (1), X (11)	—

(+) presence of a particular type of anisakid larva(e); (—) absence of a particular type of anisakid larva(e).

^a Figures in parentheses indicate the number of specific anisakid larvae found in a fish.

detection of larval anisakids in seafood to underpin the prevention of gastrointestinal anisakidosis as well as anisakid-induced allergy in humans.

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References

- Abe, N., Ohya, N., Yanagiguchi, R., 2005. Molecular characterization of *Anisakis pegreffii* larvae in Pacific cod in Japan. *Journal of Helminthology* 79, 303–306.
- Alonso, A., Daschner, A., Moreno-Ancillo, A., 1997. Anaphylaxis with *Anisakis simplex* in the gastric mucosa. *The New England Journal of Medicine* 337, 350–351.
- Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clinical Microbiology Reviews* 21, 360–379.
- Audicana, M.T., Ansoategui, I.J., de Corres, L.F., Kennedy, M.W., 2002. *Anisakis simplex*: dangerous—dead and alive? *Trends in Parasitology* 18, 20–25.
- Bruce, N.L., 1990a. *Hysterothylacium* Ward and Magath, 1917, and *Ichthyascaris* Wu, 1949, ascaridoid nematodes from Australian demersal fishes. *Memoirs of the Queensland Museum* 28, 389–426.
- Bruce, N.L., 1990b. Redescription of the ascaridoid nematode *Hysterothylacium scomberomori* (Yamaguti) from Australian Spanish mackerel *Scomberomorus commerson* (Lacépède). *Memoirs of the Queensland Museum* 28, 427–434.
- Bruce, N.L., Cannon, L.R.G., 1989. *Hysterothylacium*, *Iheringascaris* and *Maricostula* new genus, nematodes (Ascaridoidea) from Australian pelagic marine fishes. *Journal of Natural History* 23, 1397–1441.
- Cannon, L.R., 1977a. Some ecological relationships of larval ascaridoids from south-eastern Queensland marine fishes. *International Journal for Parasitology* 7, 227–232.
- Cannon, L.R., 1977b. Some larval ascaridoids from south-eastern Queensland marine fishes. *International Journal for Parasitology* 7, 233–243.
- Cribb, T.H., Bray, R.A., 2010. Gut wash, body soak, blender and heat-fixation: approaches to the effective collection, fixation and preservation of trematodes of fishes. *Systematic Parasitology* 76, 1–7.
- Damin, L., Heqing, H., 2001. *Heliconema minnanensis* n. sp. (Physalopteridae: Physalopteridae) and *Raphidascaris trichiuri* (Yin and Zhang) n. comb. (Ascaridoidea: Anisakidae) in marine fishes. *The Journal of Parasitology* 87, 1090–1094.
- Daschner, A., Alonso-Gomez, A., Cabanas, R., Suarez-de-Parga, J.M., Lopez-Serrano, M.C., 2000. Gastroallergic anisakiasis: borderline between food allergy and parasitic disease—clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by *Anisakis simplex*. *The Journal of Allergy and Clinical Immunology* 105, 176–181.

- Deardorff, T.L., Overstreet, R.M., 1981. Larval *Hysterothylacium* (= *Thynnascaris*) (Nematoda: Anisakidae) from fishes and invertebrates in the Gulf of Mexico. *Proceedings of the Helminthological Society of Washington* 48, 113–126.
- del Pozo, V., Arrieta, I., Tunon, T., Cortegano, I., Gomez, B., Cardaba, B., Gallardo, S., Rojo, M., Renedo, G., Palomino, P., Tabar, A.I., Lahoz, C., 1999. Immunopathogenesis of human gastrointestinal infection by *Anisakis simplex*. *Journal of Allergy and Clinical Immunology* 104, 637–643.
- Dominguez-Ortega, J., Cimarra, M., Sevilla, M., Alonso Llamazares, A., Moneo, I., Robledo Echarren, T., Martinez-Cocera, C., 2000. *Anisakis simplex*: a cause of intestinal pseudo-obstruction. *Revista Española de Enfermedades Digestivas* 92, 132–139.
- Dominguez-Ortega, J., Alonso-Llamazares, A., Rodriguez, L., Chamorro, M., Robledo, T., Bartolome, J.M., Martinez-Cocera, C., 2001. Anaphylaxis due to hypersensitivity to *Anisakis simplex*. *International Archives of Allergy and Immunology* 125, 86–88.
- Doupe, R.G., Lymbery, A.J., Wong, S., Hobbs, R.P., 2003. Larval anisakid infections of some tropical fish species from north-west Australia. *Journal of Helminthology* 77, 363–365.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Gasser, R.B., Hu, M., Chilton, N.B., Campbell, B.E., Jex, A.J., Otranto, D., Cafarchia, C., Beveridge, I., Zhu, X., 2006. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nature Protocols* 1, 3121–3128.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.
- Hooper, J.N.A., 1983. Parasites of estuarine and oceanic flathead fishes (Family Platycephalidae) from northern New South Wales. *Australian Journal of Zoology* 30 (5), 1–69.
- Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755.
- Jabbar, A., Khoo, A.T., Hui, T.X., Schaeffner, B.C., Jex, A.R., Nolan, M.J., Lopata, A., Gasser, R.B., Beveridge, I., 2012a. Mutation scanning-based analysis of anisakid larvae from *Sillago flindersi* from Bass Strait, Australia. *Electrophoresis* 33, 499–505.
- Jabbar, A., Asnoussi, A., Norbury, L.J., Eisenbarth, A., Shamsi, S., Gasser, R.B., Lopata, A.L., Beveridge, I., 2012b. Larval anisakid nematodes in teleost fishes from Lizard Island, northern Great Barrier Reef, Australia. *Marine and Freshwater Research* 63, 1283–1299.
- Kijewska, A., Dzido, J., Shukhgalter, O., Rokicki, J., 2009. Anisakid parasites of fishes caught on the African shelf. *The Journal of Parasitology* 95, 639–645.
- Lopata, A.L., Lehrer, S.B., 2009. New insights into seafood allergy. *Current Opinions in Allergy and Clinical Immunology* 9, 270–277.
- Lymbery, A.J., Doupe, R.G., Munshi, M.A., Wong, T., 2002. Larvae of *Contracaecum* sp. among inshore fish species of southwestern Australia. *Diseases of Aquatic Organisms* 51, 157–159.
- Mattiucci, S., Nascetti, G., 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. *Parasite* 13, 99–113.
- Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Advances in Parasitology* 66, 47–148.
- Mattiucci, S., Paoletti, M., Borri, F., Palumbo, M., Palmieri, R.M., Gomes, V., Casati, A., Nascetti, G., 2011. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. *BMC Infectious Diseases* 11, 82.
- Nadler, S.A., D'Amelio, S., Dailey, M.D., Paggi, L., Siu, S., Sakanari, J.A., 2005. Molecular phylogenetics and diagnosis of *Anisakis*, *Pseudoterranova*, and *Contracaecum* from northern Pacific marine mammals. *The Journal of Parasitology* 91, 1413–1429.
- Nash, J.A., 1998. The Ecology of Endoparasites of Whiting, *Sillago flindersi*. The University of New England, Armidale, NSW, Australia.
- Nieuwenhuizen, N., Lopata, A.L., Jeebhay, M.F., Herbert, D.R., Robins, T.G., Brombacher, F., 2006. Exposure to the fish parasite *Anisakis* causes allergic airway hyperreactivity and dermatitis. *The Journal of Allergy and Clinical Immunology* 117, 1098–1105.
- Oshima, T., 1987. Anisakiasis — is the sushi bar guilty? *Parasitology Today* 3, 44–48.
- Posada, G., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Quiazon, K.M., Yoshinaga, T., Santos, M.D., Ogawa, K., 2009. Identification of larval *Anisakis* spp. (Nematoda: Anisakidae) in Alaska pollock (*Theragra chalcogramma*) in northern Japan using morphological and molecular markers. *The Journal of Parasitology* 95, 1227–1232.
- Quiazon, K.M., Yoshinaga, T., Ogawa, K., 2011. Distribution of *Anisakis* species larvae from fishes of the Japanese waters. *Parasitology International* 60, 223–226.
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.
- Shamsi, S., 2007. Morphologic and genetic characterisation of selected ascaridoid nematodes. PhD thesis. The University of Melbourne, Australia.
- Shamsi, S., Butcher, A.R., 2011. First report of human anisakidosis in Australia. *Medical Journal of Australia* 194, 199–200.
- Shamsi, S., Gasser, R., Beveridge, I., Shabani, A.A., 2008. *Contracaecum pyripapillatum* n. sp. (Nematoda: Anisakidae) and a description of *C. multipapillatum* (von Drasche, 1882) from the Australian pelican, *Pelecanus conspicillatus*. *Parasitology Research* 103, 1031–1039.
- Shamsi, S., Norman, R., Gasser, R., Beveridge, I., 2009a. Genetic and morphological evidences for the existence of sibling species within *Contracaecum rudolphii* (Hartwich, 1964) (Nematoda: Anisakidae) in Australia. *Parasitology Research* 105, 529–538.
- Shamsi, S., Norman, R., Gasser, R., Beveridge, I., 2009b. Redescription and genetic characterization of selected *Contracaecum* spp. (Nematoda: Anisakidae) from various hosts in Australia. *Parasitology Research* 104, 1507–1525.

- Shamsi, S., Eisenbarth, A., Saptarshi, S., Beveridge, I., Gasser, R.B., Lopata, A.L., 2011a. Occurrence and abundance of anisakid nematode larvae in five species of fish from southern Australian waters. *Parasitology Research* 108, 927–934.
- Shamsi, S., Gasser, R.B., Beveridge, I., 2011b. Mutation scanning-coupled sequencing of nuclear ribosomal DNA spacers as a tool for the specific identification of different *Contracaecum* (Nematoda: Anisakidae) larval types. *Molecular and Cellular Probes* 25, 13–18.
- Szostakowska, B., Myjak, P., Kur, J., Sywula, T., 2001. Molecular evaluation of *Hysterothylacium auctum* (Nematoda, Ascaridida, Raphidascarididae) taxonomy from fish of the southern Baltic. *Acta Parasitologica* 46, 196–201.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.
- Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2007. Molecular identification of the etiological agent of the human anisakiasis in Japan. *Parasitology International* 56, 211–215.
- Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2008. Multiplex PCR for the identification of *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and the other anisakid nematodes. *Parasitology International* 57, 49–53.
- Van Thiel, P.H., 1962. Anisakiasis. *Parasitology* 52, 16–17.
- WHO, 2006. The global burden of foodborne diseases : taking stock and charting the way forward: WHO Consultation to Develop a Strategy to Estimate the Global Burden of Foodborne Diseases, Geneva, 25–27 September 2006.
- WHO, 2007. First formal meeting of the Foodborne Disease Burden Epidemiology Reference Group: WHO Initiative to Estimate the Global Burden of Foodborne Diseases, Geneva, 26–28 November 2007.
- Yagi, K., Nagasawa, K., Ishikura, H., Nakagawa, A., Sato, N., Kikuchi, K., Ishikura, H., 1996. Female worm *Hysterothylacium aduncum* excreted from human: a case report. *Japanese Journal of Parasitology* 45, 12–23.
- Zhang, L., Hu, M., Shamsi, S., Beveridge, I., Li, H., Xu, Z., Li, L., Cantacessi, C., Gasser, R.B., 2007. The specific identification of anisakid larvae from fishes from the Yellow Sea, China, using mutation scanning-coupled sequence analysis of nuclear ribosomal DNA. *Molecular and Cellular Probes* 21, 386–390.
- Zhu, X., Gasser, R.B., Podolska, M., Chilton, N.B., 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *International Journal for Parasitology* 28, 1911–1921.
- Zhu, X., D'Amelio, S., Hu, M., Paggi, L., Gasser, R.B., 2001. Electrophoretic detection of population variation within *Contracaecum ogmorhini* (Nematoda: Ascaridoidea: Anisakidae). *Electrophoresis* 22, 1930–1934.
- Zhu, X.Q., Podolska, M., Liu, J.S., Yu, H.Q., Chen, H.H., Lin, Z.X., Luo, C.B., Song, H.Q., Lin, R.Q., 2007. Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. *Parasitology Research* 101, 1703–1707.