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Larval anisakid nematodes in teleost fishes from Lizard Island, northern Great Barrier Reef, Australia

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Abstract. A survey was undertaken to characterise larval anisakid nematodes present in teleosts at Lizard Island on the northern Great Barrier Reef. In total, 464 fish were examined from 32 families, 62 genera and 107 species. Anisakid nematodes were found in 46 (9.9%) of the fish examined. Infections in Atherinidae, Lethrinidae, Lutjanidae and Serranidae were moderately prevalent, with the intensities of infection ranging from 1 to 80; whereas in the Sphyraenidae and Scombridae, the prevalence of infection was very high, with intensities ranging from 1 to >375 anisakids. A combined morphological and molecular-phylogenetic approach was employed to identify larval anisakid nematodes to species and/or genotypes. The nematodes examined were identified as *Anisakis typica* (three genotypes based on molecular characterisation), *Terranova* Types I (five genotypes) and II (five genotypes) and *Hysterothylacium* Types IV, V (four genotypes), VI and X. The findings of the present study provide some insights into the distribution of larval anisakid nematodes in coral-reef fishes and a basis for future investigations of anisakid populations in marine fishes.

Additional keywords: Anisakidae, first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA, Nematoda, single-strand conformation polymorphism (SSCP) analysis.

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Introduction

Various groups of marine internal parasites (nematodes, cestodes, trematodes) utilise trophic transmission to complete their complex life cycles, with definitive hosts being vertebrates near the apex of food chains and feeding on smaller vertebrates or invertebrates at lower trophic levels (Marcogliese 2002; Lafferty *et al.* 2008). In the case of anisakid nematodes, larval stages may be present in small crustaceans, which are ingested by small fishes, then by larger fishes, and finally reaching their definitive hosts, which include sharks, birds and marine mammals. For example, for species of *Anisakis*, the definitive hosts are dolphins and whales, for species of *Contracaecum*, fish-eating birds and pinnipeds, for species of *Terranova*, sharks, and for species of *Hysterothylacium*, large predatory pelagic teleosts (Anderson 2000).

The life cycles of parasites are potentially informative in establishing trophic pathways within marine systems, as well as discovering how parasites themselves may influence host abundance in invertebrate and fish communities in marine systems (Marcogliese 2002; Lafferty *et al.* 2008). Parasites constitute a significant but frequently unrecognised component of marine biodiversity (Marcogliese 2004). However, the life cycles of anisakid nematodes have been difficult to elucidate using traditional techniques of experimentally infecting hosts raised worm-free, although some anisakids cause severe gastroenteritis in humans if ingested in raw seafood (e.g. Van Thiel 1962; Kasuya *et al.* 1990; Yagi *et al.* 1996; Audicana *et al.* 2002; Shamsi and Butcher 2011). In addition, these parasites contribute to allergic reactions to seafood (Audicana *et al.* 2002; Nieuwenhuizen *et al.* 2006; Audicana and Kennedy 2008; Lopata and Lehrer 2009).

In Australia, information on the prevalence and abundance of different species of anisakid nematodes is extremely limited. Although larval stages are found commonly in species of

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teleosts, frequently in species used for human consumption, identification beyond the level of genus is virtually impossible using morphological methods, resulting in the larvae being assigned to various morphotypes designated by Roman numerals (Cannon 1977*a*, 1977*b*; Bruce and Cannon 1989, 1990; Bruce 1990*a*, 1990*b*; Nash 1998; Lymbery *et al.* 2002; Doupé *et al.* 2003; Muñoz *et al.* 2007; Shamsi 2007; Shamsi *et al.* 2008, 2011*a*, 2011*b*, 2012; Jabbar *et al.* 2012*a*, 2012*b*). The more recent introduction of molecular techniques has allowed the identification of some larval stages in teleosts and, as a consequence, the identification of life-cycle patterns (Mattiucci and Nascetti 2006, 2008). In addition to being able to elucidate the life cycles of parasites, the ability to specifically identify the larval stages of anisakid nematodes also provides an opportunity to investigate how these nematodes utilise intermediate hosts at various trophic levels to complete their life cycles. Cannon (1977*a*, 1977*b*) provided initial data on the biology of larval anisakid nematodes in teleost fishes in south-eastern Queensland, but was hampered by the limitations of morphological methods, describing his morphological forms as different numerical types, but being unable to confidently establish their relationships with adult forms. Nonetheless, this pioneering work suggested substantial ecological differences among the various genera of anisakid nematodes described (*Anisakis*, *Contracaecum*, *Terranova* and *Hysterothylacium* (as *Thynnascaris*)). Subsequently, it has been shown that some of the morphotypes identified by Cannon (1977*b*) in fact constitute more than one species (Shamsi *et al.* 2011*b*). This information indicates that molecular techniques of larval identification can provide considerable insights into nematode life cycles and, in addition, into the complex food webs that exist in marine systems, by elucidating the various intermediate host species in a food chain.

In the present study, we surveyed larval anisakid nematodes present in teleosts at Lizard Island, on the northern Great Barrier Reef, Australia. The aim of the study was to identify larval anisakids by using a combined morphological and molecular approach and in as large a range of teleosts as could be examined. It was hoped that such data would indicate the pattern of infection in coral reef fishes and, by attempting to relate the data to the ecology of the reef-fish community, would provide basic information on possible pathways in the food webs that might facilitate the completion of parasite life cycles.

Materials and methods

Study area and collection of fishes

Fish were collected during two trips to Lizard Island (145°28′S, 14°40′E) in the northern Great Barrier Reef, Queensland, Australia, in April 2008 and August–September 2010. Teleosts were collected by line fishing, seine netting, spear fishing and by anaesthetising small fish with clove oil. An attempt was made to examine as many fish species as possible, as part of a preliminary study of anisakids infecting reef fishes.

Fish were photographed to confirm identifications and, in most instances, tissue samples were collected for genetic identification. Representatives of any fish species that proved difficult to identify in the field were preserved and returned to the Queensland Museum, Brisbane, for definitive identification.

Isolation of anisakid larvae and their morphological identification

Nematodes were removed from the body cavity and were fixed in ethanol for subsequent examination. In cases where relatively small numbers of nematodes were present, all nematodes were collected. In fish infected with hundreds of nematodes (*Scomberomorus commerson* and *Grammatocyrnus bicarinatus*), a subsample of 100–200 nematodes was collected. Prevalence (the percentage of fish infected with parasites) and intensity of infection (the numbers of parasites in infected hosts) are reported following the definitions of Bush *et al.* (1997).

In the laboratory, all nematodes were prepared in a similar fashion. The anterior and posterior ends of each nematode were excised with a scalpel and preserved in lactophenol for morphological identification, whereas the mid-section of the body was utilised for molecular studies.

Each nematode was examined morphologically and assigned to a larval 'type' following Cannon (1977*b*) and Shamsi (2007). Representatives of each morphological nematode type, from each host species, were retained and have been deposited in the Queensland Museum, Brisbane (G233918–79).

Drawings of each morphological type of nematode, and brief descriptions are provided. More detailed descriptions of each of these nematode types can be found in Cannon (1977*b*) and Shamsi (2007). Drawings were made with a drawing tube attached to an Olympus BH microscope (Shinjuku Tokyo, Tokyo, Japan) and measurements were made with an ocular micrometer; the latter are presented in mm, as the mean followed by the range; the number of measurements made is represented by '*n*'. As the mid-region of the nematodes had been removed for molecular studies, no total lengths were available.

Molecular characterisation of anisakid larvae

Total genomic DNA was extracted from individual larvae by sodium dodecyl–sulfate/proteinase K treatment and purified using a mini-column (WizardDNA Clean-Up System, Promega, Fitchburg, WI, USA), according to the manufacturer's protocol. The first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear rDNA (rDNA) were amplified from 10–50 ng of genomic DNA by PCR using the primer sets SS1/NC13R and SS2/NC2 (Zhang *et al.* 2007; Shamsi *et al.* 2008), respectively, under the same conditions as described previously (Jabbar *et al.* 2012*a*). For each set of PCRs, negative (no-DNA) and known positive controls were included. Following PCR, 5 µL of each amplicon was examined on a 1.5% w/v agarose gel stained with ethidium bromide, and photographed.

ITS-1 and ITS-2 amplicons were subjected (separately) to single-strand conformation polymorphism (SSCP) analysis (Gasser *et al.* 2006, Protocol B) to display sequence variation within and among amplicons, as described previously (Jabbar *et al.* 2012*a*). For each locus, amplicons representing each unique SSCP profile were selected, treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., Glen Burnie, MD, USA), and subjected to bi-directional, automated sequencing (BigDye Terminator v.3.1, Applied Biosystems, Foster City, California, USA) using (separately) the same primers as employed in PCR. The quality of each sequence was assessed by appraising its electropherogram using the

Table 1. Fish collected and examined for larval anisakid nematodes on Lizard Island, Queensland, including prevalence of infection (i.e. no. of individuals infected)
Family totals are given in parentheses

Order	Family	Genus and species	Common name	No. examined	Prevalence
Perciformes	Acanthuridae	*Ctenochaetus binotatus*	Twospot surgeon fish	1	0
		Zebrasoma veliferum	Sailfin tang	6	0
		Acanthurus sp. *14366*		1	0
				(8)	
	Apogonidae	*Apogon compressus*	Ochre-striped cardinal fish	5	0
		Apogon exostigma	Narrowstripe cardinalfish	2	0
		Apogon angustatus	Broadstriped cardinalfish	2	0
		Zoramia leptacantha	Threadfin cardinalfish	117	0
		Apogon properuptus	Southern orange-lined cardinal fish	2	0
		Apogon rubrimacula	Redspot cardinalfish	5	0
		Archamia fucata	Orangelined cardinalfish	8	0
		Archamia zosterophora	Blackbelted cardinalfish	1	0
		Cheilodipterus artus	Wolf cardinalfish	10	0
		Cheilodipterus intermedius	Intermediate cardinalfish	8	1
		Cheilodipterus quinquelineatus	Five-lined cardinalfish	17	0
		Nectamia fusca	Samoan cardinalfish	19	0
		Rhabdamia gracilis	Luminous cardinalfish	10	0
				(206)	
	Atherinidae	*Atherinomorus endrachtensis*	Eendracht Land silverside	11	5
				(11)	
	Blenniidae	*Ecsenius stictus*	Great Barrier Reef blenny	1	0
		Plagiotremus tapeinosoma	Piano fangblenny	1	0
		Salarias alboguttatus	White-spotted blenny	1	0
		Salarias fasciatus	Jewelled blenny	2	0
				(5)	
	Bothidae	*Bothus pantherinus*	Leopard flounder	1	0
				(1)	
	Carangidae	*Caranx papuensis*	Brassy trevally	2	2
				(2)	
	Chaetodontidae	*Chaetodon plebeius*	Blueblotch butterflyfish	2	0
		Chaetodon citrinellus	Speckled butterflyfish	10	1
		Chaetodon baronessa	Eastern triangular butterflyfish	1	0
		Ctenochaetus binotatus	Twospot surgeonfish	1	0
		Chaetodon auriga	Threadfin butterflyfish	6	0
		Chaetodon lineolatus	Lined butterflyfish	3	0
		Chaetodon ephippium	Saddle butterflyfish	3	0
		Chaetodon rafflesii	Latticed butterflyfish	3	0
		Chaetodon lunulatus	Oval butterflyfish	2	0
		Chaetodon pelewensis	Sunset butterflyfish	2	0
		Chaetodon ulietensis	Pacific double-saddle butterflyfish	8	1
		Chaetodon melannotus	Blackback butterflyfish	8	0
		Chaetodon unimaculatus	Teardrop butterflyfish	2	0
		Chaetodon kleinii	Sunburst butterflyfish	2	0
		Chaetodon vagabundus	Vagabond butterflyfish	9	0
		Chelmon rostratus	Copperband butterflyfish	4	0
		Forcipiger flavissimus	Longnose butterfly fish	3	0
		Heniochus chrysostomus	Threeband pennantfish	4	0
		Heniochus varius	Horned bannerfish	2	0
				(75)	
	Cirrhitidae	*Paracirrhites forsteri*	Blackside hawkfish	1	1
	Lethrinidae	*Lethrinus atkinsoni*	Pacific yellowtail emperor	4	0
		Lethrinus harak	Thumbprint emperor	6	0
		Lethrinus nebulosus	Spangled emperor	4	3
				(14)	
	Lutjanidae	*Caesio cuning*	Redbelly yellowtail fusilier	3	3
		Lutjanus carponotatus	Spanish flag snapper	2	2

(*Continued*)

Table 1. (Continued)

Order	Family	Genus and species	Common name	No. examined	Prevalence
		Lutjanus ehrenbergii	Blackspot snapper	4	0
		Lutjanus fulviflamma	Dory snapper	1	1
		Lutjanus fulvus	Blacktail snapper	1	0
		Lutjanus monostigma	One-spot snapper	1	0
		Lutjanus russellii	Russell's snapper	2	0
				(14)	
	Microdesmidae	*Gunnellichthys monostigma*	Onespot wormfish	1	0
				(1)	
	Mullidae	*Parupeneus trifasciatus*	Doublebar goatfish	4	0
		Parupeneus ciliatus	Whitesaddle goatfish	1	0
		Parupeneus indicus	Indian goatfish	1	0
		Mulloidichthys vanicolensis	Yellowfin goatfish	2	0
				(8)	
	Muraenidae	*Gymnothorax pseudothyrsoideus*	Highfin moray	1	0
				(1)	
	Nemipteridae	*Scolopsis monogramma*	Monogrammed monocle bream	4	1
		Scolopsis margaritifera	Pearly monocle bream	1	0
		Scolopsis affinis	Peters' monocle bream	2	0
				(7)	
	Pinguipedidae	*Parapercis hexophthalma*	Speckled sandperch	1	0
				(1)	
	Pomacanthidae	*Centropyge bicolor*	Bicolor angelfish	1	0
				(1)	
	Pomacentridae	*Abudefduf septemfasciatus*	Banded sergeant	1	0
		Abudefduf sordidus	Blackspot sergeant	1	0
		Acanthochromis polyacanthus	Spiny chromis	1	1
		Dascyllus reticulatus	Reticulate dascyllus	1	0
				(4)	
	Pseudochromidae	*Pseudochromis fuscus*	Brown dottyback	1	0
		Stegastes apicalis	Australian gregory	1	1
				(2)	
	Scaridae	*Scarus oviceps*	Dark capped parrotfish	1	0
				(1)	
	Scombridae	*Grammatorcynus bicarinatus*	Shark mackerel	6	6
		Scomberomorus commerson	Narrow-barred Spanish mackerel	2	2
				(8)	
	Serranidae	*Cephalopholis boenak*	Chocolate hind	3	1
		Cephalopholis cyanostigma	Bluespotted hind	5	2
		Epinephelus ongus	White-streaked grouper	1	1
		Plectropomus areolatus	Squaretail coral grouper	1	0
		Plectropomus leopardus	Leopard coral grouper	8	6
				(18)	
	Siganidae	*Siganus corallinus*	Blue-spotted spinefoot	1	0
		Siganus doliatus	Barred spinefoot	1	0
		Choerodon schoenleinii	Blackspot tuskfish	1	0
		Coris batuensis	Batu coris	1	0
		Halichoeres scapularis	Zigzag wrasse	1	0
		Oxycheilinus diagrammus	Cheeklined wrasse	1	0
		Thalassoma lunare	Moon wrasse	2	0
				(8)	
	Sillaginidae	*Sillago* sp. *14308*		1	0
				(1)	
	Sphyraenidae	*Sphyraena jello*	Pickhandle barracuda	1	0
		Sphyraena forsteri	Bigeye barracuda	2	2
				(3)	
Beloniformes	Belonidae	*Tylosurus crocodilus*	Hound needlefish	4	1
				(4)	
	Hyporhamphidae	*Hyporhamphus affinis*	Tropical halfbeak	7	1
				(7)	

(*Continued*)

Table 1. (Continued)

Order	Family	Genus and species	Common name	No. examined	Prevalence
Mugiliformes	Mugilidae	*Valamugil buchanani*	Bluetail mullet	1	0
		Liza vaigiensis	Squaretail mullet	8	1
				(9)	
Gonorynchiformes	Chanidae	*Chanos chanos*	Milkfish	3	0
				(3)	
Tetraodontiformes	Balistidae	*Balistapus undulatus*	Orange-lined triggerfish	1	0
		Rhinecanthus aculeatus	White-banded triggerfish	16	0
		Sufflamen chrysopterus	Halfmoon triggerfish	4	0
				(21)	
	Monacanthidae	*Oxymonacanthus longirostris*	Harlequin filefish	1	0
		Paraluteres prionurus	False puffer	1	0
				(2)	
	Ostraciidae	*Ostracion cubicus*	Yellow boxfish	3	0
				(3)	
	Tetraodontidae	*Arothron hispidus*	White-spotted puffer	3	0
		Arothron manilensis	Narrow-lined puffer	2	0
		Arothron mappa	Map puffer	1	0
		Arothron nigropunctatus	Blackspotted puffer	1	0
		Canthigaster bennetti	Bennett's sharpnose puffer	2	0
		Canthigaster solandri	Spotted sharpnose	1	0
		Canthigaster valentini	Valentin's sharpnose puffer	2	0
				(12)	
Beryciformes	Holocentridae	*Neoniphon sammara*	Sammara squirrelfish	2	0
				(2)	
Total				464	46 (9.9%)

program BioEdit (Hall 1999). Polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes.

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; accessed 27 April 2012) to establish the 'top hits' to all nucleotide sequences available in the current databases and identities (%) calculated by pairwise comparisons. Subsequently, the consensus sequence was aligned with a selected subset of closely related reference sequences (*Anisakis typica*, *Hysterothylacium* spp., *Pseudoterranova* spp., *Terranova* spp. and *Raphidascaris trichiuri*) by using the program Clustal X (Thompson *et al.* 1997) and alignments were adjusted manually. Phylogenetic analyses were performed on individual or concatenated sequence datasets. Each concatenated (ITS-1+ITS-2) sequence was derived from the same individual nematode. Phylogenetic analyses 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 Modelltest v3.7 (Posada and Crandall 1998). The 'best' model for the ITS-1 dataset using AIC was the general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR+Γ+I), whereas that for ITS-2 as well as for concatenated (ITS-1+ITS-2) sequence datasets was the transversion model of evolution, with gamma distribution and a proportion of invariable sites (TVM+Γ+I). 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 on the basis of the final 75% of trees generated by BI. Phylogenetic trees constructed using different datasets were examined for concordance in topology.

Results

In total, 464 fish were examined from 32 families, 62 genera and 107 species. Anisakid nematodes were found in 46 (9.9%) of the fish examined (Table 1). Although sampling across fish families was very uneven, few or no anisakid nematode larvae were found in the following families (only those families with five or more fish species examined were included): Acanthuridae, Apogonidae, Balistidae, Blenniidae, Chaetodontidae, Hyporhamphidae, Mugilidae, Mullidae, Nemipteridae, Siganidae and Tetraodontidae (Table 1). By contrast, nematodes were found commonly in members of the families Lethrinidae, Lutjanidae, Scombridae and Serranidae.

The nematodes examined were identified as *Anisakis typica*; two morphotypes of *Terranova*, corresponding closely with *Terranova* Types I and II of Cannon (1977*b*), and four morphotypes of *Hysterothylacium* corresponding with Type IV of Cannon (1977*b*) and Types V, VI and X of Shamsi (2007).

Diagnoses of larvae

Anisakis typica (= *Anisakis* Type I of Cannon, 1977*b*, in part)

Diagnosis (Fig. 1)

Third-stage larvae with prominent ventral tooth. Oesophagus 1.32–1.57 (1.47, $n = 10$) long; nerve ring 0.22–0.28 (0.26, $n = 10$) from anterior end; deirids 0.35 ($n = 1$) from anterior end; ventriculus 0.48–0.88 (0.73, $n = 10$) long; tail blunt, 0.09–0.12 (0.11, $n = 10$) long with prominent mucro.

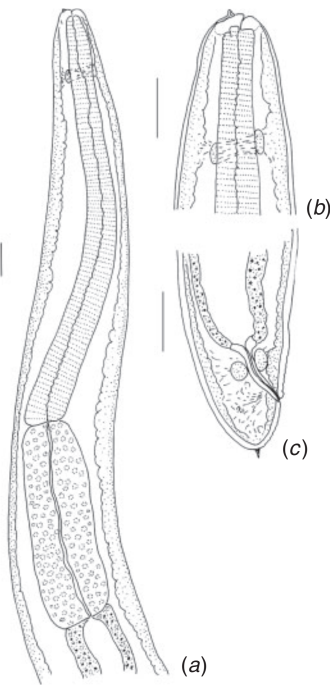

Fig. 1. Third-stage larva of *Anisakis typica*: (*a*) anterior end; (*b*) cephalic extremity; and (*c*) tail. Scale bars = 0.1 mm.

Remarks

The measurements presented here of larvae identified definitively by molecular methods as being those of *A. typica* correspond closely with those of larvae of *Anisakis* Type I of Cannon (1977*b*), which he described from various Queensland fishes. However, the description also potentially encompasses the features of the third-stage larva of *A. pegreffii* (see Shamsi *et al.* 2011*a*).

In the larva of *A. pegreffii*, the oesophagus was longer 2.16 (1.67–2.66 mm), whereas the mean length of the ventriculus (0.84 mm) was similar to that of *A. typica* (see Shamsi *et al.* 2011*a*), as was the mean length of the tail (0.12 mm). Consequently, the principal potential distinguishing feature of these larval stages appears to be the length of the oesophagus. However, because total lengths of larvae were not available, the ratios of these organs to total lengths could not be compared, which needs to be done before using the data as differential criteria.

In all, 155 larvae of this type were subjected to PCR-coupled SSCP analysis. On the basis of SSCP profiles, five and three representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms (if any), G+C content and respective sequence accession numbers are given in Table 2. In the ITS-1, sequence polymorphism was detected (see Table 2; Genotypes A–C) and, on pairwise comparison, the percentage difference among all of the different ITS-1 sequence types ranged from 0.3% to 1.2%. No sequence polymorphism was detected in the ITS-2.

Terranova (Type I of Cannon 1977*b*)

Diagnosis (Fig. 2)

Third-stage larvae with prominent ventral tooth. Body with numerous prominent longitudinal striations. Oesophagus 1.08–1.65 (mean 1.16, $n = 10$) long; nerve ring 0.25–0.33 (mean 0.30, $n = 10$) from anterior extremity; deirids 0.30–0.37 (0.34, $n = 3$) from anterior extremity; ventriculus 0.95–1.96 (1.32, $n = 10$) long; intestinal diverticulum 0.73–2.08 (1.52, $n = 10$) long, extending just anterior to oesophago–intestinal junction; tail with prominent transverse cuticular striations, 0.16–0.20 (0.18, $n = 10$) long, blunt tipped.

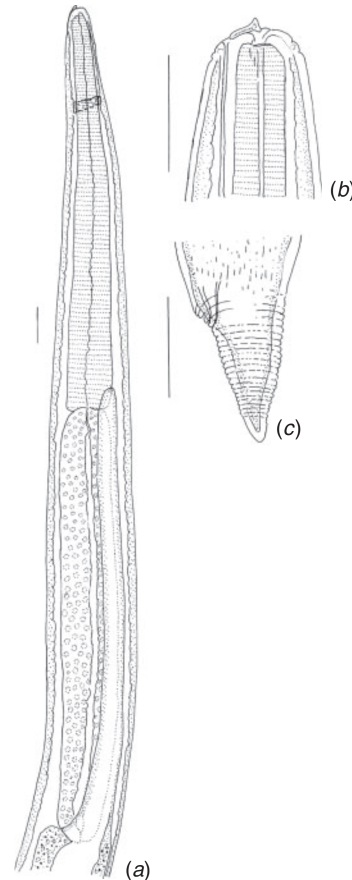

Fig. 2. Third-stage larva of *Terranova* Type I: (*a*) anterior end; (*b*) cephalic extremity; and (*c*) tail. Scale bars = 0.1 mm.

Table 2. The classification of anisakid nematode specimens on the basis of 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 and polymorphism and G+C content. Mean nucleotide frequencies for the main anisakids are also provided. Polymorphism for each sequence type was assessed by aligning these sequences with the reference sequences (see Table 2). R = A/G; K = G/T; S = C/G; Y = C/T

Genotype	ITS-1									ITS-2								
	SSCP profile (no. of samples with a unique profile)	Accession number	Length (bp)	Polymorphism (alignment position)	G+C content (%)	Mean nucleotide frequency				SSCP profile (no. of samples with a unique profile)	Accession number	Length (bp)	Polymorphism (alignment position)	G+C content (%)	Mean nucleotide frequency			
						A	C	G	T						A	C	G	T
A (*Anisakis typica*)	AT-1.1 (153)	(JX848663)	348	–	50.86	0.23946	0.23705	0.27107	0.25241	AT-2 (155)	(JX848680)	355	–	47.32	0.25070	0.20282	0.27042	0.27606
B (*A. typica*)	AT-1.2 (1)	(JX848664)	348	Y(201)	50.57					-do-	-do-	-do-	–					
C (*A. typica*)	AT-1.3 (1)	(JX848665)	348	G (52) T (201) T (249) G (252)	50.86					-do-	-do-	-do-	–					
D (*Terranova* I)	TI-1.1 (37)	(JX848666)	437	–	46.68	0.25057	0.21510	0.25400	0.28032	TI-2.1 (35)	(JX848681)	266	T (43)	51.13	0.24812	0.19838	0.31517	0.23833
E (*Terranova* I)	-do-	-do-	-do-	–						TI-2.2 (1)	(JX848682)	266	S (94)	51.13				
F (*Terranova* I)	-do-	-do-	-do-	–						TI-2.3 (1)	(JX848683)	266	Y (43)	51.13				
G (*Terranova* I)	TI-1.2 (3)	(JX848667)	439	G (175) G (176)	46.92					TI-2.4 (2)	(JX848684)	266	C (43)	51.50				
H (*Terranova* I)	-do-	-do-	-do-	-do-						TI-2.5 (1)	(JX848685)	266	K (67)	51.13				
I (*Terranova* II)	TII-1.1 (184)	(JX848668)	437	–	47.60	0.24939	0.21440	0.26110	0.27511	TII_2.1 (187)	(JX848686)	252	–	46.43	0.27183	0.18651	0.27778	0.26389
J (*Terranova* II)	TII-1.2 (1)	(JX848669)	437	M (207)	47.37					-do-	-do-	-do-	–					
K (*Terranova* II)	TII-1.3 (1)	(JX848670)	437	R (243)	47.37					-do-	-do-	252	–					
L (*Terranova* II)	TII-1.4 (1)	(JX848671)	437	W (359)	47.60					-do-	-do-	-do-	–					
M (*Terranova* II)	TII-1.5 (1)	(JX848672)	437	W (28) W (29) W (399)	47.60					TII_2.2 (1)	(JX848687)	253	A (152)	46.25				
N (*Hysterothylacium* Type IV)	HIV-1 (1)	(JX848673)	438	–	51.14	0.21689	0.22374	0.28767	0.27169	HIV-2 (1)	(JX848688)	345	–	54.20	0.16812	0.23478	0.30725	0.28986
O (*Hysterothylacium* Type VI)	HVb-1.1 (11)	(JX848674)	437	–	49.66	0.23717	0.22883	0.26855	0.26545	HVb-2 (13)	(JX848689)	272	–	50	0.19118	0.20588	0.29412	0.30882
P (*Hysterothylacium* Type VI)	HVb-1.2 (1)	(JX848675)	437	R (64)	-do-					-do-	-do-	-do-	–					
Q (*Hysterothylacium* Type VI)	HVb-1.3 (1)	(JX848676)	437	R (383)	-do-					-do-	-do-	-do-	–					
R (*Hysterothylacium* Type V)	HVc-1 (14)	(JX848677)	434	–	49.54	0.24424	0.22581	0.26959	0.26307	HVc-2 (14)	(JX848690)	270	–	49.26	0.21111	0.21111	0.28148	0.29630
S (*Hysterothylacium* Type V)	HVI-1 (8)	(JX848678)	431	–	50.35	0.23898	0.22738	0.27610	0.25754	HVI-2 (8)	(JX848691)	267	–	49.06	0.19476	0.20225	0.28839	0.31461
T (*Hysterothylacium* Type X)	HX-1 (5)	(JX848679)	436	–	51.15	0.22018	0.22706	0.28440	0.26835	HX-2 (5)	(JX848692)	276	–	54.35	0.15942	0.22101	0.32246	0.29710
Total				424									424					

Remarks

The features of these larvae conform to the description of Cannon (1977*b*), being distinguished by an elongate ventriculus and the intestinal diverticulum reaching the oesophago–intestinal junction. Sequence data are provided for this larval type for the first time. Cannon (1977*b*) suggested that they were the larvae of *T. chyloscylii*. However, because of the absence of sequence data for the adults of this species, this suggestion cannot be confirmed.

Thirty-seven specimens of this larval type were subjected to PCR-coupled SSCP analysis. On the basis of the SSCP profiles, six and eight representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms, G+C content and respective sequence accession numbers are given in Table 2. Sequence polymorphism was detected (see Table 2; Genotypes D–H) both in ITS-1 and ITS-2 regions. On pairwise comparison, the percentage difference among all of the different sequence types of ITS-1 ($n = 2$) and those of ITS-2 ($n = 5$) was 0.5% and 0.4–0.8%, respectively.

Terranova (Type II of Cannon 1977*b)*

Diagnosis (Fig. 3)

Third-stage larvae with prominent ventral tooth. Body with numerous prominent longitudinal striations. Oesophagus 0.78–1.10 (0.94, $n = 10$) long; nerve ring 0.26–0.36 (0.28, $n = 10$) from anterior extremity; deirids 0.32–0.36 (0.34, $n = 7$) from anterior extremity; ventriculus 0.35–0.45 (0.38, $n = 10$) long; intestinal diverticulum 0.60–0.87 (0.72, $n = 10$) long, extending well anterior to oesophago–intestinal junction; tail with prominent transverse cuticular striations, 0.12–0.18 (0.15, $n = 10$) long, blunt tipped.

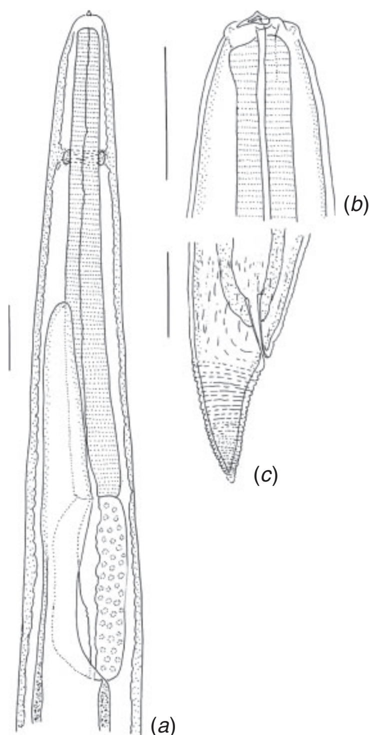

Fig. 3. Third-stage larva of *Terranova* Type II: (*a*) anterior end; (*b*) cephalic extremity; and (*c*) tail. Scale bars = 0.1 mm.

Remarks

The larvae corresponded closely to that described as *Terranova* Type II by Cannon (1977*b*), also from fishes in Queensland. Cannon (1977*b*) suggested that they may be the larvae of *T. galeocerdonis* or *T. scoliodontis*. However, the lack of sequence data from adults prevents the testing of this hypothesis.

In all, 180 larvae of this type were subjected to PCR-based SSCP analysis. On the basis of the SSCP profiles, seven and four representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms, G+C content and respective sequence accession numbers are given in Table 2. Sequence polymorphism was detected (see Table 2; sequence Types I–M) both in ITS-1 and ITS-2 regions. On pairwise comparison, the percentage difference among all of the different sequence types of the ITS-1 ($n = 5$) and those of the ITS-2 ($n = 2$) ranged from 0.3% to 1% and 0.5%, respectively.

Hysterothylacium *(= Thynnascaris* Type IV of Cannon 1977*b)*

Diagnosis (Fig. 4)

Fourth-stage larvae with three well developed lips and prominent interlabia. Oesophagus 0.85–1.25 (0.94, $n = 10$) long;

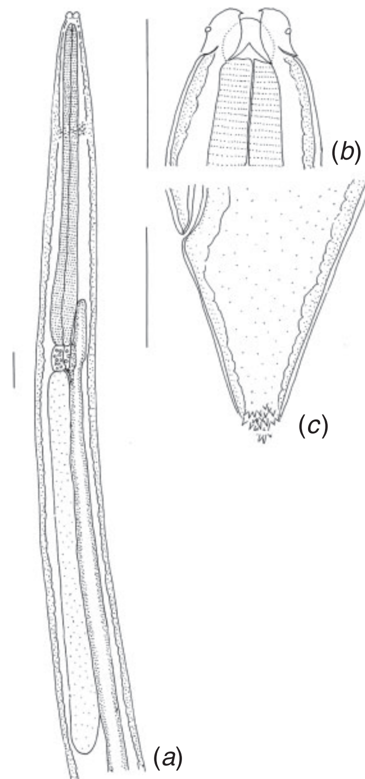

Fig. 4. Fourth-stage larva of *Hysterothylacium* Type IV: (*a*) anterior end; (*b*) cephalic extremity; and (*c*) tail. Scale bars = 0.1 mm.

nerve ring 0.45–0.46 (0.46, $n = 2$) from anterior end; ventriculus 0.10–0.15 (0.12, $n = 4$) long; ventricular appendix 1.3 ($n = 1$) long; intestinal diverticulum extending into posterior oesophageal region, 0.20 ($n = 1$) long; tail 0.11–0.24 (0.18, $n = 6$) long, terminating in numerous spikes.

Remarks

The features of this larva agree well with the description of *Hysterothylacium* (= *Thynnascaris*) larval Type IV of Cannon (1977*b*) and also with the description of similar larval stages by Shamsi (2007). The multiple spikes on the tail are characteristic of this larval form. Shamsi (2007) recognised two genotypes with similar morphological features, with Type A in fishes from the Great Barrier Reef and Type B from Victorian fishes.

Because the morphological characteristics of this larval type are highly distinctive, only a single larva from *Scomberomorus commerson* was subjected to PCR-based SSCP analysis. On the basis of the SSCP profiles, the ITS-1 and ITS-2 regions of one amplicon were sequenced. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 2 (sequence Type N).

Hysterothylacium (Type V of Shamsi 2007)

Diagnosis (Fig. 5)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.38–0.46 (0.42, $n = 6$); nerve ring 0.22–0.29 (0.25, $n = 4$) from anterior end; excretory pore 0.30 ($n = 1$) from anterior end; ventriculus 0.04–0.06 (0.05, $n = 4$) long; ventricular appendix 0.20–0.38 (0.31, $n = 4$) long; intestinal diverticulum extending into posterior oesophageal region, 0.13–0.20 (0.15, $n = 3$) long; tail 0.10–0.19 (0.13, $n = 6$), conical with tiny mucro at tip.

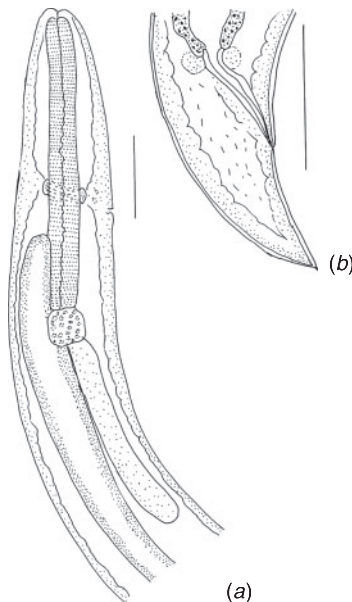

Fig. 5. Third-stage larva of *Hysterothylacium* Type V: (*a*) anterior end; and (*b*) tail. Scale bars = 0.1 mm.

Remarks

Morphologically these larvae resemble *Hysterothylacium* Type V of Shamsi (2007) by possessing a conical tail with a mucro at the tip and having the ventricular appendix longer than the intestinal caecum. Two different genotypes were recognised within this morphotype, both of which were closely related to but not identical to that described by Shamsi (2007). The three genotypes have been designated 'a', 'b' and 'c', with type 'a' being that described by Shamsi (2007).

Twenty-seven larvae of this type were subjected to PCR-coupled SSCP analysis. On the basis of the SSCP profiles, eight and six representative PCR amplicons were selected for the sequencing of the ITS-1 and ITS-2, respectively. Two different genotypes were identified and designated 'HVb' and 'HVc', because they differed slightly from Type V of Shamsi (2007), here considered to represent Type HVa. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms, G+C content and respective sequence accession numbers are given in Table 2 (see Sequences O–R). For the sequence HVb, sequence polymorphism was detected in the ITS-1 region only (Table 2; Genotypes O–Q); on pairwise comparison, the percentage difference among all of the five sequence types of the ITS-1 ranged from 0.3% to 0.5%. No sequence polymorphism was detected in the ITS-2. For the sequence HVc, no sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

Hysterothylacium (Type VI of Shamsi 2007)

Diagnosis (Fig. 6)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.43–0.65 (0.56, $n = 5$); nerve ring 0.32–0.36 (0.34, $n = 3$) from

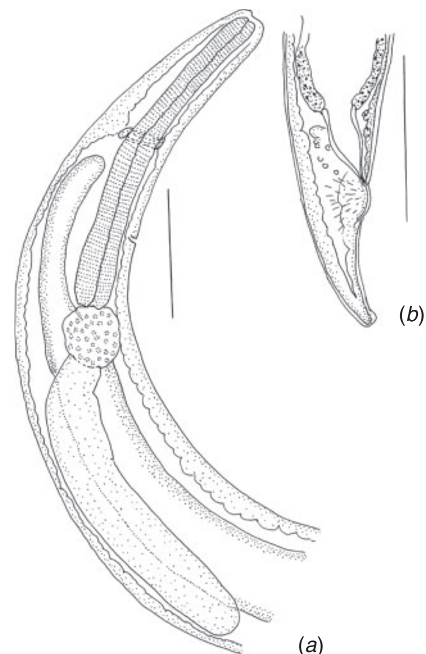

Fig. 6. Third-stage larva of *Hysterothylacium* Type VI: (*a*) anterior end; and (*b*) tail. Scale bars = 0.1 mm.

anterior end; excretory pore 0.40–0.48 (0.44, $n = 1$) from anterior end; ventriculus 0.09–0.10 (0.10, $n = 2$) long; ventricular appendix 0.60 ($n = 1$) long; intestinal diverticulum extending into posterior oesophageal region, 0.31–0.33 (0.32, $n = 2$) long; tail 0.12–0.21 (0.16, $n = 5$), conical with enlarged blunt tip.

Remarks

This larva is similar to *Hysterothylacium* Type VI of Shamsi (2007) in that it possesses a broad, blunt tail and the ventricular appendix is longer than the intestinal diverticulum (see Shamsi 2007).

Eight larvae of this type were subjected to PCR-based SSCP analysis. On the basis of the SSCP profiles, the ITS-1 and ITS-2 regions of three PCR amplicons were sequenced. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 2 (Genotype S). No sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

Hysterothylacium (Type X of Shamsi 2007)

Diagnosis (Fig. 7)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.40–0.60 (0.48, $n = 3$); nerve ring 0.23 ($n = 1$) from anterior end; tail 0.12–0.20 (0.16, $n = 2$), terminal extremity directed posteriorly with prominent spikes.

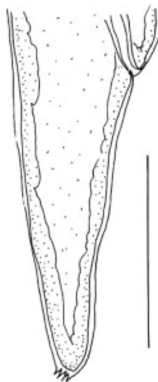

Fig. 7. Third-stage larva of *Hysterothylacium* Type X, tail. Scale bar = 0.1 mm.

Remarks

Few specimens of this larval type were available and those examined were in a poor state of preservation. The characteristic features of the tail with the posteriorly directed spines as well as the sequence data allowed the identification of *Hysterothylacium* Type X. However, other morphological features were not clearly discernible. Shamsi (2007) reported this type only from *Sphyraena novaehollandiae* from Victoria; on the basis of the distinctive features of the tail, this type is now reported from reef fishes in northern Queensland. Shamsi (2007) reported that the ITS-1 and ITS-2 sequence of this larval type was identical to that of the adult of *H.* cf. *pelagicum*.

Five larvae of this type were subjected to PCR-coupled SSCP analysis. On the basis of the SSCP profiles, the ITS-1 and ITS-2 regions were sequenced from three amplicons. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 2 (Genotype T). No sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

Host associations

Infections in Atherinidae, Lethrinidae, Lutjanidae and Serranidae were more prevalent, with intensities of infection ranging from 1 to 80, whereas for the Sphyraenidae and Scombridae, the prevalence of infection was very high, with intensities ranging from 1 to >375 (Table 3). However, in the latter case, the number of nematodes recorded is only a subsample of those present, because the number of nematodes was too high to quantify accurately and only a proportion of the entire infection was collected.

Anisakis typica was found in a wide range of host species (Table 3), being most common in scombrids, serranids and sphyranids. Among the smaller fish examined, prevalence was highest in atherinids compared with mullids, pseudochromids, chaetodontids and cirhittids.

Terranova Type I was the less prevalent of the two species of this genus identified in the present study and was found in scombrids, serranids, lutjanids, lethrinids and sphyraenids, all being predatory fish of a medium to large size. *Terranova* Type II was very common and was found in large numbers in sphyraenids, serranids and scombrids. Smaller numbers were found in lethrinids and lutjanids, with occasional infections in a wide range of families, including Apogonidae, Atherinidae, Carangidae, Cirrhitidae, Chaetodontidae and Nemipteridae. The larger carnivorous species harboured more nematodes than did the smaller fish species.

Only small numbers of *Hysterothylacium* Type IV were recovered from a disparate range of families, including Carangidae, Cirrhitidae, Chaetodontidae, Pseudochromidae, Scombridae and Serranidae. *Hysterothylacium* Type V larvae were found in lethrinids and lutjanids in small numbers, with a single specimen collected from *Tylosurus* (Belonidae) (Table 3). The few specimens of *Hysterothylacium* Type VI larvae were found in Pomacentridae, Pseudochromidae, Chaetodontidae (small species) and, in one instance, in a scombrid. The least frequently encountered nematode, *Hysterothylacium* Type X was found only in an atherinid, a serranid and a hyporhamphid (Table 3).

Phylogenetic analyses

To determine the relationships of the anisakid larvae reported herein with other closely related anisakid nematodes, phylogenetic analyses were performed using concatenated ITS-1 and ITS-2 sequence data for all 20 genotypes detected in the present study (GenBank accession numbers JX848663–JX848692), together with key reference data (comprising concatenated sequences from previous studies representing *Anisakis typica*, *Hysterothylacium* spp., *Pseudoterranova* spp., *Terranova* sp., and using *Raphidascaris trichiuri* as the outgroup; see Table 4).

The concatenated sequences ($n = 59$) were aligned over 877 positions and subjected to phylogenetic analysis. The analysis

Table 3. Intensity (no. of parasites per individual fish) of infection in the examined fish and the association of anisakid larvae with their hosts
Presence (+) or absence (–) of a particular type of anisakid larva(e)

Family	Fish species	Intensity of infection (range (mean, *n*))	*Anisakis typica*	*Terranova* Types I or II	*Hysterothylacium* Types IV, V, VI, X
Apogonidae	*Cheilodipterus intermedius*	6 (*n* = 1)	–	II	–
Atherinidae	*Atherinomorus endrachtensis*	1–4 (2.2, *n* = 5)	+	II	X
Cirrhitidae	*Paracirrhites forsteri*	3 (*n* = 1)	+	II	IV
Lethrinidae	*Lethrinus nebulosus*	5–43 (26.7, *n* = 3)	–	I, II	Vb
Lutjanidae	*Caesio cuning*	5–53 (26.0, *n* = 3)	–	I, II	Vb, Vc
	Lutjanus carponotatus	4 (*n* = 2)	–	II	–
	Lutjanus fulviflamma	1 (*n* = 1)	–	II	–
Chaetodontidae	*Chaetodon ulietensis*	1 (*n* = 1)	+	–	IV
	Chaetodon citrinellus	11(*n* = 1)	–	II	VI
Pseudochromidae	*Stegastes apicalis*	7 (*n* = 1)	+	–	IV, VI
Scombridae	*Grammatorcynus bicarinatus*	2–197 (88.8, *n* = 6)	+	I, II	IV
	Scomberomorus commerson	19–254 (137, *n* = 2)	+	I, II	IV, VI
Serranidae	*Cephalopholis boenak*	1 (*n* = 1)	+	–	IV
	Cephalopholis cyanostigma	5–18 (11.5, *n* = 2)	+	I, II	X
	Epinephelus ongus	1 (*n* = 1)	+	–	IV
	Plectropomus leopardus	5–80 (42, *n* = 6)	+	I, II	–
Belonidae	*Tylosurus crocodilus*	1 (*n* = 1)	–	–	Vc
Mugilidae	*Liza vaigiensis*	1 (*n* = 1)	+	–	–
Hyporhamphidae	*Hyporhamphus affinis*	2 (*n* = 1)	–	–	X
Nemipteridae	*Scolopsis monogramma*	3 (*n* = 1)	–	II	Vb
Carangidae	*Caranx papuensis*	2–4 (3, *n* = 2)	+	II	IV
Sphyraenidae	*Sphyraena forsteri*	211–357 (284, *n* = 2)	+	I, II	–
Pomacentridae	*Acanthochromis polyacanthus*	28 (*n* = 1)	–	–	Vc, VI

grouped three genotypes (designated as A–C) with the published sequences representing *A. typical*, with strong nodal support (pp = 1.0) (Fig. 8). Five genotypes (D–H) constituted a monophyletic group representing *Terranova* larval Type I, whereas five other genotypes (I–M) grouped with the previously reported sequence representing *Terranova* larval Type II, with a strong statistical support (Fig. 8). Genotype N clustered with *Hysterothylacium* Type IV of Shamsi (2007), whereas three genotypes (O–Q) grouped with *Hysterothylacium* Type VI of Shamsi (2007) (Fig. 8). Genotypes R and S formed one clade and grouped with *Hysterothylacium* Type V, whereas Genotype T clustered with *Hysterothylacium* Type X, with strong nodal support (Fig. 8). With the exception of Genotypes R and S, separate analyses of ITS-1 (56 sequences aligned over ITS-1 = 459 bp) and ITS-2 (52 sequences aligned over 283 bp) sequence datasets produced trees with topologies identical to that generated for the concatenated ITS-1 and ITS-2 dataset (data not shown). Phylogenetic analyses revealed that Genotypes R and S formed one clade, based on the analyses of the ITS-1 as well as concatenated ITS-1 and ITS-2 datasets (Fig. 8), whereas on the basis of the ITS-2 dataset analysis, these form monophyletic groups (see inset Fig. 8).

Discussion

Our understanding of the biodiversity and systematics of parasites has been revolutionised through the use of molecular approaches (Mattiucci and Nascetti 2008). These methods have been utilised not only for delimiting and identifying anisakid nematodes, but also to reveal cryptic species, such as the members of *Anisakis simplex* complex, including *A. simplex C* and *A. pegreffii* (see Mattiucci and Nascetti 2006, 2008). In the present study, we used both molecular-phylogenetic and morphological approaches to characterise larval anisakid nematodes in fishes collected from a single location, Lizard Island. The genetic analysis of larvae has allowed the specific and genotypic identification and differentiation of larvae within single morphotypes, on the basis of matching their sequences with those available for morphologically defined specimens of species of *Anisakis*, *Terranova* and *Hysterothylacium*.

Although more than 450 individual teleost fishes and 107 species were sampled during the present study, this represents a relatively limited portion of the teleost biodiversity occurring around Lizard Island and at other reefs on the GBR. Moreover, many of the species (12 species) were represented by only a single individual. The following discussion is therefore presented within these acknowledged limitations in mind.

The present epidemiological survey revealed eight morphotypes representing *A. typica*, *Terranova* larval Types I and II, *Hysterothylacium* larval Types IV, Vb, Vc, VI and X; whereas, mutation scanning analysis revealed 20 profiles (see Table 3) representing 20 genotypes following sequencing. On the basis of the polymorphism in the ITS-1 region of *A. typica*, three genotypes were defined (designated as Genotypes A–C) and the comparison of these sequences with the published ITS-1 sequences showed that Genotypes A and B had almost identical sequences (except for a Y at Position 201 in Genotype B), as reported previously from China (GenBank Accession number AM706345) and Indonesia (EU346093 and JN968932) (Zhang *et al.* 2007; Palm *et al.* 2008; Kuhn *et al.* 2011), whereas Genotype C matched other *A. typica* ITS-1 sequences available

Table 4. Anisakid internal transcribed spacer (ITS-1 and ITS-2) sequence data determined in the present study, together with reference sequences (see GenBank accession numbers) from previous studies used in the phylogenetic analyses (Fig. 8)
*Voucher number

Parasite species/genotype	Stage of parasite	Host species	Location	Accession numbers (ITS-1)	Accession numbers (ITS-2)	References
A (*Anisakis typica*)	Third-stage larvae -L(3)	*Atherinomorus endrachtensis*, *Caranx papuensis*, *Cephalopholis boenak*, *C. cyanostigma*, *Chaetodon ulietensis*, *Epinephelus ongus*, *Grammatorcynus bicarinatus*, *Liza vaigiensis*, *Paracirrhites forsteri*, *Plectropomus leopardus*, *Scomberomorus commerson*, *Sphyraena forsteri*, *Stegastes apicalis*	Lizard Island, Australia	JX848663	JX848680	This study
B (*A. typica*)	L(3)	*S. forsteri*	Lizard Island, Australia	JX848664	-do-	This study
C (*A. typica*)	L(3)	*S. apicalis*	Lizard Island, Australia	JX848665	-do-	This study
D (*Terranova* larval Type I)	L(3)	*Caesio cuning*, *C. cyanostigma*, *G. bicarinatus*, *Lethrinus nebulosus*, *P. leopardus*, *S. commerson*, *S. forsteri*	Lizard Island, Australia	JX848666	JX848681	This study
E (*Terranova* I)	L(3)	*G. bicarinatus*	Lizard Island, Australia	-do-	JX848682	This study
F (*Terranova* I)	L(3)	*G. bicarinatus*	Lizard Island, Australia	-do-	JX848683	This study
G (*Terranova* I)	L(3)	*G. bicarinatus*	Lizard Island, Australia	JX848667	JX848684	This study
H (*Terranova* I)	L(3)	*G. bicarinatus*, *S. forsteri*	Lizard Island, Australia	-do-	JX848685	This study
I (*Terranova* larval Type II)	L(3)	*A. endrachtensis*, *C. cuning*, *C. papuensis*, *C. cyanostigma*, *Chaetodon citrinellus*, *Cheilodipterus intermedius*, *G. bicarinatus*, *L. nebulosus*, *Lutjanus carponotatus*, *P. forsteri*, *P. leopardus*, *S. commerson*, *S. forsteri*	Lizard Island, Australia	JX848668	JX848686	This study
J (*Terranova* II)	L(3)	*G. bicarinatus*	Lizard Island, Australia	JX848669	-do-	This study
K (*Terranova* II)	L(3)	*Scolopsis monogramma*	Lizard Island, Australia	JX848670	-do-	This study
L (*Terranova* II)	L(3)	*P. leopardus*	Lizard Island, Australia	JX848671	-do-	This study
M (*Terranova* II)	L(3)	*P. leopardus*	Lizard Island, Australia	JX848672	JX848687	This study
N (*Hysterothylacium* larval Type IV)	L(3)	*C. papuensis*, *C. boenak*, *C. ulietensis*, *E. ongus*, *G. bicarinatus*, *P. forsteri*, *S. commerson*, *S. apicalis*, *Tylosurus crocodilus*	Lizard Island, Australia	JX848673	JX848688	This study
O (*Hysterothylacium* larval Type VI)	L(3)	*A. polyacanthus*, *C. cuning*, *L. nebulosus*, *S. monogramma*, *T. crocodiles*	Lizard Island, Australia	JX848674	JX848689	This study
P (*Hysterothylacium* VI)	L(3)	*C. cuning*	Lizard Island, Australia	JX848675	-do-	This study
Q (*Hysterothylacium* VI)	L(3)	*C. cuning*	Lizard Island, Australia	JX848676	-do-	This study
R (*Hysterothylacium* larval Type V)	L(3)	*Acanthochromis polyacanthus*, *C. cuning*, *T. crocodiles*	Lizard Island, Australia	JX848677	JX848690	This study
S (*Hysterothylacium* V)	L(3)	*A. polyacanthus*, *C. citrinellus*, *S. commerson*, *S. apicalis*	Lizard Island, Australia	JX848678	JX848691	This study
T (*Hysterothylacium* larval Type X)	L(3)	*A. endrachtensis*, *C. cyanostigma*, *Hyporhamphus affinis*	Lizard Island, Australia	JX848679	JX848692	This study
Anisakis typica	Adult (A)	*Stenella longirostris*	Brazil	AY826724	AY826724	(Nadler *et al.* 2005)

A. typica	A	*Sotalia guianensis*	Brazil	EU327686	EU327686	(Iñiguez *et al.* 2009)
A. typica	L(3)	*Auxis thazard*	Brazil	EU327689	EU327689	(Iñiguez *et al.* 2009)
A. typica	L	*Auxis rochei rochei*	Indonesia	EU346092	EU346092	(Palm *et al.* 2008)
Anisakis cf. *typica* 1	L	*Auxis r. rochei*	Indonesia	EU346091	EU346091	(Palm *et al.* 2008)
Anisakis cf. *typica* 2	L	*Auxis r. rochei*	Indonesia	EU346093	EU346093	(Palm *et al.* 2008)
A. typica	L	*Scomber japonicas*	Japan	AB432908	AB432908	(Umehara *et al.* 2010)
A. typica	L	*Trichiurus* sp.	Japan	AB551660	AB551660	(Umehara *et al.* 2010)
A. typica	A	*Steno bredanensis*	Florida, USA	AB479120	AB479120	(Umehara *et al.* (2010)
A. typica	L	*Selar crumenophthalmus*	Guangdong province, China	AM706345	AM706345	(Zhu *et al.* 2007)
A. typica	L	*C. cuning*	Indonesia	JN968932	JN968932	(Kuhn *et al.* 2011)
A. typica	L	*Trichiurus lepturus*	Indonesia	JN968944	JN968944	(Kuhn *et al.* 2011)
A. typica	L	*Selar crumenophthalmus*	Hawaii	JN968912	JN968912	(Kuhn *et al.* 2011)
A. typica	L	*Katsuwonus pelamis*	Hawaii	JN968906	JN968906	(Kuhn *et al.* 2011)
A. typica	L	*K. pelamis*	Moorea, Polynesia	JN968964	JN968964	(Kuhn *et al.* 2011)
A. typica	L(3)	*Merluccius polli*	Morocco	EU718476	EU718476	(Kijewska *et al.* 2009)
Hysterothylacium aduncum	L(3)	*Clupea pallasi*, *M. cephalus*	China	AM503955	AM503956	(Zhang *et al.* 2007)
H. aduncum	L(3)	*Platichthys flesus*	Poland	AJ225068	AJ225069	(Zhu *et al.* 1998)
H. aduncum	L(3)	*Hypomesus pretiosus japonicas*	Japan	AB277826	AB277826	(Umehara *et al.* 2008)
H. aduncum	L(3)	*P. flesus*	Poland	AJ937672	AJ937672	(Zhu *et al.* 2007)
H. aduncum	L(3)	*Zoarces viviparous*	Poland	AJ937673	AJ937673	(Zhu *et al.* 2007)
Hysterothylacium auctum	L(3), A	*Z. viviparous*	Baltic sea	AF115571	AF115571	(Szostakowska *et al.* 2001)
H. auctum	A	*Z. viviparous*	Finland	AF226591	AF226591	(Nadler *et al.* 2000)
H.pelagicum	A	*Coryphaena hippurus*	USA	AF226590	AF226590	(Nadler *et al.* 2000)
Hysterothylacium cf. *pelagicum*	A	*Seriolala lalandi*	Australia	175-5-18*	175–2-2*	(Shamsi 2007)
Hysterothylacium larval Type III	L(3)	*Lutjanus argentimaculatus*, *Lutjanus fulviflammus*	Australia	336-8-43*	336–8-2*	(Shamsi 2007)
Hysterothylacium larval Type IVA	L(4)	*C. cuning*, *L. argentimaculatus*	Australia	311-1-1*	311–1-2*	(Shamsi 2007)
Hysterothylacium larval Type IVB	L(4)	*Sillago flindersi*, *S. australasicus*	Australia	11-2-1–4*	11–2-2–3*	(Shamsi 2007)
Hysterothylacium larval Type V	L(3)	*Lutjanus carponotatus*	Australia	305-1-1*	305–1-2*	(Shamsi 2007)
Hysterothylacium larval Type VI	L(3)	*Chaetodon lineolatus*	Australia	314-1-1*	314–1-2*	(Shamsi 2007)
Hysterothylacium larval Type VII	L(3)	*C. cuning*	Australia	306-4*	306–4*	(Shamsi 2007)
Hysterothylacium larval Type VIII	L(3)	*Engraulis australis*, *Sardinops neopilchardus*	Australia	141-3-1–4*	141–3-2–3*	(Shamsi 2007)
	L(3)	*Seriola hippos*, *S. lalandi*	Australia			
	L(3)	*S. flindersi*	Australia			
	L(3)	*S. australasicus*	Australia			
Pseudoterranova azarasi	A	*Eumetopias jubata*	Iwanai, Japan	AJ413973	AJ413974	(Zhu *et al.* 1998)
P. bulbosa	A	*Erignathus barbatus*	Newfoundland, Canada	AJ413969	AJ413971	(Zhu *et al.* 1998)
P. cattani	A	*Otaria byronia*	Central-southern, Chile	AJ413982	AJ413984	(Zhu *et al.* 1998)
P. decipiens	A	*Phoca vitulina*	Newfoundland, Canada	AJ413968	AJ413967	(Zhu *et al.* 1998)
P. krabbei	A	*Halichoerus grypus*	Newfoundland, Canada	AJ413965	AJ413966	(Zhu *et al.* 1998)
Terranova II	L	*Abduefduf whitleyi*, *C. cuning*, *Carangoides fulvoguttatus*, *Caranax ignobilis*, *C. melampygus*, *Epinephelus cyanopodus*, *G. bicarinatus*, *L. argentimaculatus*, *L. buhar*, *L. fulviflammus*, *S. australasicus*	Australia	301-1-1*	301–1-2	(Shamsi 2007)
Raphidascaris trichiuri	A	*Muraenesox cinereus*	Taiwan	FJ009682	FJ009682	(Damin and Heqing 2001)

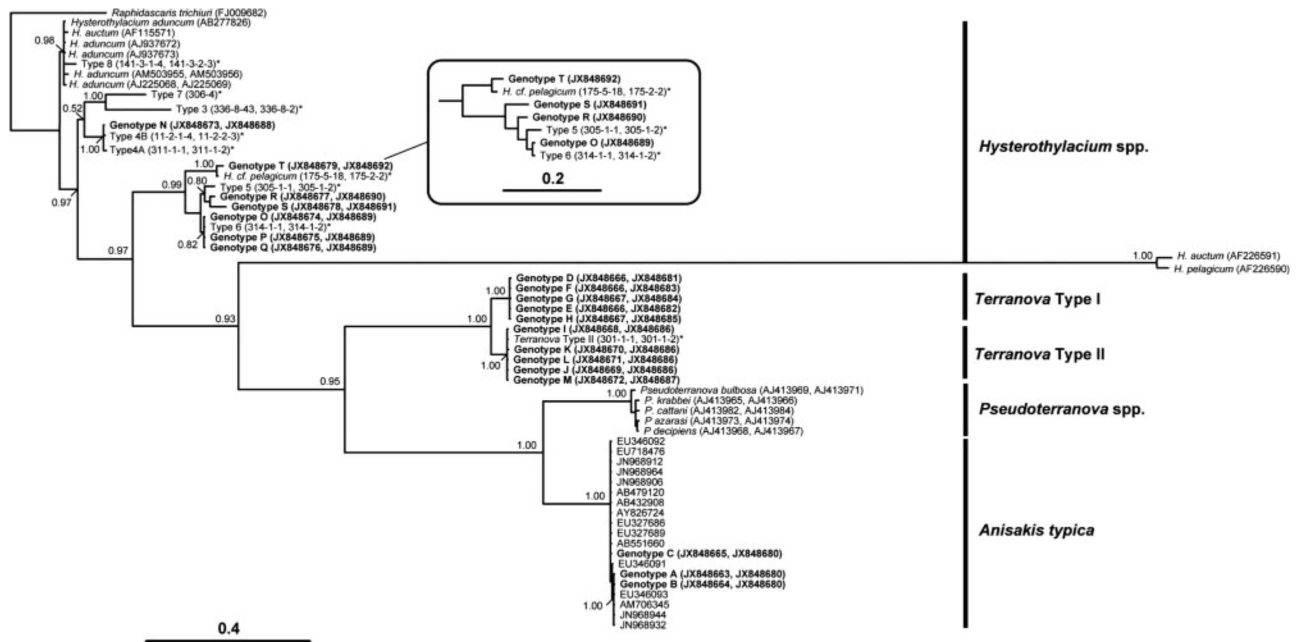

Fig. 8. Phylogenetic relationships of anisakid nematodes representing Genotypes A–T, on the basis of concatenated ITS-1 and ITS-2 sequence data determined herein, together with selected reference sequences for closely related anisakid nematodes. An inset box shows the difference in the topology of Genotypes R and S on the basis of the analysis of ITS-2 dataset. The sources and accession numbers of sequences are listed in Table 4. For each branch of the tree, a parasite species/genotype name is followed by GenBank accession or voucher (*) number. Relationships were inferred on the basis of analyses employing Bayesian inference (BI) method. Nodal support is given as a posterior probability for BI (below the line). Scale bars show the rate of substitution.

on the GenBank database (see Fig. 8). Two larval Types (I and II) of *Terranova* spp. were defined on the basis of their morphological characteristics; molecular methods showed that each of these larval types was composed of five genotypes (see Fig. 8). Comparison of the ITS-1 and ITS-2 sequences of both *Terranova* spp. with the published sequences revealed that those of *Terranova* larval Type I (Genotypes D–H) reported herein are new sequences, whereas those of *Terranova* larval Type II (Genotypes I–M) matched *Terranova* larval Type II (301-1-1, 301-1-2) of Shamsi (2007).

In the present study, five types of *Hysterothylacium* were identified on the basis of their morphological characteristics; however, their molecular characterisation allowed the identification of seven genotypes (N–T). Sequence data for *Hysterothylacium* larval Type IV reported herein (Genotype N) matched with those of *Hysterothylacium* larval Type IVB of Shamsi (2007). Although *Hysterothylacium* larval Types Vb and Vc reported herein resembled, on the basis of 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 Vb (Genotypes O–Q) matched with those of *Hysterothylacium* larval Type VI of Shamsi (2007) (see Fig. 8). This discrepancy between morphological and molecular identification of the same larval type in two different studies cannot be readily explained, because we ensured that the sequence types being reported in the present study corresponded to the correct morphotypes. In the present study, we also found that there were at least three genotypes (O–Q) for *Hysterothylacium* larval Type Vb. Morphologically, *Hysterothylacium* larval Type X reported herein (Genotype T) resembled that reported by Shamsi (2007). Phylogenetic analysis revealed that this larval type grouped with *H.* cf. *pelagicum* of Shamsi (2007) (see Fig. 8). On the basis of morphological characteristics, *Hysterothylacium* larval Type Vc matched *Hysterothylacium* larval Type V of Shamsi (2007); however, phylogenetic analysis of the ITS-1 and concatenated ITS-1 and ITS-2 datasets revealed that Genotype R formed a clade with *Hysterothylacium* Type VI (Genotype S), with *Hysterothylacium* larval Type V of Shamsi (2007) as the sister group, whereas the phylogenetic analysis of the ITS-2 dataset revealed that all *Hysterothylacium* larval Type Vc (Genotype R), *Hysterothylacium* Type VI (Genotype S) and *Hysterothylacium* larval Type V of Shamsi (2007) formed monophyletic clades (see inset Fig. 8). This might be attributed to the percentage of sequence difference in the two ITS regions because pair-wise comparisons between *Hysterothylacium* larval Types Vb and Vc revealed differences for ITS-1 and ITS-2 of 2.6% and 3.0%, respectively; whereas comparison between *Hysterothylacium* larval Types Vc and VI revealed differences for the ITS-1 and the ITS-2 of 4.4% and 9.2%, respectively. It is possible that the groups currently recognised as *Hysterothylacium* Types V and VI each comprises two or more distinct species; however, additional collections are required to confirm this hypothesis.

The data presented here suggest that anisakid infections are relatively uncommon in some families of small reef fishes that were extensively sampled; these fishes included the Apogonidae (cardinal fishes) and Chaetodontidae (butterfly fishes), whereas the infections occurred in very large numbers in members of the Scombridae (mackerels) and Sphyraenidae (barracudas), although

the numbers of fish sampled in the latter families was small. In the case of the scombrids and sphyraenids, the infestations were too heavy to determine the precise numbers of nematodes present; the numerical data presented here therefore underestimate the abundance of larval nematodes present. Anisakid nematodes occurred at a moderate to high abundance in members of the Serranidae (gropers), although, again, only small numbers of fish in this family were examined for parasites. Prevalence in other predatory fish families, such as the Lethrinidae (emperors) and Lutjanidae (snappers), was modest; however, this may reflect limited sampling of the relevant families. Considering these limitations, anisakid nematodes do appear to be more prevalent and more abundant in higher-order predator teleosts than in smaller teleosts occupying a lower position in the food web, a pattern to be expected on the basis of the known or presumed life cycles of these nematodes as summarised by Anderson (2000).

One particular obstacle with parasite life cycles using trophic transmission is how parasites might avoid so-called 'dead-end' hosts that are not consumed by higher-order predators. Such a group of fishes might be the Tetraodontiformes which includes species of the Tetraodontidae (puffer fish, toad fish) that are highly toxic and, therefore, are unlikely to be consumed by higher-order predators. In the present study, none of the 38 tetraodontiform fishes examined was infected with anisakids. Although a relatively small sample size was used, the data do suggest not only that members of this order of fishes are unlikely to be significant contributors to anisakid transmission but that the 'avoidance' of these hosts, however achieved, removes or reduces the possibility of aniskaid larvae infecting 'dead-end' hosts that are unlikely to contribute to the completion of the life cycle. The lack of infection in potential 'dead-end' hosts belonging to the order Tetraodontiformes was intriguing, and requires additional sampling to confirm this preliminary finding. Members of this order of fish are omnivores, algivores or feed on invertebrates. Given this spectrum of diets, the reasons for their lack of infection with anisakid larvae could prove to be of interest.

The scombrids and sphyraenids examined were infected with large numbers of *A. typica* and two species of *Terranova*. Adults of *A. typica* are found primarily in small, piscivorous cetaceans (Mattiucci and Nascetti 2006), whereas species of *Terranova* mature primarily in sharks (Bruce and Cannon 1990). Large scombrids and sphyraenids are likely to be suitable prey species for both of these groups of definitive hosts, thereby facilitating the completion of the life cycle. Serranids were infected with *A. typica* and species of *Terranova*, whereas lutjanids and lethrinids were also infected with species of *Terranova*, although the numbers recovered in all three families were smaller than those seen in scombrids and sphyraenids. All of these species represent potential prey for cetaceans and large elasmobranchs.

Of the smaller fishes examined, both *A. typica* and *Terranova* Type II were recovered from *Atherinomorus*. The extent to which definitive hosts might prey on this species is not known, but they may be preyed on by the larger species of teleosts, such as the scombrids and sphyraenids, which act as effective 'accumulators' of larvae. The various types of *Hysterothylacium* encountered occurred in a wide range of species but predominated in medium- to smaller-sized fishes likely to be consumed as prey by higher-order teleost predators in which species of this genus mature (Deardorff and Overstreet 1980).

The present preliminary study attempted to map the distribution of larval anisakids across a community of reef fishes and identified some broad features that should guide future investigations to provide greater definition of this distribution. The scarcity of anisakid larvae in small fishes, such as apogonids and chaetodontids, was not surprising, given their position in the food chain with the apogonids feeding primarily on zooplankton (Marnane and Bellwood 2002), whereas chaetodontids are omnivorous or corallivores (Morand *et al.* 2000; Cole *et al.* 2008). By contrast, the higher-order predators, such as the serranids, scombrids and sphyraenids, harboured large numbers of anisakid larvae, dominated by *A. typica* and species of *Terranova*. More extensive sampling of these families is clearly warranted to confirm their role in the transmission of anisakids. The species of *Hysterothylacium* detected occurred predominantly in smaller- or medium-sized fishes, but the numbers recovered were low and the range of families extremely broad, preventing any general conclusions from being drawn. However, given their apparent diversity, which was lower in the present study than that reported by Shamsi (2007) from Heron Island in the southern Great Barrier Reef, a greater emphasis needs to be placed on the distribution of species of this genus in future studies of reef teleosts.

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