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Description and genetic characterisation of *Hysterothylacium* (Nematoda: Raphidascarididae) larvae parasitic in Australian marine fishes ☆

Shokoofeh Shamsi [a,*], Robin Gasser [b], Ian Beveridge [b]

[a] School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, New South Wales 2678, Australia
[b] Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia

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ABSTRACT

Nematodes belonging to the genus *Hysterothylacium* (family Raphidascarididae) infect various species of marine fish in both the larval and adult stages. Humans can be accidentally infected upon eating infected seafood. In spite of their importance, relatively little is known of their occurrence and systematics in Australia. An examination of various species of marine teleosts in Australian waters revealed a high prevalence of *Hysterothylacium* larval types. In the present study, seven previously undescribed *Hysterothylacium* larval morphotypes (V to VII and IX to XII) were discovered. In total we found 10 different morphotypes and we genetically characterised nine morphotypes identified. A morphological dichotomous identification key has been established to differentiate these morphotypes. Since some larvae of *Hysterothylacium* from marine fishes cannot be differentiated morphologically from other nematode larvae, such as *Paraheterotyphlum*, *Heterotyphlum*, *Iheringascaris* and *Lapetascaris*, the first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) of these larvae were characterised to confirm their taxonomic status. This genetic characterisation implied that some distinct morphotypes belong to different developmental stages of the same species. In addition, it revealed that some morphotypes can comprise distinct genotypes. No match was found between ITS-1 and ITS-2 sequences obtained from larvae in the present study and those from adults available in the GenBank, highlighting the lack of knowledge on occurrence of adult nematodes infecting Australian fish.

1. Introduction

Hysterothylacium species are gastro-intestinal parasites of predatory teleost fish [1] which also infect various species of marine teleosts in their larval stages. These parasites are not host specific in the larval stages and infect a broad range of fish species; thus they are widely distributed throughout the marine ecosystem and exhibit a global distribution. They can also accidentally infect humans after eating infected seafood [2]. However, compared with many other genera of ascaridoid nematodes, data on the systematics and biology of *Hysterothylacium* spp. are limited.

The genus *Hysterothylacium*, currently consisting of ~67 species, is considered one of the largest of the ascaridoid genera parasitising fish [1,3–5]. In Australia, the genus *Hysterothylacium* is represented by 16 species [6–9]. Reports of the larval stages of *Hysterothylacium* are fewer than those for adults in Australia. Thus far, five types of *Hysterothylacium* larvae, numbered I to IV, and VIII, have been described from fishes in Victoria and Queensland waters [10,11]. Subsequently, some of these larvae have been reported from various fishes in additional regions of Australia [11–15]. Nothing is known about specific identity of larvae, their life cycle or other biological aspects in Australia. In the present study, we describe 10 different morphotypes of *Hysterothylacium* found in Australian marine fish, seven of them (V to VII and IX to XII) being described for the first time, and we genetically characterize nine morphotypes identified.

2. Materials and methods

Eighty-one fish of 20 species, including *Abudefduf whitleyi*, *Caesio cuning*, *Chaetodon auriga*, *Chaetodon aureofasciatus*, *Chaetodon flavirostris*, *Chaetodon lineolatus*, *Chaetodon melanotus*, *Chaetodon minutus*, *Engraulis australis*, *Heniochus monoceros*, *Heniochus singularius*, *Lutjanus argentimaculatus*, *Lutjanus carponotatus*, *Lutjanus fulviflammus*, *Sardinops neopilchardus*, *Scomber australasicus*, *Seriola hippos*, *Seriola lalandi*, *Sillago flindersi* and *Sphyraena novaehollandiae* were examined fresh for infection with parasites. Fish were collected from Heron Island, Queensland (23°26′S, 151°54′E); Port Phillip Bay, Victoria (38°4′S, 144°52′E) and Clarries Reef, Greenly Island (34°38′S, 134°47′E) and Port Lincoln (34°42′S, 135°51′E) in South Australia.

Parasites were washed in saline after collection. A small piece of the midbody of each nematode was excised for molecular study, and the rest of the nematode was cleared in lactophenol for morphological examination, as described previously [16–19]. Nematodes were identified using the morphology of the labia, the position of the excretory pore,

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under the GenBank ID: FN811678–FN811767.

* Corresponding author. Tel./fax: +61 2 69334887.
E-mail address: sshamsi@csu.edu.au (S. Shamsi).

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the oesophageal venticulus, ventricular appendix and the tail [10]. All measurements are given in millimetres unless otherwise stated as the mean followed by the range in parentheses. Specimens were washed in saline and then fixed in 70% ethanol and deposited in South Australian Museum, Adelaide (SAM) (Table 1).

Genomic DNA was isolated from individual larvae by sodium dodecyl-sulphate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega) and eluted into 40 μl of water [17]. Host DNA was also isolated from the musculature of fishes using the same method. PCR was used to amplify the ITS-1 and ITS-2 regions using primers and cycling conditions described previously [16–19]. Samples with fish DNA or without genomic DNA were included in the PCR as negative controls; no amplicons were produced in the PCR from these samples. An aliquot (4 μl) of each amplicon was examined on a 1.5% w/v agarose gel, stained with ethidium bromide and photographed using a gel documentation system.

Amplicons were purified over mini-columns (Wizard™ PCR Prep, Promega, WI, USA), eluted in 30 μl H_2O and then subjected to automated sequencing (BigDye® chemistry, Applied Biosystems), in both directions, using the same primers as for PCR. Sequences were aligned using the computer programme ClustalX [20] and then adjusted manually. Polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC). The pairwise comparison of sequence differences were calculated by formula $D = 1 - (M/L)$ [21].

3. Results

All eighty-one fish examined in the present study were infected with at least one *Hysterothylacium*-type larva. In total, 10 different morphotypes were found. Larvae numbered III, IV and VIII are those previously described [10,11]. Morphologically different *Hysterothylacium* larval types discovered in the present study were numbered V to VII and IX to XII.

3.1. Morphology

3.1.1. Hysterothylacium larval type III

Description (Fig. 1a, b): third stage larvae. Body length 13.3 (11.4–15.1), width 0.29 (0.096–0.32). No developed labia. A minute cephalic boring tooth located anteroventrally. Nerve ring 0.33 (0.31–0.36) from anterior end. Excretory pore at level of nerve ring, 0.41 (0.33–0.48) from anterior end. Oesophagus 1.2 (1.1–1.4) long, 9% of body length. Ventriculus 0.09 (0.07–0.10) long. Ventricular appendix 1.3 (1.1–1.4) long, approximately same length as oesophagus. Intestinal caecum 0.25 (0.20–0.30) long, 20% (19–22%) of oesophageal length and 0.20 (0.18–0.21) length of ventricular appendix. Tail very short, 0.11 (0.09–0.12) long, 0.8% of body length, rounded with single prominent spine.

Material examined: two larvae (AHC46080-1) were in good morphological conditions, three larvae were found in three species of fish.

Hosts: *L. argentimaculatus* (Forsskål, 1775), *L. carponotatus* (Richardson, 1842) and *L. fulviflammus*. (Forsskål, 1775) (family: Lutjanidae).

Localization in host: intestine.

Locality: Heron Island, Queensland.

3.1.2. Hysterothylacium larval type IV (Fig. 1c–h)

Larvae identified as *Hysterothylacium* type IV were morphologically similar to those previously described by Cannon [10]. In the present study, it was shown that these larvae comprised at least two distinct genotypes named herein as A and B (see below). Therefore, the morphological description and genetic characterisation of the two groups are given separately.

3.1.2.1. Hysterothylacium larval type IV genotype A. Description: fourth stage larvae. Body length 6.4 (5.5–7.5), width 0.14 (0.06–0.18). Body annulated. Three small labia, one dorsal, two subventral. Tooth absent. Nerve ring 0.24 (0.20–0.27) from anterior end. Excretory pore at level of nerve ring, 0.32 from anterior end. Muscular oesophagus 0.67 (0.46–0.80) long, 10% (8–13%) of body length. Ventriculus 0.12 (0.06–0.2) long. Ventricular appendix longer than intestinal caecum, 0.8 (0.7–0.9), 1.25 (1.05–1.74) times oesophageal length. Small intestinal caecum 0.16 (0.08–0.20) long, 23% (17–26%) of oesophageal length, 20% (5–25%) of ventricular appendix length. Tail relatively short, 0.13 (0.08–0.18) long, 2% (1–2%) of body length; tip covered with cluster of spines, with single enlarged spine at tip.

Material examined: four larvae (AHC34987, 34988).

Hosts: Whitley's sergeant, *A. whitleyi* Allen & Robertson, 1974 (family: Pomacentridae); Redbelly yellowtail fusilier, *C. cuning* (Bloch, 1791) (family: Caesionidae); Mangrove red snapper, *L. argentimaculatus* (Forsskål, 1775) (family: Lutjanidae).

Localization in host: encapsulated on the surface of internal organs and connective tissues.

Locality: Heron Island, Queensland.

Table 1
Accession numbers of individuals of *Hysterothylacium* larval types studied in the present study and their ITS data.

Hysterothylacium larval type	Museum accession no	ITS region							
		1				2			
		GenBank accession no.	Length (bp)	G + C content (%)	Polymorphism	GenBank accession no.	Length (bp)	G + C content (%)	Polymorphism
III	AHC46080-1	FN811721–FN811723	434	51.4–51.8%	Alignment positions 310 and 397	FN811678–FN811681	365	53.7	No polymorphism
IV-A	AHC34987 -8	FN811724–FN811729	438	51.8	No polymorphism	FN811690–FN811698	346, 347*	54.3–54.6	Positions 97, 98 and 126
IV-B	AHC34989-92	FN811730–FN811737	438	51.1	No polymorphism	FN811682–FN811689	345	53.9–54.5	36, 37, 172 and 199
V	AHC46083	FN811738–FN811739	440	50.7	No polymorphism	FN811699–FN811700	275	50.9	No polymorphism
VI	AHC46084	FN811740–FN811748	437	49.6	No polymorphism	FN811701–FN811708	272	50.0	No polymorphism
VII	AHC46085	FN811749	433	51.7	No polymorphism	FN811709	356	57.0	No polymorphism
VIII	AHC46086-93, 34190	FN811750–FN811760	431	49.9	No polymorphism	FN811710–FN811713	348	50.3	No polymorphism
X	AHC46077	FN811761–FN811762	436	51.4	No polymorphism	FN811714–FN811716	286	53.5	No polymorphism
XI	AHC46095	FN811763	436	51.4	No polymorphism	FN811717	286	53.5	No polymorphism
XII	AHC46094	FN811764–FN811767	437	49.6	No polymorphism	FN811718–FN811720	272	50.0	No polymorphism