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ORIGINAL PAPER

Morphological and molecular identification of *Hysterothylacium longilabrum* sp. nov. (Nematoda: Anisakidae) and larvae of different stages from marine fishes in the South China Sea

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Abstract A new ascaridoid nematode *Hysterothylacium longilabrum* sp. nov. collected from the intestine and stomach of the marine fishes *Siganus fuscescens* (Houttuyn) and *Siganus canaliculatus* (Park) (Perciformes: Siganidae) in the South China Sea is described and illustrated. The new species differs from its congeners by the unusually long lips, the very short intestinal caecum and relatively long ventricular appendix (ratio of intestinal caecum to ventricular appendix, 1:2.38–5.50), the long spicules (1.96–3.28 mm long, representing 7.42–11.4% of the body length), the number and arrangement of male caudal papillae [38–43 pairs in total, arranged as: 31–34 pairs precloacal, 1 pair of paracloacal and 4–6 pairs postcloacal (the second or fourth pair double)] and the presence of a particular medioventral precloacal papilla in the male. Molecular analyses by sequencing and comparing the internal transcribed spacer (ITS) of the ribosomal DNA of *H. longilabrum* sp. nov. with the closely related nematode sequences seem to support the validity of the new species based on the morphological observation. In addition, the third- and fourth-stage larvae of the new species are also exactly identified and described by analysing and comparing the ITS sequence with the adult, and the result is a substantial step toward elucidating its life cycle.

Introduction

With 67 described species worldwide, *Hysterothylacium* Ward & Magath, 1917 is one of the largest groups of ascaridoid nematodes (Bruce et al. 1994; Moravec et al. 1996, 1997; Moravec and Nagasawa 1998, 2000; Torres et al. 1998; Torres and Soto 2004; Gopar-Merino et al. 2005; Li et al. 2007a, b, 2008; Raffel and Anderson 2009; Rossin et al. 2011). Adults of *Hysterothylacium* commonly occur in the digestive tract of marine, estuarine and freshwater fishes (Deardorff and Overstreet 1980; Bruce and Cannon 1989; Bruce et al. 1994). However, *Hysterothylacium burtti* Raffel and Anderson 2009 was also reported parasitic in amphibian (Raffel and Anderson 2009). Up to 2012, a total of ten species of *Hysterothylacium* were recorded from marine fishes in the Chinese waters (Hsü 1933; Parukhin 1966; Yin and Zhang 1982, 1983; Pan et al. 1990; Li et al. 2007a, b, c, 2008). During a helminthological survey of Chinese marine fishes from May 2005 to June 2011, large numbers of larvae and adults of ascaridoid nematodes were collected from marine fishes in the Yellow Sea, East China Sea, Taiwan Strait and South China Sea. The results of systematic evaluations of some of this material have already been published (Zhang et al. 2007; Li et al. 2007a, b, c, 2008, 2011, 2012; Du et al. 2010). In the present paper, a new species *Hysterothylacium longilabrum* sp. nov. collected from the marine fishes *Siganus fuscescens* (Houttuyn) and *Siganus canaliculatus* (Park) in the South China Sea is described and illustrated by light and scanning electron microscopy.

Accurate identification of a parasite at any stage of its development has very important implications for studying parasite epidemiology and resolving systemic problems (Kijewska et al. 2002). However, proper identification of adults of ascaridoid nematodes, only based on morphological characters, is sometimes problematic because of the existence of sibling or cryptic species appearing to be ubiquitous in ascaridoid nematodes, which nearly cannot be distinguished from each other only by morphological

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studies (Mattiucci et al. 1997; Zhu et al. 2002; Martín-Sánchez et al. 2003; Li et al. 2005; Mattiucci et al. 2008, 2010). In addition, morphological identification of ascaridoid larvae is also unreliable and unpractical as they are usually very similar to each other morphologically, and many significant taxonomic morphological features of the larvae are not fully developed (Moravec 2009). Recently, molecular techniques, utilising the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) as a genetic marker, have proved to be particularly useful for the accurate identification of ascaridoid nematodes at the species level for eggs, larvae and adults (Zhu et al. 2007; for a review see Mattiucci and Nascetti 2008; Mašová et al. 2010; Fang et al. 2010; Testini et al. 2011; Li et al. 2011). Consequently, specimens of the adults, together with the identified morphologically as putative third- and fourth-stage larvae, of the new species collected from the two different hosts *S. fuscescens* and *S. canaliculatus* are characterised using molecular methods by sequencing and analysing the internal transcribed spacer (ITS) of the ribosomal DNA to assess the validity of the new species genetically, and to exactly recognize the larvae of different stages.

Materials and methods

Light and scanning electron microscopy

Fishes collected from the South China Sea were examined for parasites. Nematodes recovered from the digestive tract of fishes were washed in physiological saline and then fixed and stored in 70 % ethanol until studied. For light microscopical studies, nematodes were cleared in lactophenol. Drawings were made with the aid of a Nikon microscope drawing attachment. For scanning electron microscopy, specimens were fixed in 4 % formaldehyde solution, post-fixed in 1 % OsO4, dehydrated via an ethanol series and acetone, and then critical point dried. The specimens were coated with gold and examined using a Hitachi S-570 scanning electron microscope at an accelerating voltage of 15 kV.

Measurements (the range, followed by the mean in parentheses) are given in micrometres unless otherwise stated. Type specimens are deposited in the College of Life Science, Hebei Normal University, Hebei Province, China.

Molecular procedures

Twenty-four nematodes (details in Table 1) were subjected to molecular analysis. Genomic DNA from individual worms was extracted using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. DNA was eluted in an elution buffer and kept at −20 °C until use. The ITS region was amplified by PCR using the primers A (forward: 5′-GTCGAATTCG TAGGTGAACCTGCGGAAGGATCA-3′) and B (reverse: 5′-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3′) (D'Amelio et al. 2000). PCR was performed in 50 μl of PCR reaction buffer with 10 mM Tris–HCl at pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, 250 μM of each dNTP, 50 pmol of each primer and 1.5 U of *Taq* polymerase (Takara) in a thermocycler (2720, Applied Biosystems) under the following conditions: 94 °C, 5 min (initial denaturation), followed by 30 cycles of 94 °C, 30 s (denaturation), 55 °C, 30 s (annealing), 72 °C, 70 s (extension), and a final extension of 72 °C for 7 min. PCR products were checked on GoldView-stained 1.5 % agarose gel and purified by the Column PCR Product Purification Kit (Shanghai Sangon, China). Sequencing was carried out using a DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, CA, USA) and an automated sequencer (ABI PRISM 377). Sequencing for each sample was carried out for both strands. Sequences were aligned using ClustalW2 (Thompson et al. 1994) and adjusted manually. The ITS sequences determined were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Results

Hysterothylacium longilabrum sp. nov.

Description

Medium to large, whitish nematodes with finely transversely striated cuticle. Maximum width of body at about mid-body. Lateral alae absent (Fig. 4a). Anterior end with three very long lips, approximately equal in size, with deep post-labial grooves and prominent lateral flanges (Figs. 1a, 3c, 4b). Proximal part of each lip with four lobes (Fig. 3a). Dorsal lip with two lateral double papillae (Figs. 1a, 3c), ventrolateral lips each with one lateral amphid, one single papilla and one double papilla (Figs. 1c, 4b). Interlabia well developed, about 1/3 length of lips (Figs. 1b, 4b). Oesophagus relatively long, slightly broader posteriorly than anteriorly. Nerve ring at 20–35 % of oesophageal length. Excretory pore just posterior to nerve ring (Fig. 1c). Ventriculus oval to oblong, slightly narrower than posterior region of oesophagus. Intestinal caecum very short, slightly longer or shorter than ventriculus (Fig. 1d, e). Ventricular appendix usually narrower and longer than intestinal caecum (Fig. 1d, e). Rectum hyaline, tubular, surrounded by three large, unicellular rectal glands. Tail of both sexes conical, tip with numbers of nodular protuberances (Figs. 1g, k, l, 3d, g).

Table 1 Specimens of *H. longilabrum* sp. nov. selected for molecular analysis

Hosts	Nematode individuals for molecular analysis	Nematode number
Siganus fuscescens	Adults randomly selected	3
Siganus canaliculatus	Adults randomly selected	3
Siganus fuscescens	Identified morphologically as putative third-stage larvae	3
Siganus fuscescens	Identified morphologically as putative fourth-stage larvae	3
Siganus fuscescens	Adults with relatively long intestinal caecum (as Fig. 1e)	3
Siganus fuscescens	Adults with short intestinal caecum (as Fig. 1d)	3
Siganus fuscescens	Adults with four postcloacal papillae (as Fig. 3)	3
Siganus fuscescens	Adults with six postcloacal papillae (as Fig. 3d)	3

Male (based on 18 mature specimens)

Body 26.4–31.5 (27.6) mm long; maximum width of 637–833 (755). Dorsal and ventrolateral lips almost equal in size, 392–490 (441) long, 196–275 (237) wide. Interlabia 98–147 (127) long, 196–245 (223) wide. Oesophagus 2.65–3.43 (2.98) mm long, 343–441 (402) in maximum width, representing 9.02–12.6 (10.8) % of body length. Nerve ring and excretory pore 833–1,058 (947) and 882–1,098 (1,007), respectively, from anterior extremity. Ventriculus 196–314 (241) long, 225–294 (270) wide. Ventricular appendix 882–1,274 (1,053) long, 49–98 (68.6) wide. Intestinal caecum 225–392 (280) long, 98–176 (139) wide, representing 7.81–13.8 (9.47) % oesophageal length. Ratio of intestinal caecum to ventricular appendix 1:2.50–5.20 (1:3.74). Posterior end of body curves ventrally (Fig. 1i, j). Ejaculatory duct 1.86–2.74 (2.33) mm long. Spicules slender, alate, subpointed apically, of almost equal or slightly unequal length, 1.96–3.28 (2.72) mm long, representing 101–129 (108) % of ejaculatory duct and 7.42–11.4 (9.59) % of body length (Figs. 1i, 3e). Gubernaculum absent. Caudal papillae very small, 38–43 pairs in total, arranged as follows: 31–34 pairs precloacal, 1 pair of paracloacal and 4–6 pairs postcloacal (the second or fourth pair double) (Figs. 1j, 2, 3b, d, f, 4c). Medioventral precloacal papilla present (Figs. 3b, 4d, e). Tail 167–245 (206) long. Small lateral phasmids present at base of tail tip (Fig. 3b, d, g).

Female (based on 20 gravid specimens)

Body 23.4–47.2 (35.6) mm long; maximum width 588–1,274 (850). Dorsal and ventrolateral lips almost equal in size, 441–637 (497) long, 245–372 (307) wide. Interlabia 147–245 (181) long, 225–392 (298) wide. Oesophagus 2.55–5.68 (3.88) mm long, 372–539 (448) in maximum width, representing 10.0–12.0 (10.8) % of body length. Nerve ring 804–1,372 (1,003) and excretory pore 882–1,519 (1,105) from anterior extremity, respectively. Ventriculus 245–392 (315) long, 245–392 (312) wide. Ventricular appendix 882–1,078 (973) long, 58–98 (86.8) wide. Intestinal caecum 196–392 (307) long, 127–245 (185) wide, representing 6.67–13.5 (8.64) % oesophageal length. Ratio of intestinal caecum to ventricular appendix 1:2.38–5.50 (1:3.40). Vulva slit-like, situated anteriorly, 6.27–11.8 (9.47) mm from anterior extremity, at 25.0–28.0 (26.6) % of body length (Fig. 1f). Vagina muscular, directed posteriorly. Uteri form coils in region posterior to vagina, extend posteriorly to level of rectum. Eggs suboval to almost rounded, 29–49 (41.2) long, 29–49 (37.2) wide (Fig. 1h). Tail 490–853 (670) long. Small lateral phasmids present at base of tail tip (Fig. 1g).

Type host and type locality: *S. fuscescens* (Houttuyn) (Perciformes: Siganidae); South China Sea, off Sanya (109°30′E; 18°12′N), Hainan Province, China.

Other host and locality: *S. canaliculatus* (Park) (Perciformes: Siganidae); South China Sea, off Sanya (109°30′E; 18°12′N), Hainan Province, China.

Site of infection: Intestine and stomach.

Type specimens: Holotype: male collected from *S. fuscescens*, South China Sea, off Sanya (HBNU-F1143); allotype: female collected from *S. fuscescens*, South China Sea, off Sanya (HBNU-F1144); paratypes: 133 males and 324 females collected from *S. fuscescens*, South China Sea, off Sanya (HBNU-F1145); 10 males and 11 females collected from *S. canaliculatus*, South China Sea, off Sanya (HBNU-F1146).

Prevalence and intensity of infection: *S. fuscescens*: 87.5 % (35 out of 40 fishes) were infected off Sanya with intensity of 1–35 (mean=13.1) specimens; *S. canaliculatus*: 33.3 % (one out of three fishes) were infected off Sanya with intensity of 21 specimens.

Etymology: The specific epithet is derived from a combination of the Latin words *longus*- (long) and *labrum*- (lip), and refers to the unusually long dorsal and ventrolateral lips.

Fourth-stage larvae (based on 14 specimens)

Body 16.2–18.9 (17.5) mm long; maximum width 490–588 (549). Dorsal and ventrolateral lips distinctly shorter than lips of adult, almost equal in size, 157–196 (180) long, 118–147 (135) wide (Fig. 2g, h). Interlabia 59–78 (70.6) long, 98–147 (113) wide (Fig. 2h). Oesophagus almost cylindrical, muscular, slightly broader posteriorly than anteriorly, 1.81–2.16 (1.96) mm long, 196–245 (229) in maximum width,

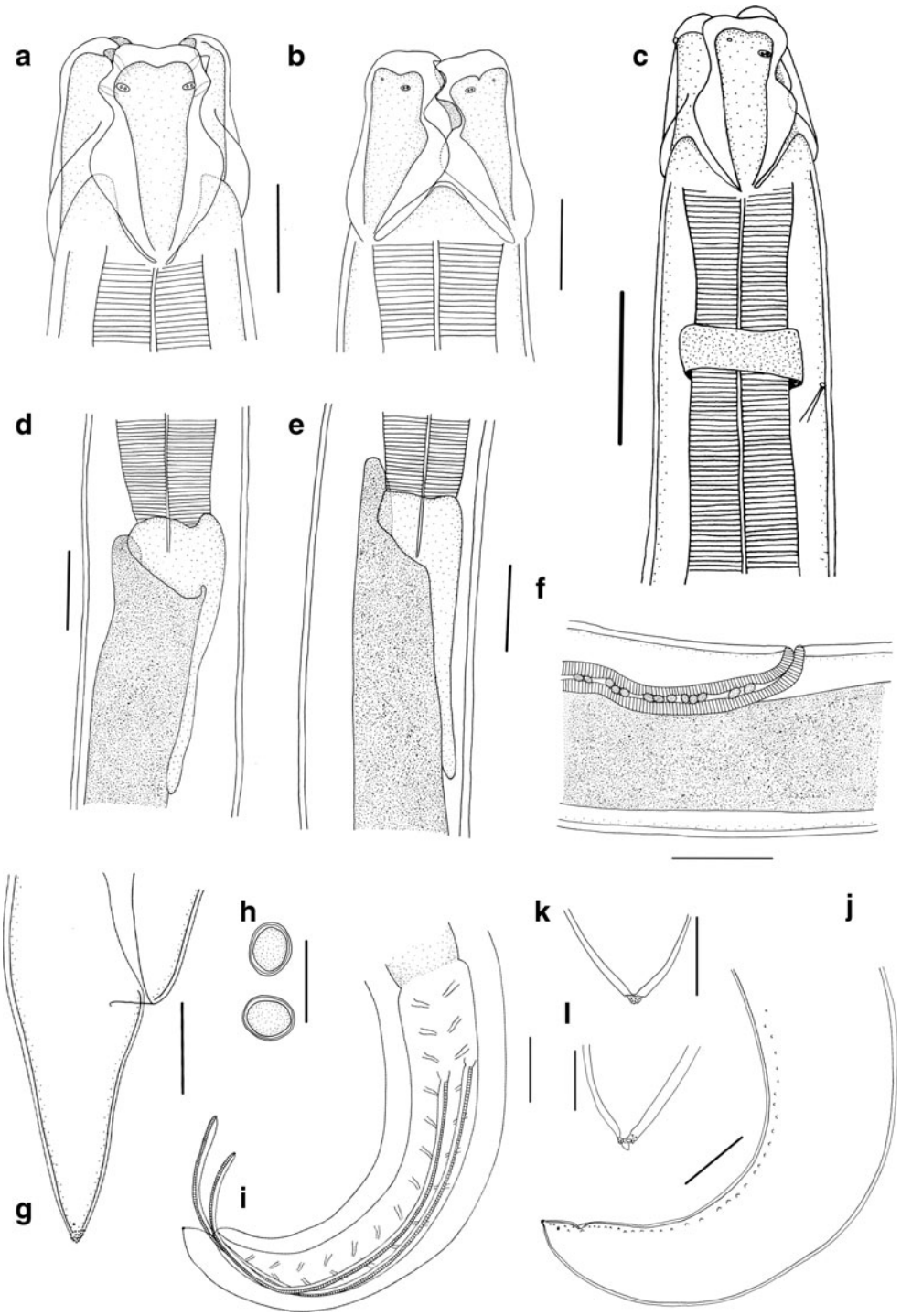

Fig. 1 *H. longilabrum* sp. nov. from *S. fuscescens* (Houttuyn) in the South China Sea: **a** cephalic end of male, dorsal view; **b** cephalic end of male, ventral view; **c**. anterior part of male, lateral view; **d, e** region of ventriculus; **f** region of vulva; **g** posterior end of female, lateral view; **h** eggs; **i**. posterior end of male (showing ejaculatory duct and spicules), lateral view; **k** posterior end of male (showing caudal papillae), lateral view; **k, l** tip of male tail. *Scale bars*: **a**, **b** 200 μm; **c, d, e, f, i, j** 400 μm; **g** 500 μm; **h** 50 μm; **k**, **l** 100 μm

representing 9.84–12.6 (11.3) % of body length (Fig. 2f). Nerve ring 490–519 (505) and excretory pore 539–568 (546) from anterior extremity, respectively (Fig. 2f). Ventriculus 147–245 (176) long, 163–245 (182) wide. Ventricular appendix 588–785 (667) long, 49–69 (56.8) wide (Fig. 2j). Intestinal caecum slightly longer than ventriculus, 98–294 (186) long, 78–137 (98.0) wide, representing 5.26–13.9 (9.31) % oesophageal length. Ratio of intestinal caecum to ventricular appendix 1:2.20–7.00 (1:4.25) (Fig. 2j). Tail 353–412 (388) long, tip with numbers of nodular protuberances (Fig. 2i). Small lateral phasmids not observed.

Host and locality of fourth-stage larvae: *S. fuscescens* (Houttuyn) (Perciformes: Siganidae); South China Sea, off Sanya (109°30′E; 18°12′N), Hainan Province, China.

Site of infection: Intestine.

Prevalence and intensity of infection: 20.0 % (8 out of 40 fishes) were infected with intensity of 3–25 (mean=14.8) specimens.

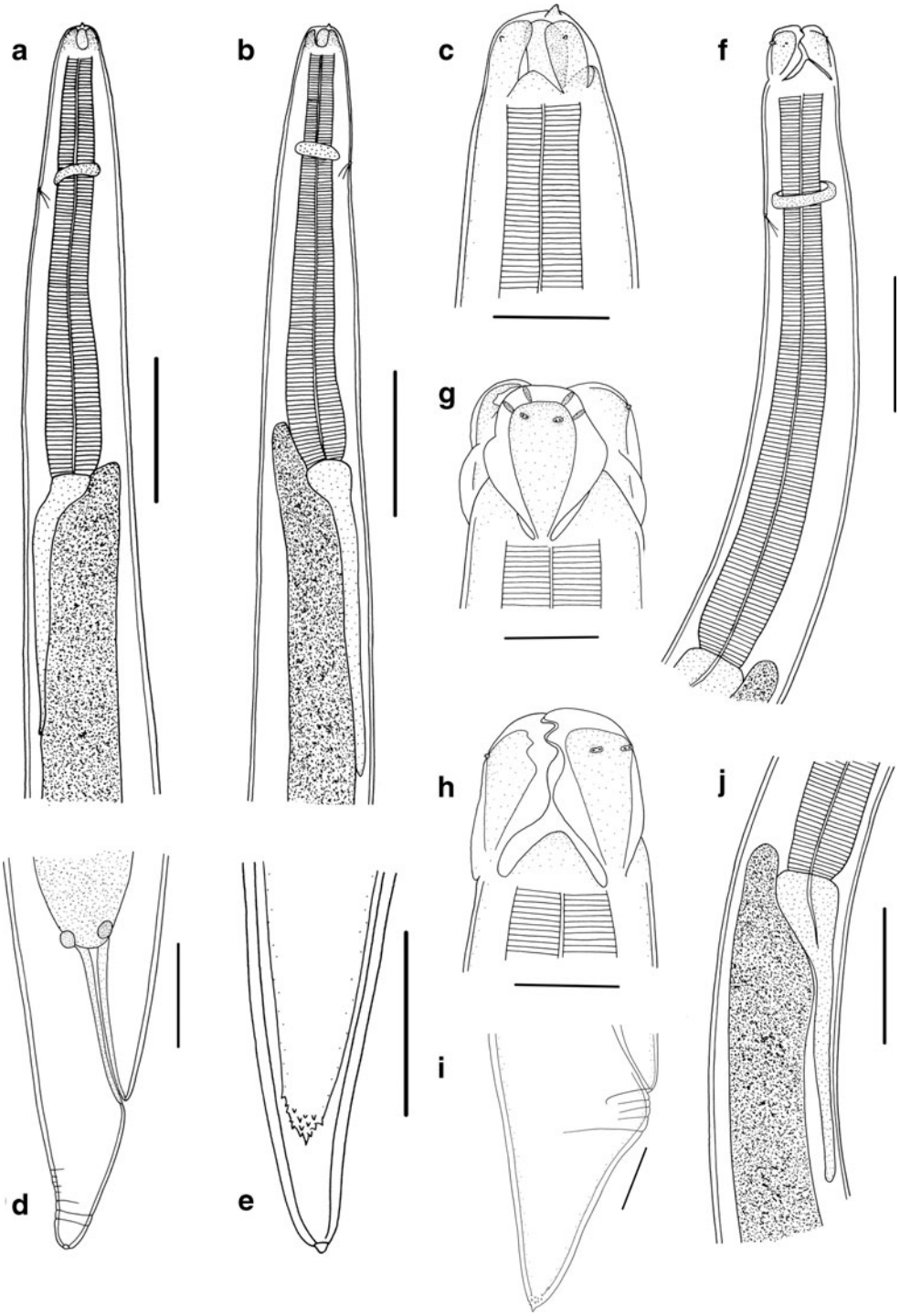

Fig. 2 Third- and fourth-stage larvae of *H. longilabrum* sp. nov. from *S. fuscescens* (Houttuyn) in the South China Sea: **a**, **b** anterior part of third-stage larva, lateral view; **c** cephalic end of third-stage larva, lateral view; **d** posterior end of third-stage larva, lateral view; **e** tip of tail of third-stage larva (showing tip of tail of fourth-stage larva inside); **f** anterior part of fourth-stage larva, lateral view; **g** cephalic end of fourth-stage larva, dorsal view; **h** cephalic end of fourth-stage larva, lateral view; **i** posterior end of fourth-stage larva, lateral view; **j** region of ventriculus. *Scale bars*: **a**, **b**, **f**, **j** 400 μm; **c**, **d**, **e**, **g**, **h**, **i** 100 μm

Voucher specimens: 118 specimens (HBNU-F1147).

Third-stage larvae (based on ten specimens)

Body 8.20–13.1 (10.2) mm long; maximum width 196–392 (304). Cephalic end rounded, with prominent ventral cuticular tooth, sharply pointed (Fig. 2a–c). Anlagen of lips weakly developed (Fig. 2c). Oesophagus almost cylindrical, muscular, slightly broader posteriorly than anteriorly. Oesophagus almost cylindrical, muscular, slightly broader posteriorly than anteriorly, 931–1,274 (1,098) long, 98–176 (124) in maximum width, representing 10.1–11.4 (10.8) % of body length (Fig. 2a, b). Nerve ring 294–490 (392) and excretory pore 314–539 (426) from anterior extremity, respectively (Fig. 2a, b). Ventriculus 59–98 (78.6) long, 98–127 (108) wide. Ventricular appendix 488–841 (704) long,

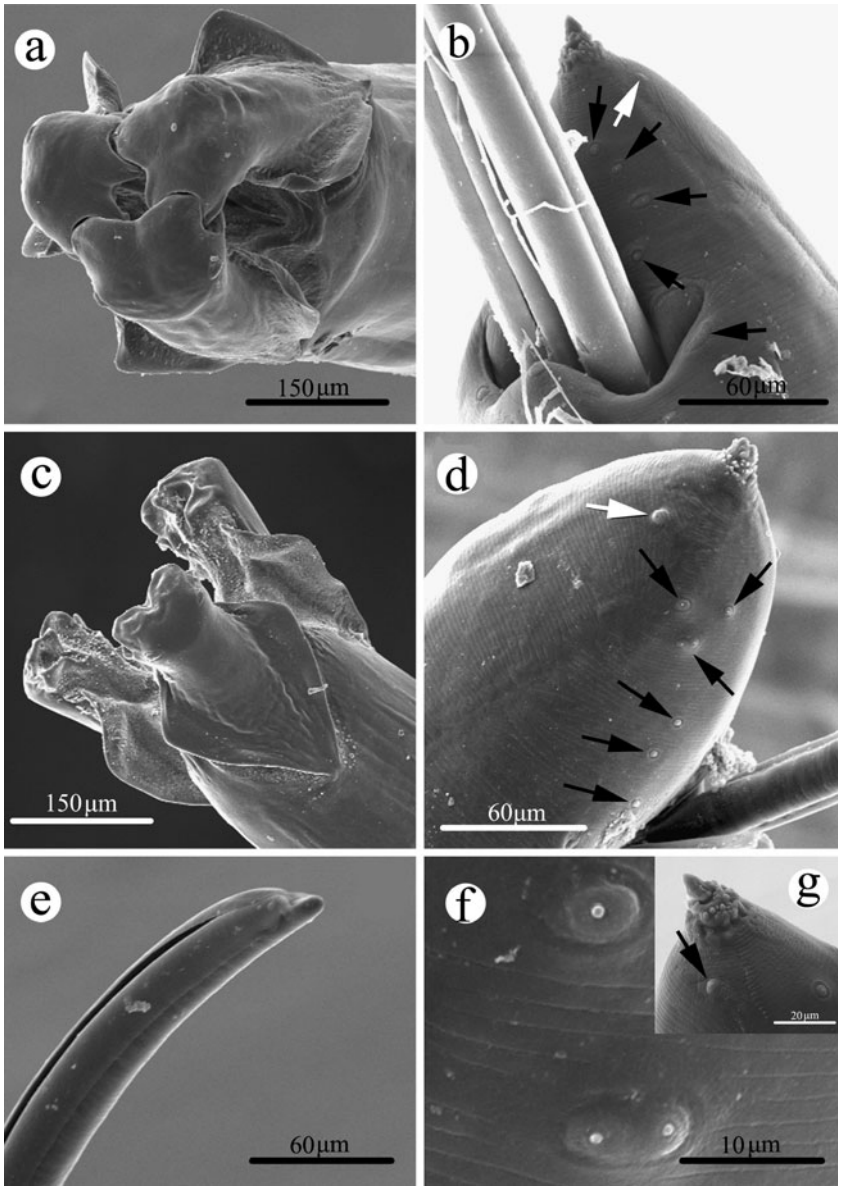

Fig. 3 Scanning electron micrographs of *H. longilabrum* sp. nov. from *S. fuscescens* (Houttuyn) in the South China Sea: **a** cephalic extremity of male, apical view; **b** posterior end of male, ventral view, showing four postcloacal and single paracloacal papillae (*black arrows*) and lateral phasmid (*white arrow*); **c** cephalic extremity of male, dorsal view; **d** posterior end of male, lateral view, showing six postcloacal papillae (*black arrows*) and lateral phasmid (*white arrow*); **e** distal end of left spicule; **f** postcloacal double papilla; **g** tip of male tail, showing lateral phasmid (*black arrow*)

29–49 (35.3) wide. Intestinal caecum slightly longer than ventriculus, 108–196 (135) long, 58–98 (76.4) wide, representing 9.47–12.9 (11.3) % oesophageal length (Fig. 2a, b). Ratio of intestinal caecum to ventricular appendix 1:2.28–5.78 (1:4.30). Tail 140–245 (196) long, tip without nodular protuberances (Fig. 2d, e). Small lateral phasmids not observed.

Host and locality of third-stage larvae: *S. fuscescens* (Houttuyn) (Perciformes: Siganidae); South China Sea, off Sanya (109°30′E; 18°12′N), Hainan Province, China.

Site of infection: Abdominal cavity.

Prevalence and intensity of infection: 12.5 % (5 out of 40 fishes) were infected with intensity of 1–5 (mean=2.8) specimens.

Voucher specimens: 14 specimens (HBNU-F1148).

DNA characterisation

The sequences of the samples of adult and identified morphologically as putative third- and fourth-stage larvae, obtained for the ITS region of the partial rDNA, were all 1,007 bp in length and showed no intraspecific nucleotide variability among the different developmental specimens collected from the two different hosts examined. There are nine species of *Hysterothylacium* ITS sequences registered in GenBank and pairwise comparison between *H. longilabrum* sp. nov. and the other species of *Hysterothylacium* (expect *Hysterothylacium* sp. JYW-2010, HM545895) displayed 9.59 (JQ520158) to 27.27 % (AM706344) nucleotide differences (details in Table 2). The low level of sequence variation was found between *H. longilabrum* sp. nov. and *Hysterothylacium* sp. JYW-2010 (HM545895) (i.e.

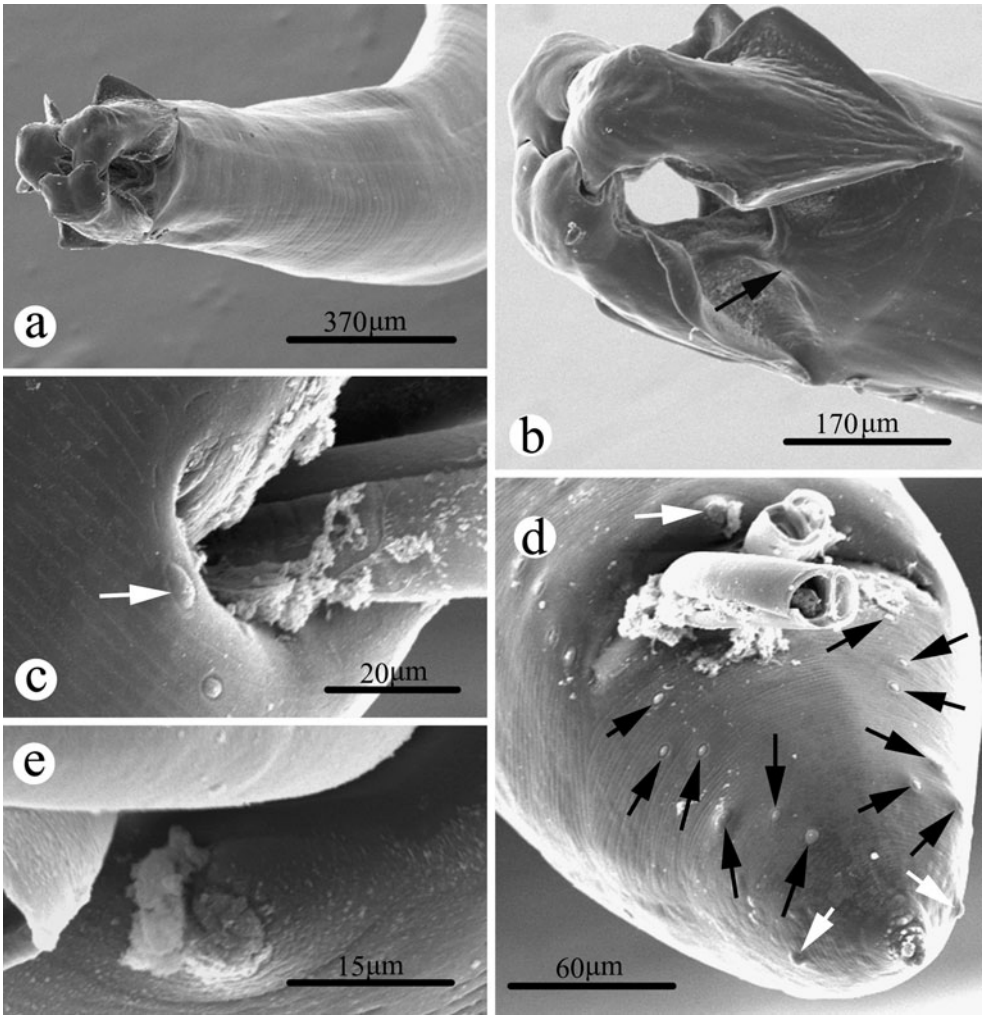

Fig. 4 Scanning electron micrographs of *H. longilabrum* sp. nov. from *S. fuscescens* (Houttuyn) in the South China Sea: **a** anterior part of male, apical view (lateral alae absent); **b** cephalic end of male, ventral view, showing interlabium (*black arrow*); **c** paracloacal papilla (*white arrow*); **d** posterior end of male, ventral view, showing six postcloacal papillae (*black arrows*), single medioventral precloacal papilla and lateral phasmid (*white arrows*); **e** medioventral precloacal papilla

only two nucleotide alterations were detected at alignment positions 197 and 318, respectively). The ITS sequence of *H. longilabrum* sp. nov. is deposited in the GenBank database (http://www.ncbi.nlm. nih.gov) under accession number (JQ520159).

Discussion

The gross morphology of the specimens from *S. fuscescens* and *S. canaliculatus*, especially the position of the excretory pore and the presence of both intestinal caecum and ventricular appendix, clearly shows they should belong to *Hysterothylacium*. *H. longilabrum* sp. nov. remarkably differs from all other congeners in *Hysterothylacium* by the unusually long lips. As far as we are aware, *Hysterothylacium ogcocephali* (Olsen 1952) appears most similar to *H. longilabrum* sp. nov. in having the elongated lips (Olsen 1952; Deardorff and Overstreet 1980). But the lips of *H. longilabrum* sp. nov. are much larger than *H. ogcocephali* (0.44–0.64 mm in the former vs 0.17–0.31 mm in the latter, in the female). From *H. ogcocephali*, it also differs by having much longer spicules (1.96–3.28 mm long, representing 7.42–11.4 % of body length in the new species vs 0.33–0.66 mm long, representing 1.0–2.0 % of body length in *H. ogcocephali*), a much greater number of postcloacal papillae (four to six pairs in *H. longilabrum* sp. nov. vs three pairs in *H. ogcocephali*) and the absence of lateral alae (presence of distinct lateral alae in *H. ogcocephali*). Within *Hysterothylacium*, the new species is similar to the following species in having a very short intestinal caecum (slightly longer or shorter than ventriculus) and relatively long ventricular appendix (about two to six times longer than intestinal caecum): *Hysterothylacium habena* (Linton, 1900) from *Opsanus tau* (Linneaus) (Batrachoidiformes: Batrachoididae) in the American waters (Norris and Overstreet 1975); *Hysterothylacium rhacodes* (Deardorff and Overstreet 1978) from *Pelates quadrilineatus* (Blochin) (Perciformes: Terapontidae), *Solea aegyptiaca* Chabanaud (Pleuronectiformes: Soleidae), *Boops boops* (Linnaeus) (Perciformes: Sparidae), *Diplodus sargus sargus* (Linnaeus) (Perciformes: Sparidae), *Diplodus vulgaris* (Geoffroy Saint-Hilaire) (Perciformes:

Table 2 *Hysterothylacium* spp. compared with *H. longilabrum* sp. nov. genetically in the present study and their ITS data

Nematode species	GenBank nos.	Length of ITS	G + C content (%)	Nucleotide difference from *H. longilabrum* sp. nov. (%)
H. longilabrum sp. nov.	JQ520159	1,007	52.83	0
H. auctum	AF115571	1,579	50.47	11.82
H. bidentatum	AY603539	926	50.54	11.45
H. amoyense (as *Contracaecum muraenesoxi* in GenBank)	EU826125	998	51.50	23.24
	EU828749	990	51.62	23.87
Hysterothylacium sp. JYW-2010	HM545895	1,032	53.20	0.20
Hysterothylacium sp. CHJ-2010	HM437224 HM437225	937	50.27	11.74
Hysterothylacium sp. PB-2010	JN005769	1,033	51.60	9.93
Hysterothylacium sp. HS1	AM706344	957	50.99	27.27
H. fabri	JQ520158	1,001	51.45	9.59
H. aduncum	HM598666 HQ270425–HQ270433	877–1,427	50.27–51.52	11.12–12.54
	HQ702732			
	HQ702733			
	JF683733			
	JF683734			
	HM598666			
	AB277826			
	AJ937672			
	AJ937673			

Sparidae), *Lithognathus mormyrus* (Linnaeus) (Perciformes: Sparidae), *Oblada melanura* (Linnaeus) (Perciformes: Sparidae) and *Sparus aurata* Linnaeus (Perciformes: Sparidae) in the eastern Mediterranean Sea (Deardorff and Overstreet 1978); *Hysterothylacium pelagicum* Deardorff and Overstreet 1982 from *Coryphaena hipputus* Linnaeus (Perciformes: Coryphaenidae) in the Pacific and Atlantic oceans (Deardorff and Overstreet 1982; Bruce and Cannon 1989); *Hysterothylacium physiculi* Moravec and Nagasawa 2000 from *Physiculus japonicus* Hilgendorf [as *Physiculus maximowiczi* (Herzenstein)] (Gadiformes: Moridae) in the Japanese waters (Moravec and Nagasawa 2000); *Hysterothylacium cornutum* (Stossich, 1904) from *Thunnus thynnus* (Linneaus), *Thunnus maccoyii* (Castelnau), *Thunnus albacares* (Bonnaterre) and *Thunnus alalunga* (Bonnaterre) (Perciformes: Scombridae) in the Pacific and Atlantic oceans (Yamaguti 1941; Bruce and Cannon 1989; Moravec and Nagasawa 2000); *Hysterothylacium sebae* Bruce 1990 from *Lutjanus sebae* (Cuvier) (Perciformes: Lutjanidae) in the Australian waters (Bruce 1990); *Hysterothylacium reliquens* (Norris and Overstreet 1975) from *Archosargus probatocephalus* (Walbaum) (Perciformes: Sparidae), *Halichoeres bivittatus* (Bloch) (Perciformes: Labridae), *Opsanus beta* (Goode & Bean) (Batrachoidiformes: Batrachoididae), *Chilomycterus schoepfii* (Walbaum) (Tetraodontiformes: Diodontidae), *Micropogonias undulatus* (Linnaeus) [as *Micropogon undulatus* (Linnaeus)] (Perciformes: Sciaenidae), *Gymnothorax nigromarginatus* (Girard) (Anguilliformes: Muraenidae), *Sciaenops ocellatus* (Linnaeus) (Perciformes: Sciaenidae) and *Ogcocephalus cubifrons* (Richardson) (Lophiiformes: Ogcocephalidae) in the American waters, and *Batrachoides surinamensis* (Bloch & Schneider) (Batrachoidiformes: Batrachoididae) in the Brazilian waters (Norris and Overstreet 1975; Deardorff and Overstreet 1980); *Hysterothylacium scomberoidei* Bruce and Cannon 1989 from *Scomberoides commersonianus* Lacepède (Perciformes: Carangidae) in the Australian waters (Bruce and Cannon 1989); *Hysterothylacium fortalezae* (Klein 1973) from *Scomberomorus brasiliensis* Collette, Russo & Zavala-Camin and *Scomberomorus cavalla* (Cuvier) (Perciformes: Serranidae) in the Brazilian waters (Klein 1973), and *Scomberomorus maculates* (Mitchill) (Perciformes: Scombridae), *Mycteroperca bonaci* (Poey) (Perciformes: Serranidae) and *Oligoilites saurus* (Bloch & Schneider) (Perciformes: Carangidae) in the American waters and Mediterranean Sea (Deardorff and Overstreet 1980); *Hysterothylacium fabri* (Rudolphi 1819) from *Uranoscopus scaber* Linnaeus (Perciformes: Uranoscopidae) and *Zeus faber* Linnaeus (Zeiformes: Zeidae) in the Mediterranean Sea (Petter and Maillard 1987; Bruce et al. 1994), and *Trachurus japonicus* (Temminck & Schlegel) (Perciformes: Carangidae), *Pennahia argentata* (Houttuyn) [as *Argyrosomus argentatus* (Houttuyn)](Houttuyn (Perciformes: Sciaenidae), *Conger myriaster* (Brevoort) [as *Astroconger myriaster* (Brevoort)] (Anguilliformes:

Congridae) and *Chelidonichthys kumu* (Cuvier) in the Chinese waters (Li et al. 2008). *H. longilabrum* sp. nov. can be readily distinguished from *H. physiculi*, *H. scomberoidei*, *H. fortalezae*, *H. sebae*, *H. pelagicum* and *H. fabri* by having much longer spicules (representing 7.42–11.4 % of body length in the former vs 1.8–5.0 % of body length in the latter six species) and the absence of lateral alae (presence of distinct lateral or caudal alae in the latter six species). The new species differs from *H. cornutum* by different arrangement of caudal papillae (31–34 pairs precloacal, 1 pair of paracloaca and 4–6 pairs postcloacal in the former vs 22–24 pairs precloacal, 1 pair of paracloacal and 7–10 pairs postcloacal in the latter) and different morphology of tail tip (absence of nodular protuberances in *H. cornutum*). From *H. reliquens* and *H. habena*, the new species differs by the presence of medioventral precloacal papilla and the absence of lateral alae (lateral alae prominent at posterior end of body in the latter two species). *H. longilabrum* sp. nov. is different from *H. rhacodes* by the presence of one pair of paracloacal papillae and a single particular, medioventral precloacal papilla and much longer spicules (representing 7.42–11.4 % of body length in the former vs 3.0–6.0 % of body length in the latter species).

Although over 60 species of *Hysterothylacium* have been described until now (Bruce et al. 1994; Moravec et al. 1996, 1997; Moravec and Nagasawa 1998, 2000; Torres et al. 1998; Torres and Soto 2004; Gopar-Merino et al. 2005; Li et al. 2007a, b; Raffel and Anderson 2009; Rossin et al. 2011), the life cycles of most species are still poorly known. In our opinion, this is mainly due to our very limited knowledge of the development, morphogenesis and identification of the larvae. The larvae of *Hysterothylacium* commonly parasitise various tissues of numerous fishes and invertebrates (Norris and Overstreet 1976; González 1998). During this helminthological survey of *S. fuscescens*, the prevalence and intensity of infection with third-stage larvae are very low [(12.5 % (5 out of 40 fishes) were infected with intensity of 1–5 (mean=2.8) specimens], in contrast to the high prevalence and intensity of infection with adults [87.5 % (35 out of 40 fishes) were infected with intensity of 1–35 (mean=13.1) specimens]. In addition, the third-stage larvae collected from the abdominal cavity of *S. fuscescens* are all aged because of the anlagen of lips and the nodular protuberances of tail tip well developed in most of specimens. The result may indicate that *S. fuscescens* only acts as the natural definitive host of *H. longilabrum* sp. nov.. *S. fuscescens* is a herbivorous fish and mainly feeds on filamentous algae, leafy algae and seagrasses, and sometimes also eats crustaceous invertebrates, such as gastropods and amphipods in the wild (Lavina and Alcala 1973; Wassef and Hady 1997). Consequently, we speculate the crustaceous invertebrates may be the intermediate hosts of *H. longilabrum* sp. nov..

The accurate identification of anisakid larvae is a key step for understanding their life cycles, epidemiology and disease surveillance and control (Zhang et al. 2007). However, it is often problematic to exactly recognize the different larvae developmental stages of *Hysterothylacium* spp. only based on morphological characters as there are usually remarkable morphological differences between the different larvae and adult developmental stages; for example, the tail tip of the third-stage larvae and the lips of the fourth-stage larvae of *H. longilabrum* sp. nov. are distinctly different from the adults. In addition, the larvae always lack some morphological features of taxonomic significance including the number and arrangement of cloacal papillae, the length of spicules and the position of the vulva, which result to a difficulty in distinguishing the different larvae of *Hysterothylacium* spp. morphologically. Therefore, in the present paper, the internal transcribed spacer (ITS) of the ribosomal DNA of the samples identified morphologically as putative third- and fourth-stage larvae of *H. longilabrum* sp. nov. are sequenced and compared with the sequence of adult, and no nucleotide difference was detected. The result strongly proved that the samples identified morphologically as putative third- and fourth-stage larvae and the adults are homogeneous genetically and all belong to the same species *H. longilabrum* sp. nov.. Although there are some morphological varieties between some individuals of *H. longilabrum* sp. nov., for instance, the length of intestinal caecum (Fig. 1d, e), the number of postcloacal papillae (Fig. 3b, d) and the morphology of tip of male tail (Fig. 1k, l), the molecular analyses for adult specimens of *H. longilabrum* sp. nov. collected from the two different fish hosts (*S. fuscescens* and *S. canaliculatus*, respectively) using ITS of rDNA showed only one genotype (i.e. no intraspecific variation was detected). This result was predictable when we considered that all the morphological varieties between individuals were just the intraspecific variation. Interspecific variation between the ITS sequences of *H. longilabrum* sp. nov. herein generated and those species of *Hysterothylacium* available in the GenBank (expect *Hysterothylacium* sp. JYW-2010, HM545895) was detected from 9.59 (JQ520158) to 27.27 % (AM706344) nucleotide differences, which is a very high level of interspecific sequence variation. This result supported the validity of this new nematode species based on the morphological observation and suggested that it is a useful approach to utilise the ITS of rDNA as a genetic marker for the distinction between species of *Hysterothylacium* spp.. The low level of ITS sequence variation (only two nucleotide alterations) detected between *H. longilabrum* sp. nov. and *Hysterothylacium* sp. JYW-2010 (HM545895) was also expected because the specimen of *Hysterothylacium* sp. JYW-2010 was collected from the same fish host *S. fuscescens*, in a neighbouring region of the South China Sea (off Zhanjiang, Guangdong

Province, China). Therefore, we have no hesitation in considering the species *Hysterothylacium* sp. JYW-2010 (HM545895) to be conspecific with *H. longilabrum* sp. nov., and the two nucleotide differences should be considered as intraspecific variation because of the different geographical locations.

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