DOI: 10.2478/s11686-012-0037-2

Acta Parasitologica, 2012, 57(3), 316–322; ISSN 1230-2821

VERSITA

Morphological and molecular characterization of *Raphidascaris (Ichthyascaris) lophii* (Wu, 1949) (Nematoda, Anisakidae) from marine fishes from China, with a key to the species of the subgenus *Ichthyascaris*

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Abstract

The little known ascaridoid nematode *Raphidascaris (Ichthyascaris) lophii* (Wu, 1949) is redescribed and illustrated based on newly collected specimens from the five different marine fishes: *Lophius litulon* (Jordan) (Lophiiformes: Lophiidae), *Lophiomus setigerus* (Vahl) (Lophiiformes: Lophiidae), *Antennarius hispidus* (Bloch et Schneider) (Lophiiformes: Antennaridae), *Zeus faber* Linnaeus (Zeiformes: Zeidae) and *Ostichthys japonicus* (Cuvier) (Beryciformes: Holocentridae) from the East and South China Sea. This species differs from all congeners in the subgenus *Ichthyascaris* by the length of the ventricular appendix (0.52–0.98 mm long), the number and arrangement of caudal papillae (26–32 pairs of precloacal, 3–4 pairs of paracloacal and 8–11 pairs of postcloacal) and the length of the spicules (0.49–0.88 mm long, representing 3.08–4.70% of body length). In addition, nematodes collected from these five different fishes have been characterized using molecular methods by sequencing and analysing the internal transcribed spacer (ITS) of ribosomal DNA. No variation in size and nucleotide polymorphisms is detected within the target sequence among all samples analysed. These data contribute to facilitate an accurate diagnosis of this poorly known nematode. An identification key to the species of the subgenus *Ichthyascaris* is also provided.

Keywords

Ascaridida, marine fishes, *Raphidascaris*, *Ichthyascaris*, internal transcribed spacer (ITS)

Introduction

Wu (1949) established the genus *Ichthyascaris* and designated *I. lophii* Wu, 1949 from the intestine of *Lophius litulon* (Jordan) in the East China Sea as the type species. The genus was established based on the simple lips without lateral membranous flanges, the lack of interlabia and an intestinal caecum, the presence of a ventricular appendage and the lateral alae uniting close the ventrolateral lips (Wu 1949; Bruce 1990). Later, Hartwich (1957), Chabaud (1965) and Moravec (1994) placed the genus in synonymy with the genus *Raphidascaris*. However, Bruce (1990) resurrected *Ichthyascaris* as an independent genus. Recently, Moravec and Nagasawa (2002) and Moravec and Justine (2005) considered *Ichthyascaris* as a subgenus of *Raphidascaris*. We follow this taxonomic treatment in this paper.

Up to 2011, the subgenus *Ichthyascaris* included 10 species (Yamaguti 1935; Wu 1949; Olsen 1952; Santos 1970; Hooper 1983; Lèbre and Petter 1983; Yin and Zhang 1983; Bruce 1990; Bruce *et al.* 1994; Luo and Huang 2001; Moravec and Nagasawa 2002; Moravec and Justine 2005). Of all these species, only two species had been reported to occur in China: *R.* (*I.*) *lophii* (Wu, 1949) from Shanghai and *R.* (*I.*) *trichiuri* (Yin et Zhang, 1983) from Zhejiang and Fujian (Wu 1949, Yin and Zhang 1983, Luo and Huang 2001). However, *R.* (*I.*) *lophii* is still a poorly studied species and the original description is insufficient in detail to allow recognition or clear separation from other species now in this group (Smith 1984, Bruce 1990). Redescription and comparison of this species with similar species are necessary to confirm its validity.

Recently, the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) has been successfully employed as a molecular marker for the accurate identification of ascaridoid nematodes (Zhang *et al.* 2007; Zhu *et al.* 2007; Du *et al.* 2010; Fang *et al.* 2010; Testini *et al.* 2011; Li *et al*. 2012). Thus, the

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present paper reports the use of PCR-based methods utilising the internal transcribed spacer (ITS) of ribosomal DNA (including internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2) for molecular characterization of *R.* (*I.*) *lophii* and provides a detailed redescription of this little known species based on newly collected specimens from the five different marine fishes: *Lophius litulon* (Jordan) (Lophiiformes: Lophiidae), *Lophiomus setigerus* (Vahl) (Lophiiformes: Lophiidae), *Antennarius hispidus* (Bloch et Schneider) (Lophiiformes: Antennaridae), *Zeus faber* Linnaeus (Zeiformes: Zeidae) and *Ostichthys japonicus* (Cuvier) (Beryciformes: Holocentridae) from the East and South China Sea. An identification key to the species of the subgenus *Ichthyascaris* is also provided.

Materials and methods

Light and scanning electron microscopy

Fishes collected from the East and South China Sea were examined for parasites. Nematodes recovered from the digestive tract of fishes were washed in physiological saline, and then stored in 70% ethanol. For light microscopic studies, nematodes were cleared in lactophenol. Drawings were made with the aid of Nikon microscope drawing attachment. For scanning electron microscopy (SEM) studies, specimens were fixed in 4% formaldehyde solution, post-fixed in 1% OsO_4, dehydrated through ethanol and acetone, then critical point dried. The specimens were coated with gold and examined with a Hitachi S-570 scanning electron microscope at an accelerating voltage of 15 kV. Measurements (minimum, maximum, followed by mean in parentheses) are given in micrometers, unless otherwise stated. Specimens have been deposited in College of Life Sciences, Hebei Normal University, Hebei Province, China (see Table I).

Molecular procedures

Twenty-four nematodes (i.e., six individuals randomly selected from each host *L. litulon*, *L. setigerus* and *A. hispidus*; four individuals from *O. japonicus* and two individuals from *Z. faber*) were subjected to molecular analysis. Genomic DNA from individual worms was extracted by Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. DNA was eluted in elution buffer and kept at −20°C until use. The ITS region was amplified by PCR using the primers A (forward 5'-GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA-3') and B (reverse: 5'-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3'). PCR was performed in 50 µl of PCR reaction buffer with 10 mM Tris HCl, pH 8.4, 50 mM KCl, 3.0 mM MgCl2, 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 were purified by the Column PCR Product Purification Kit (Shanghai Sangon, China). Sequencing was carried out using DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, Cali-

Table I. The specimens of *Raphidascaris (Ichthyascaris) lophii* (Wu, 1949) collected from the East and South China Sea in this study

Hosts	Site of infection	Date of collection	Locality of collection	Prevalence and intensity	Voucher specimens
Lophius litulon (Jordan)	Intestine and stomach	14–19.X. 2010	Zhoushan Island, China Sea, Zhejiang Province	23.1% (3 infected/13 examined), 3–7 (5.0) specimens	6 males, 9 females (HBNU-F1101)
Lophiomus setigerus (Vahl)	Intestine and stomach	24.IV. 2010	Shanwei, South China Sea, Guangdong Province	50.0% (2 infected/4 examined), 11–13 (12.0) specimens	7 males, 17 females (HBNU-F1102)
	Intestine and stomach	24–28.XI. 2010	Sanya, South China Sea, Hainan Province	15.0% (3 infected/20 examined), 2–49 (18.7) specimens	19 males, 37 females (HBNU-F1103)
Antennarius hispidus Bloch et Schneider	Intestine and stomach	24.XI. 2010	Sanya, South China Sea, Hainan Province	33.3% (1 infected/3 examined), 6.0 specimens	1 male, 5 females (HBNU-F1104)
Zeus faber Linnaeus	Intestine	25.IV. 2010	Shanwei, South China Sea, Guangdong Province	20.0% (1 infected/5 examined), 2.0 specimens	2 females (HBNU-F1105)
Ostichthys japonicus (Cuvier et Valenciennes)	Intestine	21.IV. 2010	Shanwei, South China Sea, Guangdong Province	14.3% (1 infected/7 examined), 2.0 specimens	1 male, 3 females (HBNU-F1106)

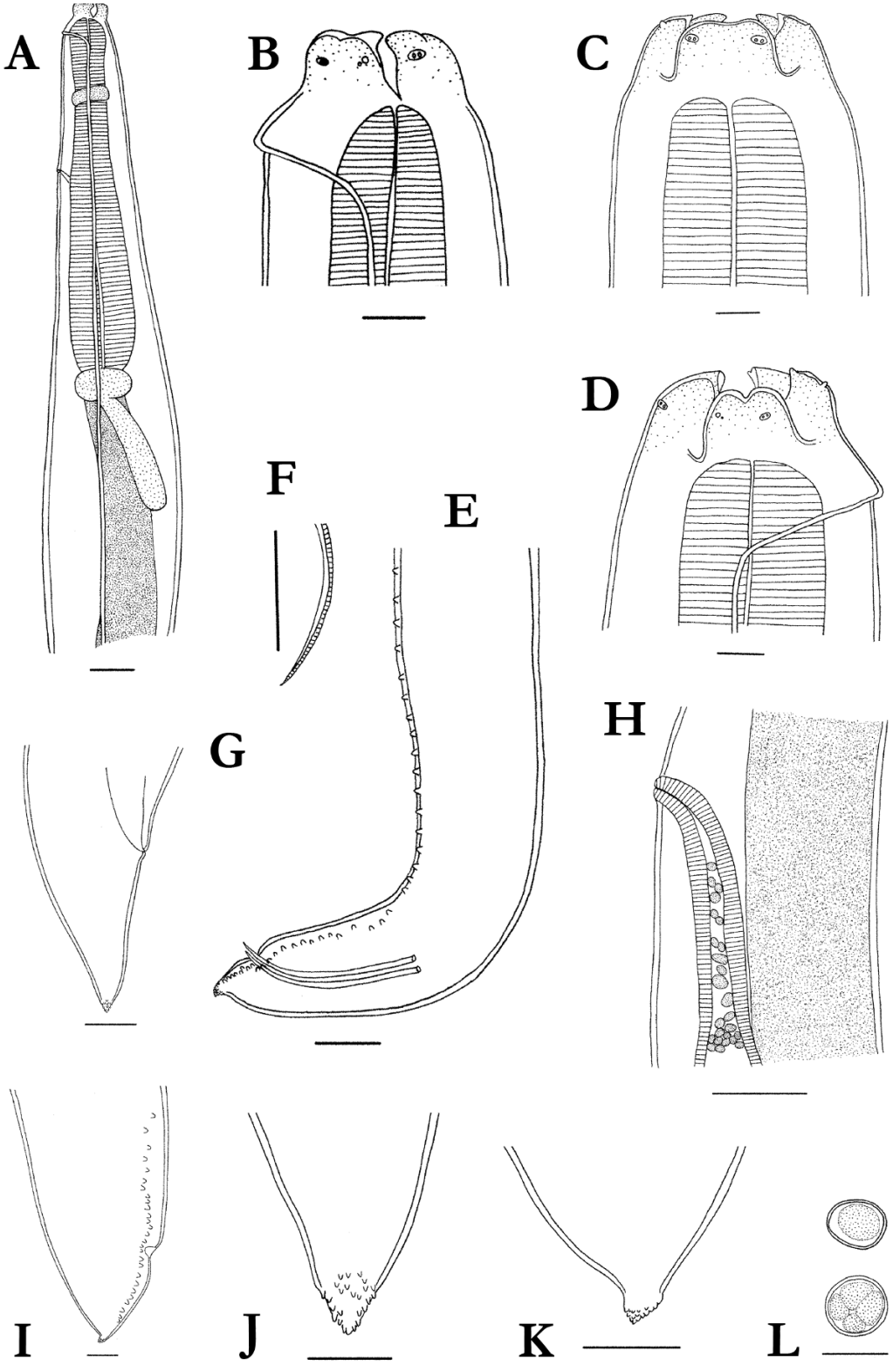

Fig. 1. *Raphidascaris (Ichthyascaris) lophii* Wu, 1949 collected from *Lophius litulon*. **A** – anterior part of female body, lateral view, scale bar 0.2 mm; **B** – cephalic end of female, lateral view, scale bar 0.1 mm; **C** – cephalic end of female, dorsal view, scale bar 0.05 mm; **D** – cephalic end of female, sublateral view, scale bar 0.05 mm; **E** – posterior end of male, lateral view, scale bar 0.2 mm; **F** – apical part of left spicule, scale bar 0.2 mm; **G** – posterior end of female, lateral view, scale bar 0.2 mm; **H** – region of vulva, scale bar 0.4 mm; **I** – caudal region of male, lateral view, scale bar 0.05 mm; **J** – tip of female tail, scale bar 0.03 mm; **K** – tip of male tail, scale bar 0.04 mm; **L** – two stages of egg, scale bar 0.04 mm

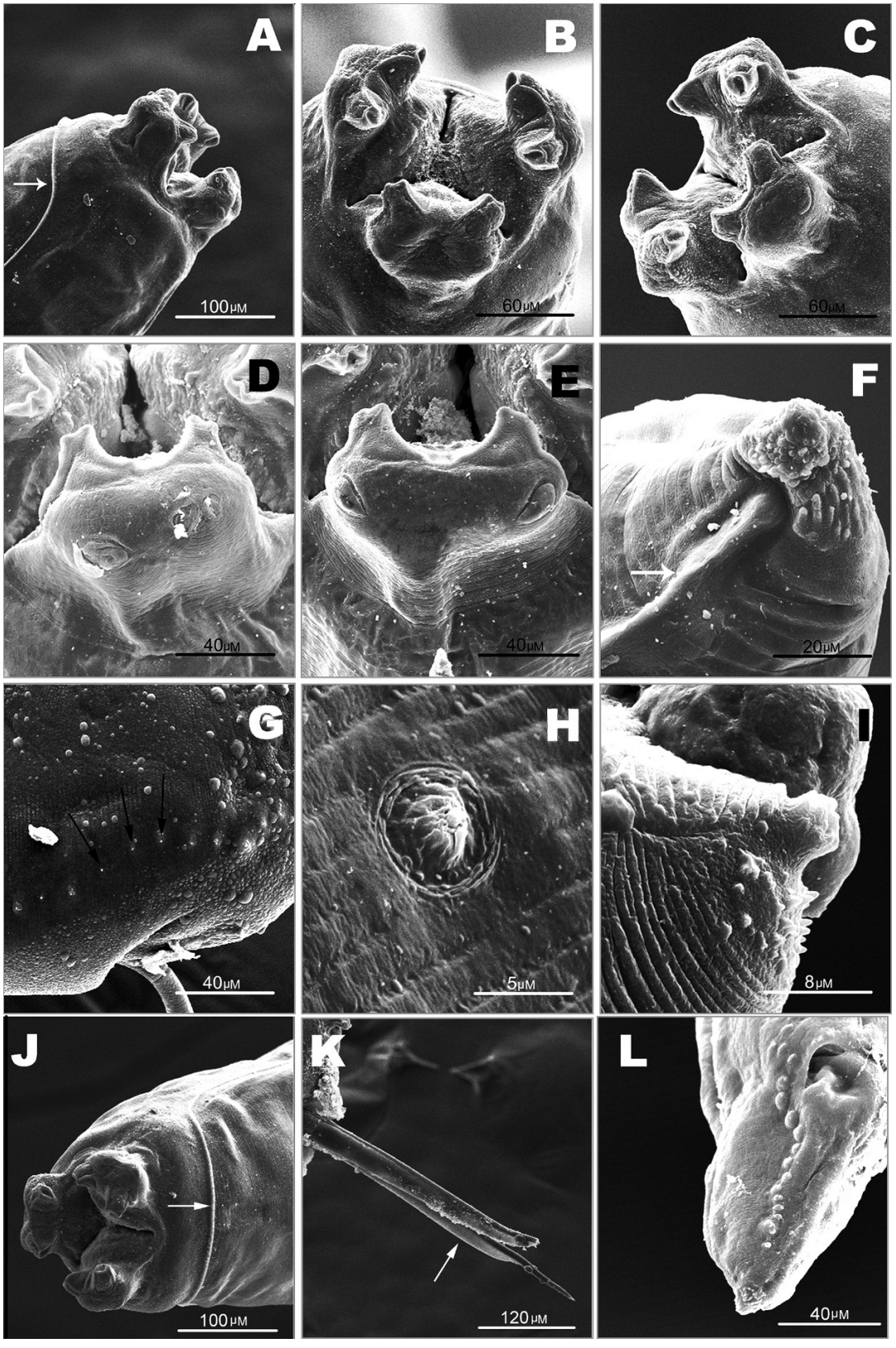

Fig. 2. *Raphidascaris (Ichthyascaris) lophii* Wu, 1949 collected from *Lophius litulon*. **A** – anterior end of male, lateral view (arrow–alae); **B** – cephalic extremity of male, apical view; **C** – cephalic extremity of male, lateral view; **D** – ventrolateral lip; **E** – dorsal lip; **F** – posterior end of female, lateral view (arrow – alae); **G** – paranal papillae (arrows), lateral view; **H** – preanal papillae; **I** – tip of male tail; **J** –anterior end of male, ventral view (arrow – alae uniting close the ventrolateral lips); **K** – spicules (arrow – alae of spicule); **L** – posterior end of male, ventrolateral view paracloacal

fornia, 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 (NCBI) database (http://www.ncbi.nlm. nih.gov).

Results

***Raphidascaris* (*Ichthyascaris*) *lophii* (Wu, 1949)** (Figs 1, 2)

Wu 1949: 51 (*Ichthyascaris*; type locality: Shanghai, China); Smith 1984: 692 (*Raphidascaris*; discussion of species validity); Bruce *et al*. 1994: 605 (*Ichthyascaris*; checklist of species); Moravec and Justine 2005: 105 (*Raphidascaris*; subgenus *Ichthyascaris*).

The morphometrics of specimens collected from these five different marine fishes are strikingly similar to each other (i.e., no notable morphological variation was detected among specimens from different hosts). The collection details for the specimens of *R.* (*I.*) *lophii* from these five different marine fishes are provided (see Table I).

Description

Medium sized, whitish nematodes, cuticle with fine, transverse striations. Maximum width of body at region just posterior to oesophagus. Anterior end with 3 lips (Figs 1B-D); lips nearly equal in size or dorsal lip slightly smaller than ventrolateral lips, without lateral membranous flanges. Proximal part of each lip with two lobes (Figs 2B, C). Dorsal lip with two lateral double papillae at approximately anterior 1/3 of its length; ventrolateral lips each with one double papilla, one small single papilla and amphid (Figs 2D, E). Interlabia absent. Narrow lateral alae extend along whole body length, united anteriorly close to ventrolateral lips on one side of body and form cordons on tail (Figs 2A, F, J). Oesophagus short, slightly broader posteriorly than anteriorly. Nerve ring encircling oesophagus between first and second sixths of its length (Fig. 1A). Ventriculus transversely oval, narrower than posterior part of oesophagus. Ventricular appendix relatively short. Excretory pore well posterior to nerve ring. Tail of both sexes conical, relatively short (Figs 1G, I).

Male (based on 18 mature specimens): Body 12.5–28.6 (19.4) mm long, maximum width 490–931 (723). Dorsal lip 58.6–88.2 (72.1) long, 68.6–88.2 (83.6) wide. Ventrolateral lips 68.6–88.2 (73.5) long, 78.4–88.2 (81.8) wide. Oesophagus 1.23–2.21 (1.85) mm long, 294–490 (392) in maximum width, representing 7.72–10.0 (8.94)% of body length (BL). Nerve ring and excretory pore 392–588 (539) and 566–852 (764), respectively, from anterior extremity. Ventriculus 127–206 (132) long by 196–314 (245) wide. Ventricular appendix 518–980 (782) long, 88.2–147 (113) wide. Posterior end of body curved ventrally. Ejaculatory duct 1.03–2.25 (1.50) mm long, representing 6.98–8.70 (7.84)% of BL. Spicules slender, alate, almost equal length (Figs 1E, F, 2K), 490–882 (728) long, representing 39.1–57.1 (48.7)% of ejaculatory duct (ED) and 3.08–4.70 (3.79)% of BL. Gubernaculum absent. Caudal papillae very small, 37–47 pairs in total, arranged as follows: 26–32 pairs of precloacal papillae, 3–4 pairs of paracloacal papillae and 8–11 pairs of postcloacal papillae (Figs 1E, I, 2G, H, L). Tail bluntly pointed, 127–196 (157) long, base of tip with numerous nodular protuberances (Figs 1K, 2I).

Female (based on 24 gravid specimens): Body 16.2–29.3 (24.6) mm long, maximum width 588–931 (784). Dorsal lip 58.8–98.0 (78.4) long, 58.8–88.2 (73.5) wide. Ventrolateral lips 68.6–108 (88.2) long, 58.8–98.0 (83.3) wide. Oesophagus 1.47–2.50 (1.88) mm long, 294–392 (353) in maximum width, representing 7.96–10.3 (9.20)% of BL. Nerve ring 441–588 (490) and excretory pore 588–882 (735), respectively, from anterior extremity. Ventriculus 147–294 (225) long by 245–392 (323) wide. Ventricular appendix 539–980 (784) long, 118–196 (157) wide. Vulva slit-like, situated in anterior region of body, 1.86–4.36 (3.48) mm from anterior extremity, at 13.7–20.5 (16.7)% of BL. Vagina muscular, directed posteriorly (Fig. 1H). Uteri forms coils in region posterior to vagina, extending posteriorly to level of rectum. Eggs subspherical (Fig. 1L), 19.6–49.0 (34.3) long by 29.4–49.0 (38.7) wide. Tail 372–686 (607), tip with numerous nodular protuberances (Figs 1J, F).

DNA characterization

ITS region. The sequences obtained of the partial rDNA were all 938 bp in length and showed no intraspecific nucleotide variability within different individuals collected from different hosts here examined. There is no *R.* (*I.*) *lophii* ITS sequence registered in GenBank; thus, pairwise comparison was possible only with one *R.* (*I.*) *trichiuri* sequence (FJ009682) and one *R.* (*R.*) *acus* sequence (AY603537) available in GenBank, which displayed 0.45% (FJ009682) and 11.2% (AY603537) nucleotide differences, respectively. The ITS sequence of *R.* (*I.*) *lophii* was deposited in GenBank database (http://www.ncbi.nlm. nih.gov) under accession number JF809816.

Discussion

Wu (1949) reported the species *R.* (*I.*) *lophii* from *Lophius litulon* (Jordan) (Lophiiformes: Lophiidae) from the East China Sea (off Shanghai) and this species has not been recorded since. Because Wu (1949) did not state where the type material is deposited in the original description (although the type material described by Wu was usually deposited in Institute of Hydrobiology, Chinese Academy of Science; the type specimens of this species were not available); it is practically impossible to re-examine the type specimens. This situation can be solved only through new nematode collections and their

exact taxonomical identification. The morphology of the specimens collected from the East and South China Sea in our study is almost identical with the original description of *R.* (*I.*) *lophii* including body length, lip shape, oesophagus, ventricular appendage and spicule length, position of vulva and tail morphology, and some of the present specimens and the type material are both collected from the same host species *L. litulon* from the same locality (the East China Sea). Therefore, we have not hesitated to make the identification. However, in the original description of this species, Wu (1949) did not mention the critical diagnostic characters of the number and arrangement of caudal papillae. The caudal papillae are very small in some specimens under light microscopy, especially the paracloacal and postcloacal papillae. In our material we observed 26–32 pairs of precloacal, 3–4 pairs of paracloacal and 8–11 pairs of postcloacal papillae.

In the subgenus *Ichthyascaris*, the species *R.* (*I.*) *lophii* is similar to the following congeners in having a relative long oesophagus and ventricular appendix: *R.* (*I.*) *trichiuri* from *Trichiurus haumela* from the East China Sea, *R.* (*I.*) *fisheri* (Hooper, 1983) from *Platycephalus arenarius* from Australia, *R.* (*I.*) *nemipteri* Moravec et Justine, 2005 from *Nemipterus furcosus* from New Caledonia, *R.* (*I.*) *chirocentri* Yamaguti, 1935 from *Chirocentrus dorab* from Sea of Japan. *Raphidascaris* (*I.*) *lophii* differs from *R.* (*I.*) *chirocentri* in having distinctly fewer caudal papillae in total (37–47 pairs in the former vs 63 pairs in the latter). *Raphidascaris* (*I.) lophii* is different from *R.* (*I.*) *fisheri* in having longer spicules [0.49–0.88 mm in *R.* (*I.*) *lophii* vs 0.28–0.32 mm in the latter species] and more postcloacal papillae 8–11 pairs in the former vs 2 pairs in *R.* (*I.*) *fisheri*]. *Raphidascaris* (*I.*) *lophii* can be readily distinguished from *R.* (*I.*) *nemipteri* in having 3–4 pairs of paracloacal papillae and longer spicules [0.49–0.88 mm in *R.* (*I.*) *lophii* vs 0.23–0.40 mm in the latter]. *Raphidascaris* (*I.*) *lophii* differs from *R.* (*I.*) *trichiuri* in having distinctly fewer precloacal papillae (26–32 pairs in the former vs 39 pairs in the latter) and longer spicules [0.49–0.88 mm in *R.* (*I.*) *lophii* vs 0.25–0.45 mm in *R.* (*I.*) *trichiuri*].

The molecular analyses for specimens morphologically identified as *R.* (*I.*) *lophii* collected from the five different hosts from the East and South China Sea, respectively, showed only one sequence type obtained (i.e., no intraspecific variation was detected). It confirmed that all the nematode material collected from the five different hosts was the same species; although these five different fish species belong to three different orders. This result was consistent with the morphological observations. Among the interspecific variation in ITS sequences of *R.* (*I.*) *lophii* herein generated and those of *R.* (*I.*) *trichiuri* and *R.* (*R.*) *acus* available in GenBank, the ITS sequence of *R.* (*R.*) *acus* showed the highest interspecific difference. This result can be easily understood when considering the special morphological characters of *R.* (*I.*) *lophii* including the simple lips without lateral membranous flanges and the presence of lateral alae uniting close the ventrolateral lips. The ITS sequences of *R.* (*I.*) *lophii* and *R.* (*I.*) *trichiuri* showed low interspecific difference. This result concords with the fact that *R.* (*I.*) *lophii* and *R.* (*I.*) *trichiuri* have very similar morphological characters.

Key to species of the subgenus Ichthyascaris

1. Spicules very long, 1.06–1.09 mm long ..... *R.*(*I.*) *lutjani* Olsen
   Spicules relatively short, less than 0.80 mm long 2
2. Caudal papillae 63 pairs in total ..... *R.* (*I.*) *chirocentri* Yamaguti
   Caudal papillae 17–53 pairs in total 3
3. Postcloacal papillae 2 pairs in total ..... *R.* (*I.*) *fisheri* (Hooper)
   Postcloacal papillae 7–11 pairs in total 4
4. Precloacal papillae 14–17 pairs in total ..... *R.* (*I.*) *mediterraneus* Lèbre et Petter
   Precloacal papillae 22–40 pairs in total 5
5. Spicules relatively long, 0.49–0.88 mm long ..... *R. (I.*) *lophii* (Wu)
   Spicules relatively short, less than 0.45 mm long 6
6. Paracloacal papillae 4 pairs in total ..... *R.* (*I.*) *trichiuri* (Yin et Zhang)
   Paracloacal papillae less than 2 pairs in total 7
7. Tail of male with minutely nodulose apex 8
   Tail of male sooth, without minutely nodulose apex 9
8. Spicules 17.7–19.1% of ejaculatory duct, vulva 22.8–29.0% of body length from anterior, paracloacal papillae 1 pair ..... *R.* (*I.*) *sillagoides* (Bruce)
   Spicules 26.1–44.2% of ejaculatory duct, vulva 18.2–19.0% of body length from anterior, paracloacal papillae 2 pairs ..... *R.* (*I.*) *gymnocraniae* (Bruce)
9. Caudal papillae 41–51 pairs in total; spicules 0.14–0.25 mm long ..... *R.* (*I.*) *vicentei* Santos
   Caudal papillae 30–41 pairs in total; spicules 0.23–0.40 mm long..*R.* (*I.*) *nemipteri* ..... Moravec et Justine

Acknowledgements. The authors wish to thank Professor Ruiyong An (College of Life Science, Hebei Normal University) for identifying the fishes. This study was supported by the National Natural Science Foundation of China (No. 31101615, No. 30970318), the Natural Science Foundation of Hebei Province (No. C2012205007) and the Science Foundation of Hebei Normal University (No. L2010B13).

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(Accepted May 07, 2012)