Morphology, ultrastructure and molecular characterisation of Spiroxys japonica *Morishita, 1926 (Spirurida: Gnathostomatidae) from* Pelophylax nigromaculatus *(Hallowell) (Amphibia: Ranidae)*

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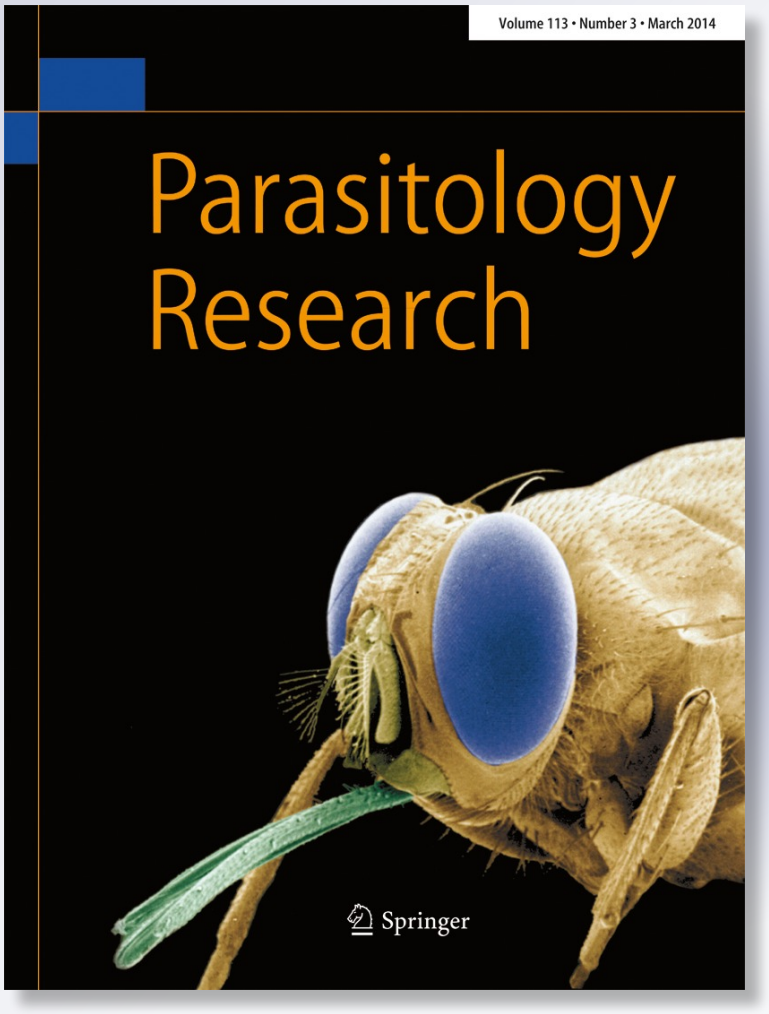

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ORIGINAL PAPER

Morphology, ultrastructure and molecular characterisation of *Spiroxys japonica* Morishita, 1926 (Spirurida: Gnathostomatidae) from *Pelophylax nigromaculatus* (Hallowell) (Amphibia: Ranidae)

Liang Li · Hideo Hasegawa · Vicente Roca · Zhen Xu · Yan-Ning Guo · Akiko Sato · Lu-Ping Zhang

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Abstract Gnathostomatid nematodes identified morphologically as *Spiroxys japonica* Morishita, 1926 were collected from the dark-spotted frog *Pelophylax nigromaculatus* (Hallowell) (Amphibia: Ranidae) in China. Light and scanning electron microscopy were used to study the morphology of this species in detail. Previously unreported morphological features are revealed and others corrected. In addition, adult nematodes of *S. japonica* collected from *P. nigromaculatus* and *Spiroxys hanzaki* Hasegawa, Miyata & Doi, 1998 collected from *Andrias japonicus* (Temminck) (Caudata: Cryptobranchidae) in China and Japan, respectively, and the third-stage larva of *S. japonica* collected from *Lithobates catesbeianus* (Shaw) (Anura: Ranidae) in Japan, were characterised using molecular methods by sequencing and analysing ribosomal [large ribosomal DNA (18S) and internal transcribed space] and mitochondrial [cytochrome c oxidase subunit 1] target regions, respectively. The new morphological and genetic data contributes to a more accurate diagnosis of this hitherto little known nematode genus.

L. Li (✉) · Y.-N. Guo · L.-P. Zhang (✉)
Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, College of Life Science, Hebei Normal University, 20 East Road of 2nd South Ring, Yuhua District, 050024 Shijiazhuang, Hebei Province, People's Republic of China
e-mail: liangliangex369@126.com
e-mail: lupingzhang0505@yahoo.com.cn

H. Hasegawa · A. Sato
Department of Biology, Faculty of Medicine, Oita University, Hasama, Yufu, Oita 879-5593, Japan

V. Roca
Departament de Zoologia, Facultat de Ciències Biològiques, Universitat de València, Dr. Moliner, 50, 46100 Burjassot, València, Spain

Z. Xu
Medical College of Hebei University of Engineering, 056002 Handan, Hebei Province, People's Republic of China

Introduction

Adult nematodes of the genus *Spiroxys* Schneider, 1866 commonly occur in the digestive tract of freshwater turtles; but also can parasitise frogs, salamanders and snakes (Baylis and Lane 1920; Morishita 1926; Hedrick 1935; Berry 1985; Hasegawa et al. 1998; Roca and García 2008). Their larvae are generally found in various freshwater copepods, fishes and tadpoles (Hedrick 1935; Hasegawa and Otsuru 1978; Hasegawa et al. 2000). To date, a total of 18 recognised species of *Spiroxys* have been reported from all zoogeographical regions of the world (Berry 1985; Baker 1987; Hasegawa et al. 1998; Roca and García 2008). For years, most of our taxonomical knowledge of *Spiroxys* has been based purely on morphological studies at the light microscope level. However, with the development of scanning electron microscopy and molecular techniques, the former enables us to observe the diagnostically significant taxonomic morphological features in more detail, and the latter enhances the accuracy of species identification and assists in our ability to reveal hidden species diversity. Recent studies have shown that it is very useful to integrate molecular data with morphology for the accurate identification of spiruridan nematodes (Otranto et al. 2007; García-Márquez et al. 2009; Ferri et al. 2009; Guerrero et al. 2011; Li et al. 2013). Consequently, *Spiroxys japonica* Morishita, 1926, based on newly collected specimens from the dark-spotted frog *Pelophylax nigromaculatus* (Hallowell) (Anura: Ranidae) in China, was studied using both light and scanning electron microscopy to supplement the existing morphological data. Furthermore, adult nematodes of *S. japonica*

collected from *P. nigromaculatus* and *Spiroxys hanzaki* Hasegawa, Miyata & Doi,1998 collected from *Andrias japonicus* (Temminck) (Caudata: Cryptobranchidae) in China and Japan, respectively, and third-stage larva of *S. japonica* collected from *Lithobates catesbeianus* (Shaw) (Anura: Ranidae) in Japan, were characterised using molecular methods by sequencing and analysing ribosomal [large ribosomal DNA (18S) and internal transcribed spacer (ITS)] and mitochondrial [cytochrome c oxidase subunit 1 (*cox*1)] target regions, respectively, in order to facilitate the future molecular identification of this poorly known genus.

Materials and methods

Light and scanning electron microscopy

Nematodes collected from the intestine of the dark-spotted frog *P. nigromaculatus* (Hallowell) (Amphibia: Ranidae) from Yingtan, Jiangxi Province, China during July 2007 were washed in physiological saline and then fixed and stored in 80 % ethanol until studied. For light microscopical studies, nematodes were cleared in lactophenol. Drawings were made with the aid of Nikon microscope drawing attachment. Scanning electron microscopical studies were prepared following the methods used by Li et al. (2012). Measurements (the range, followed by the mean in parentheses) are given in micrometres unless otherwise stated. Voucher specimens are deposited in Natural History Museum, London, UK (NHMUK) and College of Life Science, Hebei Normal University, Hebei Province, China (HBNU).

Molecular procedures

A total of six randomly selected nematodes were subjected to molecular analysis of different target regions: ITS of rDNA, mitochondrial *cox*1 and small ribosomal DNA (18S) (see Table 1 for details). 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 elution buffer and kept at − 20 °C until use. The partial 18S rDNA was amplified by PCR using the primers 18SF (forward: 5′-CGCGAATRGCTC ATTACAACAGC-3′) and 18SR (reverse: 5′-GGGCGGTATC TGATCGCC-3′) (Hasegawa et al. 2013). The *cox*1 was amplified by PCR using the primers CO1F (forward: 5′-TTTTTT GGTCATCCTG AGGTTTAT-3′) and CO1R (reverse: 5′-ACATAATGAAAATGACTAACAAC-3′) (Lazarova et al. 2006). The ITS region was amplified by PCR using the primers TW1 (forward: 5′-GTTTCCGTAGGTGAACCTGC -3′) and AB28 (reverse: 5′-ATATGCTTAAGTTCAGCGGG T-3′) (Subbotin et al. 2001) for the material of *S. japonica* from China, and the primers SSU24HF (forward: 5′-AGAG GTGAAATTCGTGGACC-3′) and AB28 (Hasegawa et al. 2009) for the material of *S. hanzaki* and *S. japonica* from Japan. All PCRs were 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, California, 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 target sequences (18S rDNA, ITS and *cox*1) 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

S. japonica Morishita, 1926 (Figs. 1, 2, and 3)

Type hosts and type locality Dark-spotted frog *Rana nigromaculata* [now *Pelophylax nigromaculatus* (Hallowell)], Japanese brown frog *Rana japonica* Guenther and wrinkled frog *Rugosa rugosa* Temminck and Schlegel (Amphibia: Ranidae), Japan.

Table 1 Randomly selected specimens of *Spiroxys* spp. for molecular analysis

Species	Hosts	Localities	Specimens number
S. japonica	*Pelophylax nigromaculatus*	China	3 adults
S. japonica	*Pelophylax nigromaculatus*	Japan	1 adult
S. japonica	*Lithobates catesbeianus*	Japan	1 third-stage larva
S. hanzaki	*Andrias japonicus*	Japan	1 adult

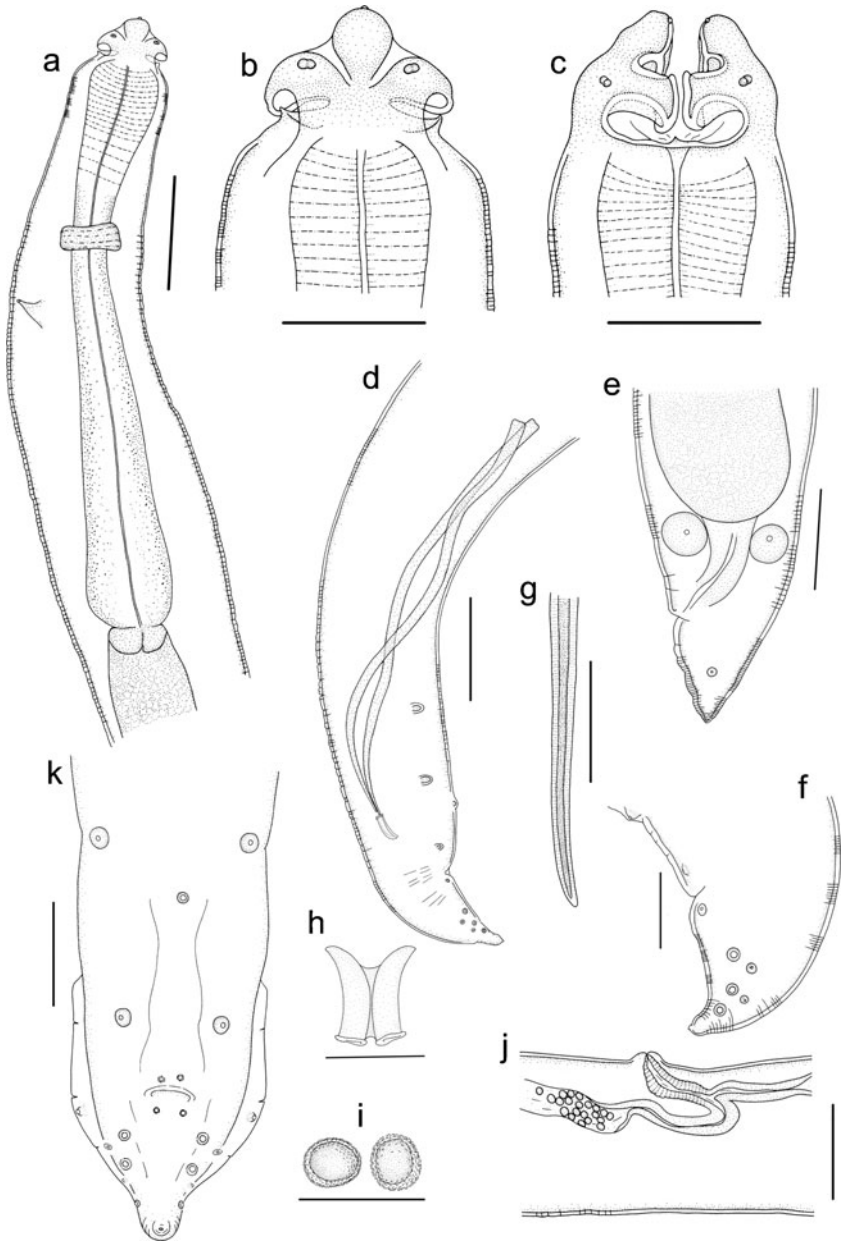

Fig. 1 *Spiroxys japonica* Morishita from *Pelophylax nigromaculatus* (Hallowell) in China: *a* anterior part of female, lateral view; *b* cephalic end of female, lateral view; *c* cephalic end of female, dorsoventral view; *d* posterior end of male, lateral view; *e* posterior end of female, lateral view; *f* caudal region of male, lateral view; *g* distal end of spicule; *h* gubernaculum, ventral view; *i* eggs; *j* region of vulva; *k* posterior end of male, ventral view. *Scale bars*—*a*, *d*, *j* 500 μm; *b*, *c*, *e*, *f*, *k* 250 μm; *g*, *h*, *i* 120 μm

Host and locality of present material Dark-spotted frog *P. nigromaculatus* (Hallowell) (Amphibia: Ranidae), Yingtan, Jiangxi Province, China.

Site of infection Duodenum.

Voucher specimens Six males, nine females (HBNU-A13041L) and one male, one female (NHMUK 2013.7.2.1-2).

Diagnosis Medium-sized, stout nematodes. Anterior region of body usually curved dorsally. Cuticle with very fine transverse striations (Figs. 1a and 3d). Maximum width of body at about mid-body. Lateral alae absent. Cephalic end with two trilobed, lateral pseudolabia, each with dorsal, ventral and middle lobes; median lobe with single apical cuticular tooth; dorsal and ventral lobes without cuticular tooth; each pseudolabium with two large, submedian double papillae and small, papilla-like lateral amphid (the latter is often indistinct in the present material; Figs. 1a–c, 2a, b, and 3c). Collar with small cuticular protrusions (Figs. 1b, c, 2a, b, and 3c). Oesophagus thickened posteriorly and divided by indistinct junction into anterior muscular and posterior glandular portions (Fig. 1a). Nerve ring is at about 2/5 of oesophageal length (Fig. 1a). Excretory pore situated slightly posterior to the nerve ring (Figs. 1a and 3a, b). Cervical papillae are not observed. Tail is very short in both sexes.

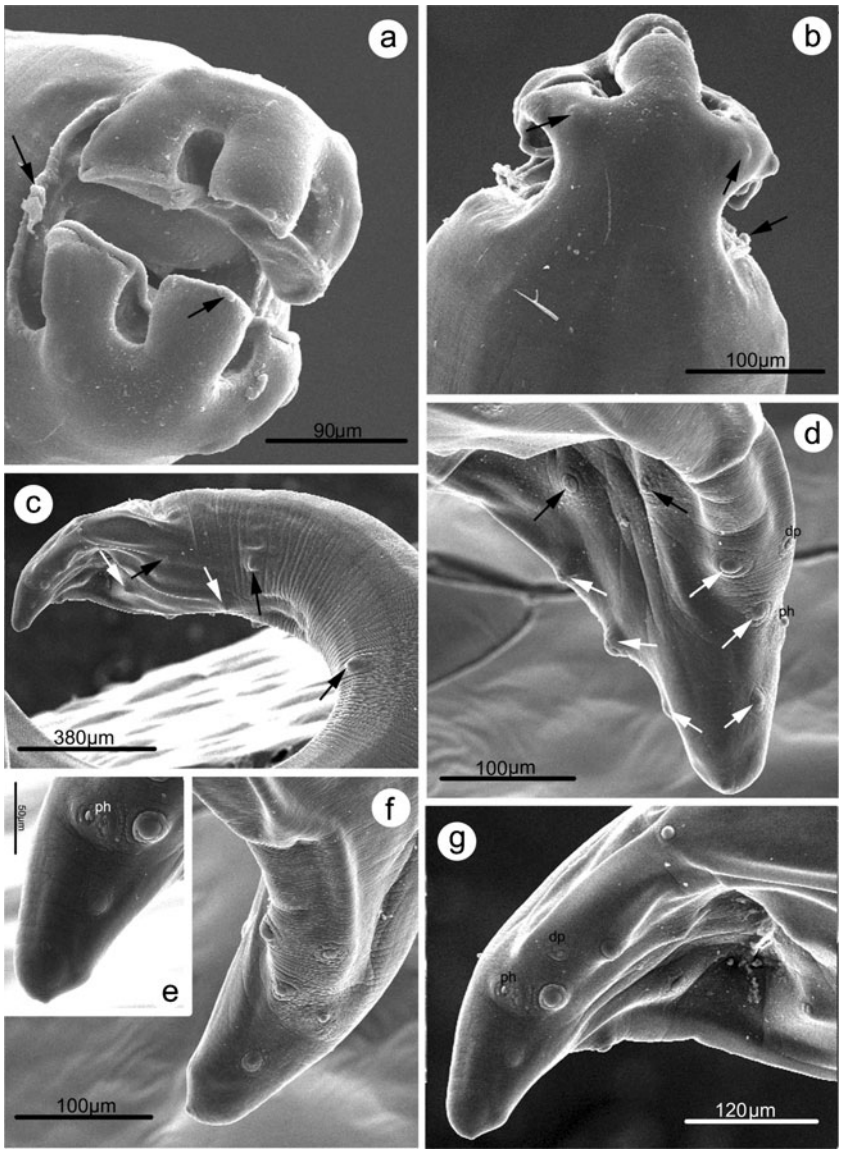

Fig. 2 Scanning electron micrographs of *Spiroxys japonica* Morishita from *Pelophylax nigromaculatus* (Hallowell) in China, male: *a* cephalic extremity, apical view (cuticular tooth of median lobe of lateral pseudolabia and small cuticular protrusion of *collar arrowed*); *b* cephalic extremity, dorsoventral view (submedian double papillae of lateral pseudolabia and small cuticular protrusion of collar arrowed); *c* posterior part of body, ventrolateral view [precloacal lateral papillae (*black arrows*) and medioventral papillae (*white arrows*) arrowed]; *d* caudal region of body, ventral view [postcloacal paired medioventral papillae (*black arrows*) and lateral papillae (*white arrows*) arrowed]; *e* tail tip, lateral view; *f* caudal region of body, lateral view; *g* caudal region of body, ventrolateral view (the other side of Fig. 2d). *ph* phasmid, *dp* postcloacal double papillae

Male [based on seven mature specimens] Body 17.2–24.1 (21.2)mm long, maximum width 735–833 (789). Pseudolabium 147–176 (160) long, 265–314 (281) wide. Oesophagus 2.25–2.74 (2.52)mm long, 196–294 (263) in maximum width, representing 10.5–13.7 (12.4)% of body length. Nerve ring and excretory pore 931–1,078 (1,000) and 1,176–1,519 (1,303), respectively, from anterior extremity. Posterior end of body curved ventrally (Figs. 1d and 2c, d, f, g). Caudal alae thick, forming vesicular swelling (Figs. 1k and 2d). Ejaculatory duct 2.65–3.09 (2.95)mm long. Spicules well sclerotised, pointed at distal end (Fig. 1g), of almost equal length, 1.96–2.30 (2.13)mm long, representing 69.9–81.5 (73.3)% of ejaculatory duct length and 8.60–12.6 (10.2)% of body length (Fig. 1d, g). Gubernaculum present, banana-shaped in lateral view, 118–166 (132) long (Fig. 1d, h). Caudal papillae arranged as follows: lateral precloacal papillae four pairs with last one pair more ventral and close to cloaca; single medioventral precloacal papilla situated posterior to the second pair of lateral papillae; and six pairs postcloacal papillae approximately arranged as follows: one pair of medioventral postcloacal papillae situated near to cloaca and five pairs of postcloacal lateral papillae (first and third pairs not in line with others; third pair double) (Figs. 1d, f, k, 2c–g, and 3g–i). Phasmids small, 120–153 (138) from tail tip (Figs. 2d–g and 3i). Tail 323–490 (394) long, with very small, nipple-like tip (Figs. 1d, f, k and 2c–g).

Female [based on nine gravid specimens] Body 18.9–24.0 (22.5)mm long, maximum width 784–980 (915). Pseudolabium 167–216 (191) long, 274–372 (328) wide. Oesophagus 2.45–2.90 (2.63)mm long, 255–314 (284) in maximum width, representing 10.7–13.1 (11.7)% of body length. Nerve ring and excretory pore 980–1,098 (1,041) and 1,225–1,519 (1,356), respectively, from anterior

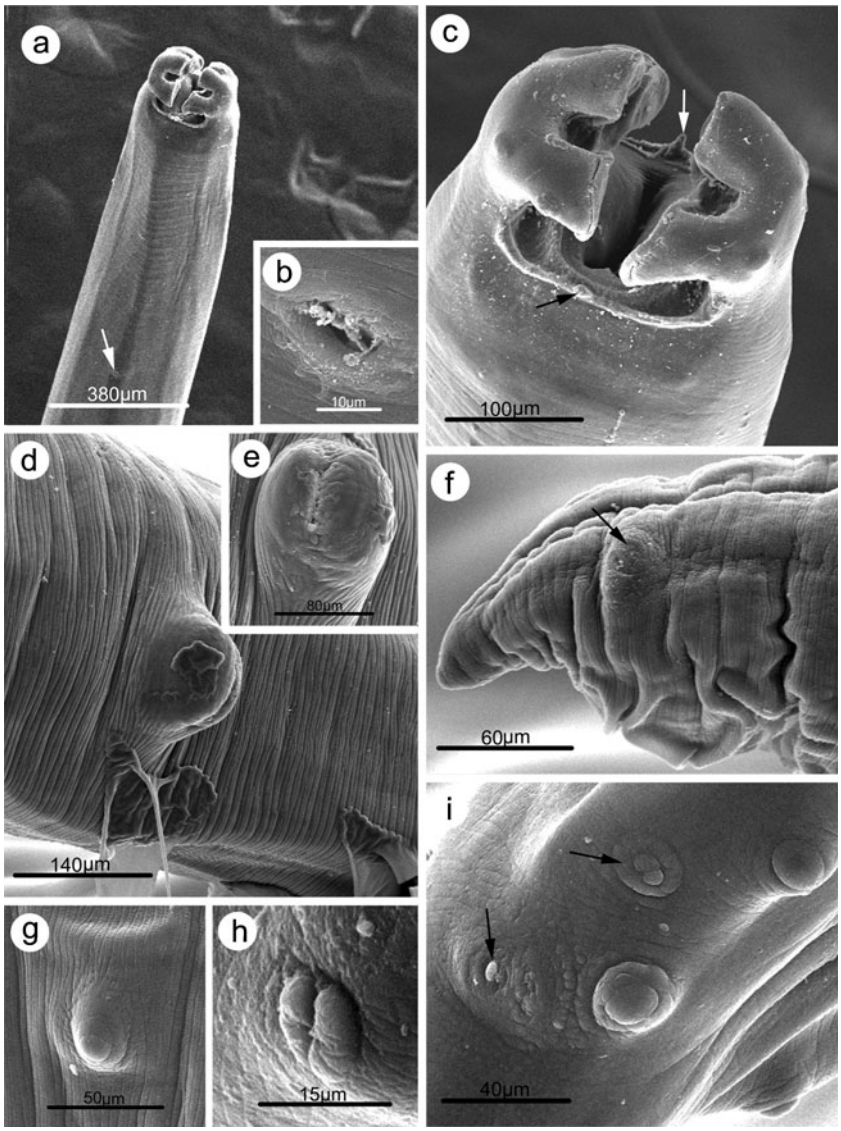

Fig. 3 Scanning electron micrographs of *Spiroxys japonica* Morishita from *Pelophylax nigromaculatus* (Hallowell) in China: *a* anterior part of female, ventral view (excretory pore arrowed); *b* magnified image of excretory pore; *c* cephalic extremity of female, dorsalventral view (small cuticular protrusions of collar arrowed); *d* region of vulva, ventral view; **e**. magnified image of vulva; *f* posterior part of female, lateral view (phasmid arrowed); *g* magnified image of the second pair of lateral precloacal papilla; *h* magnified image of medioventral precloacal papilla situated near to cloaca; *i* magnified image of region of phasmid, lateral view (postcloacal double papilla and phasmid arrowed)

extremity. Vulva slit-like, with two prominent lips; situated in posterior region of body, 14.9–15.9 (15.4)mm from anterior extremity at 64.5–66.3 (65.3)% of body length (Figs. 1j and 3d, e). Vagina muscular, very short, directed anteriorly from vulva, thick-walled and annulated (Fig. 1j). Eggs almost rounded, unembryonated, thin-shelled, with slightly rough surface, 49–59 (53.1)×49–59 (51.2) (Fig. 1i). Tail 225–314 (257) long, without nipple-like tip (Figs. 1e, 3f). Phasmids distinct, 147–167 (154) from tip of tail (Figs. 1e, 3f).

DNA characterisation

ITS region There were five different genotypes obtained for the ITS region of the partial rDNA of *S. japonica* examined herein based on the material collected from *P. nigromaculatus* and *L. catesbeianus* in China and Japan, respectively. The length of the five different ITS sequences of *S. japonica* ranged from 717 to 743 bp and showed 0.14–0.98 % nucleotide differences (see Table 2 and Fig. 4 for details). The sequence obtained for the ITS region of *S. hanzaki* was 837 bp in length. There are no ITS sequences of *Spiroxys* spp. registered in GenBank, so comparison of ITS sequence data is only be possible for the species *S. japonica* and *S. hanzaki*, where over 45.0 % nucleotide differences were detected. The ITS sequences of *S. japonica* and *S. hanzaki* are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under accession numbers (KF530321–KF530326).

Partial 18S region There were three sequences (representing only one genotype) obtained for the partial 18S region of *S. japonica* examined herein based on the material collected from *P. nigromaculatus* in China and the length were all 884 bp. The partial 18S sequences of *S. japonica* and *S. hanzaki* based on the material collected from *P. nigromaculatus* and *L.*

Table 2 The sequence polymorphisms revealed at alignment positions among the five different genotypes of *Spiroxys japonica* in the ITS region obtained in the present study

GenBank nos.	Hosts, localities	Sequence polymorphisms revealed at alignment positions											
		100	211	244	329	442	515	528	586	589	637	657–663	694
KF530321	*P. nigromaculatus*, China	T	A	**G**	T	C	C	G	G	T	T	TGCACTA	C
KF530322	*P. nigromaculatus*, China	**G**	A	A	**C**	**T**	C	G	G	T	**C**	TGCACTA	C
KF530323	*P. nigromaculatus*, China	T	A	A	T	C	C	G	G	T	T	-------	C
KF530324	*P. nigromaculatus*, Japan	T	**G**	A	T	C	**T**	G	**A**	**C**	T	TGCACTA	**A**
KF530325	*L. catesbeianus*, Japan	T	**G**	A	T	C	**T**	**T**	**A**	**C**	T	TGCACTA	**A**

------- Represents lack of one nucleotide in this site

catesbeianus in Japan, respectively, were registered in GenBank by two of the authors of the present study (Hasegawa H. and Sato A.) under accession numbers (AB818381–AB818383). There are no other partial 18S sequences of *Spiroxys* spp. registered in GenBank, and pairwise comparison between the present data and the partial 18S sequences of *S. japonica* registered in GenBank (AB818381 and AB818382) showed no intraspecific nucleotide variability. Comparison of partial 18S sequence data of *S. japonica* and *S. hanzaki* (AB818383) displayed 2.28 % nucleotide differences. The partial 18S sequences of *S. japonica* based on the material collected from *P. nigromaculatus* in China are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under accession numbers (KF844293–KF844295).

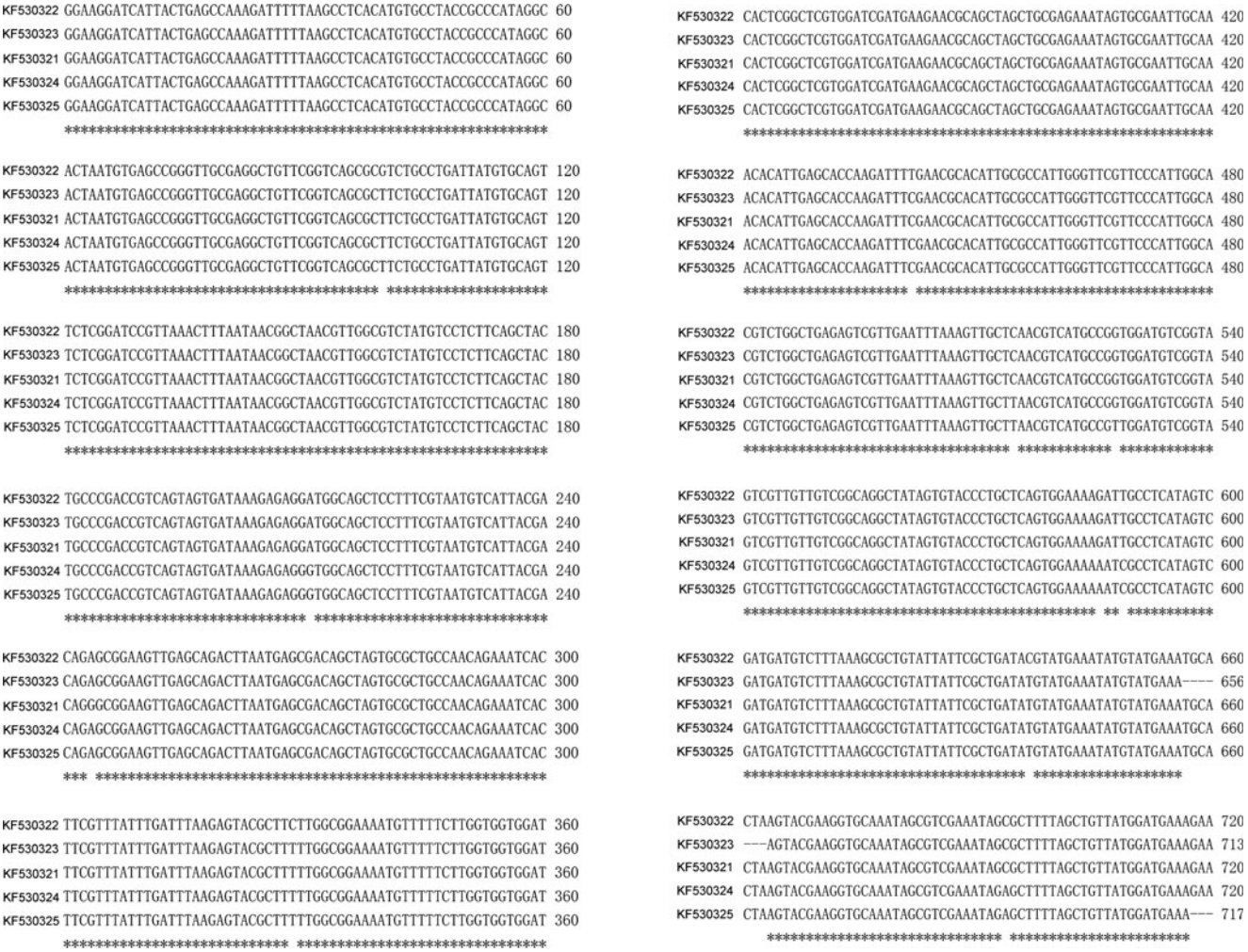

Fig. 4 Alignment of ITS sequences of *Spiroxys japonica* Morishita. *Numbers to the right of the alignment* indicate the alignment position and the same nucleotide sites were designated using the *asterisks*

cox1 region There were five sequences (representing four different genotypes) obtained for the *cox*1 of *S. japonica* examined herein based on the material collected from *P. nigromaculatus* and *L. catesbeianus* in China and Japan, respectively. The length of the five *cox*1 sequences were all 384 bp and showed 0–9.38 % nucleotide differences (see Fig. 5 for details). The *cox*1 sequence of *S. hanzaki* obtained herein was also 384 bp in length. There are no other *cox*1 sequences of *Spiroxys* spp. registered in GenBank, and pairwise comparison between *cox*1 sequences of *S. japonica* and *S. hanzaki* displayed 16.9–18.0 % interspecific nucleotide variability. The *cox*1 sequences of *S. japonica* and *S. hanzaki*

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KF844301 ATTTTGATTTTGCCGGCTTTCGGCATTATCAGCCAGAGAAGTTTGTATTTAACTGGTAAG 60
KF844299 ATTTTGATTTTGCCGGCTTTCGGCATTATCAGCCAGAGAAGTTTGTATTTAACTGGTAAG 60
KF844300 ATTTTGATTTTGCCGGCTTTCGGCATTATCAGCCAGAGGAGTCTGTATTTAACTGGTAAG 60
KF844298 ATTTTGATTTTGCCGGCTTTCGGCATTATTAGCCAGAGCAGGCTTTATTTGACAGGTAAG 60
KF844297 ATTTTGATTTTGCCGGCTTTCGGCATTATTAGCCAGAGCAGGCTTTATTTGACAGGTAAG 60
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KF844301 AAGGAGGTTTTTGGTTCTTTAGGAATGGTTTATGCTATTTTAAGTATCGGCCTTATTGGT 120
KF844299 AAGGAGGTTTTTGGTTCTTTAGGAATGGTTTATGCTATTTTAAGTATCGGCCTTATTGGT 120
KF844300 AAGGAGGTTTTTGGTTCTTTAGGAATGGTTTATGCTATTTTAAGTATCGGTCTTATTGGT 120
KF844298 AAGGAGGTTTTTGGTTCTTTGGGTATGGTTTATGCTATTTTAAGTATTGGTCTTATTGGT 120
KF844297 AAGGAGGTTTTTGGTTCTTTGGGTATGGTTTATGCTATTTTAAGTATTGGTCTTATTGGT 120
         ******************** ** ********************** ** *********

KF844301 TGTGTTGTTTGGGCTCACCATATGTATACTGTTGGTATAGATTTAGACTCTCGTGCTTAT 180
KF844299 TGTGTTGTTTGGGCTCACCATATGTATACTGTTGGTATAGATTTAGACTCTCGTGCTTAT 180
KF844300 TGTGTTGTTTGGGCTCACCATATGTATACTGTGGGTATAGATTTAGACTCTCGTGCTTAT 180
KF844298 TGTGTCGTTTGGGCCCACCATATGTATACTGTAGGTATAGATTTAGACTCTCGTGCTTAC 180
KF844297 TGTGTCGTTTGGGCCCATCATATATATACTGTAGGTATAGATTTAGACTCTCGTGCTTAC 180
         ***** ******** ** ***** ******** ************************

KF844301 TTTACTGCAGCTACCATGGTAATTGCTGTTCCTACTGGGGTGAAAATTTTTAGTTGGTTG 240
KF844299 TTTACTGCAGCTACCATGGTAATTGCTGTTCCTACTGGGGTGAAAATTTTTAGTTGGTTG 240
KF844300 TTTACTGCAGCTACTATGGTAATTGCTGTTCCTACTGGGGTGAAAATTTTTAGTTGGTTG 240
KF844298 TTTACTGCGGCTACTATGGTAATTGCTGTTCCTACGGGGGTTAAGATTTTTAGTTGGTTG 240
KF844297 TTTACTGCGGCTACTATGGTAATTGCTGTTCCTACTGGGGTTAAGATTTTTAGTTGGTTG 240
         ******** ***** ******************** ***** ** ***************

KF844301 GCTACGCTTTATGGTATAGAGGTTGTTTTTTCACCTGTTTTGTTGTGGGTTTTGGGCTTT 300
KF844299 GCTACGCTTTATGGTATAGAGGTTGTTTTTTCACCTGTTTTGTTGTGGGTTTTGGGCTTT 300
KF844300 GCTACGCTTTATGGTATAGAGGTTGTTTTTTCACCTGTTTTGTTGTGGGTTTTGGGCTTT 300
KF844298 GCTACACTTTACGGTATAGAGGTTGTTTTTTCTCCTGTTTTGTTATGGGTTTTAGGTTTT 300
KF844297 GCTACACTTTACGGTATAGAGGTTGTTTTTTCTCCTGTTTTGTTATGGGTTTTAGGTTTT 300
         ***** ***** ********************** ********** ******** ** ***

KF844301 ATTTTTCTTTTTACAGTCGGTGGGTTAACTGGTATTATGTTATCTAATTCTAGTCTGGAT 360
KF844299 ATTTTTCTTTTTACAGTCGGTGGGTTAACTGGTATTATGTTATCTAATTCTAGTCTGGAT 360
KF844300 ATTTTTCTTTTTACAGTCGGTGGGTTAACTGGTATTATGTTATCTAATTCTAGTCTGGAT 360
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KF844297 ATTTTTCTTTTTACAATTGGGGGATTAACCGGTATTATGCTTTCTAATTCTAGGCTGGAT 360
         *************** * ** ** ** ** ********* * ********** ******

KF844301 ATTATTCTTCATGATACATATTAT 384
KF844299 ATTATTCTTCATGATACATATTAT 384
KF844300 ATTATTCTTCATGATACATATTAT 384
KF844298 ATTATTCTTCATGATACCTATTAT 384
KF844297 ATTATTCTTCATGATACCTATTAT 384
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Fig. 5 Alignment of *cox*1 sequences of *Spiroxys japonica* Morishita. *Numbers to the right of the alignment* indicate the alignment position and the same nucleotide sites were designated using the *asterisks*

are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under accession numbers (KF844296–KF844301).

Discussion

Morishita (1926) described *S. japonica* from the dark-spotted frog *R. nigromaculata* (=*P. nigromaculatus*), the Japanese brown frog *R. japonica* and the wrinkled frog *R. rugosa* (=*R. rugosa*) (Amphibia: Ranidae) in Japan, but he did not designate a type host in the original description. Hsü and Hoeppli (1931), Kung and Wu (1945) and Xu (1960) reported the same species from the Güenther's frog *Hylarana guentheri* (Amphibia: Ranidae) and *P. nigromaculatus* in China, respectively. Later, Hasegawa and Otsuru (1978) elucidated the life cycle of *S. japonica*. The morphology and measurements of the present material collected from *P. nigromaculatus* in China agree well with the original description of *S. japonica* by Morishita (1926), including body size, the morphology of the head, the length of oesophagus and spicules, the position of nerve ring and excretory pore, the number and arrangement of caudal papillae, the position and morphology of vulva, and the morphology and length of the male and female tail. It should be also noted that the present material was from the same host, *P. nigromaculatus*, as the original specimens of Morishita (1926). Therefore, we consider our material to be conspecific with that of Morishita.

The original description and illustrations of *S. japonica* by Morishita (1926) are rather good according to the standards of the time. However, the present scanning electron microscopy (SEM) study revealed some errors or previously unreported morphological features. In the original description, Morishita (1926) reported four pairs of precloacal papillae (the last pair situated ventrally) and seven pairs of postcloacal papillae (the first pair situated ventrally), whereas the present SEM study showed the presence of an additional single, medioventral precloacal papilla situated posterior to second pair of precloacal lateral papillae. This situation also occurs in *S. chelodinae*, *S. hanzaki* and *S. ankarafantsika* (see Berry 1985; Hasegawa et al. 1998; Roca and García 2008). Furthermore, we also observed six pairs of postcloacal papillae and one pair of phasmids using scanning electron microscopy. In fact, it is very difficult to distinguish the phasmids and caudal papillae under the light microscope in this species. Morishita (1926) did not state whether there were any postcloacal double papillae in the male, but we observed a single pair in our material. Furthermore, in his original description, Morishita mentioned the vulva of *S. japonica* opened on a cone, whereas, using SEM, we observed the vulva to have two protruding lips. Morishita (1926) also indicated the presence of a pair of caudal papillae in the female, which, using SEM proved to be the lateral phasmids.

The genus *Spiroxys* was divided into two morpho-groups by Berry (1985) based on the presence or absence of teeth or cuticular protrusion on the pseudolabia or collar (in addition to the median tooth on each median lobe of the pseudolabia). *S. japonica*, with a prominent cuticular protrusion (tooth) on the collar, should belong to group I of Berry (1985), which currently includes 14 species (Berry 1985; Hasegawa et al. 1998). Within Berry's group I, by having only the median lobe of each pseudolabium with a single tooth and the collar region with only one dorsal and one ventral cuticular protrusion, *S. japonica* is similar to those species parasitic in amphibians. These include: *Spiroxys utahensis* Todd, 1969 from the tiger salamander *Ambystoma tigrinum nebulosum* (Hallowell) (Amphibia: Ambystomatidae) and *Spiroxys allegheniensis* Walton, 1930 from the hellbender *Cryptobranchus allegheniensis* (Daudin) (Amphibia: Cryptobranchidae) in North America; and *Spiroxys corti* Caballero, 1935 from the Montezuma leopard frog *Rana montezumae* Baird (Amphibia: Ranidae) in Mexico (Todd 1969; Caballero 1935; Walton 1930; Hedrick 1935). However, *S. japonica* can be readily distinguished from *S. utahensis* and *S. corti* by its much longer spicules (1.96–2.30 vs 0.99–1.30 mm in *S. utahensis* and 0.20 mm in *S. corti*). From *S. utahensis*, it also differs in the different morphology of the vulva (without prominent lips in *S. utahensis*). The present species can also be differentiated from *S. allegheniensis* by the different morphology of the pseudolabium (dorsal, ventral and median lobes of almost equal size vs dorsal and ventral lobes greatly reduced, much smaller than median lobe in the latter), the relatively longer spicules (representing 8.60–12.6 % of body length vs 7.10–7.40 % of body length in *S. allegheniensis*) and the presence of a well-developed gubernaculum (reduced in the latter species).

There are no previous studies on the genetic characteristics of *Spiroxys* spp., so the molecular data presented here represent an important advance in the molecular identification and differentiation of *Spiroxys* spp. In addition, the molecular data of *S. japonica* and *S. hanzaki* may contribute to future studies on population genetics and phylogenetics. The molecular analyses for the specimens of *S. japonica* collected from *P. nigromaculatus* in China and Japan showed five different genotypes and a low level of nucleotide variation in the ITS region (only 0.14–0.98 % sequence differences). We consider that this nucleotide variation represents intraspecific nucleotide differences because of their much lower level compared with the interspecific nucleotide differences (>45.0 % nucleotide differences) between *S. japonica* and *S. hanzaki*. Furthermore, the level of the intraspecific nucleotide variation of *S. japonica* in the ITS region did not appear to relate to any particular group of samples, e.g. from different geographical localities or hosts. There were no intraspecific nucleotide differences detected in the partial 18S rDNA of *S. japonica* based on the material collected from different geographical

localities and hosts. The level of the interspecific nucleotide variation between *S. japonica* and *S. hanzaki* in the partial 18S rDNA is also far lower than that of ITS region (only 2.28 % nucleotide differences). However, the *cox* 1 gene of *S. japonica* showed the highest intraspecific nucleotide differences (ranged from 0 to 9.38 %) and the level of the intraspecific variation of *S. japonica* in *cox* 1 is remarkably related with the geographical localities (1.04 % within Japanese samples, 0–1.30 % within Chinese samples vs 8.59–9.38 % nucleotide differences detected in *cox* 1 region between Japanese and Chinese samples) (see Fig. 5 for details). This result also indicates that the 18S rDNA, ITS and mtDNA *cox* 1 are useful genetic markers for the accurate identification and discrimination of *Spiroxys* species, whereas mtDNA *cox* 1 is a better candidate for studies on their population genetics in this group.

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