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Larvae of *Spirocerca lupi* and another spirurid species in the same dung beetles: notes on species identification

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Research Paper

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Abstract

Various dung beetle species of the family Scarabaeidae have been reported as intermediate hosts of *Spirocerca lupi* from different geographical locations, but there has been no data from south-east Asian countries. In the present study in Vietnam, by using morphological and molecular approaches, we identified the third-stage larvae of *S. lupi* for the first time in two dung beetle species, *Catharsius molossus* and *Copris szechouanicus*, adding them as new intermediate hosts of this nematode. In addition, both beetle species were infected with larvae of another spirurid nematode of *Physocephalus* sp. At large magnifications above 200×, these two spirurid larvae can be differentiated from each other by their body size (1880–2662 vs. 1417–1635 µm) and detailed morphological features of the anterior and posterior ends, such as the length of the buccal cavity, the position of the nerve ring and excretory pore, and tail characters. However, it is difficult to distinguish them at a lower magnification due to their minute size. Moreover, morphometric data of *S. lupi* larvae vary among reports and the body length may overlap with that of *Physocephalus* sp., thus, misidentification may occur, indicating the necessity of careful examination for accurate identification. The findings of the present study also supposed that larvae of *S. lupi* and more spirurid nematodes may be concurrently found in various dung beetle species, including *C. molossus* and *C. szechouanicus*, in other south-east Asian countries. Thus, more investigations in various countries should be conducted to identify spirurid larvae from dung beetles in order to fully understand their role as intermediate hosts of *S. lupi* and other spirurid nematodes.

Introduction

Spirocerca lupi (Spirurida: Spirocercidae) is a parasitic nematode that causes spirocercosis in canids and is associated with canine oesophageal sarcomas (Mazaki-Tovi *et al.*, 2002; van der Merwe *et al.*, 2008; Rojas *et al.*, 2020). Its indirect life cycle requires two obligatory hosts, canid as definitive hosts and dung beetles as intermediate hosts. Moreover, a variety of beetle-eating small animals can serve as paratenic hosts (Bailey *et al.*, 1963; van der Merwe *et al.*, 2008; Rojas *et al.*, 2020). The infective third-stage larvae in intermediate and paratenic hosts are similar, except for differences in their body length and size of the internal organs (Sen & Anantaraman, 1971), and are recognized by two cephalic horns at the anterior end and rosette spinous processes at the posterior end (Chhabra, 1968; Sen & Anantaraman, 1971; Chhabra & Singh, 1972). However, the cephalic horns and spinous tail are common characteristics of larvae of spirurid nematodes in dung beetle intermediate hosts (Seurat, 1915; Alicata, 1935; Nichols & Gómez, 2014), thus misidentification may occur.

Previous studies on *S. lupi* larvae from intermediate hosts usually reported this species alone (Chowdhury & Pande, 1969; Du Toit *et al.*, 2008; Gottlieb *et al.*, 2011; Mohtasebi *et al.*, 2021). So far, various dung beetle species of the family Scarabaeidae have been reported as intermediate hosts of *S. lupi* and vary among different geographical locations from the United States, Africa and Asia (Faust, 1928; Ono, 1929; Anantaraman & Jayalakshmi, 1963; Bailey *et al.*, 1963; Chowdhury & Pande, 1969; Bailey, 1972; Chhabra & Singh, 1972; Du Toit *et al.*, 2008; Gottlieb *et al.*, 2011; Mohtasebi *et al.*, 2021). In Asia, the majority of reports are from China, India, Israel and Iran (Faust, 1928; Ono, 1929; Anantaraman & Jayalakshmi, 1963; Chowdhury & Pande, 1969; Chhabra & Singh, 1972, 1973; Gottlieb *et al.*, 2011; Mohtasebi *et al.*, 2021). To the best of our knowledge, there has been no report from south-east Asian countries where the warm climate favours for development of the *S. lupi* nematode and its beetle intermediate hosts. The aim of this study is, therefore, to survey *S. lupi* larvae in dung beetle hosts in northern Vietnam, where we found adults of this nematode in dogs in a previous study (Hoa *et al.*, 2021). In addition to larvae of *S. lupi*, we also detected those of an

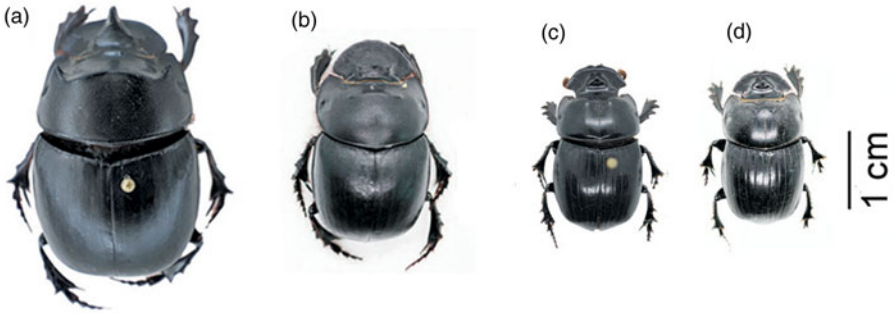

Fig. 1. Two dung beetle species collected from Dong Hy District, Thai Nguyen Province, Vietnam: (a) and (b) male and female of *Catharsius molossus*; and (c) and (d) male and female of *Copris szechouanicus*.

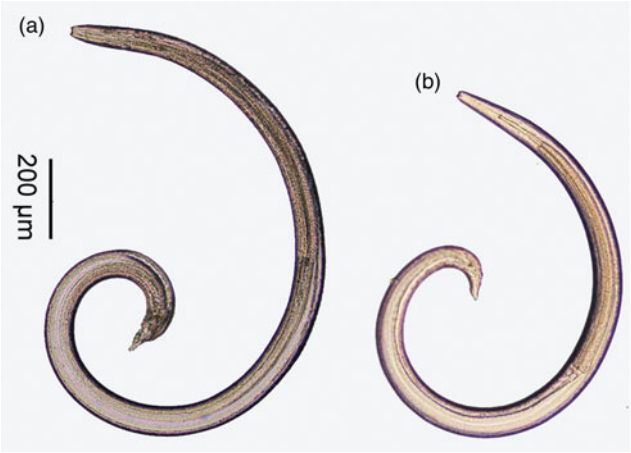

Fig. 2. Whole body of third stage larvae of two spirurid species: (a) *Spirocerca lupi*; and (b) *Physocephalus* sp.

unidentified spirurid species in different or the same individuals of two dung beetle species. Morphological variation and molecular analyses of these larvae are discussed herein.

Materials and methods

Materials

Dung beetles were collected manually while rolling dung balls of animals surrounding the residential area in Dong Hy District (21°41′10″N°105°55′43″E), Thai Nguyen Province, Vietnam, where *S. lupi* infections have been detected from domestic dogs (Hoa *et al.*, 2021). A total of 280 individuals of dung beetles were collected and transferred to the Department of Parasitology, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, using ventilated plastic boxes. They were identified at the species level following Ek-Amnuay (2008) and Bui *et al.* (2018). Two species, *Catharsius molossus* (Linnaeus, 1758) (210 individuals; fig. 1a, b) and *Copris szechouanicus* Balthasar, 1958 (70 individuals; fig. 1c, d), were identified. They were reared in separate plastic tanks containing crushed soil moistened with tap water daily and were fed with sterilized cow dung every two days (Mukaratirwa *et al.*, 2010).

Each beetle was dissected in normal saline solution in a Petri dish (90 mm) and was checked under a stereomicroscope to look for nematode larvae. For positive beetles, larvae were transferred into another Petri dish (35 mm) containing normal saline to wash until clean. Then, the larvae were treated with hot water to be straightened and were observed under a light microscope for initial morphological examination. Morphologically different types of larvae were separated from each other. One to two larvae of each species were preserved in 96% ethanol for molecular analyses, and the others were preserved in 4% formalin for morphological studies in details.

Morphological study

The larvae preserved in 4% formalin were transferred to lactophenol solution in a Petri dish until the body became transparent, then they were placed in a glass slide, covered with a coverslip, and were examined under a light microscope (Nikon Eclipse N*i*., code KST2019-KHV01, IEBR) connected to a digital camera and a computer. Measurements of the body and taxonomic internal organs were taken with the aid of NIS-Elements BR software.

Molecular analyses

Four larvae of two morphological types from the two beetle species were used for molecular identification. Larvae preserved in 96% ethanol were washed with sterile distilled water to remove ethanol. Then, DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). A partial fragment (393 base pairs) of the mitochondrial cytochrome c oxidase 1 gene (*cox*1) was amplified using primers JB3 and JB4.5 (Bowles *et al.*, 1993) following the protocol described by Rojas *et al.* (2018) and Hoa *et al.* (2021). Polymerase chain reaction (PCR) products were electrophoresed on 1.5% agarose gels and visualized using an ultraviolet transilluminator. Positive PCR products were directly sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Waltham, Massachusetts, USA). Nucleotide sequences obtained in this study were deposited in GenBank under accession numbers LC731325 to LC731328.

The obtained sequences in this study were compared with sequences available in GenBank using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences in GenBank with high coverage and identity over 80% were downloaded and analysed in MEGA7 software (Kumar *et al.*, 2016). The sequence (LC057236) of *Crassicauda giliakiana* (Spirurida: Tetrameridae) was used as an outgroup for phylogenetic analysis. Multiple sequence alignments were carried out using the ClustalW algorithm. A phylogenetic tree was reconstructed using the maximum likelihood. The best nucleotide substitution model was statistically selected by MEGA7 software (Kumar *et al.*, 2016). All positions containing gaps and missing data were eliminated. Bootstraps of 1000 replicates were used for the assessment of topology reliability of the trees.

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Table 1. Comparison of mophometric data of *Spirocerca lupi* and *Physocephalus* spp. larvae found in this study and previous report.

	S. lupi						
Measurement (μm) of	Seurat (1915)	Sen & Anantaraman (1971)	Mohtasebi *et al.* (2021)	Chhabra (1968)	This study	*Physocephalus* sp. This study	*Physocephalus sexalatus* Seurat (1915)
Body length	3400	810–1400 (1105)	2810–3150 (2950)	1810–2648	1880–2662 (2224.6 ± 227.8)	1417–1635 (1541.3 ± 61.3)	940–1728
Body width	150	70–80 (75)	100–150 (12)	54–88	66–116 (81.9 ± 12.5)	54–81 (67.1 ± 7.3)	75
buccal capsule length	35	50	51	37–46	30–40 (33.1 ± 2.7)	78–96 (88.8 ± 4.6)	91
buccal capsule width		10	13		10–16 (12.6 ± 2.6)	8–12 (9.7 ± 1.0)	
muscular oesophagus length	140	70		94–174	92–138 (110.8 ± 12.5)	85–100 (91.1 ± 4.2)	84
muscular oesophagus width		20			18–22 (20.1 ± 1.0)	14–17 (16.0 ± 1.1)	
glandular oesophagus length	1160	300		506–1050	568–883 (712.8 ± 76.2)	432–594 (506.8 ± 35.6)	601
Glandular oesophagus width		30			35–62 (45.8 ± 7.9)	24–37 (30.1 ± 3.5)	
ratio (%) of buccal capsule to muscular oesophagus length					26–34 (30.0 ± 2.0)	89–104 (98.0 ± 4.0)	
nerve ring to the anterior end	157	110	108–113 (111)	101–120	83–126 (97.6 ± 11.0)	107–128 (120.3 ± 5.9)	125
excretory pore to the anterior end	170	90	70–110 (90)	109–164	121–178 (140.1 ± 15.6)	130–159 (147.3 ± 7.9)	145
intestine length		530		1048–1460	939–1.500 (1234.6 ± 143.1)	672–925 (676.3 ± 60.6)	
rectum length		50	53	59-90	52–77 (65.4 ± 8.7)	32–50 (38.6 ± 4.4)	
anus to the posterior end					45–63 (51.0 ± 4.6)	41–61 (53.5 ± 4.2)	

Note: data in parentheses indicate average measurements and standard deviation values.

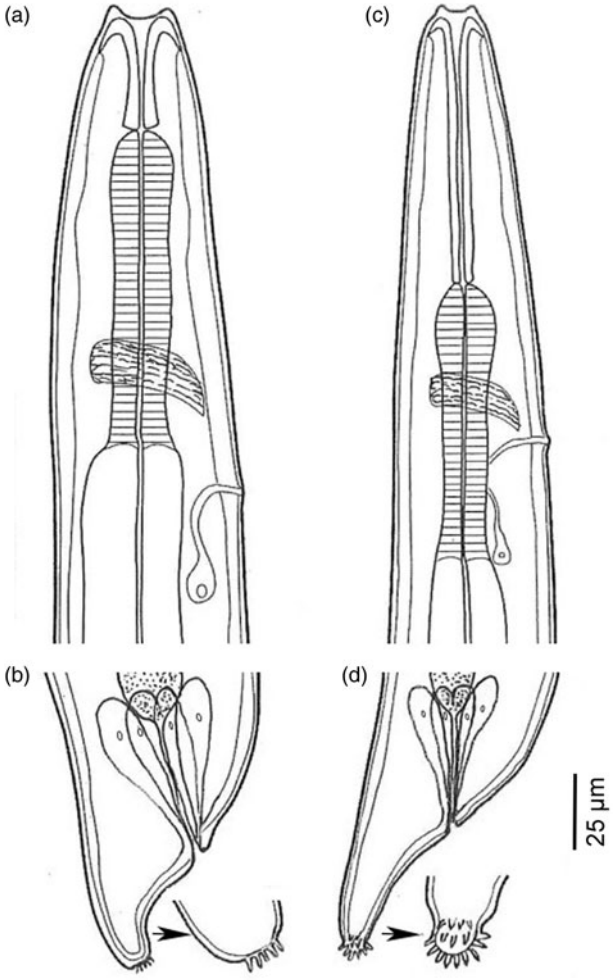

Fig. 3. Anterior and posterior parts of larvae of *Spirocerca lupi* and *Physocephalus* sp. showing differences between two species: (a) and (b) anterior and posterior parts of *Spirocerca lupi* larva; and (c) and (d) anterior and posterior parts of *Physocephalus* sp. larva.

Results

Morphological data

Nematode larvae with two cephalic horns at the anterior end and spines at the posterior end were collected from two beetle species, *C. molossus* and *C. szechouanicus*. Two morphological types of the third-stage larvae were recognized. They were distinguished from each other in their body size (fig. 2) and other morphometric data (table 1). The main morphological characteristics of the two types are as follows.

The first larval type (fig. 3a, b): third-stage larvae were found to be free or encysted in various parts of the body of the beetles. Body slender, tapering slightly anteriorly and rather abruptly posterior to anus, 1880–2662 µm in length; the anterior end has two cephalic horns and the posterior end looks like a cup bottom carrying a rosette of closely cuticular spines at the bottom of the tail. The buccal cavity is clear, the oesophagus shows the anterior muscular and posterior glandular parts. The buccal cavity is short, equal to about one-third (26–34%) of the muscular oesophagus. The nerve ring lies at the posterior end of the muscular oesophagus; the excretory pore opens slightly posterior to the nerve ring, around the conjunction between the muscular and glandular oesophagus.

The second larval type (fig. 3c, d): third-stage larvae were also free or encysted in various parts of the body of the beetles. Body slender, tapering slightly anteriorly and rather abruptly posterior to anus, 1417–1635 µm in length; the anterior end has two cephalic horns and the posterior end is a characteristic small knob, bearing small cuticular spines around the knob tail. The buccal cavity is clear, the oesophagus shows the anterior muscular and posterior glandular parts. The buccal cavity is long, near equal (89–104%) to the muscular oesophagus length. The nerve ring lies at around the middle of the muscular oesophagus; the excretory pore opens at slightly posterior to the nerve ring.

The characteristics of the first type are identical to the *S. lupi* larva (Seurat, 1915; Chhabra, 1968) and those of the other are most similar to the *Physocephalus sexalatus* larva (Seurat, 1915) (table 1).

Molecular analyses

Partial sequences from the *cox*1 gene were obtained from four larvae of the two types collected from the two beetle species. Two sequences from the larvae, which were morphologically identified as *S. lupi*, were identical to each other and also identical to *S. lupi* adults from Vietnam and India, and were highly similar (99.8%) to a Chinese sequence (KC305876), but slightly different (3.3–4.7%) from South African, Israeli and Peruvian sequences, while far distant (8.6%) from a Hungarian sequence (MH634011). In the phylogenetic tree (fig. 4), the *cox*1 sequences of *S. lupi* from Vietnam, India and China made a common clade close to the sequences from South Africa, Israel and Peru, while the Hungarian sequence made a separate group. The grouping of genotypes of *S. lupi* from different geographical origins in the present study is similar to those of previous reports (Rojas *et al.*, 2018; Hoa *et al.*, 2021).

Two sequences from the other species were also completely identical to each other but were not completely identical to any sequences available in GenBank. The result of computing pairwise distances showed that these two *cox*1 sequences were far distant from sequences of other species, showing closest to *Dirofilaria* species (*Dirofilaria* sp. 'hongkong', *Dirofilaria* sp. 'Thailand' and *Dirofilaria repens*), but at high differences from 16.1 to 17.7%. In the phylogenetic tree (fig. 4), they made a distinct group.

Thus, the morphological and molecular data confirmed the first larval type as *S. lupi*; because the second larval type is highly likely to be *P. sexalatus* by morphological identification but has not been confirmed by molecular data, we tentatively name it *Physocephalus* sp.

Prevalence

Both the dung beetle species, *C. molossus* and *C. szechouanicus*, were infected with larvae of two nematode species, *S. lupi* and *Physocephalus* sp. Prevalences of *Physocephalus* sp. infection in the two host species (7.1% in both) were slightly higher than those of *S. lupi* infection (6.2 and 5.7%, respectively). Similarly, intensities of *Physocephalus* sp. (1–31 larvae per beetle) were slightly higher than those of *S. lupi* (1–22 larvae per beetle) (table 2).

Discussion

Dung beetles play an indispensable role as intermediate hosts in the life cycle of spirurid nematodes, including *S. lupi* (Alicata,

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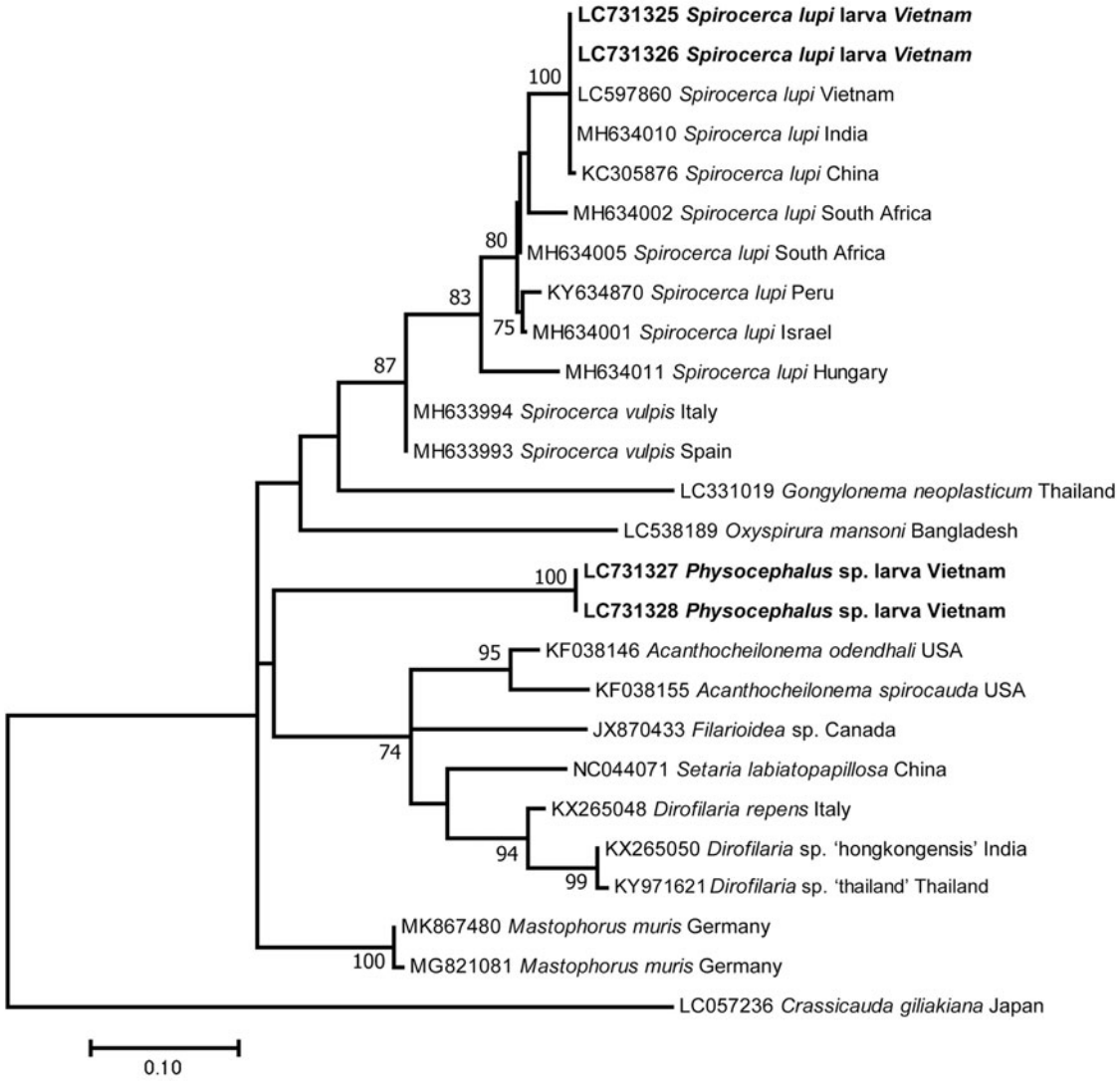

Fig. 4. Phylogenetic tree reconstructed from *cox*1 sequences by the maximum likelihood method based on the Tamura–Nei model with a discrete Gamma distribution. Bootstrap values higher than 60% based on 1,000 replicates are shown above the nodes. The accession number, species name and geographical origin of each nucleotide sequence are shown. The sequences obtained in this study are printed in boldface type while the others from GenBank database are printed in regular type. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicated 0.05 changes per nucleotide.

Table 2. Prevalence of Spirurid larvae in dung bettles from Thai Nguyen Province, Vietnam

		Spirocerca lupi larva		*Physocephalus* sp. larva	
Beetle species	Number examined	Number infected (%)	Intensity (average)	Number infected (%)	Intensity (average)
Catharsius molossus	210	13 (6.2)	1–22 (5.5)	15 (7.1)	1–31 (7.7)
Copris szechouanicus	70	4 (5.7)	1–7 (4.0)	5 (7.1)	1–11 (4.4)

1935; van der Merwe *et al.*, 2008; Nichols & Gómez, 2014; Rojas *et al.*, 2020). In this study, by using morphological and molecular approaches, we confirmed *S. lupi* larvae in two dung beetle species, *C. molossus* and *C. szechouanicus*, as new intermediate hosts. In addition, larvae of another spirurid nematode, *Physocephalus* sp., were also detected in both beetle species.

Morphologically, although the body size of *Physocephalus* sp. larvae is smaller than that of *S. lupi*, it is difficult to distinguish them at low magnification due to their minute size, especially when they are not concurrently present for comparison. Moreover, the body size and other morphometric data of *S. lupi* larvae vary among reports. Generally, its body length ranges from 1880–3400 µm (Seurat, 1915; Chhabra, 1968; Mohtasebi *et al.*, 2021; this study), longer than that of *Physocephalus* larvae (940–1728 µm). However, *S. lupi* larvae from dung beetles reported by Sen & Anantaraman (1971) is 810–1400 µm in

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length, similar to, or even smaller than that of *Physocephalus* sp. (1417–1635 µm; this study) or *P. sexalatus* (940–1728 µm; Seurat, 1915). In addition, *S. lupi* larvae reported by Seurat (1915), Chhabra, 1968 and those found in this study have a distance from the nerve ring to the anterior end of the body shorter than that from the excretory pore, whereas, it is converse in *S. lupi* larvae reported by Sen & Anantaraman (1971) and by Mohtasebi *et al.* (2021). These overlaps and differences in morphometric data suggest that misidentification may occur. This potential problem can be avoided by careful observation of their detailed morphological features under a light microscope at magnification above 200×. In the anterior end, *S. lupi* larva has a short buccal cavity in comparison to the muscular oesophagus (about one-third) while *Physocephalus* sp. larva has a much longer buccal cavity, almost equal to the length of the muscular oesophagus. The nerve ring and excretory pore of *S. lupi* are located at the base of the muscular oesophagus, while those of *Physocephalus* sp. are located at about the middle of the muscular oesophagus. In the posterior end, the tail of *S. lupi* larva looks like a cup bottom carrying a rosette of closely cuticular spines at the end, while the posterior end of *Physocephalus* sp. is a characteristic small knob, about 7 to 8 µm long, bearing small cuticular spines around the knob tail. These characteristics of the second larval type are similar to *P. sexalatus* larva described by Seurat (1915). However, morphology alone might be insufficient to identify species of nematode larvae because larvae of different genera share common characteristics (Bain *et al.*, 2014) and those of more than one spirurid species can be found in the same dung beetle species (Seurat, 1915; Alicata, 1935). Unfortunately, molecular data do not help in identifying this larva to species level due to the lack of completely identical sequences in GenBank, including the sequence of *P. sexalatus* to which the larva is morphologically similar. These shortages of necessary data point out that morphological descriptions and molecular data of more spirurid larvae should be investigated and deposited in a database for use in future studies. For morphology, detailed morphological features of the anterior and posterior ends of the body are important and need to be carefully examined.

The available data from studies on *S. lupi* larvae in beetle hosts showed that a wide variety of dung beetles serve as intermediate hosts of *S. lupi* and vary from place to place. They are: *Scarabaeus sacer*, *Scarabaeus variolosus* and *Gymnopleurus sturmi* in the United States (Bailey *et al.*, 1963); *Copris hispana* in Algeria (Seurat, 1915); *Onthophagus pugionatus*, *Onthophagus sugillatus*, *Onthophagus ebenus*, *Onthophagus obtusicornis*, *Gymnopleurus virens*, *Gymnopleurus humanus*, *Pachylomerus femoralis*, *Scarabaeus rugosus*, *Kheper nigroaeneus*, *Anachalcos convexus* and also a millipede species, *Daratoagonus crristulatus* (experimental infection) in South Africa (Du Toit *et al.*, 2008; Mukaratirwa *et al.*, 2010); *Onthophagus sellatus* in Israel (Gottlieb *et al.*, 2011); *Scarabaeus armeniacus* in Iran (Mohtasebi *et al.*, 2021); *Gymnopleurus koenigi*, *Oniticellus pallens*, *Oniticellus pallipes*, *Onthophagus deflexicollis*, *Onthophagus quadridentatus*, *Onthophagus bonasus*, *Onthophagus dama*, *Onthophagus gazella*, *Onthophagus mopsus*, *Onitis philemon*, *Catharsius pithecius*, *Euoniticellus pallipes*, *Scarites indus*, *Hybosorus orientalis*, *Hister maindronii* and *Hister lutarius* in India (Anantaraman & Jayalakshmi, 1963; Chhabra, 1968; Chowdhury & Pande, 1969); and *Gymnopleurus sinnatus*, *Gymnopleurus mopsus*, *Scarabaeus sacer* and *Cathon* spp. in China (Faust, 1928; Ono, 1929), but *Cathon* beetles reported by Faust (1928) were suggested to belong to the genus *Paragymnopleurus* (Theodorides, 1952). The detection of *S. lupi* larvae from two beetle species of *C. molossus* and *C. szechouanicus* in Vietnam in the present study seems to be the first report from south-east Asia, adding the two species as new intermediate hosts of *S. lupi*. Since dung beetles are widespread and abundant in tropical Asian regions, particularly in south-east Asia, more surveys in different countries of this region should be conducted to fully understand the diversity of intermediate hosts of *S. lupi* and other spirurid nematodes.

Concerning prevalence, previous studies showed that infection rates of *S. lupi* vary from place to place, and from beetle species to species. In India, Chowdhury & Pande (1969) reported the prevalence of *S. lupi* larvae in four beetle species, *Onthophagus deflexicollis*, *Onthophagus quadridentatus*, *Oniticellus pallens* and *Oniticellus pallipes*, these at the same study site were 3.8%, 9.9%, 15%, and 31.8%, respectively, and burdens ranged from 1–24 larvae per beetle. In a study in Iran, Mohtasebi *et al.* (2021) found an infection rate of 1.5% (3/200) in *Scarabaeus armeniacus* with an intensity of 1–6 larvae per beetle. A survey in South Africa revealed considerable variation of infection rates in different study sites: 13.5% (7/52) and burdens of 1–129 larvae per beetle in the urban area, 2.3% (3/129) and intensity of 1–10 larvae per beetle in the rural area, while beetles collected in the suburban areas were not infected (Du Toit *et al.*, 2008). In the present study, the prevalence and intensities of *S. lupi* larvae in two beetle hosts are in these ranges. In addition, the larvae of *Physocephalus* sp. were found to be concurrently present in both host species with a prevalence slightly higher than that of *S. lupi*. The genus *Physocephalus* includes nine species parasitizing suids, equids, camelids, cattle, lagomorphs and rodents (Bain *et al.*, 2014). *Physocephalus* sp. larva found in this study was similar to *P. sexalatus* larva – a parasite of pigs – described by Seurat (1915). The finding of this larva indicated the possibility of *Physocephalus* infection in pigs in the study site, suggesting that morphological and molecular studies on adult nematodes from pigs are necessary to confirm the species identification and that studies on *S. lupi* larvae in beetle hosts should also pay attention to other spirurid larvae.

Conclusion

The present study found *S. lupi* larvae from two beetle species of *C. molossus* and *C. szechouanicus* in Vietnam, adding them as new intermediate hosts of *S. lupi*. In addition, larvae of an unidentified spirurid nematode, *Physocephalus* sp., was concurrently found in both dung beetle species. Although these larvae are morphologically different from each other in detailed morphological features of the anterior and posterior ends, it is difficult to distinguish them at a low magnification due to their minute size and overlap measurements. To avoid misidentification of spirurid larvae from dung beetles, detailed taxonomic characteristics of larvae should be carefully examined.

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Conflicts of interest. None.

Ethical standards.

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