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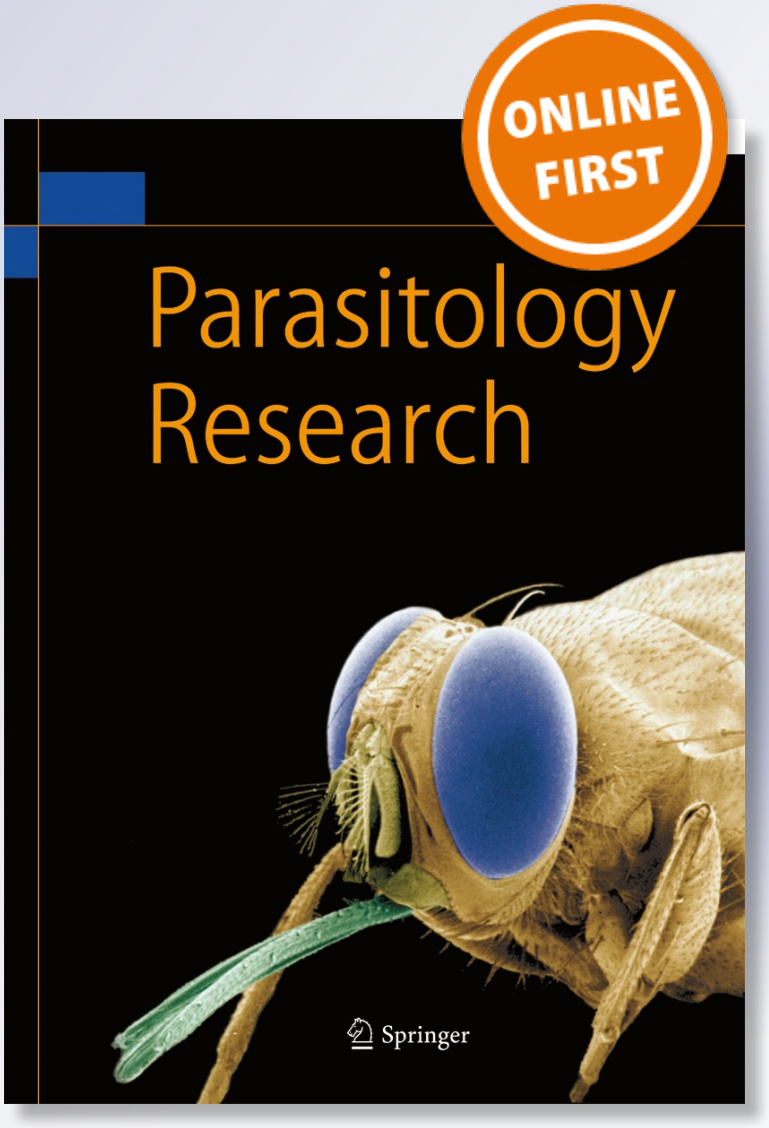

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ORIGINAL PAPER

Morphological and molecular genetic diversity of *Strongyluris calotis* (Nematoda: Ascaridida: Heterakidae) in South East and East Asian lizards

Binh Thi Tran[1] · **An Vinh Ong**[2] · **Pham Van Luc**[3] · **Hiroshi Sato**[4]

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Abstract *Strongyluris calotis* is a heterakid nematode in the large intestine of agamid lizards (Reptilia: Sauria: Agamidae) from the Oriental Region. The standard light microscopic definition of the species counts the "caudal papillae" as 10 pairs on male worms. However, previous work from our group using scanning electron microscopy (SEM) on the heterakid from agamid lizards in Japan, Taiwan, and Singapore revealed that this counting contained a pair of phasmids and that two pairs of postcloacal papillae were completely fused to form a pair of united papillae, thus resulting in "10 pairs." In the present study, we examined *S. calotis* specimens from the Emma Gray's forest lizard, *Calotes emma* (Agamidae), living in the plain forest at low altitude, and the Vietnam false bloodsucker, *Pseudocalotes brevipes* (Agamidae), living in the mountainous forest at high altitude in the northern part of Vietnam. Using SEM, the arrangement of caudal papillae in male worms from an Emma Gray's forest lizard was found to be comparable to classical *S. calotis* specimens from agamid lizards collected in Japan, Taiwan, and Singapore. However, male worms from Vietnam false bloodsuckers did not have a pair of united papillae but had 10 pairs of independent caudal papillae with a pair of phasmids. Molecular genetic analyses of the ribosomal RNA gene (rDNA) of worms of the classical *S. calotis* morphotype from Japan and Singapore and two *S. calotis* morphotypes from Vietnam demonstrated absolutely identical nucleotide sequences of partial 18S rDNA (at least 1764 base pairs (bp)) and 5.8S rDNA (158 bp). However, intraspecific differences were detected in other regions of the rDNA, related to the geographical distribution of hosts regardless of morphotype: 97.8–98.5 % identity (443–446 bp/453 bp) in the internal transcribed spacer (ITS)-1 region, 96.6–98.0 % identity (425–431 bp/440 bp) in the ITS-2 region, and 99.6–99.7 % identity (1149–1151 bp/1154 bp) in the 28S rDNA. Thus, in the future, taxonomic relationships of *S. calotis* distributed widely in the Oriental Region as well as other nominal Oriental *Strongyluris* spp., currently six in number, need to be extensively explored based on molecular genetic analyses in addition to intensive morphological characterization.

Keywords Heterakidae · *Strongyluris calotis* · *Pseudocalotes brevipes* · *Calotes emma* · Vietnam · Agamid lizard · Scanning electron microscopy (SEM) · rDNA · Genetic diversity · Morphotype

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Introduction

The genus *Strongyluris* Müller, 1894 is assigned to heterakid nematodes with lips offset from the body, notable cuticular flanges extending from each lip, a posteriorly directed precloacal sucker, and an obliquely truncate tail with a short terminal spike in male worms (Inglis 1957; Skrjabin et al. 1961; Chabaud 2009). Multiple stout pedunculated papillae support the caudal cuticular expansion, sometimes referred to as the caudal alae, of male worms. The type species of the

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genus is *S. brevicaudata* Müller, 1894 and currently, a few dozen species have been recorded from the large intestine of lizards and, rarely, amphibians (Yamaguti and Mitunaga 1943). Bursey et al. (2003, 2013), by adding several species since Baker's report (1984), documented 32 nominal *Strongyluris* spp. recorded worldwide: six species from the Australian zoogeographic region; 12 species from the Ethiopian; four species from the Nearctic; four species from the Neotropical; and six species from the Oriental. Subsequently, a few new species such as *S. amazonicus* from the Neotropical have been added to the list (Santos et al. 2013). Inglis (1957) specifically expressed his concern regarding whether every nominal species at that time, also included in the latest list by Bursey et al. (2013), could be differentiated from congeners based on the morphological descriptions provided by each research group.

As shown in our previous study (Tran et al. 2015b), *S. calotis* specimens collected from various sources often showed different spectra of morphometric values, causing potential difficulties with species identification. Furthermore, the number of "caudal papillae" in male worms, one of the most important characters used for morphological species characterization of the genus *Strongyluris*, recorded in previous studies may have incorrectly included phasmids present in the caudal region of male worms (Tran et al. 2015b). In addition, our previous study (Tran et al. 2015b) using scanning electron microscopy (SEM) found a pair of united papilla structures formed from the complete fusion of two pairs of caudal papillae in male *S. calotis* worms collected in the southern part of Japan, Taiwan, and Singapore. This finding highlights the importance of reexamining previously described *Strongyluris* spp. for clear species differentiation using more advanced techniques in addition to light microscopy.

In the present study, we collected *Strongyluris* worms from two types of agamid lizards; Vietnam false bloodsuckers, *Pseudocalotes brevipes* (Werner, 1904), distributed limitedly in northern Vietnam and southern China, and Emma Gray's forest lizards, *Calotes emma* (Gray, 1845), distributed widely in Vietnam. We compared not only the morphology of the worms but also their ribosomal RNA gene (rDNA) nucleotide sequences in order to clarify the taxonomic relationship of morphologically differentiated worms.

Materials and methods

Animals and parasitological examination

Seven Vietnam false bloodsuckers (snout-to-vent length, 56–68 mm) were collected by hand during the period from June 2012 to September 2013 from Xuan Son National Park, Phu Tho Province (21° 12′ 25.5″ N, 104° 50′ 26.2′ E) and Kim Hy Nature Reserve, Bac Kan Province (22° 11′ 05″ N, 106° 03′ 45.5″ E), Vietnam, at an altitude of 420 and 460 m above sea level, respectively. Six Emma Gray's forest lizards (snout-to-vent length, 44–58 mm) were similarly collected in September 2014 from Ba Be National Park, Bac Kan Province (22° 23′ N, 105° 57′ E), living in plain forests in the northern part of Vietnam at an altitude of 230 m above sea level. Parasitological examination of lizards and morphological observation of collected nematodes were performed as described previously (Tran et al. 2015a). Drawings were made with the aid of a camera lucida, partly referring to SEM photographs of the selected worms. Measurements are in millimeters (mm), with the range followed by the mean in parentheses. Nematodes were deposited in the Vietnam National Museum of Nature, VAST, Hanoi, Vietnam, under specimen nos. VNMN-2014-001–003.

Twelve Ryukyu tree lizards, *Japalura polygonata ishigakiensis* (Agamidae), were collected by hand on August 2 and 4, 2015 from Ishigaki Island, Okinawa, Japan (24° 26′ 14″ N, 124° 12′ 44″ E). All 12 lizards harbored *S. calotis* worms in the large intestine, 3–11 (geomean 5.6) in number/host. A portion of the collected worms from the Ryukyu tree lizards was used for morphological examination and genetic analysis. Similarly, a male *S. calotis* worm from an Emma Gray's forest lizard caught in Singapore in 2000 by Dr. C. H. Diong, National Institute of Education, Nanyang Technological University, was used for genetic analysis. Additionally, SEM photographs of a male worm of Singapore origin are shown in the present study as a representative of the classical *S. calotis* morphotype.

Scanning electron microscopy

Individual male and female worms, stored in 70 % alcohol, were cut into three longitudinally equal parts. The anterior and posterior 1/3 parts were used for SEM. Specimens were washed three times in 0.2 M Na_2HPO_4-NaH_2PO_4-buffered solution (PB), pH 7.8, then immersed in 2.5 % glutaraldehyde in PB overnight. Subsequent processing was similar to that described previously (Tran et al. 2015a).

DNA extraction, polymerase chain reaction, and sequencing

Parasite DNA extraction was performed using the middle 1/3 portion of individual worms (the two other parts were processed for SEM observation, see above). PCR amplification of overlapping DNA fragments of the 18S to 28S rDNA was performed in a 20-μl volume as described previously (Tran et al. 2015a). Primers used for PCR amplification of parasite DNA are summarized in Table 1. As a forward primer annealing to the 5′-terminus of the 18S rDNA, F-47 (5′-CCCGATTGATTCTGTCGGC-3′) was initially used;

Table 1 Primers used to amplify eight overlapping segments of rDNA of *Strongyluris* worms

Segment no.		Primer name	Sequence	Position of 5'-end[a]
1	F:	F-47	5′-CCCGATTGATTCTGTCGGC-3′	1
	R:	NSR1438/20	5′-GGGCATCACAGACCTGTTAT-3′	1434
1'	F:	Strongyluris18S_4F	5′-GATTGATTCTGTCGGCGGTT-3′	4
	R:	NSR1438/20	(see above)	1434
1"	F:	Strongyluris18S_11F	5′-TCTGTCGGCGGTTATATGCT-3′	11
	R:	NSR1438/20	(see above)	1434
1'"	F:	Strongyluris18S_15F	5′-TCGGCGGTTATATGCTTGTC-3′	15
	R:	NSR1438/20	(see above)	1434
2	F:	NSF573_Binh/19	5′-CGCGGTAATTCCAGCTCTC-3′	570
	R:	NSR1787/18	5′-CGACGGGCGGTGTGTACA-3′	1639
3	F:	NSF573_Binh/19	(see above)	570
	R:	S.r.18S-SSU18R	5′-TGATCCTTCYGCAGGTTCAC-3′	1794
4	F:	NSF1624/20	5′-TTTGTACACACCGCCCGTCG-3′	1620
	R:	NC13(ITS1)/RcNC13(ITS1)/R[b]	5′-GCTGCGTTCTTCATCGA(T)-3′	2297
5	F:	NC5(ITS1)/F	5′-GTAGGTGAACCTGCGGAAGGATCATT-3′	1771
	R:	NC2(ITS2)/R	5′-TTAGTTTCTTTTCCTCCGCT-3′	2914
6	F:	NC13(ITS2)/F	5′-ATCGATGAAGAACGCAGC-3′	2280
	R:	28S-408R/20	5′-TTCACGCCCTCTTGAACTCT-3′	3256
7	F:	B.p.28S/F	5′-AGCGGAGGAAAAGAAACTAA-3′	2895
	R:	B.p.26S-1270R/22	5′-CAGCTATCCTGAGGGAAACTTC-3′	4021
8	F:	B.p.28S/F	(see above)	2895
	R:	NLR1432-1/22	5'-GTTGTTACACACTCCTTAGCGG-3'	4329

F forward, *R* reverse

[a] Relative position of the 5′-end of each primer in an rDNA sequence of *Strongyluris calotis* from an Emma Gray's forest. Relative position of the 5′-end of each primer in an rDNA sequence of *Strongyluris calotis* from an Emma Gray's forest lizard (DDBJ/EMBL/GenBank accession no. LC133186). The 5′-end of unfunctional F-47 primer is considered as the beginning of 18S rDNA here

[b] Primer NC13(ITS1)/R might be functional when it lacked "T" at the 3′-terminus, i.e., 5′-GCTGCGTTCTTCATCGA-3′

however, no DNA amplification was obtained despite repeated trials with altered annealing temperatures. Consequently, three different forward primers, Strongyluris18S_4F, 11F, and 15F (see Table 1), were designed referring to the 18S rDNA sequence of *Aspidodera* sp. SAN-2007 (DDBJ/EMBL/GenBank accession no. EF180070) from the nine-banded armadillo, *Dasypus novemcinctus*, caught in Costa Rica (Nadler et al. 2007). These primers successfully amplified the 5′ portion of 18S rDNA. The 3′-terminus of a reverse primer used for an internal transcribed spacer (ITS)-1 region of the rDNA fragment, NC13 (ITS1)/R, did not match the final rDNA sequence, indicating that this primer worked as a degenerate primer lacking "T" at the 3′-terminus, i.e., 5′-GCTGCGTTCTTCATCGA-3′. The PCR cycling protocol for the amplification of each rDNA fragment was 94 °C for 3 min, then 40 cycles of 94 °C for 45 s, 64 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products for sequencing were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The amplicon was cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a FastGene Plasmid Mini Kit (Nippon Genetics) and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. Sequences were assembled manually with the aid of the CLUSTAL W multiple alignment program (Thompson et al. 1994). The newly obtained rDNA sequences of *S. calotis* in the present study were deposited in the DDBJ/EMBL/GenBank database (accession nos. LC133186–LC133190).

Phylogenetic analysis

For phylogenetic analysis, the newly obtained rDNA sequences of *S. calotis* from agamid lizards caught in Vietnam,

Singapore, and Japan were aligned using the CLUSTAL W multiple alignment program and demonstrated to contain no nucleotide insertions/deletions in the sequences compared. After removal of ambiguous nucleotide positions in any sequence, maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the "phylogeny.fr" website (http://www.phylogeny.fr/). The probability of inferred branch was assessed by the approximate likelihood ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

Results

Parasite recovery

Seven Vietnam false bloodsuckers, three from Xuan Son National Park, Phu Tho Province and four from Kim Hy Nature Reserve, Bac Kan Province, were examined. All three lizards collected at the former locality and one at the latter locality were infected with *S. calotis* in the large intestine. Infected lizards harbored one to six worms/host, and one to four male and one or two female worms were collected from individual lizards. One of six Emma Gray's forest lizards from Ba Be National Park, Bac Kan Province was infected with *S. calotis* in the large intestine.

Morphological characterization of *S. calotis* isolates

The *Strongyluris* worms were medium-sized nematodes, 8–16 mm in length, and worms of either sex had tapering anterior ends and stout posterior ends with a small terminal spike (Fig. 1). Worms had three lips offset from the body and distinct cuticular flanges extended from the upper part of the internal surface of each lip (Fig. 2). An esophagus, following a pharynx, consisted of a corpus and a tri-valved posterior bulb. Male worms had a posteriorly directed precloacal sucker, two non-alate spicules of equal length and shape, but no gubernaculum. Around an obliquely truncate tail of male worms, numerous stout pedunculated papillae appeared to support the caudal cuticular expansion. These morphological characters were coincident with the definition of the genus *Strongyluris* (Inglis 1957; Skrjabin et al. 1961; Chabaud 2009).

Either male or female worms collected from Vietnam false bloodsuckers and an Emma Gray's forest lizard had almost identical morphological features, close to *S. calotis* except for a minor but critical difference in the number and arrangement of caudal papillae in male worms (Table 2). They had three pairs of stout pedunculated papillae ventrally on both lateral sides of the precloacal sucker, two pairs of small adcloacal papillae on both lateral sides of the cloacal opening, and two pairs of ventrolateral papillae on both sides around the level of the cloaca. In addition, near the posterior end around the terminal spike, four pairs and three pairs of small papillae were observed by light microscopy for specimens collected from Vietnam false bloodsuckers and an Emma Gray's forest lizard, respectively (Fig. 1). By SEM, it was demonstrated that four pairs or three pairs of small terminal papillae, observed by light microscopy, included a pair of phasmids (Fig. 3). This finding is in accordance with recent work by our group (Tran et al. 2015b). Therefore, to be precise, male specimens in Vietnam false bloodsuckers and an Emma Gray's forest lizard had three or two pairs of caudal papillae around the terminal spike, respectively. Furthermore, all terminal papillae in the specimens from Vietnam false bloodsuckers were individual ones, whereas one of two pairs of terminal papillae in the specimens from an Emma Gray's forest lizard was a united papilla structure (Fig. 3). No other critical morphological differences between male worms of the two hosts were detected by SEM. Light microscopy showed that female worms of both sources had a pair of papillae at the lateral sides of the tail; however, in line with our earlier work (Tran et al. 2015b), SEM revealed them to be a pair of phasmids.

Molecular genetic characterization of *S. calotis* isolates

The rDNA nucleotide sequences of three isolates of *S. calotis* from Vietnam, including the two morphotypes mentioned above, one isolate from Singapore, and one isolate from Japan were successfully sequenced. Nearly complete lengths of the 18S rDNA with 1760–1771 base pairs (bp), ITS-1 region with 453 bp, 5.8S rDNA with 158 bp, ITS-2 region with 440 bp, and partial 28S rDNA with at least 1154 bp, except for the isolate from Singapore, were obtained. The rDNA nucleotide sequences of two isolates of *S. calotis* from Vietnam false bloodsuckers at Xuan Son National Park, Phu Tho Province and Kim Hy Nature Reserve, Bac Kan Province (DDBJ/EMBL/GenBank accession nos. LC133189 and LC1331909) were absolutely identical to one another except for one nucleotide in the 18S rDNA (Table 3). The rDNA nucleotide sequences of two *S. calotis* isolates from Emma Gray's forest lizards in Vietnam and Singapore, 4284 and 3025 bp, respectively, were also absolutely identical to one another as far as they were able to be compared (DDBJ/EMBL/GenBank accession nos. LC133186 and LC133187). The *S. calotis* isolate from a Ryukyu tree lizard, 4284 bp, displayed several nucleotide substitutions from the same morphotype of *S. calotis* from Emma Gray's forest lizards in Vietnam and Singapore as well as another morphotype from Vietnam false bloodsuckers in Vietnam (Table 3).

Different isolates of *S. calotis* showing two morphotypes had absolutely identical nucleotide sequences of 18S rDNA, except for one nucleotide substitution within the same

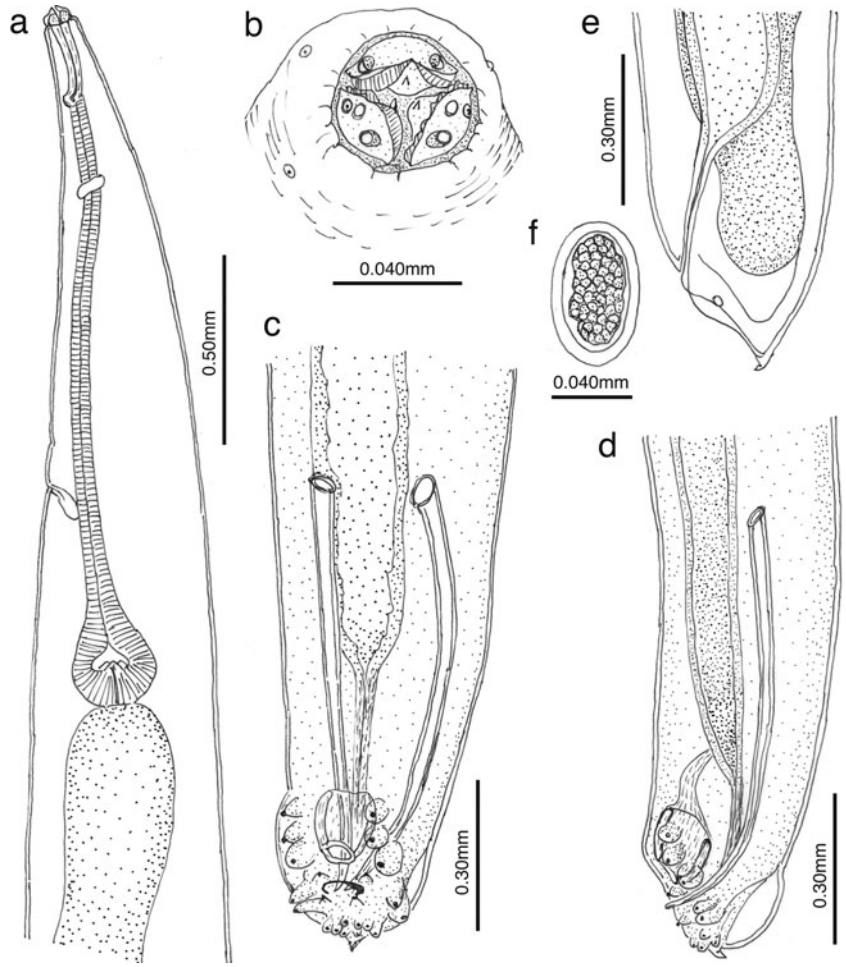

Fig. 1 *Strongyluris calotis* in *Pseudocalotes brevipes*. **a** Lateral view of the anterior end of a male worm. **b** Nearly *en face* view of the anterior end of a male worm. **c** Ventral view of the posterior end of a male worm. **d** Lateral view of the posterior end of a male worm. **e** Lateral view of the posterior end of a female worm. **f** Egg in the uterus

morphotype, and 5.8S rDNA. However, they showed several intraspecific differences in other regions: 97.8–98.5 % identity (443–446 bp/453 bp) in the ITS-1 region, 96.6–98.0 % identity (425–431 bp/440 bp) in the ITS-2 region, and 99.6–99.7 % identity (1149–1151 bp/1154 bp) in the 28S rDNA. The relationships of the different isolates examined in the present study are illustrated in Fig. 4.

Discussion

The Vietnam false bloodsucker, *P. brevipes*, has a limited distribution in the Tonkin region of northern Vietnam and Guangxi region of southern China, adjacent to Vietnam (Ananjeva et al. 2007; Nguyen et al. 2009). This agamid lizard is found on trees or bushes in tropical mountain forests, generally at an altitude of more than 1000 m above sea level. In

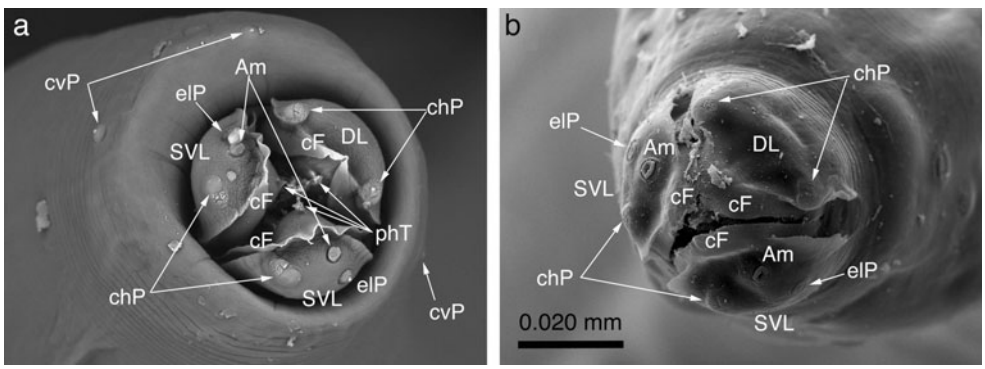

Fig. 2 SEM view of the anterior end of a male *Strongyluris calotis* worm. Specimen from *Pseudocalotes brevipes* in Vietnam (**a**) and from *Calotes emma* in Singapore (**b**). Photographs are at the same magnification and the scale is shown in **b**. Abbreviations: *Am*, amphid; *cF*, cuticular flange; *chP*, cephalic papilla; *cvP*, cervical papilla; *DL*, dorsal lip; *elP*, external labial papilla; *phT*, pharyngeal teeth; and *SVL*, subventral lip

Table 2 Comparison of morphometrics of different isolates of *Strongyluris calotis* (measurement in millimeter)

Morphotype	Without a pair of united papillae	With a pair of united papillae			
Host	*Pseudocalotes brevipes*	*Calotes emma*	*Japalura swinhonis*	*Japalura polygonata*	*Japalura polygonata*
Locality	Vietnam (Kanton)	Vietnam (Kanton)	Taiwan (Taipei)	Japan (Yonakuni, Okinawa Is.)	Japan (Kunigami, Okinawa Is.)
Reference	The present study	The present study	Tran et al. (2015b)	Tran et al. (2015b)	Tran et al. (2015b)
Male	(*n* = 6)	(*n* = 1)	(*n* = 4)	(*n* = 5)	(n=5)
Worm length	8.1—13.1 (10.4)	11.4	6.6—7.0 (6.9)	6.7—8.1 (7.4)	9.1—10.8 (10.0)
Worm width	0.33—0.56 (0.42)	0.52	0.30—0.38 (0.35)	0.29—0.34 (0.32)	0.35—0.41 (0.37)
Pharynx length	0.19—0.23 (0.21)	0.25	0.16—0.19 (0.18)	0.17—0.20 (0.18)	0.18—0.22 (0.20)
Esophagus length	1.29—1.96 (1.62)	2.13	1.25—1.33 (1.29)	1.22—1.36 (1.28)	1.27—1.39 (1.33)
Bulb length	0.20—0.32 (0.24)	0.23	0.16—0.19 (0.17)	0.17—0.19 (0.18)	0.19—0.21 (0.20)
Bulb width	0.19—0.30 (0.23)	0.27	0.21—0.22 (0.22)	0.18—0.22 (0.20)	0.21—0.22 (0.21)
Nerve ring from anterior end	0.34—0.50 (0.42)	0.62	0.36—0.41 (0.38)	0.37—0.42 (0.40)	0.38—0.42 (0.40)
Excretory pore from anterior end	0.78—1.36 (1.11)	1.38	0.86—1.05 (0.98)	0.81—0.95 (0.89)	0.85—0.94 (0.88)
Spiculae Length	0.72—1.00 (0.85)	ca. 1.15	0.56—0.68 (0.62)	0.56—0.59 (0.57)	0.54—0.68 (0.61)
Caudal papillae number and arrangement[a]	22 (6 : 4 : 4: 8)	20 (6 : 4 : 4 : 6)	20 (6 : 4 : 4 : 6)	20 (6 : 4 : 4 : 6)	20 (6 : 4 : 4 : 6)
Female	(*n* = 4)	(*n* = 1)	(*n* = 4)	(*n* = 5)	(n=5)
Worm length	11.0—16.2 (14.0)	13.1	7.1—8.8 (7.8)	7.9—9.2 (8.4)	11.3—12.3 (11.9)
Worm width	0.68—0.82 (0.74)	0.82	0.35—0.45 (0.39)	0.31—0.38 (0.35)	0.46—0.56 (0.52)
Pharynx length	0.22—0.27 (0.24)	0.30	0.18—0.22 (0.20)	0.20—0.21 (0.20)	0.21—0.23 (0.22)
Esophagus length	1.68—2.05 (1.90)	2.14	1.40—1.51 (1.45)	1.37—1.58 (1.45)	1.46—1.62 (1.53)
Bulb length	0.24—0.27 (0.25)	0.29	0.20—0.22 (0.21)	0.18—0.22 (0.20)	0.21—0.22 (0.22)
Bulb width	0.25—0.27 (0.26)	0.32	0.24—0.25 (0.25)	0.22—0.25 (0.24)	0.22—0.25 (0.23)
Nerve ring from anterior end	0.48—0.60 (0.54)	0.65	0.42—0.47 (0.44)	0.38—0.42 (0.40)	0.42—0.46 (0.43)
Excretory pore from anterior end	1.24—1.56 (1.41)	1.34	0.79—0.98 (0.92)	0.83—1.02 (0.93)	0.93—1.01 (0.97)
Valva					
Distance from anterior end	6.15—9.70 (8.13)	8.15	4.3—5.5 (4.8)	4.8—5.7 (5.3)	6.6—7.4 (7.1)
Position[b]	0.56—0.60 (0.58)	0.62	0.61—0.63 (0.62)	0.60—0.66 (0.62)	0.58—0.60 (0.60)
Tail length	0.23—0.26 (0.27)	0.15	0.14—0.16 (0.15)	0.13—0.16 (0.14)	0.13—0.18 (0.16)
Egg length	0.068—0.080 (0.077)	0.080—0.084 (0.083)	0.076—0.084 (0.079)	0.072—0.084 (0.080)	0.068—0.080 (0.077)
Egg width	0.040—0.044 (0.041)	0.040—0.044 (0.043)	0.040—0.044 (0.041)	0.040—0.044 (0.041)	0.040

[a] Total in number (precloacal/adcloacal/ventrolateral/terminal). Total number and terminal number of papillae contain two phasmids

[b] Distance between anterior end and vulva/worm length

addition to *P. brevipes*, the country is also populated by *Pseudocalotes microlepis* (Boulenger 1887) found widely in Vietnam, southern China, Myanmar, Laos, Thailand, and India (Assam) as well as *Pseudocalotes ziegleri* (Hallermann et al. 2010) distributed only in the mountain rainforest of central Vietnam (Kon Tum Province) (Ananjeva et al. 2007; Hallermann et al. 2010). All these agamid lizards of the genus *Pseudocalotes* are rare, with most of them known from only a few specimens (Ananjeva et al. 2007; Hallermann et al. 2010). Consequently, to date, very few of their helminth faunas have been investigated.

According to Bursey et al. (2013), with the addition of *S. amazonicus* by Santos et al. (2013), 33 nominal *Strongyluris* spp. have currently been recorded worldwide. Specifically, six species have been described from the Oriental Region, namely, *S. chamaeleonis* (Baylis and Daubney 1922), *S. calotis* (Baylis and Daubney 1923), *S. bengalensis* (Chakravorty 1936), *S. karawirensis* (Karve 1938), *S. bufonis* (Yamaguti and Mitunaga 1943), and *S. japalurae* (Jiang and Lin 1980). Except for *S. bufonis*, the five other species were recorded from lizards. Baylis and Daubney (1923) described *S. calotis* concisely as a new species from the rectum of *Calotes*

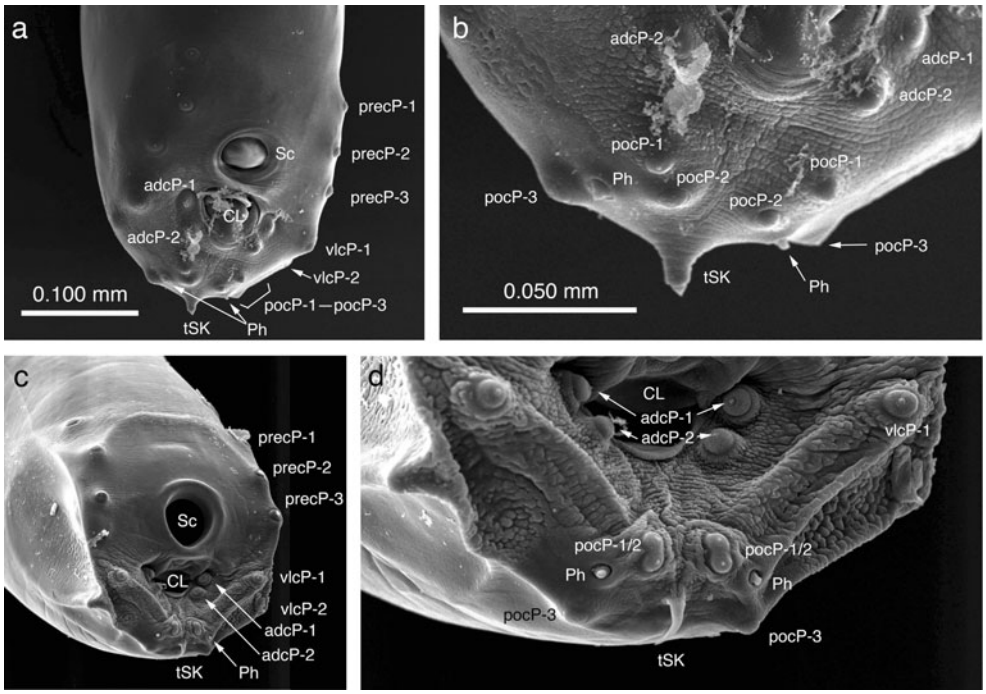

Fig. 3 SEM view of the posterior end of a male *Strongyluris calotis* worm. Specimen from *Pseudocalotes brevipes* in Vietnam (**a**, **b**) and from *Calotes emma* in Singapore (**c**, **d**). Photographs on the right (**b**, **d**) are three times higher magnification of a part of each photograph on the left (**a**, **c**), respectively. Photographs on the same side are at the same magnification and scales are shown in **a** and **b**. Abbreviations: *adcP*, adcloacal caudal papilla; *CL*, cloaca; *Ph*, phasmid; *pocP*, postcloacal caudal papilla around the terminal spike; *precP*, precloacal caudal papilla; *Sc*, precloacal sucker; *tSK*, terminal spike; and *vlcP*, ventrolateral caudal papilla around the cloaca. *pocP-1/2* denotes united papillae

nigrilabris in Sri Lanka and reported 10 pairs of caudal papillae in male worms—seven postcloacal and three at the sides of the precloacal sucker—without morphological drawings. As indicated to some extent by Soota and Chaturvedi (1971) who recorded *S. calotis* from a *Calotes* sp. in India, *S. chamaeleonis*, *S. bengalensis*, and *S. karawirensis*, all of which were described from *Calotes versicolor* in India (Baylis and Daubney 1922; Chakravorty 1936; Karve 1938), have the possibility to be congeners of *S. calotis*, although the author(s) for each species based their differentiation on morphological criteria referring to morphometric values and arrangements of caudal papillae in male worms. Difficulties with the light microscopic observation of caudal papillae, particularly those in the tail area around the terminal spike, may be an explanation for the current taxonomic complications. Although the descriptions of different *Strongyluris* spp. referred to morphometric differences as one of the critical points for species differentiation (see Table 2 of Tran et al. 2015b), *S. calotis* specimens with different origins examined in our studies (Tran et al. 2015b and the present study) also showed such variation, sometimes with no overlapping of some morphometric values (Table 2).

All previous records before Tran et al. (2015b) may have counted the number of caudal papillae without considering the presence of phasmids in the terminal region of male and female worms of *Strongyluris* spp. due to the difficulty of differentiating the phasmids from genuine caudal papillae by light microscopy. SEM observation of *S. calotis* specimens from different sources divided them into two morphotypes: classical *S. calotis* morphotype from agamid lizards at lower altitude with a pair of united papillae in the terminal area around the spike in male worms as observed by Tran et al. (2015b) and a new *S. calotis* morphotype from Vietnam false bloodsuckers living at high altitude in Vietnam without a pair of united papillae in the aforementioned part of male worms. As shown in the previous study (Tran et al. 2015b), *S. calotis* specimens collected in Japan (*Japalura polygonata*), Taiwan (*Japalura swinhonis*), and Singapore (*Calotes emma*) consistently demonstrated the same number and arrangement of caudal papillae in male worms beyond their remarkable morphometric variations. All of them had nine pairs of caudal papillae (three pairs of precloacal ones; two pairs of adcloacal ones; two pairs of ventrolateral ones around the level of the cloaca; and two pairs of terminal ones) with a pair of phasmids, and the first pair of terminal papillae was a structure of fused papillae. Similarly, a male *S. calotis* specimen from *Calotes emma* in the plain forest at low altitude in Vietnam showed the same morphological characteristics.

Since PCR amplification of any rDNA fragments was not possible on the *S. calotis* specimens collected in Japan and Taiwan in the previous study (Tran et al. 2015b), possibly due to a long duration of worm preservation for nearly 30 years, new specimens were collected from Ryukyu tree lizards in the southernmost part of Japan, close to Taiwan, for genetic analysis. Intriguingly, molecular genetic analyses based on the rDNA nucleotide sequences failed to divide the *S. calotis* isolates examined in the present study into two groups of different morphotypes. Genetic distances based on

Table 3 Nucleotide changes observed in the rDNA of *Strongyluris calotis* of different origins

Host	Locality	DDBJ/EMBL/GenBank accession no.	Nucleotide position where any changes in rDNA are observed[a]													
			18S	ITS-1												
			1474	77	89	152	179	191	213	301	310	383	389	392	408	451
Calotes emma	Bac Kan Province, Northern Vietnam	LC133186	A	T	A	T	C	C	A	C	T	G	A	A	A	C
Calotes emma	Singapore	LC133187	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Japalura polygonata ishigakiensis	Ishigaki Is., Okinawa, Japan	LC133188	·	·	T	·	·	T	G	·	A	A	G	·	·	·
Pseudocalotes brevipes	Phu Tho Province, Northern Vietnam	LC133189	G	G	·	C	T	T	·	T	A	·	G	C	G	T
Pseudocalotes brevipes	Bac Can Province, Northern Vietnam	LC133190	·	G	·	C	T	T	·	T	A	·	G	C	G	T

Host	Nucleotide position where any changes in rDNA are observed[a]																								
	ITS-2																			28S					
	46	49	50	51	58	125	187	188	241	266	284	343	357	378	383	387	412	418	448	530	594	662	765	1068	1187
Calotes emma	G	A	T	G	G	A	A	C	T	G	A	G	T	C	A	G	G	G	C	G	A	G	A	C	A
Calotes emma	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·							
Japalura polygonata ishigakiensis	T	G	C	A	T	·	G	T	·	·	G	A	C	·	G	A	·	·	·	A	T	·	A/G	·	C
Pseudocalotes brevipes	·	G	C	A	T	G	G	T	A	A	G	A	C	T	·	·	A	A	T	A	T	C	·	A	
Pseudocalotes brevipes	·	G	C	A	T	G	G	T	A	A	G	A	C	T	·	·	A	A	T	A	T	C	·	A	

[a] Nucleotide position is expressed relative to the rDNA sequence of *S. calotis* collected from an Emma Gray's forest lizard in Vietnam (DDBJ/EMBL/GenBank accession no. LC133186). Dots denote an identical base to that of *S. calotis* collected from an Emma Gray's forest lizard in Bac Kan Province, Vietnam, and blanks indicate no data

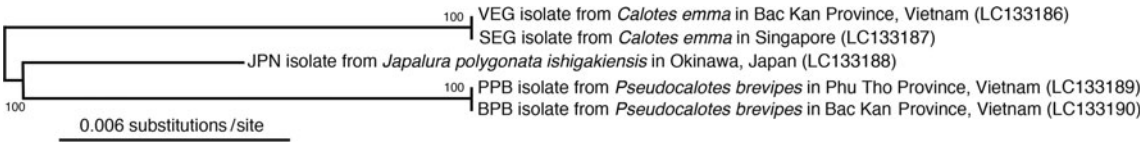

Fig. 4 Unrooted ML phylogenetic tree based on concatenated ITS-1 and ITS-2 nucleotide sequence of 892-bp length

the rDNA nucleotide sequences were almost equal among isolates from agamid lizards at low altitude in South East Asia (plain forest lizards in Vietnam and Singapore), isolates from agamid lizards at high altitude in Vietnam (mountainous forest lizards in Vietnam), and an isolate from plain forest lizards in Japan (Table 3 and Fig. 4).

As briefly discussed above, the taxonomic status of *Strongyluris* spp. in Oriental lizards and possibly lizards worldwide remains highly complicated. Inglis (1957) described the taxonomic state of *Strongyluris* spp. in the world as "being complicated by the description of virtually every sample collected as a new species so that almost the only specimens available for study are types of one kind or another." Today, technical difficulties with the microscopic observation of truncated posterior ends of male worms can be surmounted by using SEM. Additionally, as in the present study, DNA sequencing can assist the taxonomic differentiation of parasites. Sampling of *Strongyluris* spp. widely in the Oriental Region and consequent intensive SEM observation and DNA sequencing following routine light microscopic observation may resolve their current taxonomic complications.

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