ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Morphological and molecular description of *Armillifer moniliformis* larvae isolated from Sri Lankan brown palm civet (*Paradoxurus montanus*)

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Abstract

We report *Armillifer moniliformis* species infecting the endemic Sri Lankan brown palm civet (*Paradoxurus montanus*) from the Knuckles Range Forest Conservation Area, Sri Lanka. Larval stages of *A. moniliformis* were found during the postmortem of three civet cats found dead. Morphological studies were done by a light microscope and a scanning electron microscope (SEM). Histopathological examination was conducted using tissue samples obtained from the liver. For the molecular analysis, DNA was extracted from the isolated third-stage larvae. The NADH dehydrogenase subunit 5 (ND5) and the second internal transcribed spacer region (ITS-2), a portion of the large subunit nuclear ribosomal DNA (28S), a portion of 18S ribosomal rRNA gene (18S), and cytochrome c oxidase subunit 1 gene (COX1) were amplified using polymerase chain reaction (PCR). Excysted third-stage larvae were observed in the lungs, omentum, the pleural cavity, the abdominal cavity, and the surface of the spleen and the pericardium. Around 88 third-stage larvae were isolated from three civet cats. First-stage larvae in the liver were surrounded by outer fibrous layer over the inner germinal layer and filled with clear fluid. Slight hemorrhage, leukocyte infiltration, and mild hepatocellular degeneration in the liver were observed. The SEM examination indicated the unique oral apparatus comprises the oval-shaped mouth opening in between two pairs of curved, retractile hamuli. The sequences obtained for ND5, ITS-2, 28S, 18S, and COX1 were 301, 382, 325, 414, and 644 bp in length respectively. Morphology, sequence similarity search, sequence alignment, and phylogenetic analysis identified this parasite as *A. moniliformis*.

Keywords *Armillifer moniliformis* · Sri Lankan brown palm civet (*Paradoxurus montanus*) · ND5 · ITS-2 · 28S · 18S · COX1 · Sri Lanka

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Introduction

Pentastomids are parasites of reptiles and other vertebrates (Paré 2008). About 130 species of pentastomids have been identified thus far (de Oliveira Almeida and Christoffersen 1999). The systematic classification of Pentastomida has been previously discussed (Lavrov et al. 2004; Waloszek et al. 2005). Subclass Pentastomida is placed into the subphylum Crustacea in phylum Arthropoda (Richter et al. 2009; Chen et al. 2010). *Armillifer* species belong to the order Porocephalida and family Porocephalidae (Meyers and Neafie 2011).

The definitive host of the adult *Armillifer* is usually a snake (Riley 1986; Paré 2008). The most common intermediate host is a rodent (Self 1972; Anjos et al. 2007; Meyers and Neafie 2011). Male and female adult *Armillifer* attach to the respiratory epithelium of snakes using their two pairs of retractile hooks. Approximately 4 to 8 months later, impregnated

females deposit embryonated eggs. The eggs are carried by respiratory secretions to the snake's oral cavity from where they are either discharged or swallowed and excreted in feces (Riley 1986; Dakubo et al. 2008; Chen et al. 2010). Intermediate hosts get infected by the consumption of water or vegetation contaminated with *Armillifer* eggs (Chen et al. 2010).

In intermediate hosts, ingested eggs hatch, liberating the first-stage larvae into the digestive tract. The first-stage larvae may migrate by passing through the entire thickness of the gut wall and enter the abdominal cavity. Another way is to migrate hematogenously (Chitwood and Lichtenfels 1972; Field et al. 1991) or via bone perforations (Cho et al. 2017). At this stage, larvae lose their appendages and encyst in or on any organ. After several molts, the third-stage larvae excyst and migrate in the body. The infective third-stage larvae (nymphs) (Lavarde and Fornes 1999; Paré 2008) are morphologically similar to the adult but smaller in size (Meyers and Neafie 2011). The life cycle is maintained when a snake preys upon an infected intermediate host (Paré 2008). In the snake, infective third-stage larvae migrate from the stomach up to the esophagus and into the lungs, where they mature into adults and complete the life cycle. In accidental intermediate hosts, the ingestion of eggs results in visceral larval invasion (Meyers and Neafie 2011).

Several mammal species, including humans, become intermediate hosts accidentally (Steinbach and Johnstone 1957; Paramananthan 1962; Field et al. 1991; McCarthy and Moore 2000; Brookins et al. 2009). Pentastomiasis is among the earliest zoonotic human infections described (Meyers and Neafie 2011). Two families of the order Porocephalida, Porocephalidae, and Linguatulidae infect humans (Lavrov et al. 2004; Meyers and Neafie 2011).

There have been several reports of pentastomids in Sri Lanka (Fernando 1953; Fernando and Fernando 2014). Civet cats have been recorded as intermediate hosts (Paramananthan 1962). Palm civets are a group of small mammals in the order Carnivora. They are solitary, nocturnal animals and tend to be mainly arboreal (Joshi et al. 1995). Palm civet species are important seed dispersers (Nakashima et al. 2010). There are at least three endemic civet cat species in Sri Lanka: golden wet-zone palm civet (*Paradoxurus aureus*), Sri Lankan brown palm civet (*Paradoxurus montanus*), and golden dry-zone palm civet (*Paradoxurus stenocephalus*) (Groves et al. 2009).

In this study, we isolated the third-stage larvae of *Armillifer moniliformis* from the Sri Lankan brown palm civet (*Paradoxurus montanus*). Given the possibility of zoonotic transmission of pentastomids, identification and characterization of these parasites are important. Thus, we used molecular and morphological findings to give a detailed account of the *A. moniliformis* species isolated.

Materials and methods

Study site

This study focused on the Knuckles Range Forest Conservation Area. This mountain range is located at 7° 21′ N, 81° 45′ E in the Central Province of Sri Lanka. The range covers approximately 2.1×10^4 ha in the intermediate climate zone of the island (Cooray 1984) and includes at least 35 peaks rising above 900 m (Ekanayake and Bambaradeniya 2001). Three infected Sri Lankan brown palm civets were found dead in the Illukkumbura Forest area, Knuckles Range (Fig. 1) between the years 2010 and 2014.

Postmortem and sample collection

Postmortem examinations of the first two dead palm civets were performed in the field, and the presence of *Armillifer* larvae in the abdominal cavity was recorded. We were able to transport the third dead civet cat to the Department of Parasitology, Faculty of Medicine, University of Peradeniya and conduct the postmortem. Samples of the third-stage larvae and infected tissue samples were obtained for further investigation. Using five isolated third-stage larvae, the lengths of the cephalothorax, anterior widths, midpoint widths, widths of the

Fig. 1 Location in Sri Lanka where three dead palm civets were found

posterior region, and number of annulations were measured. Mean values of each parameter were calculated.

Staining of parasites

Fixation was done in the field by collecting the third-stage larvae into 70% ethanol. The method described by Garcia and Ash (1979) was used with modification for preserving and staining parasites. The specimens were flatted by placing them between slides secured with rubber bands. The specimens stored in 70% ethanol were transferred to distilled water through a graded series of ethanol with several changes in 70, 50, and 30% distilled water, respectively. The specimens were then stained with aceto-alum carmine. Overstained specimens were destained using acid alcohol. After washing the specimens with distilled water, they were dehydrated in an ascending series of ethanol concentrations (30, 50, 70, 80, 90%, absolute ethanol). The dehydrated specimens were placed in clove oil for clearing. The well-cleared specimens were mounted in DPX.

Histopathology

Parts of the liver were excised and fixed in 10% buffered formalin. Portions of the fixed livers were thoroughly washed with distilled water and were dehydrated through an ascending concentration series of ethanol from 25 to 100%, allowing sufficient time to dehydrate the tissues. These tissues were then immersed in xylene for 1 h and kept overnight in molten paraffin wax. They were then embedded in paraffin wax and sectioned with a rocking microtome (LR-85, Osaka, Japan) to obtain 8-μm-thick sections. The sections were mounted on glass slides coated with egg albumen and glycerin and allowed to dry at 37 °C. The sections were dewaxed with xylene and passed through a descending series of alcohol. The sections were then washed in distilled water and placed in hematoxylin for 10 min. Excess stain was removed by washing in distilled water, and the slides were passed through an ascending series of ethanol up to 70%. The sections were then immersed in eosin stain for 10 min, and excess stain was removed by placing them in 70% ethanol. The sections were then passed through 90% and absolute ethanol followed by xylene. Finally, the sections were mounted with a DPX medium and examined using light microscopy (RL 3002, Nikon, Japan) at × 100 and × 400.

Scanning electron microscopy

Ethanol-fixed (70%) parasite specimens were air-dried to evaporate ethanol. Then, each specimen was cut into three parts (anterior end, middle part, and posterior end). Each part was fixed separately on to a stub using a double-sided conducting carbon tape and the stubs loaded into a sputter coater (SC7620, Quorum Technologies, UK). Samples were coated using gold palladium for 60 s. Finally, the gold-coated samples were loaded into a scanning electron microscope (EVO/LS15, Carl Zeiss, Germany), and photos were taken at various magnifications.

Genomic DNA extraction

DNA was extracted from third-stage larvae preserved in absolute alcohol using a commercial kit (Wizard® Genomic DNA Purification Kit; Promega Corporation, USA) following the manufacturer's protocol. The extracted DNA from each sample was eluted separately with DNA rehydration solution for a 100 μl final volume.

PCR amplification and DNA sequencing

The polymerase chain reaction (PCR) was performed in a thermal cycler (Nyx Technik, Amplytronix series, USA). The amplified regions, the PCR profiles, and the primers are given in Table 1. Each reaction had a total volume of 25 μl containing a 10x PCR buffer, 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate (dNTP), 10-pmol primers, and Taq DNA polymerase (5U/μl). Electrophoresis of 5 μl of PCR product through a 1.5% agarose gel was carried out to confirm successful amplification and for a rough quantification. The amplified DNA was subsequently purified using the Invitrogen™ PureLink™ Quick Gel Extraction and PCR Purification Combo Kit. The purified PCR products were subjected to direct DNA sequencing at Macrogen Corporation, Korea and Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, using PCR primers as sequencing primers.

Sequence alignment and phylogenetic analysis

Sequences were edited manually in BioEdit version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence similarity search was done using the NCBI BLAST (www.ncbi.nlm.nih.gov/blast) search program. The sequences obtained for the ND5, ITS-2, COX1, and 28S regions were not used for sequence alignment because there were inadequate number of reference sequences available in the GenBank for phylogenetic analysis. For the 18S sequence, multiple sequence alignment was obtained with default gap penalties in the ClustalW v2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignment contained 13 sequences including 12 reference nucleotide sequences available in the NCBI GenBank (Acc. no: HM048870, HM756289, KJ877183, KJ877184, KM023155, FJ607339, MG559612, MG559607, JX088397, KF029439, MK103080, KF543342). This alignment was subsequently used for the phylogenetic analysis.

Phylogenetic analysis was performed to understand the relationships of *Armillifer* species identified in this study with other

Table 1 List of primers and profiles used for PCR amplification and sequencing

Amplified region	Primers	PCR conditions	Reference
NADH dehydrogenase subunit 5 (ND5)	F: ASL forward (5′-TTA CTC CAA CCA AAG GTA TA -3′) R: ASL reverse (5′-TCG TCG ACT CTT GTG ACC TC-3′)	94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, a final extension of 72 °C for 5 min	Present study
Second internal transcribed spacer region (ITS-2)	F: 3S (5′-CGG TGG ATC ACT CGG CTC GT-3′) R: 28A (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′)	94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, a final extension of 72 °C for 5 min	Bowles et al. 1995
Large subunit nuclear ribosomal DNA (partial) (28S)	F: LSU5 (5′-ACC CGC TGA ATT TAA GCA-3′) R: LSU3 (5′-TCC TGA GGG AAA CTT CGG-3′)	94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, a final extension of 72 °C for 5 min	Littlewood and Johnston 1995
18S rRNA gene (partial) (18S)	F: Pent629F (5′-CGG TTA AAA AGC TCG TAG TTG G-3′) R: Pent629R (5′-GGC ATC GTT TAT GGT TAG AAC TAG GG-3′)	94 °C for 12 min, 10 cycles at 94 °C for 40 s, touchdown 62–52 °C for 40 s, increment − 1 °C, 72 °C for 1 min, 30 cycles at 94 °C for 40 s, 52 °C for 40 s, 72 °C for 1 min final extension of 72 °C for 5 min	Tappe et al. 2011
Cytochrome c oxidase subunit 1 (COX 1)	F: LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) R: HCO-2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT C-3′)	94 °C for 15 min, 45 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, a final extension of 72 °C for 5 min	Folmer et al. 1994

selected pentastomids. Maximum likelihood (ML) tree was obtained using the RAxML 8.2.10 (Stamatakis, 2014) in the Cyber Infrastructure for Phylogenetic Research project (CIPRES) Science Gateway v.3.3 (https://www.phylo.org/). Model of evolution was obtained using jModelTest v.2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012), which was the general time reversible model of sequence evolution with gamma-distributed rate variation with a proportion of invariant sites (GTR + I + G). Rapid bootstrapping algorithm was implemented with 100 bootstrap replicates in the RAxML version; GTR and the CAT approximation of rate heterogeneity were applied. The tree was rooted with *Fasciola gigantica* (KF543342). Then, the phylogenetic tree was visualized and edited in FigTree v.1.4. 3 (http://tree.bio.ed.ac.uk/software/figtree/).

Bayesian inference as implemented in MrBayes (Huelsenbeck and Ronquist 2011), with the model as GTR + I + G, was done. Four Markov Chain Monte Carlo (MCMC) chains were run for one million generations. They were applied as three heated chains and one cold chain. The four chains reached burn-in time by 250,000 generations. The frequency of clades in trees was sampled for every 100 generations.

Results

Postmortem and morphological examination

Excysted third-stage larvae were observed in the omentum, the pleural cavity, the abdominal cavity, and the surface of the spleen and the pericardium. Samples of the third-stage larvae (Fig. 2) were taken from the surface of the spleen and the pericardium. Around 88 third-stage larvae were isolated from three civet cats (36, 24, and 28 respectively).

The mean length of five third-stage larvae was 10.97 ± 0.49 mm (10.45–11.76 mm). The mean length of the cephalothorax was 1.09 ± 0.09 mm (1.00–1.04 mm). The anterior width of the pseudo-annulated body ranged from 1.08 to 1.35 mm with a mean value of 1.14 ± 0.11 mm. The mean midpoint width of the body was 1.24 ± 0.06 mm (1.2–1.36 mm). The mean width of the posterior region of the body was 0.93 ± 0.16 mm (0.82–1.08 mm). Number of annulations ranged from 27 to 31 with a mean value of 28.0 ± 1.4.

Histopathology

The first-stage larvae were encysted with an outer fibrous layer over the inner germinal layer and filled with clear fluid (Fig. 3). Histologically, slight hemorrhage, leukocyte infiltration, and mild hepatocellular degeneration in the liver were noticed. The adjacent hepatic parenchyma showed atrophy, variable degeneration, and infiltration. The parenchyma adjacent to the cysts was markedly congested and showed multiple small hemorrhagic areas.

SEM examination

The cephalothorax of the third-stage larva was crowned with the oral apparatus (Fig. 4a). The oval-shaped mouth

Fig. 2 **a** Third-stage *A. moniliformis* larvae on the spleen (s), and pericardium (p) of the infected palm civet. **b** Isolated third-stage *A. moniliformis* larvae

opening was positioned ventrally with two curved, retractile hamuli on either side of it (Fig. 4b). Each hamulus was placed in a hamulus pit. The cuticle was penetrated by the openings of the ducts of subcuticular glands (Tappe and Büttner 2009), which appear as small, circular, ring-like structures (Fig. 4c). The distance between any two openings ranged from 24.32 to 31.35 μm. The surface of the pseudo-annulated, elongated abdomen was finely corrugated (Fig. 4d). Annulations ranged in width from 222.8 to 246.1 μm. The posterior end of the body was tapered (Fig. 4e). The excretory pore (Fig. 4f: a) was located terminally, behind the gonopore (Fig. 4f: b).
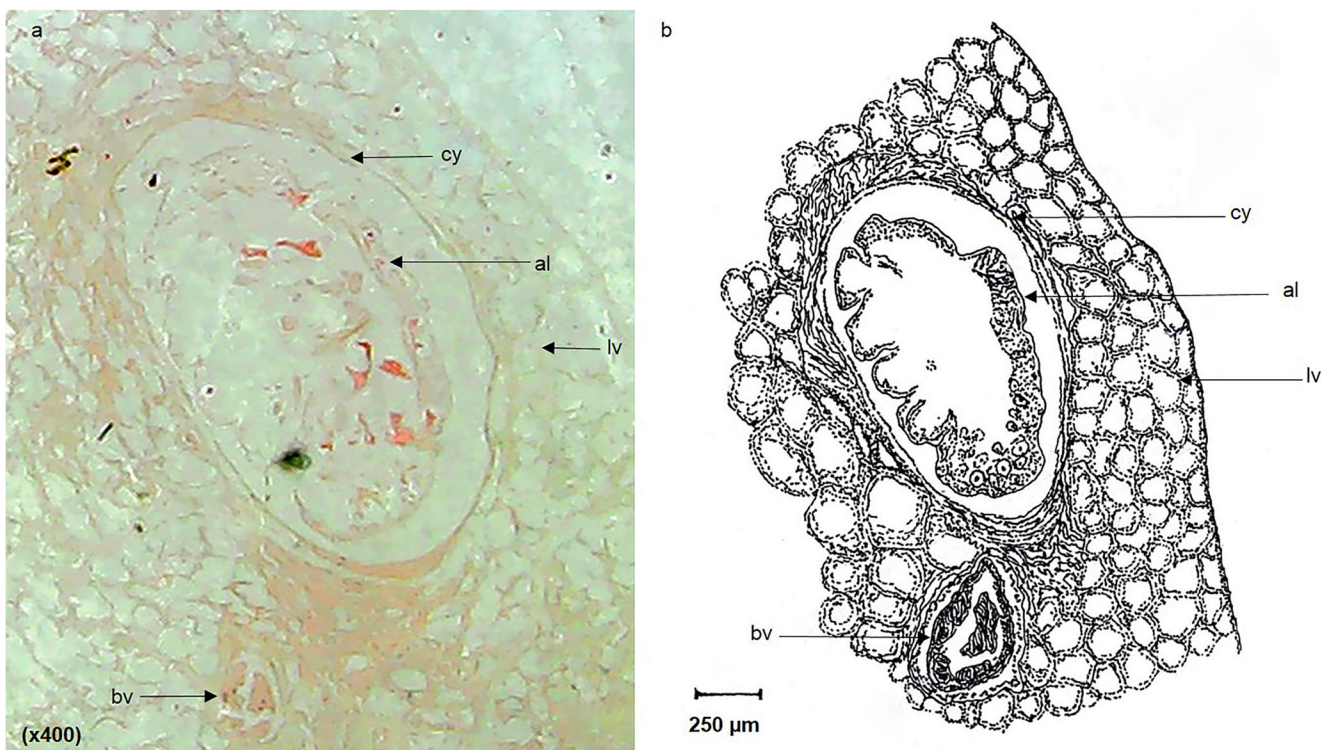
Fig. 3 Microscopic view (**a**) and line drawing (**b**) of the encysted first-stage *A. moniliformis* larva. cy outer fibrous layer of the cyst, al the encysted first-stage larva, lv palm civet's liver tissue, bl blood vessel
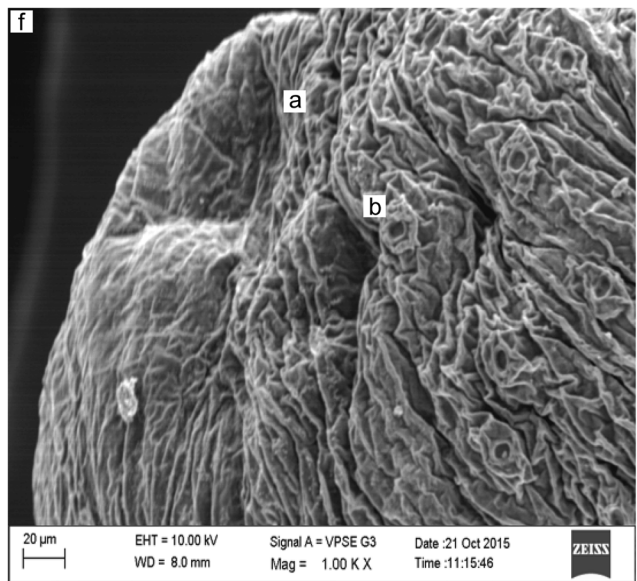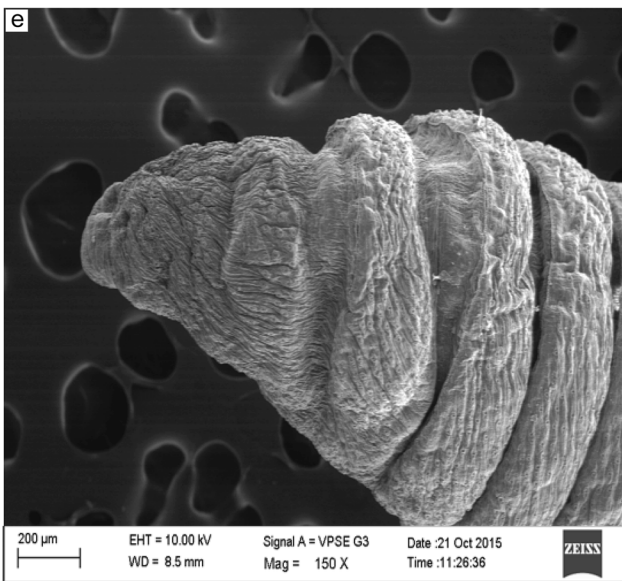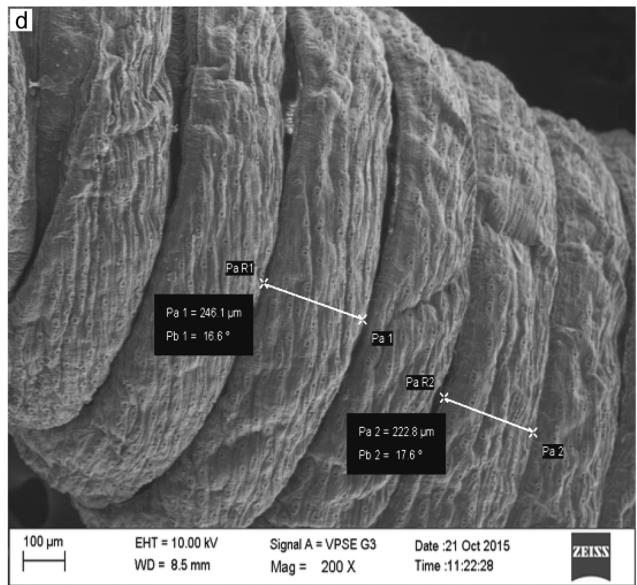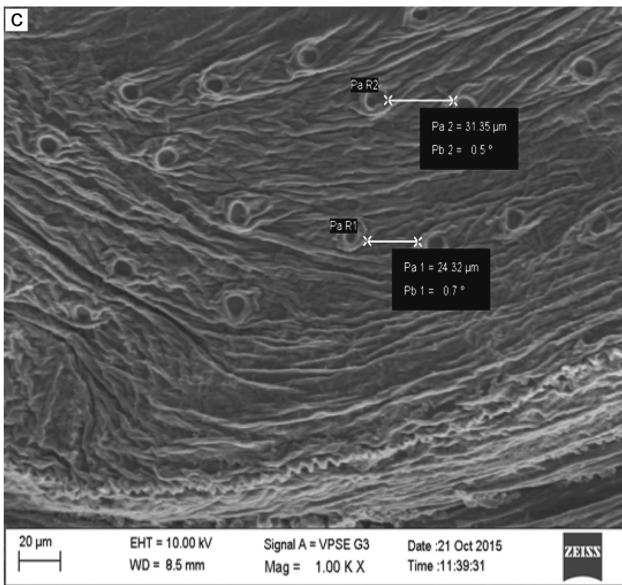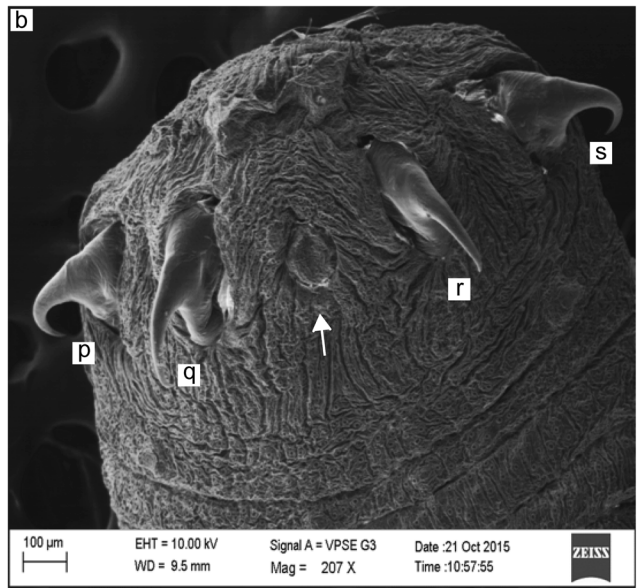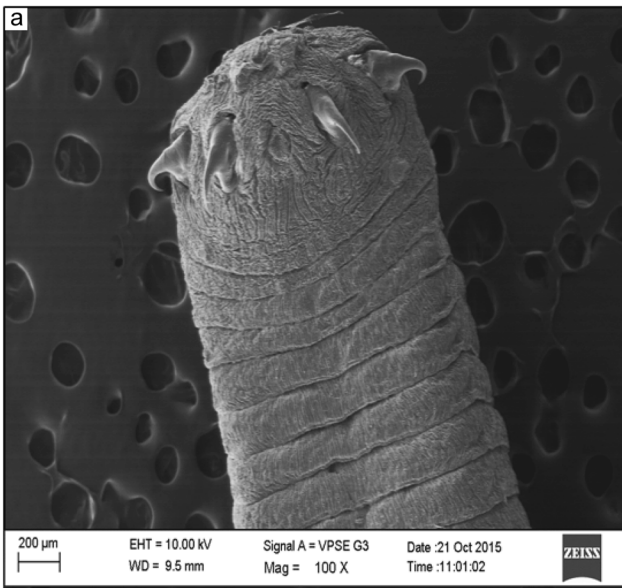
◀ **Fig. 4** SEM photographs of third-stage *A. moniliformis* larvae. **a** Position of the oral apparatus in the cephalothorax. **b** The oval-shaped mouth (arrowhead) located between two pairs of curved, retractile hamuli (p, q, r, s). **c** Ring-like cuticle openings of the ducts of subcuticular glands. Distances between two adjacent openings are shown. **d** Corrugated surface of the pseudo-annulated abdomen. Widths of two annuli are shown **e** Tapered posterior end of the body. **f** Terminally positioned excretory pore (a) and the gonopore (b)

PCR amplification and DNA sequencing

ND5 sequence

The sequence obtained for the ND5 region using ASL forward and ASL reverse primers was 301 bp in length.

ITS-2 sequence

The length of the sequence obtained for the ITS-2 region using 3S and 28A primers was 382 bp.

28S sequence

A 325-bp length sequence was obtained for the 28S region using LSU5 and LSU3 primers.

COX1 sequence

A 644-bp sequence was obtained for the COX1 region using LCO1490 and HCO2198 primers.

18S sequence

A 414-bp length sequence was obtained for the portion of 18S rRNA gene using Pent629F and Pent629R primers.

The nucleotide sequence data reported for 28S, ITS-2, ND5, COX1, and 18S in this paper are available in the NCBI GenBank database under the accession numbers MK089920, MK063888, MK 063889, MN531845, and MN524183 respectively.

Sequence alignment and phylogenetic analysis

The NCBI BLAST sequence similarity search for the 18S sequence (partial) showed 100% similarity with *A. moniliformis*. The ML tree and the Bayesian inference had a similar tree topology. Therefore, in Fig. 5, the ML tree is given with the rapid bootstrap values and posterior probability values. The ML tree of the 18S sequence alignment contains a total of 13 taxa (Fig. 5). The analyzed sample was identified as *A. moniliformis* by the sequence similarity search, sequence alignment, and phylogenetic analysis.

Discussion

There is little information regarding *Armillifer* spp. in Sri Lanka. In this study, we have provided details of the morphology of the larval stages, molecular characteristics, and phylogenetics of *A. moniliformis* isolated from the endemic Sri Lankan brown palm civet. Morphological features of *Armillifer* spp. nymphs described during several previous cases (Chen et al. 2010; Meyers and Neafie 2011; Tappe et al. 2013), such as the unique oral apparatus, were observed in *A. moniliformis* species identified in this study (Fig. 4). Encysted first-stage larvae (Du Plessis et al. 2007; Paré 2008) were detected during the histopathological examination.

Molecular phylogenetics showed the phylogenetic relationship of the isolated *A. moniliformis* with other pentastomes. In the ML tree, all the *Armillifer* species formed a separate clade from other pentastomes. Furthermore, *A. moniliformis* formed a monophyletic clade with *A. agkistrodontis*. Also, *A. moniliformis* formed a polyphyletic group with *P. crotali*, which is another pentastome in the order Porocephalida that infects snakes. Pentastomida species also infect birds, mammals, reptiles, and fish (Fig. 5). The bird parasite *Raillietiella* sp. is in the order Cephalobaenida. *Linguatula* spp. that mainly infect dogs and other mammals, and *S. mississippiensis* in fish and alligators belong to the order Porocephalida (Tappe 2011; Poore 2012; Woodyard et al. 2019).

In Sri Lanka, a previous study has identified *A. moniliformis* from civet cats (*Viverricula indica mayori*) solely based on morphological analysis of the nymphs (Paramananthan 1962). However, this host species (*Viverricula indica mayori*) is not endemic to Sri Lanka. In the present study, we identified an endemic palm civet species (*Paradoxurus montanus*) as a mammal intermediate host for *A. moniliformis*. This parasite might be the cause of death of the palm civets found. Further studies should be carried out to verify that opinion. *Armillifer* species could be a serious threat for the endemic and native palm civet species in Sri Lanka. The real extent of this parasitic infection in the wild and its effect on biodiversity is yet to be revealed. Further studies are required to identify other possible intermediate hosts.

There has been an increasing number of documented human infections caused by *Armillifer* species in many countries (Meyers and Neafie 2011; Tappe et al. 2014; Tappe et al. 2016; Vanhecke et al. 2016; Beltrame et al. 2017; Cho et al. 2017). In 2011, a study identified human pentastomiasis in Malaysian Borneo caused by *A. moniliformis* (Latif et al. 2011). Human visceral pentastomiasis is a zoonosis transmitted to humans by handling or eating undercooked snakes as practiced in certain cultures and by drinking contaminated water (Beltrame et al. 2017). The major pathology induced by these parasites is tissue damage at the site of their attachment to the host (Paré 2008). In Sri Lanka, human visceral
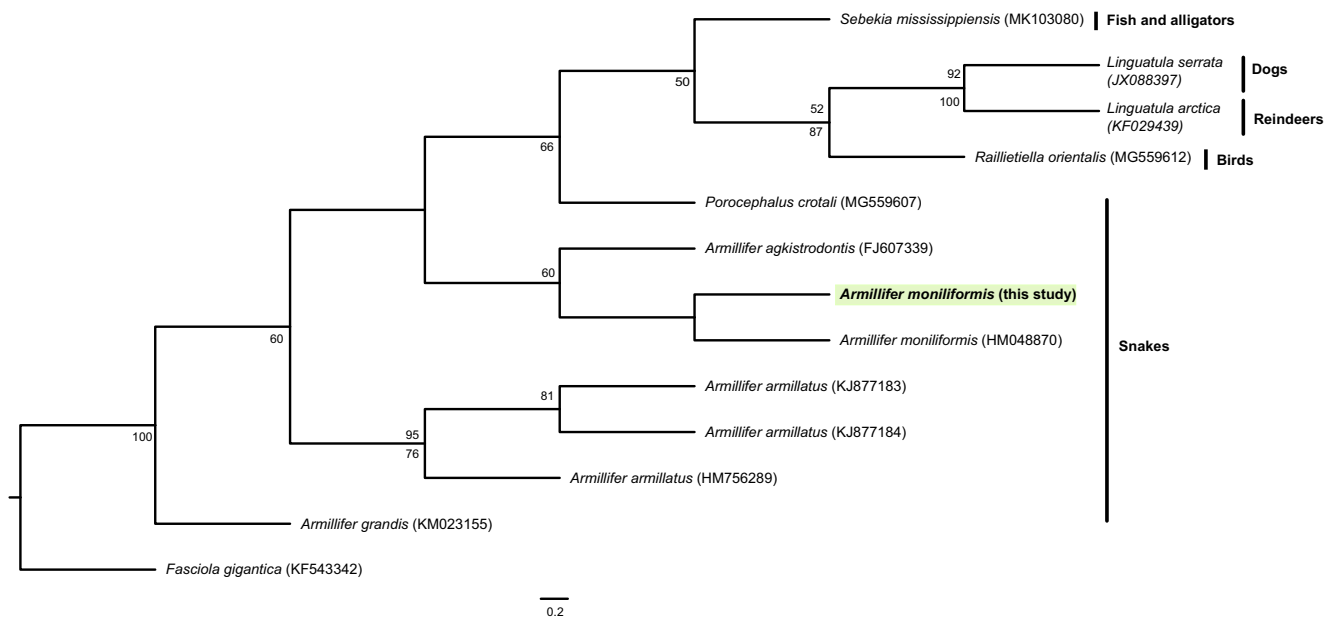
Fig. 5 Maximum likelihood tree obtained for the pentastomid species using partial sequence of 18S rRNA gene. Numbers above and below the nodes indicate rapid bootstrap values (> 50) and Bayesian posterior probabilities respectively. Scale bar represents 0.2 nucleotide divergence. The *A. moniliformis* identified in this study is highlighted. The relevant host species of the pentastome is given with the clade

pentastomiasis has not yet been reported. However, certain snakes and intermediate host species commonly occur in the vicinity of human habitation (Fernando and Fernando 2014). Therefore, water and vegetation can be contaminated in these areas. Hence, the zoonotic potential of this parasite in Sri Lanka should not be overlooked.

Conclusions

We have provided details on the larval stages of the *A. moniliformis* found in the Sri Lankan brown palm civet (*Paradoxurus montanus*). The species was identified by examining the morphology of the larvae, the sequence similarity search, the alignment of the partial sequence of the 18S rRNA gene, and the phylogenetic analysis. This study identified Sri Lankan brown palm civet as a potential accidental or intermediate host of *A. moniliformis* species.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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