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Molecular characterization of *Fasciola jacksoni* from wild elephants (*Elephas maximus maximus*) of Sri Lanka: a taxonomic evaluation

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Research Article

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Abstract

Fasciola jacksoni is a significant contributor to the health and mortality of Asian elephants, particularly those in Sri Lanka. Despite the impact of fascioliasis on elephant populations, it is a neglected veterinary disease with limited taxonomic understanding. Molecular characterization and phylogenetic analysis of *F. jacksoni* were carried out to evaluate its suggested basal position in the Fasciolidae. Adult worms were collected during post-mortem of elephants, and eggs were collected from living elephants in National parks across Sri Lanka. Using the mitochondrial genes nicotinamide dehydrogenase subunit 1 (*nad*1) and cytochrome oxidase subunit 1 (*cox*1), and a partial 28S ribosomal DNA (28S rDNA), DNA sequences were generated from the *F. jacksoni* adult and egg material. Maximum likelihood (ML) phylogenetic analyses did not resolve *F. jacksoni* to be basal to the Fasciolidae. Furthermore, the ML analyses showed that the genus *Fasciola* was not monophyletic and that *F. jacksoni* was a sister species to the deer liver fluke *Fascioloides magna*. A clear framework is required to determine the taxonomic status of *F. jacksoni* and this current study provides the first detailed application of molecular techniques from multiple hosts across Sri Lanka with the production of reference DNA sequences for this important parasite.

Introduction

Fasciola jacksoni (Cobbold, 1869) is one of four recognized species within the genus *Fasciola* Linnaeus, 1758 (Cobbold, 1882; Mas-Coma *et al*., 2009) and a major health problem for elephants in Sri Lanka, causing fatal fascioliasis in wild elephant populations. The size of the natural populations of Sri Lankan Asian elephants (*Elephas maximus maximus*) is estimated to be 2500–3000 individuals; approximately 15% of the total number of elephants found across Asia. Sri Lankan elephant herds vary in size with some made up of a few adults, juveniles and calves through to those composed of several hundred individuals in the Forest Reserves, Sanctuaries, National Parks and Orphanages distributed across the country. However, elephants are one of the most endangered large animal species in the world, in part associated with their continued long-term conflict with people for land. In Sri Lanka there is intense human-wild elephant conflict (HEC), resulting in the death of an elephant every 2 days and a human death every week (Fernando *et al*., 2005). Each year elephants destroy human properties and crops valued at millions of rupees leaving thousands of people living in financial uncertainty and insecurity (Perera, Rajapakse, 2009; Lorimer, 2010).

Owing to continued HEC elephant numbers have dramatically decreased due to an ever increasing lack of space, as well as infectious diseases (Fernando, 2000; Lorimer, 2010; Perera *et al*., 2015). Consequently, emerging parasitic diseases, including *F. jacksoni* are now major factors contributed to the dramatic decline of elephant populations (Bhalerao, 1933; Alahakoon, 1994; Perera *et al*., 2015). Such flukes contribute to the high burden of gastrointestinal parasitism in elephants, which has led to high mortality and morbidity of calves and adults alike across Sri Lanka (Alahakoon, 1994). Despite *F. jacksoni* being one of the major risk factors contributing to the decrease of wild Asian elephants, the parasite is completely neglected, regardless of continued reports of infection in India, Sri Lanka and Malaysia causing severe pathology (Windsor and Scott, 1976; Caple *et al*., 1978; Alahakoon, 1994; Islam, 1997; Perera and Rajapakse, 2009; Hing *et al*., 2013).

The systematics of *Fasciola* and the Fasciolidae has been fiercely debated over the past decade with the majority of studies depending on tenuous and plastic morphological characteristics of both adult flukes and eggs, which has ultimately affected the accuracy of identification and diagnosis. Molecular phylogenetic approaches have been employed to

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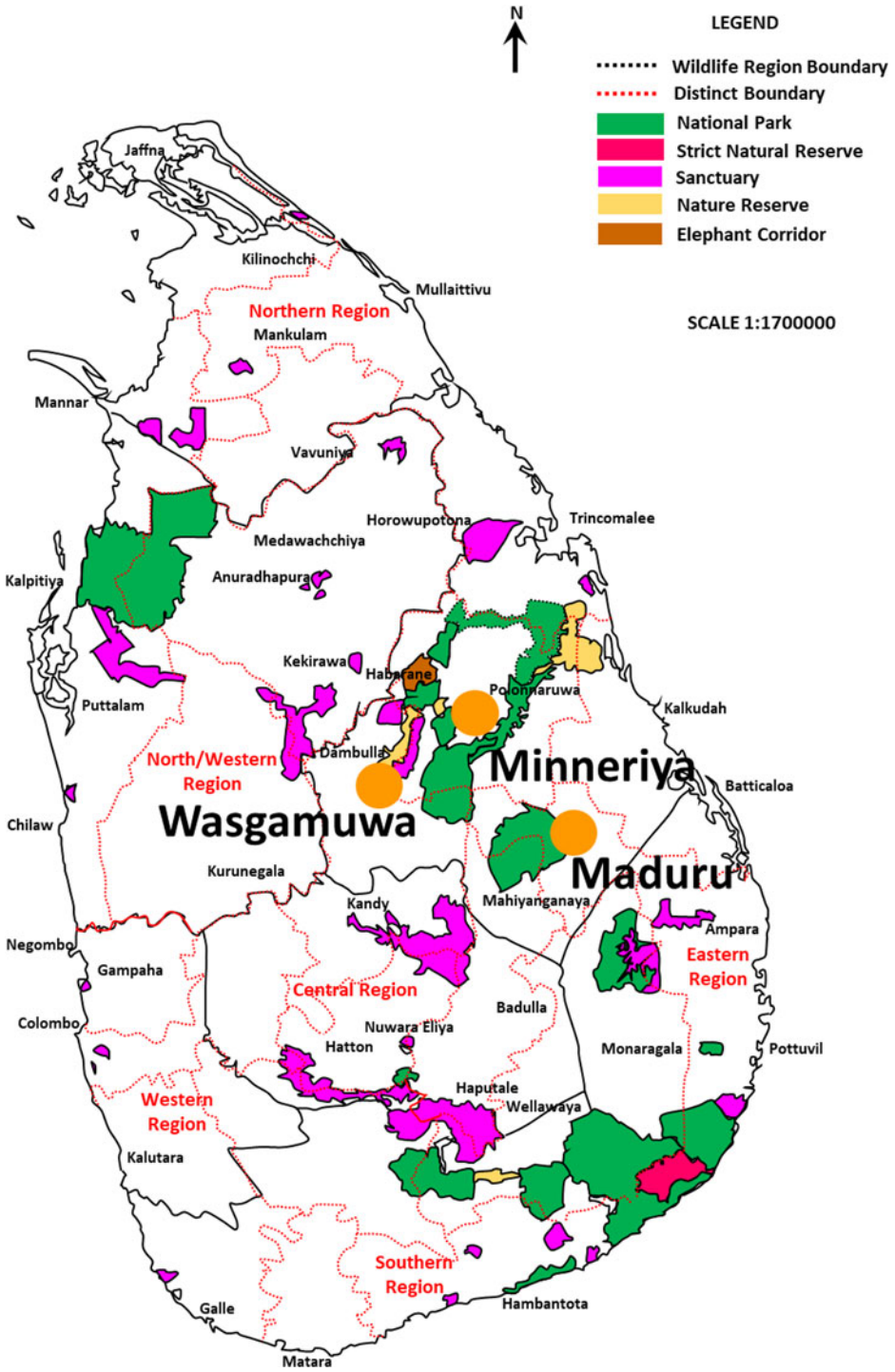

Fig. 1. Schematic map of living elephants in Sri Lanka and the study localities of National Parks where *Fasciola jacksoni* samples were collected (indicated by solid circles).

resolve the relationships between members of the Fasciolidae illustrating the complex taxonomic associations between members of the genus *Fasciola* and their association with the genus *Fascioloides* (Nolan and Cribb, 2005; Wey-Fabrizius *et al.*, 2013; Tkach *et al.*, 2016; Le *et al.*, 2017). Recently, such approaches have suggested that *F. jacksoni* should be moved to the genus *Fascioloides* and thus renamed as *Fascioloides jacksoni* (Lotfy *et al.*, 2008; Heneberg, 2013). Phylogenetic analyses of the Fasciolidae family have been dependant on the mitochondrial markers cytochrome oxidase subunit 1 gene (*cox1*) and the NADH dehydrogenase subunit 1 gene (*nad1*) as well as the nuclear ribosomal transcription units particularly ITS1, ITS2, 18S rDNA and partial 28S rDNA. These genetic markers have greatly contributed to molecular identification, diagnostic, epidemiological, phylogenetic and evolutionary studies of the Fasciolidae, especially the highly pathogenic *Fasciola hepatica* and *Fasciola gigantica*, the invasive *Fascioloides magna*, and the neglected but re-emerging *Fasciolopsis buski* (Králová-Hromadová *et al.*, 2008; Lotfy *et al.*, 2008; Heneberg, 2013; Mucheka *et al.*, 2015). Yet, despite their economic importance and the impact of fascioliasis on the health of wild elephant populations to date, molecular data associated with *F. jacksoni* is limited (Lotfy *et al.*, 2008; Singh *et al.*, 1994; Perera and Rajapakse, 2009; Heneberg, 2013).

Thus, we present one of the first detailed applications of molecular approaches for the identification and phylogenetic analysis of *F. jacksoni* from wild elephants in Sri Lanka. This study was part of a larger epidemiological survey of *F. jacksoni* infection

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Table 1. Information of geographical localities with estimated living elephants for *Fasciola jacksoni* infection survey

Geographical localities	Provinces/areas	Estimated living elephants[b,c]	Geographical coordinates
Kaudulla national park	North central	++	8.161111 N, 80.905 E
Maduru ova national park[a]	Eastern/Uva	+++	7.575833 N, 81.142778 E
Minneriva national park[a]	North central	+++	7.978889 N, 80.848889 E
Somawathiva national park	North central	+++	8.120833 N, 81.168889 E
Udawalawe national park	Sabaragamuwa/Uva	+++	6.438333 N, 80.888333 E
Wasgamuwa national park[a]	North central/Central	++	7.716667 N, 80.933333 E
Yala national park	Southern/Uva	++	6.372778 N, 81.516944 E
Lunugamwehera national park	Southern	++	6.383333 N, 81.233333 E

[a]Geolocalities where *Fasciola jacksoni* samples (adult and eggs) were collected.
[b]Scientific name: *Elephas maximus maximus*.
[c]Estimated numbers of living elephants in the area: +: <50; ++: 50–250; +++: >250 elephants based on the information in public media and statistics (see the map of living areas for elephants in Fig. 1), geographical coordinates provided in latitude and longitude.

in National Parks in Sri Lanka and aimed to address the taxonomic position of *F. jacksoni* species in the Fasciolidae and ultimately the Trematoda.

Materials and methods

Post-mortem pathological examination was performed on a total of 47 elephants during a large-scale epidemiological study carried out from January 2000 to April 2003 (39 months) across six districts of Sri Lanka (Fig. 1; Table 1) (Perera and Rajapakse, 2009). The liver was opened and separated with scissors, and the bile ducts were examined for the presence of adult *Fasciola*. Flukes were collected from the infected animals, washed with saline several times and fixed with 70% ethanol. Morphological species identification was performed by microscopy after adult flukes were stained with carmine (Fig. 2). Total worm burden within the liver was used to assess the intensity of the parasitic infestation in the affected animals (Perera and Rajapakse, 2009). In addition, fecal samples from 48 living elephants were also collected randomly from the Maduruoya, Minneriya and Wasgamuwa National Parks (see map in Fig. 1), and *Fasciola* eggs were harvested for identification by filtration and examined using standard microscopy and fecal egg count techniques. Eggs were washed and centrifuged several times in normal saline (0.9% NaCl), then a further three times in phosphate buffered saline before storage at −20 °C until use. A total of three to four adult worms from each post-mortem elephant from each locality were individually fixed in 70% molecular grade ethanol. One ethanol-fixed worm and eggs samples from the selected localities were separately subjected to genomic DNA extraction and molecular analysis (Table 1).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from approximately 10 mg lateral tissue from individual adult flukes or pooled eggs (about 1000 eggs) using the GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., MA, USA), according to the manufacturer's instructions. Genomic DNA was eluted into 50 μL of the elution buffer provided in the kit and stored at −20 °C. Complete gene fragments of both the mitochondrial markers *cox1*, 1.9 kb and *nad1*, 1.2 kb, where amplified using novel primers designed during this current study (Table 2). These were coupled with the amplification of a 1.4 kb portion of 28S ribosomal DNA and additional internal primers were designed and used for sequencing (see Le *et al.*, 2017) (Table 2). The PCR reactions were performed in a 50 μL volume containing 25 μL of DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc.) and 2 μL DNA template (50 ng μL^{-1}), 2 μL of each primer (10 pmol μL^{-1}), 2 μL DMSO (dimethyl sulfoxide) and 17 μL H_2O. All PCRs were performed in an MJ PTC-100 thermal cycler with the following cycling conditions: initiation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94 °C, annealing for 30 s (at 52 °C for *nad1* and *cox1*; at 56 °C for 28S rDNA), extension at 72 °C for 3 min; and a final extension at 72 °C for 7 min. The PCR products (10 μL of each) were examined on a 1% agarose gel, stained with ethidium bromide and visualized under UV light (Wealtec, Sparks, NV, USA). All the purified or gel-extracted PCR amplicons were sent for sequencing (Macrogen Inc., South Korea) using amplifying/flanking and internal primers (Table 2) in both directions. All sequences obtained from adult or eggs of *F. jacksoni* samples were identical, regardless of the adult or eggs stage or locality.

Sequence analyses and phylogenetic reconstruction

An alignment of 21 concatenated *nad1* and *cox1* DNA sequences, approximately 2439–2457 nucleotides in length, containing representations of trematode isolates from the Fasciolidae including *F. jacksoni* from three localities, the Echinostomatidae and *Schistosoma haematobium* as the outgroup (Supplementary Table S1) was constructed using GENEDOC 2.7 (available at: http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). The final alignment was 2470 nucleotide (nt) long of which 21 nt positions were trimmed from either end, leaving 2449 characters for analyses. Edited alignments were used to perform phylogenetic reconstructions using the maximum likelihood (ML) method in MEGA 7 (Kumar *et al.*, 2016). Pairwise distance analysis, was also performed on the concatenated *nad1* and *cox1* alignment as a measure of genetic distances (*p*-distance) between 12 representative species across three families of the Echinostomatoidea (Fasciolidae, Echinochamidae, Echinostomatidae) (Supplementary Table S1). DNA sequences of 28S rRNA genes (listed in Supplementary Table S2) were also aligned using GENEDOC 2.7. The resultant alignment of 1349 nucleotide long was edited by the removing 282 nt positions from both ends leaving to a total of 1067 characters for analyses. The final 28S rDNA alignment was composed of 43 species/isolates and MEGA 7 was again used to perform ML phylogenetic reconstruction construction (Kumar *et al.*, 2016). For both alignments, MEGA 7 identified the general time reversible GTR + G + I model (γ rate heterogeneity and a proportion of invariant sites) as the most appropriate model for phylogenetic reconstruction based on the lowest Bayesian information criterion score. Thus phylogenetic analyses for both alignments were performed under

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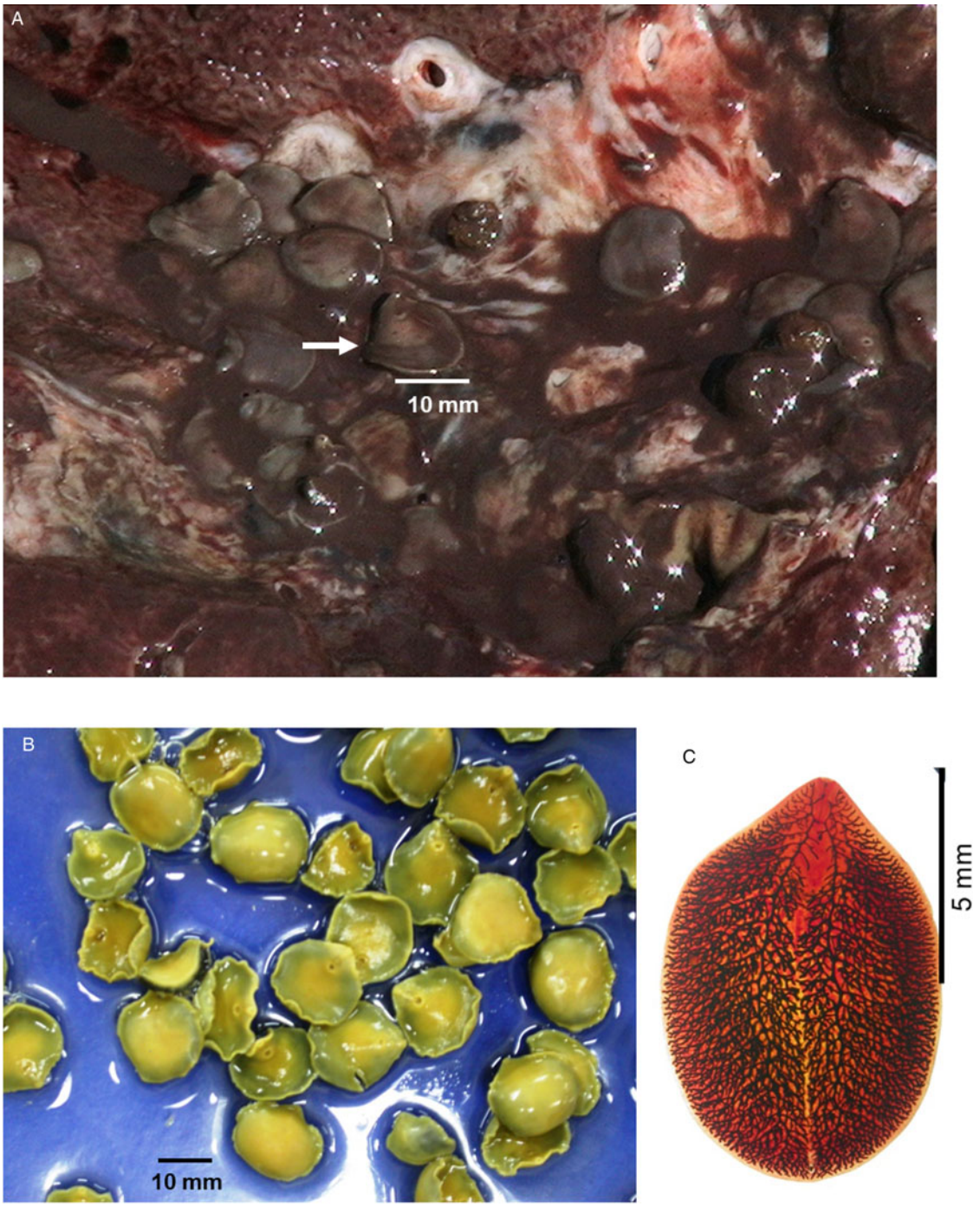

Fig. 2. Adult *Fasciola jacksoni* collected from elephants. Where (A) are adult *Fasciola jacksoni* flukes (indicated by arrow) in the bile duct of the infected liver in a dead elephant. Bar (10 mm) indicates the size of the flukes; (B) are adult flukes collected from bile duct of the liver from a post-mortem elephant in Sri Lanka and illustrate gross morphology; (C) an adult *F. jacksoni* fluke stained (5X) with carmine. Bars indicate the size of the flukes. Bars (100 and 5 mm) indicate the size of the flukes.

Table 2. Primers for amplification and sequencing of the mitochondrial protein-coding and nuclear ribosomal genes used in this study

Primer name	Sequence (5′–3′)	Target gene	Amplicon by PCR	Length of Sequence used	Reference
FJND1F	CATTGCGAGGACGGTGTAGT	*nad*1	1.2 kb	903 bp	This study
FJND1R	AATACCGTACACGGGCAACA				
FJCO1F	CGGGGGTATGTTCGTTGGAG	*cox*1	1.9 kb	1,554 bp	This study
FJCO1R2[a]	AAGTGAGCCACCACAAACCA				
FJCO1R	ATCAGTATCCTTCGGATACCCC				
U28SF	CTAACAAGGATTCCCTTAGTAAC	28S	1.3 kb	~1100 bp	Le *et al*., 2017
U28SR	GTCTTTCGCCCCTATACTCAC				

F, forward; R, reverse.
[a]Internal primer used for sequencing.

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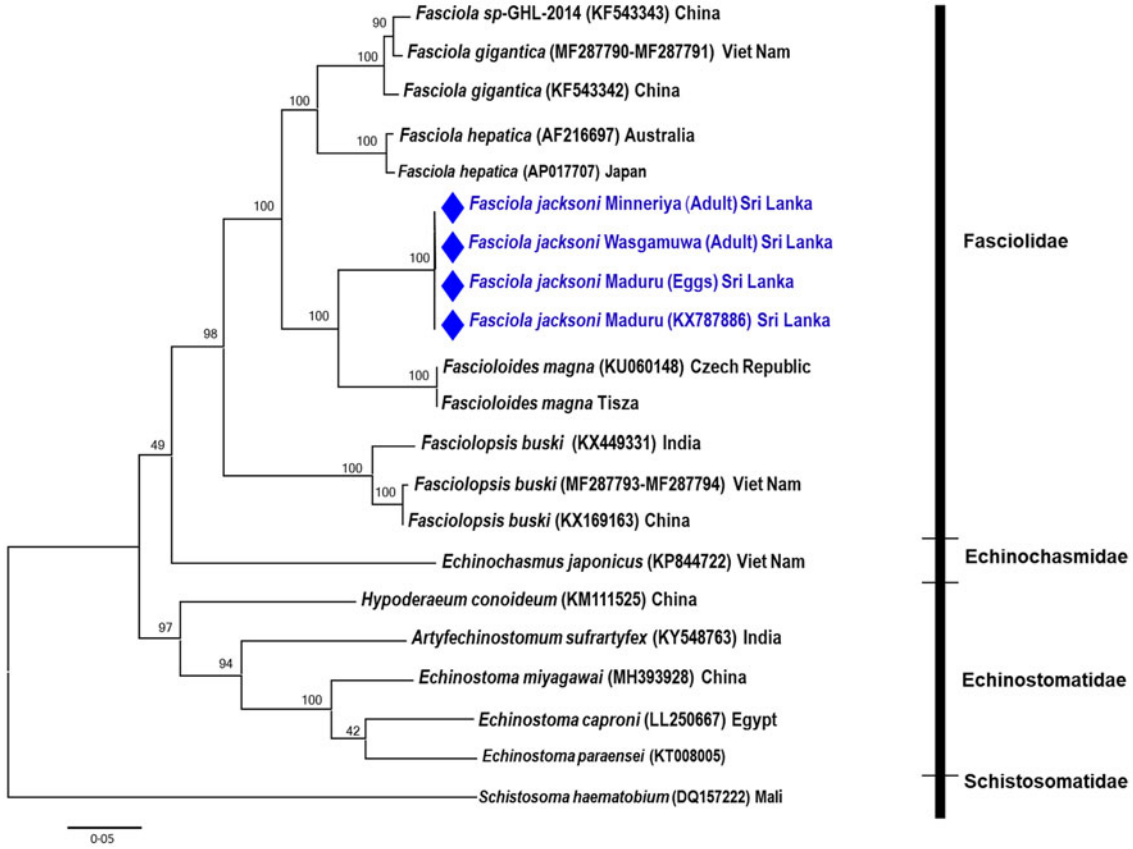

Fig. 3. Maximum likelihood phylogenetic reconstruction showing the position of *Fasciola jacksoni* within the Fasciolidae based on concatenated cox1 and nad1 DNA sequences. Three trematode families are represented Fasciolidae, Echinostomatidae, Echinochasmidae, and the position of *F. jacksoni* indicated by a diamond symbol. Nodal support values are shown based on 1000 bootstrap replicates.

the conditions of the above mentioned model with nodal support values calculated using 1000 bootstrap replicates (Kumar *et al.*, 2016).

Results

Cause of mortality of elephants, description and incidence of Fasciola jacksoni

The survey was performed on a total of 47 post-mortems of elephants across all age groups over the duration of the 4-year study period. There were a variety of causes of fatalities of these elephants which included septicaemia associated with gunshot wounds ($n = 23$), gunshot leading to severe damage of the brain and lungs ($n = 7$), electrocution ($n = 9$), drowning ($n = 1$), suffocation through airway obstruction ($n = 1$), severe pneumonia ($n = 2$), old age ($n = 1$), unknown causes ($n = 1$) and fatal infection with *F. jacksoni* ($n = 2$) (Perera and Rajapakse, 2009).

The parasitological investigation revealed that 27 out of the 47 elephant post-mortems were infected with *F. jacksoni*, with worm burdens varying from seven to 325 flukes (Fig. 2A). Livers of the affected animals showed cholangitis and fibrous tissue proliferation on the bile duct wall. The liver flukes present in the elephants were identified as typical for *F. jacksoni* species, according to the morphological characters described by Bhalerao (1933). The average size of each *F. jacksoni* fluke was 12–14 mm × 9–12.5 mm (Fig. 2A–C) and the severity of infection varied greatly between individual elephants. There were two elephants with heavy worm burdens (>100 flukes), four with moderate (51–100 flukes), seven with mild (10–50 flukes) and 14 with low burden (<10 flukes). It was clear that the severity of the *Fasciola* infection was higher in the weaker animals compared to those considered to be healthy. Fecal examination of 48 live animals revealed that 60% of the dung samples harboured eggs of *F. jacksoni* and fecal egg counts in the infected animals were varied from six to 30 EPG (eggs per gram).

Phylogenetic analyses of F. jacksoni *isolates*

Four of each representative *F. jacksoni* adult flukes from three localities (Maduruoya, Minneriya and Wasgamuwa National Parks) and fecal eggs from live elephants were subjected to molecular characterization. The *p*-distance calculations for the concatenated nad1 + cox1 nucleotide sequences showed the lowest level of divergence to be between *F. jacksoni* and *Fa. magna* at 12.9%. This was contrasted to the 15.0–15.6% divergence seen when *F. jackosoni* was compared to other members of the *Fasciola* genus, including *F. gigantica*, *F. hepatica* and an intermediate *Fasciola* sp. Within the Fasciolidae *F. jacksoni* had the highest rate of divergence with *F. buski* at 21.9%, however, this rate of divergence increased substantial to 26.2–27.9% among echinostomids; and with the highest rate at 28.2% with *Echinochasmus japonicus*, within the superfamily Echinostomoidea (Table 3).

To examine the phylogenetic position of *F. jacksoni* in the family Fasciolidae and in the superfamily Echinostomatoidea, an ML tree was constructed from 21 complete *nad1* and *cox1* nucleotide sequences from 13 trematode species belonging to four families including Fasciolidae, Echinochasmidae, Echinostomatidae and *Schistosoma haematobium* of the Schistosomatidae as the outgroup (Fig. 3; Supplementary Table S1). Sequences of *nad1* and *cox1* from all *F. jacksoni* samples were identical regardless of being generated from adults or eggs and no geographical

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Table 3. Estimation of pairwise genetic distances (%) between *Fasciola jacksoni* and the published or GenBank deposited representative species of the superfamily Echinostomatoidea (Fasciolidae, Echinochasmidae, Echinostomatidae) inferred from the concatenated nucleotide sequence of mitochondrial *nad1* and *cox1*

			No. of base substitutions/site in sequences[b]													
Nucleotide sequences[a]		Accession No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Fjac-Maduru-LK	KX787886		0.007	0.010	0.009	0.009	0.008	0.012	0.012	0.013	0.014	0.015	0.015	0.015	0.015
2	Fmag-Kokorinsko-CZ	KU060148	0.129		0.010	0.008	0.008	0.008	0.013	0.013	0.012	0.015	0.016	0.016	0.016	0.016
3	Fbus-Jiangxi-CN	KX169163	0.221	0.224		0.010	0.011	0.009	0.012	0.013	0.012	0.015	0.016	0.016	0.016	0.016
4	Fgig-Guangxi-CN	KF543342	0.152	0.150	0.205		0.003	0.006	0.011	0.012	0.011	0.014	0.014	0.015	0.015	0.015
5	Fsp-GHL-CN	KF543343	0.151	0.147	0.210	0.025		0.006	0.011	0.012	0.012	0.014	0.014	0.015	0.015	0.015
6	Fhep-Geelong-AU	AF216697	0.157	0.153	0.197	0.096	0.100		0.011	0.012	0.013	0.015	0.015	0.016	0.015	0.016
7	Ecap-SAMEA-EG	AP017706	0.265	0.263	0.270	0.250	0.252	0.259		0.009	0.013	0.014	0.016	0.015	0.016	0.015
8	Epar-UNM-MX	KT008005	0.269	0.286	0.279	0.252	0.252	0.257	0.144		0.013	0.015	0.016	0.016	0.017	0.016
9	Hcon-Hubei-CN	KM111525	0.257	0.261	0.265	0.246	0.245	0.236	0.257	0.250		0.015	0.017	0.016	0.016	0.016
10	Cmic-Hunan-CN	KR337555	0.347	0.365	0.346	0.339	0.340	0.332	0.369	0.371	0.367		0.008	0.008	0.008	0.007
11	Eexp-Hunan-CN	KT198989	0.352	0.358	0.362	0.334	0.332	0.340	0.372	0.383	0.392	0.134		0.009	0.007	0.008
12	Ostr-Tianmen-CN	KM659177	0.367	0.371	0.354	0.343	0.347	0.351	0.389	0.387	0.396	0.152	0.153		0.008	0.009
13	Pcer-Qinghai-CN	KF475773	0.333	0.339	0.350	0.331	0.330	0.324	0.364	0.376	0.371	0.125	0.131	0.154		0.008
14	Pley-Nimu-CN	KP341657	0.344	0.346	0.350	0.330	0.332	0.337	0.373	0.379	0.380	0.142	0.130	0.152	0.129	

[a]Sequence abbreviation is listed in Supplementary Table S1.

[b]The percentage representing the number of base substitutions per site from all sequence pairs between species is shown. Genetic distances were inferred by the analysis of 2,439–2,457 nucleotides of the *nad1* and *cox1* genes, conducted using the maximum likelihood method in MEGA 7 (Kumar *et al.*, 2016). Nodal values were obtained by a bootstrap procedure (1000 replicates).

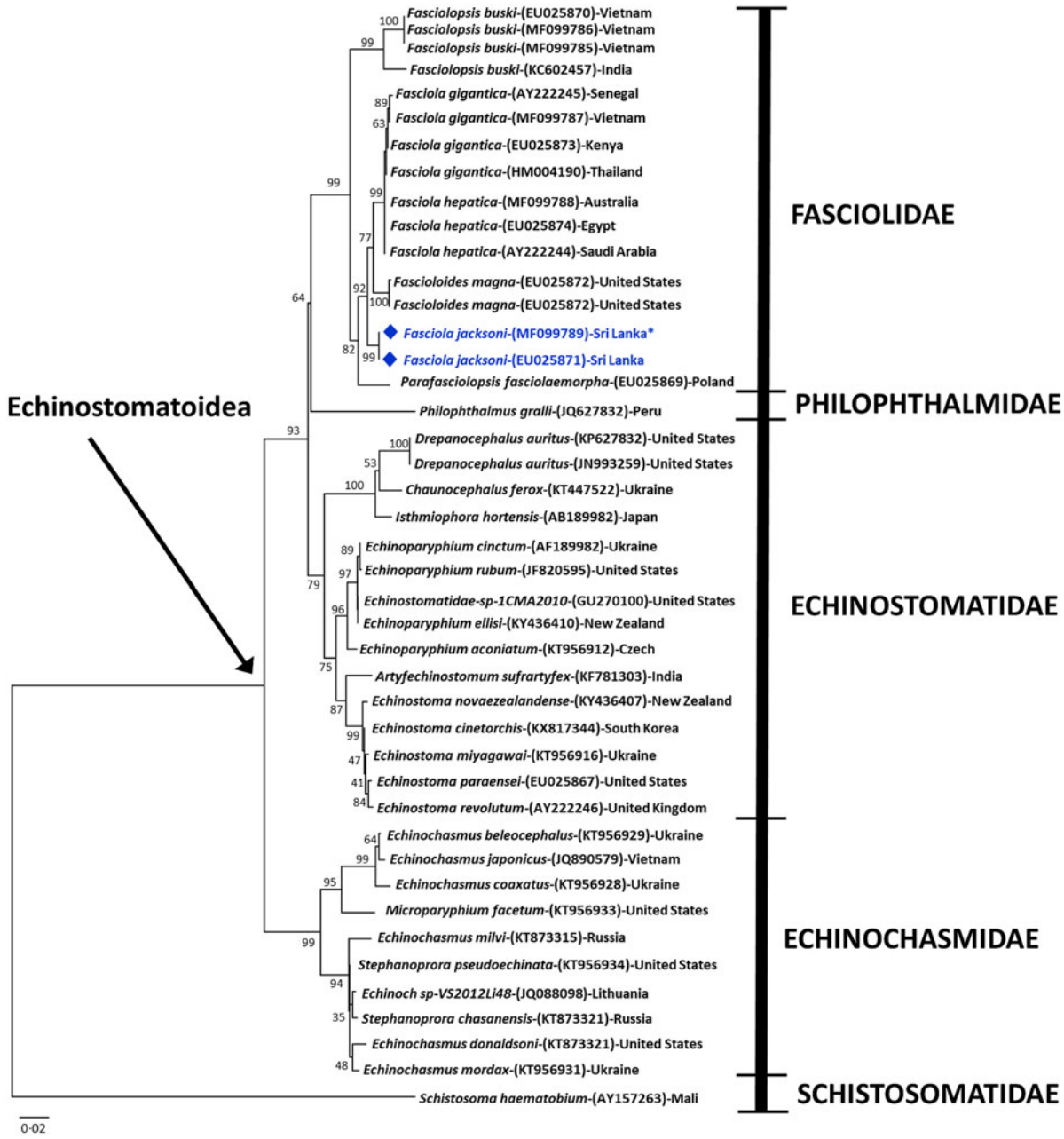

Fig. 4. Maximum likelihood phylogenetic reconstruction showing the position of *Fasciola jacksoni* within the Fasciolidae based on partial 28S rDNA sequences. Four families of the superfamily Echinostomatoidea (Fasciolidae, Philophthalmidae, Echinostomatidae, Echinochasmidae) are represented and the position of *Fasciola jacksoni* is indicated by a diamond symbol. Nodal support values are shown based on 1000 bootstrap replicates.

differentiation was shown. The ML analyses clustered *F. jacksoni* firmly within the Fasciolidae and closely associated with trematodes of ruminants. It was placed in a monophyletic clade as a sister taxon to the European isolate of *Fa. magna* from the Czech Republic and Hungary; while *Fasciola* species and *F. buski* clustered into separate groups, respectively, with strong nodal support of 98–100% (Fig. 3). This rendered the genus *Fasciola* polyphyletic as all other species within the genus formed a *Fasciola* specific clade, which contained *F. gigantica*, *F. hepatica* and the intermediate form of *Fasciola* (Fig. 3).

The 28S rDNA alignment produced a well-supported phylogeny of four families in Echinostomatoidea produced topology for 43 sequences of 33 species, with *S. haematobium* as an outgroup (Fig. 4; Supplementary Table S2). Sequences of 16 fasciolid species/isolates were consistently grouped together, forming a discrete Fascioloidae clade, distinct from Philophthalmidae, Echinostomatidae and Echinochasmidae (Fig. 4). *Fasciola jacksoni* sequences generated in this study clustered into a single group with a published reference sequence of the same species (Acc: EU025871) (Lotfy *et al*., 2008). However, the topology of the 28S rDNA placed *F. jacksoni* basal to the *Fasciola* genus, which also included *Fa. magna* as the paraphyletic sister taxon to a distinct *F. gigantica*/*F. hepatica* clade, again illustrating a lack of monophyly in the genus *Fasciola* (Fig. 4). In Echinostomatoidea, broadly, the family Echinochasmidae clearly is distinct from the cluster formed by Echinostomatidae; Fasciolidae and Philophthalmidae. The *sensu lato Philophthalmus gralli* is closer to Fasciolidae than other echinostomids and echinochasmids with a high nodal support (Fig. 4).

Discussion

To date, *F. jacksoni* has been found to be highly host specific residing in the bile ducts of the liver and causing severe fascioliasis

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only in Asian elephants (*E. maximus maximus* and *E. maximus indicus*) (Cobbold, 1882; Islam, 1997; Heneberg, 2013; Perera *et al.*, 2015). In this current study, among the 27 infected dead elephants examined, there were 22.2% of individuals with more than 50 flukes (6/27) and only 50% with low worm burdens with less than 10 flukes (14/27). There was a high rate of infection of *F. jacksoni* in living elephants with 60% of the 48 elephants examined, although egg shedding seemed to be moderate, with 6–30 EPG. Despite the high burden of infection, generally *F. jacksoni* has low egg counts and has also been shown to have reduced numbers of rediae and cercariae relative to those of *Fasciola* spp infecting ruminants, indicating a low rate of transmission (Islam, 1997; Perera and Rajapakse, 2009). Such low levels of transmission could possibly be related to the infrequent consumption of food contaminated with metacercariae owing to the wide-ranging habit of the elephant, with herds taking advantage of large territories and transmission sites being visited infrequently (Lorimer, 2010; Hing *et al.*, 2016). However, it could be argued that even with low infection and transmission rates, *F. jacksoni* has evolved to by highly virulent with infections of elephants being highly pathogenic causing lesions not only in the liver parenchyma and bile ductules, but also in other organs such as lungs and kidneys leading to fatalities, particularly in calves (Singh *et al.*, 1994; Islam, 1997; Perera and Rajapakse, 2009; Heneberg, 2013; Perera *et al.*, 2015).

The family Fasciolidae Railliet, 1895 comprises of nine recognized species of which only three are found in the genus *Fasciola* including *F. jacksoni*, *F. hepatica* and *F. gigantica* which are considered to be taxonomically valid (Mas-Coma *et al.*, 2009). Although listed in the genus *Fasciola*, taxonomic consideration of *F. jacksoni* is limited and its phylogenetic position within the Fasciolidae continues to be debated (Lotfy *et al.*, 2008; Heneberg, 2013). In both molecular phylogenetic reconstructions in this study the members of the genus *Fasciola* collected from ruminants and humans always clustered together in one monophyletic clade that did not include *F. jacksoni*, placing it with other fasciolid species (Lotfy *et al.*, 2008; Le *et al.*, 2008; Nguyen *et al.*, 2009; Mas-Coma *et al.*, 2009; Heneberg, 2013; Tkach *et al.*, 2016). Using three ribosomal markers, 28S rDNA, ITS1, ITS2 and a single mitochondrial *nad1* marker Heneberg (2013) indicated the high similarity of *F. jacksoni* to *Fa. magna* rather than to the other ruminant *Fasciola* spp. group and suggested re-classification of *F. jacksoni* as *Fascioloides jacksoni* comb. nov. In this current study mitochondrial and ribosomal 28S DNA analyses based on increased numbers and longer sequences of the *nad1* and 28S, as well as representations of *cox1*, once again illustrated the close evolutionary relationships of *F. jacksoni* to *Fa. magna* (Figs 3 and 4). Similarly, evolutionary distance analyses (shown in Table 3) in this study found the lowest rate of divergence (12.9%) between *F. jacksoni* and *Fa. magna,* rather than 15.0–15.6% among the ruminant *Fasciola* spp. and other echinostomatids (around 27%) in Echinostomatoidea. Interestingly, the close phylogenetic relationship between *F. jacksoni* and *Fa. magna* is also supported by several shared morphological characteristics such as a thick body, lack of distinct cephalic cone and have long extensive median intestinal branches as highlighted by Lotfy *et al.* (2008).

The close evolutionary relationship between *F. jacksoni* and *Fa. magna* makes it therefore challenging to disentangle the origins of these two species. *Fascioloides magna* is a parasite of North American cervids and it has been argued that proboscideans brought liver fluke with them into the Neoarctic as they moved from Asia into North America (Lotfy *et al.*, 2008). Eventually, the parasite would have possibly undergone a host shift into North American cervids and persisted in deer after the North American elephants became extinct (Lotfy *et al.*, 2008). However, only by sampling liver flukes from elephants, antelope and deer across Asia and cervids in the Americas will provide a deeper understanding of the evolutionary relationships of *F. jacksoni* and *Fa. magna* and ultimately resolve the taxonomic relationship between *F. jacksoni*, the ruminant *Fasciola* spp. and monotypic *Fa. magna* of the genus *Fascioloides*.

The present study revealed the utility of molecular taxonomic approaches for the accurate identification of highly pathogenic liver flukes in Asian elephants from different localities in Sri Lanka. However, despite the accuracy of the species identification it was challenging to resolve the taxonomic relationships of *F. jacksoni* within the Fasciolidae. Molecular data analysed in this study suggested the reappraisal of the family Fasciolidae and recognition of the right taxonomic position of *F. jacksoni* in the superfamily Echinostomatoidea. Such molecular approaches not only provide vital DNA sequence reference data to develop tools but also provide a foundation for a detailed reconciliation of the taxonomy of these parasites, which is crucial to aid in accurate diagnostics and monitoring of the spread of such an important, but a neglected disease of wild and domestic elephants in Asia.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019000519.

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Ethical standards. The study had ethical approval from the Sri Lankan Government. Appropriate permission was obtained from the commune authorities before the collection of parasite specimens from elephants.

References

Alahakoon J (1994) Presence of liver fluke *Fasciola jacksoni* in a Sri Lankan elephant. *Gajah* **12**, 46–47.

Bhalerao GD (1933) The trematode parasites of the Indian elephant, *Elephas indicus*. *Indian Journal of Veterinary Science and Animal Husbandry* **3**, 103–155.

Caple IW, Jainudeen MR, Buick TD and Song CY (1978) Some clinicopathologic findings in elephants (*Elephas maximus*) infected with *Fasciola jacksoni*. *Journal of Wildlife Diseases* **14**, 110–115.

Cobbold T. S. (1869). *Fasciola jacksoni*. *Quarterly Journal of Microscopical Science; Supplement to Entozoa*. p. 80.

Cobbold TS (1882) VII. The parasites of elephants. *Transactions of the Linnean Society of London. 2nd Series: Zoology* **2**, 223–258.

Fernando P (2000) Elephants in Sri Lanka: past present and future. *Loris* **22**, 38–44.

Fernando P, Wikramanayake E, Weerakoon D, Jayasinghe L, Gunawardene M and Janaka H (2005) Perceptions and patterns of human–elephant conflict in old and new settlements in Sri Lanka: insights for mitigation and management. *Biodiversity and Conservation* **14**, 2465–2481.

Heneberg P (2013) Phylogenetic data suggest the reclassification of *Fasciola jacksoni* (Digenea: Fasciolidae) as *Fascioloides jacksoni* comb. nov. *Parasitology Research* **112**, 1679–1689.

Hing S, Othman N, Nathan S, Fox M, Fisher M and Goossens B (2013) First parasitological survey of Endangered Bornean elephants *Elephas maximus borneensis*. *Endangered Species Research* **21**, 223–230.

Hing S, Narayan EJ, Thompson RCA and Godfrey SS (2016) The relationship between physiological stress and wildlife disease: consequences for health and conservation. *Wildlife Research* **43**, 51.

Islam S (1997) Studies on some aspects of fascioliasis in Asian elephants (*Elephas maximus*). *Journal of Veterinary Parasitology* **11**, 109.

Králová-Hromadová I, Špakulová M, Horáčková E, Turčeková L, Novobilský A, Beck R, Koudela B, Marinculić A, Rajský D and Pybus M (2008) Sequence analysis of ribosomal and mitochondrial genes of the giant liver fluke *Fascioloides magna* (Trematoda: Fasciolidae): intraspecific variation and differentiation from *Fasciola hepatica*. *Journal of Parasitology* **94**, 58–67.

Kumar S, Stecher G and Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Le TH, De NV, Agatsuma T, Nguyen TGT, Nguyen QD, Mcmanus DP and Blair D (2008) Human fascioliasis and the presence of hybrid/introgressed forms *of Fasciola hepatica* and *Fasciola gigantica* in Vietnam. *International Journal for Parasitology* **38**, 725–730.

Le TH, Nguyen KT, Nguyen NTB, Doan HTT, Dung DT and Blair D (2017) The ribosomal transcription units of *Haplorchis pumilio* and *H. taichui* and the use of 28S rDNA sequences for phylogenetic identification of common heterophyids in Vietnam. *Parasites & Vectors* **10**. doi: 10.1186/s13071-017-1968-0.

Liu G-H, Gasser RB, Young ND, Song H-Q, Ai L and Zhu X-Q (2014) Complete mitochondrial genomes of the 'intermediate form' of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica*. *Parasites & Vectors* **7**, 150.

Lorimer J (2010) Elephants as companion species: the lively biogeographies of Asian elephant conservation in Sri Lanka. *Transactions of the Institute of British Geographers* **35**, 491–506.

Lotfy WM, Brant SV, DeJong RJ, Le TH, Demiaszkiewicz A, Rajapakse RPVJ, Perera VBVP, Laursen JR and Loker ES (2008) Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). *American Journal of Tropical Medicine and Hygiene* **79**, 248–255.

Mas-Coma S, Valero MA and Bargues MD (2009) Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology* **69**, 41–146.

Mucheka VT, Lamb JM, Pfukenyi DM and Mukaratirwa S (2015) DNA sequence analyses reveal co-occurrence of novel haplotypes of *Fasciola gigantica* with *F. hepatica* in South Africa and Zimbabwe. *Veterinary Parasitology* **214**, 144–151.

Nguyen TGT, De NV, Vercruysse J, Dorny P and Le TH (2009) Genotypic characterization and species identification of *Fasciola* spp. with implications regarding the isolates infecting goats in Vietnam. *Experimental Parasitology* **123**, 354–361.

Nolan MJ and Cribb TH (2005) The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology* **60**, 101–163.

Perera BVP and Rajapakse RPVJ (2009). Mortality and morbidity of wild elephants (*Elephas maximus maximus*) of Sri Lanka, as a result of liver flukes (*Fasciola jacksoni*) infestation. In Wibbelt G, Kretschmar P, Hofer H and Seet S (eds), *Proceedings of the International Conference on Diseases of Zoo and Wild Animals*. Leibniz Institute for Zoo and Wildlife Research, Beekse Bergen, The Netherlands, 20–24 May 2009, p. 191.

Perera BVP, Rajapakse RPVJ, Silva-fletcher A, Thewarage LD and Jinadasa HRN (2015) Emerging parasitic and infectious diseases of wild Asian Elephants (*Elephas Maximus*) in Udawalawe National Park, Sri Lanka. *American Association of Zoo Veterinarians*. Oregon, USA.

Singh KP, Srivastava VK, Prasad A and Pandey AP (1994) Pathology due to *Fasciola jacksoni* in Indian elephants (*Elephas indicus*). *Indian Journal of Animal Science* **648**, 802–804.

Tkach VV, Kudlai O and Kostadinova A (2016) Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). *International Journal for Parasitology* **46**, 171–185.

Wey-Fabrizius AR, Podsiadlowski L, Herlyn H and Hankeln T (2013) Platyzoan mitochondrial genomes. *Molecular Phylogenetics and Evolution* **69**, 365–375.

Windsor R and Scott W (1976) Fascioliasis and Salmonellosis in African elephants in captivity. *British Veterinary Journal* **132**, 313–317.

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