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Description and phylogenetic analyses of ribosomal transcription units from species of Fasciolidae (Platyhelminthes: Digenea)

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Abstract

Many members of Fasciolidae are common trematodes in cattle, buffaloes, sheep, elephants, pigs, with some capable of infecting humans also. In this study, the complete or near-complete sequences of ribosomal transcription unit (rTU or rDNA), each of *Fasciola hepatica* (Australia), *Fascioloides jacksoni* (Sri Lanka), *Fasciolopsis buski* (Vietnam) and three isolates of *F. gigantica* (Vietnam), were obtained and characterized. The full length of rDNA for each *F. hepatica*, 'hybrid' *Fasciola* sp., *Fas. jacksoni* and *Fa. Buski*, was 7657 bp, 7966 bp, 7781 bp and 8361 bp, with the complete intergenic spacer region (IGS) (862 bp, 1170 bp, 987 bp and 561 bp), respectively. The rDNA of two 'pure' *F. gigantica* isolates from Vietnam was 6794 bp with unsequenced IGS. For 28S rRNA genes the *Fasciola* spp. are equal, 1958 bp for 18S, 160 bp for 5.8S, 3863 bp and 454 bp for ITS1 but ITS2 differ by one nucleotide (Thymine) (359 or 360 bp). The ITS1 of the *sensu lato Fa. buski* has some distinguishable features, 286 bp for ITS2, 3862 bp for 28S and four repeat units of 356–361 bp each found in ITS1. The 28S rDNA analysis showed the lowest level of divergence (0–0.57%) between *F. hepatica* and *F. gigantica* and higher (2.23–2.62%) and highest (6–6.42%) for *Fas. jacksoni* and *Fasciolopsis*, respectively. The tree of 43 strains/species clearly produced a well-supported phylogeny, where 18 fasciolids consistently grouped, forming a discrete Fasciolidae clade, distinct from Philophthalmidae, Echinostomatidae and Echinochasmidae in Echinostomatoidea. *Fascioloides jacksoni* is outside *Fasciola* spp.: basal with *Fas. magna*, as previously demonstrated.

Introduction

The family Fasciolidae Raillet, 1895 (Platyhelminthes: Trematoda) comprises six distinct genera including *Fasciola*, *Fascioloides*, *Fasciolopsis*, *Tenuifasciola*, *Parafasciolopsis* and *Protofasciola*, all of which predominantly parasitize ungulates across the globe (Mas-Coma *et al.*, 2019). The Fasciolodae are of interest as several species are zoonotic and of medical importance, including the closely related parasites of cattle and sheep, *Fasciola hepatica* Linnaeus, 1758, from Europe and Asia, and *Fasciola gigantica* Cobbold, 1855, from Africa and Asia (Le *et al.*, 2008; Mas-Coma *et al.*, 2019). Similarly, the intestinal fluke of pigs *Fasciolopsis buski* (*sensu lato*) Lankester, 1857 has also been shown to cause disease in humans in South-East Asia (Mas-Coma *et al.*, 2019). This family of flukes is also renowned for its impact on wildlife and importance in conservation, especially *Fascioloides magna* Ward, 1917, which is predominantly a fluke of North American deer populations but has now been shown to be invasive in Central Europe (Králová-Hromadová *et al.*, 2008; Heneberg, 2013). *Fascioloides jacksoni* (syn. *Fasciola jacksoni* Cobbold, 1869) has also been shown to have a considerable impact on wild elephant populations across South-East Asia especially India, Sri Lanka, Nepal, Myanmar and peninsular Malaysia (Lotfy *et al.*, 2008; Heneberg, 2013; Rajapakse *et al.*, 2019). Despite their medical, veterinary and environmental importance there is still a deficit in detailed evolutionary and molecular genetic studies of the Fasciolidae and their relationship within the Echinostomatoidea superfamily.

DNA markers have been shown to be crucial in resolving taxonomic issues and provide a basis for the development of accurate diagnostic tools for parasitic worms (Blair, 2006; Tkach *et al.*, 2016). Recently, reference DNA sequence data for complete mitochondrial genomes have been obtained for several species within the Fasciolidae family, including *F. hepatica*, *F. gigantica*, *Fa. buski, Fas. magna* and *Fas. jacksoni* as well as other species across the Echinostomatoidea superfamily (Le *et al.*, 2001; Liu *et al.*, 2014; Ma *et al.*, 2016, 2017;

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Rajapakse *et al*., 2019). However, to date, there have been no complete ribosomal gene, nuclear ribosomal transcription units (rTUs) and reference sequences generated that would allow detailed comparisons between species of the Fasciolidae (Králová-Hromadová *et al*., 2008; Ichikawa & Itagaki, 2010; Rajapakse *et al*., 2019). The rTU consists of three coding regions, the 18S, 5.8S and 28S rDNA genes, and are separated by two internal transcribed spacer regions, ITS1 and 2. The rTUs are sequentially repeated units arranged into hundreds of copies with the 28S gene being followed by a further non-transcribed intergenic spacer region (IGS) which connects one rTU to another (Zhao *et al*., 2011; Cerqueira, Lemos, 2019; Qiu *et al*., 2019). In trematodes each copy unit has a length of 7–9 kb with the typical structure of the rTU as 5′ 18S-ITS1-5.8S-ITS2-28S-IGS 3′ (Cerqueira, Lemos, 2019).

Historically, only short fragments of 18S and 28S rDNA, and both ITS1 and 2, have been utilized in phylogenetic and population genetic studies. The analyses of short fragments have been extensively used for molecular classification, species identification and evolutionary studies both within and between trematode families (Weider *et al*., 2005; Blair, 2006). In fact, the rDNA sequences have been shown to be valuable markers in the identification of hybrids and measuring introgression between populations of sympatric fluke species (Blair, 2006; Nguyen *et al*., 2018). However, until recently very few complete rDNA reference sequences for trematodes have been available which could have been used to develop and refine such molecular markers further (Weider *et al*., 2005; Blair, 2006).

In this study, we provide complete and near-complete rDNA sequences obtained from six species/strains within the Fasciolidae. We have determined the structure and arrangement of the rTU and provide a detailed account of the characteristics of each ribosomal gene and the intergenic regions. We also provide a detailed comparative phylogenetic analysis of the rDNA sequences, emphasizing their utility as molecular markers for diagnostics, classification and molecular evolutionary studies of the Echinostomatoidea.

Material and methods

Parasite material

Six strains of three species from the three genera, *Fasciola*, *Fasciolopsis* and *Fascioloides*, were used to represent the Fasciolidae in this study. These included: *Fasciola hepatica* (isolate GL) collected from cattle in Geelong, Australia (38°9′0.0072″S; 144°21′0.0216″E), designated Fhep-GL (AU) (Le *et al*., 2001); *F. gigantica* (isolate T4V) from cattle in Thua Thien-Hue Province, Vietnam (16°22′7.932″N; 107°37′31.584″E), designated Fgig-T4V (VN); *F. gigantica* (isolate NB) from cattle in Ninh Binh Province (20°15′28.764″N; 105°58′33.3732″E), designated Fgig-NB (VN); 'hybrid' *Fasciola* sp. (isolate DL11) from buffalo in Dak Lak Province (12°47′19.284″N; 108°14′23.892″E), designated Fsp-DL11 (VN) (Nguyen *et al*., 2018); *Fas. jacksoni* (isolate Madu) from post mortem wild elephants in Maduru, Sri Lanka (7°34′33″N; 81°08′34″E), designated Fjac-Madu (LK) (Rajapakse *et al*., 2019); *Fa. buski* (isolate HT) from human in former Ha Tay Province, Vietnam (21°01′42″N 105°51′15″E), designated Fbus-HT (VN). Adult worms of each species were collected from necropsied animals and either frozen or preserved in 70% ethanol before storage at −20°C. Subsequently, the worms were identified to species based on morphology using the standard measures of body length (BL) and width (BW) which have been used to distinguish between species. The average size for *F. hepatica* was 20–36 mm (BL) × 13 mm (BW), for 'pure' *F. gigantica* was 42–65 mm (BL) × 12 mm (BW), for *Fas. jacksoni* was 12–14 mm (BL) × 9–12.5 mm (BW) and for the *Fa. buski* fluke was 41 mm (BL) × 13 mm (BW), with typical morphological features for each species/form described in previous studies (Le *et al*., 2004; Lotfy *et al*., 2008; Periago *et al*., 2008; Nguyen *et al*., 2018; Rajapakse *et al*., 2019). However, the shape of the hybrid form of *Fasciola* sp. (DL11) resembled *F. hepatica* in size and could not be distinguished based on morphology alone. Each worm species identification was accurately confirmed using molecular approaches, using both mitochondrial and rDNA sequences, identifying each species as suspected. However, it was only possible to identify the hybrid status of DL11 genetically as it possessed the mitochondrial sequences from *F. gigantica* and showed to have nuclear ITS2 sequences of *F. hepatica*.

PCR amplification

Total genomic DNA was extracted from ~10-mg sections of an adult worm using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., MA, USA) as instructed, eluted in 100 µl and stored at −20°C until use. The majority of the rTU universal primers used were previously reported (Le *et al*., 2017) and some were added for use in this study (supplementary table S1). PCR reactions of 50 µl were prepared using 25 µl of DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc., MA, USA) and 2 µl of DNA template (50 ng/µl), 2 µl of each primer (10 pmol/µl), 2 µl DMSO (dimethyl sulphoxide) and 17 µl of water, performed in an MJ PTC-100 thermal cycler. Initiation was at 94°C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing at 52°C for 30 s, extension at 72°C for 6 min and a final extension at 72°C for 10 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). The amplicons underwent direct sequencing by primer-walking in both directions. Flanking and primer-walking sequencing was applied until the complete sequence for the whole fragment was obtained.

The entire rDNA sequence for each Fasciolidae species/isolate was obtained after editing chromatograms (Chromas 2.6.6; http://technelysium.com.au/wp/chromas/) and 18S, 5.8S and 28S rRNA genes were determined by using the previously published reference sequences and those available in GenBank. These reference sequences were obtained from several trematode species, including *Euryhelmis costaricensis* (GenBank: AB521797); *Isthmiophora hortensis* (GenBank: AB189982); *Paragonimus kellicotti* (GenBank: HQ900670); *Paramphistomum cervi* (GenBank KJ459934; Zheng *et al*., 2014); *Brachycladium goliath* (GenBank: KR703279; Briscoe *et al*., 2016); *Eurytrema pancreaticum* (5 isolates, GenBank: KY490000−KY490004; Su *et al*., 2018); *Clonorchis sinensis* (five isolates, GenBank: MK450523−MK450527; Qiu *et al*., 2019) and *Metorchis orientalis* (five isolates, MK482051−MK482055; Qiu *et al*., 2019). For intergenic regions, ITS1 was recognized as located between 18S and 5.8S; ITS2 as between 5.8S and 28S; and the IGS as between 3′ 28S and 5′ 18S sequences, respectively. Repeat units (RUs) were detected in the ITS1 or ITS2 or in IGS using the Tandem Repeat Finder v3.01 (Benson, 1999).

Forty-three partial 28S rDNA sequences representing 33 species from across the four families (Fasciolidae, Philophtalmidae, Echinostomatidae, Echinochasmidae) of the Echinostomatoidea

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Table 1. Position of ribosomal RNA genes, internal transcribed spacers (ITS) and non-transcribed intergenic spacer (IGS) in the ribosomal transcriptional unit of trematodes in the family Fasciolidae in this study

Species[a]	18S	ITS1	5.8S	ITS2	28S	IGS
Fasciola hepatica (GL (AU))	1–1958 (1958 bp)	1959–2412 (454 bp)	2413–2572 (160 bp)	2573–2932 (360 bp)	2933–6795 (3863 bp)	6796–7657 (862 bp)
Fasciola gigantica (T4 V (VN)) 'pure isolate'	1–1958 (1958 bp)	1959–2412 (454 bp)	2413–2572 (160 bp)	2573–2931 (359 bp)	2932–6794 (3863 bp)	NA
Fasciola gigantica (NB (VN)) 'pure isolate'	1–1958 (1958 bp)	1959–2412 (454 bp)	2413–2572 (160 bp)	2573–2931 (359 bp)	2932–6794 (3863 bp)	NA
Fasciola gigantica (DL11 (VN)) ('hybrid' isolate)	1–1958 (1958 bp)	1959–2412 (454 bp)	2413–2572 (160 bp)	2573–2932 (360 bp)	2933–6795 (3863 bp)	6796–7966 (1170 bp)
Fascioloides jacksoni (Madu (LK))	1–1958 (1958 bp)	1959–2412 (454 bp)	2413–2572 (160 bp)	2573–2931 (359 bp)	2932–6794 (3863 bp)	6795–7781 (987 bp)
Fasciolopsis buski (HT (VN))	1–1958 (1958 bp)	1959–3492 (1534 bp)	3493–3652 (160 bp)	3653–3938 (286 bp)	3939–7800 (3862 bp)	7801–8361 (561 bp)
		2054–2414 (RU1: 361 bp)				
		2415–2775 (RU2: 361 bp)				
		2776–3136 (RU3: 361 bp)				
		3137–3492 (RU4: 356 bp)				

[a]*Fasciola hepatica* (GL (AU)): *F. hepatica*, strain GL, Australia; *Fasciola gigantica* (T4V (VN)), *F. gigantica,* strain T4V, Vietnam; *Fasciola gigantica* (NB (VN)), *F. gigantica,* strain NB, Vietnam; *Fasciola gigantica* (DL11 (VN)), 'hybrid' *Fasciola* sp., strain DL11, Vietnam; *Fascioloides jacksoni* (Madu (LK)), *Fas. jacksoni*, strain Madu, Sri Lanka; *Fasciolopsis buski* (HT (VN), *Fa. buski*, strain HT, Vietnam. ITS, internal transcribed spacer; IGS, non-transcribed intergenic spacer; RU, repeat unit. NA: not available.

were used for phylogenetic analysis. The 28S rDNA sequence of *Schistosoma haematobium* (family: Schistosomatidae) was used as an outgroup (supplementary table S2). The final 28S rDNA alignment was composed of 43 species/isolates and MEGA 7.0 was used to perform maximum likelihood phylogenetic reconstruction with 1000 bootstrap resamplings (Kumar *et al*., 2016). MEGA 7.0 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. Pairwise distance analysis, was also performed on the final 28S rDNA alignment as a measure of genetic divergence (*P*-distance) between 17 strains of six species, *F. gigantica*, *F. hepatica*, hybrid *Fasciola* sp., *Fa. buski*, *Fas. jacksoni* and *Fas. magna* within the Fasciolidae of the Echinostomatoidea (supplementary table S3).

Results and discussion

The complete rDNA sequence was obtained for *F. hepatica* (7657 bp), *Fas. jacksoni* (7781 bp), the DL11 hybrid *Fasciola* sp. (7966 bp) and *Fa. buski* (8361 bp); and near-complete sequence for T4V and NB Vietnamese strains of *F. gigantica* at 6794 bp each, lacking the unsequenced IGS region (table 1) (GenBank accession numbers: MN97005–MN970010). The rDNA genes of T4V and NB strains of *F. gigantica*, *F. hepatica*, DL11 hybrid *Fasciola* and *Fas. jacksoni* were all shown to be of equal length 1958 bp for 18S, 160 bp for 5.8S and 3863 bp for 28S. The 18S of *Fa. buski* was also shown to be 1958 bp but the 28S was lengthened to 3862 bp by a single extra-base pair. In all *Fasciola* spp., the length of ITS1 was 454 bp and 286 bp in *Fa. buski*. The ITS2 was shown to be 360 bp for *F. hepatica* and DL11 hybrid *Fasciola*, but 359 bp for both strains of *F. gigantica* as well as *Fas. jacksoni* which lacked a Thymine (T). This T is a differential marker for paternal discrimination of pure *F. gigantica* and *F. hepatica* and their subsequent hybrid offspring as previously explored by a number of studies (Le *et al*., 2008; Ichikawa & Itagaki, 2010; Nguyen *et al*., 2018) (table 1). There were no repeats in any intergenic regions of all *Fasciola* spp.; however, three repeat units of 361 bp each and one of 356 bp were found in ITS1 of *Fa. buski*. The IGS sequences did not contain any repeat elements and varied in length across species with *F. hepatica* having an IGS of 862 bp, DL11 hybrid *Fasciola* at 1170 bp, *Fas. jacksoni* at 987 bp and *Fa. buski* at 561 bp (table 1). The *P*-distance calculations for the pairwise genetic divergence of 17 rTU sequences, nine of *Fasciola* spp., four of *Fa. buski*, two of *Fas. jacksoni* and two of *Fas. magna* indicated the lowest level of divergence (0.0 to 0.57%) between *F. hepatica* and *F. gigantica*, higher (2.23–2.62%) and the highest (6–6.42%) to *Fas. jacksoni* and *Fa. buski,* respectively (supplementary table S3).

Both *Fasciola* species and the DL11 hybrid shared common generic features in length and nucleotide composition of the ITS1 and two with only one nucleotide change being identified and a lack of the repetitive structures. The single mutational change within the ITS1 region was characterized as the previously identified GTAC haplotype in *F. hepatica* (*Rsa*I(+)) and the GTAT haplotype in *F. gigantica* (*Rsa*I(−)). Within the ITS2 region, there was a presence/absence of T (T/−) at position 327 with T being present in *F. hepatica* and the substitution being absent in both *F. gigantica* and the DL11 hybrid (Le *et al*., 2008; Ichikawa & Itagaki, 2010; Nguyen *et al*., 2018).

Interestingly, *Fa. buski* showed the most striking differences in rDNA relative to the other Fasciolidae members with the 28S and IGS of being distinctly different lengths and the ITS1 also contained four repeat units measuring 356–361 bp each. The variation in repeat number, size and location of the repetitive sequences within the ITS and IGS regions have been shown to be responsible for the high polymorphism seen in these intergenic regions found in many trematode species including the Schistosomatidae, the Opisthorchiidae, the Heterophyidae and

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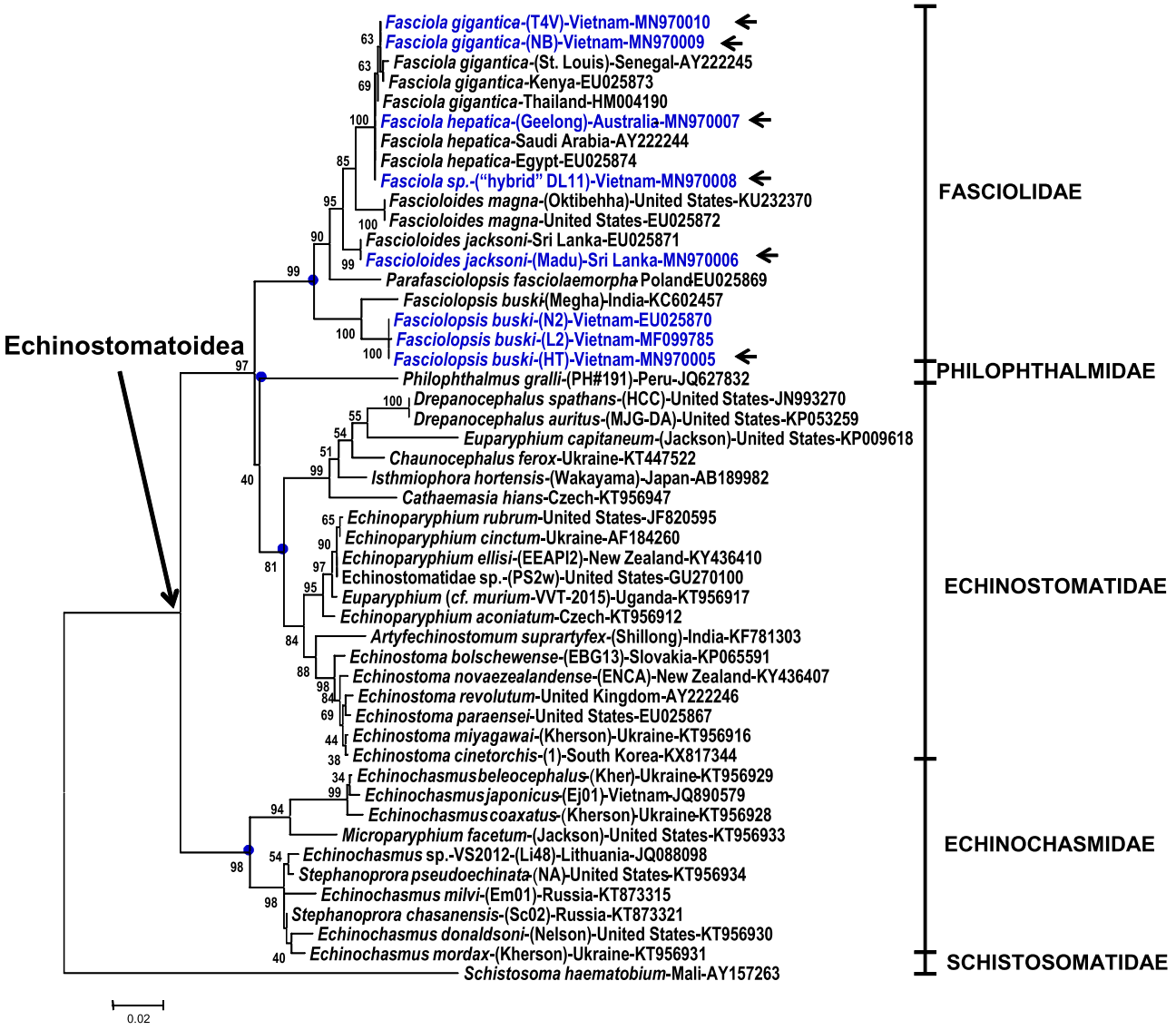

Fig. 1. Maximum likelihood phylogenetic tree showing the position of *Fasciola* spp., *Fascioloides magna*, *Fascioloides jacksoni*, *Parafasciolopsis fasciolaemorpha* and *Fasciolopsis buski* within the Fasciolidae based on partial 28S rDNA sequences. Four families of the superfamily Echinostomatoidea (i.e. Fasciolidae, Philophthalmidae, Echinostomatidae, Echinochasmidae) are separately represented (marked by a point at each node). The species/strains used in this study are indicated by arrows. Full names of each species, followed by abbreviations/designation of strains, where available (in brackets) and country origin are provided. Accession numbers are given for each species/strain at the end of each sequence. Nodal support values are shown based on 1000 bootstrap replicates. The scale bar represents the number of substitutions per site.

the Paramphistomatidae (Lockyer *et al.*, 2003; Tatonova *et al.*, 2012; Zheng *et al.*, 2014; Le *et al.*, 2017; Qiu *et al.*, 2019). Owing to the extensive divergence of the ITS1 in *Fa. buski* we argue that the ITS markers would be inappropriate for interspecies comparisons with other fasciolids. Other studies on trematodes have concluded that these markers are inappropriate for interspecies phylogenetic studies between distantly related genera as both the ITS1 and two acquire repetitive elements and have high mutation rates causing nuclear alignment data to reach substitutional saturation rapidly, diluting the phylogenetic signal that allows accurate taxonomic comparisons (Blair, 2006; Zheng *et al.*, 2014; Le *et al.*, 2017). However, although caution is required when using ITS regions, they have been shown to be useful in disentangling relationships between closely related species within a genus (Lockyer *et al.*, 2003; Blair, 2006; Tatonova *et al.*, 2012; Le *et al.*, 2017).

Phylogenetic analyses

Forty-nine 28S rDNA sequences of ~1100 bp of 33 species across four families of Echinostomatoidae, (supplementary table S2), including the sequences generated within this study, and sequences of *S. haematobium* as an outgroup, were aligned and used to perform maximum likelihood phylogenetic analyses (fig. 1). The alignment produced a well-supported phylogeny of four families in Echinostomatoidea, illustrating that the family Echinochasmidae are distinct from the cluster formed by Echinostomatidae; Fasciolidae and Philophthalmidae broadly agreeing well with previous findings (Olson *et al.*, 2003; Tkach *et al.*, 2016; Rajapakse *et al.*, 2019). Sequences of 18 fasciolid species/isolates were consistently grouped together, in a discrete Fasciolidae clade, distinct from the Philophthalmidae, the Echinostomatidae and the Echinochasmidae. Interestingly, the Echinostomatidae, Fasciolidae and Philophthalmidae, resolved

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by grouping together in the tree, rendering Echinochasmidae completely distinct. The paraphyletic position of the Echinochasmidae with respect to the Echinostomatidae within the Echinostomatoidea demonstrated clear distinctness between these two families.

Within the Fasciolidae clade, *F. gigantica* and the DL11 hybrid *Fasciola* sp. clustered together into a single group, forming a monophyletic branch with *F. hepatica* sequences. As in previous studies based on mitochondrial markers, the topology of the 28S rDNA placed *Fas. jacksoni* basal to the *Fasciola* genus, which also included *Fas. magna,* as the paraphyletic sister taxon to a distinct *F. gigantica*/*F. hepatica* clade further confirming the validity of the *Fas, jacksoni* as a member of the *Fascioloides* genus rather than *Fasciola* as originally described (fig. 1). *Fasciolopsis buski* of Vietnam and India grouped together and was rendered paraphyletic to the *Fasciola* and other groups.

The phylogenetic analyses of the 28S sequences indicate that the entire superfamily Echinostomatoidea presents broad systematic and taxonomic challenges to be met in the future using combined morphological and molecular analyses, both mitochondrial and ribosomal genomic data, of more species (Heneberg, 2013; Le *et al.,* 2016; Tkach *et al.,* 2016; Rajapakse *et al.*, 2019). As illustrated in this and other studies, the complete rTU provides excellent markers to disentangle phylogenetic associations between parasite species, with its unique properties of having regions, such as the 18S and 28S, which can resolve interspecies relationships within and between families but also regions, such as ITS1 and 2, which are more suited to disentangling relationships between species within a single genus. Such properties could be exploited for the development of accurate molecular diagnostic approaches which are crucial for the monitoring and treatment of these socioeconomically important yet neglected parasites.

Supplementary material. To view supplementary material for this article, please visit 10.1017/S0022149X20000164

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Conflicts of interest. None.

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