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Evolutionary Origins, Diversification, and Biogeography of Liver Flukes (Digenea, Fasciolidae)

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Abstract

Fasciolid flukes are among the largest and best known digenetic trematodes and have considerable historical and veterinary significance. *Fasciola hepatica* is commonly implicated in causing disease in humans. The origins, patterns of diversification, and biogeography of fasciolids are all poorly known. We have undertaken a molecular phylogenetic study using 28S, internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) of nuclear ribosomal DNA, and mitochondrial nicotinamide dehydrogenase subunit 1 (*nad*1) that included seven of the nine recognized species in the family. The fasciolids examined comprise a monophyletic group with the most basal species recovered from African elephants. We hypothesize fasciolids migrated from Africa to Eurasia, with secondary colonization of Africa. Fasciolids have been conservative in maintaining relatively large adult body size, but anatomical features of their digestive and reproductive systems are available. These flukes have been opportunistic, with respect to switching to new snail (planorbid to lymnaeid) and mammalian hosts and from intestinal to hepatic habitats within mammals.

INTRODUCTION

One of the first infectious agents to be discovered and implicated in causing disease was no doubt the liver fluke, *Fasciola hepatica* Linnaeus, 1758, causative agent of liver rot disease in domestic ruminants. This species is also increasingly implicated in causing disease in humans.[1] *Fasciola hepatica* also holds a special place in the history of parasitology by virtue of being the first fluke for which a complete life cycle was shown.[2,3] It is today one of the most identifiable of all invertebrates and remains a common subject of study in invertebrate zoology and parasitology classes. Surprisingly, after decades of study, the evolutionary history of *F. hepatica* and other members in the Fasciolidae Railliet, 1895, remains virtually unexplored.

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The Fasciolidae is a relatively small family comprised of only nine recognized species. Flukes in this family are nonetheless well known for their large body size and considerable public health and veterinary significance. Although fasciolids mostly parasitize large herbivorous mammals, three species *Fasciola hepatica*, *F. gigantica* Cobbold, 1856, and *Fasciolopsis buski* Lankester, 1857, regularly infect humans.[4] Other species such as *Fascioloides magna* Bassi, 1875, one of the largest of all digenetic trematodes, can cause considerable morbidity and mortality when infecting abnormal hosts.[5] Infections by other species, such as *Fasciola jacksoni* Cobbold, 1869, or *Protofasciola robusta* Lorenz, 1881, can kill even their massive mammalian hosts, Asian and African elephants, respectively.[6,7]

Whereas some fasciolids have retained circumscribed geographic distributions, others such as *F. hepatica* have become cosmopolitan.[8] The spread of this species is in part caused by its adaptability to different lymnaeid snail hosts and to introduction of infected livestock or of susceptible snail hosts into new areas. In its new environments, for example, in the altiplano of Bolivia, *F. hepatica* can be responsible for significant public health problems.[9] To gain a better perspective on the biology of fasciolid species of medical and/or veterinary importance and to more fully understand the evolutionary history in this and other fluke families, comparative studies that include a greater breadth of fasciolid flukes are needed.

Although the taxonomy of fasciolid flukes has been reviewed recently and a classification scheme has been proposed based on adult morphology,[10] hitherto lacking have been inclusive phylogenetic studies using molecular characters to provide perspective on the taxonomy of the family. Molecular phylogenetic studies will also help us to better understand the origins, radiation, evolution, and patterns of host use of these important trematodes. Accordingly, the aim of this study is to use molecular DNA sequence data to reconstruct the phylogenetic relationships among the species in the Fasciolidae to 1) test the monophyly of the family, 2) investigate the relationships between the different species and establish hypotheses for their origins and diversification; 3) examine how host switching may have influenced the evolution of fasciolids; and 4) synthesize some of the morphological trends that have taken place among the adult flukes in this family.

MATERIALS AND METHODS

Specimens

Samples from seven of the nine recognized species of Fasciolidae were collected during this study (Table 1). One or two individuals from each species or strain were used for the molecular study.

Molecular analysis

Genomic DNA was extracted from a small part of the apical zone, to avoid inclusion of female genitalia likely to include foreign sperm, by the alkaline-lysis (Hot-SHOT) method,[11] in a final volume of 400 mL of storage buffer. For some samples, we used an Aqua-Pure Genomic DNA kit (Bio-Rad Laboratories, Hercules, CA). The extracted DNA was used either immediately for PCR amplification or stored at -20°C until used. Nucleotide sequences of the internal transcribed spacer regions of the small subunit (SSU) ribosomal DNA (rDNA) gene have proven to be helpful in delineating genera and species in the Fasciolidae.[12] Also, mitochondrial genomes have been found useful to investigate phylogenetic relationships among trematodes at many levels.[13] Thus, in this study, nuclear rDNA 28S[14] and internal transcribed spacer regions (ITS1 and ITS2), and partial mitochondrial NADH dehydrogenase subunit 1 (*nad*1) were sequenced and analyzed to study the phylogenetic relationships among the seven species of fasciolids we obtained. The volume of each amplification reaction was 20 μL with 200 ng

of DNA, 0.8 mmol/L dNTPs, 2 mmol/L $MgCl_2$, 0.5 μmol/L of each primer, 0.5 units *Taq* DNA Polymerase (Promega, Madison, WI), and 2 μL 10× PCR buffer.

To amplify the ITS1 region, we used the primers[15] BD1 [5′-GTC GTA ACA AGG TTT CCG TA-3′] and 4S [5′-TCT AGA TGC GTT CGA AGT GTC GAT G-3′]. The ITS2 region was amplified using the primers GA1 [5′-AGA ACA TCG ACA TCT TGA AC-3′][16] and BD2 [5′ TAT GCT TAA ATT CAG CGG GT 3′].[17] The mitochondrial DNA region *nad*1 was amplified using the primers of Bowles and McManus[18]: JB11 [5′-AGA TTC GTA AGG GGC CTA ATA-3′] and JB12 [5′-ACC ACT AAC TAA TTC ACT TTC-3′]. Polymerase chain reaction (PCR) cycles were performed on Eppendorf Mastercycler epigradient machines. For ITS1 and ITS2, the thermocycler was programmed as follows, with a 1°C/s rate of change: 1 cycle of 95°C for 1 minute, 55°C for 2 minutes, and 74°C for 1 minute 30 seconds, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute 30 seconds, plus a final extension step for 7 minutes. For *nad*1, the conditions were identical, except the annealing temperature was 45°C. Amplified products were visualized by electrophoresis in 1.5% agarose gels and detected by staining with GelRed nucleic acid gel stain (Biotium, Hayward, CA). PCR products were purified using PCR Microcon columns (Millipore, Billerica, MA), and both strands were sequenced using an Applied Biosystems 3100 automated sequencer (BigDye terminator cycle sequencing kit; Applied Biosystems, Foster City, CA).

Sequence alignment and phylogenetic analysis

Sequences for 28S, ITS1 and ITS2 rDNA, and *nad*1 mtDNA were assembled and edited using Sequencher ver. 4.6 (Gene Codes, Ann Arbor, MI). Alignments were made by eye and edited in Se-Al (A Rambaut, 1996, Se-Al: Sequence Alignment Editor, http://evolve.zoo.ox.ac.uk). Sequences generated in this study were submitted to GenBank (Table 1). Phylogenetic analyses using standard methods of maximum parsimony (MP), maximum likelihood (ML), and minimum evolution (ME) were carried out using PAUP* ver. 4.0b10[19] and Bayesian inference using the program MrBayes.[20] Modeltest[21] was used to determine the best nucleotide substitution model based on Akaike Information Criteria (AIC) for the combined data to use for ML and ME analyses.

To place members of the family in a larger phylogenetic context of the superfamily, the 1,255 base pairs of the 28S region were sequenced, and outgroups were selected from the Echinostomatoidea.[22] The GTR+G model from Modeltest was used for this 28S dataset with the following parameters: base frequencies = 0.20, 0.21, and 0.31; rate matrix = 0.60, 3.8, 1.9, 0.12, 6.4, and 1.0; pinvar = 0.42; gamma distribution = 0.9. Gaps were treated as missing data. The GTR+G model from Modeltest was used for the combined dataset of ITS and *nad*1 with the following parameters: base frequencies = 0.19, 0.18, and 0.27; rate matrix = 0.60, 2.9, 1.3, 0.25, 2.0, and 1.0; gamma distribution = 0.5. Gaps were treated as missing data. To explore the data for inconsistencies and test the usefulness of the genes used, each gene was analyzed independently, and the genes were combined. For all datasets, parsimony trees were reconstructed using heuristic searches (500 replicates), random taxon-input order, and tree-bisection and reconnection (TBR) branch swapping. Optimal ME and ML trees were determined from heuristic searches (500 replicates for ME, 100 replicates for ML), random taxon-input order, and TBR. Nodal support was estimated by bootstrap (500 replicates) and was determined for the MP and ME trees using heuristic searches (10 replicates); 100 replicates were used for a bootstrap using ML, each with random taxon-input order.

For the Bayesian analysis, 28S commands were the following: Nst = 6 rates = gamma ngammacat = 4, followed by the following parameter settings: unlink shape = (all) pinvar = (all) statefreq = (all) revmat = all; preset ratepr = variable. In the combined dataset, there were two partitions, ITS1 and ITS2 (Nst = 6 rates = gamma ngammacat = 4) and *nad*1 codon positions codon1, codon2, and codon3 (Nst = 6 rates-invgamma ngammacat = 4), followed by

the following parameter settings: unlink shape = (all) pinvar = (all) statefreq = (all) revmat = all; preset ratepr = variable. For each dataset, four chains were run simultaneously for 3×10^5 generations, trees were sampled every 100 cycles, the first 3,000 trees with preasymptotic likelihood scores were discarded as burn-in, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities.

RESULTS

A phylogenetic tree based on the 28S (548 bp) DNA sequence data was reconstructed (Figure 1) to place members of the Fascioloidae in broader context relative to its hypothesized closest relative digenean families.[22] Tree topology of the MP, ME, and Bayesian analyses were the same. However, only the Bayesian analysis resulted in moderate node support for a basal position of *Protofasciola* (Protofasciolinae). The ML analysis placed *Protofasciola* as unresolved. To explore relationships within the Fascioloidae, a dataset of ITS (367 bp) and *nad*1 (488 bp) was analyzed. For the ITS and *nad*1 combined dataset, all nodes in the tree presented in Figure 2 were robust and recovered the following relationships: a basal position in the family for *P. robusta*, an intermediate position for members of the Fasciolopsinae (*F. buski* and *P. fasciolaemorpha*), and a derived position for the Fasciolinae (*Fasciola* and *Fascioloides*), similar to the 28S dataset. *Fasciola* as currently defined seems to represent a paraphyletic assemblage.

DISCUSSION

This study is the most complete phylogenetic analysis of the Fasciolidae thus far undertaken and includes molecular data for representatives of five of the six known fasciolid genera and seven of nine described species, retrieving a monophyletic group. The comments provided below are the first attempt that we know of to synthesize the evolutionary history across the family.

Evolutionary relationships among the fasciolids

In general, our molecular trees were in agreement and retrieved *P. robusta* as the basal fasciolid (Figure 3). This species, the sole member of the Protofasciolinae, is an inhabitant of the small intestine of the African elephant. There has been one report of *P. robusta* in Asian elephants, but this report was in error (M. E. Fowler, personal communication).[23] Some of the prominent morphological characters that distinguish it from other fasciolids are an unspined tegument, testes and ovaries that are entire as opposed to dendritic, and simple non-sinuous and unbranched caeca.[10] The life cycle and snail host for this basal fasciolid unfortunately remain unknown.

Fasciolopsis buski is the next most basal member of the Fasciolidae. This species is one of two representatives of the Fasciolopsinae and is noteworthy for inhabiting the small intestine of suids and humans in Eastern Asia (Figure 3). This fluke has a large linguiform body with sinuous but unbranched intestinal caeca and dendritic testes and ovary.[10] This fluke parasitizes small planorbid snails (*Segmentina* spp. and *Hippeutis* spp.) in Asia.[24]

The next branch on the molecular tree is occupied by the other member of the Fasciolopsinae, *Parafasciolopsis fasciolaemorpha*, which occurs in the liver and bile ducts of cervids in Europe (Figure 3). This species also possesses sinuous, unbranched intestinal caeca and dendritic testes, but the ovary is entire.[10] This is the most basal fasciolid to possess the cephalic cone that is prominently developed in most derived fasciolids. This fasciolid parasitizes the planorbid snail *Planorbarius corneus*.[25]

The remaining clade on the tree is occupied by members of the two genera available for molecular study within the Fasciolinae: *Fasciola* (represented by three species) and the monotypic *Fascioloides* (represented by *F. magna*). All members of the Fasciolinae are united by having branched intestinal caeca and dendritic testes and ovaries, and all colonize either the bile ducts or liver parenchyma of their definitive hosts (Figure 3). Where the molluscan intermediate hosts are known (*F. hepatica, F. gigantica, F. nyanzae*, and *F. magna*), lymnaeid snails are implicated in transmission.[26] *Fasciola hepatica* and *F. gigantica* are retrieved as sister species, which is not surprising given that they share several morphological features. Moreover, these two species hybridize in nature.[27-30]

Interestingly, the fasciolid of Indian elephants, *Fasciola jacksoni*, groups more closely with *F. magna*, a parasite of North American cervids, than with other species of *Fasciola* (except in the Bayesian analysis where *F. jacksoni* is sister to the clade containing *F. gigantica, F. hepatica*, and *F. magna*). Both flukes are relatively thick-bodied, lack a distinctive cephalic cone, and have long median (internal) intestinal branches, which are relatively short in *F. hepatica* and *F. gigantica*. Based on our data, placement of *F. jacksoni* in *Fascioloides* should be considered: *Fasciola* as currently defined is paraphyletic. *F. jacksoni* is well known as an Asian elephant fasciolid, and although there is one anecdotal reference to this species occurring in African elephants,[30] there is no published reference supporting that report.

The two remaining fasciolid species that were unavailable for study are *Tenuifasciola tragelaphi* from the sitatunga[31] and *Fasciola nyanzae* from the hippo,[32] both of which inhabit the livers of their respective hosts. In both species, the body is elongated compared with other fasciolids, but they have the morphological features characteristic of the Fasciolinae (cephalic cone, branched intestinal caeca, and reproductive organs), suggesting they are derived fasciolids.

Snail hosts

The results of this study are suggestive of certain trends that seem to have characterized the evolution of this relatively small family of flukes, although more complete information is required, particularly regarding the life cycles of several species, including for the most basal species, *P. robusta*. Given that *F. buski* and *P. fasciolaemorpha* (both of the Fasciolopsinae) are the closest relatives of *P. robusta* and that they use planorbid snails as first intermediate hosts, the Planorbidae would be the most reasonable family in which to begin to search for the intermediate host for *P. robusta* (Figure 3). Also needed for this species is a determination that its cercariae encyst on vegetation, thus making its life cycle dependent on ingestion of metacercariae on vegetation, a constant feature across the Fasciolidae for which life cycles are known.

Of the six species in the more derived Fasciolinae, four are well known for their use of lymnaeid snails as first intermediate hosts; the snail hosts for the remaining two species are unknown. Thus, it seems clear that there has been a major host switch from one basommatophoran family, the Planorbidae, to another, the Lymnaeidae, as the fasciolids diversified (Figure 3).

The likely ancestral snail host for *F. hepatica* and certainly the most important snail host of this species today is *Lymnaea truncatula* of Europe, Africa, and South West Asia.[26] *Fasciola gigantica* is transmitted by the more fully aquatic lymnaeid species *Radix auricularia* in Asia and *Lymnaea natalensis* in Africa. The latter species also transmits *F. nyanzae*.[32] *Fasciola hepatica* has been more successful than *F. gigantica* in exploiting diverse lymnaeid lineages.[33] Interestingly, these diverse lymnaeid snails are united by their preference for shallow water or muddy banks, raising the possibility that habitat preference more than phylogenetic affinity may help to explain patterns of snail host use in this most cosmopolitan of fasciolids.

Biogeography of Fasciolidae

The presence of the most basal fasciolid in African elephants is suggestive of an origin of fasciolids in proboscideans that originated in Africa ~50 million years ago (MYA).[34] Proboscideans dispersed toward and within Eurasia from ~18.5 to 0.8 MYA,[35] where they underwent extensive radiation. Perhaps as a result of the northward migration and radiation of proboscideans, the next most basal species, *F. buski* and *P. fasciolaemorpha*, are also found in Eurasia. It is noteworthy that the extant Asian elephant lacks a species comparable to the ancestral fasciolid, *P. robusta*. Both suids, as future hosts for *F. buski*, and cervids, as future hosts for *P. fasciolaemorpha*, would have been present in Eurasia at the time proboscideans first colonized this land mass.[36-38] Proboscideans are usually found to be one of the most diverse orders of mammals in fossil deposits.[39-42] Similarly, this might suggest that their fasciolid parasites radiated with them. Subsequently, it also suggests that with the widespread extinctions of these potential proboscidean hosts, followed a wide spread extinction of a majority of fasciolid diversity and might explain the relatively small number of extant species of fasciolids and the odd mix of current definitive hosts.

The origins of members of the Fasciolinae are more problematic to discern: two of the six known species, *F. nyanzae* from hippos and *T. tragelaphi* from sitatunga, are today exclusively African. However, although hippos probably originated in Africa, ~16 million years ago, they radiated extensively in Eurasia where they are now extinct.[43] *Fasciola gigantica* is common and widespread in Asia, Africa, and Hawaii, and *F. jacksoni* is a parasite of the Indian elephant. *Fasciola hepatica* is likely of Eurasian origin given its clear host preference for *L. truncatula* of that region.[26] It seems likely that a host switch from planorbids to lymnaeids occurred in Eurasia and that this favored the emergence of the Fasciolinae, with colonization of Africa occurring secondarily, both by *F. gigantica* and an apparent ancestor of *F. nyanzae* in hippos and *T. tragelaphi* in sitatungas, both hosts sharing common habitats.

Fasciola jacksoni is of particular interest for two reasons. Its presence in Indian elephants is suggestive of a second, independent colonization of proboscideans by fasciolids. Also of interest is its relationship to the North American cervid parasite, *Fascioloides magna*. One possibility is that proboscideans brought a fasciolid with them to the Nearctic and that descendents of this parasite that shifted into cervids in North America were able to persist after the extinction of proboscideans in the Americas. However, it should be kept in mind that the first descriptions of *F. magna* were from Italy.[44] Although these reports came from North American cervids imported to that country and it is therefore likely that the animals acquired their *F. magna* infections in North America, another possibility that should be kept in mind is that *F. magna* is Eurasian in origin and later colonized North America where it became common and widespread, in contrast to Eurasia where it persisted at low levels.

Habitat use by adult fasciolids

The fasciolids are also of particular interest with respect to the evolution of body form and habitat use among their adult life cycle stages. The two most basal species in the phylogeny, *P. robusta* and *F. buski*, both colonize the small intestine of their respective hosts. The next species retrieved in the phylogeny, *P. fasciolaemorpha*, inhabits both the duodenum and the bile ducts[25] of its cervid hosts. All three species are characterized by unbranched caeca and a ventral sucker that is large relative to the oral sucker. The remaining species, all of which have an extensively branched gut and relatively small ventral suckers, live either in the biliary tree (*F. hepatica, F. gigantica, F. jacksoni F. nyanzae*, and *T. tragelaphi*) or in the liver parenchyma in a fibrous capsule that communicates with bile ducts (*F. magna*). Thus, two prominent trends in the evolution of the family seem to be a switch in habitat within the definitive host from the small intestine to the liver and arborization of the parasite gut. The habitat switch is presumably accompanied by a shift in diet of adult worms from the partially digested contents of the small

intestine to blood cells or parenchyma or epithelial cells of the liver.[45] The complex parasite gut architecture may have allowed the worms to digest more complex dietary substrates found in the liver. Dendritic gut architecture would also have the general benefit of reducing diffusion distances for both nutrients and waste products for these large-bodied flukes. Alternatively, branching of the gut may have allowed development of large body size, rather than being necessary to accommodate dietary changes. Also, the reduction in size in the ventral sucker noted for fasciolids that inhabit the liver may have been in response to the lack of pronounced peristaltic action characteristic of the mammalian intestine that does not occur in the liver.

The adoption of branched ovaries and testes also reaches its highest manifestation in the Fasciolinae, although clearly evolution in this direction has already occurred in the more basal and large-bodied *F. buski* that inhabits the small intestine. A branched structure for the ovary and testes would again have the general benefit of facilitating diffusion of nutrients to these metabolically active organs. Adoption of highly branched organs may be indicative of the approach of fasciolids to a body size that can be supported without a circulatory system, particularly given that they live in endothermic hosts that would also increase their own metabolic rates.

A discussion of the evolution of the fasciolids must also consider the extraordinary size achieved by the adult worms, all of which are parasites of large-bodied herbivorous or omnivorous mammals (Figure 3). One of the more derived fasciolids, *F. magna*, has body dimensions of 3-7.3 × 2-3 cm and thickness 0.2-0.45 cm, making it one of the largest of all fluke species. Large body size is not a feature unique to liver-inhabiting representatives, however, because the basal, intestine-inhabiting species *P. robusta* is itself a large, thick-bodied fluke that is 2 cm in length. Perhaps big body size as a family-wide trait reflects conservative retention of this trait from an ancestral fasciolid that inhabited a very large definitive host such as a proboscidean.

Long-range host switches

The Fasciolidae shares with two other relatively well-known digenean families, the Schistosomatidae and Paragonimidae,[46] evidence for having undergone prominent host switches with respect to the molluscan host, but the patterns observed in each case differ. In the Schistosomatidae, shifts from marine to freshwater gastropod lineages have occurred, as have shifts from caenogastropods to pulmonates and from pulmonates to opisthobranchs.[46, 47] In the Paragonimidae, host shifting has been less extensive, but nonetheless, two different caenogastropod superfamilies have been exploited.[46] In the Fasciolidae, the shift between two basommatophoran families is by comparison modest but still noteworthy given the small size of this fluke family and that this switch has undoubtedly played a major role in the emergence of fasciolids of both veterinary and public health importance.

The fasciolids provide yet another example of digeneans that exhibit host specificity with respect to their snail hosts and show an evolutionary history in which long-range shifts with respect to the snail host have clearly occurred.[47] The beginnings of a resolution to this paradox may lie in the work of Abrous and others,[48] who noted that the planorbid snail *Planorbis leucostoma* Millet, 1813, could support cercariae-producing infections of *F. hepatica* if first infected with another trematode, *Paramphistomum daubneyi*.[24] This is of interest for two reasons: it may be indicative of retention of some ability of fasciolids to infect planorbid snails, which, as noted above, are the snail hosts of more basal fasciolid species, and second, this provides a potential mechanism to explain how long-range host shifts might occur within particular digenean families.

CONCLUSIONS

The Fasciolidae is a relatively small group of digeneans that are hypothesized to have originated in African proboscideans and that later radiated in Eurasian herbivores. They likely all share a similar life cycle pattern with the vertebrate host becoming infected by ingesting metacercariae on vegetation, although this remains to be shown in a few pivotal species. Host shifts have occurred in both molluscan and mammalian hosts as the family diversified, and a trend toward abandoning the intestine for the liver is evident. Fasciolids are large flukes that also show a trend toward arborization of internal organs, and they may approach the upper limit of body size without a separate circulatory system. *Fasciola* as currently conceived is paraphyletic, and *F. jacksoni* might be considered a representative of *Fascioloides*. Acquisition of life cycle details for *F. jacksoni* should instruct any changes in nomenclature.

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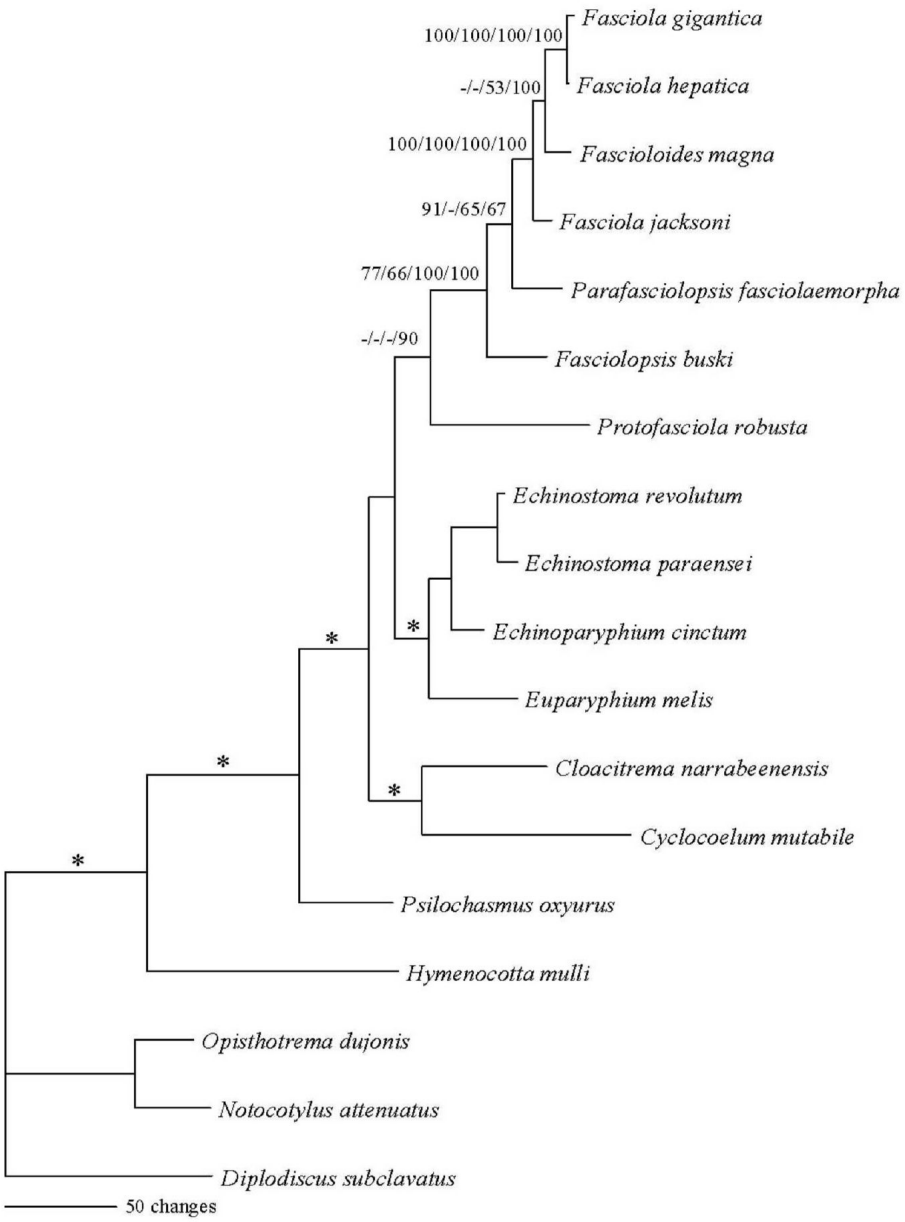

Figure 1.

Bayesian estimated tree from partial 28S rDNA. Nodal support values are indicated on the branch as bootstrap values for MP/ME/ML/Bayesian posterior probabilities. *More than 95% branch support for all four analyses for the outgroup taxa.

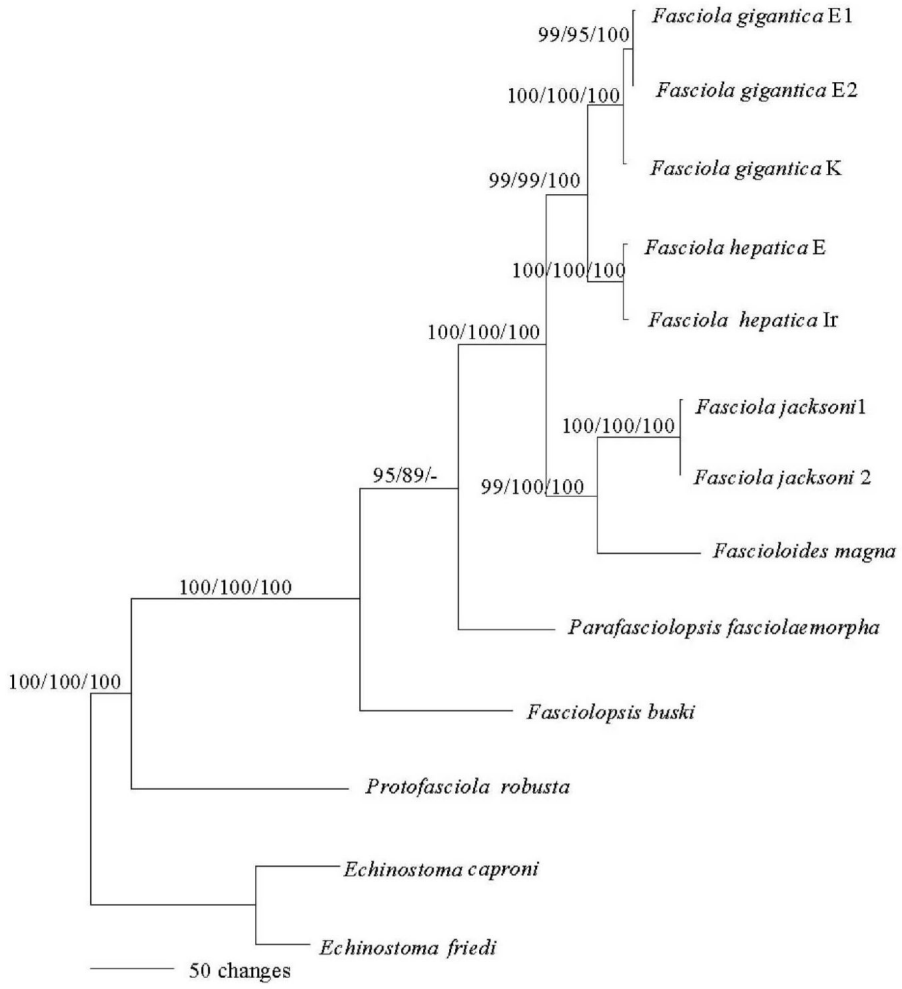

Figure 2.

Bayesian estimated tree from the combined data partitions of ITS1, ITS2, and partial *nad*1 genes. Nodal support values are indicated on the branch as bootstrap values for MP/ME/ Bayesian posterior probabilities (E, E1; E2, Egyptian; K, Kenyan; Ir, Iranian).

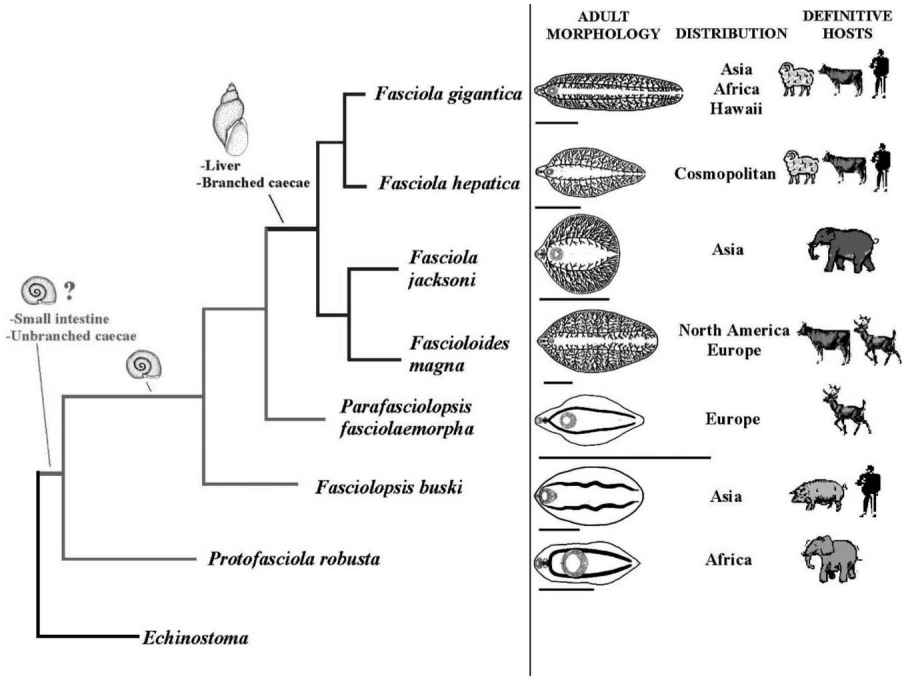

Figure 3.

Tree simplified from Figure 2 to map significant patterns and changes in hosts, habitat, morphology, and distribution. Snail at base of the tree represents the family Planorbidae, the other snail family is Lymnaeidae. Each illustrated fluke indicates the intestinal caeca (scale bar: 10 mm).

Table 1

The fascioloid specimens and outgroups used in the present study, their hosts, geographical origins, and the GenBank accession number for each sequenced DNA region

Species	Host	Location	GenBank accession number			
			28S	ITS1	ITS2	*nad*1
Fasciola hepatica	Domestic water buffalo	Alexandria, Egypt	EU025874	EF612467	EF612479	EF612491
F. hepatica	Domestic water buffalo	Alexandria, Egypt		EF612468	EF612480	EF612492
F. hepatica	Domestic sheep	Tehran, Iran		EF612469	EF612481	EF612493
F. gigantica	Domestic water buffalo	Alexandria, Egypt		EF612470	EF612482	EF612494
F. gigantica	Domestic water buffalo	Alexandria, Egypt		EF612471	EF612483	EF612495
F. gigantica	Domestic cow	Njiru, Kenya	EU025873	EF612472	EF612484	EF612496
F. jacksoni	Asian elephant	Sri Lanka	EU025871	EF612473	EF612485	EF612497
F. jacksoni	Asian elephant	Sri Lanka		EF612474	EF612486	EF612498
Fascioloides magna	White-tailed deer	Minnesota, U.S.A.	EU025872	EF612475	EF612487	EF612499
Parafasciolopsis fasciolaemorpha	European bison	Białowieża, Poland	EU025869	EF612476	EF612488	EF612500
Fasciolopsis buski	Domestic pig	Hanoi, Vietnam	EU025870	EF612477	EF612489	EF612501
Protofasciola robusta	African forest elephant	Africa	EU025868	EF612478	EF612490	EF612502
Diplodiscus subclavatus			AY222212			
Notocotylus attenuatus			AF184259			
Opisthotrema dujonis			AY222223			
Hymenocotta mulli			AY222239			
Psilochasmus oxyrurus			AF151940			
Cloacitrema narrabeenensis			AY222248			
Cyclocoelum mutabile			AY222249			
Euparyphium melis			AF151941			
Echinostoma paraensei			EU025867			
E. caproni				AJ564382	AJ564382	AJ564378
E. friedi				AJ564383	AJ564383	AJ564379
Echinoparyphium cinctum			AF184260			