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Substantial genetic divergence between morphologically indistinguishable populations of *Fasciola* suggests the possibility of cryptic speciation★

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★This study contains GenBank submissions JX236561-JX236579; JX236580-JX236590; JX236591-JX236639; JX236643-JX236655; JX236508-JX236560; and JX236640-JX236642.

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Abstract

The liver flukes, *Fasciola hepatica* and *Fasciola gigantica*, are considered to be sister species and between them present a major threat worldwide to livestock production. In this study sequence data have been employed from informative regions of the nuclear and mitochondrial genomes of over 200 morphologically *F. hepatica*–like or *F. gigantica*–like flukes from Europe, sub-Saharan Africa and South Asia to assess genetic diversity. Evidence is presented for the existence of four well-separated clades: African *gigantica*-like flukes, Indian *gigantica*-like flukes, European *hepatica*-like flukes and African high-altitude *hepatica*-like flukes. Application of the Biological Species Concept to trematodes is problematic; however, the degree of separation between these groups was sufficient for them to be considered as distinct species using the four times rule for speciation.

Keywords: *Fasciola*, Genetic diversity, Cryptic species
1. Introduction

Fasciolosis, a food-borne infection by fasciolid trematodes (most commonly Fasciola hepatica or Fasciola gigantica) causes losses to agriculture estimated at US $2,000 million per annum (McManus and Dalton, 2006). It is also an emerging zoonotic disease with up to 17 million people thought to be infected worldwide (Hopkins, 1992) and more than 90 million (Keiser and Utzinger, 2005) at risk of infection. In the developing world the economic impact of fasciolosis may be especially severe as there is a heavy reliance on buffalo and oxen as draught animals.

The spread and increased incidence of fasciolosis due to climate change, drug resistance and intensification of agriculture have already been noted with F. hepatica in Europe and may be expected to occur with F. gigantica in the developing world (Mas Coma et al., 2009).

The evolution of F. hepatica and F. gigantica, together with other members of the Fasciolidae, has recently been studied in detail (Lotfy et al., 2008) and the analysis presented by these authors resulted in F. hepatica and F. gigantica being grouped as sister species. Fasciola hepatica has a cosmopolitan distribution, being reported from Europe, the Americas, Australasia and the more temperate regions of Africa and Asia, whereas F. gigantica appears to be more restricted in its range, being reported from the tropical regions of Asia and Africa, and Hawaii (introduced in the 19th century) (Torgerson and Claxton, 1999). It is possible that outside Eurasia, the distribution of F. hepatica is the result of anthropogenic introductions in historical times to the Americas and Oceania (Mas Coma et al., 2009). Irrespective of the region, the successful establishment of either F. hepatica or F. gigantica is dependent on the distribution of its preferred intermediate host (Walker et al., 2008). Although domestic bovine and ovine herbivores sustain the bulk of the fasciolid population,
both *F. hepatica* and *F. gigantica* appear to be generalist parasites with regard to their mammalian hosts and, thus, they are capable of infecting a wide range of species (Torgerson and Claxton, 1999). This lack of specialisation with regard to the definitive host, in which sexual reproduction takes place, might be expected to restrict their evolutionary divergence (Maynard Smith, 1966). The phylogenetic proximity of *F. hepatica* and *F. gigantica* has been recently underscored by the finding that the transcriptomes of *F. gigantica* and *F. hepatica* showed homology (Blastx, $E < 1E^{-05}$) for almost 90% of the protein sequences examined (Young et al., 2011). Where intermediate hosts are present which can support both *F. hepatica* and *F. gigantica*, such as in eastern Asia, hybrids between the species have been reported (reviewed in Mas Coma et al., 2009). Such hybrids may be diploid, mixoploid or triploid and are generally aspermic. Some diploid isolates of *F. hepatica* may also be aspermic, suggesting that parthenogenic reproduction is relatively common in wild populations of fasciolids (Itagaki et al., 2009; Hanna et al., 2008). In studies where both mitochondrial and nuclear genes have been examined (Agatsuma et al., 2000; Itagaki et al., 2005a, b) it has been shown that the mitochondrial genomes of hybrid flukes from Japan and Korea may be of either *F. hepatica* or *F. gigantica* in origin as may their nuclear genes. In contrast, with flukes from Vietnam the mitochondrial sequences were exclusively of *F. gigantica* origin (Le et al., 2008). These differences indicate that hybridisation events are relatively common and have occurred independently on several occasions. Recently infra-populations of fasciolid flukes from Egypt and Iran have also been shown to contain individuals bearing nuclear genes derived from both *F. hepatica* and *F. gigantica* (Amer et al., 2011; Amor et al., 2011). Taken together, this body of work seems to indicate that where *F. hepatica* and *F. gigantica* are found in the same infra-population, hybridisation is a common
occurrence and the ability of these hybrids to reproduce parthenogenically allows the establishment of essentially clonal hybrid field populations. The aim of much of this work has been to determine whether flukes from a particular region should be regarded as *F. hepatica* or *F. gigantica* as this distinction is of importance with regard to epidemiology (Mas-Coma et al., 2005). However, in view of the range of reproductive strategies which may be operating in fasciolid populations (Fletcher et al., 2004) it is questionable how well the species concept (as defined by Mayr, 1963) can be applied to these trematodes (Kunz, 2002; de Meeûs et al., 2003). Moving from theoretical to practical matters, control and therapy of fasciolosis in the foreseeable future is likely to remain dependent on anthelmintic chemotherapy (Fairweather, 2011) or the development of a vaccine (McManus and Dalton, 2006). For these strategies to be applied successfully in the different regions where fasciolosis is a problem it is important that we have as full an understanding as possible of the variability inherent in the target liver fluke populations.

Working with mitochondrial genomic material it has been shown that *F. hepatica* populations can exhibit a high level of “intraspecific” diversity/divergence within a relatively confined geographic region (Walker et al., 2007, 2011) and that *F. hepatica*-like flukes can be found in geographical proximity to *F. gigantica* in highland regions of eastern Africa where the local climatic conditions favour the establishment of its preferred intermediate host, *Lymnaea (Galba) truncatula* (Walker et al., 2008). The most parsimonious explanation for the origin of the highland eastern African flukes is that they have been introduced relatively recently with livestock of European origin. In order to determine more precisely the relationship of these flukes to other populations of *F. hepatica* and *F. gigantica* a portion of the of their lsrRNA (nLSU/28S rRNA) (Teofanova et al., 2011) was sequenced as an
example of nuclear genomic material. This has been supplemented with sequences from the highly informative mitochondrial genome region coding for Cox III, tRNA-His and Cob (Walker et al., 2007) to determine mitochondrial lineages. Phylogenetic tools have been applied to determine whether the fasciolids in our African samples and in additional material from Europe and India could be characterised as either *F. hepatica* or *F. gigantica*. We believe that this is the first time that such an extensive dataset, in terms of geographic origin and number of samples (*n* = 200) has been analysed in this manner. Both nuclear and mitochondrial datasets indicate that the division of these fasciolids into two species is simplistic and may conceal the occurrence of cryptic speciation.

2. Materials and methods

2.1. Sources of fasciolid specimens

All samples of adult fasciolid worms were collected from abattoirs and thus represent “wild-type” specimens. The African material was obtained from Iringa (Tanzania), Mbeya (Tanzania), Kitulo (Tanzania), Njombe (Tanzania) and Amin el Ghaysu (Egypt). Details of the Tanzanian collection sites have been presented elsewhere (Walker et al., 2008). Indian samples were collected from Chennai (southern India) and Aligarh (northern India). Flukes of (ultimately) European origin were obtained from Ireland, The Netherlands, Greece and Australia. All flukes from regions below 2,500 metres in Tanzania originated from cattle: three from Iringa, three from Mbeya and one from Njombe. Highland (>2,500 m) Tanzanian flukes were from a single infrapopulation found in a cow from the Kitulo region. All adult flukes collected from India originated from water buffalo; one from Aligarh and one
from Chennai. Only one infrapopulation was sampled from Egypt, which was harboured in a donkey. Flukes from Ireland were obtained from a cow whereas fluke samples from The Netherlands (one), Greece (two) and Australia (one) were from sheep. Where sufficient numbers of flukes were available, up to 24 flukes from each infra-population were used for subsequent molecular analysis. All material was initially washed in distilled water prior to storage in 99% molecular grade ethanol.

2.2. Identification of fasciolid specimens

Flukes were initially classified on a morphological basis and their body length and width recorded (Walker et al., 2008). Flukes morphologically similar to *F. gigantica* had a mean body width to body length ratio of 2.85 whereas those morphologically similar to *F. hepatica* had a ratio of 1.59. Fasciolid species were later identified according to the PCR-Restriction Fragment Length Polymorphism protocol of Marcilla et al. (2002), based on the 28S rDNA gene.

2.3. DNA extraction from adult liver flukes

Approximately 25 mm² of fluke tissue were placed into 500 μl of 10% Chelex® (Fluka) solution incorporating 10 μl of Proteinase K (Sigma) at a concentration of 20 mg/ml. This was then heated in a heat block at 55°C for 1 h, followed by gentle vortexing and a further incubation at 95°C for 30 min. The mixture was then gently vortexed and centrifuged at 13,000 g for 10 s. A 200 μl sample of the supernatant was removed and diluted 1:10 in deionised water before storage at -20°C.

2.4. mtDNA analysis
Details of the region used for the identification of liver fluke mitochondrial haplotypes, their amplification by PCR and the subsequent sequencing of the amplicons have been given elsewhere (Walker et al., 2011). Briefly, this comprised 1,400 bp of contiguous mtDNA enclosing the regions coding for cytochrome c oxidase subunit III (cox III), transfer RNA histidine (tRNA-His) and cytochrome b (cob). Two primer sets were used to generate overlapping fragments in PCR: Primer set 1: Fhmt1.1F 5'-gcttgtggtttcttaggg-3', Fhmt1.1R 5'-caaccaaacctaacaacct-3'; Primer set 2: Fhmt1.2F 5'-tgtggtgctggagtctcttg-3' and Fhmt1.2R 5'-taaccatagctctgta-3. Fragment 1 consisted of nucleotides 77 to 881 of the complete *F. hepatica* mitochondrial sequence (Le et al., 2001), whilst fragment two ran from nucleotides 681 to 1,480. As the second primer set failed to amplify the morphologically *F. gigantica*-like flukes, an additional primer set was designed, Fgmt 1.2F 5'-ggtgtcggagagttctgttg-3' and Fgmt1.2R 5'-accaaatcaggaaaccaaa-3'. PCR products were purified as detailed elsewhere (Walker et al., 2011) and sequenced commercially by Macrogen (Korea). The sequences comprising the non-African *F. hepatica* dataset have been published previously (Walker et al., 2011; Teofanova et al., 2011) and accession numbers may be obtained from those sources. The Indian and African datasets have the following accession numbers: Aligarh, **JX236561-JX236579**; Chennai, **JX236580-JX236590**; Iringa, **JX236591-JX236639**; Kitulo, **JX236643-JX236655**; Mbeya, **JX236508-JX236560**; Njombe, **JX236640-JX236642**.

### 2.5. rDNA analysis

Primers 28SF 5' - agctgattacccgtgaact-3’ and 28SR 5’-ctgagaaagtgcactgacaag-3’ were used for amplification of the region from 15 bp to 632
bp (618 bp in length), with GenBank accession number **AJ440788** (*F. hepatica* partial
28SrRNA gene, Bolivia: Northern altiplano) (Marcilla et al., 2002) using the PCR
conditions described by Teofanova et al. (2011). PCR products were purified and
sequenced as above.

2.6. Data Analysis

Raw sequencing data (both directions in each case) were initially assembled
using ChromasPro (Technelysium Pty. Ltd, Australia) software and subsequent
alignments were carried out in BioEdit (Hall, 1999). All sequencing variants were
double checked on chromatogram traces before the derived sequences were allowed to
progress for further analysis. DNAsp (Rozas et al., 2003) was used to calculate the
haplotype diversity, average number of nucleotide differences between sequences and
nucleotide diversity (Nei, 1987) for each sample. To investigate the phylogenetic
relationships and relative frequency among the resulting mtDNA haplotypes, taking
into consideration the geographic origin of the samples, a haplotype network was
constructed using the median-joining method (Bandelt et al., 1999), implemented in
Network 4.5.0.2 (fluxus-engineering.com, Fluxus Technology Ltd., UK, 2004). A
Bayesian phylogenetic analysis on the resulting mtDNA haplotypes was also
performed using MrBayes version 3.2 (Ronquist and Huelsenbeck, 2003) using the
GTR+G model of DNA substitution, estimated using MODELTEST v3.0, Akaike
Information Criterion (Posada and Crandall, 1998). Four replicates of the Markov
chain Monte Carlo (MCMC) search were run with four chains of 10,000,000
generations each. Trees were sampled every 1,000 generations following a burn-in of
25,000 generations for each replicate. A *Fasciola jacksoni* sequence was used as an
outgroup for the analysis. In an attempt to provide a quantitative assessment of
whether the differentiation between the mtDNA groups/clades identified from the phylogenetic analyses was sufficient to confirm the existence of distinct species, the data were analysed according to the “4x rule” species criterion suggested by Birky et al. (2010). This approach is designed to identify clusters that are separated by $t \geq 4N_e$ generations (where $t$ is the time to most recent common ancestor and $N_e$ is the effective population size), which is equivalent to the upper 95% confidence limit of the coalescent time and the depth of separation formed by random drift. For two such clades the ratio of divergence between individuals from each clade ($K$) and $\theta$, the Watterson estimator of population mutation rate, is given by the equation $K/\theta = 2t\mu/2N_e\mu \geq 8N_e\mu/2N_e\mu = 4$. Although we do not have an accurate measure of $N_e$ or the mutation rate ($\mu$), these can be assumed to be the same for each clade and can thus be eliminated from the equation. Speciation may therefore be considered to have occurred (by this criterion) when the mean sequence divergence ($K$) between individuals in two candidate clades is greater than $4 * \theta$, where $\theta = \pi/(1-4\pi/3)$ is derived from the mean sequence difference between individuals within a clade, $\pi$; this parameter can be calculated from our sequence dataset.

3. Results

3.1. Differentiation according to morphology and 28S rDNA

Morphological criteria assigned all the Indian flukes to *F. gigantica*. All of the European flukes were classified as *F. hepatica* as were the African flukes from Kitulo (Tanzania) and Egypt. All other African flukes were of the *F. gigantica* phenotype.
The analysis of the 618 bp fragment from 28S rDNA is shown in Table 1. There were four positions at which nucleotide variation was observed. These occurred at positions 105, 130, 283 and 547. This variation was not in complete accordance with the morphological determination of species. At position 105, a substantial minority of the European and Egyptian flukes carried a G, as did all of the Indian and most of the Tanzanian flukes. At locus 130, all but one of the European, Australian and Egyptian flukes carried an A as did the flukes from Kitulo whilst the Indian and other African flukes had a G at this position. However, one European and one Tanzanian fluke were heterogeneous at this site. Position 283 distinguished between African “F. gigantica” and Indian “F. gigantica”, the former having a G as opposed to the A seen in the other populations. Again, two heterogeneous flukes were observed. Position 547 gave the best agreement with morphological classification with the F. hepatica-like flukes bearing a C at this position and the F. gigantica-like samples a T, although again two aberrant samples were noted. The heterogeneity seen at positions 130, 283 and 547 in the European and Iringa populations was due in each case to an individual fluke showing two alleles at each position.

3.2. Mitochondrial haplotype analysis

The fluke samples which provided mitochondrial sequence data are shown in Table 2 together with statistical data relating to the sequences. In general, this region of the mitochondrial genome was extremely variable with the number of haplotypes observed being approximately one-third of the sample size. The population from the highland region of Tanzania (Kitulo) was the most diverse with a very high average number of nucleotide differences between paired sequences and the highest levels of
nucleotide diversity; this was in contrast to the population from the lowland regions of Tanzania which was the least diverse.

A Bayesian tree constructed using the sequences of the unique mitochondrial haplotypes and rooted using the homologous region from *F. jacksonii* is shown in Fig. 1. The *F. gigantica*- and *F. hepatica*-like flukes were clearly separated into two deeply rooted clades. Within the “*gigantica*” clade there was a further division between the *F. gigantica*-like flukes from Africa and those from India. A division of comparable or greater depth existed between a subset of the highland (Kitulo) flukes and the rest of the *F. hepatica*-like population. There was strong posterior probability support (P=1) for each of these groupings. Within the *F. gigantica* African clade and the “European” *F. hepatica* clade there was little geographical structuring.

In order to substantiate these findings through the use of an alternative analytical tool, a Median Joining network was constructed using the mitochondrial dataset (Fig. 2). This employs a different algorithm and allows for the simultaneous existence of ancestral and derived haplotypes in the network – this is not possible using trees. This analysis indicated that there were four clearly separated clades based on geographic origin but with a subset of the highland Tanzanian flukes being more closely associated with the European population than others from the Kitulo region. The two *F. hepatica*-like clades were separated by almost three times the number of nucleotide substitutions separating the Indian and African *F. gigantica*-like clades.

### 3.3. Application of the 4x rule for speciation

The population statistics used to test for speciation in accordance with the 4x rule model are presented in Table 3. With the exclusion of the subset of highland
Tanzanian flukes, which clustered with the European *F. hepatica* population, it can be seen that each clade meets this criterion for speciation.

### 4. Discussion

This study was prompted by our interest in the morphologically *F. hepatica*-like liver flukes found in association with *Lymnaea truncatula*, the favoured intermediate host for *F. hepatica*, in areas of the Tanzanian highlands above 2,500 m (Walker et al., 2008). rDNA genes have been extensively employed as representatives of the nuclear genome in studies of speciation in digeneans (Lotfy et al., 2008) and the partial 28S subunit has been reported to be suitable for use in distinguishing between *F. hepatica* and *F. gigantica* (Marcilla et al., 2002). Liver flukes from eastern Europe are known to contain two 28S genotypes (Tefanova et al., 2011) and the relative proportions of these has been shown to correlate with environmental temperature when used in ecological niche modeling (Kantzoura et al., 2011). Analysis of the partial 28S sequences (Table 1) at the 130 position gave good discrimination between morphologically *hepatica*-like and *gigantica*-like flukes with *hepatica*-like flukes carrying the 130A genotype whilst the *gigantica*-like flukes carried a 130G transition. Position 283 was of interest in that it distinguished between the *gigantica*-like flukes of African origin and those from India, and position 547 was comparable to position 130 in its ability to discriminate between *hepatica*-like flukes and *gigantica*-like flukes. Taken in toto the results from the analysis of the variable positions in 28S rDNA indicate that the nuclear genome of the highland Tanzanian flukes is related to that of the subset of *F. hepatica* of European origin which has been associated with tolerance of high temperatures (Kantzoura et al., 2011) but they also reveal that *F. gigantica* from Africa and India may differ in their nuclear genomes.
This may be of importance as to date most research on *F. gigantica* has been undertaken with material from Asia with its applicability to African *F. gigantica* being assumed.

As expected, the mitochondrial genome region analysed was highly informative with regard to intra-population differences. The statistics presented in Table 2 indicate that the *F. hepatica*-like flukes from the Tanzanian highland region were by far the most diverse genetically. This was in contrast to the *F. gigantica*-like Tanzanian flukes which provided the least genetically diverse group. It has been proposed that *F. gigantica* originated in the east African lowlands and spread to Asia following the domestication of bovids (Mas Coma et al., 2009). In general, more recently colonised areas have lower genetic variability (Hewitt, 2000). The ranking order of liver fluke genetic diversity seen in Table 2 (European>Indian>African lowland) might be taken to imply that African populations of *F. gigantica* are less ancient than those of India but our results may be influenced more by the size of the geographic region from which the samples were collected than their relative age. A confounding factor may be the frequency with which population bottle-necks may have occurred – for example, Europe, southern Africa and India differ greatly in their history of glacial cycles. An explanation of the high level of genetic diversity seen in the highland Tanzanian flukes was provided by the analyses presented in Figs. 1 and 2 which show that this population is made up of two distinct clades. One of these was 20 nucleotide differences removed from the European *F. hepatica* clade whereas the other was over 70 substitutions distant (Fig. 2).

The mitochondrial data supported the distinction seen with the nuclear 28S rDNA gene in the African and Indian *F. gigantica*-like flukes in that these populations were separated into two well supported clades. There was a deep separation between
the morphologically *F. hepatica*-like and the *F. gigantica*-like flukes, with the former clade showing further sub-division into the flukes of ultimately European origin (Netherlands/Ireland/Greece/Australia/Egypt (Mas Coma et al., 2009)) compared with those from the Tanzanian highlands. Four of the five haplotypes present in the Tanzanian highland population formed a clade separated from the European flukes by at least 71 nucleotide substitutions (Fig. 2). Relating such data to a molecular clock is hazardous for reasons discussed in detail elsewhere (Walker et al., 2011), but assuming a rate of change for *F. hepatica* comparable with that reported for *Schistosoma mansoni* (Morgan et al., 2005) of 4% per million years, these 71 substitutions would be equivalent to approximately 1 million years, ruling out the possibility that they could have been introduced as parasites present in domesticated herbivores. The remaining haplotype present in the highland Tanzanian fluke population was represented by four flukes and was within the range of nucleotide diversity seen in European *F. hepatica*. As such it may have been introduced together with the cattle and sheep known to have been brought into this area in recent times (Walker et al., 2008).

Little evidence was seen that would indicate the presence of hybrid flukes in our populations. In all but two individuals the mitochondrial haplotype was consistent with the nuclear 28S rDNA genotype. These aberrant flukes from the European and Iringa populations were heterozygous at 28S rDNA positions 130, 283 and 547 which could indicate hybridization between an *F. hepatica*-like fluke and an African *F. gigantica*-like fluke in previous generations. Whilst this is possible for the Tanzanian fluke, it seems less likely in the case of the European fluke as it was from an infrapopulation present in a Dutch sheep and would thus be distant from known sources of *F. gigantica*. 
The results presented in this study show that the four geographical populations of *Fasciola* spp. can be assigned to four clades which are well separated genetically. Application of the 4x rule for speciation (Table 3) suggested that these clades are sufficiently divergent to be regarded as species under the definition forming the basis of this model (Birky et al., 2010). However, it has been proposed that in the absence of an internationally agreed definition of a species that is applicable to parasites we should refrain from naming new species “just on the basis of a certain number of base exchanges within their ribosomal DNA sequence” (Kunz, 2002). *Fasciola gigantica* and *F. hepatica* are well established as separate species and can be distinguished by a number of characteristics - morphologically (Periago et al., 2006), by their preferences for intermediate hosts (*Radix natalensis* does not appear to be capable of supporting the growth of *F. hepatica* (Boray, 1985)), the minimal temperature required for egg hatching (Grigoryan, 1958; Ross and McKay, 1929) and by the ability of *F. hepatica* to evade immune responses in the Indonesian Thin-Tailed sheep (Roberts et al., 1997). If the Tanzanian *F. hepatica*-like flukes and African *F. gigantica*-like flukes are separated from these populations due to the process of speciation we might expect to discover differences of comparable importance to their biology on closer examination (Nicolalde-Morejon et al., 2009; Nadler and Perez-Ponce de Leon, 2011). Indeed, prior to 1965 (when it was synonymised with *F. gigantica* (Kendall, 1965)) liver flukes from India and elsewhere in south Asia were classified as *Fasciola indica* (Varma, 1953). This distinction was made on morphological grounds, with their spines and eggs in particular showing distinct differences from both European *F. hepatica* and African *F. gigantica*. With further research on the four clades identified in this study it may become apparent that there
are distinct differences in their physiology, behaviour or morphology. If this is the case then it may be pragmatic at that stage to designate them as separate species.

In conclusion, it has been shown that four populations of *Fasciola* spp. from hosts located in geographically and climatically different environments form divergent lineages. It is to be presumed that the selective pressures which have brought about this situation may also have led to the evolution of other adaptive differences between the lineages. These may be expressed as biological characteristics such as host preference, tolerance to high or low temperatures, sensitivity to anthelmintics and immunoevasive mechanisms, all of which could be of importance to the future spread and control of fasciolosis.

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**Figure legends**

**Fig. 1.** Bayesian phylogenetic tree of liver fluke (*Fasciola gigantica* and *Fasciola hepatica*) mtDNA haplotypes (branch lengths are proportional to the number of inferred changes). Different colours/shades represent the geographic origin of clades/groups identified in this study. Nodal values represent posterior probability support for particular groups. Only values higher than 0.52 are shown. Taxon name codes: Fh, *Fasciola hepatica*; Fg, *Fasciola gigantica*; Du, Netherlands; In, India; S, south; N, north; Tz, – Tanzania; I, Iringa; M, Mbeya; Eur, Europe (Ireland/Greece); Ob, Australia. *Fasciola jacksonii* served as an outgroup.

**Fig. 2.** Median Joining Network for liver fluke (*Fasciola gigantica* and *Fasciola hepatica*) mtDNA haplotypes. Different colours/shades represent the geographic origins of clades/groups identified in this study. Sizes of nodes are proportional to frequency of the haplotype within the dataset and distance between nodes within a group is approximately proportional to the number of nucleotide changes between haplotypes. For clarity of presentation the minimal number of nucleotide substitutions between groups is indicated rather than displayed to scale.
Highlights

*Fasciola hepatica* and *Fasciola gigantica* from Africa, Europe and India have been compared. The analyses support the existence of four distinct clades. Cryptic speciation may have occurred, masking significant biological differences.
**Table 1**

28S rDNA diversity for fasciolids from different geographic regions.

<table>
<thead>
<tr>
<th>28S Position</th>
<th>Origin of Flukes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>European</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
</tr>
<tr>
<td>130</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>283</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>547</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T</td>
</tr>
</tbody>
</table>

Numbers indicate the number of flukes within a set (numerator) and the number from the respective geographic region sampled (denominator).

- a Due to the same Dutch fluke.
- b Due to the same Iringa fluke.
Table 2
Population and mitochondrial genome genetic statistics for fasciolids from different geographic regions.

<table>
<thead>
<tr>
<th>Populations</th>
<th>No. sequences</th>
<th>No. polymorphic sites</th>
<th>No. haplotypes</th>
<th>Ave no. nucleotide differences</th>
<th>Nucleotide diversity</th>
<th>Standard deviation</th>
<th>Haplotype diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>All African <em>F. gigantica</em>-like</td>
<td>109</td>
<td>49</td>
<td>32</td>
<td>3.947</td>
<td>0.00346</td>
<td>0.00041</td>
<td>0.854</td>
</tr>
<tr>
<td>All Indian <em>F. gigantica</em>-like</td>
<td>30</td>
<td>39</td>
<td>16</td>
<td>7.533</td>
<td>0.00662</td>
<td>0.00096</td>
<td>0.945</td>
</tr>
<tr>
<td>All European <em>F. hepatica</em></td>
<td>50</td>
<td>28</td>
<td>12</td>
<td>8.579</td>
<td>0.00748</td>
<td>0.00051</td>
<td>0.836</td>
</tr>
<tr>
<td>Kitulo TZ <em>F. hepatica</em>-like</td>
<td>13</td>
<td>80</td>
<td>5</td>
<td>37.641</td>
<td>0.03435</td>
<td>0.00705</td>
<td>0.731</td>
</tr>
</tbody>
</table>

Australian and Egyptian flukes are consolidated with European flukes.
Table 3

Variables associated with the populations used to test compliance with the “4x rule” for speciation.

<table>
<thead>
<tr>
<th>Population</th>
<th>Nucleotide diversity ((\pi))</th>
<th>0</th>
<th>0 x 4</th>
<th>Sequence divergence between clades (K)</th>
<th>K (\geq 40)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>All <em>Fasciola</em> spp.</td>
<td></td>
<td>0.10151</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European <em>F. hepatica</em></td>
<td></td>
<td>0.00748</td>
<td>0.00755</td>
<td>0.0302</td>
<td>Yes</td>
</tr>
<tr>
<td>All <em>Fasciola gigantica</em></td>
<td></td>
<td>0.01283</td>
<td>0.01283</td>
<td>0.05128</td>
<td>Yes</td>
</tr>
<tr>
<td>All <em>F. hepatica</em>-like</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European <em>F. hepatica</em></td>
<td></td>
<td>0.00748</td>
<td>0.00755</td>
<td>0.0302</td>
<td>Yes</td>
</tr>
<tr>
<td>Highland Tanzanian <em>F. hepatica</em></td>
<td></td>
<td>0.0029</td>
<td>0.0029</td>
<td>0.0116</td>
<td>Yes</td>
</tr>
<tr>
<td>All <em>F. gigantica</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African <em>F. gigantica</em></td>
<td></td>
<td>0.00346</td>
<td>0.00346</td>
<td>0.01384</td>
<td>Yes</td>
</tr>
<tr>
<td>Indian <em>F. gigantica</em></td>
<td></td>
<td>0.00662</td>
<td>0.00663</td>
<td>0.02652</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*a* Highland Tanzanian samples clustering with the European clade \(n = 4\) were excluded.