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

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1 Substantial genetic divergence between morphologically indistinguishable
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20 ★This study contains GenBank submissions JX236561-JX236579; JX236580-
21 JX236590; JX236591-JX236639; JX236643-JX236655; JX236508-JX236560; and
22 JX236640-JX236642.

23 **Abstract**

24 The liver flukes, *Fasciola hepatica* and *Fasciola gigantica*, are considered to be sister
25 species and between them present a major threat worldwide to livestock production.

26 In this study sequence data have been employed from informative regions of the
27 nuclear and mitochondrial genomes of over 200 morphologically *F. hepatica* –like or
28 *F. gigantica* –like flukes from Europe, sub-Saharan Africa and South Asia to assess
29 genetic diversity. Evidence is presented for the existence of four well-separated
30 clades: African *gigantica*-like flukes, Indian *gigantica*-like flukes, European *hepatica*-
31 like flukes and African high-altitude *hepatica*-like flukes. Application of the
32 Biological Species Concept to trematodes is problematic; however, the degree of
33 separation between these groups was sufficient for them to be considered as distinct
34 species using the four times rule for speciation.

35
36 **Keywords:** *Fasciola*, Genetic diversity, Cryptic species

37

38 1. Introduction

39 Fasciolosis, a food-borne infection by fasciolid trematodes (most commonly
40 *Fasciola hepatica* or *Fasciola gigantica*) causes losses to agriculture estimated at US
41 \$2,000 million per annum (McManus and Dalton, 2006). It is also an emerging
42 zoonotic disease with up to 17 million people thought to be infected worldwide
43 (Hopkins, 1992) and more than 90 million (Keiser and Utzinger, 2005) at risk of
44 infection. In the developing world the economic impact of fasciolosis may be
45 especially severe as there is a heavy reliance on buffalo and oxen as draught animals.
46 The spread and increased incidence of fasciolosis due to climate change, drug
47 resistance and intensification of agriculture have already been noted with *F. hepatica*
48 in Europe and may be expected to occur with *F. gigantica* in the developing world
49 (Mas Coma et al., 2009).

50 The evolution of *F. hepatica* and *F. gigantica*, together with other members of
51 the Fasciolidae, has recently been studied in detail (Lotfy et al., 2008) and the analysis
52 presented by these authors resulted in *F. hepatica* and *F. gigantica* being grouped as
53 sister species. *Fasciola hepatica* has a cosmopolitan distribution, being reported from
54 Europe, the Americas, Australasia and the more temperate regions of Africa and Asia,
55 whereas *F. gigantica* appears to be more restricted in its range, being reported from
56 the tropical regions of Asia and Africa, and Hawaii (introduced in the 19th century)
57 (Torgerson and Claxton, 1999). It is possible that outside Eurasia, the distribution of
58 *F. hepatica* is the result of anthropogenic introductions in historical times to the
59 Americas and Oceania (Mas Coma et al., 2009). Irrespective of the region, the
60 successful establishment of either *F. hepatica* or *F. gigantica* is dependent on the
61 distribution of its preferred intermediate host (Walker et al., 2008). Although
62 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

163 Details of the region used for the identification of liver fluke mitochondrial
 164 haplotypes, their amplification by PCR and the subsequent sequencing of the
 165 amplicons have been given elsewhere (Walker et al., 2011). Briefly, this comprised
 166 1,400 bp of contiguous mtDNA enclosing the regions coding for cytochrome c
 167 oxidase subunit III (cox III), transfer RNA histidine (tRNA-His) and cytochrome b
 168 (cob). Two primer sets were used to generate overlapping fragments in PCR: Primer
 169 set 1: Fhmt1.1F 5'-gcttggtgggttttcttaggg-3', Fhmt1.1R 5'-caaccaaactcaacaacct-3';
 170 Primer set 2: Fhmt1.2F 5'-tgtggtgctggagagttctg-3' and , Fhmt1.2R 5'-
 171 taaccatagatccgctga-3. Fragment 1 consisted of nucleotides 77 to 881 of the
 172 complete *F. hepatica* mitochondrial sequence (Le et al., 2001), whilst fragment two
 173 ran from nucleotides 681 to 1,480. As the second primer set failed to amplify the
 174 morphologically *F. gigantica*-like flukes, an additional primer set was designed, Fgmt
 175 1.2F 5'-ggtgctggagagttctgtg-3' and Fgmt1.2R 5'-accaaatacaggaaacaccaa-3'. PCR
 176 products were purified as detailed elsewhere (Walker et al., 2011) and sequenced
 177 commercially by Macrogen (Korea). The sequences comprising the non-African *F.*
 178 *hepatica* dataset have been published previously (Walker et al., 2011; Teofanova et
 179 al., 2011) and accession numbers may be obtained from those sources. The Indian
 180 and African datasets have the following accession numbers: Aligarh, **JX236561-**
 181 **JX236579**; Chennai, **JX236580-JX236590**; Iringa, **JX236591-JX236639**; Kitulo,
 182 **JX236643-JX236655**; Mbeya, **JX236508-JX236560**; Njombe, **JX236640-**
 183 **JX236642**.

184

185 2.5. rDNA analysis

186 Primers 28SF 5'- agctgattaccgctgaact-3' and 28SR 5'-
 187 ctgagaaagtgcactgacaag-3' were used for amplification of the region from 15 bp to 632

188 bp (618 bp in length), with GenBank accession number **AJ440788** (*F. hepatica* partial
 189 28SrRNA gene, Bolivia: Northern altiplano) (Marcilla et al., 2002) using the PCR
 190 conditions described by Teofanova et al. (2011). PCR products were purified and
 191 sequenced as above.

192

193 2.6. Data Analysis

194 Raw sequencing data (both directions in each case) were initially assembled
 195 using ChromasPro (Technelysium Pty. Ltd, Australia) software and subsequent
 196 alignments were carried out in BioEdit (Hall, 1999). All sequencing variants were
 197 double checked on chromatogram traces before the derived sequences were allowed to
 198 progress for further analysis. DNAsp (Rozas et al., 2003) was used to calculate the
 199 haplotype diversity, average number of nucleotide differences between sequences and
 200 nucleotide diversity (Nei, 1987) for each sample. To investigate the phylogenetic
 201 relationships and relative frequency among the resulting mtDNA haplotypes, taking
 202 into consideration the geographic origin of the samples, a haplotype network was
 203 constructed using the median-joining method (Bandelt et al., 1999), implemented in
 204 Network 4.5.0.2 (fluxus-engineering.com, Fluxus Technology Ltd., UK, 2004). A
 205 Bayesian phylogenetic analysis on the resulting mtDNA haplotypes was also
 206 performed using MrBayes version 3.2 (Ronquist and Huelsenbeck, 2003) using the
 207 GTR+G model of DNA substitution, estimated using MODELTEST v3.0, Akaike
 208 Information Criterion (Posada and Crandall, 1998). Four replicates of the Markov
 209 chain Monte Carlo (MCMC) search were run with four chains of 10,000,000
 210 generations each. Trees were sampled every 1,000 generations following a burn-in of
 211 25,000 generations for each replicate. A *Fasciola jacksoni* sequence was used as an
 212 outgroup for the analysis. In an attempt to provide a quantitative assessment of

213 whether the differentiation between the mtDNA groups/clades identified from the
 214 phylogenetic analyses was sufficient to confirm the existence of distinct species, the
 215 data were analysed according to the “4x rule” species criterion suggested by Birky et
 216 al. (2010). This approach is designed to identify clusters that are separated by $t \geq 4N_e$
 217 generations (where t is the time to most recent common ancestor and N_e is the
 218 effective population size), which is equivalent to the upper 95% confidence limit of
 219 the coalescent time and the depth of separation formed by random drift. For two such
 220 clades the ratio of divergence between individuals from each clade (K) and θ , the
 221 Watterson estimator of population mutation rate, is given by the equation $K/\theta =$
 222 $2t\mu/2N_e\mu \geq 8N_e\mu/2N_e\mu = 4$. Although we do not have an accurate measure of N_e or
 223 the mutation rate (μ), these can be assumed to be the same for each clade and can thus
 224 be eliminated from the equation. Speciation may therefore be considered to have
 225 occurred (by this criterion) when the mean sequence divergence (K) between
 226 individuals in two candidate clades is greater than $4 * \theta$, where θ [$\theta = \pi/(1-4\pi/3)$] is
 227 derived from the mean sequence difference between individuals within a clade, π ; this
 228 parameter can be calculated from our sequence dataset.

229

230 3. Results

231

232 3.1. Differentiation according to morphology and 28S rDNA

233 Morphological criteria assigned all the Indian flukes to *F. gigantica*. All of
 234 the European flukes were classified as *F. hepatica* as were the African flukes from
 235 Kitulo (Tanzania) and Egypt. All other African flukes were of the *F. gigantica*
 236 phenotype.

237 The analysis of the 618 bp fragment from 28S rDNA is shown in Table 1.
238 There were four positions at which nucleotide variation was observed. These
239 occurred at positions 105, 130, 283 and 547. This variation was not in complete
240 accordance with the morphological determination of species. At position 105, a
241 substantial minority of the European and Egyptian flukes carried a G, as did all of the
242 Indian and most of the Tanzanian flukes. At locus 130, all but one of the European,
243 Australian and Egyptian flukes carried an A as did the flukes from Kitulo whilst the
244 Indian and other African flukes had a G at this position. However, one European and
245 one Tanzanian fluke were heterogeneous at this site. Position 283 distinguished
246 between African "*F. gigantica*" and Indian "*F. gigantica*", the former having a G as
247 opposed to the A seen in the other populations. Again, two heterogeneous flukes
248 were observed. Position 547 gave the best agreement with morphological
249 classification with the *F. hepatica*-like flukes bearing a C at this position and the *F.*
250 *gigantica*-like samples a T, although again two aberrant samples were noted. The
251 heterogeneity seen at positions 130, 283 and 547 in the European and Iringa
252 populations was due in each case to an individual fluke showing two alleles at each
253 position.

254 3.2. Mitochondrial haplotype analysis

256 The fluke samples which provided mitochondrial sequence data are shown in
257 Table 2 together with statistical data relating to the sequences. In general, this region
258 of the mitochondrial genome was extremely variable with the number of haplotypes
259 observed being approximately one-third of the sample size. The population from the
260 highland region of Tanzania (Kitulo) was the most diverse with a very high average
261 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

286 Tanzanian flukes, which clustered with the European *F. hepatica* population, it can be
287 seen that each clade meets this criterion for speciation.

288

289 4. Discussion

290 This study was prompted by our interest in the morphologically *F. hepatica*-
291 like liver flukes found in association with *Lymnaea truncatula*, the favoured
292 intermediate host for *F. hepatica*, in areas of the Tanzanian highlands above 2,500 m
293 (Walker et al., 2008). rDNA genes have been extensively employed as
294 representatives of the nuclear genome in studies of speciation in digeneans (Lotfy et
295 al., 2008) and the partial 28S subunit has been reported to be suitable for use in
296 distinguishing between *F. hepatica* and *F. gigantica* (Marcilla et al., 2002). Liver
297 flukes from eastern Europe are known to contain two 28S genotypes (Tefanova et al.,
298 2011) and the relative proportions of these has been shown to correlate with
299 environmental temperature when used in ecological niche modeling (Kantzoura et al.,
300 2011). Analysis of the partial 28S sequences (Table 1) at the 130 position gave good
301 discrimination between morphologically *hepatica*-like and *gigantica*-like flukes with
302 *hepatica*-like flukes carrying the 130A genotype whilst the *gigantica*-like flukes
303 carried a 130G transition. Position 283 was of interest in that it distinguished between
304 the *gigantica*-like flukes of African origin and those from India, and position 547 was
305 comparable to position 130 in its ability to discriminate between *hepatica*-like flukes
306 and *gigantica*-like flukes. Taken in toto the results from the analysis of the variable
307 positions in 28S rDNA indicate that the nuclear genome of the highland Tanzanian
308 flukes is related to that of the subset of *F. hepatica* of European origin which has been
309 associated with tolerance of high temperatures (Kantzoura et al., 2011) but they also
310 reveal that *F. gigantica* from Africa and India may differ in their nuclear genomes.

311 This may be of importance as to date most research on *F. gigantica* has been
312 undertaken with material from Asia with its applicability to African *F. gigantica*
313 being assumed.

314 As expected, the mitochondrial genome region analysed was highly
315 informative with regard to intra-population differences. The statistics presented in
316 Table 2 indicate that the *F. hepatica*-like flukes from the Tanzanian highland region
317 were by far the most diverse genetically. This was in contrast to the *F. gigantica*-like
318 Tanzanian flukes which provided the least genetically diverse group. It has been
319 proposed that *F. gigantica* originated in the east African lowlands and spread to Asia
320 following the domestication of bovids (Mas Coma et al., 2009). In general, more
321 recently colonised areas have lower genetic variability (Hewitt, 2000). The ranking
322 order of liver fluke genetic diversity seen in Table 2 (European>Indian>African
323 lowland) might be taken to imply that African populations of *F. gigantica* are less
324 ancient than those of India but our results may be influenced more by the size of the
325 geographic region from which the samples were collected than their relative age. A
326 confounding factor may be the frequency with which population bottle-necks may
327 have occurred – for example, Europe, southern Africa and India differ greatly in their
328 history of glacial cycles. An explanation of the high level of genetic diversity seen in
329 the highland Tanzanian flukes was provided by the analyses presented in Figs. 1 and 2
330 which show that this population is made up of two distinct clades. One of these was
331 20 nucleotide differences removed from the European *F. hepatica* clade whereas the
332 other was over 70 substitutions distant (Fig. 2).

333 The mitochondrial data supported the distinction seen with the nuclear 28S
334 rDNA gene in the African and Indian *F. gigantica*-like flukes in that these populations
335 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

ACCEPTED MANUSCRIPT

Figure 1 colour

Table 1

28S rDNA diversity for fasciolids from different geographic regions.

28S Positi on	Origin of Flukes								
	Europ ean	Austral ian	Egypt ian	Sout h Indi an	Nort h Indi an	Tanzan ian Iringa	Tanzan ian Kitulo	Tanzan ian Mbeya	Tanzan ian Njomb e
105									
G	12/32	6/6	1/4	10/1	20/2	52/52		61/61	4/4
A	20/32		3/4	0	0		15/16 1/16		
G/A									
130									
G	31/32	6/6	4/4	10/1	20/2	51/52		61/61	4/4
A	1/32 ^a			0	0	1/52 ^b	16/16		
G/A									
283									
G	31/32	6/6	4/4	10/1	20/2	51/52		60/61	4/4
A	1/32 ^a			0	0	1/52 ^b	16/16	1/61	
G/A									
547									
C	31/32	6/6	4/4				16/16		
T	1/32 ^a			10/1	20/2	51/52		61/61	4/4
				0	0	1/52 ^b			
C/T									

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.

623

624

625 **Table 2**

626 Population and mitochondrial genome genetic statistics for fasciolids from different geographic regions.

627

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

628

629 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.

Population	Nucleotide diversity (π)	θ	$\theta \times 4$	Sequence divergence between clades (K)	$K \geq 4\theta$?
All <i>Fasciola</i> spp.				0.10151	
European <i>Fasciola hepatica</i>	0.00748	0.00755	0.0302		Yes
All <i>Fasciola gigantica</i>	0.01283	0.01283	0.05128		Yes
All <i>F. hepatica</i> -like				0.06595	
European <i>F. hepatica</i>	0.00748	0.00755	0.0302		Yes
Highland Tanzanian <i>F. hepatica</i> ^a	0.0029	0.0029	0.0116		Yes
All <i>F. gigantica</i> spp.				0.03052	
African <i>F. gigantica</i>	0.00346	0.00346	0.01384		Yes
Indian <i>F. gigantica</i>	0.00662	0.00663	0.02652		Yes

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