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Insight into the Genetic Relationship of *Fasciola gigantica* Inferred from LSU and ITS1 Ribosomal DNA

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Abstract
Current understanding of the level of genetic diversity in Fasciola gigantica is limited. For the first time we explore the level of molecular diversity of the flukes using all available LSU and ITS1 genes on GenBank. The LSU datasets revealed a higher molecular diversity in F. gigantica (H, 14; S, 195; Hd, 0.816; π, 0.13663; K, 55.19786) in comparison to the ITS1 datasets (H, 12; S, 53; Hd, 0.237; π, 0.00570; K, 2.04531). Overall intraspecific variability based on the ITS1 marker was 0.006 while the LSU was 0.142. Network analysis based on the LSU revealed two major F. gigantica lineages; China-Niger lineage which showed >172 mutational changes in relation to a lineage comprising of sequences from nine other countries.

Introduction
Fasciola gigantica (Cobbold, 1856) is one of the two aetiological agents of fascioliasis in most African and Asian countries were they are reported to negatively impact productivity in livestock management (1). Cases of human infection with *F. gigantica* have been reported in some countries thus highlighting the zoonotic importance of the fluke (2-6). Advances in molecular techniques within the last two decades, have seen the rise in the use of DNA sequences in the study of *F. gigantica* worldwide. Several genetic markers such as nuclear (large ribosomal subunit [LSU] and internal transcribed spacers 1 and 2 [ITS1/ITS2]) and/or mitochondrial (cytochrome c oxidase subunit 1 [*cox*1] and nicotinamide dinucleotide dehydrogenase subunit 1 [*nad*1]) have been widely used mainly for discriminating between *F. gigantica* and its sister species *F. hepatica* and their hybrid forms (reviewed in 1; 7).
Generally, the various gene markers that have been used to study digeneans have their strength and weakness when investigating inter or intraspecific variations (8; 9). The ITS1 and/or ITS2 spacer regions within the rDNA operon have been suggested as the most preferable marker for the identification of both *F. gigantica* and *F. hepatica* (1; 10). However, few studies have explored the level of intraspecific variation within ITS in *F. gigantica.* The only detailed intraspecific variations study till date on *F. gigantica* was conducted by Mas-Coma *et al.* (1) using populations from four African countries. The study by revealed only one haplotype based on both ITS1 and ITS2. Few studies have characterized the partial fragments of the LSU of *F. gigantica* which when compared with *F. hepatica* showed few nucleotide differences (11; 12). Although Marcilla *et al*. (11) reported no intraspecific variations within the *F. gigantica* samples collected from three African countries, Raina *et al.* (13) recently reported a single nucleotide polymorphism in the LSU rDNA sequences from *F. gigantica* samples collected from four geographical regions in India.
In this investigation, for the first time we explore the genetic diversity and genealogical relationship of *F. gigantica* populations from several countries in Africa, Asia and Iran using LSU and ITS1 gene markers.

Materials and Methods
LSU and ITS1 datasets of F. gigantica
Sequences used in this study (LSU and complete ITS+ITS1) were retrieved from GenBank (Table 1). Also included in the analysis were novel sequences from Nigeria (2 LSU and ITS1 sequences respectively) reported by Enabulele and Imasuen (14) and (3 ITS1 sequences) by Enabulele and Awharitoma (15). All sequences were aligned using the online alignment tool (http://www.ebi.ac.uk/Tools/msa/muscle). Aligned sequences were further edited in the software BioEdit (16) were necessary minor adjustments were performed particularly for complete ITS sequences which were trimmed to the appropriate ITS1 length (i.e. ≈ 450 base pairs).
Molecular diversity analysis
Due to the limited sequences from most of the countries, molecular diversity analysis was determined at the continental level (i.e. Africa and Asia) including the sequences from Iran. Molecular diversity indices inferred from each gene marker was estimated in Dnasp v5.0 (17). The indices calculated were as described (18) which includes: number of haplotypes (*H*), number of polymorphic sites (*S*), haplotype diversity (*Hd*: a measure of the frequencies

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and numbers of haplotypes among individuals), nucleotide diversity (π: average weighted sequence divergence between haplotypes) and mean number of pairwise differences (*K*).

Haplotype network analysis

To visualize the relationship between genealogy of haplotypes and geography, haplotype networks were inferred using statistical parsimony algorithm (19) as implemented in PopART (20). The haplotype network images were enhanced and annotated using the Paint™ software in Microsoft Windows™ for computers. Uncorrected p-distance between various haplotypes was calculated using MEGA v6 (21).

Results

Molecular diversity analysis

The LSU sequences produced an alignment of ≈699 base pairs (bp) with several gaps introduced by sequences from China and Niger. In contrast, the ITS1 sequences produced an alignment of ≈441 bp without gaps. Although the sequences analysed were few, some insight was gained on the genetic diversity in *F. gigantica*. Interestingly, the LSU datasets revealed a higher molecular diversity (*H*, 14; *S*, 195; *Hd*, 0.816; π, 0.13663; *K*, 55.19786) (Table 2) in comparison to the ITS1 datasets (*H*, 12; *S*, 53; *Hd*, 0.237; π, 0.00570; *K*, 2.04531) (Table 3).

Table 1 *Fasciola gigantica* LSU, complete ITS and ITS1 sequences retrieved from GenBank

Geographical region/Country	GenBank accession of LSU sequences (n = 34)	GenBank accession of complete ITS and ITS1 sequences (n = 103)
Africa		
Burkina Faso	AJ440785	AJ853848
Cameroon	NA	JF295000
Cape Verde	AJ439739	NA
Egypt	AJ440786 & KF791537	AB553651 - AB553673, AB555691 - AB555693, EF612470, JF295001, KF425321, KP099943
Kenya	EU025873	KP760871, EF612472
Mauritania	Not available	HQ197358 & HQ197359
Niger	JF708020 - JF708022	AM850108
Nigeria	+	+
Senegal	AY222245	NA
Zambia	NA	AB207142, AB514855
Asia		
Bangladesh	NA	KC424482-KC424484, KC476163 - KC476171
China	JF708017 - JF708019	AB477354, AJ628043, AJ628425, JF496708 - JF496715, JF708038, JN974306, KF543340, KJ789332 - KJ789345
India	HM126479, HM776945, JF323865, JF323866, KC602458 & KP064093	EF027104 & EF198867
Indonesia	NA	AB207143
Thailand	HM004190	AB207144, AB514853 & AB514854
Vietnam	NA	AB385614, AB514856 & AB514857
Pakistan	NA	KF638561
Iran	AB674551, AB674553, AB674554, AB674558, AB674560 - AB674562, AB674564, JN811689, JQ999968, LC076384, LC120687 & LC120688	HM746787, HM746788, JF432073, JF432074, JN828953, JN828955 - JN828958

NA, not available; +, not yet submitted on GenBank

Table 2 Genetic diversity of *Fasciola gigantica* from Africa, Asia and Iran based on LSU sequences. n = number of sequences, *H* = number of haplotypes, *S* = number of polymorphic sites, *Hd* = haplotype diversity, π = average weighted sequence divergence between haplotypes, *K* = mean number of pairwise differences (*K*).

Geographical region/Country	*n*	*S*	*H*	*Hd*	π	*K*
Africa	11	217	6	0.727 (SD± 0.144)	0.19252 (SD± 0.05856)	93.56364
Asia	10	196	5	0.667 (SD±0.163)	0.20998 (SD± 0.05919)	90.71111
Iran	13	58	7	0.833 (SD±0.086)	0.01873 (SD± 0.01223)	9.91026
All Population	**34**	**195**	**14**	**0.816 (SD±0.051)**	**0.13663 (SD± 0.03670)**	**55.19786**

Table 3 Haplotype distribution of *Fasciola gigantica* from Africa, Asia and Iran based on LSU sequences

Haplotype	Country/number of sequences in each haplotype	GenBank accession numbers of sequences in each haplotypes
HAP1	Burkina Faso (1), Nigeria (1), Kenya (1), Iran (5), Egypt (2), Senegal (1) & Cape Verde (1)	AJ440785, +, EU025873, AB674560, AB674553, AB674558, AB674554, AB674564, KF791537, AJ440786, AY222245 & AJ439739
HAP2	Iran	LC120687
HAP3	China (1), Niger (1)	JF708017, JF708020
HAP4	Niger (1)	JF708021
HAP5	Niger (1)	JF708022
HAP6	China (1)	JF708018
HAP7	China (1)	JF708019
HAP8	Iran (1)	JN811689
HAP9	Iran (1)	LC120688
HAP10	Iran (1)	LC076384
HAP11	Iran (1)	JQ999968
HAP12	Thailand (1), India (5), Iran (3)	HM004190, JF323866, JF323865, HM776945, HM126479, KP064093, AB674561, AB674551, AB674562
HAP13	India	KC602458
HAP14	Nigeria	+

+, not yet submitted on GenBank

Based on the LSU data, population from Africa had the highest molecular diversity (*S*, 217; *Hd*, 0.727; π, 0.19252; *K*, 93.56364) (Table 2). Of the 14 haplotypes characterized in the LSU analysis (Table 3), 11 were exclusive to five of the 12 countries analysed (Iran, 5; China, 2; Niger, 2; Nigeria and India, 1 each). Three haplotypes were shared by two or more countries; the first and largest had sequences from seven countries (Burkina Faso, 1; Nigeria, 1; Kenya, 1; Iran, 5; Egypt, 2; Senegal, 1; Cape Verde, 1), the second had nine sequences from three countries (Thailand, 1; India, 5; Iran, 3) while the last haplotype had two sequences, one for each country (China and Niger).

The ITS1 marker though showing an overall low molecular diversity revealed that the population from Asia had the highest molecular diversity (*S*, 46; *Hd*, 0.394; π, 0.00687; *K*, 2.82449) while the African population had (*S*, 14; *Hd*, 0.132; π, 0. 0.00489; *K*, 1.77907) (Table 4). All nine sequences from Iran did not show any diversity. Of the 12 haplotypes characterized, nine were unique to some geographical locations: (Bangladesh, 6; Nigeria 1; India, 1 and China, 1). There were only three shared haplotypes; the first and largest consisted of 90 sequences from 16 countries (Nigeria, 2; China, 25; India, 1; Bangladesh, 6; Vietnam, 3; Thailand, 3; Indonesia, 1; Egypt, 30; Zambia, 2; Kenya, 2; Mauritania, 2; Cameroon, 1; Niger, 1; Burkina Faso, 1; Pakistan, 1 and Iran, 9. The other two shared sequences comprised of two sequences each from Nigeria and China.

Table 4 Genetic diversity of *Fasciola gigantica* from Africa, Asia and Iran based on ITS1 sequences. n = number of sequences, *H* = number of haplotypes, *S* = number of polymorphic sites, *Hd* = haplotype diversity, π = average weighted sequence divergence between haplotypes, *K* = mean number of pairwise differences (*K*), SD = Standard Deviation.

Geographical region/Country	*n*	*S*	*H*	*Hd*	π	*K*
Africa	44	14	3	0.132 (SD± 0.068)	0.00489 (SD± 0.00248)	1.77907
Asia	50	46	11	0.394 (SD±0.089)	0.00687 (SD± 0.00269)	2.82449
Iran	9	0	1	0 (SD±0)	0 (SD± 0)	0
All Population	**103**	**53**	**12**	**0.237 (SD±0.056)**	**0.00570 (SD± 0.00191)**	**2.04531**

Haplotype network analysis

The relationship between the various haplotypes as illustrated in the network analysis shows that in the LSU network (Figure 1) there are three main groups (A, B and C). Haplotype group A comprised of eight haplotypes (HAP1 & HAP2, HAP8 - HAP10, HAP12 - HAP14) connected by < 10 mutational changes. Only one sequence from Iran was in Haplotype group B which though connected to the largest shared haplotype (HAP1) was genetically diverged by 29 mutational changes. There was evidence of some structuring by haplotype group C which consisted of only sequences from Niger and China (HAP3 - HAP7). Haplotype group C was diverged from haplotype groups A and B by >172 mutational changes. In the ITS1 network (Figure 2), there was no structuring, the largest haplotype (HAP1) consisted of 90 (87%) of the haplotypes from16 countries (Table 5). The most genetically diverse haplotype (HAP8) from Bangladesh was connected to the largest haplotype by 24 mutational changes.

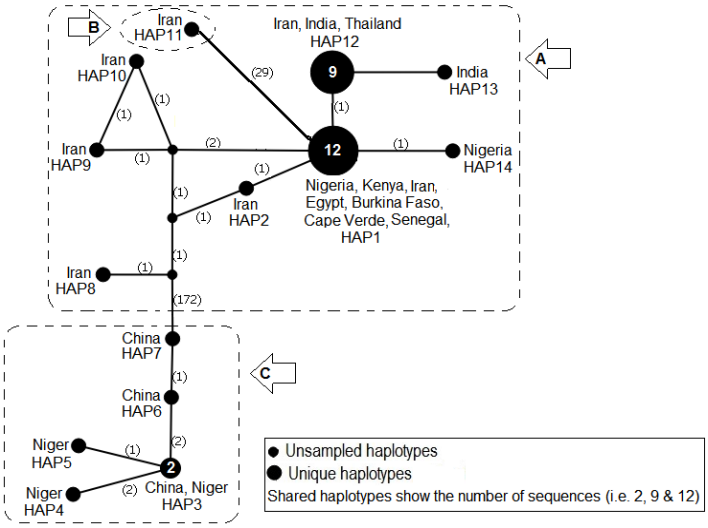

Figure 1: Haplotype network generated from 34 Fasciola gigantica LSU rDNA sequences. Numbers of mutational changes are in parenthesis. A – C, the major haplotype groups; HAP, haplotypes.

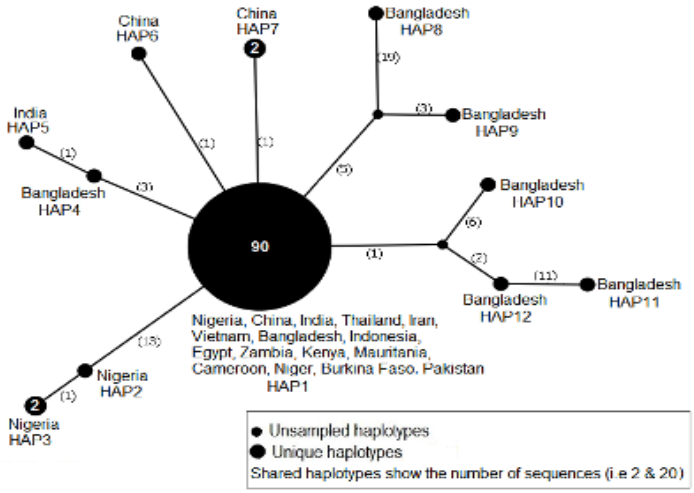

Figure 2: Haplotype network generated from 103 Fasciola gigantica ITS1 rDNA sequences. Numbers of mutational changes are in parenthesis. A – C, the major haplotype groups; HAP, haplotypes.

Table 5 Haplotype distribution of *Fasciola gigantica* from Africa, Asia and Iran based on ITS1 sequences

Haplotype	Country/number of sequences in each haplotype	GenBank accession numbers of sequences in each haplotypes
HAP1	Nigeria (2), China (25), India (1), Bangladesh (6), Vietnam (3), Thailand (3), Indonesia (1), Egypt (30), Zambia (2), Kenya (2), Mauritania (2), Cameroon (1), Niger (1), Burkina Faso (1), Pakistan (1) & Iran (9)	+; AB477354, AJ628043, AJ628425, JF496708 - JF496711, JF496714 & JF496715, JF708038, KF543340, KJ789332 - KJ789345; EF027104; KC476171, KC476168, KC476164 - KC476166 & KC424483; AB385614, AB514856 & AB514857; AB207144, AB514853 & AB514854; AB207143; AB553651 - AB553673, AB555691 - AB555693, EF612470, JF295001, KF425321 & KP099943; AB207142 & AB514855; KP760871 & EF612472; HQ197358 & HQ197359; JF295000; AM850108; AJ853848; KF638561; HM746787, HM746788, JF432073, JF432074, JN828953, JN828955 - JN828958.
HAP2	Nigeria (1)	+
HAP3	Nigeria (2)	+
HAP4	Bangladesh (1)	KC424482
HAP5	India (1)	EF198867
HAP6	China (1)	JN974306
HAP7	China (2)	JF496713, JF496712
HAP8	Bangladesh (1)	KC476163
HAP9	Bangladesh (1)	KC476170
HAP10	Bangladesh (1)	KC424484
HAP11	Bangladesh (1)	KC476169
HAP12	Bangladesh (1)	KC476167

+, not yet submitted on GenBank

Discussion

Current understanding of the level of genetic diversity in *Fasciola gigantica* is inferior to that of its sister species *F. hepatica* which has been widely investigated (22-24). Generally, an insight into the genetic diversity of parasitic organism is an important factor in understanding their pathogenicity, epidemiology, and resistance to anthelmintic drugs (25). In this investigation our analysis of the level of genetic diversity and interrelatedness of *F. gigantica* from different parts of the world based on available LSU and ITS1 sequences provided some evidence of genetic structuring.

The partial LSU sequences of *F. gigantica* proved to be a better genetic marker at studying genetic diversity in the flukes (Table 2) in comparison to ITS1 (Table 4). Although only 34 LSU sequences of *F. gigantica* from 11 countries were available for analysis, two major lineages were observed (Figure 1). The sequences from Niger and China (Haplotype group C) formed a separate lineage which was highly genetically diverged (> 172 mutational changes) from the second lineage comprising of sequences from nine other countries; Burkina Faso, Nigeria, Kenya, Iran, Egypt, Senegal, Cape Verde, India and Thailand (Haplotype group A). It is possible that the Niger-China haplotype group is a more recent lineage of *F. gigantica* since most of the sequences (79%) were located in Haplotype group A (Table 3).

Evidence of gene flow between populations was also revealed in the LSU network analysis (Figure 1) which shows the sharing of haplotypes between sequences from three different geographical regions (Africa, Asia and Iran) and (China-Niger). However, considering the huge geographical distance between some of the countries sharing haplotypes, livestock trade is the most likely source of introduction of the identified shared haplotypes in such countries. The role of cattle domestication and international livestock trade in both the speciation and phylogeography of *F. gigantica* and *F. hepatica* has been reviewed (1). Although the complete LSU in both *F. gigantica* and *F. hepatica* is about 4,171 base pairs long and highly conserved (1), the partial fragments ≈ 620 base pairs appear to be suitable for investigating genetic diversity in *F. gigantica* as has been demonstrated in this study. The LSU as an alternative or complimentary barcode marker to the popular *cox*1 mitochondrial gene for species diagnosis has been proposed (26). The study by Sonnenberg *et al*. (26) has also successfully showed the potential of LSU in detecting hybrids species.

The ITS1 and ITS2 genes have been widely used in the identification of *Fasciola* species (7) and other species of flatworms (8; 10). However, genetic diversity analysis of *F. gigantica* based on the nuclear ITS1 gene shows that the gene marker is unsuitable for such investigation due to low intraspecific variability (Table 4). Although some unique haplotypes were identified from some countries (Nigeria, Bangladesh, India and China), 87% of the 103 sequences analysed were located in the main haplotype (Figure 2). Similar low genetic diversity in ITS1 has been reported in some other trematodes *Opisthorchis felineus* (27) and *Clonorchis sinensis* (28).

This investigation has further confirmed the unsuitability of ITS1 in investigating genetic diversity in *F. gigantica* populations. In contrast, the LSU has been shown to provide some insight into the molecular diversity of *F. gigantica*. In future, as more *F. gigantica* LSU sequences from more countries become available, it would be possible to better understand the utility of the gene in *F. gigantica* population studies.

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