Molecular characterization of *Fasciola gigantica* infecting cattle in Benin City, southern Nigeria, based on LSU and ITS1 ribosomal DNA sequences

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Abstract

Fasciola gigantica is the widely reported aetiological agent of fascioliasis in bovines and ovines in Nigeria. Prior to this investigation there has not been any molecular confirmation of the *Fasciola* species infecting livestock in the country. Fasciolid flukes isolated from livers of two cows from an abattoir in Benin City, Edo State, were characterized by sequencing the partial fragment of the large subunit ribosomal nuclear DNA (LSU rDNA) and the first internal transcribed spacer region (ITS1). Phylogenetic analysis based on Maximum Likelihood show a close relationship between the novel sequences from this investigation with *F. gigantica* sequences from different countries in Africa (*p*-distance: 0.000, ITS1; 0.000 and 0.002, LSU). Comparison of *p*-distance reveal that the sequences within the *F. gigantica* clade diverge from its sister species *F. hepatica* by values ranging between 0.008-0.015 for LSU rDNA while the values for the ITS1 was 0.010 or 0.011. The novel sequences in this study are the first for *F. gigantica* from cattle in Nigeria and thus serves as baseline data for molecular identification of the species in the country.

Keywords: *Fasciola gigantica*; DNA sequences; cattle; Benin City; Nigeria.

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Introduction

Two fasciolid species *Fasciola gigantica* (Cobbold, 1855) and *F. hepatica* (Linnaeus, 1758) are the known aetiological agents of fascioliasis also known as liver rot infecting both ovines and bovines worldwide. The disease is of huge economic importance in livestock management where they can result in low milk production, poor beef quality and in some cases death (Mas-Coma *et al* 2005). In Nigeria only *F. gigantica* has been confirmed to cause fascioliasis based on morphological identification of the parasite's egg and adult stages (Ogunrinade and Ogunrinade, 1980; Ogunrinade, 1984).

Morphological characters has traditionally been used in the differentiation of *F. gigantica* and its sister species *F. hepatica*. However, the application of molecular techniques has shown that morphology can be misleading due to the known occurrence of an "intermediate" *Fasciola* sp. which shares morphological features with *F. gigantica* and *F. hepatica*. Accurate diagnosis of which *Fasciola* spp. is responsible for fascioliasis in a location is crucial in understanding the disease epidemiology (Mas-Coma *et al* 2009; Ai *et al* 2011).

Within the past two decades, molecular approach involving DNA sequencing of nuclear and/or mitochondrial genes has provided insights into the taxonomy/systematics, evolution, hybridization, population genetics and phylogeography of *Fasciola* spp. (Mas-Coma *et al* 2009). In Nigeria, there have been studies on some aspects of *F. gigantica* (Olusi, 1997; Dipeolu *et al* 2000; Olusi and Amuta, 2001; Ekwunife and Eneanya, 2006; Idowu *et al* 2007; Aliyu *et al* 2014). However, there is no published record of molecular confirmation of the parasite in any part of the country.

Ali *et al* (2008) used ITS rDNA to show the existence of both *F. hepatica* and *F. gigantica* in Niger. The study by Ali *et al* (2008) highlights the need for a molecular approach in investigating *Fasciola* species infecting cattle in Nigeria as it shares a border with Niger. The aim of this investigation therefore was to molecularly characterized fasciolid isolated from cows in one of the local abattoirs in Benin City, Edo State, using nuclear LSU rDNA and ITS1 genes.

Materials and methods

Collection of flukes

Adult fasciolid worms were collected from the only two infected cattle slaughtered during a helminth survey visit to an abattoir in Benin City, Edo State. Only one and two flukes respectively were recovered from the cattle suggesting a recent infection. It was impossible to

determine the exact origin of the cattle, although most cattle sold in southern Nigeria originate from the north where cattle rearing is a major occupation. It is now common for herdsmen to rear their cattle in southern Nigeria from calves to adult before selling to local butchers in abattoirs. Two of the isolated flukes labelled *as F. gigantica* A and *as F. gigantica* B (1 each from infected cattle) were preserved separately in 96% ethanol prior to DNA extraction.

DNA extraction and sequencing

Genomic DNA was extracted from small tissue snip of the worms using the QIAGEN DNeasy blood and tissue kit (Qiagen Inc. UK) according to the manufacturer's instructions and then stored at -20°C until use. Partial fragments of the Large subunit ribosomal DNA (LSU rDNA or 28S) were amplified by the following primer sets forward: (5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′) and reverse (5′-GCTATCCTGAGGGAAACTTCG-3′). PCR conditions: initial heating at 94°C for 3 minutes, followed by 35 amplification cycles consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 2 minutes, elongation at 72°C for 2 minutes and final extensions for 7 minutes at 72°C. The primer sets used for the ITS1 region were; forward: (5′-GTCGTAACAAGGTTTCCGTAGGTG-3′) reverse (5′-TATGCTTAAATTCAGCGGGTAATC-3′) with the following PCR protocol: initial heating at 95°C for 10 minutes, followed by 35 amplification cycles, consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 minute, elongation at 72°C for 90 seconds and final extensions for 10 minutes at 72°C. Quantification of the amplified PCR amplicons was determined by running 5 µl of each product in 1% agarose gel electrophoresis using TAE buffer and then visualized under UV illumination. DNA size marker Hyperladder I (Bioline™) was used to determine the size of the amplified gene region and 20 ìl of each amplicon were sequenced using the same PCR amplification primers with Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems™); sequencing reactions were run on an Applied Biosystems™ 3730XL automated sequencer.

Molecular phylogenetic analysis

The novel contiguous sequences were assembled in BioEdit v.7.5.0.2 (Hall, 1999) and submitted for BLAST search using blastn (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to enable species identification which indicated 99% similarity to *F. gigantica*. The sequences were then aligned using the online MUSCLE sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/muscle) with sequences of some *F. gigantica*, *F. hepatica* and other species within the Fasciolidae family retrieved from GenBank™. Necessary minor adjustments of the alignment were then performed in BioEdit prior to phylogenetic analysis.

The phylogenetic relationships of the novel fasciolid sequences were inferred based on Maximum Likelihood (ML) phylogenetic method implemented in the software MEGA v6 (Tamura *et al* 2013). Prior to phylogenetic analysis, MEGA v6 was used to determine the appropriate evolutionary models and the Kimura 2 Parameter + Gamma distribution model was suggested as the best model. MEGA v6 was used to calculate the uncorrected genetic distance (*p*-distance) between sequences used in the phylogenetic analysis. Nodal support for the phylogenetic reconstruction was assessed based on 1000 bootstrap replicates.

Results

The generated novel LSU sequences of the two fasciolids were between 642 and 659 base pairs long with average nucleotide composition of A, 22.8%; C, 21.9%; G, 31.4% and T, 24%. A single transversion (G/T) at site 491 differentiated both sequences. The ITS1 fragments which were identical was 450 base pairs long with average nucleotide composition of A, 20.71%; C, 26.06%; G, 26.06% and T, 27.17%. The Maximum Likelihood (ML) phylogenetic trees constructed using both genetic markers shows the fasciolid from this study grouping with other *F. gigantica* sequences and supported with a bootstrap of 87% and 94% in both the LSU and ITS1 trees (Figures 1 and 2). In both trees, *Protofasciola robusta* was recovered as the basal genus while *Fasciola* species were recovered as a paraphyletic assemblage. *Fasciola gigantica* and *F. hepatica* are depicted in a sister relationship with *p*-distance ranging between 0.008-0.015 based on LSU (Table 1) and 0.010 or 0.011 for ITS1 (Table 2). *Fasciola jacksoni* was more genetically related to *Fascioloides magna* with a *p*-distance of 0.015 (Table 1) while genetic distance based on ITS1 indicates it is more related to *F. hepatica* with *p*-distance of 0.035 (Table 2) though associating with *F. magna* than to the two other *Fasciola* species.

. . . . Figures 1 and 2 are overleaf.

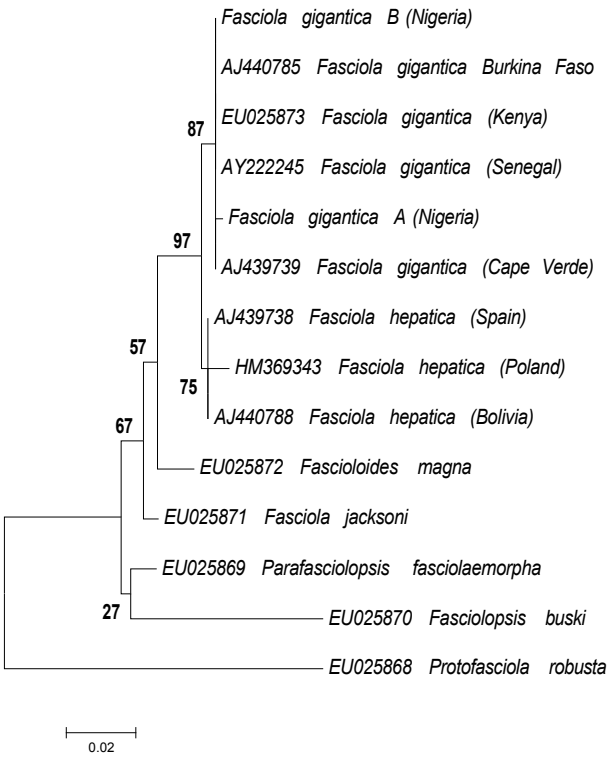

Figure 1. Maximum Likelihood phylogenetic reconstruction based on LSU rDNA sequences of fasciolids within the family Fasciolidae. Specimens from this study are in the dotted squares and located within *Fasciola gigantica* sub-clade. The scale shows the number of nucleotide substitutions per site between DNA sequences.

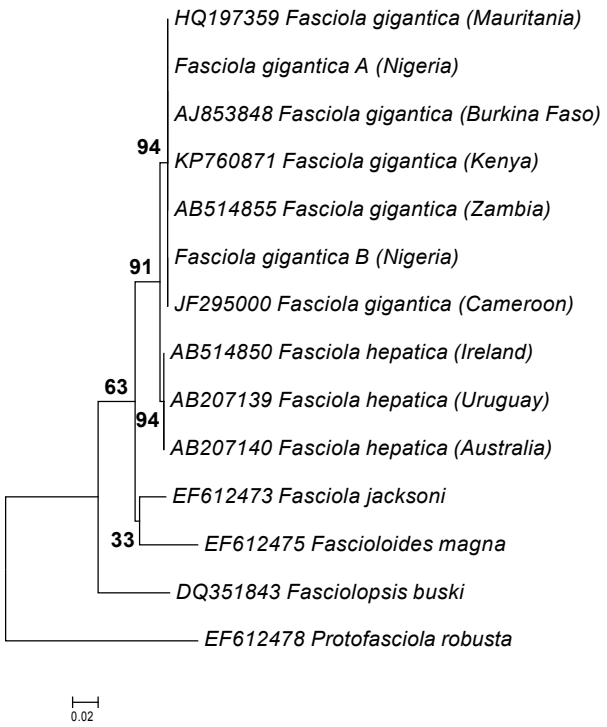

Figure 2. Maximum Likelihood phylogenetic reconstruction based on ITS1 sequences of fasciolids within the family Fasciolidae. Specimens from this study are in the dotted squares and located within *Fasciola gigantica* sub-clade. The scale shows the number of nucleotide substitutions per site between DNA sequences.

Table 1. Close genetic distance between fasciolid specimens from Nigeria with *Fasciola gigantica* sequences based on LSU rDNA. Bold values, genetic distance closest to the fasciolid specimens from Nigeria.

	Accession	Fasciolidae	1	2	3	4	5	6	7	8	9	10	11	12	13
1	EU025868	*Protofasciola robusta*													
2	EU025869	*Parafasciolopsis fasciolaemorpha*	0.094												
3	EU025872	*Fascioloides magna*	0.105	0.029											
4	EU025871	*Fasciola jacksoni*	0.096	0.021	0.015										
5	EU025870	*Fasciolopsis buski*	0.107	0.050	0.056	0.050									
6	HM369343	*Fasciola hepatica* (Poland)	0.100	0.038	0.029	0.028	0.068								
7	AJ440788	*Fasciola hepatica* (Bolivia)	0.098	0.032	0.023	0.023	0.062	0.006							
8	AJ439738	*Fasciola hepatica* (Spain)	0.098	0.032	0.023	0.023	0.062	0.006	0.000						
9	AY222245	*Fasciola gigantica* (Senegal)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008					
10	EU025873	*Fasciola gigantica* (Kenya)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	0.000				
11	AJ440785	*Fasciola gigantica* (Burkina	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	0.000	0.000			
12	AJ439739	*Fasciola gigantica* (Cape Verde)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	0.000	0.000	0.000		
13	This study	*Fasciola gigantica* A (Nigeria)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	**0.000**	**0.000**	**0.000**	**0.000**	
14	This study	*Fasciola gigantica* B (Nigeria)	0.101	0.034	0.025	0.023	0.064	0.015	0.009	0.009	**0.002**	**0.002**	**0.002**	**0.002**	0.002

Table 2. Close genetic distance between fasciolid specimens from Nigeria with *Fasciola gigantica* sequences based on ITS1 rDNA. Bold values, genetic distance closest to the fasciolid specimens from Nigeria.

	Accession	Fasciolidae	1	2	3	4	5	6	7	8	9	10	11	12	13
1	EU025868	*Protofasciola robusta*													
2	EU025869	*Parafasciolopsis fasciolaemorpha*	0.094												
3	EU025872	*Fascioloides magna*	0.105	0.029											
4	EU025871	*Fasciola jacksoni*	0.096	0.021	0.015										
5	EU025870	*Fasciolopsis buski*	0.107	0.050	0.056	0.050									
6	HM369343	*Fasciola hepatica* (Poland)	0.100	0.038	0.029	0.028	0.068								
7	AJ440788	*Fasciola hepatica* (Bolivia)	0.098	0.032	0.023	0.023	0.062	0.006							
8	AJ439738	*Fasciola hepatica* (Spain)	0.098	0.032	0.023	0.023	0.062	0.006	0.000						
9	AY222245	*Fasciola gigantica* (Senegal)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008					
10	EU025873	*Fasciola gigantica* (Kenya)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	0.000				
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13	This study	*Fasciola gigantica* A (Nigeria)	0.099	0.032	0.025	0.023	0.064	0.013	0.008	0.008	0.000	0.000	0.000	0.000	
14	This study	*Fasciola gigantica* B (Nigeria)	0.101	0.034	0.025	0.023	0.064	0.015	0.009	0.009	0.002	0.002	0.002	0.002	0.002

Discussion

Fascioliasis in Nigeria has always been attributed to *F. gigantica* which is believed to be the only *Fasciola* sp. occurring in the country (Mas-Coma *et al* 2009). However, within the last two decades it has become vital to accurately identify which species is infecting livestock as both *F. gigantica* and *F. hepatica* have been reported in some countries in Africa and Asia which were previously considered to have only one species (Mas-Coma *et al* 2009). Molecular tools involving DNA sequencing of nuclear and/or mitochondrial genes have been useful in identifying *F. gigantica* and *F. hepatica* and the "intermediate" *Fasciola* sp. which is a hybrid between both species (Ai *et al* 2011). Molecular analysis of the LSU rDNA sequences of the flukes from this investigation confirmed they were *F. gigantica* as illustrated in the phylogenetic trees generated from both LUS and ITS1 sequences (Figures 1 and 2). In both phylogenetic trees the sequences from this study grouped with sequences from Kenya, Senegal, Burkina Faso, Cape Verde, Mauritania and Zambia where only *F. gigantica* is known to occur (Mas-Coma *et al* 2009).

Historically, *F. gigantica* and *F. hepatica* have been differentiated by morphological characters of the adult and egg; however misidentification can arise from variations in the sizes of the adult stages. This can be influenced by factors such as age of the flukes, host from where the flukes were isolated and fixation/staining protocols prior to light microscopy identification (Kendall, 1965). Also, the morphological features of the "intermediate" *Fasciola* sp. which share features with *F. gigantica* and *F. hepatica* can obscure accurate identification. Ashrafi *et al* (2006) and Periago *et al* (2006) have however demonstrated that body length/body width ratio and the length between the ventral suckers to posterior end are the most reliable characters for distinguishing *F. gigantica* and *F. hepatica* particularly in countries where only one species occurs. Analysis of molecular data is currently the best means of unequivocally distinguishing between the species and their hybrid (Ai *et al* 2011).

The relationship between *F. gigantica* and *F. hepatica* as revealed in the phylogenetic trees shows both species as sister sub-clades with high bootstrap support of 87% and 94% in the LSU and ITS1 trees respectively. This is in agreement with an earlier report by Lotfy *et al* (2009). The genetic distance determined between *F. gigantica* and *F. hepatica* in this study though small sufficiently distinguishes both species. A recent whole mitochondrial genome study by Liu *et al* (2014) showed that the genome sequence for *F. gigantica* is longer than *F. hepatica* by 16 base pairs and the *p*-distance between both species amounts to 11.4%. The few sequences of *F. gigantica* compared in this investigation indicate that the LSU data shows more intraspecific variation than observed in the ITS1 sequences. Comparison of more *F. gigantica* sequences from different countries will be essential in determining the degree of intraspecific variation revealed by both genetic markers.

Studies have shown that in situations where both *F. hepatica* and *F. gigantica* or hybrid *Fasciola* sp. are suspected, a combination of a nuclear (ITS1 or ITS2) and a mitochondrial (*cox*1 or *nad*1) gene markers are essential for correct diagnosis (Ai *et al* 2011). Although *F. hepatica* is not known to occur in Nigeria, Ali *et al.* (2008) using molecular approach reported the occurrence of both *F. gigantica and F. hepatica* in Niger. Since Niger shares international boundary with some northern states in Nigeria, there is the possibility of *F. hepatica* being introduced into the country as a result of cross border cattle rearing and trade. Extensive sampling of livestock in different regions of Nigeria and analysing the data by several DNA loci will be indispensable to providing insight into the level of molecular diversity in *F. gigantica* within the country and perhaps unravel the presence of both *F. hepatica* and hybrid *Fasciola* species.

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