



DNA barcode identification of a tropical liver fluke (*Fasciola gigantica*) in cattle from Ilorin, Northcentral Nigeria

Oluyinka A. Iyiola¹ · Rahmat D. Shaibu¹ · Olalere Shittu² · Musbau O. Alayande³ · Musa Rabi⁴ · Mujidat K. Sulaiman⁵ · Grace T. Obarombi¹

Received: 25 June 2020 / Accepted: 14 July 2021
© Institute of Zoology, Slovak Academy of Sciences 2021

Abstract

Fascioliasis is a parasitic disease caused by liver flukes. It affects both domesticated and undomesticated ruminants. It causes reduced livestock productivity and it is no doubt of public health importance. There is limited documented information on the genetic diversity of liver flukes among cattle in Northcentral, Nigeria. Information on genetic diversity of *Fasciola* species will assist in disease surveillance and control measures by health agencies in the studied area. This study therefore aimed at investigating genetic diversity of *Fasciola gigantica* in the liver of slaughtered cattle. A total of eighty (80) flukes were collected from livers of fifty-seven (57) slaughtered cattle at four different local abattoirs within Ilorin, Nigeria. A molecular-based approach was used to analyse partial DNA sequences of the mitochondrial cytochrome C oxidase subunit I (*COI*) gene regions. Aligned *COI* sequences containing 381 base pairs were analysed by maximum likelihood method. Phylogenetic trees revealed that there is similarity among Nigerian *F. gigantica* and those from China, Iran and Egypt. High proportions of the DNA nitrogenous base substitution observed in the samples was C to T transitions and the least DNA base substitution observed was from A to G transition. Our findings revealed that *COI* gene region is very informative for investigating intraspecific variations amongst *F. gigantica* isolates. Our results revealed that the *F. gigantica* populations sampled are genetically similar. Taken together, these results revealed that mitochondrial DNA sequence could be a very useful marker for correct identification and characterization of various populations of *Fasciola* species from different ruminants.

Keywords Molecular identification · Polymerase chain reaction · *Fasciola gigantica* · Diversity

Introduction

Fasciola gigantica (Cobbold, 1855) is a trematode that causes fascioliasis, a zoonotic disease mostly associated with animals and in some cases humans. Fascioliasis

occurs in several areas of the globe and a bulk of the infections is caused by *F. gigantica*, which is a predominant liver fluke found in ruminant. Fascioliasis has been reported in Asia and Africa (Mas-Coma et al. 2009) and it is caused mainly by two common species of parasitic trematodes namely *Fasciola hepatica* (Linnaeus, 1758) and *F. gigantica*. These trematodes are morphologically similar and inhabit the liver and other associated organs. Fascioliasis is reported to be among the major neglected tropical diseases and has been the major cause of low yield in livestock and reduction in the economic value of the animals affected (Alajmi 2019). At least, 2.4 million people are infected in more than 70 countries worldwide, with several millions reported to be at risk, especially in some parts of the world with intensive sheep and cattle production (Tolan Jr. 2011). Recent review of bovine fascioliasis by Elelu and Eisler (2018) reported that bovine fascioliasis and other trematode infections in Nigeria may exert negative effect on livestock and humans.

✉ Oluyinka A. Iyiola
iyiola.aa@unilorin.edu.ng; iyiolagenetics@gmail.com

¹ Cell Biology and Genetics Unit, Department of Zoology, University of Ilorin, P.M.B 1515, Ilorin, Kwara state, Nigeria

² Parasitology Unit, Department of Zoology, University of Ilorin, P.M.B 1515, Ilorin, Kwara state, Nigeria

³ Department of Veterinary Parasitology and Entomology, Usman Dan Fodio University, Sokoto, Nigeria

⁴ Department of Veterinary Parasitology and Entomology, University of Ilorin, P.M.B 1515, Ilorin, Kwara state, Nigeria

⁵ Ministry of Agriculture and Natural Resources, Ilorin, Kwara state, Nigeria

Identifications of *Fasciola* species have relied upon microscopic analysis using morphometric parameters of the eggs and the adult fluke (Mas-Coma et al. 2005; Periago et al. 2006; Thanh 2012). However, the shortcomings of morphological method have made way for the use of molecular methods for accurate identification of *Fasciola* samples. Recent studies have shown that there are intermediate forms of *Fasciola* spp. (Iyiola et al. 2018; Sumruayphol et al. 2020). The two common *Fasciola* species are distinguished morphologically by size and shape but these measurements are not dependable (Reaghi et al. 2016) because of the discrepancies in morphology and the existence of intermediate forms (Shafiei et al. 2014; Sumruayphol et al. 2020).

There is a dire need for improved methods of species identification. This will assist and appropriately guide health officers to develop workable strategies towards eradication of the disease in affected communities. Due to difficulty in differentiating these two species, molecular techniques have been employed to help distinguish them at species and subspecies levels (Itagaki et al., 2005; Ichikawa et al. 2011; Ichikawa and Itagaki 2012; Mohanta et al. 2014; Tadayon et al. 2015; Sumruayphol et al. 2020). DNA barcoding is very informative, has proven to be a reliable tool for precise identification and has consistent discriminatory power for distinguishing closely related biological samples. In addition, previous studies have also given much attention to the molecular characterisation and genetic structure of *Fasciola* species (Itagaki et al. 2005; Ichikawa et al. 2011; Sharifiyazdi et al. 2011; Ichikawa and Itagaki 2012; Mohanta et al. 2014; Tadayon et al. 2015). Molecular distinction between *F. hepatica* and *F. gigantica* has been previously investigated using Polymerase Chain Reaction (PCR) and Restriction Length Polymorphism (RFLP) assay (Marcilla et al. 2002). Relatively few studies have focused on molecular analysis of *Fasciola* using *COI* sequence throughout Nigeria even though *F. gigantica* is considered the major pathogen causing fasciolosis in Africa (Ichikawa-Seki et al. 2017). Mitochondrial genes are known for precise identification, genotyping, assessing intra- and inter-specific variations and appropriate phylogenetic analyses of these parasites (Alajmi 2019).

Recent studies have shown that there are intermediate forms of *Fasciola* spp. (Iyiola et al. 2018; Sumruayphol et al. 2020). To get a deeper understanding of expansion history of *Fasciola* flukes within the African continent, (Ichikawa-Seki et al. 2017) phylogenetically analysed *NAD1* and *COI* sequences of Nigerian *Fasciola* flukes and then compared with those from Egypt. DNA barcoding is a reliable method for interspecies identification. Mitochondrial cytochrome C oxidase subunit I is the marker for animal DNA barcoding because it shows inter and intra specific differences in animals. In addition to providing species-specific information on organisms, barcodes are

important for disease vector identification (Kaur 2015). We have previously carried out morphometric and molecular analysis of *Fasciola* species using PCR-RFLP approach (Iyiola et al. 2018) but we suggested DNA sequence analysis which undoubtedly, will discriminate the diversity pattern and elucidate the dispersal routes in the *Fasciola* species studied. There is paucity of information on DNA barcoding and phylogenetic relationships of *F. gigantica* in North-central Nigeria. In this study we have explored the use of DNA barcoding approach as a reliable molecular tool for precise species identification and molecular differentiation of *F. gigantica* samples.

Materials and methods

Sample collection

In this study, a total of eighty (80) *Fasciola* flukes were collected from infected livers of fifty-seven (57) slaughtered cattle at four different local abattoirs within Ilorin, Northcentral Nigeria. Map of the geographical locations of the collection sites is shown in Fig. 1. The collected *Fasciola* flukes were washed extensively in physiological saline solution, fixed in 70% ethanol, and transferred to the Cell Biology and Genetics Laboratory at the University of Ilorin, Nigeria for morphometric and molecular studies.

Morphometric analysis

Fasciola worms were identified using parameters previously described by (Periago et al. 2006, 2008). The flukes were transferred into a Petri-dish and forceps was used to hold down the oral sucker from the ventral sucker. A binocular compound microscope was used to observe the flukes properly and one of the pictures taken is shown in Fig. 2. Profiles of *F. gigantica* isolated from cattle during this study with details of number of flukes amplified are shown in Table 1.

Genomic DNA extraction

Genomic DNA (gDNA) of the *Fasciola* samples were extracted according to DNA Miniprep kit from Omega Bio-tek, United States of America with slight modifications to manufacturer's instructions. The presence of gDNA was detected by 1% agarose gel electrophoresis. Concentrations of the extracted DNA were quantified using NanoDrop spectrophotometer. The confirmed gDNA from *Fasciola* samples were stored at -20°C . DNA concentrations ranged from 80 to 300 ng/ μL .

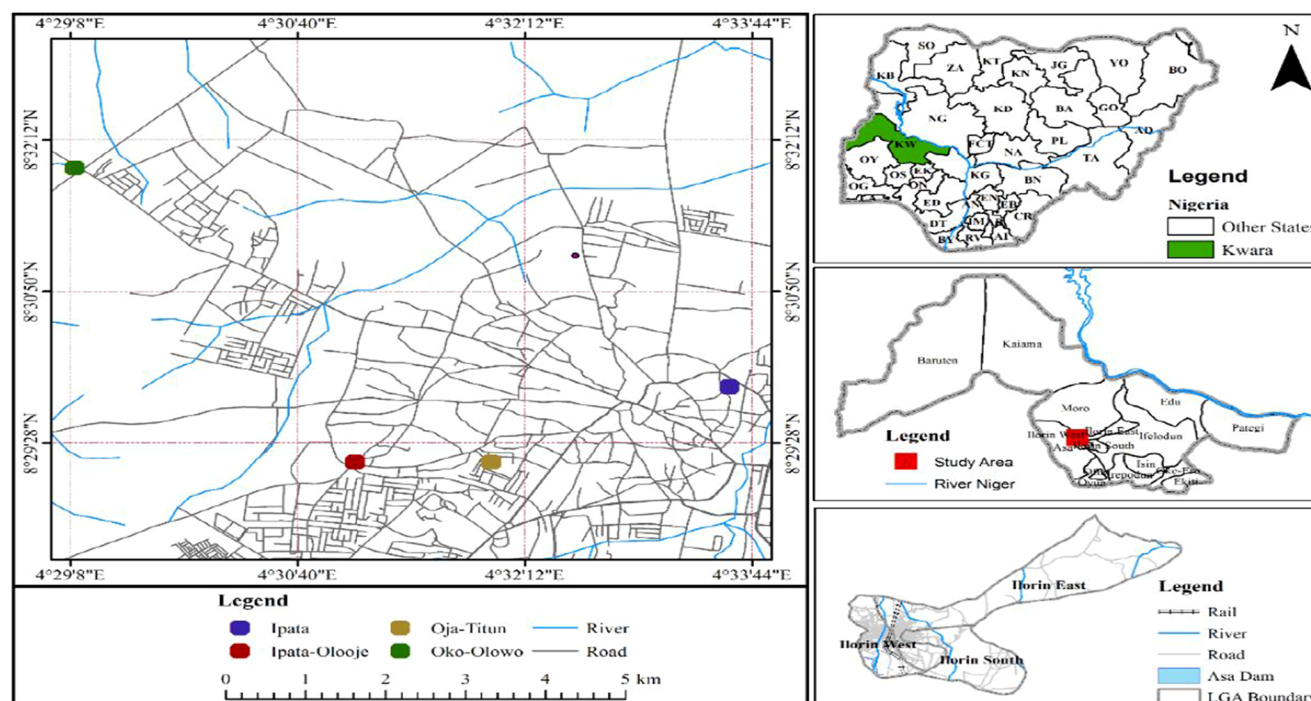


Fig. 1 Map showing the geographical location of the sample collection sites

Polymerase chain reaction (PCR)

The 5' portion of mitochondrial cytochrome c oxidase subunit I gene (*COI-5P*) was amplified for *Fasciola* species DNA barcoding using a pair of highly conserved primers (forward and reverse) adopted from previously published study by Itagaki et al. (2005) (Table 2). For PCR amplification, a 25 µL reaction mix was prepared containing 14.5 µL of nuclease-free water, 5 µL of 5X Firepol master mix with 12.5 mM MgCl₂ from Solis Biodyne, Estonia, 0.25 µL of each primer and 5 µL of DNA template. The PCR condition used was initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min, extension at 72 °C for 1 min. The final elongation was run at 72 °C for 5 min. PCR product was confirmed using 2% agarose gel electrophoresis prepared with 1X TBE. Ethidium Bromide (EtBr) was used to stain the DNA gel and bands were viewed with a UV transilluminator system.

Mitochondrial DNA sequencing

The DNA purification was carried out using Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, (Catalogue No. D4050). The purified products were obtained by automated sequencer at Inqaba Biotech™ (Pretoria, South Africa) using the Big Dye terminator kit (Applied Biosystems, ThermoFisher Scientific, USA). The base trimming and other improvement of the sequence quality was carried out on Sequencher 5.4.6.

Analysis of the *COI* sequence similarity

The *COI* sequence results were queried on the BLASTn feature on the NCBI database. Search for sequence similarity of ≥97% was carried out in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the use of Megablast search. The sequences showed percentage similarities ranging from 97.97% to 100% with other *Fasciola gigantica* sequences that

Table 1 Profiles of *Fasciola* isolates from cattle in Ilorin, Northcentral Nigeria

Cattle breeds	No. of flukes collected	No. of <i>Fasciola gigantica</i> isolates	No. of <i>Fasciola gigantica</i> analysed by PCR
Red Bororo	37	37	8
White Fulani	31	31	8
Sokoto Gudali	12	12	4
Total	80	80	20

Fig. 2 *Fasciola gigantica* sample isolated from cattle in Ilorin, Northcentral, Nigeria



were queried. FASTA sequences of each of *COI* sequences were inserted into the Barcoding of Life Database (BOLD) Identification Request tool (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Sequences were submitted for species level identification under option “Species Level Barcode Record.” Barcode Index Number (BIN) was estimated from the BOLD database. When the sequences were run in BOLD, they showed similarity in percentages ranging from 93.88% to 96.34% with other *F. gigantica* sequences on the database. The sequences are similar with those with Barcode Identification number (BIN) ACQ1693. The *COI* sequence similarities were obtained by aligning the sequences from this study with some homologous sequences from NCBI to evaluate the accuracy of the morphological identification. The sequences were aligned using the Clustal W multiple alignment feature in BioEdit. Pairwise genetic distance and maximum likelihood analysis were inferred using the MEGA7 software with default parameters and 1000 bootstrap replicates (Kumar et al. 2016). The sequences were uploaded to the GenBank and they were assigned with accession numbers MW555812.1 - MW555821.1.

Results and discussion

Details of the coordinates global positioning system (GPS) and representative pictures of the *F. gigantica* analyzed microscopically are indicated in Figs. 1 and 2, respectively. Interestingly, all the *Fasciola* worms isolated and analysed

in this study were *F. gigantica* (Table 1). Molecular phylogenetic analysis by maximum likelihood method inferred by Tamura-Nei method is presented in Fig. 3. From the phylogenetic tree, it was observed in that the bootstrap values at every node were low (51%) and only two distinct clades were formed which are closely related to the *COI* DNA sequences of *Fasciola* isolates from China, Egypt and Iran available in the Genbank. Similarly, the evolutionary divergences among *F. gigantica* between the *COI* sequences ranged from 0.000 to 0.114. It was observed that *COI* sequence of Nigerian *F. gigantica* species was identical with *F. gigantica* obtained from Iran and China. *F. hepatica* (accession number MF88121.1) was used as an outgroup (Table 3). After trimming the ambiguous bases, 470 base pairs of the 15 *COI* sequence data were aligned. Out of the 472 bases aligned, 407 were conserved regions and there were three (3) parsimony informative sites. The nucleotide frequencies of the 6127 bases analyzed were T (50.3%), G (25.9%), A (13.2%) and C (10.6%). Nucleotide base substitutions data showed that base transition mutation was lowest between Adenine (A) and Guanine (G) and was highest between Thymine (T) and Cytosine (C) (Table 4). Estimates of evolutionary divergence between the *COI* sequences obtained from the *F. gigantica* obtained in this study and the *F. gigantica* from GenBank are shown in Table 3.

In this study, we obtained *Fasciola* samples from slaughtered cattle in Ilorin, Nigeria. These were morphologically identified using parameters reported in our previous study (Iyiola et al. 2018). Molecular characterization of the

Table 2 Details of the primers specific for the *COI* gene used for detecting the *Fasciola gigantica* polymorphism

S/NO	Primer names	Nucleotide sequences	% GC content	Expected PCR product size(bp)
1	Forward	5' ACGTTGGATCATAAGCGTGT 3'	45	485
2	Reverse	5' CCTCATCCAACATAACCTCT 3'	45	485

Table 3 Estimates of evolutionary divergence of *COI* sequences of *Fasciola gigantica* samples collected and analysed during this study

S/No Samples	Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>F. gigantica</i> _ (This study)	MW555812.1	–															
2. <i>F. gigantica</i> _ (China)	KF543342.1	0.004	–														
3. <i>F. gigantica</i> _ (This study)	MW555813.1	0.000	0.004	–													
4. <i>F. gigantica</i> _ (This study)	MW555814.1	0.004	0.000	0.004	–												
5. <i>F. gigantica</i> _ (This study)	MW555815.1	0.004	0.008	0.004	0.008	–											
6. <i>F. gigantica</i> _ (This study)	MW555816.1	0.000	0.004	0.000	0.004	0.004	–										
7. <i>F. gigantica</i> _ (This study)	MW555817.1	0.004	0.008	0.004	0.008	0.008	0.004	–									
8. <i>F. gigantica</i> _ (This study)	MW555818.1	0.000	0.004	0.000	0.004	0.004	0.000	0.004	–								
9. <i>F. gigantica</i> _ (This study)	MW555819.1	0.000	0.004	0.000	0.004	0.004	0.004	0.004	0.000	–							
10. <i>F. gigantica</i> _ (This study)	MW555820.1	0.004	0.000	0.004	0.000	0.008	0.004	0.008	0.004	0.004	–						
11. <i>F. gigantica</i> _ (This study)	MW555821.1	0.004	0.000	0.004	0.000	0.008	0.004	0.008	0.004	0.004	0.000	–					
12. <i>F. gigantica</i> _ (Nigeria)	LC142785.1	0.000	0.004	0.000	0.004	0.004	0.000	0.004	0.000	0.000	0.004	0.004	–				
13. <i>F. gigantica</i> _ (Nigeria)	LC142775.1	0.000	0.004	0.000	0.004	0.004	0.000	0.004	0.000	0.000	0.004	0.004	0.000	–			
14. <i>F. gigantica</i> _ (Egypt)	AB553790.1	0.004	0.000	0.004	0.000	0.008	0.004	0.008	0.004	0.004	0.000	0.000	0.004	0.004	–		
15. <i>F. gigantica</i> _ (Iran)	KX021298.1	0.004	0.000	0.004	0.000	0.008	0.004	0.008	0.004	0.004	0.000	0.000	0.004	0.004	0.000	–	
16. <i>F. hepatica</i> (Outgroup)	MF88121.1	0.110	0.106	0.110	0.106	0.114	0.110	0.106	0.110	0.110	0.106	0.106	0.110	0.110	0.106	0.106	–

The accession numbers of *F. gigantica* obtained from the GenBank are indicated in bold

Table 4 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Base Substitution

Nucleotide bases	A	T	C	G
A	–	2.54	0.51	0.28
T	0.68	–	14.98	1.4
C	0.68	74.33	–	1.4
G	0.14	2.54	0.51	–

The values in normal font represent transversions while those in bold represent transitions

Fasciola samples was carried on the genomic DNA isolated from the *Fasciola* isolates. Cytochrome oxidase subunit I is very informative for intraspecific variation amongst *F. gigantica* samples. Previous studies showed that the first internally transcribed spacer of ribosomal DNA (ITS1) and the two highly polymorphic genes of *COI* and *ND1* are suitable markers to help elucidate the origin and source of the infection as well as *Fasciola* species identification (Itagaki et al. 2005). We amplified the *COI* sequences of the mitochondrial region of the flukes. The *COI* sequences were then compared with GenBank sequences of *Fasciola* spp. from different geographical regions of China, Iran and Nigeria.

This study revealed that all the *Fasciola* worms analyzed were *F. gigantica* and similar observation was reported (Ichikawa-Seki et al. 2017). Interestingly, we observed in this study that the haplotype of the *Fasciola* collected from Nigeria exhibited comparatively close relationship with *Fasciola* samples from previously reported studies in Iran, Egypt and China haplogroups. However, the genetic diversity of the *F. gigantica* in Nigeria is extremely low, probably because it is not indigenous to Nigeria and was probably introduced from other

regions of the world by any means of various dispersal routes. Taken together, it sounds reasonable to suggest that the *Fasciola* spp. from our study arose from the same origin when compared with documented GenBank sequences of *Fasciola* spp. from different geographical regions of the world. Molecular phylogenetic results showed that the *F. gigantica* collected and analyzed in this study were similar with the *F. gigantica* samples on the GenBank database. Mitochondrial markers are mostly used because of their high evolution rates and are very useful in differentiating closely related organisms since they are maternally inherited and do not undergo any recombination (Tadayon et al. 2015). In this present study, we investigated intraspecific sequence variations in *F. gigantica* population and observed sequence similarities in the *COI* region. They showed no evolutionary divergence in the *COI* region (Table 3). Findings from previous study revealed high levels of genetic diversity in *F. gigantica* collected from Iran (Moazeni et al. 2012). Previous genetic diversity based on *COI* sequences among *Fasciola* species isolates from Northern Iran in comparison with southwestern isolates suggested that the differences observed could be related to definitive and intermediate host species (Sakari et al. 2017).

A high proportion of the nucleotide base substitution observed in our study was Cytosine to Thymine base transition mutations and the least base substitution mutation was Adenine to Guanine transitions. From the phylogenetic analysis using Maximum Likelihood method (Fig. 3), it can be observed that the *F. gigantica* samples with GenBank accession numbers (LC142785.1 & LC142775.1) both from Nigeria; MW555812.1, MW555813.1, MW555815.1, MW555816.1, MW555818.1 and MW555819.1 from this study formed a distinct clade (monophyletic groups) suggesting similar dispersal route. *F. gigantica* samples with

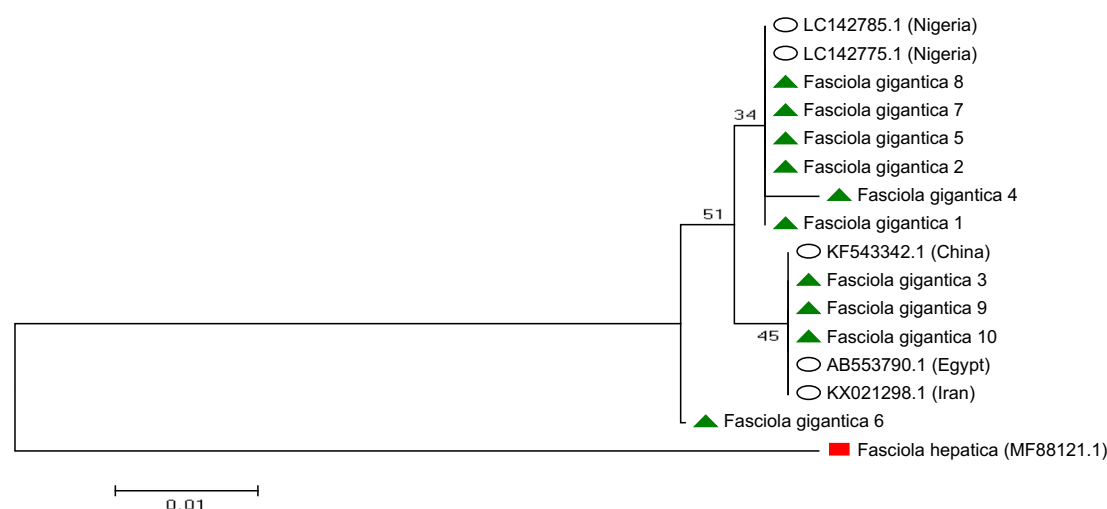


Fig. 3 Molecular phylogenetic analysis by Maximum Likelihood method inferred by Tamura-Nei method with 1000 bootstrap replications. The triangles represent the *Fasciola gigantica* samples sequenced for this study, the circles represent *F. gigantica* samples collected from the

GenBank database and the square represents the *Fasciola hepatica* sequence retrieved from the GenBank database and used as an outgroup. Nodes are labelled with bootstrap values

GenBank accession numbers KF543342.1 (China), AB553790.1 (Egypt), KX021298.1 (Iran); MW555814.1, MW555820.1 and MW555821.1 from this study formed another distinct clade (monophyletic group). The separation of the haplotypes into two distinct monophyletic groups is a true reflection of their phylogeographical origin, evidence of similar dispersal route and it may be suggested that genetic diversity of sampled *F. gigantica* is very low. In this study, our ClustalW analysis and phylogenetic tree showed minimum level of genetic variability between individuals of the same species. Only the *F. gigantica* with GenBank accession number MW555817 diverged separately from the other samples. This study revealed that the *F. gigantica* DNA sequences from this study are closely related with those sequences of previously studied *Fasciola gigantica* in Nigeria available in the GenBank database and those found in Iran, China and Egypt. This suggests that the global genetic diversity of *F. gigantica* is low. The variations observed in DNA sequences of the *F. gigantica* sample could be due to genetic mutation that have occurred over a long period of time and possibly induced by drugs as suggested by Amer et al. (2011). This may also probably be due to point mutations observed in this study as shown in Table 4.

Conclusion

This study concludes that there is low genetic diversity among *Fasciola* samples obtained for this study when compared with those from other parts of the world as shown in the phylogenetic tree results. There is an urgent need to reduce the incidence of fascioliasis in Nigeria and authorities in charge of animal health must develop appropriate control strategies to mitigate economic loss due to fascioliasis among cattle and other ruminants.

Acknowledgments The authors wish to thank the authorities of the Department of Zoology, University of Ilorin, Ilorin, Nigeria and Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, Lagos state, Nigeria for providing technical assistance and facilities used for this study.

Authors' contributions OAI conceived and designed this study; OAI, MR and MKS collected and preserved the samples; OAI and RDS performed the molecular laboratory experiments; OAI, OS, MOA and RDS analysed the data; OAI wrote the first draft of the manuscript with significant contributions from all authors. All authors read and approved the final version of the manuscript.

Declarations

Ethical approval This research does not require any ethical approval because the flukes were collected from fresh livers obtained from the slaughter slabs after the animals were sacrificed at the abattoirs.

Research involving human participants and/or animals Not Applicable.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Alajmi RA (2019) Molecular characterization of *Fasciola* flukes using mitochondrial 28S rRNA gene in Naimi Saudi sheep. Saudi J Biol Sci 26:112–117. <https://doi.org/10.1016/j.sjbs.2017.06.010>
- Amer S, Dar Y, Ichikawa M, Fukuda Y, Tada C, Itagaki T, Nakai Y (2011) Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS 1 and 2) and mitochondrial (ND1 and *COI*) gene markers. Parasitol Int 60(1):5–12. <https://doi.org/10.1016/j.parint.2010.09.003>
- Elelu N, Eisler M (2018) A review of bovine fasciolosis and other trematode infections in Nigeria. J Helminth 92(2):128–141. <https://doi.org/10.1017/S0022149X17000402>
- Ichikawa M, Itagaki T (2012) Molecular analysis of aspermic *Fasciola* flukes from Korea on the basis of the nuclear ITS1 region and mitochondrial DNA markers and comparison with Japanese aspermic *Fasciola* flukes. J Vet Med Sci 74:899–904. <https://doi.org/10.1292/jvms.11-0523>
- Ichikawa M, Bawn S, Maw NN, Htun LL, Thein M, Gyi A, Sunn K, Katakura K, Itagaki T (2011) Characterization of *Fasciola* spp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers. Parasitol Int 60:474–479. <https://doi.org/10.1016/j.parint.2011.08.007>
- Ichikawa-Seki M, Tokashiki M, Opara MN, Iroh G, Hayashi K, Kumar UM, Itagaki T (2017) Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from Nigeria. Parasitol Int 66:893–897. <https://doi.org/10.1016/j.parint.2016.10.010>
- Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K (2005) Genetic characterization of parthenogenic *Fasciola* spp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. Parasitol 131(5):679–685. <https://doi.org/10.1017/S0031182005008292>
- Iyiola OA, Shittu O, Owolodun OA, Animasaun DA, Udeze AO (2018) Morphometric phenotypes and molecular identification of *Fasciola* species isolated from cattle in Ilorin, north-Central Nigeria. Sri Lankan J Biol 3(2):9–23. <https://doi.org/10.4038/slj.v3i2.23>
- Kaur S (2015) DNA barcoding and its applications. Int J Eng Res Gen Sci 3:602–604
- Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Marcilla A, Bargues MD, Mas-Coma S (2002) A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *F. gigantica*. Mol Cell Probes 16:327–333. <https://doi.org/10.1006/mcpr.2002.0429>
- Mas-Coma S, Bargues MD, Valero MA (2005) Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 35:1255–1278. <https://doi.org/10.1016/j.ijpara.2005.07.010>
- Mas-Coma S, Valero MA, Bargues MD (2009) *Fasciola*, lymnaeids and human fascioliasis, a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol 69:41–146. [https://doi.org/10.1016/S0065-308X\(09\)69002-3](https://doi.org/10.1016/S0065-308X(09)69002-3)
- Moazeni M, Sharifiyazdi H, Izadpanah A (2012) Characterization of *Fasciola hepatica* genotypes from cattle and sheep in Iran using cytochrome C oxidase gene (*COI*). Parasitol Res 110(6):2379–2384. <https://doi.org/10.1007/s00436-011-2774-9>
- Mohanta UK, Ichikawa-Seki M, Shoriki T, Katakura K, Itagaki T (2014) Characteristics and molecular phylogeny of *Fasciola* flukes from

- Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitol Res* 113:2493–2501. <https://doi.org/10.1007/s00436-014-3898-5>
- Periago MV, Valero MA, Panova M, Mas-Coma S (2006) Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitol Res* 99(4):368–378. <https://doi.org/10.1007/s00436-006-0174-3>
- Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, Desquesnes M, Curtale F, Mas-coma S (2008) First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from human endemic area of the Nile Delta, Egypt. *Infect Genet Evol* 8:51–58. <https://doi.org/10.1016/j.meegid.2007.10.001>
- Reaghi S, Haghigi A, Harandi MF, Spotin A, Arzamani K, Rouhani S (2016) Molecular characterization of *Fasciola hepatica* and phylogenetic analysis based on mitochondrial (Nicotinamide adenine dinucleotide dehydrogenase subunit 1 and cytochrome oxidase subunit 1) genes from the north-east of Iran. *Vet World* 9(9):1034–1038. <https://doi.org/10.14202/vetworld.2016.1034-1038>
- Sakari B, Parhoode M, Abdolahi Khabisi S, Shafiei R, Mohammadi-Ghalehbin B (2017) Genetic diversity of *Fasciola* spp. isolates from northern part of Iran: comparison with southwestern isolates. *J Parasit Dis* 41(3):768–772. <https://doi.org/10.1007/s12639-017-0886-6>
- Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A (2014) Molecular and morphological characterization of *Fasciola* spp. isolated from different host species in a newly emerging focus of human fascioliasis in Iran. *Vet Med Int* 405740:1–10. <https://doi.org/10.1155/2014/405740>
- Sharifiyazdi H, Moazeni M, Rabbani F (2011) Molecular characterisation of human *Fasciola* samples in Gilan province, northern Iran on the basis of DNA sequences of ribosomal and mitochondrial DNA genes. *Comp Clin Pathol* 21(5):889–894. <https://doi.org/10.1007/s00580-011-1193-8>
- Sumruayphol S, Siribat P, Dujardin J-P, Dujardin S, Komalamisra C, Thaenkham U (2020) *F. gigantica*, *F. hepatica* and *Fasciola* intermediate forms: geometric morphometrics and an artificial neural network to help morphological identification. *Peer J* 8:e8597 <https://doi.org/10.7717/peerj.8597>
- Tadayon S, Sharifiyazdi H, Moazeni M, Divar MR (2015) Molecular differentiation of *Fasciola* species and characterization of genetic diversity of *F. gigantica* using NADH dehydrogenase I (ND1) gene in the endemic areas of Iran. *Iran J Parasitol* 10(1):9–18. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4403545/>
- Thanh NT (2012) ‘Zoonotic fasciolosis in Vietnam: molecular identification and geographical distribution’, Doctoral Dissertation, Tesis de Doctorado, Ghent University, Bélgica
- Tolan RW Jr (2011) Fascioliasis due to *Fasciola hepatica* and *F. gigantica* infection: an update on this ‘neglected’ neglected tropical disease. *Lab Med* 42(2):107–116. <https://doi.org/10.1309/LMLFBB8PW4SA0YJI>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.