Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 84: 35-41 Jan 2025
DOI: 10.5455/ajvs.207412

Molecular Characterization of *Fasciola gigantica* from Cattle in Ibadan, Nigeria

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Key words:
Cattle, Fasciola gigantica, polymerase chain reaction, multiple sequence alignment

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Article History
Received: 28 Jun 2024.
Accepted: 03 Aug 2024

ABSTRACT

Fasciolosis is a major parasite infestation in ruminant livestock that causes public health issues and large financial losses for livestock businesses worldwide. Although additional species have been reported within the genus *Fasciola*, *Fasciola hepatica* and *Fasciola gigantica* have been acknowledged taxonomically as the primary causes of fasciolosis in animals and humans. However, it is limiting to differentiate the two isolates based on morphological features only. Identification and characterization using molecular techniques are important. Polymerase chain reaction (PCR) was used to detect and amplify 500 bp mitochondrial Cytochrome c Oxidase subunit 1 (COX1), 420 bp NADH Dehydrogenase Subunit 1 (NAD1), and 1100 bp nuclear ribosomal Internal Transcribed Spacers (ITS) regions of the adult *Fasciola* samples from the bile ducts of the infected liver. PCR, multiple sequence alignment, and phylogenetic tree reconstruction confirmed the presence of *Fasciola gigantica* (*F. gigantica*) and further confirmed the species. All the sequences phylogenetically cluster in *F. gigantica* clade for COX1, NAD1, and ITS-2 genes. The level of diversity in mitochondrial COX1 and NAD1 genes was significantly higher than that in the nuclear ITS region. The multiple sequence alignment also revealed genetic sites that can be employed to distinguish *F. gigantica* from other species. All sequenced *Fasciola* isolates from Ibadan were identified as *F. gigantica*. Sequence analysis revealed relatedness between isolates from Nigeria and sub-Saharan countries. Molecular techniques are very important and reliable in the diagnosis of parasitic diseases. This will help not only differentiate between *F. gigantica* and *Fasciola hepatica* (*Fasciola hepatica*), but also distinguish these from other related *Fasciola species*, and endemic from imported species.

1. INTRODUCTION

Fasciolosis, caused by the digenetic trematodes *F. hepatica* and *F. gigantica*, is an important parasitic disease of livestock and a reemerging zoonotic disease affecting humans globally. A prevalence of 20% was recorded in ruminants in Nigeria (Banwo et al., 2023) with more than 70% in some countries (WHO, 2007). Traditionally, *Fasciola* flukes have been identified by examination of adult morphology and morphometrics under microscopy. However, accurate species delineation can be challenging due to overlapping measurements and intermediate forms (Valero et al., 2001; Anawat et al., 2014). While *F. gigantica* occurs mainly in the tropics, *F. hepatica* predominates in temperate regions. Both species can however be found in subtropical zones (Banwo et al., 2023). Additionally, a poorly taxonomically defined "intermediate *Fasciola* species", has been reported in humans, cattle and buffaloes (Le et al., 2008; Ai et al., 2011).

Despite fasciolosis being enzootic in Nigeria, and of great economic importance there are limited studies on the genetic characterization and phylogenetic analysis of *Fasciola* in the country. Also, cattle and more recently goats have been imported from North America, Europe and South Africa into Nigeria to improve local breeds. These imported animals could be sources of foreign and potentially drug-resistant

Fasciola species. Hence, genetic characterization of *Fasciola* species in Nigeria is fundamental for a robust epidemiological study of the parasite in the country and also to monitor the transboundary spread of *Fasciola spp*.

Molecular techniques utilizing genomic deoxyribonucleic acid (DNA) provide an alternative means of *Fasciola spp*. detection and differentiation (Prastowo et al., 2022). Targeting ribosomal and mitochondrial gene sequences such as NAD1 and COX1, PCR-based methods can rapidly and reliably discriminate *F. hepatica* from *F. gigantica* and identify intermediate hybrid forms and evaluate the genetic diversity and variability of *Fasciola species* (Levy et al., 2023). Molecular assays in this study also enable the investigation of genetic diversity and population variation within *Fasciola spp.* to understand transmission patterns. These PCR-based diagnostic capabilities support frontline surveillance, informed treatment regimens, and control measures in the face of increased globalization and climate change impacting the spread of fasciolosis. The aim of this study was to detect *Fasciola* isolates from cattle using molecular techniques, and the genetic characterization of the nuclear ITS, and mitochondrial COX1 and NAD1 genes with the sequence analysis.

2. MATERIALS AND METHODS

2.1 Location of the Study

The study was conducted at the Bodija Municipal Abattoir in Ibadan North Local Government Area of Oyo State, Nigeria. The abattoir is located at a latitude of 7°42′34″N and a longitude of 3°90′33″E (Fig. 1). The abattoir is the biggest in the state. Cattle, sheep, goats and pigs are slaughtered and processed in the abattoir.

2.2. Sample Collection

Adult *Fasciola* samples (n=14) were obtained at random, each from the liver of 340 infected cattle. Infected livers appear grossly enlarged, firm, and irregular, with necrotic tracts and hemorrhages on the surface and bile ducts containing flukes. Flukes were recovered by portal perfusion, and perfusate rinse to recover worms without damage using standard perfusion technique. Each fluke was thoroughly washed individually; in a 0.9% saline solution to remove debris and contamination. Flukes were identified as *F. gigantica* according to existing morphological keys and descriptions given by Soulsby (1982) and Periago et al., (2006). Flukes were then stored at -20°C until the extraction of the genomic DNA.

2.2.1. DNA Extraction

Total genomic DNA was extracted using a DNA Tissue Extraction kit, ZR Genomic DNA™-Tissue MiniPrep (Zymo Research Corp, California, USA) according to the manufacturer's instructions. Extracted DNA was stored at −20°C until use.

2.2.2. PCR and Sequencing Analysis

All PCR were carried out using OneTaq® Quick – Load 2X Master Mix (New England BioLabs Inc®, UK), in 50 µl of total volume which contained 25 µl Master Mix, 18 µl of nuclease-free water, 5 µl of DNA and 1 µl of 0.2 µM of respective forward and reverse primers.

PCR was used to amplify a 500 bp portion of mitochondrial cytochrome c oxidase subunit 1 (COX1) gene using specific primers COX1F: 5’-TTGGTTTTTTGGGCATCCT-3’) and COX1R: 5’-AGGCCACCACCAAATAAAAGA-3’ (Semyenova et al., 2006.

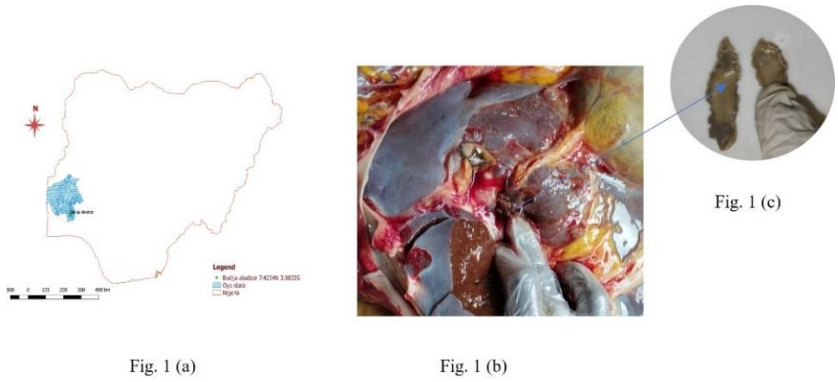

Fig. 1 (a) Fig. 1 (b) Fig. 1 (c)

Fig..1 (a) Map of Nigeria showing the location of Bodija abattoir, Ibadan. (b) A photograph of the liver showing the presence of Fasciola worm (c) *F. gigantica* adult worm.

A 420 bp portion of the mitochondrial NADH dehydrogenase subunit 1 (NAD1) gene was amplified using PCR with the following pair of primers NAD1F5´-TATGTTTTGTACGGGATGAG-3´ and NAD1R:5´-AACAACCCCAACCAACACTTA-3´ (Semyenova et al., 2006). Also, the nuclear ITS-2 of ribosomal DNA (rDNA) using the Primers BD1 (forward: 5′- GTCGTAACAAGGTTTCCGTA -3′) and BD2 (reverse: 5′-TATGCTTAAATTCAGCGGGT -3′) (Ali et al., 2008).

PCR amplification was performed in an MJ PTC-100 Thermal Cycler (MJ Research) programmed using the following temperature conditions: 94°C for 2 min for initial denaturation, annealing (35 cycles of 95°C for 20 s, (depending on the primer, 47°C COX1, 44°C NAD1, 52°C *ITS-2*) for 30 s, 72°C for 1 min), and final extension at 72°C for 7 min. The PCR products were electrophoresed on 2% agarose gels (Sigma, St. Louis, MO, USA), stained with SYBR Green and visualized under ultraviolet light. Amplified DNA was purified using DNA Clean and Concentrator™ Kit (Zymo Research Corp, California, USA).

Automated nucleotide sequencing was performed on an ABI 3130XL for five purified samples, for each of the genes which have been deposited at the GenBank. The nucleotide sequences of each gene Multiple sequence alignment of the partial *F. gigantica* COX1, NAD1 and ITS-2 gene sequences from this study, were compared with their respective published gene sequences in the GenBank database using BLAST search (Altschul et al., 1990) *via* the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) was carried out. This was done using the Clustal W algorithm in the CLC Main Workbench computer application (Qiagen, Valencia, CA). Phylogenetic analysis was computed using maximum likelihood and Kimura 2-parameter model, and bootstrap analysis at 1000 replicates on the MEGA computer application, version 11 (Tamura et al., 2011).

3. RESULTS

3.1 PCR and sequence analysis

PCR amplification of *F. gigantica* COX1, NAD1 and ITS genes generated PCR products of 500 bp, 420 bp and 1100 bp, respectively (Figures. 2, 3, and 4). A BLAST search showed that sequence analysis performed on the sequenced PCR products for the three genes corresponded to *F. gigantica* published sequences. They were designated as Fg-IB-1-NGA, Fg-IB-2-NGA, Fg-IB-3-NGA, and Fg-IB-4-NGA for each gene and have been deposited in the GenBank with the accession numbers MN608173-MN608169; MN586872-MN586866.

Multiple sequence analysis of the four COX1 and three NAD1 nucleotide sequences from this study, and other individual sequences retrieved from the NCBI online repository distinguishes *F. gigantica* from *F. hepatica*. Also, the COX1 gene nucleotide substitutions A771G, differentiate *F. gigantica* in Ibadan from that of China and Thailand. Maximum likelihood phylogenetic trees were constructed using COX1, NAD1 and ITS-2 gene sequences retrieved from the GenBank including sequences from this study. All the sequences phylogenetically cluster in *F. gigantica* clade for COX1, NAD1 and ITS-2 genes (Figures. 7, 8 and 9, respectively).

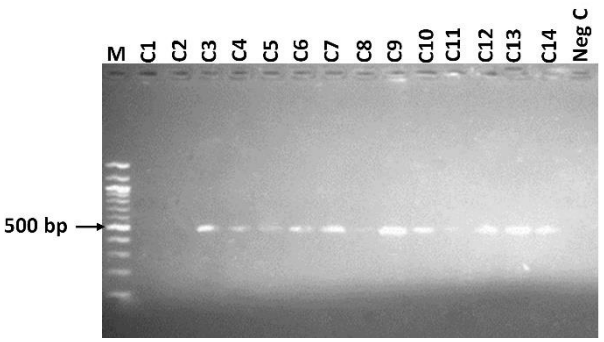

Fig. 2. Agarose gel electrophoresis of *Cox 1* gene PCR products 1 to 14. Lane M: 100 bp Molecular marker (M), Lane 1-14, Lane 15: Negative control. 5 µl of each PCR amplicon was used for agarose gel for electrophoresis. The 500 bp PCR amplicons are shown.

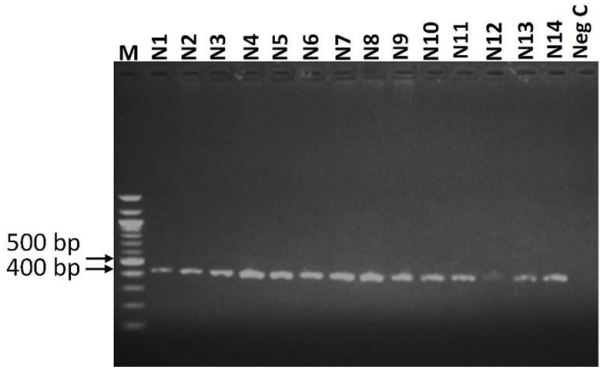

Fig. 3. Agarose gel electrophoresis of *F. gigantica Nad 1* gene PCR products 1 to 14. Lane M: 100 bp Molecular marker (M), Lane 1-14, Lane 15 served as the Negative control. 5 µl of each PCR amplicon was used for agarose gel for electrophoresis. The 400 bp PCR amplicons are shown.

In Fig. 5 and 6. The mutations differentiate the samples in a way that provides taxonomic or phylogenetic information between the two major species of *Fasciola.*

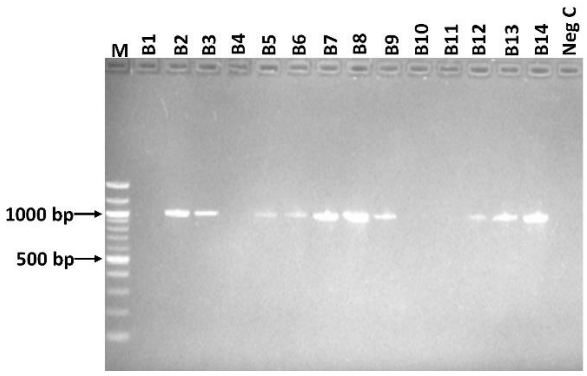

Fig. 4. Agarose gel electrophoresis of *ITS-2* region of PCR products 1 to 14. Lane M: 100-bp Molecular marker (M), Lane 1-14, Lane 15: Negative control. 5 µl of each PCR amplicon was used for agarose gel for electrophoresis. The 1000bp PCR amplicons are shown.

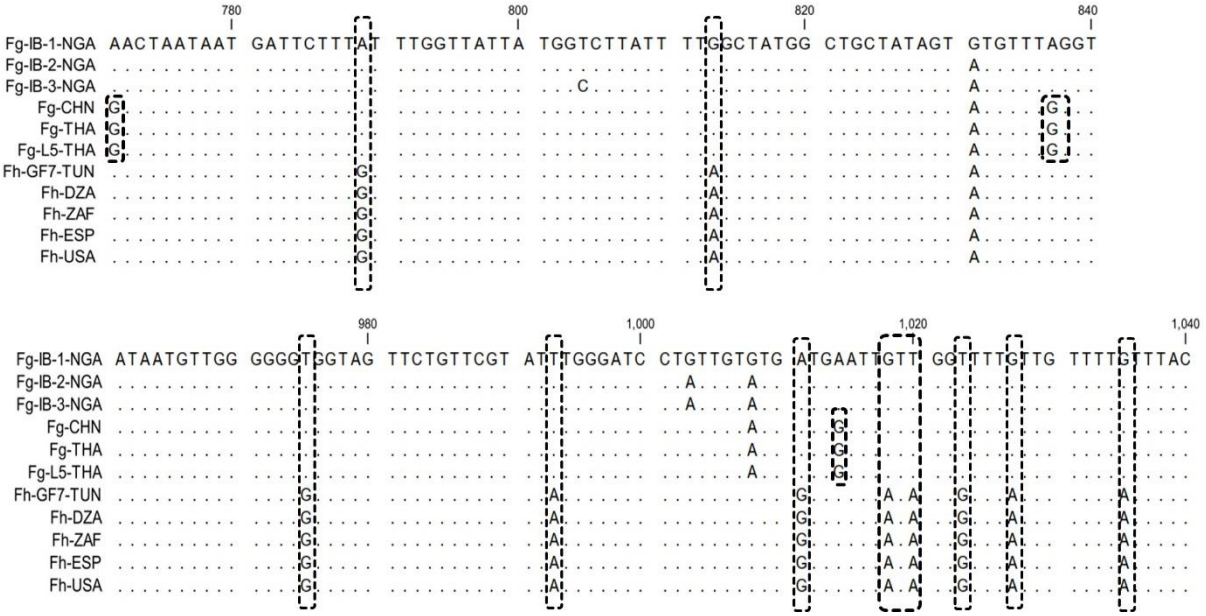

Fig.5 Multiple sequence alignment for the COX1 gene highlighting the regions of the alignment that show single nucleotide polymorphisms (SNPs) with evident mutation hotspots from samples from China, Thailand and *F. hepatica* in other countries.

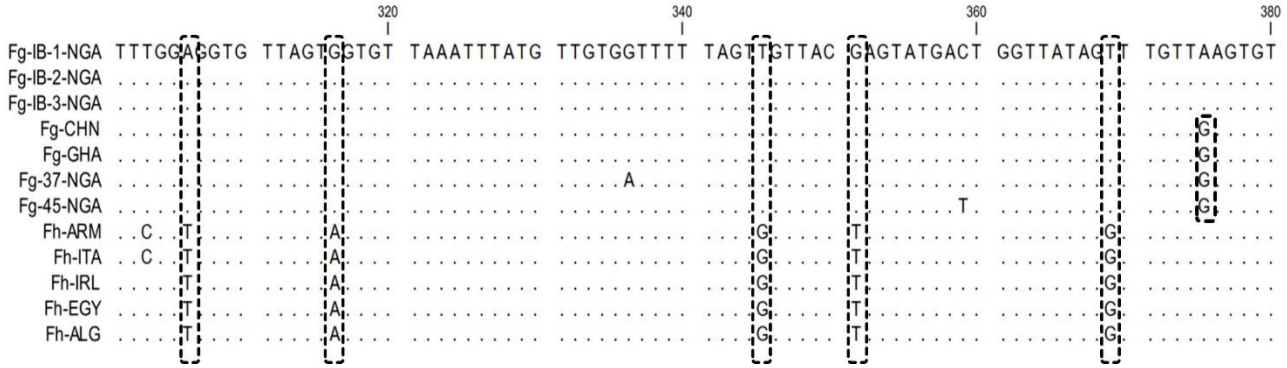

Fig.6. Multiple sequence alignments for the NAD1 gene with patterns of mutation concentrated in certain areas to identify variable regions. Samples from China and Germany show a conserved sequence with a single variable region.

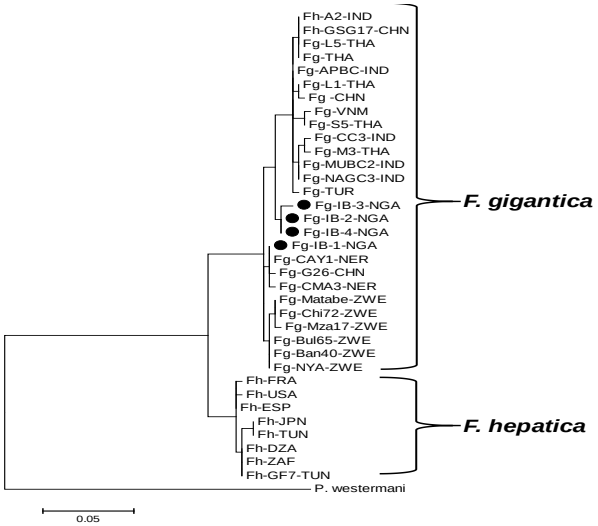

Fig.7. Phylogenetic analysis of *Fasciola spp* based on COX1 gene nucleotide sequences from different geographical locations. The phylogenetic tree was constructed via multiple alignments of the nucleotide sequence of the COX1 gene from Nigeria *Fasciola spp* and other sequences retrieved from the GenBank. *Paragonimus westermani* (*P. westermani*) COX1 gene was used as the out-group. The tree was analyzed by the maximum likelihood method with bootstrapping (1000). *Fasciola spp* clusters, *F. gigantica* and *F. hepatica* are labelled. Bar, 0.05 nucleotide substitutions per site**.** *Fasciola spp* sequences from this study have black circles whereas Nigeria *Fasciola* sequences retrieved from the GenBank have white circles.

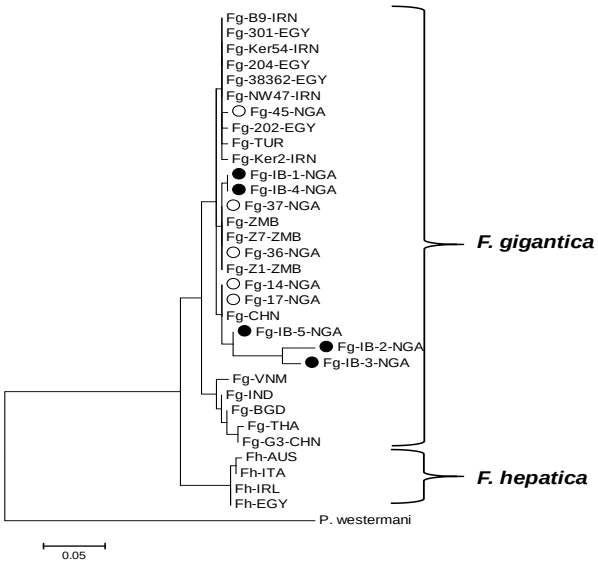

Fig.8. Phylogenetic analysis of *Fasciola spp* based on NAD1 gene nucleotide sequences from different geographical locations. *Fasciola spp* clusters *F. gigantica* and *F. hepatica* are labelled. Bar, 0.05 nucleotide substitutions per site.

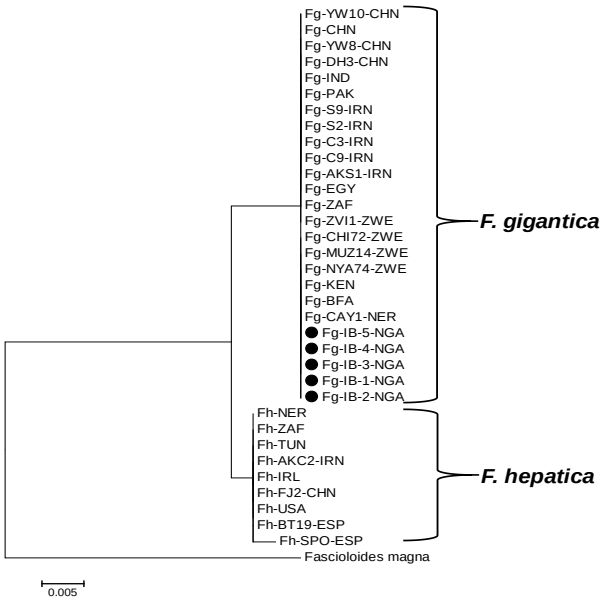

Fig.9 Phylogenetic analysis of *Fasciola spp* based on ITS-2 region nucleotide sequences from different geographical locations. The phylogenetic tree was computed using the multiple alignments of the nucleotide sequence of the NAD1 gene from Nigeria *Fasciola spp* and other sequences retrieved from the GenBank. *Fascioloides magna* ITS-2 Region was used as the outgroup.

4. DISCUSSION

Genetic information is essential for managing parasitic diseases, as it allows for the easy identification of the specific parasites infecting the host (Palevich et al., 2018). Molecular data provides valuable markers that can be used for species description and determining the genetic makeup of different populations. Despite the many steps forward, most of the helminth genomic data still consists of partial draft assemblies and numerous hypothetical genes that remain uncharacterized, and not understood (Quinzo et al., 2022). Morphological characteristics can often be sufficient for distinguishing these two *Fasciola species*, however, the inclusion of molecular data through the use of molecular markers enhances the diagnostic accuracy. The COX1 gene sequence exhibits significant differences among various cestode and nematode species. Amplification and sequence analyses of mitochondrial genes COX1 and NAD1 can enhance species identification and characterization. Also, due to the abundance of mitochondria in cells. While COX1 is often considered a reliable marker for species-level identification in many taxa (Poon et al., 2017), including trematodes, the inclusion of a second mitochondrial gene NAD1, an independent marker can provide additional confidence in species

identification, especially in cases where morphological characteristics are ambiguous or when dealing with potential hybrid or cryptic species (Bennett and Robinson, 2021).

The advent of molecular techniques has facilitated the identification and genetic characterization of morphologically similar parasites (Teofanova et al., 2011). Therefore, in this study, mitochondrial COX1 and NAD1 genes as well as the nuclear ITS region of *Fasciola* species were amplified and characterized. Phylogenetic analysis of the COX1 and NAD1 genes as well as the nuclear ITS region of *Fasciola spp* from different countries yielded two broad divisions representing *F. gigantica* and *F. hepatica*. All sequenced isolates in this study clustered with *F. gigantica* and thus identified as *F. gigantica.* The level of diversity in mitochondrial COX1 and NAD1 genes was significantly higher than that in the nuclear ITS region. Several sub-clusters were seen on the phylogenetic trees of mitochondrial COX1 and NAD1 genes, unlike the nuclear ITS region. This is in line with previous studies that reported the nuclear ITS region to be more conserved than mitochondria regions (Amer et al., 2011).

Multiple sequence analysis of the COX1 and NAD1 nucleotide sequences from this study and other individual sequences retrieved from the NCBI online repository as shown in Figures 5 and 6 distinguishes *F. gigantica* from *F. hepatica*. The mutations show differences that provide taxonomic or phylogenetic information between the two major species of *Fasciola*. These may have a functional impact or be associated with putative functional domains. The mutations may be responsible for differences in species phenotypes which may be associated with pathogenicity. Also, the COX1 gene substitutions A771G, differentiate *F. gigantica* in Ibadan from that of China and Thailand.

On the COX1 gene phylogenetic tree, *Fasciola* isolates in this study were found on two clusters: a cluster containing only Nigerian samples from this study and another cluster shared with isolates from Niger. On the NAD1 gene phylogenetic tree, Zambian isolates were found in the Nigerian cluster. Interestingly, Chinese isolates from Buffalo were found on Nigerian clusters on both NAD1 and COX1 phylogenetic trees. However, other isolates from China were found in Asian clusters. The relatedness of isolates from Nigeria and the sub-Saharan countries of Niger and Zambia can be explained due to transboundary movement and spread of livestock and wildlife. Besides infesting livestock, such as cattle, sheep and goats, *F. gigantica* infests an array of wildlife as well as draught animals; camels, donkeys and horses, all of which roam sub-Saharan Africa freely. North African isolates were more commonly related to isolates from Mediterranean and Middle Eastern countries highlighting greater import and export of livestock within this region. This study contributes to the growing body of knowledge on the genetic diversity and population structure of *Fasciola spp*. This information is essential for understanding the epidemiology and control of these important parasites. Further studies would be necessary to investigate the relatedness of Chinese isolates with Nigerian isolates and correlate mutations to phenotypes if applicable, especially in the area of drug resistance and pathogenicity.

5. CONCLUSIONS

This study shows the diagnostic specificity of the molecular method used and the appropriateness of genes COX1, NAD1 and ITS in the confirmation of *F. gigantica*. The dominant *Fasciola species* in this region is *F. gigantica*. Hence, monitoring transboundary movement, export, and import of livestock and wildlife that host *Fasciola* is important in the control of fasciolosis. This study opens up a new field of research into fasciolosis in Nigeria upon which other studies can be based and confirms the presence of *F. gigantica* in fasciolosis in Nigeria, using molecular techniques. Further studies should be done on the genetic diversity, and population structure of different species so researchers can gain insights into transmission dynamics, host specificity, drug resistance, and the potential for interspecies hybridization. This knowledge can underscore the need for the development of effective control strategies and targeted interventions.

Compliance with Ethical Standards *Fasciola* samples were obtained from the bile ducts of the infected liver of already slaughtered animals at the government abattoirs.

Authors' declarations

Publication consent

Each author has demonstrated their consent for the publication of the current manuscript.

Conflict of interest declaration

All authors declare no financial, academic, commercial, or personal conflict of interest.

Funding This study received no financial aid and was self-funded.

Authors' contributions.

O.G.B: Conceptualization, writing – review and editing.
B.T.F: Data collection, processing, analysis, and writing.
R.D.A: Data curation, and analysis.
O.T.J: Conceptualization, materials and supervision
O.A.F: Conceptualization, supervision, review and editing.

REFERENCES

Ai, L., Weng, Y.B., Elsheikha, H.M., Zhao, G.H., Alasaad, S., Chen, J.X., et al. 2011. Genetic diversity and relatedness of *Fasciola spp*. isolates from different hosts and geographic regions revealed by analysis of mitochondrial DNA sequences. Vet. Parasitol. 181: 329-334.

Ali, H., Ai, L., Song, H.Q., Ali, S., Lin, R.Q., Seyni, B. et al. 2008. Genetic characterization of Fasciola samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. Parasitol. 102: 1021–1024.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215: 403-410.

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 (ITS1 and ITS2) and mitochondrial (NDI and COI) gene markers. Parasitol. Int. 60: 5-12.

Anawat, P., Chalobol, W. 2014. Prevalence of infection and molecular confirmation by using ITS-2 region of *Fasciola gigantica* found in domestic cattle from Chiang Mai province, Thailand. Asian Pac J Trop Med. 7: 207-211.

Banwo, O.G., Oyedokun, P.O., Akinniyi, O.O., Jeremiah, O.T. 2023. Bovine Fasciolosis in Slaughtered Cattle at Akinyele, Ibadan, Nigeria. J. Appl. Vet. Sci. 8: 104-110.

Bennett, A. P. S., Robinson, M. W. 2021. Trematode Proteomics: Recent Advances and Future Directions. Pathogens. 10(3): 348.

Le, T.H., Van De, N., Agatsuma, T., Nguyen, T.G., Nguyen, Q.D., McManus, D.P. et al. 2008. Human fasciolosis and the presence of hybrid/introgressed forms of *Fasciola* in Vietnam. Int. J. Parasitol. 38: 725-730.

Levy, S., Calado, M., Mateus, T.L., Vieira-Pinto, M. 2023. First Molecular Identification of Fasciola gigantica in Slaughtered Cattle in Cape Verde: Prevalence, Gross Pathological Lesions, Genetic Identification and Coprological Analysis. Pathogens. 12: 75.

Palevich, N., Britton, C., Kamenetzky, L., itreva, M., de Moraes Mourão, M., Bennuru, S., Quack, T., Scholte, L. L. S., Tyagi, R., Slatko, B. E. 2018. "Tackling hypotheticals in helminth genomes," Trends Parasitol. 34:179–183.

Periago, M.V., Valero, M.A., 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: 368-378.

Poon, R. W. S., Tam, E. W. T., Lau, S. K. P., Cheng, V. C. C., Yuen, K. Y., Schuster, R. K., Woo, P. C. Y. 2017. Molecular identification of cestodes and nematodes by cox1 gene real-time PCR and sequencing. Diagn. Microbiol. Infect. Dis. 89(3): 185–190.

Prastowo, J., Priyowidodo, D., Sahara, A., Nurcahyo, W., Nugraheni, Y.R., Awaludin A. 2022. Molecular identification of cercaria *Fasciola gigantica* in lymnaeid snails in Kulon Progo, Yogyakarta. Vet. Parasitol. Reg. St Rep. 30: 100707

Quinzo, M. J., Perteguer, M. J., Brindley, P. J., Loukas, A., Sotillo, J. 2022. Transgenesis in parasitic helminths: a brief history and prospects for the future. Parasit. Vectors. 15(1): 110.

Semyenova, S.K., Morozova, E.V., Chrisanfova, G.G., Gorokhov, V.V., Arkhipov, I.A., Moskvin, A.S. et al. 2006. Genetic differentiation in eastern European and Western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial *Nad*1 and *Cox*1 genes. J. Parasitol. 92: 525-530.

Soulsby, E.J.L. 1982. Helminth, arthopods and protozoa of domesticated animals, 7th Ed. ELBS, Bailliaerc Tindall and Cassel. London.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Teofanova, D., Kantzoura, V., Walker, S., Radoslavov, G., Hristov, P., Theodoropoulos, G. et al. 2011. Genetic diversity of liver flukes (*Fasciola hepatica*) from Eastern Europe. Infect. Genet. Evol. 11: 109-115.

Valero, M.A., Darce, N.A., Panova, M., Mas-Coma, S. 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplanohyper endemic region. Vet. Parasitol. 102: 85-100.

WHO World Health Organization 2007. Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control Bolivian Altiplanohyper endemic region. Vet. Parasitol. 102: 85-100.