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Research Article **Open Access**

MORPHOMETRIC PHENOTYPES AND MOLECULAR IDENTIFICATION OF *FASCIOLA* SPECIES ISOLATED FROM CATTLE IN ILORIN, NORTH-CENTRAL NIGERIA

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Abstract

Fascioliasis is a serious infectious parasitic disease of human and domestic ruminants. It is caused by the common liver flukes *Fasciola hepatica* and *Fasciola gigantic* and it is of public health concern in some countries of the world. Species-level identification of these species using only morphological methods is unconvincing, unreliable and sometimes difficult. Molecular methods enhance the prospects of species-level identifications using a well established and highly reliable DNA-based approach. We used PCR-RFLP analysis for precise identification of *Fasciola* species collected from Nigeria. One hundred *Fasciola* adult worms were collected from selected abattoirs in Ilorin, North-central Nigeria. The parasitic worms were first identified by morphometric analysis using AmScope digital camera fastened to the ocular eye piece of the light microscope. For the molecular identifications, genomic DNA was extracted, Polymerase Chain Reaction (PCR) technique and RFLP were used to characterise the *Fasciola* adult worms. Morphometric data showed that 70 out of the 100 *Fasciola* adult worms have characteristic features similar with that of *F. gigantica* as described from previous studies while the remaining 30 adult worms have characteristics that deviate from that of *F. gigantica* but did not confirm with characteristic features of *F. hepatica*. Agarose gel electrophoresis result confirmed successful amplification of two gene regions of Sodium Dehydrogenase I (*ND1)* from mitochondrial DNA of *Fasciola* spp. The PCR-RFLP analysis did not show any noticeable genetic variation in the *Fasciola* adult worms. We suggest more studies to be done using DNA sequence analysis and other molecular markers such as random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) to better understand the genetic variability and population structure within *Fasciola* spp. in Nigeria.

Keywords: *Fasciola gigantica, Fasciola hepatica*, PCR-RFLP assay, molecular characterization, genomic DNA

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1. Introduction

Fasciola gigantica and *Fasciola hepatica* are common liver flukes causing fascioliasis, one of the most important helminth infections of human and livestock (Mas-Coma, 2005). Fascioliasis is a disease of veterinary and economic importance all over the world including Nigeria because it causes morbidity, weight loss and sometimes mortality among cattle (Tsegaye *et al*., 2011). *Fasciola* is very cosmopolitan in distribution, being found throughout all regions of the world, including temperate, tropical and subtropical regions. Infection is found in temperate and tropical areas where sheep and cattle are raised and in humans, typically where they consume raw watercress (Mas-Coma *et al.,* 2009). Infection with *F. gigantica*, on the other hand, is found more commonly in tropical and subtropical regions of the world (Mas-Coma *et al.,* 2014). The presence of both *F. gigantica* and *F. hepatica,* and the existence of intermediate forms, has been reported in livestock from Iran (Rokni *et al.,* 2010; Amer *et al.,* 2011), Egypt (Marcilla *et al.,* 2002; Periago *et al.*, 2008; Dar *et al*., 2012; El-Rahimy *et al*., 2012), Niger (Ali *et al.,* 2008), Japan (Itagaki *et al.,* 1998, 2005; Ichikawa and Itagaki, 2010), Korea (Agatsuma *et al.,* 2000), China (Huang *et al.,* 2004; Liu *et al.,* 2014) and Vietnam (Le *et al.,* 2008). A number of studies revealed single species infections of *F. hepatica*, reported in Tunisia, Algeria and Italy (Garippa, 2009; Farjallah *et al.,* 2013) and *F. gigantica* has been reported in India (Velusamy *et al.,* 2006; Prasad *et al.,* 2008; Raina *et al.,* 2013) and Mauritania (Amer *et al.,* 2011). In addition, fascioliasis has been recognized as a major threat to public health in human and farm animals. However to reduce the risk of contracting fascioliasis, enhance its management and control, accurate identification of these species is a crucial and important step towards the disease control strategy and management. Species-level identification of these two common *Fasciola* species using only morphological methods is unconvincing, unreliable and sometimes difficult to discriminate by taxonomists (Marcilla *et al.*, 2002). Periago *et al*. (2008) investigated phenotypic analysis of *Fasciola hepatica/Fasciola gigantica* intermediate forms in Egypt and Iran; the first of its kind in Africa. This study reported intermediate forms otherwise referred to as *Fasciola* species-like in Egypt and Iran where they used grouping variables such as body roundness, body length divided by maximum body width, and distance between the ventral sucker and the posterior end of the body (BR, BL/BW and VS-P criteria).

Afshan *et al.,* (2014) reported on the phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* from Central Punjab, Pakistan. Fasciolid populations from the studied area were grouped according to the maximum and minimum values of given differentiating morphological measurements. Periago *et al.,* 2006 also observed an intermediate form of *Fasciola* species. Ghavami *et al*. (2009) and Baran *et al.,* (2016) elucidated that the mean of morphometric values in flukes from sheep was greater than those of cattle in Iran and Baran, respectively. Genotypic characterisation to support established phenotypic intermediate forms were carried out as reported by other authors and it was discovered that ITS2 fragment of 535bp amplified specimens, showed no variation at the species-specific nucleotide sites 230, 340 and 341

(Ghavami *et al*., 2009). DNA-based methods have been used as a reliable tool to identify biological diversity between the two common *Fasciola* species (Shafiei *et al*., 2013; Barker *et al*., 1993; Yakhchali *et al.,* 2015; Itagaki and Tsutsumi, 1998; Marcilla, *et al*., 2002). Le *et al*. (2008) reported that in Vietnam, *Fasciola gigantica* is the dominating species, while an "intermediate *Fasciola* species" or "*F. hepatica*-like fluke", has been reported in humans, cattle and buffaloes. Molecular techniques using PCR-RFLP assay have been previously used for genetic identification of *Fasciola* species obtained from Japan on the basis of DNA sequences of ribosomal and mitochondrial DNA markers (Itagaki *et al*., 1998; 2005; Marcilla *et al*., 2002; Shafiei *et al*., 2013). Yakhchali *et al*. (2015) observed that PCR-RFLP analysis was able to differentiate *F. hepatica* from *F. gigantica*. While the phylogenetic reconstruction justified, to some extent, the morphological diagnosis, it failed to segregate *F. hepatica* from *F. gigantica* identified in previous studies. For effective drug and ecological intervention in Nigeria, there is an urgent need to differentiate between the fasciolid species, due to their distinct epidemiological, pathological and control characteristics. Unfortunately, coprological, immunological and pathological diagnosis may be unreliable, hence the need to establish phenotypic and genotypic distinctions in *Fasciola* species in Nigeria. In addition, there is limited documentation on molecular identification of these two common *Fasciola* spp in Nigeria. This knowledge gap hinders the understanding of the prevalence and dynamic transmission of fascioliasis. In the present study, we used standard morphological and molecular methods to identify and characterize *Fasciola* spp obtained from slaughtered cattle in Ilorin, Nigeria with the aim to investigate phenotypic and genotypic divergence existing between the hepatic flukes, *Fasciola hepatica* and *F. gigantic*a.

2. Material and Methods

2.1 Study Populations

The fasciolid populations collected from liver of slaughtered cattle at various abattoirs in the four municipal local government areas in Ilorin, Nigeria were grouped according to the maximum and minimum values of the morphological measurements as proposed by Periago *et al.* (2006) into *Fasciola hepatica, Fasciola gigantica* and *Fasciola* sp.

2.2 Parasite sample

Fasciola adult worms (n=100) were collected from four local abattoirs in Ilorin, Northcentral Nigeria during post-slaughter inspection from livers of naturally infected cattle, sheep and goat. Parasitic flukes were examined in collaboration with the veterinary doctors assigned to the abattoirs. Fresh adult worms were washed thoroughly in physiological saline, fixed in 95% ethanol and stored at -20°C until further use for morphometric analysis, genomic DNA extraction and other molecular analyses.

2.3 Slide Preparation

A single adult fluke was chosen at random from each specimen bottle (a specimen bottle contains adult flukes collected from each infected animal). The selected adult worms were fixed for 20 minutes in

sucrose hypotonic solution. The worms were then introduced into a petri dish containing prepared aceto-orcin standardized media for 30 mins, to allow proper staining of the internal organelles for easy identification and measurement. Each stained worm was spread between two glass slides and held firmly with cellophane tapes at both edges (Hussein and Khalifa, 2010).

2.4 Observation and Measurement Techniques

The slides were examined under a stereo microscope and the images viewed on a computer screen with the aid of an AmScope digital camera (AmScope Microscope Digital Camera 3.1MP APTINA Colour CMOS) fastened to the ocular eye piece of the microscope.

2.5 Morphometric Identification

Biometric characters were measured according to Periago *et al.,* (2006). The parameters measured include: Body length (BL), Maximum body width (BW), Body width at ovary level (BWOv), Body perimeter (BP), Body roundness (BR), Cone length (CL), Cone width (CW), Maximum diameter of oral sucker (OS max) and Minimum diameter of oral sucker (OS min). Also, Maximum diameter of ventral sucker (VS max), Minimum diameter of ventral sucker (VS min), Distance between the anterior end of the body and the ventral sucker (A-VS), Distance between the oral sucker and the ventral sucker (OS-VS), Distance between the ventral sucker and the union of the vitelline glands (VS- Vit) and Distance between the union of the vitelline glands and the posterior end of the body (Vit-P) Distance between the ventral sucker and the posterior end of the body (VS-P). Other morphometric features considered are Pharynx length (PhL), Pharynx width (PhW), Testicular space (taking both testes together) length (TL), Testicular space width (TW), Testicular space perimeter (TP), Body area (BA), Oral sucker area (OSA), Ventral sucker area (VSA), Pharynx area (PhA) and Testicular space area (TA).

2.6 Derived Body Ratio Parameters

Ratios of certain body parameters were determined, which includes: Body length to body width (BL/BW), Body width at ovary level to cone width (BWOv/CW). Oral sucker area to ventral sucker area (OSA/VSA) and the ratio of Body length to the distance between the ventral sucker and the posterior end of the body (BL/VS-P).

2.7 Measurement of body roundness

The body roundness measurement was determined using the relation BR = BP2/4πBA. (Ohnuma *et al.,* 2006; Periago *et al.,* 2008). All preliminary measurements and images were subsequently analyzed by image analysis software.

2.8 DNA Extraction

Two methods of DNA extraction were used i.e. Phenol-chloroform method and the use of Zymo Research genomic DNA Miniprep kit (Catalog No: D3050).

2.9 DNA Extraction Using Phenol-Chloroform Method

For genomic DNA extraction, freshly collected worms were extensively washed in physiological saline and fixed in 70% ethanol. A portion of the apical and lateral

zone of adult *Fasciola* sample was removed and crushed. DNA from the crushed materials was extracted following the listed procedures: 500 µl of lysis buffer and 8 µl of proteinase K were added to the sample and incubated overnight at 37 °C. Subsequently, 100µl of phenol–chloroform was added and centrifuged at 1000 revolutions/10 min (rpm) at 25°C. Top aqueous phase was removed and absolute alcohol was used to precipitate the DNA. Extracted DNA was diluted in TE buffer and stored at -20°C until used for PCR analysis.

2.10 DNA Extraction Using Zymo-Spin Kit

Genomic DNA was extracted using Zymo-Spin™ kit (Zymos Research, USA) following the manufacturer's instructions. The DNA concentration and purity were checked by evaluating the absorbance ratio A260 nm/A280 nm using a Beckman DU-640B Spectrophotometer. The eluted DNA was stored below -20°C until the next stage of molecular analyses.

2.11 Polymerase chain reaction (PCR)

In this study, the PCR experiments were carried out using 50 µl final reaction volume consisting of 2x PCR master mix (QIAGEN, Hilden, Germany), 300 nM of forward and reverse primers. Primer sequences used are listed in Table 3. Genomic DNA (300-500 ng) from *Fasciola spp* was used as the template. The PCR cycling conditions was 96˚C for 1 min. followed by 35 cycles of: 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 s. Reactions were terminated with a final extension of 72˚C for 7 min. The PCR cycling steps were optimized for product length and primer annealing temperature according to the manufacturer's guidelines.

2.12 Agarose Gel Electrophoresis

The isolated DNA was assessed according to standard agarose gel electrophoresis protocol on 1% (w/v) agarose in 1X TBE buffer. The success of the DNA extraction process and the integrity of the isolated DNA were assessed on 1% (w/v) agarose in the buffer. The isolated DNA samples were prepared for loading into the well created by the comb in the set agarose by mixing 4 µl of the extracted DNA sample with 1µl of the 5X loading dye. The 5 µl mixture of sample and loading dye was loaded into well formed by the comb in the gel (a sample per well). The electrophoresis was run at 80 volts for 45 mins, after which the gel was viewed and photographed under gel documentation.

2.13 Restriction fragment length polymorphism (RFLP) analysis

A PCR mixture of 20µl was digested using restriction enzyme (New England Biolabs) at the recommended temperature for 2h. The restriction fragments were separated on 1% (w/v) agarose in 1X TBE buffer and the size of DNA fragments was determined in comparison with a 100 bp ladder (New England Biolabs).

3. Results

3.1 Morphometric Analysis

The specimens from the four abattoirs were grouped into *Fasciola hepatica*-like, *Fasciola gigantica*-like and *Fasciola* sp based on morphometric features such as Body Roundness (BR), Body Length to

Body Width ratio (BL/BW) as well as the Distance between Ventral Sucker and Posterior End of the Body (VS-P). Comparing the range of values obtained from each abattoir, none of the fluke observed could be described as *Fasciola hepatica.* The samples were only tropical species *Fasciola gigantica* and the intermediate species *Fasciola* sp. based on the standard descriptor and criteria (Table 1). Comparison of the maximum values in each of the body measurements between *Fasciola gigantica* and *Fasciola* sp. showed that *F. gigantica* had the highest value. We observed that 66.67% of the measurements are greater in the specimens grouped as *F. gigantica* among the samples collected from Ipata while, 85.19% of the measurements were greater in Oja-Titun. In terms of percentage of total flukes examined, 74.07% was higher in Ipata-Oloje and 60% in Oko-Olowo. The intermediate populations from the four abattoirs differed to a great extent; whereas specimens from Ipata had low values of BL, BW, BP, CW, OS max, OS min, A-VS, OS-VS, VS-Vit, Vit-P, VS-P, TL, TW, TP, BA, OSA, TA as well as BL/BW, those from Oja-Titun had low maximum values of BL, BW, BWOv, BP, BR, CL, CW, OS max, OS min, VSMax, VSMin, A-VS, OS-VS, VS-Vit, Vit-P, PhL, TL, TW, TP, BA, OSA, VSA and BL/BW. While the specimens from Ipata-Oloje had the lowest maximum values of BL, BW, BWOv, BP, BR, CW, OS max, OS min, VSMax, VSMin, OS-VS, Vit-P, TP, BA, OSA, VSA, PhA, TA with BL/BW, the ones from Oko-Olowo had the lower maximum values of BL, BW, BP, BR, CL, CW, OS max, OS min, VSMax, VSMin, TL, TP, BA, OSA, VSA, TA and BL/BW as shown in Table 1.

Table 1. Comparative morphometric data of adults *Fasciola gigantic* and *Fasciola spp* isolated from cattle at four different abattoirs in Ilorin, North-Central, Nigeria

Abattoirs	**Ipata**		**Oja-titun**		**Ipata-Oloje**		**Oko-Olowo**	
Fasciola species	*Fasciola gigantica*	*Fasciola sp.*	*Fasciola gigantica*	*Fasciola sp.*	*Fasciola gigantica*	*Fasciola sp.*	*Fasciola gigantica*	*Fasciola sp.*
Number of adult flukes	16	9	18	7	19	6	15	10
Body Length (BL)	30.01-42.32 33.93± 3.29	22.88-30.01 26.78±2.90	30.46-42.32 35.51± 2.60	23.23-30.46 27.12± 4.67	30.01-36.39 32.56± 1.97	24.12-29.92 28.18± 2.07	32.86-42.32 34.88± 2.42	17.98-30.01 25.64± 3.87
Maximum Body Width (BW)	4.04-10.52 6.51± 1.48	5.22-9.29 6.51± 1.42	4.04-10.52 6.98± 1.82	4.29-9.29 6.63± 1.78	4.40-8.48 6.15± 1.18	5.59-6.01 6.48 ± 0.57	4.04-10.23 6.15± 1.40	4.29-8.41 6.39± 1.16
Body Width At Ovary Level (BWOv)	2.75-5.37 4.02± 0.90	2.27-5.70 4.19 ± 1.13	2.75-5.60 4.07 ± 0.88	3.01-5.01 3.99 ± 0 .86	2.87-6.91 4.38 ± 0 .96	3.86-5.70 4.82 ± 0.77	2.75-5.64 3.90 ± 0.83	2.27-5.70 3.68 ± 1.13
Body Perimeter (BP)	64.29-83.50 67.96± 4.84	62.67-70.98 65.72± 1.82	64.29-91.52 72.25± 8.07	64.69-76.98 67.62± 7.44	62.15-91.10 69.10± 7.14	61.86-84.92 70.69± 8.97	63.50-91.52 72.57± 10.21	62.35-84.92 67.67± 6.41
Body Roundness (BR)	1.08 - 3.54 2.24± 0..59	1.84 - 3.55 2.62± 0.56	1.08-4.31 2.32± 0.83	1.84-4.22 2.79± 0.90	1.55-4.64 2.53 ± 0.75	1.99-4.06 2.83 ±0.73	1.19-4.31 2.61 ± 0.77	1.84-4.22 3.01± 0.90
Cone Length (CL)	1.11-2.24 1.51± 0.32	0.79-2.29 1.37± 0.46	1.17-3.07 1.85± 0.51	0.79-2.49 1.71± 0.53	1.09-2.18 1.41± 0.29	1.19-1.44 1.32± 0.096	1.09-2.90 1.80± 0.46	1.06-2.29 1.30 ± .371
Cone Width (CW)	1.07-3.25 1.85± 0.53	1.17-2.45 1.74± 0.50	1.07-3.12 1.73± 0.45	1.17-2.25 1.64± 0.45	1.15-3.25 1.85± 0.54	2.08-2.75 2.39± 0.22	1.17-3.12 1.70± 0.46	1.25-2.75 1.94± 0.49

Key: Minimum – Maximum Measurements in Millimetres. **Mean ± SD

Table 1cont'd. Comparative morphometric data of adults *Fasciola gigantic* and *Fasciola* spp isolated from cattle at four different abattoirs in Ilorin, North-Central, Nigeria

Abattoirs	Ipata		Oja-Titun		Ipata-Oloje		Oko-Olowo	
Fasciola species	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*
Number of adult flukes	16	9	18	7	19	6	15	10
Distance Between Union of Vitelline Glands And Posterior End (Vit-P)	5.95-15.98	5.75-13.65	6.05-18.45	5.75-11.49	5.95-19.05	7.15-16.97	6.07-17.40	7.15-16.97
	10.40± 3.61	9.03 ± 2.67	11.95± 4.27	8.69 ± 4.04	11.32± 3.93	12.59± 3.90	12.55± 4.28	10.24± 2.89
Distance Between VS And Posterior End of Body (VS-P)	9.01-22.82	11.07-9.76	9.01-22.82	11.07-17.11	10.10-1.61	14.51-2.14	13.61-18.92	8.93-20.67
	16.76± 3.60	14.47± 2.43	16.22± 2.92	4.17 ± 2.82	15.30±3.04	19.09 ±3.05	16.39 ±1.98	15.26 ±3.41
Pharynx Length (PhL)	0.49-1.30	0.39-1.37	0.76-1.45	0.74-1.45	0.49-1.30	0.39-1.34	0.78-1.17	0.39-1.37
	0.93 ± 0.22	0.97 ± 0.31	1.00 ± 0.16	1.07 ± 0.19	1.02 ± 0.20	1.06 ± 0.34	1.02 ± .14	1.10 ± 0.27
Pharynx Width (PhW)	0.10-0.19	0.11-0.25	0.15-0.23	0.14-0.25	0.10-0.26	0.11-0.19	0.13-0.19	0.11-0.25
	0.15 ± 0.02	0.17 ± 0.04	0.17 ± 0.01	0.19 ± 0.02	0.15± 0.04	0.14 ± 0.03	0.17 ± 0.02	0.15 ± 0.04
Testicular Space Length (TL)	11.55-19.72	11.05-13.05	11.54-25.75	11.05-15.32	11.64-21.75	11.67-24.86	11.64-25.75	11.67-24.86
	14.06 ± 2.89	12.39 ± 0.77	15.14 ± 4.09	12.99 ± 3.64	14.91 ± 3.16	17.43 ± 5.38	15.23 ± 4.47	14.85 ± 4.59
Testicular Space Width (TW)	4.79-6.49	4.76-5.96	4.90-8.64	4.79-6.05	4.78-6.73	4.98-7.56	4.90-7.53	4.76-7.56
	5.48 ± 0.55	5.12 ± 0.45	6.18 ± 0.97	5.30 ± 0.94	5.56 ± 0.60	6.20± 0.97	5.94 ± 0.76	5.45 ± 1.00
Testicular Space Perimeter (TP)	32.53-52.50	27.51-42.98	32.49-50.75	33.05-42.51	32.62-52.50	27.51-43.70	32.49-52-50	33.10-41.74
	38.34 ± 5.99	31.01 ± 10.94	39.60 ± 6.12	35.21 ± 5.77	39.85 ± 5.53	32.51 ± 15.22	39.89 ± 7.67	33.60 ± 11.15

Key: Minimum – Maximum Measurements in Millimetres. **Mean ± SD

3.2 PCR-RFLP Results

3.2.1 Restriction digestion and agarose gel electrophoresis analysis

In order to investigate possible restriction fragment differences and genetic variation in the samples of *Fasciola* species collected, the amplified *NDI* sequences by PCR were analysed with *AluI* restriction endonucleases. When amplified PCR products of *NDI* gene were digested with the restriction enzyme *AluI*, sites in both species, and so we selected it to distinguish the two species without false negative. However, the enzyme produced similar restriction fragment patterns (the number and size of fragments) for *Fasciola spp*. This further confirmed that all the *Fasciola* adult worms collected and analysed by both morphometric and molecular methods in this study were *F. gigantica*.

3.3 Molecular Analysis Results

Our data *NDI* gene sequences of the PCR products showed that all of the worms were *F. gigantica* (Fig 1, 2 and 3). Therefore we inferred from our observation that it is very likely that *F. gigantica* is the predominant *Fasciola* species in Nigeria. It is not surprising therefore that the molecular data corroborated the morphometric results which both showed the predominance of *F gigantica* in Nigeria.

However, this is not supported by the studies performed by Alasaad *et al*. (2011) in Spain and Ghavami *et al.,* (2009) in Northwestern Iran, in which only *Fasciola hepatica* was reported. The results of this study is consistent with previous studies by Lin *et al*.,(2007); Le *et al*., (2008) and Ichikawa and Itagaki., (2010).who also reported intermediate species among *Fasciola* sp.

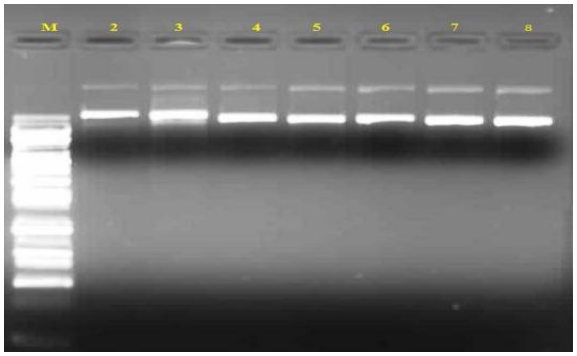

Figure 1. Agarose gel electrophoresis of genomic DNA isolated from Nigerian *Fasciola* adults worms. Lane 1: DNA ladder (M); Lane 2-8. Genomic DNA of *Fasciola* adult worms isolated from slaughtered cattle

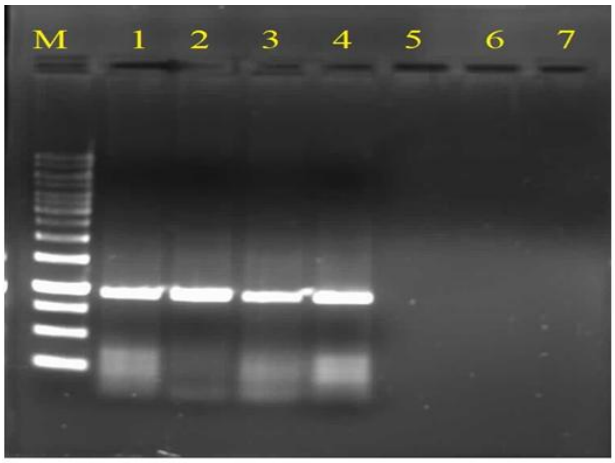

Figure 2. Agarose gel electrophoresis of mitochondrial NDI gene isolated from Nigerian *Fasciola* species. Lane 1: DNA ladder (M); Lane 1-4. PCR products of NDI gene (the samples isolated from slaughtered cattle); Lane 5-7 (negative control); Amplicon size:348bp

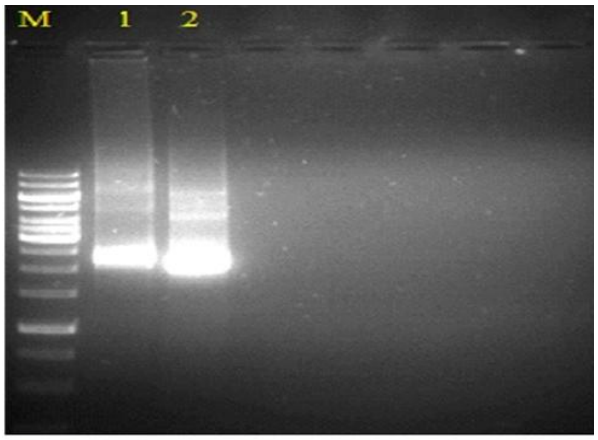

Figure 3. Agarose gel electrophoresis of mitochondrial NDI gene isolated from Nigerian *Fasciola* species. Lane 1: DNA ladder (M); Lane 1and 2 PCR products of NDI gene (the samples isolated from slaughtered cattle); Amplicon size:535bp

PCR analysis of the NADH dehydrogenase subunit I *(NDI*) gene of *Fasciola* species did not show length heterogeneity between *F. hepatica* and *F. gigantica*. This is suggesting that there are no length polymorphisms among *Fasciola* mitochondrial genomes of samples collected from Ilorin. This may further suggest that all the *Fasciola* samples are *F. gigantica* as we expected that although *F. hepatica* and *F. gigantica* are closely related species, there are overwhelming evidences from previous findings that a high intraspecific variation exist between these *Fasciola* species (Karimi, 2008; Shafiei *et al*., 2013; Saki *et al*., 2011; Yakhchali *et al.,* 2015; Itagaki and Tsutsumi, 1998; Marcilla *et al*., 2002).

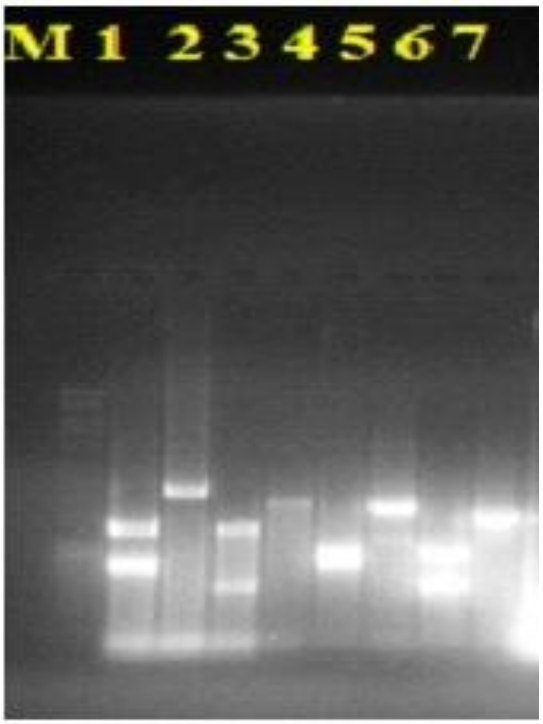

Figure 4. RFLP pattern of PCR products of *Fasciola* species digested with *AluI* (M) is 100bp DNA ladder. Lane 2, 4 and 6 are PCR products for control, lane 1,3,5, and 7 are *Fasciola* species.

We therefore suggest that based on our result, the isolated samples are all *Fasciola gigantica* and in the tropical region of the world because they were all obtained from cattle. PCR-RFLP result of the restriction endonuclease did not show any noticeable distinction between *F. hepatica* and *F. gigantica*. This is not surprising as previous study by Huang *et al*., 2004 showed no variation in restriction patterns among multiple individuals from the same geographical locations. Similarly, restriction endonuclease analysis of Japanese *Fasciola* species showed that samples of *F. hepatica* were identical in restriction map with *F. gigantica* and no intraspecific variations were detected in the restriction maps of both species of *Fasciola* (Marcilla *et al*., 2002).

4. Discussion

The present study showed remarkable differences in morphometric indices among the liver flukes isolated from slaughtered cattle in four different abattoirs in Ilorin metropolis, Kwara state. Considering the variations observed within the parameters like BL, BW and VS-P and others measured in the studied samples, they are likely to be grouped into *F. gigantica* and *Fasciola* species (i.e. other forms). In terms of body width, *F. gigantica* showed the highest measurement of body width in the four abattoirs with the highest of 10.52 mm in Ipata and Oja-tuntun while the least body width of 4.04 mm was recorded in Ipata, Oja-titun and Oko-Olowo abattoirs for *Fasciola* sp. Also, body width at ovary level was higher in *F. gigantica* than *Fasciola* sp. group. Body perimeter was

found to be higher for the *F. gigantica* group in all the abattoirs, with the highest of all measuring 91.52mm found both in Oko-Olowo and Oja-Titun, however, the lowest measurement was also found in the *F. gigantica* group, measuring 61.86 mm as observed in the specimen for Ipata-Oloje abattoir. Body roundness and cone length were higher in Oja-Titun, Ipata-Oloje and Oko-Olowo abattoirs for *F. gigantica,* although, *Fasciola* sp. had the highest value in Ipata, it measured 3.55 mm for body roundness and 2.29 mm for cone length compared to the 3.54 mm and 2.24 mm observed, respectively for the market. The lowest body roundness and cone length were observed in Ipata and Oja-Titun for *Fasciola* spp. at 1.08mm and 0.79mm respectively, with the highest body roundness and cone length values being 4.64mm in Ipata-Oloje and 3.07mm in Oja-Titun.

The values obtained from both categories (i.e. *F. gigantica* and *Fasciola sp*.) overlap to a great extent. However, such overlapping was not observed for the grouping criteria (BR, VS-P and BL/BW), this is in agreement with the result reported by Periago *et al*. (2008). There were overlapping measurements between the two categories in some parameters but this was not found in the standard parameters (i.e. BL, CW, BA and BP) used for the grouping as postulated by Periago *et al.* (2006). Therefore, morphometric analysis done as the first step of identification has been able to show the phenotypes of these flukes.

Table 2. Morphometric comparison using ratios of body parameters

Abattoirs	Ipata		Oja-titun		Ipata-Oloje		Oko-Olowo	
Fasciola species	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*	*Fasciola gigantica*	*Fasciola spp.*
Number of adult flukes	16	9	18	7	19	6	15	10
Ratio Between Sucker Areas (OSA/VSA)	0.22-1.16	0.24-0.91	0.32-1.16	0.44-0.84	0.18-1.16	0.59-0.90	0.28-1.04	0.24-0.93
	0.73± 0.26	0.54± 0.27	0.66± 0.24	0.63± 0.22	0.60± 0.33	0.80± 0.12	0.56± 0.18	0.53± 0.25
Ratio Between BWOv and CW (BWOv/CW)	1.22-4.95	1.62-3.25	1.58-4.95	1.83-3.25	1.22-3.89	1.51-2.52	1.58-3.66	1.35-2.40
	2.32± 0.85	2.48± 0.61	2.48± 0.80	2.51± 0.72	2.53 ±0.79	2.03± 0.41	2.37± 0.55	1.92± 0.38
Ratio Between BL and VS-P (BL/VS-P)	1.47-3.98	1.48-2.24	1.69-3.98	1.66-2.24	1.47-3.24	1.27-2.00	1.74-2.68	1.45-2.24
	2.14± 0.63	1.88± 0.26	2.27± 0.52	1.93± 0.49	2.21± 0.48	1.51± 0.26	2.17± 0.36	1.72± 0.27

Key: Minimum – Maximum Measurements in Millimetres. **Mean ± SD

Table 3. Gene of interest, gene location, name and primer sequence used PCR analysis

S.N	Gene	Gene Location	Name	Primer Sequence for PCR	Expected PCR product size (bp)
1	*ND1*	Mitochondria DNA	Nd1 F	5-GTGGGGTCTGTTGCAGAGAT-3	348
2	*ND1*	Mitochondria DNA	Nd1 R	5-CCCCAAACCAAAGCAACTAA-3	
3	*NDI*	Mitochondria DNA	Nd1 F	5-AAGGATGTTGCTTTGTCGTGG-3	535
4	*NDI*	Mitochondria DNA	Nd1 R	5-GGAGTACGGTTACATTCACA-3	

5. Conclusion

In conclusion, this present study demonstrated that the liver flukes isolated from cattle-at-slaughter from the four different abattoirs in Ilorin metropolis represented *Fasciola* species which were categorized into two; *Fasciola gigantica* and *Fasciola* sp. due to the varying sizes of the flukes extracted from livers of infected cattle. Although, morphometric data from liver flukes supports that some were in the *Fasciola* sp. category, but our molecular analysis confirmed that all studied flukes were *Fasciola gigantica* as there was no variation in length of the *ND1* gene sequences among specimens collected from hosts in different abattoirs in the studied LGA of Ilorin, North-Central, Nigeria. PCR-RFLP also showed that there were no variations in RFLP pattern to indicate a clear distinction between the two morphologically identified species of *Fasciola* suggesting that they might have identical restriction map.

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References

Afshan, K., Valero, M.A., Qayyum, M., Peixoto, R.V., Magraner, A., Mas-Coma, S. (2014) Phenotypes of intermediate forms of *F. hepatica* and *F. gigantica* in buffaloes from Central Punjab, Pakistan. *Journal of Helminthology* **88(4)**: 1-10.

Agatsuma, T., Arakawa, Y., Iwagami, M., H onzako, Y., Cahyaningsih, U., Kang, S.Y., Ho ng, S.J. (2000) Molecular evidence of

natural hybridization between *Fasciola hepatica* and *F. gigantica*. *Parasitology International* **49:** 231-238.

Alasaad, S., Soriguer, R.C., Abu-Madi, M., El-Behairy, A., Baños, P.D., Píriz, A., Fickel, J. Zhu, X.Q. (2011) A fluorescence-based polymerase chain reaction-linked single-strand conformation polymorphism (F-PCR-SSCP) assay for the identification of *Fasciola* spp*. Parasitology Research* **108**: 1513-1517.

Ali, H., Ai, L., Song, H.Q., Ali, S., Lin, R.Q., Seyni, B., Issa, G., Zhu, X.Q. (2008) Genetic characterisation of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. *Parasitology Research* **10(5)**: 1021-1024.

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*. Parasitology International* **60(1)**: 5-12.

Baran, A.I., Akbari, H., Shahbazi, P. (2016) Field screening of *Fasciola hepatica* seroprevalence in sheep by indirect ELISA in East Azarbaijan province. *Iranian Journal of Veterinary Clinical Sciences* **10(2):** 47-55.

Barker, S.C., Blair, D., Garrett, A.R., Cribb, T.H. (1993) Utility of the D1 domain of nuclear 28S rRNA for phylogenetic inference in the Digenea. *Systematic Parasitology* **26**: 181-188

Chaudhry, U., Van Paridon, B., Shabbir, M.Z., Shafee, M., Ashraf, Yaqub, K.T. Gilleard, J. (2016) Molecular evidence shows that the liver fluke *Fasciola gigantica* is the predominant *Fasciola* species in ruminants from Pakistan. *Journal of Helminthology*, Cambridge University Press: 1-8.

Dar, Y., Amer, S., Mercier, A., Courtioux, B. Dreyfuss, G. (2012) Molecular identification of Fasciola spp. (Digenea: Fasciolidae) in Egypt. *Parasite* **19:** 177-182.

ElRahimy, H.H., Mahgoub, A.M., ElGebaly, N.S., Mousa, W.M. Antably, A.S. (2012) Molecular biochemical, and morphometric characterisation of *Fasciola* species potentially causing zoonotic disease in Egypt. *Parasitology Research* **111**: 1103-1111.

Farjallah, S., Ben, S., limane, B., Piras, C., Amer, N., Garippa, G. Merella, P. (2013) Molecular characterisation of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers. *Experimental Parasitology* **135**: 471-478.

Garippa, S.F. (2009) Genetic characterisation of *Fasciola hepatica* from Tunisia and Algeria base. *Parasitology Research* **105**: 1617-1621.

Ghavami, M.B., Rahimi, P., Haniloo, A., Mosavinasab, S.N. (2009) Genotypic and Phenotypic Analysis of *Fasciola* Isolates. *Iranian Journal of Parasitology* **4(3)**: 61-70.

Huang, W.Y. He, B., Wang, C.R. Zhu, X.Q. (2004) Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. *Veterinary Parasitology* **120**: 75-83.

Hussein, A.A. and Khalifa, R.M.A. (2010) Phenotypic description and prevalence of Fasciola species in Qena Governorate, Egypt with special reference to a new strain of Fasciola hepatica. *Journal of King Saud University (Science)* **22**: 1- 8.

Ichikawa, M., Itagaki, T. (2010) Discrimination of the ITS1 types of *Fasciola spp.* based on a PCR-RFLP method. *Parasitology Research* **106**: 757-761.

Itagaki, T. Tsutsumi, K. (1998a) Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantic* determined by ITS-2 sequence of nuclear rDNA. *International Journal of Parasitology* **28(5)**: 777-781.

Itagaki, T., Kikawa, M., Sakaguchi, K., Shimo, J., Terasaki, K., Shibahara, T., Fukuda, K., (2005) Genetic characterisation of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology* **131(5)**: 679-685.

Itagaki, T., Tsutsumi, K.I., Ito, K., Tsutsumi, Y. (1998b) Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and COl sequences with *F. hepatica* and *F. gigantica*. *Journal of Parasitology* **84(2)**: 445-448.

Karimi, A (2008) Genetic diagnosis of *Fasciola* species based on 18S ribosomal DNA sequences. *Journal of Biological Science* **8**: 1166-1173.

Le, T.H., De, N.V., Agatsuma, N.T., Thi, T., Nguyen, Q.D., McManus, D.P., Blair, D. (2008) Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. *International of Journal of Parasitology* **38(6)**: 725-730.

Lin, R.Q., Dong, S.J., Nie, K., Wang, C.R., Song, H.Q., Li, A.X., Huang W.Y. and Zhu, X.Q. (2007) Sequence analysis of the first internal transcribed spacer of rDNA supports the existence of the intermediate Fasciola between F. hepatica and F. gigantica in mainland China. *Parasitology Research* **101(3):** 813-817.

Liu, G.H., Gasser, R.B., Young, N.D., Song, H. Q., Ai, L. Zhu, X.Q. (2014) Complete mitochondrial genomes of the 'intermediate form' of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica* . *Parasites and Vectors* **7**: 150-159.

Marcilla, A., Bargues, M.D., Mas-Coma, S. (2002) A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *F. gigantica*. *Molecular and Cellular Probes* **16(5):** 327-333.

Mas-Coma, S. (2005) Epidemiology of fascioliasis in human endemic areas. *Journal of Helminthology* **79(3)**: 207-216.

Mas-Coma, S., Valero, M.A., Bargues, M.D. (2009) *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology* **69(2)**: 141-146.

Mas-Coma, S., Barques, M.D., Valero, M.A. (2005) *Fasciola*sis and other plant–borne trematode zoonoses;. *International Journal of Parasitology* **35(11-12)**: 1255-1278.

Mas-Coma, S., Valero, M.A., Bargues, M.D. (2014) Fascioliasis. *Advances in Experimental Medicine and Biology* **766**: 177-114.

Ohnuma, K., Yomom, T.M., Asashima, M., Kaneko, K. (2006) Sorting of cells of the same size, shape, and cell cycle stage for a single cell level assay without staining. *BioMed Central Cell Biology* **7**: e25.

Periago, M.V., Valero, M.A., El Sayed, M., Ashrafi, K., El-Wakeel, A., Mohamed, M.Y., Desquesnes, M., Curtale, F., Mas-Coma, S. (2008) First phenotypic description of *Fasciola hepatica/Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infection, Genetics & Evolution* **8(1)**: 51-58.

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). *Parasitology Research* **99(4)**: 368-378.

Prasad, P.K., Tandon, V., Biswal, D.K., Goswami, L.M. Chatterjee, A. (2008) Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions. *Parasitology Research* **103**: 1247-1255.

Raina, O.K., Jacob, S.S., Sankar, M., Bhattacharya, S., Bandyopadyay, S., Varghese, A., Chamuah, J.K. Lalrinkima, H. (2013) Geneticcharacterisation of *Fasciola gigantica* from different geographical regions of India by ribosomal DNA markers. *Journal of Parasitic Diseases* **39**: 1-6.

Rokni, M.B., Mirhendi, H., Mizani, A., Mohebali, M., Sharbatkhori, M., Kia, E.B., Abdoli, H., Izadi, S. (2010) Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. *Experimental Parasitology* **124(2)**: 209-213.

Saki, J., Khademvatan, S. and Yousefi, E. (2001) Molecular identification of animal *Fasciola* isolates in Southwest of Iran. *Australian Journal of Basic and Applied Sciences* **5**: 1878-1883.

Shafiei, R., Sarkari, B., Moshfe, A. (2013) A Consistent PCR-RFLP Assay Based on ITS-2 Ribosomal DNA for Differentiation of Fasciola Species. *Iranian Journal of Basic Medical Sciences* **16**: 1266-1269.

Tsegaye, E., Begna, F., Mulugeta, S (2011) Prevalence of Bovine Fasciolosis and its Economic Significance in and Around Assela, Ethiopia. *Global Journal of Medical Research* **11(3):** 1-7.

Velusamy, R., Singh, B.P., Ghosh, S., Chand ra, D., Raina, O.K., Gupta, S.C., Jayraw, A.K. (2006) Prepatent detection of *Fasciola gigantica* infection in bovine calves using metacercarial antigen. *Indian Journal of Experimental Biology* **44**: 749-753.

Yakhchali, M., Viayeh, R.M., Baran, A.I., Mardani, K. (2015) Morphological and Molecular discrimination of *Fasciola* species isolated from domestic ruminants of Urmia city, Iran. *Iranian Journal of Parasitology* **10(1)**: 46-55.

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