Molecular identification and phylogenetic analysis of Philippine *Fasciola* spp. isolates

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ABSTRACT

Fascioliasis is a foodborne neglected tropical disease (NTD) caused by trematodes in the genus *Fasciola*. It is a major cause of morbidity and mortality in cattle and carabaos worldwide. Despite the substantial agricultural impact of bovine fascioliasis in the Philippines, genetic studies on species identification and phylogeny remain limited. This study describes the morphological and genetic characteristics of adult *Fasciola* sp. collected from swamp buffaloes in the agricultural provinces of Sorsogon and Batangas, Philippines. Morphological analysis was based on body length, cone length, and the presence or absence of shoulders while genetic identification used partial COX1-16S rRNA sequences. Among 33 intact worms, initial morphological assessment on unstained specimens suggested 11 *Fasciola gigantica*-like (33.3%), 3 *Fasciola hepatica*-like (9.1%) and 19 *Fasciola* sp. intermediate forms (57.6%). PCR amplification of a partial COX1-16S rRNA sequence identified *F. gigantica* in 32 specimens with successful DNA extraction. Phylogenetic analysis of 30 high-quality sequences showed clustering of the Philippine isolates with *F. gigantica* samples from Basrah, Iraq, and Lanzhou, China. Notably, our analysis is consistent with previous reports suggesting that Philippine swamp buffaloes originated from mainland China. These findings demonstrate the value of molecular approaches for characterizing fasciolid populations and provide supporting evidence on genetic relatedness and possible dispersal patterns.

INTRODUCTION

Fascioliasis is a parasitic disease caused by trematodes from the genus *Fasciola* (also known as liver flukes) that mainly affects ruminants such as cattle, water buffaloes, sheep and goats. Infection occurs when an animal eats watercress or aquatic plants with encysted *Fasciola* metacercariae, or by ingesting water contaminated with *Fasciola* metacercariae **(Centers for Disease Control and Prevention, 2019; Portugaliza et al. 2019; Yoshihara and Ueno, 2004)**. Once ingested, the immature worms travel from the duodenum to the liver by crossing the peritoneal cavity and penetrating the Glisson's capsule of the liver. The flukes mature within the bile ducts, feeding on host tissue cells and blood **(Lalor et al. 2021)**. Ectopic cases may also occur, wherein the adult flukes are found within the heart, brain, lungs, and subcutaneous tissues **(Wattanagoon and Bunnag, 2013)**. Fascioliasis may also occur among humans. As of 2019, the number of humans affected by fascioliasis has reached two million globally **(Centers for Disease Control and Prevention, 2019)**. Fascioliasis has been reported to affect global agriculture, causing annual losses of approximately US$3.2 billion **(Hoang Quang et al. 2022)**.

Because agriculture plays a significant role in the Philippine economy, the effect of fascioliasis on ruminants are substantial **(Lumain and Balala, 2019; Gordon et al. 2015)**. Fascioliasis is the leading cause of bovine mortality and morbidity in the country, which greatly reduces the production potential of affected livestock **(Gordon et al. 2015)**. *Fasciola hepatica* and *Fasciola gigantica* have been recorded in the Philippines **(Portugaliza et al. 2019)**, and of the two, *F. gigantica* is the more common species causing infection **(Molina et al. 2005)**. Despite this, the true distribution of *Fasciola* species in the Philippines is unknown, as few studies have been conducted on the topic. The areas with known distributions are limited to Isabela Province, Nueva Ecija, Leyte, Northern Samar, and Southern Mindanao **(Bermudez et al. 2024; Portugaliza et al. 2019; Valino et al. 2017; Gordon et al. 2015; Molina et al. 2005)**. There is currently little to no information for provinces in Southern Luzon, despite their substantial carabao

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populations, with more than 70,000 head in Sorsogon and 20,000 head in Batangas **(Philippine Statistics Authority, 2012)**.

The presence of *Fasciola hepatica* in the Philippines has long been disputed, as the study indicating its presence was based solely on morphology and lacked supporting molecular data. Recent studies have not been able to confirm the presence of this species. Confimation of *F. hepatica* in the Philippines would place smaller ruminants like goats and sheep at higher risk for infection, as unlike *F. gigantica,* which is largely associated with larger ruminants like buffaloes and cows, *F. hepatica* is more likely to infect goats and sheep **(Çelik and Çelik, 2018)**. Previous studies have also cited the unreliability of morphological analysis alone in species identification due to overlapping and poorly defined morphological distinctions and documentation of cryptic parasites **(Valino et al. 2017; Walker et al. 2012)**. Morphological identification is also limited by lower sensitivity, operator dependence, and is influenced by the fluke's freshness, timing of collection and preservatives where the worms are submerged, as opposed to molecular identification through nucleic acid amplification tests (NAATs) that are highly sensitive, regardless of sample quality, and are also capable of detenting infection in various biological samples (blood, stool, etc.) even with low parasite burden **(Wong et al. 2014)**. This study determined the *Fasciola* species present in Sorsogon and Batangas using both morphometric and molecular analyses.

MATERIALS AND METHODS

Sample Acquisition and Morphological Assessment

A total of 33 intact *Fasciola* specimens were collected from four swamp buffaloes (*Bubalus kerabau*) obtained from two abattoirs: one in Batangas (n = 10 from three carabaos) and one in Sorsogon (n = 23 from one carabao). Worms were received in a vial with 70% ethanol and stored at −20 °C until further use. Initial morphological assessment was following the methods of **Aryaeipour et al. (2017)** and **Shykat et al. (2022).** Adult worms were thawed and rehydrated in lukewarm distilled water until each sample became pliable and uncurled. Calipers were used to measure cone length, body width, and body length for morphological assessment. Species identification was performed based on the ranges of (1) body length (BL), (2) cone length (CL) specified in Table 1 of Valino et al. (2017), and (3) presence or absence of shoulders **(Valino et al. 2017)**. For classification as either *F. hepatica* or *F. gigantica*, all three variables must be consistent. Specimens exhibiting inconsistency in any one of these variables were designated as specimens with intermediate body forms. Body width and cone width were excluded from the criteria, as these measurements are reported to overlap between the two species **(Valino et al. 2017)**. Morphometric data collected for each variable and population were summarized using means, standard deviations, and confidence intervals. Normality was then assessed using the Shapiro-Wilk test in RStudio Build 402 "Ocean Storm" Release for Windows (RStudio Team, 2019). An F-test was conducted to compare the variances of the Batangas and Sorsogon populations for each variable. To compare the means of the two populations, an independent two-sample t-test was employed. Statistical significance was determined at the 0.05 level, and p-values were reported for each comparison.

Table 1: Species identification based on morphology and nucleotide BLAST results for partial 16S rRNA gene sequences.

Sample Code	Identification based on BLAST match			GenBank Number	Accession
	Closest match	% Identity	Species		
Morphological identification: *Fasciola hepatica*-like forms					
S-6	NC_024025	99.48	*Fasciola gigantica*	PQ409541	
S-10	NC_024025	99.48	*F. gigantica*	PQ409545	
S-15	NC_024025	99.48	*F. gigantica*	PQ409548	
Morphological identification: *Fasciola gigantica*-like forms					
S-18	NC_024025	99.29	*F. gigantica*	PQ409551	
S-19	NC_024025	99.48	*F. gigantica*	PQ409552	
BGB-1	NC_024025	99.48	*F. gigantica*	PQ409557	
BGB-4	NC_024025	99.12	*F. gigantica*	PQ409559	
BGB-6	NC_024025	99.48	*F. gigantica*	PQ409561	
BGB-8	NC_024025	99.48	*F. gigantica*	PQ409563	
BGB-9	NC_024025	99.12	*F. gigantica*	PQ409564	
BL-10	NC_024025	99.47	*F. gigantica*	PQ409565	
Morphological identification: *Fasciola* sp. with intermediate body forms					
S-1	NC_024025	99.48	*F. gigantica*	PQ409536	

S-2	NC_024025	99.48	*F. gigantica*	PQ409537
S-3	NC_024025	99.48	*F. gigantica*	PQ409538
S-4	NC_024025	99.30	*F. gigantica*	PQ409539
S-5	NC_024025	99.48	*F. gigantica*	PQ409540
S-7	NC_024025	99.48	*F. gigantica*	PQ409542
S-8	NC_024025	99.48	*F. gigantica*	PQ409543
S-9	NC_024025	99.48	*F. gigantica*	PQ409544
S-11	NC_024025	99.46	*F. gigantica*	PQ409546
S-13	NC_024025	99.48	*F. gigantica*	PQ409547
S-16	NC_024025	99.12	*F. gigantica*	PQ409549
S-17	NC_024025	99.48	*F. gigantica*	PQ409550
S-20	NC_024025	99.65	*F. gigantica*	PQ409553
S-21	NC_024025	99.48	*F. gigantica*	PQ409554
S-22	NC_024025	99.48	*F. gigantica*	PQ409555
S-23	NC_024025	99.48	*F. gigantica*	PQ409556
BGB-2	NC_024025	99.48	*F. gigantica*	PQ409558
BGB-5	NC_024025	99.82	*F. gigantica*	PQ409560
BGB-7	NC_024025	99.47	*F. gigantica*	PQ409562

Molecular Analysis

DNA was extracted from the 1.5 mm posterior portion of each worm using Monarch® Genomic DNA Purification Kit (New England BioLabs® Inc., USA) following the manufacturer's protocol with the minor modification of reducing the elution volume to 50 µl. Endpoint PCR was done to amplify the target COX1-16S rRNA region using the following primer pairs optimized for species-specific detection of *Fasciola hepatica* and *Fasciola gigantica.* The FHF-FHGR primer pair amplifies a partial 1031 bp sequence of the *COX1* region of *F. hepatica*, while the FGF- FHGR primer pair amplifies the 615 bp 16S rRNA region of *F. gigantica* **(Le et al. 2012).** Each PCR reaction consisted of: 12.5 µl of GoTaq® G2 Hot Start Green Master Mix (Promega), 0.1 µM of FHF (5′-GTT TTT TAG TTG TTT GGG GTT TG-3′) and FGF (5′-TGT TAT GAT TCA TTG TTT GTA G-3′) primers each, 0.2 µM of FGHR (5′-ATA AGA ACC GAC CTG GCT CAC-3′) primer, 20–250 ng of parasite DNA, and nuclease-free PCR-grade water up to 25 µl. Every PCR run included a blank control and a positive control, using the DNA extracted from a reference *F. gigantica* tissue, to ensure reliability of amplification. The PCR conditions were as follows: 95°C for 2 min (initial denaturation), 30 cycles of 95°C for 30 s (denaturation), 52°C for 30 s (annealing), 72°C for 1 min and 30 s (extension), then 72°C for 10 min (final extension).

Agarose gel electrophoresis (AGE) was performed to visualize the PCR product using a 1% agarose gel prepared in TBE and stained with 2 µL of GelRed® Nucleic Acid Gel Stain, 10, 000X in water (Biotium, USA). A 100 bp DNA ladder (New England BioLabs® Inc., USA) was used to approximate the length of each band. The agarose gel was run at 100 V for 20–30 min and then visualized using ProteinSimple AlphaImager Mini Imaging System. Bands of the expected size were excised and purified using QiaQuick® Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol, with the minor modification of reducing the elution volume to 35 µl. The purified PCR products were sent to the Philippine Genome Center Core Sequencing facility for bidirectional sequencing.

Phylogenetic Analysis

Consensus sequences were generated by aligning the forward and reverse reads using STADEN package version 2.0.0b11-2016 **(Staden et al. 2000)**. BioEdit version 7.7.1 was used to manually confirm the quality of the consensus sequences **(Hall, 2021)**. Resulting consensus sequences were compared with available *Fasciola* sequences in GenBank using Nucleotide BLAST for species identification **(Altschul et al. 1990)**. The sequences of all specimens were aligned with each other using a combination of manual inspection and automated alignment using ClustalW in BioEdit version 7.7.1 **(Hall, 2021; Thompson et al. 1994)**.

The alignment was then examined, and unique haplotypes were identified manually. Pairwise sequence comparisons were performed in RStudio Build 402 "Ocean Storm" Release for Windows **(RStudio Team, 2019)** to calculate percent identity and p-distance values, providing quantitative measures of sequence similarity and divergence between groups. The most appropriate nucleotide substitution model for phylogenetic analysis was identified through jMODELTEST version 2.1.10 **(Darriba et al. 2012; Guindon and Gascuel, 2003)**. For phylogenetic analysis, a maximum likelihood (ML) tree was then generated using MEGA software version 12 and visualized with MEGA 12's TreeExplorer (**Kumar et al. 2024**). Bootstrapping was also carried out in MEGA12 based on 1,000 bootstrap samples (**Felsenstein, 1983**). The phylogenetic analysis was conducted using available 16S rRNA sequences (21 sequences) of *F. gigantica* from GenBank, along with one *F. hepatica* and one *Fasciolopsis buski* sequence, the latter serving as the outgroup **(Supplemental Table 3)**. Finally,

haplotype analysis in PopArt version 1.7 was performed on the sequences using a median-joining network (**Bandelt et al. 1999**).

RESULTS AND DISCUSSION

Traditional classification distinguishes *F. gigantica* by its elongated and slender body, uniform body width, and absence of a prominent shoulder. In contrast, *F. hepatica* is described as broader and stouter, with prominent shoulders that distinctly protrude outward from the median of the body, resulting in a more tapered body outline **(Narva et al. 2011)**. Of the 33 intact specimens, morphological assessment identified 11 *Fasciola gigantica*-like, 3 *Fasciola hepatica*-like, and 19 intermediate forms classified as *Fasciola* sp. **(Supplementary Figure 1; Supplementary Table 1)**.

Morphometric Analysis

The Batangas isolates were generally larger than the Sorsogon isolates, showing a significant difference in means between populations for all four variables measured in the study **(Table 2)**. The mean body lengths of adult worms from both provinces were larger than previously reported. **Narva et al. (2011)** described Philippine *F. gigantica* samples as having average body lengths of only 16–39 mm, while another paper reported **(Kimura *et al*. 1984)** a body length range of 25-37 mm for the Philippine *F. gigantica*. For body width, both sample populations fit the body width range and body width-to-length ratio of *F. gigantica* as described by **Kimura et al. (1984)**. A more recent report by **Narva et al. 2011** noted overlapping body-width ranges for *F. gigantica* (4-10 mm) and *F. hepatica* (7-11 mm), such that both sample populations fell within both reported ranges.

However, it should be noted that the morphological examinations were conducted on flukes that had been frozen and stored in ethanol prior to analysis. Freezing is generally considered unsuitable for morphological assessment, as it can compromise specimen integrity and alter external features. For accurate morphological characterization, fresh samples or specimens preserved in an appropriate fixative are strongly recommended, although this was not feasible in the present study because of logistical constraints. Morphometric analyses may also be improved by documenting the instruments used to retrieve the flukes, the host's condition during and after slaughter, and the time interval between host death and worm collection. Hence, while the present findings are substantiated with previous reports of *Fasciola* in the Philippines, the morphological evaluation was limited with specimen integrity and in such cases, molecular analysis is essential for further evaluation and species identification **(Thorstad *et al*. 2007; Haridwal *et al*. 2021; Valino *et al*. 2017; El-Rahimy *et al*. 2012)**.

Table 2: Mean recorded and computed values (with 95% confidence intervals shown in parentheses after each mean) for the two sample populations from Batangas and Sorsogon. P-values indicated represent the results of the independent two sample t-tests conducted at a confidence level of 95% (α = 0.05).

Sample Population	Body length (mm)	Body width (mm)	Body width-to-length ratio	Cone length (mm)
Batangas	44.47 (41.43-47.49)	9.75 (9.18-10.31)	0.277 (0.20-0.24)	4.16 (3.63-4.68)
Sorsogon	26.58 (23.93-29.23)	7.38 (6.69-8.06)	0.279 (0.26-0.29)	3.18 (2.89-3.46)
p-value	1.743e-09	3.184e-06	5.641e-05	0.0005732

Molecular Analysis

DNA was successfully extracted from 32 of 33 specimens. All 32 samples with valid DNA extracts yielded amplicons of approximately 600 bp, consistent with identification as *Fasciola gigantica* (**Supplementary Figure 2**). Of these samples, 30 yielded good quality sequences for downstream analysis. The resulting 543-bp consensus sequences matched *F. gigantica* in GenBank, with percent identities ranging from 99.08-99.82% over a query coverage of 100% **(Table 1)**. The Philippine isolate sequences were deposited in the GenBank repository, with accession numbers PQ409536-PQ409565. The samples were most similar to isolates from Basrah-Iraq (GenBank OR063931) and Lanzhou, China isolates (GenBank NC_024025).

Phylogenetic analysis showed that Philippine isolates clustered with those from China and Iraq (**Figure 1**). Although bootstrap support was 62%, indicating only moderate confidence, this value also suggests that phylogeographic interpretations should be made cautiously. Nevertheless, haplotype and sequence similarity/divergence analyses supported this pattern, providing evidence of genetic similarities among these populations (**Figure 2**). These findings are consistent with the phylogenetic analysis of **Itagaki et al. (2022)** which used the *pepck*, *pold*, and *ND1* molecular markers, which also demonstrated the clustering of Philippine *F. gigantica* strain with *F. gigantica* strain isolated from China.

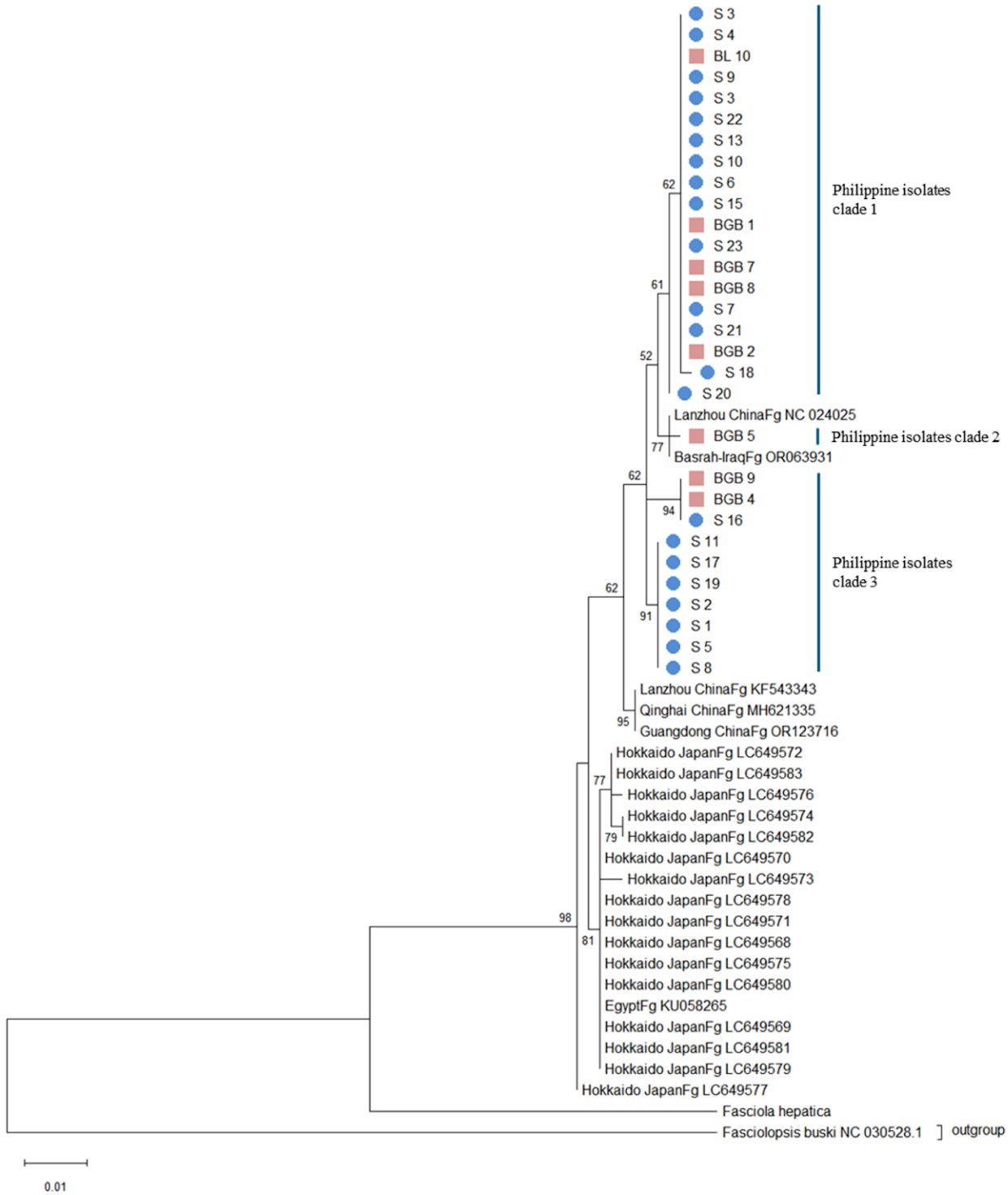

Figure 1: A maximum likelihood phylogenetic tree based on 16S rRNA sequences of *Fasciola gigantica*, inferred under the General Time Reversible model with gamma-distributed rate variation among sites (GTR+G). *F. hepatica* was used as a reference, while *Fasciolopsis buski* (NC_030528.1) served as the outgroup. The analysis included 54 nucleotide sequences with 548 positions in the final dataset. Evolutionary analyses were conducted in MEGA12. Samples generated in this study originated from Sorsogon (●S) and Batangas (■BGB/BL). Node values represent bootstrap support based on 1,000 replicates.

Previous genetic studies suggest that the host, the Philippine carabaos are of Chinese origin, with the native carabao population having the closest affinity with Taiwan population **(Villamor et al. 2021)**. The close clustering of Philippine and Chinese *F. gigantica* sequences is consistent with this historical movement pattern finding and may have facilitated parasite introduction and subsequent establishment. The similarity of the Philippine samples with the Basrah-Iraq isolate (GenBank OR063931) may be attributed to the latter's genetic similarity with isolates from Lanzhou, China. Both sequences share a haplotype, a finding that is corroborated in a previous report by **Rekani and Mero (2023)**.

The Egyptian sequences were also closely related to those of Japan, sharing a similar haplotype. The median joining network created in PopArt version 1.7 reflected the relationships shown in the phylogenetic tree **(Figure 2)**. The Philippine haplotypes are closely associated with Chinese haplotypes, having fewer mutational changes among them. Similarly, haplotypes from Japanese isolates only had a single mutation that separates them from the Egypt

haplotype. Pairwise sequence comparisons revealed high similarity within the Japan-Egypt cluster, with an average identity of 99.77% and a p-distance of 0.0023, and within the Philippines-Iraq-China cluster, with an average identity of 99.47% and a p-distance of 0.0053. In contrast, comparisons between these two groups showed lower identity (98.39%) and greater divergence (p-distance 0.0161), supporting a meaningful genetic separation between the two groups (**Figure 2; Supplementary Table 2**).

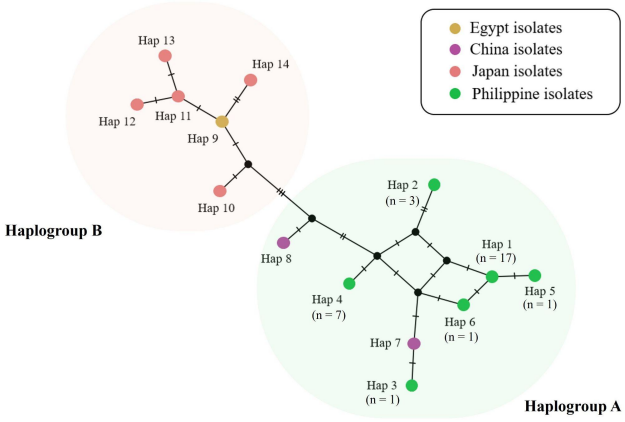

Figure 2: Median-joining network of *Fasciola gigantica* haplotypes based on 543 nucleotides of the 16S rRNA gene, using sequences downloaded from GenBank together with Philippine isolates. All sequences were collapsed into unique haplotypes prior to generating the median-joining network. Fourteen unique haplotypes were identified, six of which belong to the Philippine isolates (Hap 1 - 6). The sample sizes of Philippine isolates are indicated in their respective haplotype nodes. Hatch marks represent mutational changes. Haplogroup A comprises Philippine, Chinese, and Iraqi (no unique color, but included in haplotype 7) isolates, whereas Haplogroup B includes Japanese and Egyptian isolates.

The identification of all specimens as *Fasciola gigantica* reinforces the zoonotic relevance of fascioliasis in endemic Philippine provinces, such as Sorsogon and Batangas, where human–livestock interactions remain common (**Gordon et al. 2015**). Human infection can occur through ingestion of metacercariae on raw aquatic plants (such as kangkong or water spinach) or contaminated water. This leads to hepatic and biliary disease with both acute and chronic manifestations, including abdominal pain, fever, eosinophilia, and long-term complications such as anemia and malnutrition. Reports of human infection, especially in the Philippines, remain scarce but may be underreported due to asymptomatic or chronic presentations of the disease, as well as the clinical limitations in endemic areas (**Tenorio and Molina, 2021**).

Given the parasite's life cycle involving aquatic lymnaeid snails and livestock, together with the cultural practice of consuming potentially contaminated vegetables in agricultural, these findings highlight the need to refine local diagnostic protocols beyond conventional morphology and microscopy. Molecular and serological methods to improve sensitivity and specificity in animal, human, and environmental surveillance. Enhanced surveillance strategies incorporating One Health approaches, such as regular monitoring of livestock and snail populations, targeted health education on safe consumption of freshwater vegetables, and integration with existing neglected tropical disease control programs could better characterize the true burden of zoonotic fascioliasis and mitigate human risk in endemic communities (**Tenorio and Molina, 2021**).

The present study used a single mitochondrial marker (COX1-16S rRNA), which is widely used in species identification due to its conserved regions that enable discrimination among taxa. However, this marker has limitations, including lower resolution for distinguishing very closely related species, and it can be affected by introgression, particularly for *Fasciola hepatica* and *Fasciola gigantica* which are known to hybridize (**Agatsuma et al. 2000**). Therefore, it is recommended to explore additional gene markers (e.g. ITS1, 28S rRNA) in future studies to complement the limitations of the mitochondrial marker.

For more reliable morphometric analysis, staining should be performed to have a better view of the internal structures present, which may provide additional morphological characters useful for species identification. However, it should be noted that staining will degrade the sample and will affect further molecular analysis. The study did not perform staining to preserve the specimens for further analysis using different molecular markers. Morphological assessment is also recommended to be done in fresh samples, to avoid significant changes in the fluke's morphology. Increasing the sample size and number of sample sources per province would improve resolution in assessing the occurrence and distribution of *Fasciola* spp. in the Philippines. In the future, researchers should also consider distinguishing juveniles from adult flukes using staining and microscopy, which could affect the morphological comparison between individuals.

CONCLUSION

This study provides important new genetic information on the 16S rRNA region of *F. gigantica* in the Philippines. This study observed the varying and overlapping morphologies of *Fasciola* spp. across two endemic provinces, Batangas and Sorsogon, but these results should be interpreted cautiously due to specimen integrity. Molecular analysis using the COX1-16S rRNA marker reliably identified all 32 specimens as *F. gigantica*. Molecular data also provided phylogenetic insights into the genetic relatedness of Philippine isolates with those from China and Iraq, consistent with previously reported phylogeographic findings and host movement patterns. Although bootstrap support was moderate, haplotype and sequence similarity analyses supported these associations and highlighted the divergence from Japan and Egypt isolates. The findings underscore the limitation of morphological analysis and highlights the value of incorporating molecular approaches for more reliable species identification. Molecular data may also be used to further support the elucidation of genetic relationships and parasite dispersal patterns and their implications for public health and livestock management. In future studies, morphological examinations may be further strengthened by using fresh samples with appropriate preservatives and by incorporating staining techniques alongside molecular analysis to reduce uncertainty in identification.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

IKCF, ACK, and CAUR conceived and designed the experiments; IKCF and ACK supervised the experiments; CAUR performed all experiments; CAUR and JGGP processed and analyzed the sequencing data. DLM collected the samples. CAUR and ACK wrote the manuscript. All authors edited and contributed to the manuscript and approved the submitted version.

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SUPPLEMENTARY DATA

Supplementary Table 1: Species identification based on morphological characteristics (Body Length, Cone Length, Presence or Absence of Shoulders) (Valino *et al*. 2017)

Sample Code	Morphological Characteristics			Final Identification based on Morphology
	Body Length (mm)	Cone Length (mm)	Shoulders (Presence/Absence)	
Locality: Sorsogon, Philippines				
S-1	31.25	4.20	Present	*Fasciola* sp.
S-2	28.90	3.70	Present	*Fasciola* sp.
S-3	23.70	2.20	Absent	*Fasciola* sp.
S-4	19.70	2.60	Absent	*Fasciola* sp.
S-5	20.50	2.90	Absent	*Fasciola* sp.
S-6	18.00	2.50	Present	*Fasciola hepatica*
S-7	25.50	3.10	Absent	*Fasciola* sp.
S-8	18.90	2.10	Absent	*Fasciola* sp.
S-9	22.40	2.60	Absent	*Fasciola* sp.
S-10	22.60	2.85	Present	*F. hepatica*
S-11	21.10	3.15	Absent	*Fasciola* sp.
S-13	30.40	3.90	Present	*Fasciola* sp.
S-15	29.05	3.00	Present	*F. hepatica*
S-16	27.95	3.18	Absent	*Fasciola* sp.
S-17	30.10	3.30	Absent	*Fasciola* sp.
S-18	36.35	3.15	Absent	*Fasciola gigantica*
S-19	42.00	3.90	Absent	*F. gigantica*
S-20	27.30	2.80	Absent	*Fasciola* sp.
S-21	29.10	4.70	Present	*Fasciola* sp.
S-22	28.30	3.30	Present	*Fasciola* sp.
S-23	20.60	3.15	Present	*Fasciola* sp.
Locality: Batangas, Philippines				
BGB-1	48.15	4.45	Absent	*F. gigantica*
BGB-2	45.10	3.70	Present	*Fasciola* sp.
BGB-4	45.20	3.45	Absent	*F. gigantica*
BGB-5	43.00	3.80	Present	*Fasciola* sp.
BGB-6	45.50	4.45	Absent	*F. gigantica*
BGB-7	39.55	2.75	Absent	*Fasciola* sp.
BGB-8	49.20	4.65	Absent	*F. gigantica*
BGB-9	46.95	4.65	Absent	*F. gigantica*

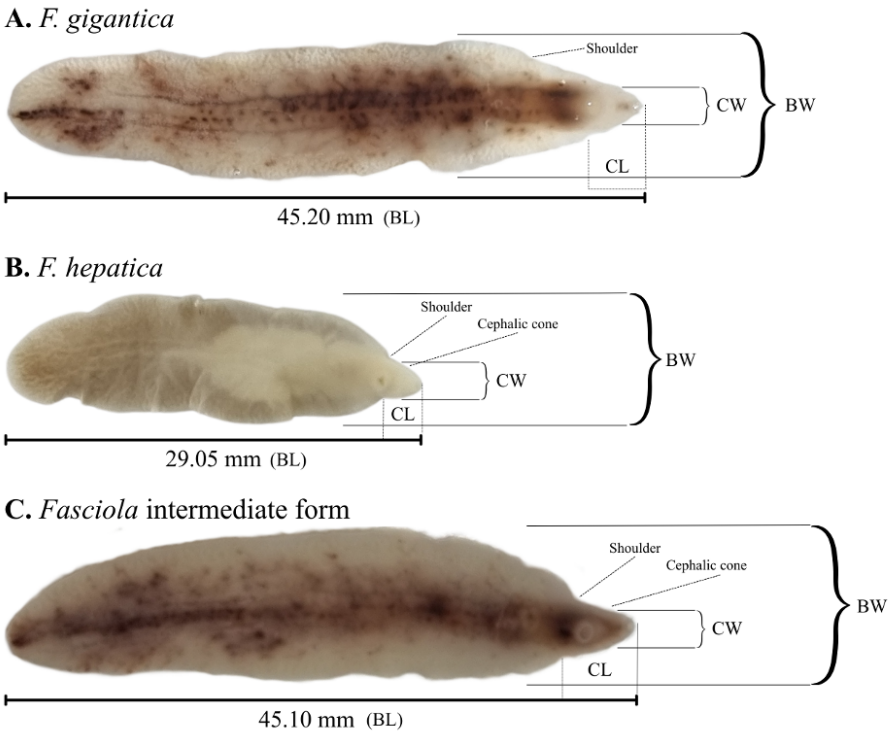
BL-10	46.80	4.35	Absent	*F. gigantica*

Supplementary Table 2: Average percent identity and p-distance values for within- and between-cluster comparisons of Japan–Egypt and Philippines–Iraq–China groups

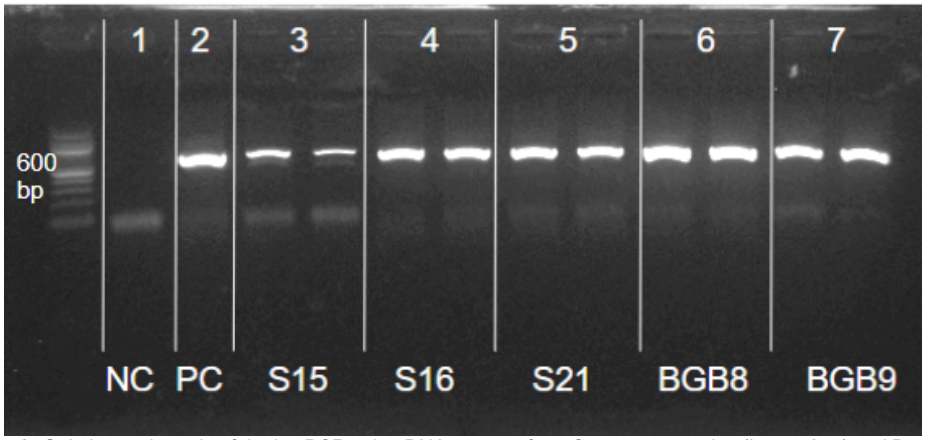
Cluster	% Identity	p-distance
Japan-Egypt (within)	99.77	0.0023
Philippines-Iraq-China (within)	99.47	0.0053
Between clusters	98.39	0.0161

Supplementary Table 3: List of reference sequences retrieved from GenBank

Species	Country	GenBank Accession Number
Fasciola gigantica	China	NC_024025
	China	MH621335
	China	OR123716
Fasciola sp.	China	KF543343
Fasciola gigantica	Iraq	OR063931
Fasciola gigantica	Egypt	KU058265
Fasciola gigantica	Japan	LC649581
	Japan	LC649580
	Japan	LC649579
	Japan	LC649577
	Japan	LC649575
	Japan	LC649571
	Japan	LC649569
	Japan	LC649568
	Japan	LC649578
	Japan	LC649572
	Japan	LC649583
	Japan	LC649576
	Japan	LC649574
	Japan	LC649573
	Japan	LC649582
	Japan	LC649570
Fasciola hepatica	Algeria	MK372243
Fasciolopsis buski	China	NC_030528



Supplementary Figure 1: Photographs of unstained representative *Fasciola* specimens from the study. A) Sample BGB-4, showing a typical *F. gigantica* morphology with an elongated body shape and less prominent shoulders. B) Sample S-15 is identified as *F. hepatica*, with a stout and short body and prominent shoulders. C) Sample BGB-2 is designated as an intermediate form; it has an elongated body shape but is broader in the anterior position, with a prominent shoulder or cone region. BW: body width; CW: cone width; CL: cone length; BL: body length



Supplementary Figure 2: Gel electrophoresis of duplex PCR using DNA extracts from Sorsogon samples (Lanes 3–5) and Batangas samples (Lanes 6–7); Lane 1, negative control (no DNA template); Lane 2, positive control (DNA from an adult *Fasciola* specimen)