OPEN ACCESS
Journal of Animal Health and Production

Research Article

# Morphological and Phylogenetic Characterization of *Fasciola* species isolated From Cows and Buffaloes in Thi-Qar province, Iraq

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**Abstract** |The current study carried out to identify the *Fasciola* species causing Fascioliosis in cows and buffaloes in Thi-Qar province, Iraq based on morphological measurements and sequences analysis of *28S rRNA* gene. *Fasciola* flukes were isolated from the livers of cows and buffaloes slaughtered in the municipality of AL-Nassiriyah abattoir during July to November 2022. Sixty *Fasciola* flukes including (30 from cows and 30 from buffaloes) were used in morphological study. Genomic DNA was extracted from 30 *Fasciola* flukes (15 from cows and 15 from buffaloes) and used to amplified the *28S rRNA* gene and amplicons were sequenced. Based on the morphological study and sequences analysis of *28S rRNA* gene (618 bp), all *Fasciola* flukes were identified as *Fasciola gigantica* with 99 – 100% similarity in the current study when compared with other international samples in the GenBank. 7 sequences of *28S rRNA* gene of *F. gigentica* from cows were placed in the database of the GenBank with accession numbers (LC731384, LC731385, LC731386, LC731387, LC731388, LC731389, LC731390), and 10 other sequences of *F.gigentica* from buffaloes (LC730609, LC730653, LC730654, LC730655, LC730656, LC730657, LC730658, LC730659, LC730660, LC730661) were also recorded in this study. In phylogenetic analysis, Iraqi *F. gigantica* samples from cows and buffaloes revealed one distinct clade and clustered with the flukes from different countries of the world, but genetic variation within the *F. gigantica* cluster showed a considerable distance of *F. gigantica* with acc. number (LC731390) from cows, and (LC730654) and (LC730655) from buffaloes.

**Keywords** | *Fasciola* species, Phylogenetic Characterization, Thi-Qar province, Iraq.

**Received** | September 21, 2023; **Accepted** | December 26, 2023; **Published** | January 25, 2024
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**Citation** | Hansh WJ (2024). Morphological and phylogenetic characterization of ***fasciola*** species isolated from cows and buffaloes in Thi-Qar province, Iraq. J. Anim. Health Prod. 12(1): 40-47.
**DOI** | http://dx.doi.org/10.17582/journal.jahp/2024/12.1.40.47
**ISSN** | 2308-2801



## INTRODUCTION

Fascioliosis is the common parasitic disease that affects animals and human that cause critical health issues and major economic losses worldwide. It is caused by *Fasciola* spp. (Mehlhorn, 2008). A significant economic losses in infected animals with fascioliosis including decrease in production of meat and milk, high morbidity rates and liver condemnation (Mas-Coma et al., 2005). There are two major species of *Fasciola* that infect both animals and humans: *F. gigantica*, and *F. hepatica*. Although, *F. hepatica* is occurring fundamentally in temperate regions and *F. gigantica* in tropical regions, both species of *Fasciola* overlap in subtropical regions (Mas-Coma et al., 2009). The final host of *Fasciola* include wide range with mainly herbivorous animals and also some mammalian species comprising humans. The larval phases of the *Fasciola* parasite develop in Lymnaeid snails as intermediate hosts (Mas-Coma et al., 1999).

The overlapping distribution of *F. gigantica* and *F. hepatica* has resulted in a taxonomic debate regarding the identification of *Fasciola* species found in Far Eastern countries. Some specimens resemble *F. gigantica*, while others resemble *F. hepatica*. Furthermore, intermediate forms exist, and phenomena such as parthenogenesis, hybridization be-

tween different genotypes, abnormal gametogenesis, diploidy, triploidy, and mixoploidy have been observed. This complexity adds to the challenge of accurately classifying these Fasciola species in the region (Mas-Coma & Bargues, 1997; Mas-Coma et al., 2009).

At present, diagnostic techniques are only beneficial to distinguish fascioliosis from other diseases. Until now, the particular differentiation can only be made by either a morphometrical study of adult worms and eggs (Periago et al., 2006; Valero et al., 2009a, 2012c) or by using molecular methods (Marcilla et al., 2002; Mas-Coma et al., 2009). The two species have been classically classified based on their morphological characteristics, like body length and width. Due to differences in size of two species of *Fasciola*, the contradiction of morphological characteristics, and the appearance of intermediate forms, it might be difficult to differentiate the two species, merely based on these features (Valero et al., 2001). Molecular techniques depended on mitochondrial (mtDNA) and nuclear ribosomal (rDNA) genes are capable to distinguish *Fasciola* species (Itagaki et al., 2005; Mas-Coma et al., 2009; Shafiei et al., 2014).

Information about morphological and molecular characterization of *Fasciola* is beneficial for precise diagnosis of the cause of the disease and also for the prevention and monitoring of fascioliosis in every endemic area (Kuk & Erensoy, 2007; Kaya et al., 2013). Studies about phenotypic and molecular characteristics of *Fasciola* species in Thi-Qar province /Iraq are limited. Therefore, the current work aims to study some morphological measurements and molecular characterization of *Fasciola* species in cows and buffaloes based on sequence analysis of *28S rRNA* gene.

## MATERIALS AND METHODS

### Collection of Samples and Morphological Measurements

During the period from July to November 2022, adult *Fasciola* parasites were collected from naturally infected livers of cows and buffaloes through postmortem inspection process in a slaughterhouse of Al-Nassiriyah municipality, Thi-Qar province, Iraq. *Fasciola* worms after their removal from the infected liver were washed softly several times using phosphate buffers solution (PBS). 60 adult *Fasciola* worms (30 from cows and 30 from buffaloes) were used to study of morphological measurements. *Fasciola* worms were laid flat on a glass slide. Another slide was put nicely over the worm, and body length (BL), body width (BW), cone length (CL), cone width (CW), distance between ventral sucker and posterior end of body (VS–P), distance between oral and ventral suckers (OS–VS) and distance between anterior end of body and ventral sucker (A–VS) were measured using a ruler. Morphological measurements were used to identify *Fasciola* species according to Periago et al. (2006).

### Molecular study

**DNA Extraction and Polymerase Chain Reaction (PCR):** Molecular study carried out in laboratory of PCR unit in Mazaya university college on 30 *Fasciola* worm isolated from cows and buffaloes (15 from cows and 15 from buffaloes). Small piece of the anterior parts of the *Fasciola* worms were cut and genomic DNA was extracted from individual *Fasciola* worms using WizPrepTM gDNA Mini Kit (Cell/Tissue) according to the manufacturer's instructions. The genomic DNA was stored at -20 ˚C until use.

The PCR was proceeded for amplifying the *28S rRNA* gene for all samples of genomic DNA prepared from *Fasciola* worms using the primer previously described by Marcilla et al. (2002) targeting *28S rRNA* gene consisted of forward primer (5' ACGTGATTACCCGCTGAACT 3') and reverse primer (5'CTGAGAAAGTGCACTGACAAG 3'). The PCR reaction was conducted in a final volume of 25 µl comprising: PCR tubes containing 5µl of PCR PreMix (Bioneer, Korea), 1µl from each primer (Forward and Reverse), 5 µl of genomic DNA and 13 µl from nuclease free water from Bioneer Accupower® PCR PreMix. The PCR reaction was performed in Thermocycler and included an initial denaturation at 94˚C for 3 minutes, followed by 30 cycle of DNA denaturation at 94˚C for 30 seconds, annealing at 58 ˚C for 30 seconds and extension at 72˚C for 60 seconds, and one cycle of final extension at 72˚C for 5 minutes. PCR product was loaded on TBE agarose gel 1.5 %. The gel stained with 0.1 – 0.3 µl from ethidium bromide stain. Electrophoresis conducted at 70V for 90 minutes. The bands were examined for visualization amplified PCR product (*28S rRNA* gene) in UV transilluminator and digitally photographed.

**DNA Sequencing and phylogenetic construction:** PCR products of *28S rRNA* gene were sent to South Korea Public biotechnology company (Macrogen) for doing the sequencing. The sequences of samples including (10 sequence from cow and 10 from buffaloes) were sent to the national centre for biotechnology information service (NCBI) (https://www.ncbi.nlm.nih.gov). Accession numbers for 17 sequences were assigned in the current study. Blastn alignment used for comparison of sequences in this study with sequences of *Fasciola* parasite recorded in Genbank database.

Phylogenetic analysis was performed using MEGA11 (Tamura et al., 2021) and the UPGMA method was used to construct the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

## RESULTS

### Morphological Measurements

The current study showed that all *Fasciola* worms isolated from livers of cows and buffaloes were of different sizes, elongated leafy worms, the cephalic cone was well developed and the shoulders were not distinguished (Figure 1). The results of some morphological measurements of *Fasciola* worms originating from cows and buffaloes are summarized in Table 1. The present study showed that the average body length in liver flukes isolated from buffaloes was 40.03 ± 3.25 larger than that in cows 35.87 ± 5.82, while the average body width was almost equal in flukes isolated from cows and buffaloes. The average cone width was 3.34 ± 1.03 in liver flukes isolated from cows, while it was 2.66 ± 0.33 in buffaloes flukes. The mean of VS-P in liver flukes isolated from cows and buffaloes was 32.36 ± 5.56 and 36.23 ± 3.34, respectively.

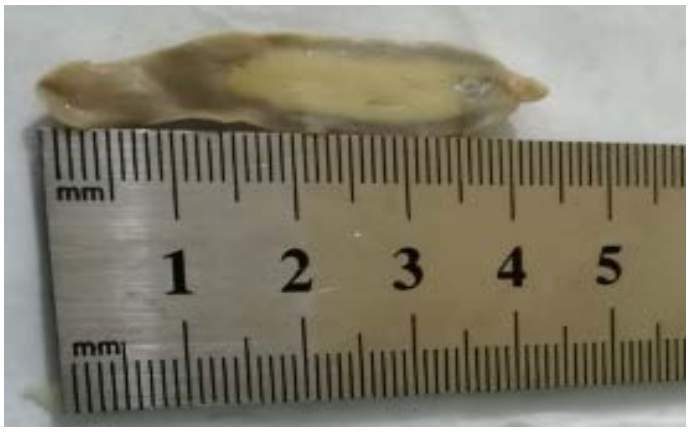


**Figure 1:** *Fasciola gigantica* isolated from buffalo's liver.

Statistical analysis of morphological measurements using T test exhibited that the significant differences ($P < 0.05$) in measured factors comprising body length, cone length, cone width and distance between ventral sucker and posterior end of body (VS–P) in *Fasciola* worms isolated from cows and buffaloes. The morphological values in the present study suggested the existence of *Fasciola gigantica* species.

### Amplification of *28S rRNA* gene

In the current study, 30 sample of *Fasciola* spp. liver flukes isolated from cows and buffaloes livers (15 from cows and 15 from buffaloes) were used in the molecular study. PCR technique targeting *28S rRNA* gene in all genomic DNA samples extracted from 30 *Fasciola* spp. samples. A single fragment of 618 bp length was successfully amplified using primer of *28S rRNA* gene when separated by 1.5 % agarose gel electrophoresis (Figure 2).

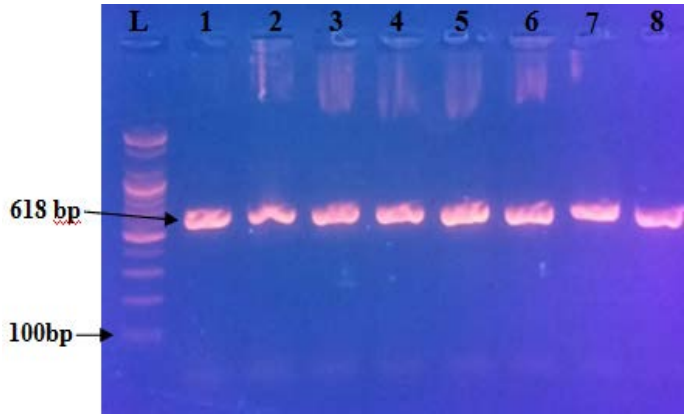


**Figure 2:** Electrophoresis of *28S rRNA* gene. L: DNA Ladder. Lane 1-4 *F. gigantica* isolated from cows. Lane 5-8 *F. gigantica* isolated from buffaloes.

### Molecular Analysis

Partial *28S rRNA gene* sequences (618 bp) of 20 PCR products in the present study were sequenced immediately and used in analysis of similarity. The *28S rRNA* gene sequences of the 20 *Fasciola* worms isolated from cows and buffaloes showed high similarity with those of *F. gigantica* using BLASTn multiple alignment with available reference sequences for *F. gigantica* parasite in Genbank. Seven sequences of *F. gigantica* isolated from cows under accession numbers (LC731384, LC731385, LC731386, LC731387, LC731388, LC731389, LC731390), and ten *28S rRNA gene* sequences of *F. gigantica* isolated from buffaloes under accession numbers (LC730609, LC730653, LC730654, LC730655, LC730656, LC730657, LC730658, LC730659, LC730660, LC730661) were deposited in the GenBank database for first time. All *28S rRNA* gene sequences of *F. gigantica* worms from cows and buffaloes were very similar and showed 99 – 100% identity. *28S rRNA* gene were used to evaluate genetic variation of *F. gigantica* worms isolated from cows and buffaloes. Alignment of the sequences of *28S rRNA* gene showed variable sites in which nucleotides at the position of 104A>C (LC731390**)** in *F. gigantica* worm isolated from cow and positions of 192C>G (LC730653**)**, 264T>G (LC730654), 187T>A (LC730655), 243G>C, 227G>C (LC730656), and 244A>G (LC730657) in *F. gigantica* worms isolated from buffaloes (Table 2).

**Table 1:** Morphological measurements (mm) of *Fasciola* spp from cows and buffaloes.

| *Fasciola* spp. measurements | *Fasciola* spp. in Cow (n=30) (Mean±SD) | *Fasciola* spp. in Buffaloes (n=30) (Mean±SD) | T-test | P value |
|---|---|---|---|---|
| BL | 35.87±5.82 (25 – 47) | 40.03±3.25 (35 – 47) | -3.43 | 0.001* |
| BW | 6.56±1.73 (4 – 11) | 6.40±1.13 (5 – 10) | 0.44 | 0.66 |
| CL | 2.60±0.86 (1.50 – 4) | 2.09±0.28 (1.50 – 3) | 3.06 | 0.003* |
| CW | 3.34±1.03 (2 – 5) | 2.66±0.33 (2 – 3.50) | 3.44 | 0.001* |
| VS-P | 32.36±5.56 (23 – 44) | 36.23±3.34 (31 – 44) | -3.25 | 0.002* |
| OS-VS | 1.66±0.44 (1 – 3) | 1.68±0.30 (1 – 2.50) | -0.17 | 0.86 |
| A-VS | 2.83±0.71 (2 – 5) | 2.80±0.36 (2 – 3.50) | 0.23 | 0.82 |

**Table 2:** New Accession Numbers Submission obtained from the present study, Host, Identity % to *F. gigantica* and Nucleotide Diversity.

| Samples of *Fasciola* spp. | New Submission to Genbank acc. no | Host | Identity % to *F.gigantica* | Nucleotide Diversity (*28S rRNA* gene) |
|---|---|---|---|---|
| 1 | LC731384 | Cows | 100% | ND |
| 2 | LC731385 | Cows | 100% | ND |
| 3 | LC731386 | Cows | 100% | ND |
| 4 | LC731387 | Cows | 100% | ND |
| 5 | LC731388 | Cows | 100% | ND |
| 6 | LC731389 | Cows | 100% | ND |
| 7 | LC731390 | Cows | 99% | 104A>C |
| 8 | LC730609 | Buffaloes | 100% | ND |
| 9 | LC730653 | Buffaloes | 99% | 192C>G |
| 10 | LC730654 | Buffaloes | 99% | 264T>G |
| 11 | LC730655 | Buffaloes | 99% | 187T>A |
| 12 | LC730656 | Buffaloes | 99% | 243G>C 227G>C |
| 13 | LC730657 | Buffaloes | 99% | 244A>G |
| 14 | LC730658 | Buffaloes | 99% | ND |
| 15 | LC730659 | Buffaloes | 100% | ND |
| 16 | LC730660 | Buffaloes | 100% | ND |
| 17 | LC730661 | Buffaloes | 100% | ND |

ND: No sequence diversity.

## Phylogenetic Analysis

Phylogenetic trees were constructed using *28S rRNA* gene sequences of *F. gigantica* isolated from cows and buffaloes livers in the present study along with other available sequences in genbank from different countries of world for analysis of phylogenetic diversity. Phylogenetic analysis demonstrated close relationship among *F. gigantica* isolated from cows in this study with accession nos. (LC731384 to LC731389) when compared with *F. gigantica* samples from other countries of world whereas the sample with acc. no LC731390 showed genetic diversity at locus 104A>C therefore took a major branch in phylogenetic tree in the present study and highly similar to *F. gigantica* samples from Santiago Island (AJ439739) and Kenya (EU025873) (Figure 3).

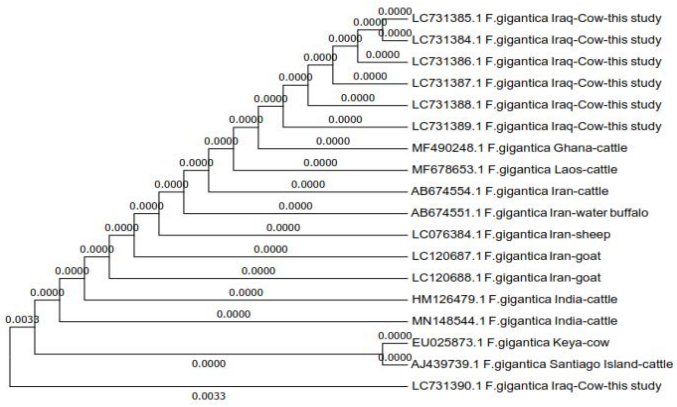


**Figure 3:** Comparison of phylogenetic analysis of 28S rRNA gene sequences of *F. gigantica* isolated from cows liver from Iraq (present study) with other sequences recorded in Genbank.

Phylogenetic relationships exhibited that all *28S rRNA* gene sequences of *F. gigantica* worms isolated from buffaloes liver in this study were placed within the same clade including accession nos: LC730609, LC730653, LC730656, LC730657, LC730658, LC730659, LC730660, LC730661. BLAST results indicated that *F. gigantica* worms in the present study possessed the sequence most identical to those from Ghana (MF490248), Laos (MF678653), Iran (AB674554), India(HM126479) and Santiago Island (AJ439739), while *F. gigantica* samples in the present study with accession nos: LC730654, LC730655 were placed within one clade in the phylogenetic tree due to a different genetic variation observed in the positions 264T>G and 187T>A, respectively, from the other sequences of the current study, therefore, an independent secondary branch was taken identical to the *F. gigantica* sequences recorded in Kenya (EU025873) (Fig 4).

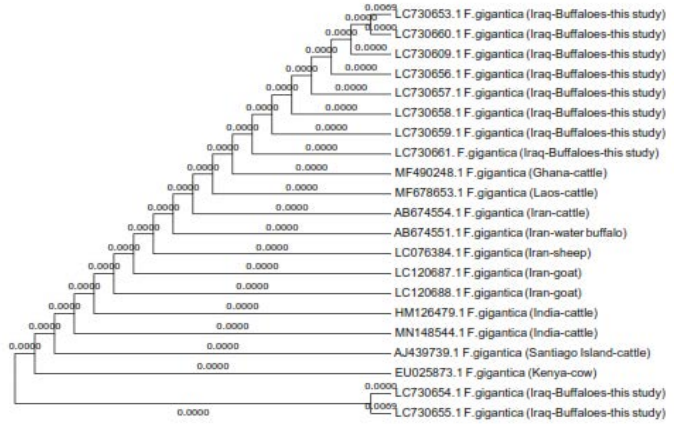


**Figure 4:** Comparison of phylogenetic analysis of 28S rRNA gene sequences of *F. gigantica* isolated from buffaloes liver from Iraq (present study) with other sequences recorded in Genbank.

## DISCUSSION

Different techniques are utilized to identify the species of *Fasciola* (Mas-coma et al., 2009). Morphological differences are one of the most reliable methods in determining the species of *Fasciola* (Valero et al., 2005; Ashrafi et al., 2006). The present study demonstrated that all *Fasciola* flukes isolated from cows and buffaloes in Thi-Qar province belong to *Fasciola gigantica* species based on morphological data. The results of this study showed that there is close similarity with previous studies such as the study of Periago et al. (2006) in Burkina Faso, which is showed that the average of BL in bovine *F. gigantica* samples was (39.72 ± 0.58), BW (8.45 ± 0.14), CL (2.67 ± 0.04), CW (3.74 ± 0.06), OS – VS (1.71 ± 0.03), VS – P (36.39 ± 0.59) and A–VS (2.36 ± 0.03), also, Shafiei *et al.*(2014) in Iran, indicated that the morphological data of *F. gigantica* isolated from cattle are BL (41.08 ± 6.12), BW (8.14 ± 0.62), CL (3.1 ± 0.35), CW (3.56 ± 0.34), OS – VS (1.79 ± 0.24), VS – P (37.67 ± 6.39) and A – VS (2.59 ± 0.24). However, all the morphological characteristics in the current study were different and higher than those of the *F. hepatica* reported by Periago et al. (2006), Shafiei et al.(2014) and Sumruayphol et al. (2020) regarding body length, body width and distance between ventral sucker and posterior end of body (VS–P), while the other characteristics were somewhat identical.

In this study, body length (BL), body width (BW) and distance between ventral sucker and posterior end of body (VS–P) have been considered as useful indices for discriminating *F. gigantica* from *F. hepatica*. Periago et al. (2008) indicated that body length, the length of ventral sucker and the posterior end of the body and the ratio of body length to body width (BL/BW) were the major characteristics for distinguishing *F. gigantica* from *F. hepatica* isolated from livestock species in Egypt.

In the current study, *F. gigantica* specimens from cows and buffaloes are significantly different in some of the morphological measurements. The cause may be due to the different hosts and the resistance that the parasite encounters during its growth in the host, especially the calcification of the bile ducts in cows. Ghavami et al. (2009) showed that the variation in the size of adult *Fasciola* worms in various hosts belong to host resistance and calcification in bile ducts of infected cow. As well, Lotfy et al. (2002) indicated that the variabilites in infection intensity, parasite phase, host species and immune reaction from previous exposure. In many studies, the phenotypic differences become apparent when populations of free-living species come from various geographical area or an announced change in their environment has occurred (Periago et al., 2008).

Molecular studies have become a prerequisite for diagnosis of *Fasciola* species, therefore, the present morphometric study was supported by a molecular study and sequence analysis of *28S rRNA* gene in order to confirm the accurate diagnosis of *Fasciola* species, determination of the genetic variation, and finding out if there are intermediate forms of *Fasciola*. Some molecular methods, utilizing various molecular targets, have been advanced for the discrimination of *F. gigantica* and *F. hepatica* (Huang et al., 2004). Molecular methods can be properly differentiated by DNA sequencing of 28S ribosomal ribonucleic acid and first internal transcribed spacers (*ITS1*), (*ITS2*) genes (Marcilla et al., 2002; Ai et al., 2011). Previous studies used *28S rRNA* gene to distinguish the *Fasciola* species from Spain (Vara-Del *et al*., 2007), Iran (Yakhchali et al., 2015) and Saudi Arabia (Alajmi, 2019).

The present results obtained from sequence analysis of *28S rRNA* gene confirmed that *F. gigantica* is main fluke of cow and buffaloes in Thi-Qar province, also sequence analysis of *28S rRNA* gene demonstrated no *F. hepatica* and intermediate forms of *Fasciola* in this study. The present study coincided with other studies that demonstrated presence of *F. gigantica* in Iraq, like, Hamoo et al. (2019) recorded *F.gigantica* in sheep in Kirkuk city using sequencing of *ITS1* gene, also, Hamoo et al. (2020) demonstrated that all flukes from cattle belong to *F.gigantica* in Aqrah city, Kurdistan region of Iraq based on sequencing of *18S rRNA* gene, While the current study differed with the study of Muhammad and Hassan (2021) who showed that all *Fasciola* worms isolated from sheep, goat and cattle belong to *F. hepatica* based on sequencing of *COX1* gene in Erbil Province, and Mohammed et al. (2021) who confirmed existence of *F. hepatica* and *F. gigantica* in cattle, sheep and goats in Duhok province by using *ITS1* and *ITS2* rDNA. In the neighboring countries of Iraq, Yakhchali et al. (2015) in Iran and Alajmi (2019) in Saudi Arabia reported presence of both *F. hepatica* and *F. gigantica* in sheep by using sequencing of *28S rRNA* gene and this differs from the present study.

The reason for the spread of *F. gigantica* in Thi-Qar province is probably due to the spread of the snail *Lymnaea auricularia*, which represents a suitable intermediate host for *F. gigantica* species in the southern areas of Iraq. Al-Mayah and Awad (2005) indicated that the *L. auricularia* snail represents appropriate intermediate host for the growth and development of *F. gigantica*. Also, Al-Qarooni (2005) showed that the *L. auricularia* snail represents the most common species in the Hammar Marsh.

In the present study, sequences analysis of *28S rRNA* gene exhibited genetic variation within *F. gigantica* in one variable site in isolate from cow and six variable site isolates from buffaloes. This study documented by Vara-Del *et al*. (2007) who described genetic variation in *F. hepatica* using the *28S rDNA* gene, Mirahmadi *et al*. (2018) showed six DNA variable sites using *ITS1 rDNA* as genetic marker. Ribosomal DNA (rDNA) is one of the most applicable indicators in molecular studies because it is available in high copy and includes variable regions separated by more conserved regions (Chilton et al., 2004), as well, *28S rDNA* gene represents a genetic evidence to the presence of natural hybridization between *F. hepatica* and *F. gigantica* in Korea (Agatsuma et al., 2000).

Genetic variation may provide information about the genetic structure of the parasite and its relationship to phenotypic variations, differences in virulence and adaptation to new definitive hosts. Amer et al. (2011) showed that the intraspecific genetic variation among *Fasciola* worms may reflects differences in virulence, drug resistance and host specificity. Mas-Coma (2005) indicated that the ability of the *Fasciola* flukes to adapt rapidly to new definitive hosts and environments is likely related to genetic variability of this parasite. Rokni et al. (2010) showed that genetic variation within and between *Fasciola* flukes contains some implications for epidemiology, control and diagnosis of fasciolosis.

DNA sequence data is considered to be a powerful method for study the phylogenetic analysis and construction of the evolutionary relationships among various groups of the parasitic trematodes (Wilson et al., 2005; Ashrafi et al., 2007; Choudhury et al., 2007). According to the findings of the phylogenetic analysis based on the *28S rRNA* gene sequence, isolated *Fasciola* species from cows and buffaloes in Thi-Qar province were clustered into one clade (*F. gigantica* clade). Phylogenetic tree showed that the *28S rRNA* gene sequences of the present study were similar with (99 – 100%) to those from different countries of world such as Ghana, Laos, Iran, India, Santiago Island and Kenya for the *F. gigantica* with a noticeable genetic variation between them. Alajmi (2019) by phylogenetic analysis of the *28S rRNA* gene discovered both *F. hepatica* and *F. gigantica* from livers of Naimi sheep slaughtered at Riyadh slaughterhouse in Saudi Arabia. The phylogenetic analysis of the *28S rRNA* gene sequence of *Fasciola* species was generally reported in Iran (Yakhchali et al., 2015). Alajmi (2019) proved that the *28S rRNA* gene is a good genetic indicator in differentiating the *Fasciola* species.

## CONCLUSION

The current study showed that the dominant *Fasciola* species in Thi-Qar province is *F. gigantica*. The use of morphological and molecular methods is a useful tool to solve the problem of *Fasciola* species taxonomy. The *28S rRNA* gene

datasets reported in the present study may be adopted as beneficial markers for other studies of taxonomy of *Fasciola* species from other hosts and geographical areas of Iraq. The current study recommends doing a survey study of *L. auricularia* snail in Thi-Qar province to control these snails and prevent fasciolosis.

## ACKNOWLEDGEMENTS

The author would like to extend special thank to the staff members of slaughterhouse of Al-Nassiriyah municipality for their assistance in collecting samples during this study.

## CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

## NOVELTY STATEMENT

This study registered new nucleotide sequences of F. gigantica isolated from cows and buffaloes in the GenBank database for the first time.

## AUTHORS CONTRIBUTION

Wesam Jasim Hansh suggested the idea of the study, completed the practical aspect, and wrote the article.

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