MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF *FASCIOLA GIGANTICA* ISOLATED FROM BUFFALOES AT AL-MUTHANNA PROVINCE, IRAQ

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ABSTRACT

The aim of this study was to identify and differentiate between *Fasciola gigamtica* and *Fasciola hepatica* infections in buffaloes, through the application of PCR technique. Also, includes investigate the characteristics of phylogenetic analysis for *Fasciola gigantica* based on partial mitochondrial DNA. The application of polymerase chain reaction technique (PCR) in molecular diagnosis was exhibited high levels of specificity and sensitivity in identifying both species of *Fasciola* depend on two different genes NADH 6 for *F. hepatica* and NADH 4 for *F. gigantica*. Moreover the percentage of infection by *F. hepatica* and *F. gigantica* were recorded using specific different primers to be 0% and 100 % respectively. The results of the sequencing and phylogenetic tree showed 99% identification with three China isolate (MH621335.1, GU121036.1, GU121037.1), and 98% identification one China isolate. Furthermore, the results showed 100% identification with two Niger isolate (GU121056.1, GU121048.1), and 99% identification with two Niger isolate (GU121047.1, FJ392263.1) based on sequence analysis of mitochondrial NADH 4 gene (ND4). Thus, the current study concluded that the phylogenetic analysis exhibited a significant genetic resemblance between the strains from Iraq and Niger.

Key words: liver fluke, pcr, sequencing, mitochondrial DNA, NADH, gene

الكشف الجزيئي والتحليل الوراثي لفاشيولا العملاقه المعزولة من الجاموس في محافظة المثنى، العراق

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المستخلص

تهدف الدراسة التعرف والتمييز بين عدوى *Fasciola hepatica* و *Fasciola gigantica* في الجاموس، من خلال استخدام تقنية تفاعل البوليميراز المتسلسل (PCR). تتضمن أيضًا التحقيق في خصائص تحليل النشوء والتطور للفاشيولا العملاقة بناءً على الحمض النووي للميتوكوندريا. أظهر التشخيص الجزيئي المعتمد على تقنية تفاعل البلمرة المتسلسل (PCR) خصوصية وحساسية عالية لتشخيص كلا النوعين *Fasciola* بالاعتماد على جينين مختلفين NADH 6 ل *F. hepatica* و NADH 4 ل *F. gigantica*، حيث سجلت نسبة الإصابة ب *F.hepatica* و *F. gigantica* باستخدام بادئات خاصة 0٪ و 100٪ على التوالي. كما اظهرت نتائج التسلسل الوراثي وشجرة النشوء والتطور تطابقا بنسبة 99٪ مع ثلاثة عزلات صينية (MH621335.1, GU121036.1, GU121037.1)، وبنسبة تطابق 98٪ مع عزلة واحدة من الصين. علاوة على ذلك، أظهرت النتائج نسبة تطابق 100 ٪ مع عزلتين من النيجر (GU121056.1, GU121048.1)، ونسبة تطابق 99٪ مع عزلتين من النيجر (GU121047.1, FJ392263.1) بناءً على تحليل تسلسل جين الميتوكوندريا NADH 4 (ND4). وبالتالي نستنتج من الدراسة الحالية إلى أن التحليل الوراثي أظهر تشابهًا جينيًا كبيرًا بين السلالات من العراق والنيجر.

الكلمات المفتاحية: ديدان الكبد، تفاعل سلسلة البلمرة، التتابع، الحمض النووي للميتوكوندريا، NADH, جين.

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INTRODUCTION

Water buffaloes possess specific physiological and anatomical adaptations that enable them to thrive in environments such as rivers, marshes, and flood-prone area (5). These animals possess a valuable trait of efficiently converting low-quality forage sources such as reed (*Phragticum communis*), bardi (*Typha latifolia*), and other vegetation found in the marshes, into high yields of milk and meat, making them a valuable resource for the local population (23, 32). Similar to other domestic animals, buffaloes are susceptible to different pathogens, infectious agents, and diseases such fasciolosis. Fasciolosis, which is caused by endoparasitic trematodes belonging to the phylum Platyhelminthes and genus *Fasciola,* is a zoonotic disease that spreads through ingestion of contaminated food. It is regarded as a significant global health concern. Various mammals, including cows, buffaloes, sheep, goats, and, sporadically, horses, are influenced by the disease, which is typically localized in the liver, bile ducts, and gall bladder. The genus Fasciola contains two important species: *Fasciola hepatica* and *Fasciola gigantica* (20, 38). The disease poses a sever risk to human health and leads to considerable financial losses in the livestock sector due to multiple factors, including mortality of infected animals, reduced productivity and growth, condemnation of affected livers and costs associated with implementing control measures and treatment in ruminants (12, 30). Geographically, fasciolosis is widely distributed *F. gigantica*, the causative agent of the large liver fluke in tropical regions, has a high incidence in Asia and Africa, primarily because of the widespread prevalence of its intermediate host, *Lymnaea natalensis*. Conversely, *F. hepatica* is commonly prevalent in areas with a temperate climate including, Americas, Australia and Europe, where its intermediate host, *Lymnaea truncatula*, has limited distribution (11, 24). Distinguishing between *F. gigantica* and *F. hepatica* infection is critical as they possess epidemiological characteristics and different transmission (27, 37). Additionally, the morphology of *F. gigantica* and *F. hepatica* can typically be used to differentiate between the two, but due to the high variability in these traits, this method has low sensitivity and efficiency to accurately distinguish both species of *Fasciola* (9, 19). Thus, a molecular technique is required for the diagnosis and differentiation of *Fasciola* spp. particularly polymerase chain reaction (PCR) with sequencing and phylogenetic analysis in order to overcome the problems encountered with conventional and serological techniques (8, 21, 39). Discrimination between the two species can be achieved through analysis of DNA sequences obtained from various regions, such as the cytochrome C oxidase I (CO1) genes and the mitochondrial NADH dehydrogenase I (ND1) (3, 18, 22). Differences in the genetic makeup of liver flukes may suggest variations in their ability to cause disease, their ability to infect specific hosts, and their susceptibility or resistance to pharmaceutical treatments (17, 34). Thus, the goal of the current investigation was to identify and diagnose both types of *Fasciola* in slaughtered buffaloes by using the PCR technique. Also, studying the characteristics of phylogenetic analysis using mitochondrial markers for *F. gigantica*.

MATERIALS AND METHODS

Study area and isolation of parasites

The research was conducted on a sample of buffaloes that were selected randomly among those slaughtered at the Rumaitha and Samawah abattoirs, situated in the southern region of Al-Muthanna Province, Iraq. The collection of samples spanned from March 2022 to December 2022, during which the abattoirs were visited three times per week, and between 2 to 5 buffaloes were slaughtered daily. The infected visceral organs with fasciolosis were collected in plastic bags and transported to the parasitology laboratory for the collection of mature *Fasciola spp.* from the bile ducts and gall bladders. The adult worms were isolated using forceps and transferred to Petri dishes containing warm water for a duration of 30 minutes to eliminate debris and contaminants, and to allow the worms to relax and prevent curling or wrinkling. Subsequently, some of the mature worms were put in a glass container filled with 70% ethyl alcohol and kept at 4°C for the purpose of DNA extraction (16).

DNA extraction and PCR assay

The genetic material (DNA) was extracted from the apical zone of adult flukes (50 samples) isolated from buffaloes infected with fasciolosis using the gSYAN DNA mini kit (tissue protocol/Geneaid/ USA). The extraction process was conducted in accordance with the directions provided by the manufacturer. On a 1% agarose gel electrophoresis, DNA integrity was examined. The PCR reaction were performed for distinguish between *F. gigantica* and *F. hepatica* using the sets of primers that are show in the Table (1). The first PCR reaction used to detect the *F. hepatica* in the infected buffaloes by use of specific primers (FHF, FHR), which amplify a 304 bp region of the mitochondrial NADH 6 gene (ND6). The PCR reactions were carried out in a total volume of 25 µl, comprising of 12.5 µl of green master mix (Promega/ USA), 5 µl of genomic DNA, 1 µl of each primer, and 5.5 µl of nucleus-free water. The amplification process was performed with an initial denaturation stage at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 66.4°C for 30 seconds, and extension at 72°C for 1 minute. The final extension stage was carried out for 5 minutes at 72°C (14). While, the second PCR reaction used to distinguish *F. gigantica* using specific primer (FGF, FGR), which amplified a 752 bp segment of the mitochondrial NADH 4 gene (ND4). The PCR procedure included an initial denaturation stage at 95°C for 5 minutes, followed by 40 cycles of amplification consisting of denaturation at 95°C for 30 seconds, annealing at 66.4°C for 30 seconds, and extension at 72°C for 1 minute. A last extension stage lasting 5 minutes at 72°C brought the amplification to an end (14). Ten µl from amplification samples were directly loaded in a 1.5% agarose gel electrophoresis and the products were visualized by UV transilluminator (4).

DNA sequencing and phylogenetic analysis

The PCR products (8 samples) of the local Fasciola gigantica isolates from infected buffaloes and primers were sending to Macrogen Corporation (Korea) for Sanger sequencing. With an Applied Biosystems 3730xl DNA Analyzer, bidirectional Sanger sequencing was accomplished (Applied Biosystems, Foster City, CA, USA). On the other hand, Phylogenetic tree was performed to study the genetic relationship between local and international isolates of *Fasciola* spp. using the maximum parsimony method utilizing the MEGA-X v10.0.5 (26).

Table 1. The sequences of the primers used in the PCR assay

Primer	Sequence	Product Size	Characterized
FHF	5′-GCT TGT TTG GCATTG TTA GGG-3′	304 bp	*F. hepatica*
FHR	5′-CAA CCA GCC CAT CAA TCCC-3′		
FGF	5′-GGGAT TCA GTC TTG GAG GGA-3	752 bp	*F. gigantica*
FGR	5′-CCG CCA TAA ACA CCA CAC CT-3′		

RESULTS AND DISCUSSION

The genomic DNA was successfully extract and purified from 50 adult liver fluke samples obtained from buffaloes infected with fasciolosis using the gSYAN DNA mini kit extraction (Geneaid/ USA), after that, each elute DNA samples were measured by using Nanodrop spectrophotometer (THERMO/ USA), to determine their concentrations, which varied between 47.2 and 226.3 ng/µl. The purity of the samples, ranging from 1.8 to 1.9 was also assessed during this analysis. In addition, by loading the genomic DNA, the purity and quality of the extracted DNA were assessed by agarose gel electrophoresis. The resulting gel image showed a distinct and intact band of genomic DNA without any indication of degradation, as shows in Figure (1). The result in the current study were line with those study by Alsulami *et al.* (7), who extracted genomic DNA from the apical zone of *Fasciola spp.* that isolated from 18 cows infected with fasciolosis and after that measured concentration of eluted DNA by using Nano-drop. On the other hand, a pair of specific primers were utilized to distinguish between both species of *Fasciola* in the buffaloes using the PCR technique. The first PCR reaction was to confirm the presence of *F. hepatica* in buffaloes, by use of specific primers (FHF, FHR) designed to amplify a 304 bp region of the mitochondrial NADH 6 gene (ND6). The PCR findings indicated that none of the 50 buffaloes tested positive for *F.*

hepatica, as seen in Table (2) and Figure (2). In contrast, the second primer (FGF, FGR) were selected to detect *F. gigantica* in infected buffaloes by amplifying a 752 bp segment of the mitochondrial NADH 4 gene (ND4). The PCR analysis revealed that all 50 buffaloes tested positive for *F. gigantica*, with a 100% positivity rate, as indicates in Table (2) and Figure (3). The polymerase chain reaction (PCR) technique, which is based on genetic markers in mitochondrial (mt) DNAs and nuclear ribosomal (r), has become widely used to overcome the problems faced with conventional and serological techniques (41). Moreover, mitochondrial genes include the mitochondrial 5.8S rRNA gene, NADH dehydrogenase subunit (NAD4) and NADH dehydrogenase subunit (ND6) gene have been previously developed for accurate identification, genotyping, genetic variations and phylogenetic analyses of these parasites (2,13). Consequently, DNA-based approaches are beneficial for identifying flukes, especially in regions where both species and maybe intermediate forms are present (1). The finding of PCR in the present study are agreement with those reported in prior studies (2, 14), who employed two primer sets, sourced from the complete mitochondrial DNA sequences of *F. gigantica* and *F. hepatica*, available in GenBank (Accession No. AF216697.1 and KF543342.1) respectively. The study utilized specific primer to amplify a non-coding DNA sequence, and the beginning of the NADH dehydrogenase subunit 6 (ND6) gene for *F. hepatica* and the gene-encoding NADH dehydrogenase subunit 4 (ND4) for *F. gigantica*, both of which are located within the mitochondrial DNA. These sets of primers were chosen because the mitochondrial genome is known to be very specific and conserved within a specific pathogenic group or species (14, 28). On the other hand, these finding are agreement with results obtained by previous studies Alshaibani *et al.* (6), who do not reported any infection by *F. hepatica* in the infected buffaloes based on PCR technique and disagreement with (14, 42), who demonstrated that the proportion of cattle infected with fasciolosis by both species is relatively equal. The observed variations may be attributed to various factors such as environmental variables, including temperature and rainfall. The survival of the intermediate host and the parasite's larval stages (miracidium and cercaria) may be significantly impacted by these factors (36). Furthermore, one possible explanation could be that buffaloes, due to their grazing behavior on grasslands, tend to graze near springs and streams where snails, which are the intermediate hosts of the parasite, are more commonly found (25). Besides, it should be noted that the intermediate host of *F. gigantica* is more aquatic than that of *F. hepatica*. This may contribute to a higher exposure to the parasite and could potentially affect the ratio of *F. hepatica* to *F. gigantica* among different types of domestic ruminants (31). Furthermore, De Agüero *et al.* (14) showed that *F. hepatica* is likely better adapted to infect sheep than *F. gigantica*. In addition, Zhang *et al.* (42) explained this as being due to the stronger cellular response elicited by the parasite and the weaker ability of *F. gigantica* to evade the immune response, which could account for the lower susceptibility of sheep to *F. gigantica* infection. On the other hand, the use of PCR and additional validation by sequencing in a molecular identification assay to reach a definitive conclusion is strongly advised. Eight samples from 50 samples were identified as *F. gigantica* by PCR based on mitochondrial NADH 4 gene (ND4). The local *F. gigantica* were submitted in NCBI-Genbank data base to get the Gene bank accession number for Iraqi *F. gigantica* isolates based on sequence analysis of mitochondrial NADH 4 gene (ND4). These isolates were sent for sequencing and submitted into the NCBI-Genbank database with the accession no (OP320519.1, OP320520.1, OP320521.1, OP320522.1, OP320523.1, OP320524.1, OP320525.1, OP320526.1). The sequencing results of the current study indicate that the Iraqi *F. gigantica* isolate (OP320519.1 and OP320520.1) are closely related to NCBI-BLAST *F. gigantica* isolate China (KF543342.1 and MH621335.1) respectively. Also, the local *F. gigantica* isolates (OP320521.1 and OP320522.1) were showed closed related to NCBI-BLAST *F. gigantica* isolate China (GU121036.1 and GU121037.1)

respectively. In addition, the local *F. gigantica* isolate (OP320523.1, OP320524.1, OP320524.1 and OP320526.1) were showed closely linked to NCBI-BLAST *F. gigantica* isolate Niger (GU121047.1, GU121056.1, GU121048.1 and FJ392263.1) respectively, (Table 3). This study represents the initial investigation in Iraq that examines the molecular aspects of partial sequences of the mitochondrial NADH 4 gene (ND4) of *F. gigantica*. These results were line with those results obtained by De Agüero *et al.* (14), which employed the mitochondrial NADH 4 gene (ND4) as the most suitable genetic marker to characterize *F. gigantica*, in addition this study was recorded that 7 DNA samples and *F. gigantica* (KF543342.1) had 98–99% similarity. Sequence alignment analysis supported our findings and demonstrated a remarkable resemblance to other isolates, which may be explained by the fact that Iraq only recently received our isolation. Furthermore, it should be noted that *F. gigantica* has expanded its range to include Asia, the Middle East, and the Far East, ultimately infecting cattle and sheep and leading to significant health concerns (35). In addition, results of phylogenetic analysis, a maximum parsimony method was employed to construct a phylogenetic tree with the aid of MEGA-X v10.0.5. The current sequences are compared to published sequences from various geographic locations to ensure an accurate phylogenetic analysis of local *F. gigantica* isolates, (Table 3). The phylogenetic analysis was recorded that the Iraqi isolate showed similarity to the *F. gigantica,* as shows in Figure (4). The sequence result for mitochondrial NADH 4 gene (ND4), partial sequence in local *F. gigantica* based on phylogenetic analyses were revealed outstanding, 98.96 % to 100%, identity matching between the current study isolates (OP320519.1 to, OP320526.1) and a strain in Gene Bank database, recognized from china and Niger respectively. These similarity may be due to the commercial movement of animals between the two countries, the high nucleotide similarity between the current Iraqi isolates with the Niger and china strains cannot be attributed to a direct transmission of this parasite from Niger or china lands into Iraq. However, this may have occurred due to an indirect commercial movement of cattle from a country other than Niger and China, such as India or Iran. This explained could be supported through the fact that the intermediate host, *Lymnaeid* family snail transmit medical and veterinary important trematodiases, mainly fasciolosis. The snail *Radix natalensis* is well known to be located In Sub-Saharan Africa and Western Africa, particularly in Niger and Egypt, and thus *F. gigantica* is predominant in many countries of Africa (15, 33, 40). Moreover, this snail was also reported to be present in Iran and India (10, 29). The Niger strain may have been re-localized from Niger to India and then to Iraq with some nucleotide modifications in the current study strains with less than 100% similarity to the Niger one because the snail presence is shared by Niger, China, India, and Iran and because there is a significant commercial movement of cattle into Iraq, especially from India.

Table 2. Results PCR of *F. hepatica* and *F. gigantica*

Type of infection	Primers		No. of examined samples	No. of positive buffaloes	%
	Upstream	Downstream			
F. hepatica	**FH-F**	**FH-R**	**50**	**0**	**0**
F. gigantica	**FG-F**	**FG-R**	**50**	**50**	**100**

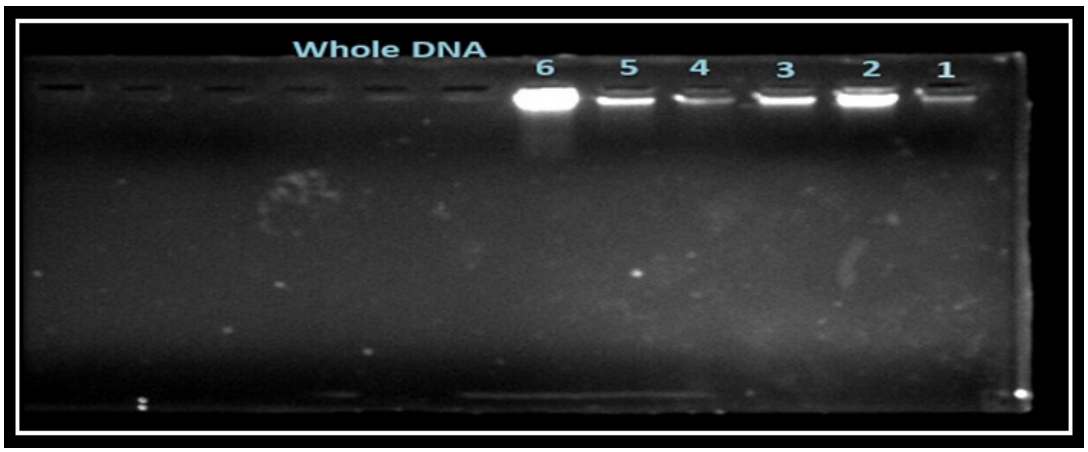

Figure 1. Total genomic DNA extracts from adult *Fasciola spp.* on 1% agarose gel electrophoresis at 60 Volt for 20 minute.

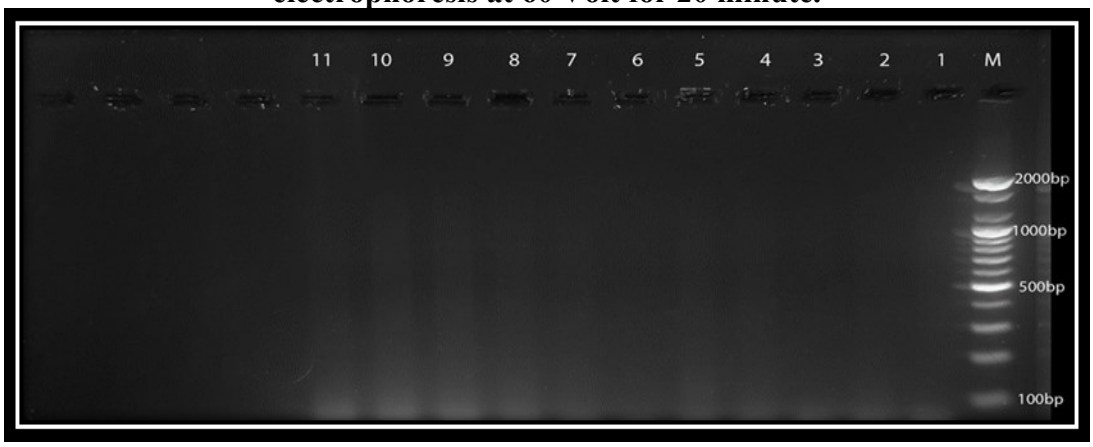

Figure 2. Agarose gel electrophoresis of PCR product obtained with *Fasciola hepatica*-specific primers. All Lanes represent negative results, Lane M represent DNA ladder.

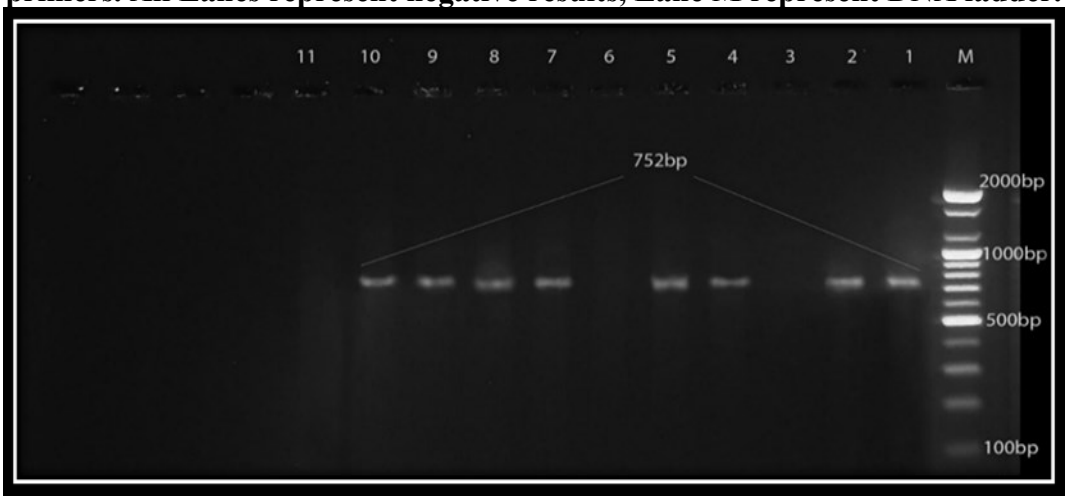

Figure 3. Agarose Gel Electrophoresis of Amplified DNA from *Fasciola gigantica* by Using Primers (FGF, FGR) Region of Mitochondrial. Lane M; represent DNA ladder, Lanes (1-10); represent Positive (752 bp), exception of 3, 6 and 11 Lanes, which represent negative isolates.

Table 3. The accessions no. that used in phylogenetic tree

F. gigantica Iraq isolate	Genbank Accession number	NCBI-BLAST Homology Sequence identity %		
		Genbank Accession number	Country	Identity (%)
F. gigantica isolate No.1	OP320519.1	KF543342.1	China	98.96
F. gigantica isolate No.2	OP320520.1	MH621335.1	China	99.87
F. gigantica isolate No.3	OP320521.1	GU121036.1	China	99.69
F. gigantica isolate No.4	OP320522.1	GU121037.1	China	99.39
F. gigantica isolate No.5	OP320523.1	GU121047.1	Niger	99.38
F. gigantica isolate No.6	OP320524.1	GU121056.1	Niger	100
F. gigantica isolate No.7	OP320525.1	GU121048.1	Niger	100
F. gigantica isolate No.8	OP320526.1	FJ392263.1	Niger	99.67

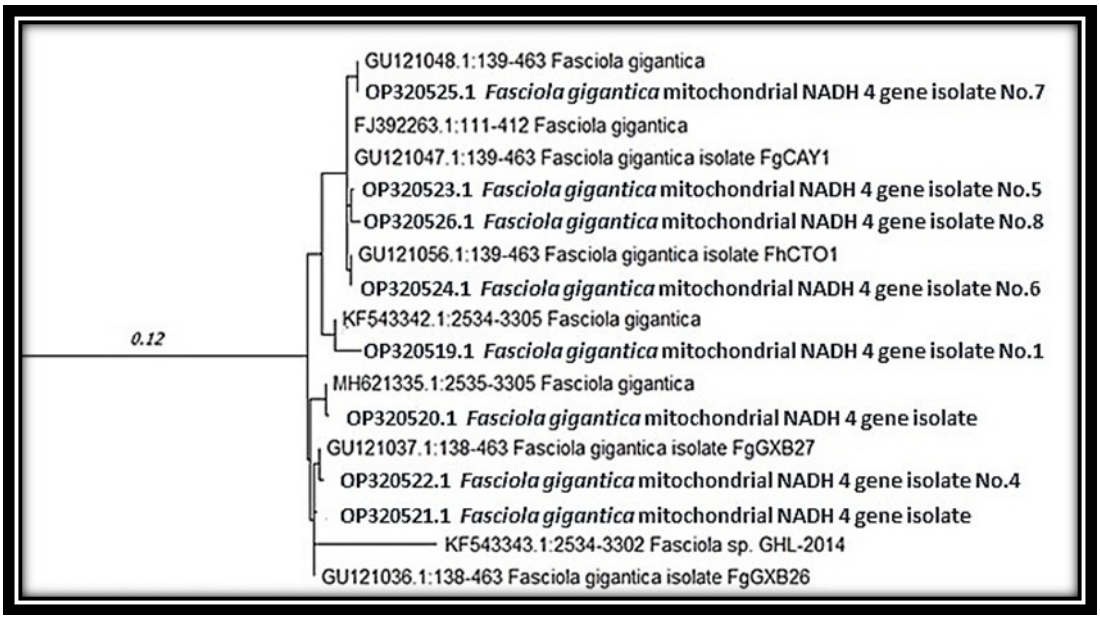

Figure 4. Phylogenetic tree analysis of eight *Fasciola* isolates based on mitochondrial NADH 4 gene (ND4) partial sequence in local *F. gigantica* isolates, a maximum parsimony method was employed to construct a phylogenetic tree with the aid of MEGA-X v10.0.5.

CONCLUSIONS

According to the results of the current study concluded that PCR technique was a high sensitive and specific for diagnosis of fasciolosis. Furthermore, the findings indicated that the phylogenetic analysis exhibited a significant genetic resemblance between the strains from Iraq and Niger.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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