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# Molecular Characterization and species differentiation of *Fasciola* species isolated from camels in Taif governorate.

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**ABSTRACT**

Camel is a very hardy animal and well adapted to harsh climatic conditions of desert. It is the principle domestic animal in Saudi Arabia. *Fasciola* sp. considered as one of the most common gastrointestinal helminthes in camels in Saudi Arabia. Understanding genetic structure and status of genetic variation of camels *Fasciola spp.* has important implications for epidemiology and effective control of fasciolosis. The aim of the present work is to obtain an accurate identification and discrimination study of *Fasciola* isolates collected from camels livers in Taif governmental slaughter house using molecular techniques. Different molecular techniques such as random amplified polymorphic DNA fragments using polymerase chain reaction (RAPD-PCR), Inter simple sequence repeats (ISSR) and mitochondrial cytochrome oxidase subunit I (*COI*) gene sequence were used for molecular characterization. The results of RAPD-PCR and ISSR analysis for the seven *Fasciola* isolates revealed approximately 223 different banding patterns, 53 of them consider as monomorphic bands with a percentage about 23.7% and other 170 fragments consider as polymorphic bands with about 76.3%. The dendogram based on RAPD-PCR and ISSR banding patterns dividing the seven *Fasciola* isolates into two clusters with about 61% genetic similarities. Our results indicate that the presence of sufficient nuclear DNA level variations among the studied *Fasciola* isolates using PCR techniques, therefore, the PCR data might be a good source of information for the diversity of native *Fasciola* isolates. Moreover, we found that *COI* gene sequence act as operative DNA genetic markers for molecular identification of *Fasciola spp*.

**Keywords:** RAPD-PCR, ISSR. *COI* gene sequence, Phylogeny tree, *Fasciola*, Camel.

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## INTRODUCTION

Camel is a very hardy animal and well adapted anatomically as well as physiologically to harsh climatic conditions of desert (Bazh et al., 2012; Elwathig et al., 2016). The camels is the principle domestic animal in Saudi Arabia and have close association with the cultural life in rural areas (Abou-Zinadah et al., 2005; Almathen et al., 2012; Hezam et al., 2016). The camels are important animals for the potential producer of meat, milk and hide (Banaja and Ghandour, 1994; Sanad and Al-Megrin, 2005). The total population of dromedary is predictable to be around 1.6 million camels inside the Arabian Peninsula, about 53% found in Saudi Arabia (source: FAO statistics, 2011; Mirzaei, 2012). Camel is suffer from various Parasitism (both endo and ecto) diseases which are main restrictions in improvement of camel health (Majidi et al., 2015). These diseases cause substantial economic losses in terms of decrease in working capacity, growth and productivity (Sanad and Al-Megrin, 2005; Al-Megrin, 2010). In Saudi Arabia, *Fasciola* sp considered as one of the most common gastrointestinal helminthes in camels (Shalaby et al., 2013). Fascioliasis is a chronic helminthic disease caused by two digenetic trematode species: *Fasciola hepatica* and *F. gigantic*. This disease is now emerging as an important chronic disease of humans due to consumption of raw and undercooked vegetables to retain the natural taste (Abou-Zinadah et al., 2005; Shalaby et al., 2013).

Molecular techniques have become widely accepted through the world. They provide a more specific method than methods conventionally employed in epidemiological studies (Hassan and Ismail, 2014; Alzahrani et al., 2016). For that reason some molecular techniques such as RAPD and ISSR markers are used for the diagnosis and identification of parasitic diseases of parasites, for the development of specific antigens for serological tests and for studying the immune response in the patients. The Polymerase Chain Reaction (PCR) amplification process enhances many templates for initial priming that allows the primers to anneal the identified conserved regions to amplify across unknown variable regions. Therefore, DNA amplification by using PCR and other techniques of sequencing have been used to support the taxonomy of various helminthes using nuclear ribosomal DNA (rDNA) like internal transcribed spacers genes (ITS1 and ITS2) and special mitochondrial DNA genetic markers like cytochrome oxidase subunit I (COI) (Li et al., 2009; Belal et al., 2013; Omar et al., 2013; Shalaby et al., 2013). Among different *Fasciola* spp. isolates from several geographical districts phylogeny and/or intra-specific variations have been categorized according to ITS2 and COI sequences (Omar et al., 2013).

The aim of the present research is to obtain an accurate identification of *Fasciola* sp. infected camels in Taif governorate using advanced molecular techniques.

## MATERIAL AND METHODS

### Isolation of *Fasciola*

Fifty liver samples from Taif government were tested for infection with *Fasciola*. Adult *Fasciola* worms were obtained from seven liver samples. The collected worms were washed several times with phosphate-buffered saline (PBS) (pH 7.4) and then incubated in the same buffer at 37°C for 3 h to eliminate any residual host matter. Afterwards the parasites were washed with PBS several times.

### DNA extraction

Genomic DNA was extracted from tissue samples of each adult flukes using a DNA extraction Promega genomic DNA purification kit (Thermo scientific kits, Germany) according to manufacturer's instructions. The DNA quality was assessed on 1.5% agarose gel then examined in the UV transilluminator and bands were visualized and photographed.

### RAPD-PCR analysis

RAPD analysis was done according to Lakhani et al., (2016), duplex RAPD-PCR was achieved using eight primers (OP-A01 to OP-A08 mixed with OP-B01 to OP-B08, respectively). Reaction volumes of 25 µl contained 1 µl DNA template, 400 µM dNTP's, 25 pmol of each primer and 1.25 units Taq DNA polymerase. The temperature profile was as follows: an initiation of 95°C for 10 min, followed by 40 cycles of 94°C for 45 s (denaturation), 35°C for 90 s (annealing), 72°C for 2:30 m (extension) and a final extension of 72°C for 7 min.

PCR products were electrophoresed on 1.5% agarose gel and the gels were stained with ethidium bromide (5 µg ml-1) and visualized by UV illumination and then photographed by a Bio-Rad Gel Doc 2000 device. 100 pb DNA Ladder (RTU, Gene Direx®) was used as a standard DNA marker.

**Inter simple sequence repeats (ISSR) analysis**

The procedure described by Hassan et al. (2014) was carried out the PCR reactions for ISSR analysis. Eight primers were used (supplied by Macrogen Inc.,1007 Seoul, 153-781, Korea). The eight ISSR primers were ISSR-7, ISSR-8, ISSR-9, ISSR-10, ISSR-11, ISSR-12, ISSR-13 and ISSR-14 (Hassan et al., 2014). The reactions were carried out in 25µl volume containing 1µl (20 ng) of genomic DNA, 12.5µl of Go Taq® Green Master Mix, Promega, USA, 1µl of primer (20 pmol), and up to a total volume of 25µl of deionized distilled water. The C1000TM Thermo Cycler BioRad, Germany, was programmed at 94°C for 10 min before adding Taq polymerase, then for 35 cycles. Each cycle consisted of 1 min at 94°C, 1.5 min at 52°C and 2.5 min at 72°C, followed by a final extension time of 7 min at 72°C. Amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel run in TAE.

**Mitochondrial COI gene amplification**

Mitochondrial COI gene was amplified from *Fasciola* isolates using two set of primers; forward as 5'-AACCACAAAGAYATYGGCA-3', and reverse as 5'- GGNGGGTCTCATTTRAT-3'. PCR was performed in a reaction volume of 25 µl according to Farjallah et al., (2009) The reactions were done in a C1000TM Thermo Cycler BioRad, Germany with denaturizing at 94 °C for 5 min, followed by 36 cycles at 94 °C for 45 sec, annealing temperature at 46 °C for 45 sec and extension at 72 °C for 1 min, then final step as extension at 72 °C for 7 min.

**Sequencing and analysis of COI gene**

PCR products **of COI gene** were purified using Jena Science PCR purification Kit, and then sequenced using an automated DNA sequencer in Macrogen Inc.,1007 Seoul, Korea. The DNA sequences undergo to further analysis by using bioinformatics tools, basic local alignment search tool (BLAST http://www.ncbi.nlm.nih.gov/blast) and nucleotide alignment using Clustal W (http://www.ebi.ac.uk/clustalw) for each DNA sequence query.

**Molecular phylogenetic analysis**

Different models were used to determine the phylogenetic tree-building consider as particular evolutionary models. Sequences of COI was entered in the MEGA6 programme (Tamura et al., 2013) for the phylogenetic trees construction by using neighbor joining methods and maximum parsimony distance methods. The distance methods were used to enhance maximum parsimony because they are less to give inaccuracy when trees contain long branches.

**Data analysis**

In order to determine the genetic relationship among studied *Fasciola* isolates, RAPD and ISSR data were scored for presence (1) or absence (0) of the bands using Gene Tools software from Syngene. A simple matching coefficient was estimated by means of the Jaccard's coefficient to construct a similarity matrix. Cluster analysis and dendrogram were produced on the basis of the unweighted average pair group method (UPGMA) using the NTSYS-PC Statistical Package (Rohlf, 2000).

## RESULTS AND DISCUSSION

**Molecular characterization of *Fasciola* by RAPD and ISSR**

Genomic diversity of *Fasciola* strains collected from Taif region was investigated using RAPD and ISSR analyses. RAPD-PCR has been used to evaluate genetic variation and taxonomic relationship in many *Fasciola* specifies (Bazh et al., 2016). This technique have been used extensively in detecting genetic variation in different *Fasciola* species because of its simplicity (Omar et al., 2013). RAPD-PCR reactions were performed among seven *Fasciola* isolates (F1 to F7) using eight different 10-mer primers, which were preselected for their

performance with *Fasciola* DNA. The RAPD results are illustrated in Table 1 and Figure 1, 2. Data showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for *Fasciola* strains. The primers yielded a total of 102 distinct bands (14.7%) of which were considered as monomorphic and 85.3% of which were considered as polymorphic. The total number of bands varied from 16 bands with primer mix OPA2- OPB2 to 9 bands with primer mix OPA1- OPB1(Table 1 and Figure 1). The total of monomorphic amplicons was 15 and the total of polymorphic amplicons was 87 (Table 1). The primer mix (OPA8- OPB8) showed the highest polymorphic pattern (15 bands) ranged from 100 bp to 1750 bp. Also, primers duplex OPA4- 8OPB4 and OPA8-OPB8 have showed 100 % polymorphism among *Fasciola* strains. So, from all these data it can be concluded that RAPD markers are effective in detecting similarity between *Fasciola* strains and, in fact, it provide a potential tool for studying the inter-strain genetic similarity and establishment of genetic relationships.

On the other hand, the eight ISSR primers produced different fragment patterns with varied number of bands (Table 2, Figures 3 and 4). The primers yielded a total of 121 distinct bands, 13.8% of which were considered as polymorphic, while 31.4% of which were considered as monomorphic. Table 2 records the number of amplified fragments scored for each *Fasciola* strains. The eight ISSR primers produced 121 fragments varied from 20 bands with primer ISSR-8 to 9 bands with primers ISSR-9 (Table 2). The total monomorphic amplicons was 38, while the total polymorphic amplicons was 83 bands (Table 2). The ISSR-13 primer showed the highest polymorphism and ISSR-9 primer showed the lowest polymorphic pattern. The lack of similarity among the fragments indicates that the primers are annealing in random regions in the genome of these *Fasciola* strains. As annealing temperatures are critical in the PCR reaction, higher annealing temperatures were tested in the amplification of some *Fasciola* isolates using the ISSR primers. Only few bands were amplified when ISSR-PCR was performed at a more restrictive temperature. Similar results were obtained for a variety of *Fasciola* sp. (Hurtres-Bousses et al., 2004; Robles-perez et al., 2015). Taken together, the results obtained in this study indicate that the RAPD and ISSR primers amplify random regions in the genome of *Fasciola* sp. and no relationships were found among the amplified regions (Omar et al., 2013; Bazh et al., 2016). Nevertheless, RAPD-PCR and ISSR-PCR are highly reproducible methods for the characterization of *Fasciola* sp. (Shalaby et al., 2013; Robles-perez et al., 2015). The ISSR-PCR approach has shown to be useful for assessing the genetic diversity of *Fasciola* isolates (Hurtres-Bousses et al., 2004; Robles-perez et al., 2015), and represents a valid alternative to the RAPD technique permitting fingerprinting of the genomes by using fewer primers. Therefore, there is a necessity to use molecular technique to understand the genetic variation within *Fasciola* strains

**Cluster analysis of *Fasciola* strains**

All fragments from RAPD and ISSR markers were used for molecular characterization of *Fasciola* strains by genetic similarities and designing the phylogenetic tree for these *Fasciola* strains. According to genetic similarity, seven *Fasciola* strains were grouped into two major clusters with about 61% genetic similarity. The Four *Fasciola* strains (Fasciola-1 to Fasciola-4) were grouped in the first cluster and only three *Fasciola* strains (Fasciola-5 to Fasciola-7) grouped in the second cluster (Figure 5). The smallest genetic distance (0.0625) was estimated between Fasciola-1 and Fasciola-5, while Fasciola-6 and Fasciola-7 relatively showed the highest genetic distance (0.8520) (Table 3). The overall genetic distance among *Fasciola* strains was relatively low. It was found that there is no correlation between genetic variability assessed by RAPD and ISSR techniques. These results are in agreement with Omar et al. (2013).

**Multiple alignment and phylogenetic analysis of COI gene nucleotide sequences**

The Mitochondrial *COI* gene of the seven *Fasciola* isolates was amplified using the primer set as mentioned above and sequenced done. The *COI* gene sequence was alignment as shown in Figure 6. The similarity matrix of the seven isolates with other GenBank accessions was found to be about 98%. The isolated *Fasciola* sequences and the available *COI* sequences for other *F. hepatica* and *F. gigantic* species from NCBI database were compared. The BLAST results showed that the query-*COI Fasciola* are more similar to the other sequences of *F. hepatica* and *F. gigantic* (Figure 7). *COI* sequences were subjected for multiple alignments by Tom Hall's offline tool BioEdit programme (Figure 6). Both intra specific and inter-specific variation was observed in the *COI* sequences. Nucleotide sequences of *COI* gene of Fasciola-1, 2, 4, 5. 6 and 7 showed 97 % similarity with *F. gigantica*, while, Fasciola isolate number 3 showed 92% similarity with other *F. hepatica* (data not shown). Additionally, the phylogeny tree depended on the *COI* nucleotide sequence databases of NCBI

using neighbor joining tree and maximum parsimony of MEGA 6 programme illustrated that our isolates Fasciola-1, 2, 4, 5, 6 and 7 are close similar to *F. gigantic* isolate CBf 201, while, Fasciol-3 are close similar to *F. hepatica* KX47058 (Figure 7). Moreover, *COI* nucleotide sequences in our study showed a higher bootstrap value of 97% confirming that it is the most conserved monophyletic group. This is in accordance with other studies (Omar et al., 2013).

**Table 1: Polymorphism level detected by eight sequence primers that have been used for RAPD-PCR analysis.**

| Primers Mix. | Total Bands | No. of Mono. Bands | No. Poly. Bands | % Monomorphic bands | % Polymorphic bands |
|---|---|---|---|---|---|
| OPA1- OPB1 | 9 | 0 | 9 | 00.00 | 100.0 |
| OPA2- OPB2 | 16 | 5 | 11 | 31.25 | 68.75 |
| OPA3- OPB3 | 16 | 6 | 10 | 37.50 | 62.50 |
| OPA4- OPB4 | 13 | 0 | 13 | 00.00 | 100.0 |
| OPA5- OPB5 | 5 | 1 | 4 | 20.00 | 80.00 |
| OPA6- OPB6 | 14 | 2 | 12 | 14.30 | 85.70 |
| OPA7- OPB7 | 14 | 1 | 13 | 07.10 | 92.80 |
| OPA8- OPB8 | 15 | 0 | 15 | 00.00 | 100.0 |
| Total | 102 | 15 | 87 | | |

**Table 2: Polymorphism level detected by the eight ISSR primers that have been used for fingerprinting PCR analysis in *Fasciola* strains collected from camels.**

| Primers Mix. | Total Bands | No. of Mono. Bands | No. Poly. Bands | % Monomorphic bands | % Polymorphic bands |
|---|---|---|---|---|---|
| ISSR-7 | 16 | 6 | 10 | 37.50 | 62.50 |
| ISSR-8 | 20 | 6 | 14 | 30.00 | 70.00 |
| ISSR-9 | 9 | 5 | 4 | 55.50 | 44.50 |
| ISSR-10 | 12 | 7 | 5 | 58.30 | 41.70 |
| ISSR-11 | 15 | 5 | 10 | 33.30 | 66.70 |
| ISSR-12 | 16 | 3 | 13 | 18.75 | 81.25 |
| ISSR-13 | 18 | 1 | 17 | 5.50 | 94.50 |
| ISSR-14 | 15 | 5 | 10 | 33.30 | 66.70 |
| Total | 121 | 38 | 83 | | |

**Table 3: Similarity coefficient between seven isolates of *Fasciola*.**

| | Fasc_1 | Fasc_2 | Fasc_3 | Fasc_4 | Fasc_5 | Fasc_6 | Fasc_7 |
|---|---|---|---|---|---|---|---|
| Fasciola_1 | 0.0000 | | | | | | |
| Fasciola_2 | 0.3811 | 0.0000 | | | | | |
| Fasciola_3 | 0.4602 | 0.2672 | 0.0000 | | | | |
| Fasciola_4 | 0.3811 | 0.2973 | 0.3023 | 0.0000 | | | |
| Fasciola_5 | 0.9375 | 0.7317 | 0.8109 | 0.7889 | 0.0000 | | |
| Fasciola_6 | 0.7030 | 0.7076 | 0.7296 | 0.6505 | 0.5166 | 0.0000 | |
| Fasciola_7 | 0.6569 | 0.6615 | 0.6293 | 0.6043 | 0.4704 | 0.1480 | 0.0000 |

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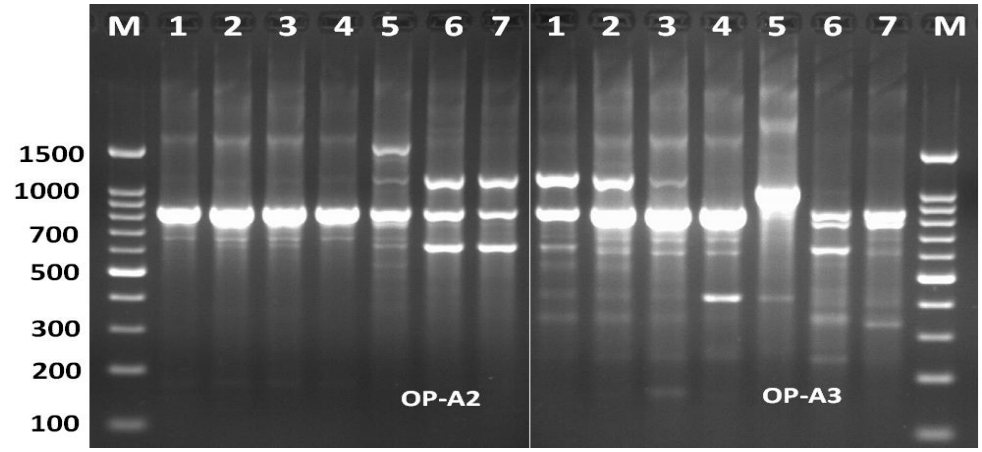


**Figure 1. RAPD-PCR profile of seven *Fasciola* strains collected from camels in Taif government generated with two mix RAPD primers. M: is 100 bp DNA ladder.**

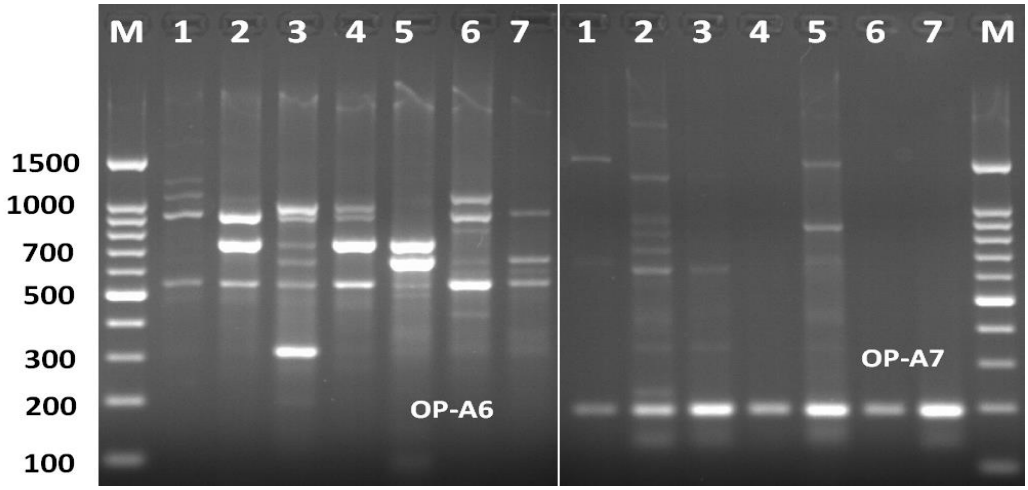


**Figure 2. RAPD-PCR profile of seven *Fasciola* strains collected from camels in Taif government generated with two mix RAPD primers. M: is 100 bp DNA ladder.**

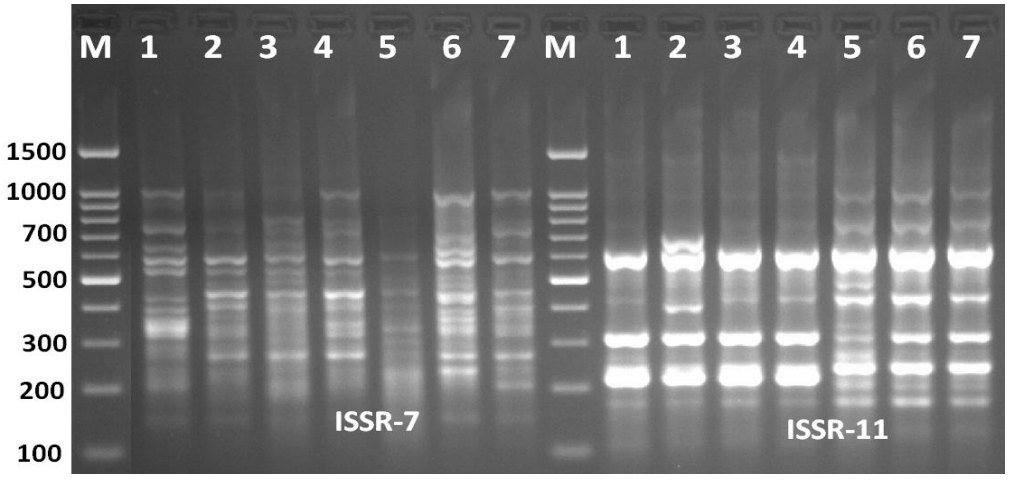


**Figure 3. ISSR profile of seven *Fasciola* strains collected from camels in Taif government generated with two ISSR primers. M: is 100 bp DNA ladder.**

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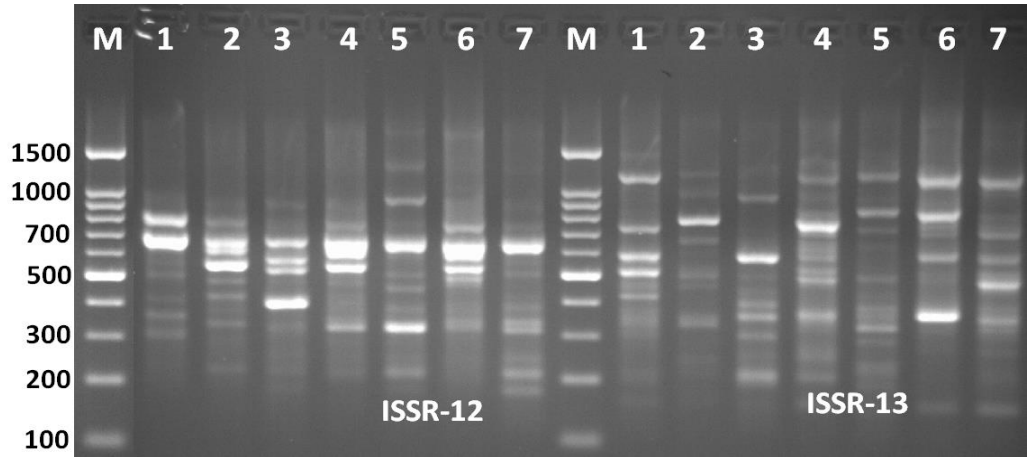


**Figure 4. ISSR profile of seven *Fasciola* strains collected from camels in Taif government generated with two ISSR primers. M: is 100 bp DNA ladder.**

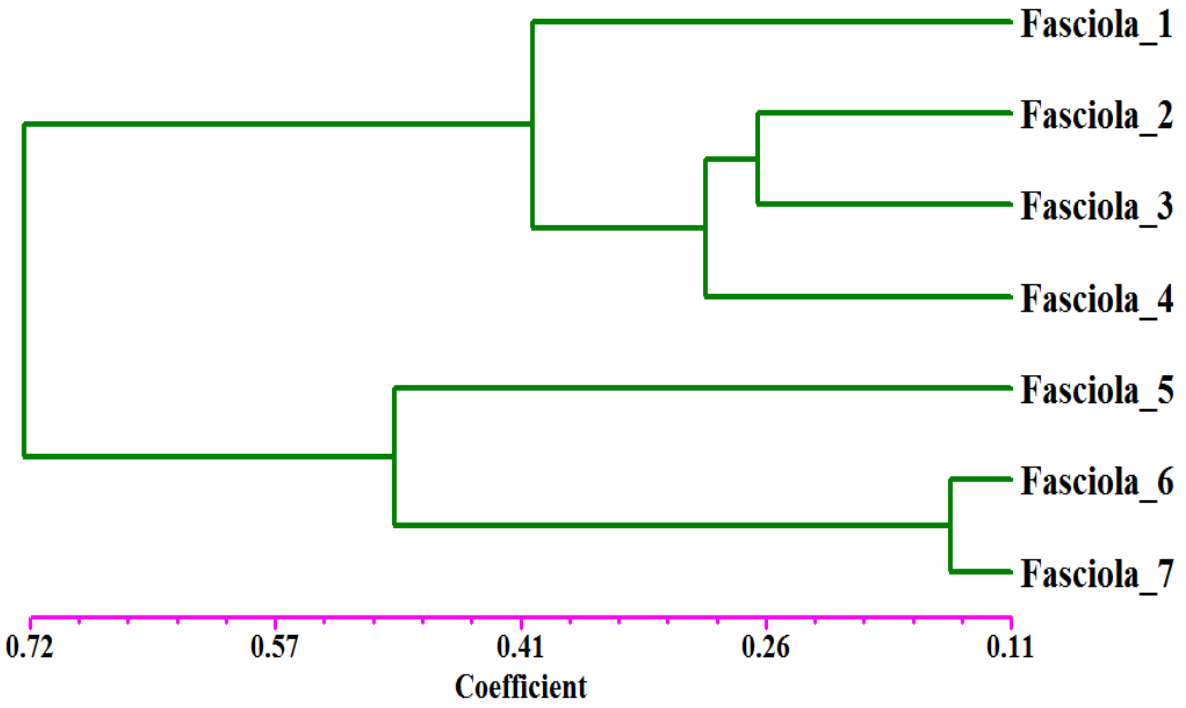


**Figure 5. UPGMA dendrogram based on cluster analysis of RAPD and ISSR data among the seven *Fasciola* isolates collected from camels in Taif region, Saudi Arabia.**

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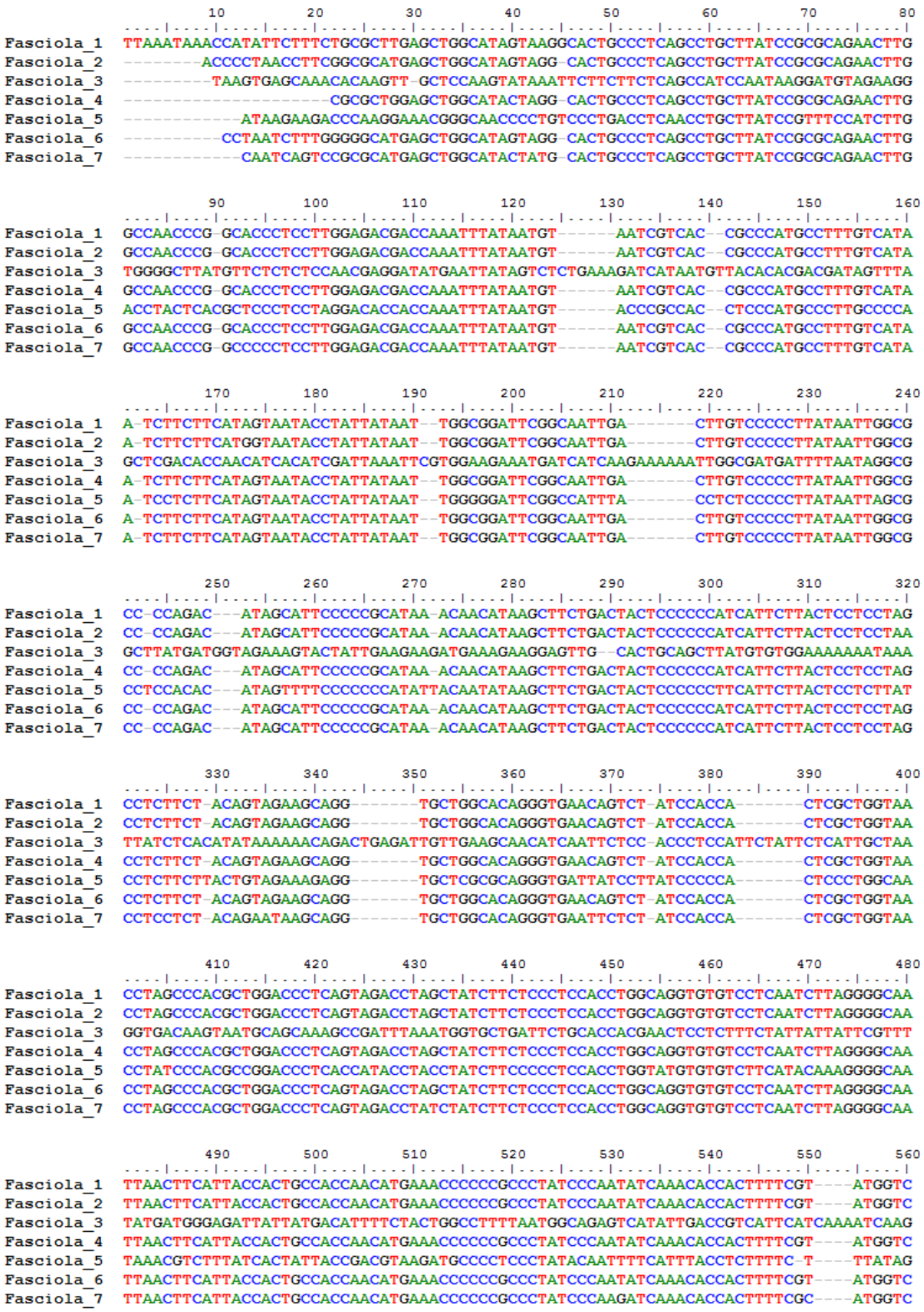


**Figure 6. Comparison aliment of nucleotide sequences of COI gene among seven *Fasciola* isolates nucleotide sequences using BioEdit programme.**

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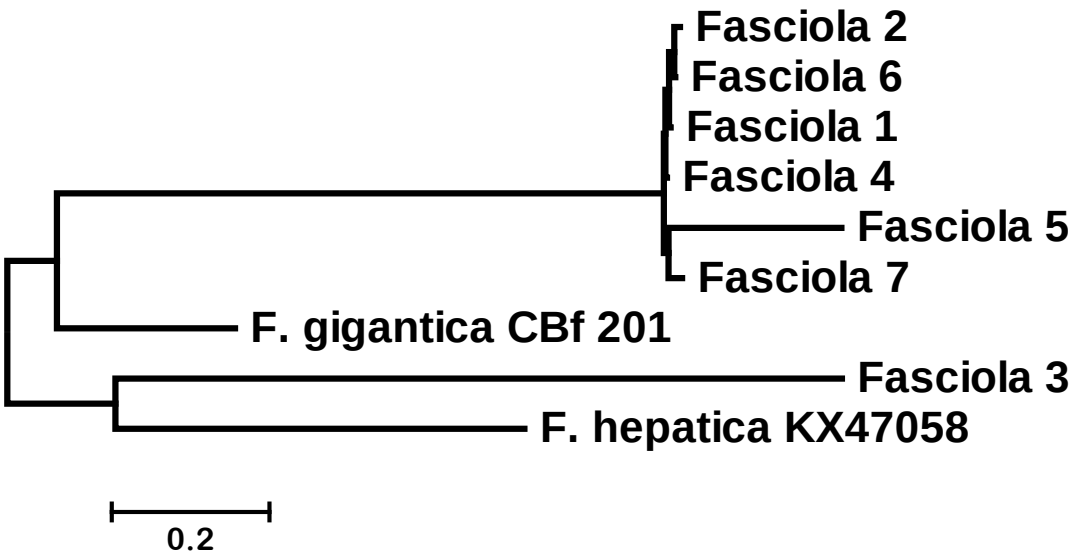


**Figure 7. Neighbor-joining phylogeny based on COI gene sequences of seven *Fasciola* isolates and related *Fasciola* strains obtained from a BLAST search of the NCBI database.**

## CONCLUSION

The *COI* gene sequence act as effective DNA genetic markers for molecular characterization of *Fasciola spp*. However, to ascertain the variations of any population structure, various geographical isolates of Fasciola from different regions and hosts need to be studied more with the usage of more molecular markers. This gives hopeful approach to make diagnosis, control and treatment of *Fasciola spp*.

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