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RESEARCH ARTICLE

Genetic Characterization of *Fasciola* Samples from Bovine Hosts in Pakistan by Sequences of Ribosomal Internal Transcribed Spacer Regions

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ABSTRACT

In present study, 50 samples representing *Fasciola* (Platyhelminthes: Trematoda: Digenea) from cattle and buffaloes of Pothwar region were characterized genetically by sequences of the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA). The ITS rDNA was amplified from individual liver flukes by polymerase chain reaction (PCR), and the amplicons were sequenced directly. The lengths of the FG ITS-1 and ITS-2 sequences were 391 and 329 bp, respectively, for all *Fasciola gigantica* samples sequenced. For *Fasciola hepatica* samples sequenced, FH ITS-1 was not amplified and length for ITS-2 sequences was 330 bp. Molecular identification using rDNA internal transcribed spacer 1 and 2 characterizes *Fasciola spp*. in Pothwar to be an intermediate/ hybrid resembling more with *F. gigantica*. The results also indicated that *Fasciola spp* in cattle and buffalo are genetically similar, as both hosts' possess *Fasciola* with the same number of base pairs in their internal transcribed spacers as proved by gel electrophoresis and sequencing. To determine the phylogenic location of *Fasciola spp*. of Pakistan based on rDNA molecular data, ribosomal ITS regions were sequenced and compared with other fasciolid species from NCBI databases for sequence homology analysis using BLAST and ClustalW programs. The phylogenetic trees revealed a close relationship with isolates of *F. gigantica* from China, India, Iran and Vietnam. This is the first demonstration of the existence of hybrid or an intermediate *Fasciola* species in Pakistan by a genetic approach, which provides foundation for further studies in different agro ecological zones of Pakistan.

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INTRODUCTION

Fasciolosis is a vital food borne zoonotic disease resulting from *Fasciola* trematode parasites, found to be prevalent across the globe affecting humans as well as animals (Fox et *al.,* 2011; Charlier *et al.,* 2012; Ali *et al.,* 2012; Bhutto *et al.,* 2012; Shahzad *et al.,* 2012). Fasciolosis causes colossal economic losses of 200 million US$ annually to the agriculture budget (Ramajo *et al*., 2001). The significant economic losses in livestock appearing in the form of mortalities, reduced fertility, abortions, slow growth and reduction of milk and meat production, infected livers and withered carcasses (Phiri *et al.,* 2006). Furthermore fasciolosis is an up-and-coming threat among the list of parasitic infections in a large number of countries as a result of environmental changes and man-made modifications. It is mostly prevalent in countries having well-developed cattle and sheep production farming. Furthermore, human fasciolosis has also been reported in developing countries, except Western Europe (Mas-Coma *et al.,* 2009a; Farooq *et al*., 2012).

A number of species have been identified within the genus *Fasciola*, but only two species, *Fasciola hepatica* and *Fasciola gigantica*, are more prevalent (Heneberg, 2013). *F. hepatica* mainly originated in the temperate zones, *F. gigantica* restricted to tropical areas, and both

these species overlapping in subtropical areas (Mas-Coma *et al.,* 2005; Mas-Coma *et al.,* 2009b). In addition to both these species an intermediate form has been identified from European and Asian countries by using the first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) as genetic markers (Choe *et al.,* 2011; Ali *et al.,* 2012; Farjallah *et al.,* 2013; Phalee and Wongsawad, 2014).

Genomic studies are very useful to explore the epidemiology, genetic variation and diagnosis of fasciolids (Liu *et al.,* 2014). Recent applications in molecular biology, particularly; amplification of specific DNA regions and direct sequencing has allowed the differentiation of closely related species (Chaichanasak *et al.,* 2012; Dar *et al.,* 2012). Previous findings have revealed that the sequences of ITS-1 and ITS-2 of rDNA are dependable genetic markers. Due to its highly repeated and conserved regions the nuclear ribosomal DNA is especially designed for molecular studies (Chilton, 2004; Choe *et al.,* 2011).

Previous studies based on ITS-1 and ITS-2 sequences of fasciolids were conducted in different Asian countries for species differentiation (Ali *et al.,* 2012; Phalee and Wongsawad, 2014). However, there had been no report on characterizing *Fasciola* from Pakistan using genetic approaches. Therefore, the present study was conducted to characterize *Fasciola* samples from Pothwar region of Pakistan from bovine hosts by sequences ITS-1 and ITS-2 of rDNA because these sequences have been shown to provide specific markers for the description of *F. hepatica, F. gigantica,* and the "intermediate *Fasciola"* (Ali *et al.,* 2012).

MATERIALS AND METHODS

Source of *Fasciola* sample: Adult *Fasciola* were collected from liver of cattle and buffaloes collected from slaughterhouses from Pothwar region, Pakistan. Worms were washed extensively in physiological saline, identified morphologically as *Fasciola* by using existing keys (Yamaguti, 1958). The flukes which were to be used for genomic DNA extraction were stored in 90% ethanol at 4°C.

Extraction of genomic DNA: Genomic DNA was extracted from 50 individual specimens and also from the apical portion of the fluke according to the method described by Semyenova *et al.* (2003), with slight modifications. DNA samples were eluted into 50 µl H_2O and stored at -20ºC, until further use.

PCR amplification for Genetic markers (rDNA ITS-1 ITS-2): Polymerase chain reaction was used to amplify the complete ITS-1 and ITS-2 genetic markers. Four pairs of primers were designed two each from the known sequences of *Fasciola gigantica* and *Fasciola hepatica*. The primers were custom synthesized (MBI-Fermentas) using the information in NCBI data base given in Table 1. PCR reactions were performed using standard protocol in a thermocycler (Applied Biosystems). Samples without genomic DNA were included in each amplification run as "negative" control. The DNA quality was checked on a DNA nanophotometer (IMPLEMEN) initially and then on 1% agarose gel. An aliquot (10 µl) of each amplicon was examined in (0.5 M) TAE buffer stained with ethidium bromide. The DNA size marker of standard 1kb was used.

PCR product purification cloning and sequencing: PCR products of FG-ITS1, FG-ITS 2 and FH-ITS2 from the six representative hosts were purified using QIA quick Purification kit (QIAGEN), according to the manufacturer's instructions. Direct sequencing of PCR products was done using PCR primers as sequencing primers. The ITS-1, ITS-2 PCR products were cloned in TA cloning vector using TA cloning kit (Invitrogen, USA). The cloned samples were sent for sequencing to Macrogen, Korea. Sequencing was done several times in order to ensure accuracy.

Sequence analysis

Sequence alignment: The alignments were constructed using CLUSTALW (Thompson *et al.,* 1994), TCoffee (Notredame *et al.,* 2000) and MUSCLE (Edgar, 2004). A comparison was done between progressive alignments and iterative alignments. The alignments considered in this exercise were of MUSCLE which works iteratively. The gaps between alignments and the regions which are identical are all discarded and blocks of relevant regions are joined together. This was done using G-blocks (Castresana, 2000; Talavera and Castresana, 2007).

Sequence comparison: Sequences obtained were analyzed using Bio-Edit software and aligned with published sequences ITS-1, ITS-2 rDNA belonging to *Fasciola spp* acquired from the GeneBank EMBL by using accession numbers. Only those sample sequences were taken which were complete and were high scoring blast sequences.

Phylogenetic interference: The trees were constructed using two different approaches. BioNJ (Gascuel, 1997) was used to construct the tree according to neighbor joining method. Bootstrap value of 1000 was used. Any branch having a less bootstrap value in the result was collapsed. The second approach used was Bayesian methods. MRBAYES 3.0 (Ronquist and Huelsenbeck, 2003) was used for analysis of the data. MRBAYES and BioNJ were integrated in a workflow using Phylogeny (Dereeper *et al*., 2008). All the trees constructed were rendered and visualized in TreeDyn (Chevenet *et al*., 2006).

RESULTS

In current study, *Fasciola* samples from cattle and buffalo from different locations in Pothwar region (Pakistan) were compared with other *Fasciola* species from ruminant and human hosts belonging to different geographical locations. The PCR products of FG ITS-1 (391 bp) and FG ITS-2 (329 bp) were subjected to direct sequencing and were also cloned in TA cloning vector (MBI, Fermentas) for sequencing. The PCR products for FH ITS-2 (330 bp) were processed similarly.

The FG ITS-1 rDNA of Pakistani *Fasciola* were compared with known sequences of other *Fasciola gigantica* obtained from GenBank. The BLAST hit results are shown in Table 2 and indicated maximum

similarity with *Fasciola*, from buffalo in China, with two nucleotide differences. A four nucleotide difference existed with isolates from Asia (Indonesia, India, Japan, South Korea, Thailand, Vietnam) and Africa (Egypt, Nigeria, Kenya, Burkina Faso and Zambia). The phylogenetic tree constructed by Bayesian method support the blast results by showing similarity between FG ITS1 (Pakistan) and the FG ITS1 species in China, India and South Korea (Fig. 1).

When FG ITS-2 rDNA sequences were compared with *F. gigantica* species (Table 3), the results showed similarity with two nucleotide differences (isolates from China) and three nucleotides difference (isolates from Egypt, Indonesia, India, Japan, Nigeria and Vietnam). A four nucleotide difference was present in isolates from Burkina Faso, Zambia and Kenya. A Phylogenetic tree using the Bayesian method (Fig. 2) and Cladogram indicated that FG ITS2 found in Pakistan is unique from the FG ITS2 found in other locations.

ITS-1 marker for *F. hepatica* could not be amplified, whereas, the ITS-2 marker was amplified successfully. The results described that FH ITS-2 from cattle and buffalo were found to be less similar with *F. hepatica* from various hosts, but more similar with *Fasciola* species (Table 4). Maximum similarity existed between Vietnam *Fasciola hepatica* in humans, with only one nucleotide difference. The other isolates showed a variable relationship with six nucleotide difference. The same relationship is also shown in the phylogenetic tree (Fig. 3). Phylogeny suggests that the closest neighbours of Pakistani FH ITS2 are Vietnam and Iran. The gel picture of *Fasciola* species from cattle and buffaloes showed the same band pairs (Fig. 4 and 5).

DISCUSSION

The identification and molecular characterization of existing *Fasciola* species in Pothwar region was done in present study by comparing sequences of ITS-1 and ITS-2 rDNA of Pakistani *Fasciola* with *Fasciola* species from other geographical locations and especially in neighboring Asian countries. Another objective was to investigate species of origin and its diversion or resemblance with ancestors. Phylogenetic analysis was performed using nuclear ITS-1, ITS-2 sequences in a number of individual worms, from buffalo and cattle and their comparison with available identified sequences in the NCBI data bank.

The Pakistani ITS-1 and ITS-2 sequences of *Fasciola* from cattle and buffalo, when compared with each other were exactly the same. FG ITS-1 resembled FG ITS-1 (*F. gigantica*) present in China, India and Egypt. The cladograms showed that Pakistani FG ITS-2 isolates to had very little ancestral relationship with the other isolates, suggesting that the isolates of FG ITS2 found in Pakistan are different from the ones already characterized in different regions of the world, and thus are unique these results are supported by strong branch support values. Whereas, the BLAST results show a resemblance of Pakistani FG ITS-2 to be unique with resemblance to *F. gigantica* isolates from China, Egypt, India, Indonesia, Japan, Nigeria and Japan.

The FH ITS2 Bayesian tree shows Pakistani FH ITS2 to be present on the same cluster as *F. gigantica* from Vietnam. The parent cluster is shared with *F. gigantica*

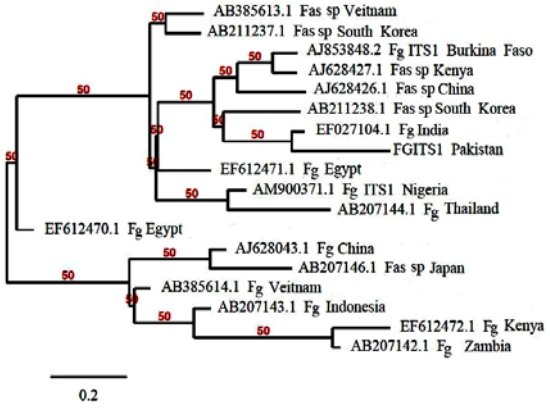

Fig. 1: Phylogenetic tree of FG ITS-1 constructed by Bayesian methods showing similarity of FG ITS1 (Pakistan) with the FG ITS1 species in India, South Korea, and China. Accession no. with country and *Fasciola* type is also given. Fg: *Fasciola gigantica*, Fh: *Fasciola hepatica* and *Fas sp*: *Fasciola* species/intermediate. Numbers in red on tree branches show the branch support values in percentages.

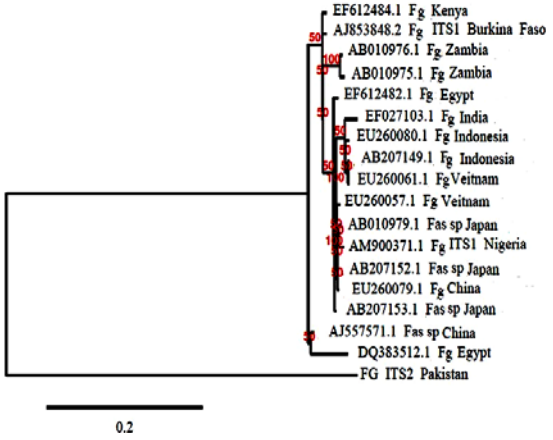

Fig. 2: Phylogenetic tree of FG ITS-2 constructed using Bayesian methods indicates that FG ITS2 found in Pakistan is unique from the FG ITS2 found at other places. Accession no. with country and *Fasciola* type is also given. Fg: *Fasciola gigantica*, Fh: *Fasciola hepatica* and Fas sp: *Fasciola* species/intermediate. Numbers in red on tree branches show the branch support values in percentages.

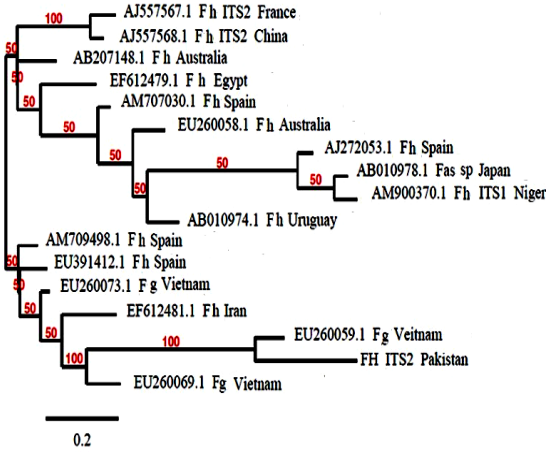

Fig. 3: Phylogenetic tree of FH ITS2 constructed using Bayesian analysis suggests that the closest neighbours of FH ITS2 in Pakistan are Vietnam and Iran. Accession no. with country and *Fasciola* type is also given. Fg: *Fasciola gigantica*, Fh: *Fasciola hepatica* and Fas sp: *Fasciola* species/intermediate. Numbers in red on tree branches show the branch support values in percentages.

Table 1: Primers pairs used for amplification of *Fasciola ITS* markers with Genebank accession Numbers.

Primer Pair		GeneBank Accession No.
FG-ITS -1 F:	GCG ACC TGA AAA TCT ACT CTT ACA CAA GCG	EF612472
FG-ITS -1 R:	GAC GTA CGT ATG GTC AAA GAC CAG GTT	
FG- ITS -2 F:	GCT TAT AAA CTA TCA CGA CGC CCC AC	EF612484
FG-ITS -2 R:	GAA GAC AGA CCA CGA AGG GTA CCG TC	
FH-ITS-1F:	CTA CTC TCA CAC AAG CGA TAC ACG	EF61246
FH-ITS-1R:	GTA CGT ATG GTC AAA GAC CAG GG	
FH-ITS-2 F:	GCT TAT AAA CTA TCA CGA CGC CC	EF612481
FH-ITS-2 R:	GAA GAC AGA CCA CGA AGG G	

Table 2: Comparison of FG ITS-1 of *Fasciola* spp hosted in bovines (present study) with *F. gigantica* from different geographical locations. The variable positions with the country and accession number are also specified.

ITS 1				Variable positions FG	
39	43	172	383	Country	Accession No
T	T	G	T	Pakistan	Present Study
C	T	T	T	China	AJ628043, AJ628425, AJ628426
C	C	T	C	Egypt	EF612470, EF612401
C	C	T	C	India	EF027104
C	C	T	C	Indonesia	AB207143
C	C	T	C	Vietnam	AB385613, AB385614
C	C	T	C	Japan	AB207146
C	C	T	C	Thailand	AB207144
C	C	T	C	S. Korea	AB211238
C	C	T	C	Burkina Faso	AJ853848
C	C	T	C	Zambia	AB207142
C	C	T	C	Nigeria	AM900371
C	C	T	C	Kenya	EF612472, AJ628427, AJ628428

Table 3: Comparison of the FG ITS-2 of *Fasciola* spp hosted in bovines (present study) with *F. gigantica* from different geographical locations. The variable positions along with its country and accession numbers are also specified.

ITS 2				Variable position FG	
105	301	306	323	Country	Accession No
A	G	G	A	Pakistan	Present study
G	T	G	A	China	EU260079, AJ557511, AJ557569
G	T	T	A	Egypt	DQ383512, EF612482, EF612483
G	T	T	A	India	EF027103
G	T	T	A	Indonesia	AB010977, AB207149, AB260080
G	T	T	A	Japan	AB207152, AB010979, AB207151
G	T	T	A	Vietnam	EU260057, EU260059, EU260060
G	T	T	A		EU260072, EU260075, EU260076
G	T	T	A		EU260078, EU260061, EU260063
G	T	T	A		EU260070, EU260074, EU260077
G	T	T	A	Nigeria	AM900371
G	T	T	G	Burkina Faso	AJ853848
G	T	T	G	Kenya	EF612484
G	T	T	G	Zambia	AB010976

species from Vietnam and *F. hepatica* species from Iran. Branch support values of 100 suggest significant results. In the master cluster containing FH ITS2 from Pakistan, *Fasciola* isolates from Iran, Vietnam, Spain, and Uruguay are also grouped together. FH ITS-2 shows common roots with FG species from many countries in this phylogenetic analysis. Therefore, phylogeny suggested that there might be a possible presence of hybrid species between *F. gigantica* and *F. hepatica* in Pakistan as reported in some other regions of the world.

The isolates of *F. hepatica* from around the world are not very similar to the Pakistani *Fasciola*, except for the Iranian and Vietnamese *F. hepatica*. The Vietnamese isolate which shows a very close resemblance, is actually a hybrid/introgressed form, existing in humans. Le *et al.* (2008) showed the existence of hybrid and/or introgressed liver flukes which possess genetic material from both the species; this phenomenon was observed especially in humans living in Vietnam. Therefore it is concluded that *Fasciola species* inhabiting the Pothwar region does not resemble any pure species but is an intermediate species, more closely related to *F. gigantica* as compared to *F. hepatica*.

A BLAST similarity search was conducted on sequence-based identification in the databases. This determined several experimental and taxonomic limitations. In addition to the identification of unknown ITS sequences based on these approaches, phylogeny studies must be performed to attain valuable results for determining closely related species. Phylogeny supports the BLAST results (Holder and Lewis, 2003). Considering these findings, a phylogenetic analysis was carried out by various distance methods and character methods like Neighbor joining method and Bayesian analysis. Results exhibited that topology is similar among the trees obtained. Though both methods were used in this study, the representation of phylogenetic trees was by Cladograms using Bayesian method.

In the present study, buffalo and cattle have the same base pairs and similar sequences. The primary sequence analysis and ITS sequences comparison with bovine liver flukes in various geographical regions indicate a high species-specific homogeneity and a close relationship between the *Fasciola* residing in Pothwar and *F. gigantica* from Asia and Africa and *F. hepatica* from Vietnam and Iran. The similarity of BLAST results of Pakistani *Fasciola* with *Fasciola* of the other geographical isolates supports the similarity to *F. gigantica* but it is not a pure species. The findings of this study are in accordance with studies conducted in Japan, Korea, China, Iran, Vietnam and Egypt indicating that an 'intermediate' *Fasciola* species exists, which is most likely a hybrid of the two species, as both FG ITS-2 and FH ITS-2 were amplified easily reconfirming that the former is a better marker for species identification (Periago *et al*., 2006; Le *et al*., 2008) and that this region is highly conserved in both species. Comprehensive studies (Mas-Coma *et al*., 2005) indicated that although *F. gigantica* mainly occurs in tropical zones and *F. hepatica* mainly in temperate areas, the distribution of both overlap in some subtropical areas.

The findings of this study are further enhanced by examining the genetic variability in *Fasciola* spp from

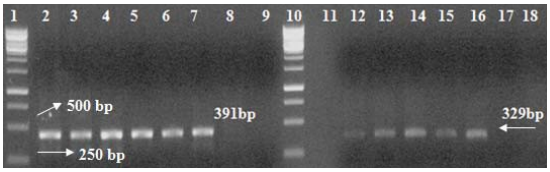

Fig. 4: PCR amplification of FG ITS1 and FG ITS 2, 391 bp bands confirms the presence of FG ITS-1 region in buffalo and cattle, 329 bp bands confirms the presence of FG ITS-2 region in buffalo and cattle. Lane 1 and 10: 1kb DNA marker, Lane 2-4: amplified product of FG ITS-1 Buffalo, Lane 5- 7: amplified product of FG ITS-1 Cattle, Lane 9 and 18: Negative control, Lane 11-13: amplified product of FG ITS-2 Buffalo, Lane 14- 16: amplified product of FG ITS-2 Cattle.

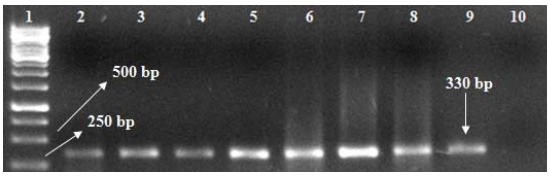

Fig. 5: PCR amplification of FH ITS 2, 330 bp bands confirms the presence of FG ITS-2 region in buffalo and cattle. Lane 1: 1 kb DNA marker, Lane 2-5: amplified product of FH ITS-2 Buffalo Lane 6-9: amplified product of FH ITS-2 Cattle, Lane 10: negative control.

Table 4: Comparison of the FH ITS-2 of *Fasciola spp* hosted in bovines (present study) with *Fasciola hepatica* from different geographical locations. The variable positions along with its country and accession number are specified.

ITS2 Variable positions FH							
167	230	236	243	294	311	Country	Accession No
C	T	T	C	A	C	Pakistan	Present Study
C	T	T	C	A	T	Vietnam	EU260059, EU260060, EU260069
T	C	C	T	G	T	Iran	EF612481
T	C	C	T	G	T	Egypt	EF612479-80
T	C	C	T	G	T	China	AJ557568, AJ557570
T	C	C	T	G	T	Japan	AB010978, AB207150
T	C	C	T	G	T	France	AJ557567
T	C	C	T	G	T	Spain	AJ272053, AM709498-99
T	C	C	T	G	T	Spain	AM709609-16, AM709618
T	C	C	T	G	T	Spain	AM709619, AM709643-49
T	C	C	T	G	T	Spain	EU391412-24, AM707030 AM709500, AM709617,
T	C	C	T	G	T	Spain	AM709620
T	C	C	T	G	T	Uruguay	AB010974
T	C	C	T	G	T	Australia	EU260058, AB207148
T	C	C	T	G	T	Niger	AM900370

different host species and in diverse geographical locations (highlands and lowlands) in Pakistan and by using additional molecular markers like mitochondrial DNA sequences. It will be an interesting aspect to carry out further studies which will highlight the genetic variability of human *Fasciola* prevalent in our region. The presence of extensive water bodies facilitate the development and survival of *Fasciola* spp. in snails and where livestock co-exist, it is a constraint to the farmer's ability to earn their livelihood which contributes significantly to the national economy. It is essential that parasite isolates should be accurately identified for effective diagnosis, treatment and control.

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REFERENCES

Ali NM, 2012. Development and evaluation of a dipstick assay in diagnosis of human fasciolosis. Parasitol Res, 110: 1649-1654.

Bhutto B, A Arijo, MS Phullan and R Rind, 2012. Prevalence of fascioliasis in buffaloes under different agro-climatic areas of Sindh Province of Pakistan. Int J Agric Biol, 14: 241-245.

Castresana J, 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol, 17: 540-552.

Chaichanasak P, M Ichikawa, P Sobhon and T Itagaki, 2012. Identification of *Fasciola* flukes in Thailand based on their spermatogenesis and nuclear ribosomal DNA, and their intraspecific relationships based on mitochondrial DNA. Parasitol Int, 61: 545-459.

Charlier J, M Hostens, J Jacobs, B Van Ranst, L Duchateau *et al.,* 2012. Integrating fasciolosis control in the dry cow management: the effect of closantel treatment on milk production. PLoS One, 7: e43216.

Chevenet F, C Brun, AL Banuls, B Jacq and R Christen, 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinformatics, 7: 439.

Chilton NB, 2004. The use of nuclear ribosomal DNA markers for the identification of bursate nematodes (order Strongylida) and for the diagnosis of infections. Anim Health Res Rev, 5: 173-187.

Choe SE, TT Nguyen, TG Kang, CH Kweon and SW Kang, 2011. Genetic analysis of *Fasciola* isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA. Parasitol Res, 109: 833-839.

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

Dereeper A, V Guignon, G Blanc, S Audic, S Buffet, F Chevenet, J F Dufayard, S Guindon, V Lefort, M Lescot, JM Claverie and O Gascuel, 2008. Phylogeny. In: robust phylogenetic analysis for the non-specialist. Nucl Acids Res, 1: 36.

Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 32: 1792-97.

Farjallah S, BB Slimane, CM Piras, N Amor, G Garippa and P Merella, 2013. Molecular characterization of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers. Exp Parasitol, 135: 471-478.

Farooq Z, S Mushtaq, Z Iqbal and S Akhtar, Shahnaz, 2012. Parasitic helminths of domesticated and wild ruminants in Cholistan desert of Pakistan. Int J Agric Biol, 14: 63-68.

Fox NJ, PC White, CJ McClean, G Marion, A Evans and MR Hutchings, 2011. Predicting impacts of climate change on *Fasciola hepatica* risk. PLoS One, 6: e16126.

Gascuel O, 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol, 14: 685–695.

Heneberg P, 2013. Phylogenetic data suggest the reclassification of *Fasciola jacksoni* (Digenea: Fasciolidae) as *Fascioloides jacksoni* comb. nov. Parasitol Res, 112: 1679-1689.

Holder M and PO Lewis, 2003. Phylogeny estimation: Traditional and bayesian approaches. Nat Rev Genet, 4: 275.

Le TH, NV De, T Agatsuma, TGT Nguyen, QD Nguyen, DP McManus and D Blair, 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola h*epatica and *Fasciola gigantica* in Vietnam. Int J Parasitol, 38: 725-730.

Liu GH, RB Gasser, ND Young, HQ Song, L Ai and XQ Zhu, 2014. Complete mitochondrial genomes of the 'intermediate form' of *Fasciola* and *Fasciola gigantica* and their comparison with *F. hepatica*. Parasit Vectors, 7: 150.

Mas-Coma S, MA Valero and MD Bargues, 2009a. Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Vet Parasitol, 163: 264-280.

Mas-Coma S, MA Valero and MD Bargues, 2009b. *Fasciola,* lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol, 69: 41-146.

Mas-Coma S, MD Bargues and MA Valero, 2005. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol, 35: 1255-1278.

Notredame C, DG Higgin and J Heringa, 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol, 302: 205-17.

Periago, MV, MA Valero, M El-Sayed, K Ashrafi, A El-Wakeel, MY Mohamed, 2006. Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. Parasitol Int, 55: 249-260.

Phalee A and C Wongsawad, 2014. Prevalence of infection and molecular confirmation by using ITS-2 region of *Fasciola gigantica* found in domestic cattle from Chiang Mai province, Thailand. Asian Pacific J Trop Med, 7: 207-211.

Phiri IK, AM Phiri and LJ Harrison. 2006. Serum antibody isotype responses of *Fasciola*-infected sheep and cattle to excretory and secretory products of *Fasciola* species. Vet Parasitol, 141: 234-242.

Ramajo V, A Oleaga, P Casanueva, GV Hillyer and A Muro, 2001. Vaccination of sheep against *Fasciola hepatica* with homologous fatty acid binding proteins. Vet Parasitol, 97: 35-46.

Ronquist F and JP Huelsenbeck, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572-1574.

Semyenova SK, EV Morozova, GG Chrisanfova, AM Asatrian, SO Movsessian and AP Ryskov, 2003. RAPD variability and genetic diversity in two populations of liver fluke, *Fasciola hepatica*. Acta Parasitol, 48: 1230-2821.

Shahzad W, K Mehmood, R Munir, W Aslam, M Ijaz, R Ahmad, MS Khan and AJ Sabir, 2012. Prevalence and molecular diagnosis of *Fasciola hepatica* in sheep and goats in different districts of Punjab, Pakistan. Pak Vet J, 32: 535-538.

Talavera G and J Castresana, 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol, 56: 564-577.

Thompson JD, DG Higgins and TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucl Acids Res, 2: 4673-4680.

Yamaguti S, 1958. Systema helminthum, vol I. The digenetic trematodes of vertebrates. Inter Science Publishers, New York.