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Molecular Characterization and Phylogenetic Analysis of Bovine Fasciolosis in Indigenous Cattle and Buffalo Populations in Okara, Pakistan

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Abstract: Illustration of distinct cause of Fasciolosis in different geographic regions has been the main wide reaching concern from veterinary medicine and public health perspectives. Present exploration was done for accurate diagnosis and evolutionary analysis of Fasciola species based on internal transcribes spacer second (ITS2), 5.8s rDNA. A total of 348 samples were used in this study, including each 87 fecal and bile samples from Sahiwal cattle and Nili Ravi buffaloes. ITS2, 5.8s rDNA gene region was amplified in 300bp through PCR from *Fasciola hepatica* eggs recovered from fecal and bile samples. Phylogenenetic analysis was concluded using maximum likelihood method and 24 different models were used. MEGA 6.0 was used to create multiple alignment of ITS1 and ITS2 gene for all Fasciola isolates, which allowed to create multiple alignments by ClustalW, using discrete Gamma distribution (+G), and evolutionary invariable (+I) and percent identity between the isolates was determined. The haplotypes structure and sequence variation indices were intended using DnaSP version 5.10.01, including nucleotide diversity, number of polymorphic sites, number of haplotypes and haplotypes diversity. Hence these investigations provide comprehensive approaches to formulate systemic searches for rising focus of human fasciolosis in Pakistan.
Keywords: Fasciolosis, Cattle, Buffalo, ITS1, ITS2, MEGA.

INTRODUCTION

Fasciolosis has traditionally been considered as pessimistic bang on livestock farming round the earth [1], caused by taxonomically valid species of *Fasciola hepatica* and *Fasciola gigantica* [2]. Prevalence of Fasciolosis in ruminants has long been perceptible in a wide range of geographical distribution, whereas human Fasciolosis has been reported in 51 countries of five continents [3], Asian countries including Pakistan, Iran, India and Iraq [4]. Almost 2.4-17 million cases of human Fasciolosis were estimated and 180 million revealed at risk, divulge the consequence of Fasciolosis [5]. As regard the health and economic losses endorsed to Fasciolosis in livestock ensue in form of mortalities, reduced growth, and diminution of milk and meat production, infected liver and withered carcasses [6]. Fasciolosis grounds colossal economic losses of 200 million US$ yearly to the agricultural assets and affect the reap of large ruminants [7]. In Pakistan various pervasiveness minutes of Fasciolosis in livestock have been documented, but defined economic losses owed by Fasciolosis are not yet on the hands [8]. Therefore, quick and truthful diagnosis of contagion is of prime importance to control Fasciolosis, as in the past effective control therapies of Fasciolosis were lacking [9]. Currently, molecular approaches based on DNA analysis, including genetic structure of parasites, gene expression, phylogenetic and evolutionary analysis has revealed advances in effective parasitic diseases control to diminish economic losses. However exploration for consistent molecular markers pertinent for low level phylogenetic analysis vestiges a taxing setback [10]. In

Asian and European countries Fasciolosis was distinguished on bases of DNA sequences of ITS1 and ITS2 as genetic markers [11, 12], as internal transcribed sequences are widely used as marker in genomic studies to investigate genetic diagnosis and diversity of Fasciolosis [13]. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is most extensively used markers to distinguish Fasciola species [14, 15], as the species of Fasciola can also be distinguished by ITS2 and 28rRNA genes [16]. Former studies revealed *F. hepatica* and *F. gigantica* in domestic animals, as well as in human based on molecular characterization of CO1 gene [17, 18]. The regions of 18S and 28S of ribosomal DNA was used in molecular studies, and ITS1 and ITS2, sequence were pertinent genetic marker for genotyping, intraspecific distinctions and phylogenetic analysis of parasites [19]. In previous studies microsatellite markers and mitochondrial genes such as portion of oxydase subunit (pcox1) and NADPH dehydrogenase subunits (pnad1) were used to reveal subsistence of genetic polymorphism and phylogenetic coalition among Fasciola species [20]. In present exploration, 28S, (ITS2, 5.8SrRNA) region was used by PCR to reveal molecular delineation and phylogenetic analysis of Fasciolosis. The intend of present exploration was to scrutinize Fasciola isolates to determine biased potential of DNA sequence analysis based on ITS2 region, dissect intra and inter-species genetic assortment and elucidate evolutionary kinship of Fasciola species isolates in Sahiwal cattle and Nili Ravi buffalo in Okara region, Punjab, Pakistan.

MATERIALS AND METHODS

Sampling and Morphological Identification

Using random sampling method a total of 348 samples were used in this study, including each 87 fecal and bile samples from Sahiwal cattle and Nili Ravi buffaloes from government and private livestock farms and various slaughter houses. Animals of all age groups and sex were included in this study, mainly those which were kept on natural grazing and other seasonal fodders. The samples were collected on the basis of history, clinical signs, and symptoms of disease and postmortem lesions of slaughtered animals. The morphological examination of fasciolosis was done by microscopically, for this purpose fecal and bile samples were processed by centrifugal sedimentation according to described protocol [21].

DNA extraction and PCR

For DNA extraction fecal and bile samples were processed by standard sieving protocol to recover Fasciola eggs [21, 22]. DNA was extracted by total genomic DNA extraction kit (Cat. #K0512, Fermentas) according to manufacturer's instructions. PCR was carried out to amplify ITS2, 5.8s rDNA region with DSJF primers (Forward; 5′-ATATTGCGGCCATGGGTTAG-3′) and DSJR (Reverse 5′ CCAATGACAAAGTGACAGCG-3′) [23] and all PCR amplification reactions were carried out in a final volume of 25 µl containing DNA template and 12.5 µl commercially available PCR master mix (Dream Taq Green PCR Master Mix #k1081, Thermo Scientific), 1µl 25mM $MgCl_2$ Thermo Scientific, 2µl forward primer, 2µl reverse primer,2µl nuclease free water #R0581 Fermentas,.5µl Taq DNA Polymerase and 5µ DNA sample. The PCR was carried out with following conditions i.e. initial denaturation at 95°C for 4 min and then for each of 30 cycles, the denaturation at 95°C for 1 min, annealing at 60 °C for 1 min and 3rd stage extension at 72°C for 2 min. PCR cycles were followed by 10 min of final extension at 72 °C. And at the end, final holding temperature was 4°C until the PCR tubes were taken out of thermal cycler and placed in refrigerator or run on agarose gel with 1kb ladder as size marker and amplification of 300bp of ITS2 gene of Fasciola species was obtained in cattle and buffalo fecal and bile samples.

SEQUENCE ANALYSIS

Nucleotide and amino acid sequences of ITS1 and ITS2, 5.8s rDNA gene of different Fasciola isolates belonging various regions were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank) and accomplished sequences of proteins were aligned through MEGA6.0 program [24], using default alignment parameters followed by manual adjustment. Phylogenetic analysis was concluded using maximum likelihood method and 24 different models were used. MEGA 6.0 was used to create multiple alignment of ITS1 and ITS2 gene for all Fasciola isolates, which allowed to create multiple alignments by ClustalW, using discrete Gamma distribution (+G), and evolutionary invariable (+I) [25]. Models with low Bayesian Information Criterion score (BIC) were considered best for substitution pattern description. For each model Akaike Information Criterion corrected (AICc), Maximum Likelihood value (LnL), and the number of parameters including branch length were also represented. Non uniformity of evolutionary rates among sites was presented by using discrete gamma distribution (+G) and by assuming that the certain fraction sites are evolutionary invariable (+I). Estimated values of trans version bases (R) were shown for each model as well. Codon positions included were first, second, third and non-coding. All positions containing gaps and missing data were eliminated.

PHYLOGENETIC ANALYSIS

Phylogenetic analysis of ITS1 and ITS2 genes of Fasciola was done by retrieving the nucleotide and amino acid sequences of Fasciola isolates from NCBI GenBank (National Centre Biotechnology Information, Rockville Pike Bethesda, USA). Phylogenetic tree was constructed with MEGA 6.0 software package and neighbor joining method. ClustalW software [26] was used for genetic sequence and correlation analysis of ITS1 and ITS2 in various Fasciola isolates belonging to

different regions. The haplotypes structure and sequence variation indices were intended using DnaSP version 5.10.01, including nucleotide diversity, number of polymorphic sites, number of haplotypes and haplotypes diversity. DnaSP results validation was done by MEGA 6.0 software package. The haplotypes were inferred using DnaSP version 5.10.01 and median joining network for these haplotypes was constructed by using Network version 4.6.1.4 [27].

RESULTS

The study was carried out at Livestock Production and Research Institute (LPRI), Bahadarnagar, Okara region, Punjab, Pakistan and Fasciola ITS2 gene was amplified from Fasciola eggs recovered from fecal and bile samples of Sahiwal cattle and Nili Ravi buffalo. A 300bp Amplification of ITS2 gene of Fasciola was obtained Fig. 1.

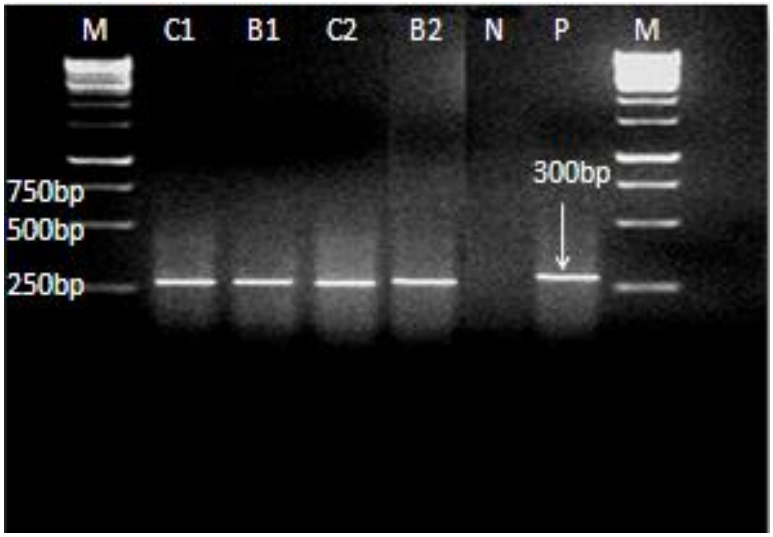

Fig-1: PCR amplification of ITS2 confirms the Fasciola hepatica in Sahiwal cattle and Nili Ravi buffalo Fasciola hepatica with specific primers of 300bp size. M serves as molecular ladder. Lane C1, C2 for cattle fecal and bile isolates, lane B1, B2 for buffalo fecal and bile isolate, lane N shows control negative and P shows control positive respectively

PHYLOGENETIC ANALYSIS

Evolutionary analysis was performed with Maximum Likelihood 24 nucleotide substitution models by using MRGA 6.0 program. There were 340 positions in final data set. The analysis involved 27 rDNA sequences and codon positions included were 1st, 2nd, 3rd and non-coding. Bayesian Information Criterion, score (BICs), Akaike Information Criterion, corrected (AICc), Maximum Likelihood (*lnL*), and parameter which includes branch length were also illustrated for all 24 substitution models. The results were simulated with four different gamma parameter values such as site variation rates, estimates of standard deviation through discrete gamma distribution values, transition/transversion ratio and the relative nucleotide frequencies. Unvarying evolutionary rates among sites were modeled by using discrete Gamma distribution (+G) with five rate categories and by evolutionary invariable sites fraction assumptions (+I) Transversion bias (R) values for each model are presented along with nucleotide frequencies (f) and substitution rate (r) . Model with lower Bayesian Information Criterion, score (BICs) were regarded as best substitution pattern. JC model presented the lowest BIC values (1471.45) amongst all 24 models with AICc value 1108.67 and R value 0.5 and Maximum Likelihood (*lnL*) for JC model was -503.04. Relative values for instantaneous r values were evaluated and the sum of r values was 1 for each model. The invariable (+I), discrete gamma distribution (+G) values and relative frequencies of each nucleotide sequence for each model are shown (Table 1). Evolutionary kinship of Fasciola isolates was determined by using ITS1 and ITS2, 5.8s rRNA sequences retrieved from Gen Bank. All sequences were aligned with Clustal W MEGA multiple sequence alignment program [25] and manual adjustment of sequences were done before alignment for all retrieved sequences. Based on open reading frame, evolutionary kinship in phylogenetic tree (Figure 2) indicate the relationship of Fasciola isolates belonging to different regions and percent identity between the isolates was determined by Crustal 2.1 shown in table 2.

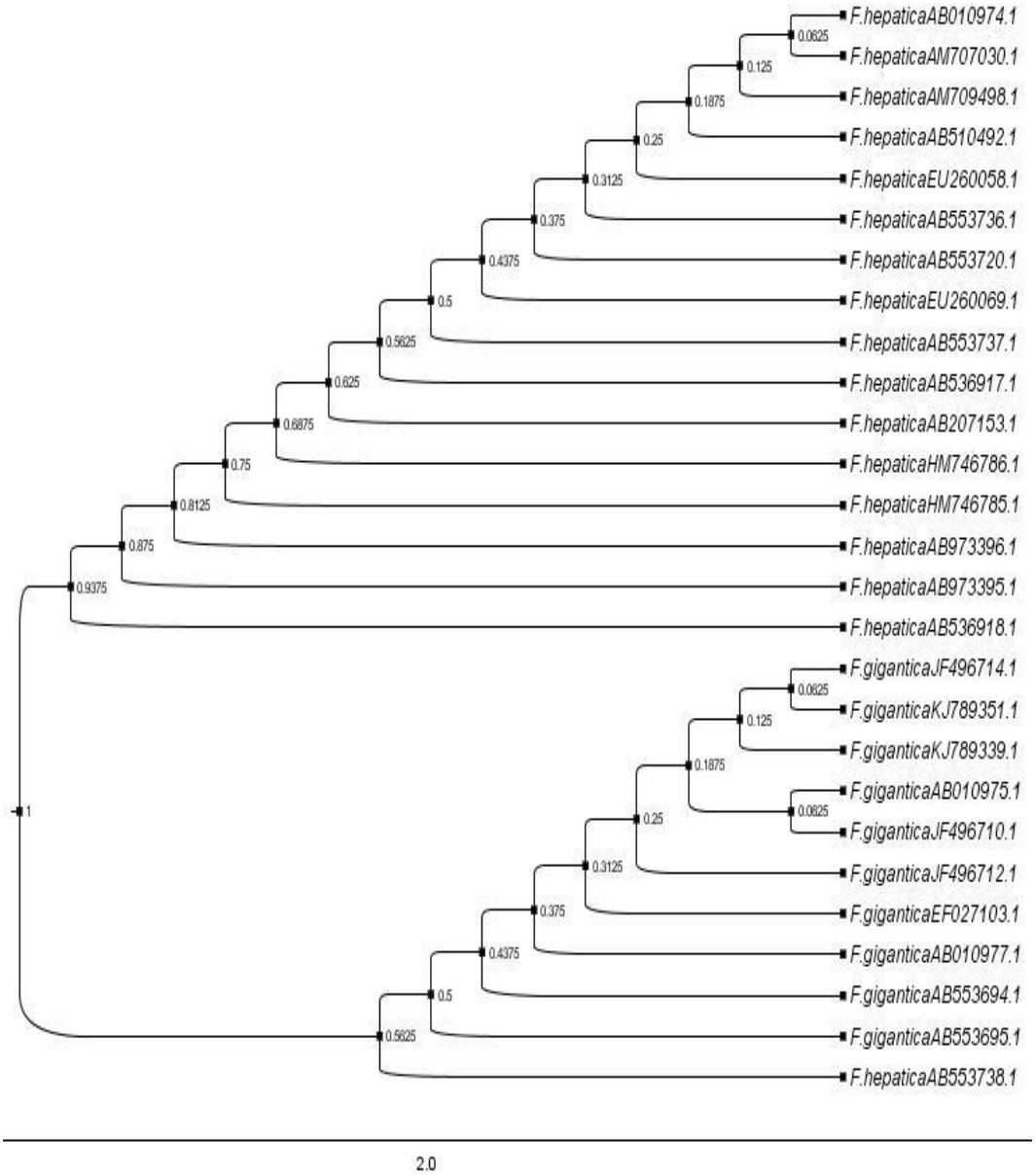

Fig-2: Phylogenetic relationship based on nucleotide sequence of ITS2, 5.8s rRNA gene by phylogeny bootstrap test using neighbor-joining method in MEGA 6.0 software. The values of major clusters are designated in the node or branch in tree, which represent the confidence tested on using 500 replicates of the data set

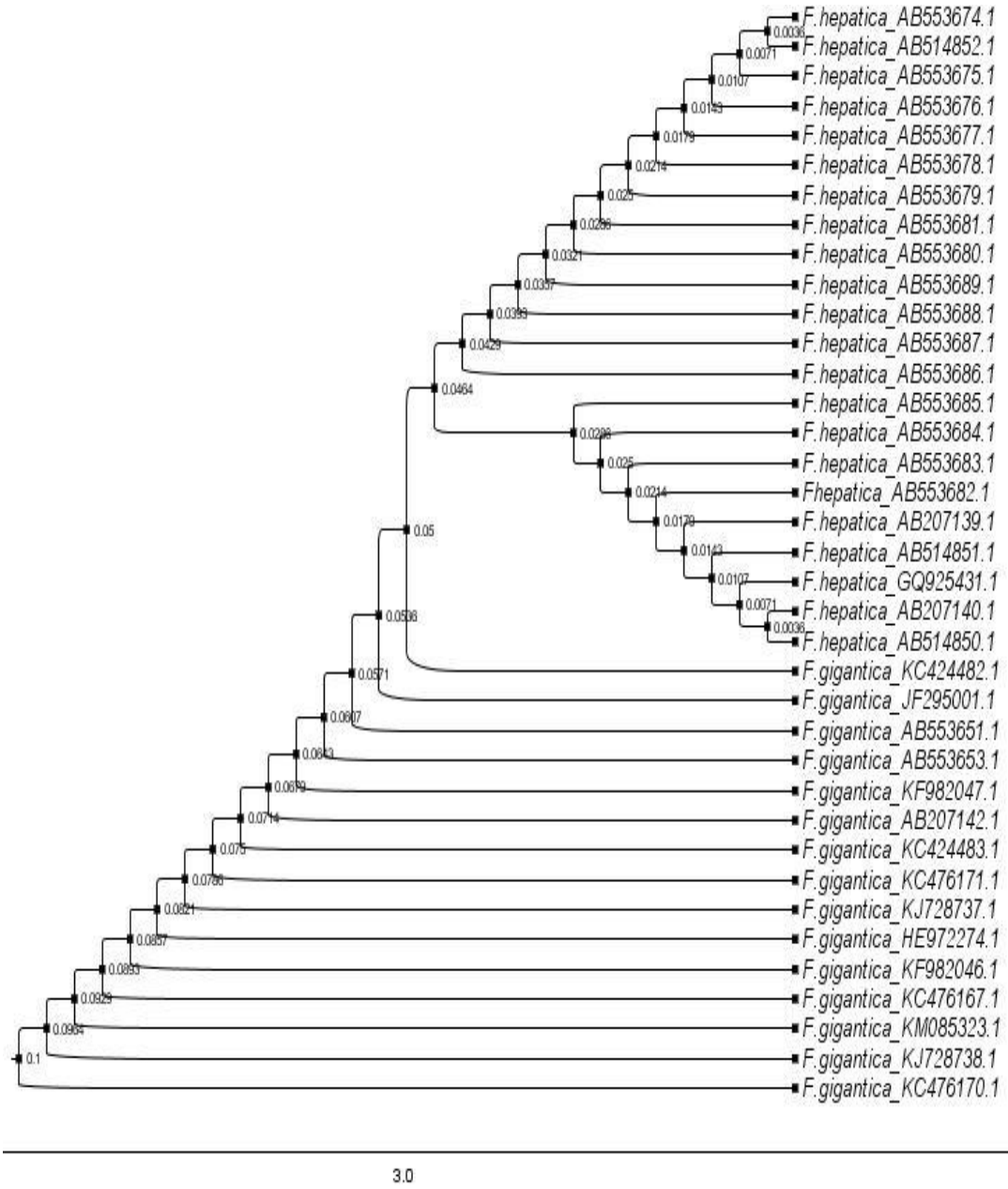

Fig-3: Phylogenetic relationship based on nucleotide sequence of ITS1, 5.8s rRNA gene by phylogeny bootstrap test using neighbor-joining method in MEGA 6.0 software. The values of major clusters are designated in the node or branch in tree, which represent the confidence tested on using 500 replicates of the data set

To expound the phylogeny of Fasciola based on ITS2 gene region, a network plot for 8 haplotypes of 27 various Fasciola isolates was drawn and the groups corresponded to taxa in phylogenetic tree were illustrated. The haplotypes were inferred by using DnaSP version 5.10.01 and median joining network of 8 haplotypes (Fig. 3) was constructed by using Network 4.6.1.4 version software.

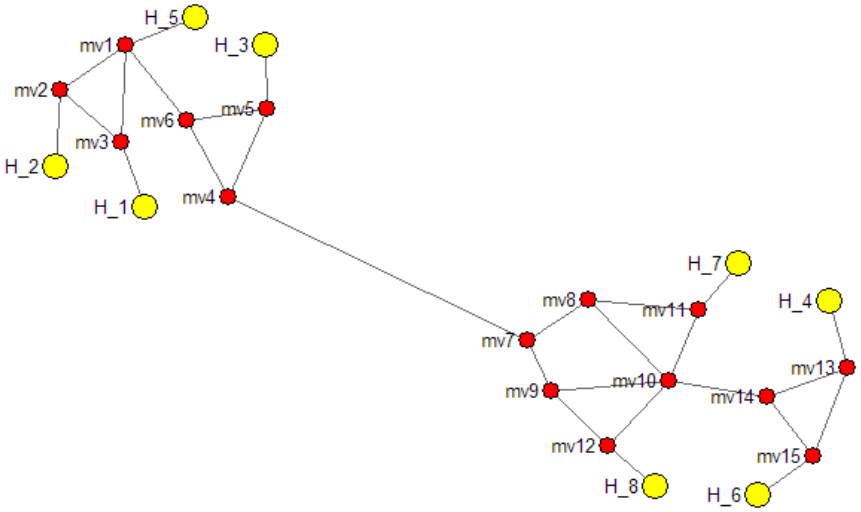

Fig-3: Phylogenetic Median-joining network based relationships among ITS2 haplotypes in various Fasciola species. The yellow circles indicate the haplotypes. A red square indicates the median vector (mv). Branch length is proportional to the number of mutations and size of node is proportional to the frequency of the haplotypes.

Table-1: Maximum likelihood based 24 nucleotide substitution models

Model	Parameter	BIC	AICc	*lnL*	Invariant	Gamma	R	f(A)	f(T)	f(C)	f(G)	A=>T	A=>C	A=>G	T=>A	T=>C	T=>G	C=>A	C=>T	C=>G	G=>A	G=>T	G=>C
JC	51	1471.5	1108.7	-503	n/a	n/a	0.5	0.25	0.25	0.3	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
K2	52	1477.7	1107.8	-501.6	n/a	n/a	3.01	0.25	0.25	0.3	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
JC+I	52	1480.6	1110.7	-503.1	0	n/a	0.5	0.25	0.25	0.3	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+G	52	1480.6	1110.7	-503.1	n/a	200	0.5	0.25	0.25	0.3	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
T92	53	1486.6	1109.6	-501.5	n/a	n/a	3.01	0.26	0.26	0.2	0.24	0.03	0.03	0.18	0.03	0.18	0.03	0.03	0.19	0.03	0.19	0.03	0.03
K2+I	53	1486.8	1109.8	-501.6	0	n/a	3.01	0.25	0.25	0.3	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
K2+G	53	1486.9	1109.9	-501.6	n/a	200	3.01	0.25	0.25	0.3	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
JC+G+I	53	1489.7	1112.8	-503.1	0	200	0.5	0.25	0.25	0.3	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
HKY	55	1495.4	1104.2	-496.7	n/a	n/a	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0.2	0.02	0.16	0.03	0.02	0.23	0.03	0.15	0.04	0.03
T92+I	54	1495.7	1111.7	-501.5	0	n/a	3.01	0.26	0.26	0.2	0.24	0.03	0.03	0.18	0.03	0.18	0.03	0.03	0.19	0.03	0.19	0.03	0.03
T92+G	54	1495.8	1111.7	-501.5	n/a	200	3.01	0.26	0.26	0.2	0.24	0.03	0.03	0.18	0.03	0.18	0.03	0.03	0.19	0.03	0.19	0.03	0.03
K2+G+I	54	1496	1111.9	-501.6	0	200	3.01	0.25	0.25	0.3	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
TN93	56	1501	1102.7	-495	n/a	n/a	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0	0.03	0.31	0.03	0.03	0.44	0.03	0	0.04	0.03
HKY+I	56	1504.5	1106.2	-496.7	0	n/a	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0.2	0.02	0.16	0.03	0.02	0.23	0.03	0.15	0.04	0.03
HKY+G	56	1504.5	1106.2	-496.8	n/a	200	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0.2	0.02	0.16	0.03	0.02	0.23	0.03	0.15	0.04	0.03
T92+G+I	55	1504.9	1113.7	-501.5	0	200	3.01	0.26	0.26	0.2	0.24	0.03	0.03	0.18	0.03	0.18	0.03	0.03	0.19	0.03	0.19	0.03	0.03
TN93+I	57	1510.1	1104.7	-495	0	n/a	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0	0.03	0.31	0.03	0.03	0.44	0.03	0	0.04	0.03
TN93+G	57	1510.2	1104.8	-495	n/a	200	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0	0.03	0.31	0.03	0.03	0.44	0.03	0	0.04	0.03
HKY+G+I	57	1513.6	1108.2	-496.8	0	200	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0.2	0.02	0.16	0.03	0.02	0.23	0.03	0.15	0.04	0.03
TN93+G+I	58	1519.3	1106.8	-495	0	200	3.01	0.2	0.31	0.2	0.27	0.04	0.03	0	0.03	0.31	0.03	0.03	0.44	0.03	0	0.04	0.03
GTR	59	1526.8	1107.2	-494.2	n/a	n/a	1.49	0.2	0.31	0.2	0.27	0.03	0.02	0	0.02	0.24	0.03	0.02	0.34	0.14	0	0.03	0.12
GTR+G	60	1535.9	1109.2	-494.2	n/a	200	1.56	0.2	0.31	0.2	0.27	0.03	0.02	0	0.02	0.25	0.03	0.02	0.35	0.14	0	0.03	0.12
GTR+I	60	1535.9	1109.2	-494.2	0	n/a	1.49	0.2	0.31	0.2	0.27	0.03	0.02	0	0.02	0.24	0.03	0.02	0.34	0.14	0	0.03	0.12
GTR+G+I	61	1545	1111.2	-494.2	0	200	1.56	0.2	0.31	0.2	0.27	0.03	0.02	0	0.02	0.25	0.03	0.02	0.35	0.14	0	0.03	0.12

Note: Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table-2: Percent identity matrix among various Fasciola isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1: F.giganticaAB010975.1	100	99	98	98.3	98.3	98	98	98	98	99	98	99	99	97	98	98	98	98	98	98	98	98.3	98	98	98	98	98.3
2: F.hepaticaAB553738.1	99	100	98	98.4	98.6	99	99	99	99	99	99	98	98	98	99	97.8	97	98	98	98	98	97.8	98	98	98	98	97.8
3: F.giganticaKJ789351.1	98	98	100	70.8	71.1	71	71	99	84	99	99	98	98	97	83	97.8	97	97	98	83	98	82.6	97	97	80	97	96.9
4: F.giganticaJF496714.1	98	98	71	100	98.3	99	98	99	91	99	99	98	98	97	95	97.8	97	97	98	93	98	95.1	98	98	92	98	97.5
5: F.giganticaJF496710.1	98	99	71	98.3	100	100	98	100	94	100	99	98	98	97	97	97.8	97	97	98	96	98	97.4	98	98	94	98	97.5
6: F.giganticaKJ789339.1	98	99	71	99.3	99.9	100	99	100	94	100	99	98	98	97	98	97.8	97	97	98	96	98	97.8	98	98	94	98	97.5
7: F.giganticaJF496712.1	98	99	71	98.2	98.4	99	100	100	93	100	99	98	98	97	97	97.8	97	97	98	95	98	97.2	98	98	94	98	97.5
8: F.giganticaAB010977.1	98	99	99	99.5	100	100	100	100	100	100	99	98	98	97	98	97.8	97	97	97	98	97	97.5	98	98	98	98	97.5
9: F.giganticaEF027103.1	98	99	84	91.2	93.6	94	93	100	100	100	99	98	98	97	95	97.8	97	97	98	95	98	94.7	98	98	94	98	97.5
10:F.giganticaAB553695.1	99	99	99	99.2	99.7	100	100	100	100	100	100	98	98	97	98	98	97	98	98	98	98	97.8	98	98	98	98	97.8
11:F.giganticaAB553694.1	98	99	99	98.9	99.5	99	99	99	99	100	100	98	98	97	98	97.8	97	97	97	98	97	97.5	98	98	98	98	97.5
12:F.hepaticaHM746786.1	99	98	98	98	98	98	98	98	98	98	98	100	100	97	98	98.6	99	99	99	99	99	98.9	99	99	99	99	98.9
13:F.hepaticaHM746785.1	99	98	98	98	98	98	98	98	98	98	98	100	100	97	98	98.6	99	99	99	99	99	98.9	99	99	99	99	98.9
14: F.hepaticaAB207153.1	97	98	97	97	97.2	97	97	97	97	97	97	97	97	100	99	98.3	98	98	99	99	99	98.6	99	99	99	99	98.6
15: F.hepaticaAB536917.1	98	99	83	94.9	97.4	98	97	98	95	98	98	98	98	99	100	98.9	99	99	99	98	99	99.2	99	99	99	99	98.9
16: F.hepaticaEU260069.1	98	98	98	97.8	97.8	98	98	98	98	98	98	99	99	98	99	100	99	99	100	100	100	99.7	100	100	100	100	99.7
17:F.hepaticaAM707030.1	98	97	97	97.2	97.2	97	97	97	97	97	97	99	99	98	99	99.5	100	100	100	100	100	99.7	100	100	100	100	99.7
18: F.hepaticaAB010974.1	98	98	97	97.2	97.2	97	97	97	97	98	97	99	99	98	99	99.4	100	100	100	100	100	99.7	100	100	100	100	99.7
19:F.hepaticaAM709498.1	98	98	98	97.5	97.5	98	98	97	98	98	97	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100
20: F.hepaticaAB510492.1	98	98	83	93.4	95.7	96	95	98	95	98	98	99	99	99	98	99.7	100	100	100	100	100	98.4	100	100	98	100	100
21: F.hepaticaEU260058.1	98	98	98	97.5	97.5	98	98	97	98	98	97	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100
22: F.hepaticaAB536918.1	98	98	83	95.1	97.4	98	97	98	95	98	98	99	99	99	99	99.7	100	100	100	98	100	100	100	100	100	100	100
23: F.hepaticaAB553736.1	98	98	97	97.5	97.5	98	98	98	98	98	98	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100
24: F.hepaticaAB553720.1	98	98	97	97.5	97.5	98	98	98	98	98	98	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100
25: F.hepaticaAB553737.1	98	98	80	91.8	93.9	94	94	98	94	98	98	99	99	99	99	99.7	100	100	100	98	100	99.8	100	100	100	100	100
26: F.hepaticaAB973396.1	98	98	97	97.5	97.5	98	98	98	98	98	98	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100
27: F.hepaticaAB973395.1	98	98	97	97.5	97.5	98	98	98	98	98	98	99	99	99	99	99.7	100	100	100	100	100	100	100	100	100	100	100

DISCUSSION

Illustration of distinct cause of Fasciolosis in different geographic region has been the main wide reaching concern from veterinary medicine and public health perspectives [28]. The development of molecular techniques has presented further prospects for researchers to study definite epidemiology and precise investigation of Fasciolosis pervasiveness in distinct geographical zones [16, 29]. Preceding studies revealed that ITS1 and ITS2 genes can be used for molecular phylogeny and can be efficiently used for accurate delineation of Fasciolosis as well as explicating the origin and source of infection [29, 30]. In present study 300bp amplification of ITS2 gene subunit of *F. hepatica* was obtained in cattle and buffalo in Okara region, Punjab, Pakistan. Our results coincides with previous study which was done in Pothwar region in Pakistan in which 391bp and 329bp amplifications of ITS1 and ITS2 gene region were obtained and ITS2 sequences from cattle and buffaloes were compared with other Fasciola isolates belonging to different geographical regions of the world [9]. Even though genetic differences of *F. hepatica* mitochondrial and ribosomal regions have been revealed previously in various countries [17], inadequate information were known about genetic variations of Fasciola isolates and among previously documented cases from Asian countries in 25 years, most were from Pakistan, India, Iran, Korea Japan and Thailand [30]. PCR-RFLP identification of Fasciola was done based on 18s rRNA, ITS1, 28s rRNA, ITS2 gene subunits and enzymes were used for RFLP, 618bp amplification was obtained and no variation was found among Fasciola species, as well as ITS2 was amplified on 361-362bp sequences of Fasciola in France and in China [31]. Moreover, 5.8s regions of ITS1 and ITS2 was used for differentiation of Fasciola species in Iran and 1000bp amplification was obtained by using restriction enzymes and these ITS1 and ITS2 regions were used for sequencing and phylogenetic studies [5]. In our study using blast sequence analysis and maximum likelihood based phylogenetic differentiation of various Fasciola isolates in MEGA 6.0 program, found no variation among Fasciola isolates only few were have genetic variations and most of species were resembled. Our findings are in agreement with previous study in which Fasciola isolate from Pakistan in Pothwar region was closely resembled with other isolates on the bases of ITS2 gene sequencing [9]. The *F. hepatica* isolates in the world have no genetic similarity except for few such as Iran and Vietnamese which exhibited close similitude as hybrid forms existing in human [32]. The results illustrating the same amplification for cattle and buffalo 300bp, 28s, ITS2 gene region as cattle and buffalo has same base pairs and alike sequences based on primary analysis of ITS1 and ITS2 in different geographical regions because these regions are highly conserved in both species [31, 32]. The results in present study correspond the findings used ITS2 28s primers in sheep, goat, cattle and buffalo for recognition and discrimination of F. hepatica from different geographical regions [21, 33]. Phylogenetic analysis of various Fasciola isolates belonging to different geographical regions using sequences for 28s, ITS2 and ITS1 genes based on maximum likelihood and minimum evolution using MEGA 6.0 program showed relationship among Fasciola species and HyPhy package was used to nucleotide substitution model based on Akaiki Information Criterion (AIC) and Bayesian Information Criterion (BIC) using discrete gamma distribution [34]. In our study 24 substitution models were used, JC and K2 models were found best substitution models for selected ITS2 data set used 51 and 52 parameters having BIC and AIC values 1471.45 and 1477.68 with maximum likelihood values -503.05, -501.59 respectively. The relative values 0.5 and 3.006 and the relative nucleotide frequencies of A, T, C, G, A-T, A-C, A-G, T-A were 0.25, 0.25, 0.3, 0.25, 0.08, 0.08, 0.08 and 0.08 respectively. Evolutionary kinship of Fasciola based on ITS1 and ITS2 was determined by MEGA 6.0 program by using median joining and maximum likelihood estimates. Phylogenetic tree was constructed using heuristic searches, 500 replicates and nodal support was by bootstrap and by Bayesian analysis of ITS1 and ITS2 gene 28s regions based on Nst=rates and gamma distribution and coding positions were included position1,2,3 and non-coding [34].

CONCLUSIONS

The existing comprehension of Fasciola species diagnosis and taxonomy has stemmed copious observational studies. However, traditional techniques for diagnosis and delineation Fasciola spp. do not emulate precise discrimination in Pakistan. The development of advance approaches for accurate perception and genetic discrimination of Fasciola spp. facilitated region wise species identification and delineation. The outcomes of this exploration improved the approaches to find out genetic variability in assorted geographical regions. The current study scrutinized the molecular delineation of Fasciolosis in a specified region and more investigation is required to conclude rising focus of human Fasciolosis in Pakistan.

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