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Molecular identification of Paramphistomum epiclitum (Trematoda: Paramphistomidae) infecting buffaloes in an endemic area of Pakistan

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Original Article

Molecular identification of *Paramphistomum epiclitum* (Trematoda: Paramphistomidae) infecting buffaloes in an endemic area of Pakistan

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Abstract

This study determined the molecular characterization of *Paramphistomum epiclitum* using the second internal transcribed spacer (ITS-2) grouping and secondary structure analysis from buffaloes in Khyber Pakhtunkhwa province, Pakistan. Paramphistomes were collected from the rumen of 25 infected buffaloes and their DNA was separated. DNA was intensified, followed by sequencing. Phylogenetic examinations were carried out using the Maximum Composite Likelihood approach. The results revealed extensive intraspecific and interspecific varieties among different paramphistomid species. The sequences in the present study (GenBank accession number: MW481321 and MW481322) were compared to 46 reference sequences of *Paramphistomum* and other trematode data from GenBank. The results revealed the existence of two genotypes of *P. epiclitum* with six intraspecific single nucleotide polymorphisms, most closely associated with Indian isolates with an evolutionary divergence in the range of 0.00-0.015. The phylogenetic tree analysis of *Paramphistomum* spp. with other representative isolates in different locations clearly showed the existence of two *P. epiclitum* genotypes in Pakistan. The derived secondary structures for both genotypes presented structural similarities with the core four-helix domain structure. Likewise, the results showed the phylogenetic utility of the ITS-2 sequence – secondary structure information for inductions at higher ordered levels and the need for accurate species identification. The results of this study also have implications for the diagnosis and control of rumen flukes in the region.

Keywords: Buffaloes, Genetic characterization, ITS-2, Pakistan, *Paramphistomum epiclitum*, Phylogenetic analysis

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Introduction

The movement of cattle and buffaloes between neighboring countries in Asia is limited or has been completely reduced. This limited movement may result in isolated helminth populations with different genetic structures. Usually, morphological structures may not reveal the existing genetic diversity within and among these populations (Dhivya Bharathi *et al*., 2018). To minimize the limitations of commonly used morphological diagnostic tools, modern sophisticated molecular analysis like PCR is useful in the process of species characterization. Having high mutational rates, ribosomal genomes of parasitic worms have become useful tools to study their presence in different animals and their phylogenetic relationships (Shylla *et al*., 2011).

Paramphistomes include more than 70 species reported as parasites of ruminants throughout the world. Most of these species cause intestinal paramphistomosis and immature paramphistomes may cause severe effects. The disease is characterized by reduced feed conversion, lowered milk production and gastroenteritis. Paramphistomes infect ruminants including sheep, goats, buffaloes and cattle and cause huge economic loss worldwide every year (Anuracpreeda *et al*., 2008). Young animals are more severely affected by the parasite and acute gastroenteritis is the primary symptom of infection. During the chronic phase, parasitized animals show a reduced nutrition conversion rate as well as reduced milk and meat production (Bansal *et al*., 2015). The prevalence rate of paramphistomosis is highest in tropical and subtropical regions.

Morphological approaches are routinely used to discriminate adult trematodes, despite being laborious and inadequate for parasitic identification at species levels (Arbabi *et al*., 2012). Phenotyping of these worms includes stumpy pear-shaped body having anterior and posterior suckers. The tegumental spines in immature flukes are main pathogenic entities, which remain present over the body of amphistomosis (Jadav *et al*., 2018). The accurate identification of *P. epiclitum* is required to provide the baseline for the study of its biology, epidemiology and genetics. To identify paramphistomes, the ITS-2 region of rDNA is used as the best genetic marker (Lotfy *et al*., 2010, Chamuah *et al*., 2016). Sequence analyses of the rDNA genes encoding for structural RNAs of ribosome have been widely used in the phylogenetic studies and identification of the trematodes (Chamuah *et al*., 2016). The ITS-2 sequence of rDNA and its secondary structure analysis are used to determine phylogenetic linkages of novel species. These molecular tools are useful in revealing the interspecific variations and structural similarities regarding core four-helix structures (Ghatani *et al*., 2012).

A study characterizing *P. epiclitum* from infected buffaloes was conducted in the Punjab region, Pakistan (Ali *et al*., 2018). However, genetic diversity was not explored in detail. Recently, molecular identity of *P. epiclitum* on the basis of ITS-1 and the 5.8S region was recorded (Khan *et al*., 2020) and further confirmation with the ITS-2 region needs to be explored .Therefore, the present study aims to determine the genetic identity of *P. epiclitum* using the ITS-2 sequences and secondary structure data in order to ascertain whether paramphistomes from Pakistan are genotypically diverse or not.

Materials and Methods

Parasite Collection Sites: *Paramphistomum* flukes were collected from naturally infected buffaloes from the Peshawar district of Khyber Pakhtunkhwa (KPK) province Pakistan from October 2016 to February 2018. More than 500 flukes were collected from the rumen of 25 buffaloes (*Bubalus bubalis*) based on necropsy when being slaughtered. The flukes were first identified based on their morphological features with standard parasitological keys (Jones *et al*., 2005) and 25 flukes, one fluke from each buffalo, were selected for sequencing.

DNA extraction and amplification, sequencing and assemblage of the ITS-2 region: The flukes were preserved in 70% ethanol and DNA was extracted through the Phenol-Chloroform extraction and Ethanol Precipitation methods (Sambrook *et al*., 1989). Pellets containing DNA were dried for 10 minutes followed by resuspension in 100 µl sterile TE buffer (pH 8.0). After overnight incubation, suspensions were stored at -20°C. To amplify the ITS-2 region of *Paramphistomum* spp. rDNA, a pair of primers forward (5′ CCTTCTTCATCTGGGCAACT 3′) and reverse (5′GCTTAAGTTCAGCGGGTATTC 3′) was designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi bin/primer/primer3_www.cgi.). A PCR reaction of 25 µl was carried out containing 2 µl of DNA template, 5 U/µl of Taq DNA Polymerase, 2.5 mM of dNTPs, 10 pmol/µl of each primer, 10x Reaction buffer B and 25 mM of $MgCl_2$. The thermocycling conditions were 95°C for 2 minutes followed by 35 cycles of 95°C for 1 minute, 62.5°C for 1 minute, 72°C for 1 minute and a final extension temperature of 72°C for 5 minutes. PCR products were purified using the Wizrep purification mini Kit (Wizbiosolutions Inc.) and sequenced through the DNA Core Facility, Centre for Applied Molecular Biology (CAMB), Lahore, Pakistan. To assemble the ITS-2 region of *P. epiclitum*, Sequencher 5.4.6 software was used.

Sequence analysis and reconstruction of phylogenetic relationships: The ITS-2 region was compared using the online GenBank Blast program, which was given an input of *Paramphistomum* species ITS-2 sequences to align them with published ITS-2 sequences of other trematodes. Chromas version 2.6.5 software and online ClustalW multiple alignment tools were used to examine the sequences and to align them with already published ITS-2 sequences, respectively. The evolutionary history was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano. Initial tree(s) for the heuristic search was obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The bootstrap consensus

tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). A total of 46 species was used for comparison, to construct phylogenetic trees *Gastrodiscoides hominis* belonging to the same family were selected as out-groups. To estimate the evolutionary divergence between the sequences, analyses were conducted using the Kimura 2-parameter model.

Secondary Structure prediction: MFOLD software version 3.2 (energy free folding algorithm) was used to predict the secondary structure of the ITS-2 region (Zuker, 2003). To analyze it, 37°C was set as its folding temperature, while the query sequence was linearized. The most stable secondary structure with the lowest free energy was selected.

Dot-plot Analysis: To compare two sequences and find similar regions between them, dot-plot analysis was used. Query sequences were arranged on an x-axis and y-axis. A dot was placed on every point where both sequences were identical or similar. Here, Dot-plot was used to find conserved sub-sequences. For this purpose, a threshold of 8 out of 10 nucleotides to be similar for one dot was set. When repetitive elements existed within the sequence, shorter lateral diagonals appeared in addition to the central one.

Results

Sequence comparison: A summary of the results obtained by comparison of 71 sequences, including 25 from the current study and 46 from trematode sequences available at GenBank is given in the supplementary Tables. *Paramphistomum* flukes (n=25) collected from native buffaloes from KPK province were sequenced and ITS-2 sequences of 286 bp length were successfully amplified through PCR using their DNA. The analysis of these *Paramphistomum* fluke sequences determined that they were divided into two groups (genotypes), i.e. *P. epiclitum* A (MW481321) and *P. epiclitum* B (MW481322), differing from each other at specific base positions of 73, 223, 225, 258, 260 and 268. The two genotypes depicted six single nucleotide polymorphisms in the form of transitions as follows: T>C at position 73 and 268, C>T at 223 and 260 and G>A at 225 and 258.

The genotypes identified herein are different from those recently reported from the Punjab province of Pakistan. The *P. epiclitum* A genotype differs showing 4 transition mutations at the specific base positions of 65, 203, 235 and 250 i.e., C>T, T>C, G>A and C>T, respectively; the *P. epiclitum* B presented 10 transition mutations at the nucleotide positions of 65, 73, 203, 223, 225, 235, 250, 258, 260 and 268, respectively.

Alignment of ITS-2 sequences of genotypes A and B revealed that both were 97.2% identical.The BLAST hit result showed that ITS-2 sequences of *P. epiclitum* A genotype were: more similar with the sequences of various geographical isolates of *P. epiclitum* (100% identical to the published sequences of GenBank accession nos. KX657875, JX678254, JF834888, KX657874 and with a 99% homology with KX840344); and showed a 96% homolgy with Genbank accession nos. KU365321 (*P. cervi*), KP341658 (*P. leydeni*) and KY068130 (*E. explanatum*). The *P. epiclitum* B genotype presented a similarity of 98% and 96% with GenBank accesion nos. of *P. epiclitum* KX840345 and KY024581, respectively, and a 98% homology with KT198990 (*E. explanatum*) and a 97% with KP341658 (*P. cervi*) as well as KP341658 (*P. leydeni*).

The intraspecific variation between *P. epiclitum* A (MW481321) and isolates from several hosts as well as geographical regions of India showed variations at 11 different nucleotide positions, in which 6 transitions, 1 transversion and 4 deletions were observed. Interspecific variation between *P. epiclitum* A genotype and various isolates of *P. cervi* was found at 18 different base positions, in which there were 17 transitions and 1 transversion. In the case of *P. leydeni*, 16 variations, i.e. 14 transitions and 2 transversions, were observed. In *E. explanatum*, 25 variations, including 20 transitions and 5 transversions, were found. In the case of *C. microbothrium* (AB817063), 11 variations were seen: 9 transitions and 2 transversions. Likewise, *Orthocoelium streptocoelium* (KT777943) presented 21 variations: 12 transitions and 9 transversions. Moreover, *P. epiclitum* A genotype showed only one base position difference with F. elongates (MG183689), i.e. C>T at site 250, and variations at 11 positions with F. cobboldi (KU530202), including 10 transitions and 1 transversion.

Phylogenetic analysis: The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All positions containing gaps and missing data were eliminated. The final dataset contained a total of 282 positions. The Neighbor-Join and BioNJ algorithms using the Maximum Composite Likelihood (MCL) approach, showed a similar topology of the phylogenetic trees. The precise topology was shown by all bootstrapping replicates of the phylogenetic tree, as shown in Fig. 1 with values higher than 50%.

Bootstrapping through Neighbor Joining (NJ) provided strong evidence for the sub-clade containing *P. epiclitum.* The phylogenetic analysis suggested a monophyletic clade of two *P. epiclitum* genotypes from Pakistan clustered with *P. cervi, P. leydeni*, *E. explanatum, C. microbothrium, O. streptocoelium, F. elongates* and *F. cobboldi*, all belonging to the family Paramphistomidae. The *P. epiclitum* A and B genotypes form a sub-clade with *P. epiclitum* belonging to different Indian locations and being closely associated. However, trematodes belonging to families Paragonomidae, Fasciolidae and Plagiorchioidea diverged into separate clades.

The estimated evolutionary divergence between other paramphistomid species and the *P. epiclitum* genotypes herein identified was 0.000-0.066 (Table 1). The result showed that *P. epiclitum* A genotype is a closer relative of *P. epiclitum* from India (KX657875) than *P. epiclitum* B genotype of Pakistan with an evolutionary divergence of 0.015.

Predicted RNA secondary structures of five paramphistomid species were constructed for the ITS-2 region following a four-helix model with calculated minimum free energies of -119.40 kcal/mol, -116.80 kcal/mol, -119.40 kcal/mol, -117.70 kcal/mol and -

118.0 kcal/mol, respectively. Helices I and IV of this model were found to be relatively short, each comprising fewer than 30 nucleotides; helix III was the longest, consisting of about 135 nucleotides, and contained a UGGU motif 50 to the apex; a U–U mismatch was found in the second helix (Fig. 2). The well-determined structural domains are identified by visual inspection of an 'energy dot plot'. Dot plots of Figure3 show all the base pairs involved in optimal and suboptimal secondary structures within the energy increment for both identified genotypes of *P. epiclitum.*

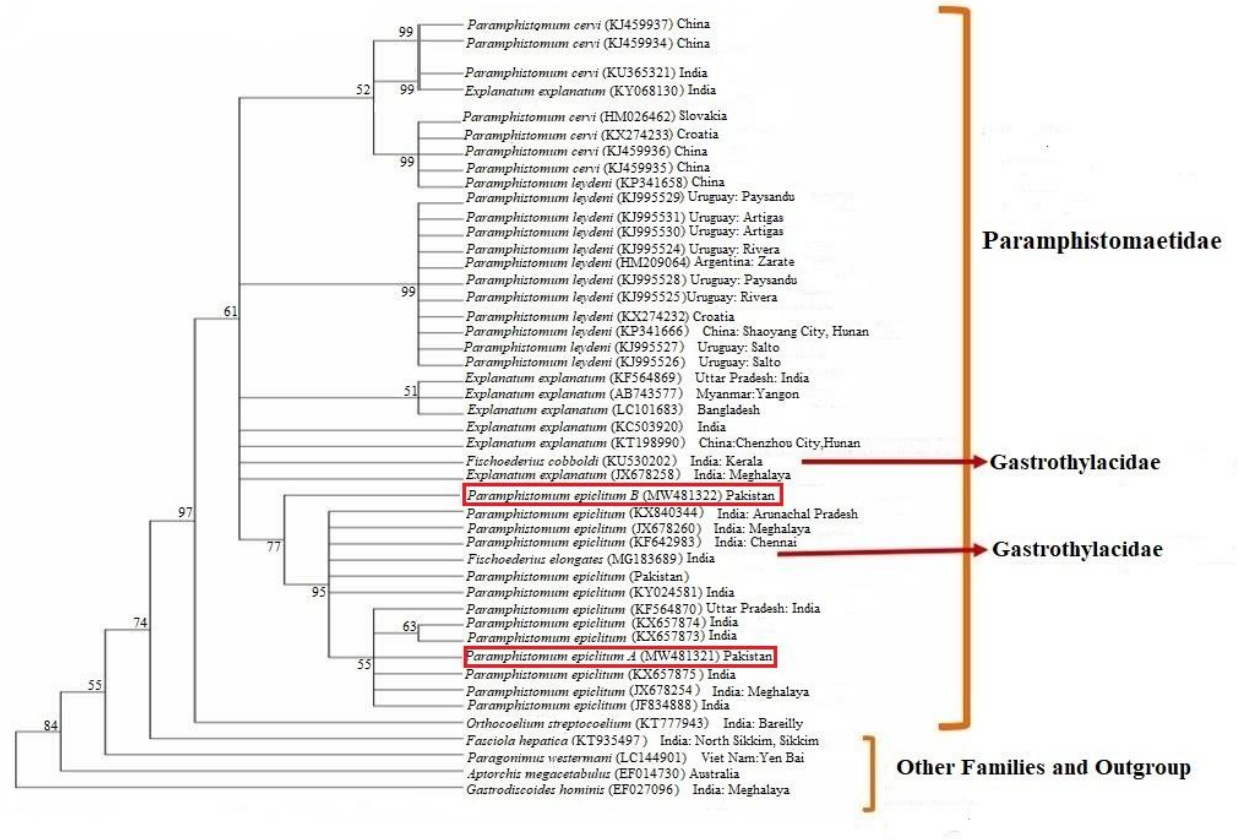


Figure 1 Molecular phylogenetic analysis of the ITS-2 sequence of trematodes by Maximum Likelihood method.

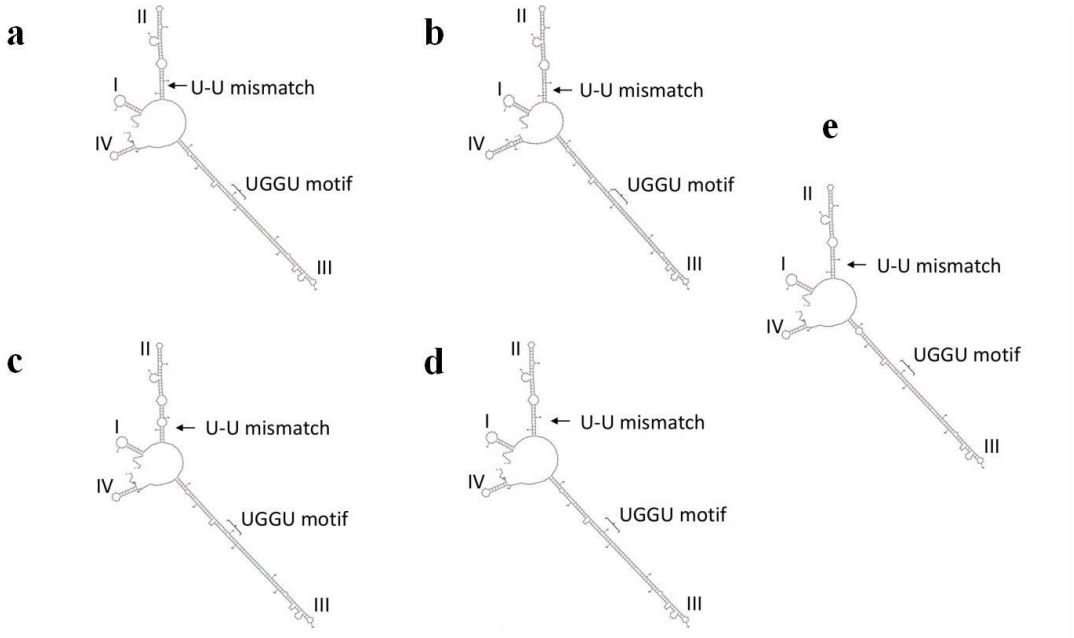
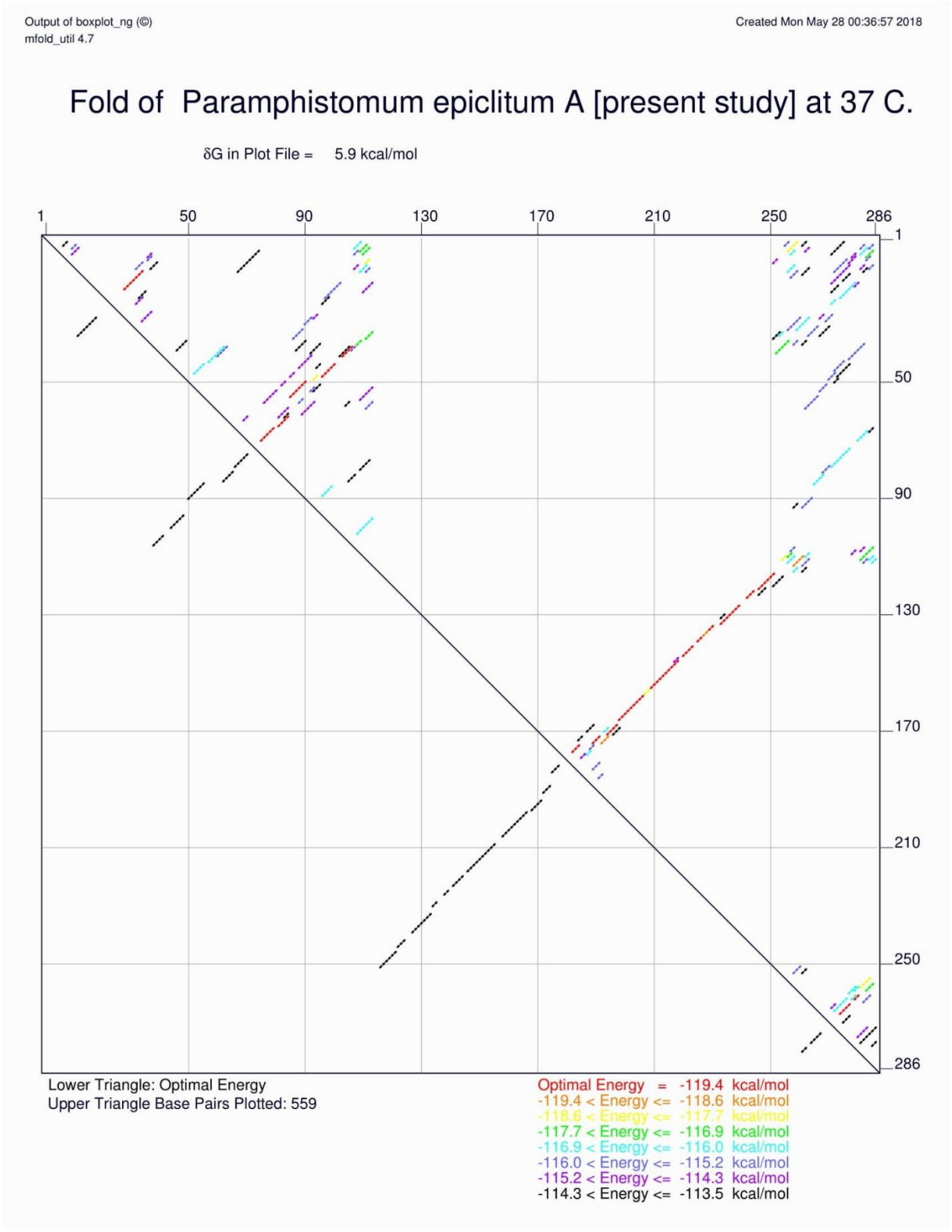


Figure 2 Predicted secondary structure of the ITS-2 rDNA region of *Paramphistomum* flukes. (a) *P. epiclitum* A (MW481321), (b) *P. epiclitum* B (MW481322), (c) *P. cervi* (KU365321), (d) *P. epiclitum* (KX657875), and (e) *P. leydeni* (KP341658).


Output of boxplot_ng (©)
mfold_util 4.7
Created Mon May 28 00:36:57 2018
Fold of Paramphistomum epiclitum A [present study] at 37 C.
δG in Plot File = 5.9 kcal/mol
1
50
90
130
170
210
250
286
Lower Triangle: Optimal Energy
Upper Triangle Base Pairs Plotted: 559
Optimal Energy = -119.4 kcal/mol
-119.4 < Energy <= -118.6 kcal/mol
-118.6 < Energy <= -117.7 kcal/mol
-117.7 < Energy <= -116.9 kcal/mol
-116.9 < Energy <= -116.0 kcal/mol
-116.0 < Energy <= -115.2 kcal/mol
-115.2 < Energy <= -114.3 kcal/mol
-114.3 < Energy <= -113.5 kcal/mol

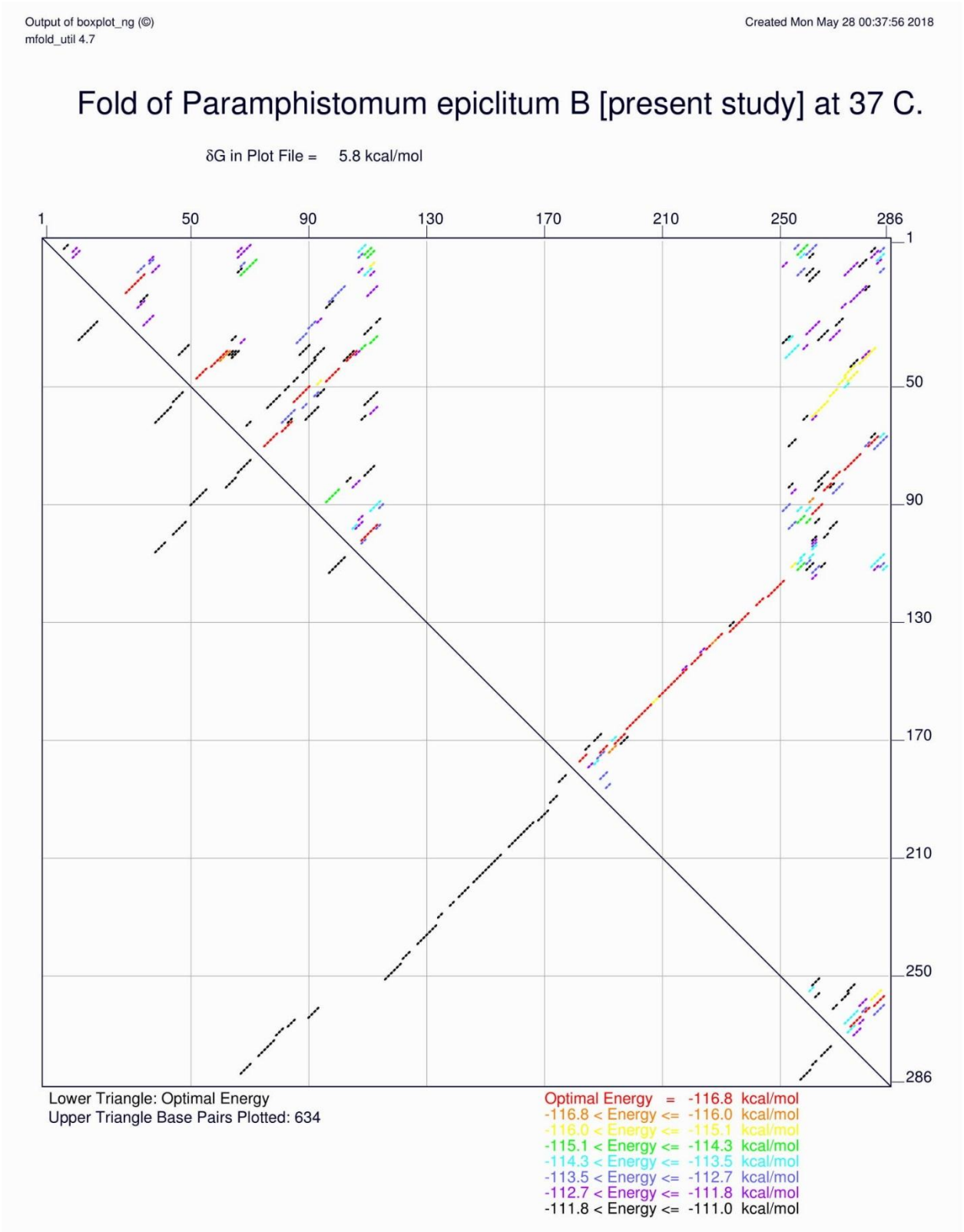


Figure 3 Dot plots show all base pairs in all optimal and suboptimal folding within the energy increment for both identified genotypes (a) *Paramphistomum epiclitum* A, and (b) *P. epiclitum* B. According to the Dot-plot analysis in the upper triangle, a dot in row *i* and column *j* represents a base pair between the i^{th} and j^{th} nucleotides. The dots represent the superposition of all possible folding within *p*% of ΔG_{mfe}, the minimum free energy, where *p* is the maximum % deviation from ΔG_{mfe}. To show different levels of sub-optimality, different colors are used. By default, two to eight is the range of colors. In *n* colors, the first color shows optimal folding. The lower left triangle shows the same base pairs but in reverse rows and columns. To show base pairs in suboptimal folding, *n*-1 colors are used. If $\Delta G_{i,j}$ is the minimum of the free energies of all possible structures containing a base pair *i.j*, and if $\Delta G_{mfe} + (k-2)\ p\Delta G/(n-1) < \Delta G_{i,j} \leq \Delta G_{mfe} + (k-1)\ p\Delta G/(n-1)$, then color *k* is used for base pair *i.j*, for $2 \leq k \leq n$. By default, when n is 8, red colors are given to optimal base pairs and black colors are used for base pairs that are least likely to form.

Table 1 Estimates of evolutionary divergence and percentage similarity between ITS-2 sequences of *Paramphistomum* species and other trematodes. The numbers of base substitutions per site between sequences are shown.

Species	Country	Accession nos.	Percentage Similarity		Evolutionary Divergence												
			P.epiclitum A (%)	*P. epiclitum* B (%)	1	2	3	4	5	6	7	8	9	10	11	12	13
P. epiclitum A	Pakistan	MW481321			-												
P. epiclitum B	Pakistan	MW481322			0.015												
C. microbothrium	Egypt	AB817063	95.8	96.8	0.038	0.034											
O. streptocoelium	India	KT777943	93.2	94.2	0.066	0.062	0.058										
F. elongates	India	MG183689	99.6	97.5	0.004	0.019	0.042	0.070									
F. cobboldi	India	KU530202	96.7	98.9	0.034	0.019	0.030	0.050	0.038								
A.megacetabulus	Australia	EF014730	88.1	88.0	0.443	0.466	0.456	0.493	0.436	0.465							
F. hepatica	India	KT935497	89.1	89.0	0.547	0.538	0.535	0.565	0.538	0.528	0.556						
P. leydeni	China	KP341658	95.8	96.8	0.038	0.034	0.023	0.062	0.042	0.030	0.442	0.520					
E. explanatum	India	KF564869	96.1	97.1	0.038	0.034	0.038	0.066	0.042	0.030	0.473	0.528	0.038				
P. westermani	Vietnam	LC144901	92.3	91.2	0.418	0.425	0.415	0.399	0.425	0.412	0.480	0.533	0.417	0.417			
P. cervi	India	KU365321	96.1	97.2	0.034	0.030	0.019	0.066	0.038	0.034	0.457	0.520	0.027	0.042	0.409		
P. epiclitum	India	KX657875	100	97.90	0.000	0.015	0.038	0.066	0.004	0.034	0.443	0.547	0.038	0.038	0.418	0.034	-

Discussion

Reports of paramphistomosis are on the increase throughout Pakistan. Recently, a study documented the existence of *P. epiclitum* from the Punjab province of Pakistan using a genetic approach (Ali *et al.,* 2018). The limitation of that study was that samples were collected from only two infected buffaloes, and *P. epiclitum* was reported to be the predominant species in Pakistan. However, prior to this study, there were no molecular investigations regarding *P. epiclitum* from Khyber Pakhtunkhwa province. Therefore, the objective of the present study was to characterize samples from different geographical locations of the province, using sequences of the second internal transcribed spacer of ribosomal DNA, as this sequence has been proven to be a reliable tool to identify digenean species and to shed light on their phylogenetic relationships (Van Van *et al*., 2009). The present results confirm the previous findings about the existence of *P. epiclitum* in Pakistan. The sequence analysis revealed that there are considerable intraspecific variations between the *P. epiclitum* genotypes already identified by Ali *et al.,* (2018).

The ITS-2 sequence analysis revealed considerable sequence divergence, with inter-specific variations ranging from 1.5 to 6.6% among *Paramphistomum* species, reinforcing the usefulness of the ITS-2 region for discriminating closely related species. It has been demonstrated that differences of as small as one nucleotide change in the ITS-2 DNA sequence alone can be used as effective genetic markers for low-level analyses to distinguish closely related species of digeneans (Nolan and Cribb, 2005).

The association between Pakistani *Paramphistomum* genotypes (*P. epiclitum* A and B) and species from other countries, including India, China, Australia etc., were revealed by the constructed tree, with the flukes studied showing a close association with geographically linked countries. As Pakistan and India are closest to each other of all the countries under study, *Paramphistomum* genotypes from the two countries present intermingled evolutionary relationships. This relationship is not surprising considering that the two countries are geographic neighbors sharing a similar geographic topology, which implies that their inhabitant species are not reproductively isolated (Worsham *et al.*, 2017). China is another geographically linked country to both Pakistan and India and its *Paramphistomum* genotypes are close relatives of sub-continental flukes as determined based on ITS-2 DNA sequences. In the case of Australian fluke species, as a different continent, are more distantly diverged from the Pakistani flukes under study.

The comparisons can also be made by means of determining secondary structure conservation of macromolecules in members of the group of interest, showing specificity at species and sub-species level. At least within the genus, the conserved regions are the basal pairings of helices I, II and IV (Coleman, 2003; Schultz *et al.*, 2005). The optimal RNA secondary structures inferred herein also agree with the four-domain model of the ITS-2 rRNA region for other digeneans (Morgan and Blair, 1998). This structurally identical nature of the ITS-2 region can be attributed to the functional similarity of rRNA biogenesis among eukaryotes, where the folding pattern of the ITS-2 region has been shown to play an important role in the correct processing of mature rRNA prior to translation of any mRNA (Joseph *et al.*, 1999).

ITS-2 DNA sequences are effective markers for phylogenetic studies at genus and species level only due to their high potential of variations and can therefore not be applied to higher level phylogenetic analyses. The phylogenetic analysis of present study genotypes forms a monophyletic clade with *P. epiclitum* species from India in a subclade. The comparative study of the ITS-2 RNA secondary structure showed a better resolution if the family Rhabditidae forms a monophyletic group well separated from Paramphistomidae, as indicated by high bootstrap values.

According to the data obtained in this study, using ITS-2 molecular markers, it can be concluded that genetic diversity exists within *P. epiclitum* isolates. Sharifiyazdi *et al.,* (2011) suggested that heterogeneity among different copies of ribosomal regions is present, which may enable cross-fertilization among digenetic trematodes (Vara-Del Río *et al*., 2007). Previous studies carried out on intraspecific differences among trematode isolates reported that host associations and geographic location are unlikely to be useful in classifying genotypes (Morgan and Blair, 1998; Gorjipoor *et al*., 2015).

In conclusion: the present study confirms the existence of two genotypes of *P. epiclitum* in buffaloes from Pakistan, being closely linked with their Indian isolates. The molecular identity of this helminthes have future implications for its diagnosis and control in Pakistan.

Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Acknowledgements

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