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Research Article

Genetic Characterization of *Gigantocotyle explanatum* from Buffaloes in Northwestern Pakistan

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Abstract

The family Paramphistomidae including *Gigantocotyle explanatum* regularly infects ruminants and causes immense economic losses to the livestock industry by decreasing dairy products and growth rates. The present study was aimed to determine the novel molecular data of *G. explanatum* in Pakistan using ribosomal DNA (ITS1-5.8S, ITS2) regions. Adult flukes, *G. explanatum*, were collected from bile ducts of infected buffaloes. The most relevant sequences from the other parts of the world were downloaded from the GenBank. High intraspecific variations were obtained at 5 end region of ITS1 gene. The 3 end of ITS1 was conserved and showed 96% similarity with *Paramphistomum cervi* (KJ459936). The nucleotide blast search of 5.8S gene revealed that 40 sequences from trematodes had 98% to 99% identity with present sequence and found genetically identical to *P. cervi* (KJ459938) and *Dicrocoelium chinensis* (KF734784) from China. The ITS2 gene of investigated isolates showed no variation with Myanmar (AB743577), while blast search revealed 96-100% similarity with isolates from Myanmar, India, Bangladesh and China. This study demonstrates the utility of ITS2 and 3´ end ITS1 sequences as a valuable tool for elucidating species phylogenetic relationship in south Asia. This sequencing data will facilitate more accurate identification of *G. explanatum,* enabling future work to resolve many ambiguities in the literature regarding this species.

Keywords: *Gigantocotyle explanatum; Water buffaloes, Pakistan, Genetic characterization*

Kuzeybatı Pakistan'daki Buffalo'lardan *Gigantocotyle explanatum*'un Genetik Karakterizasyonu

Öz

Gigantocotyle explanatum'un da dahil olduğu Paramphistomidae familyası ruminantları sistemli olarak enfekte ederek süt üretimi ve büyüme oranlarını azaltır ve hayvancılık endüstrisinde büyük ekonomik kayıplara neden olur. Bu çalışmada Pakistan'da *G. explanatum*'un yeni moleküler verilerinin ribozomal DNA (ITS1-5.8S, ITS2) bölgeleri kullanılarak belirlenmesi amaçlandı. Yetişkin *G. explanatum* kurtçukları, enfekte buffaloların safra kanallarından toplandı. Dünyanın diğer bölgelerindeki en yakın gen dizileri GenBank'tan indirildi. ITS1 geninin 5'- uç bölgesinde yüksek intraspesifik varyasyonlar elde edildi. ITS1'in 3'- ucu korundu ve *Paramphistomum cervi* (KJ459936) ile %96 oranında benzerlik belirlendi. 5.8S geninin nükleotit BLAST araştırması, trematodlardan elde edilen 40 sekansın, mevcut sekans ile %98 ila %99 özdeşliğe sahip olduğunu ve genetik olarak Çin'den *P. cervi* (KJ459938) ve *Dicrocoelium chinensis* (KF734784) ile benzer olduğunu gösterdi. İncelenen izolatların ITS2 geni, Myanmar (AB743577) ile hiçbir farklılık göstermezken, BLAST araştırması, Myanmar, Hindistan, Bangladeş ve Çin'den elde edilen izolatlarla %96-100 oranında benzerlik gösterdiğini ortaya koydu. Bu çalışma, ITS2 ve ITS1 3'- uç dizilerinin Güney Asya'daki türlerin filogenetik ilişkisinin aydınlatılmasında değerli bir araç olduğunu göstermektedir. Bu veri dizini, *G. explanatum*'un daha doğru bir şekilde tanımlanmasını kolaylaştıracak ve bu türlerle ilgili literatürdeki birçok belirsizliğin çözülmesinde yeni araştırmalara olanak sağlayacaktır.

Anahtar sözcükler: *Gigantocotyle explanatum; Su bufalosu, Pakistan, Genetik karakterizasyon*

INTRODUCTION

Gastrointestinal parasitic infections that are caused by platyhelminths (digenean trematodes) of the family Paramphistomidae (Fishoeder, 1901), have been identified under 19 genera comprising more than 70 species [1]. *G. explanatum* causes great economic losses in terms of reduced growth rate, decline in milk and meat production and high morbidity rate of infected animals. In Pakistan its overall occurrence is still in infancy and various districts of Punjab have 17.39% to 44.44% prevalence [2].

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The morphologically similar trematodes show marked differences in epidemiological factors including pathogenicity, infectivity and response to anthelmintics. Thus molecular studies based on nuclear ribosomal DNA regions have proven to be of great importance in accurate identification of digenean parasite at different systematic levels [3]. Intraspecific variation is limited in rDNA spacer regions of animals and especially in helminth [4]. The ITS1 spacer regions contain a highly variable 5´ region which has been helpful in lower taxonomic studies while on the other hand their 3´end are more conserved that helps in high-level classifications. The sequence diversity of ITS1-5.8S rDNA has been studied in *Clonochis sinensis* in Russia [5] and *Paramphistomum cervi* in China [6]. The rDNA ITS2 is a very useful marker for correct identification of species and has been used for many Paramphistomatidae including *G. explanatum* [7] Fasciolidae [8], and Dicrocoeliidae [9]. The ITS1 and ITS2 markers are highly conserved regions, and can be used to differentiate the closely related taxa that have diverged very recently i.e. <50 million years ago [10].

Although, considerable attention has been paid to adverse pathology and epidemiology of *G. explanatum* in Pakistan, relatively little attention has been paid to the systematics of the species. So far, a single report on molecular characterization of *G. explanatum* comes out from Punjab [11]. Thus, the aim of this study was to determine the genetic structure of *G. explanatum* in Pakistan by using the complete ribosomal DNA (ITS1-5.8S-ITS2) regions. Such valuable information can be used for truthful understanding of the molecular mechanism, successful species adaptation, evolution and the maintenance of the ability of the parasite to infect.

MATERIAL and METHODS

Fluke Collection sites and Genomic DNA Extraction

A study was designed to determine the presence of *G. explanatum* species in liver and bile duct of buffaloes at various abattoirs of Peshawar Khyber Pakhtunkhwa (34.95° N 72.33° E). At least 8 visits per month were made to local buffalo slaughter houses at Peshawar from February 2018 to the last week of October 2018. Adult flukes were collected from the bile duct of infected buffaloes by forceps soon after their slaughtering. Individual liver flukes were washed several times in phosphate buffer solution (PBS) and identified by superficial morphological features [10]. Flukes of the same population were preserved in 70% ethanol and stored at -80ºC. Genomic DNA was extracted from 17 worms using the consecutive three-day protocol of Phenol Chloroform method described by Barker (1998) [12].

PCR Amplification and Sequence Analysis of ITSI-5.8S-ITS2

ITSI-5.8S gene was successfully amplified by polymerase chain reaction with universal primers: BD1F (5´GTCGT AACAAGGTTTCCGTA-3´) and BD2R (5´TATGCTTAAATTCA GCGGGT-3´); while another set of universal primers F=GGT GGATCACTCGGCTCGTG, R=TTCCTCCGCTTAGTGATATGC were used for the amplification of ITS2 gene. The PCR product had a total volume of 25 µL containing 2.5 µL dNTPs, 2 µL $MgCl_2$, 0.3 µL Taq polymerase, 0.5 µL each forward and reverse primer, 2.5 µL PCR buffer, 14.7 µL PCR water and 2 µL DNA. Thermo-cycler condition was maintained at 95ºC for 45 sec followed by 35 cycles at 95ºC for 45 sec, 61ºC for 45 sec and 72ºC for 90 sec with a final extension process at 72ºC for 10 min. PCR products were purified using Wizrep purification mini Kit (Wizbio solutions), and both DNA strands were sequenced through Macrogen Sequencing services (Korea). The rDNA ITSI-5.8S-ITS2 sequence was assembled, aligned and edited to remove the attached primer and extra poor sequences on both ends using Chromas software. Sequences relatively close to this species from other geographical regions were downloaded from NCBI GenBank. The ribosomal DNA sequences were aligned using CLUSTALW.

Phylogenetic Analysis of the rDNA ITSI-5.8S-ITS2

Once species variations for the rDNA sequences had been identified, the under study haplotypes were imported into MEGA 7 [13] to calculate the suitable model of nucleotide substitution to construct their phylogeny. The most suitable node was formulated by rooting the branches to their closely related species. The phylogenetic tree was selected by the maximum likelihood method. Primary tree(s) for the heuristic search were attained automatically by selecting Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances calculated by the maximum composite likelihood (MCL) method, and then selected the topology with superior log likelihood value.

RESULTS

Successfully sequenced *Gigantocotyle explanatum* (n=17) rDNA genes were checked for intraspecific variations. The sequence was edited to length 1089 bp *(Fig.1)* with a total length of identified ITSI is 650 bp, 5.8S 157 bp and ITS2 282 bp.

The total length of identified ITSI is 650 bp, and at 3´ end 400 bp were completely identical to each other with no intraspecific variation. All the sequences of ITS1 region of *G. explanatum were* trimmed to 400bp and aligned with 9 closely related available sequences using BLAST. ITSI sequences of current parasite showed maximum similarity with *Paramphistomum cervi* of China and varied with other digenean. The present results showed 98% similarity with *Paramphistomum cervi* of China (KJ459936) with 8 nucleotide variations at positions 40 (T>A), 44 (A>T), 110 (A>G), 149 (G>A), 180 (T>C), 181 (G>T), 211 (C>T) and 382 (A>G) respectively. A consistent sequence variation was observed with the two species of *Gastrodiscoides hominis*

Fig 1. Identified sequence of ITS1-5.8S-ITSII region of *G. explanatum* from infected buffaloes of Khyber Pakhtunkhwa Pakistan

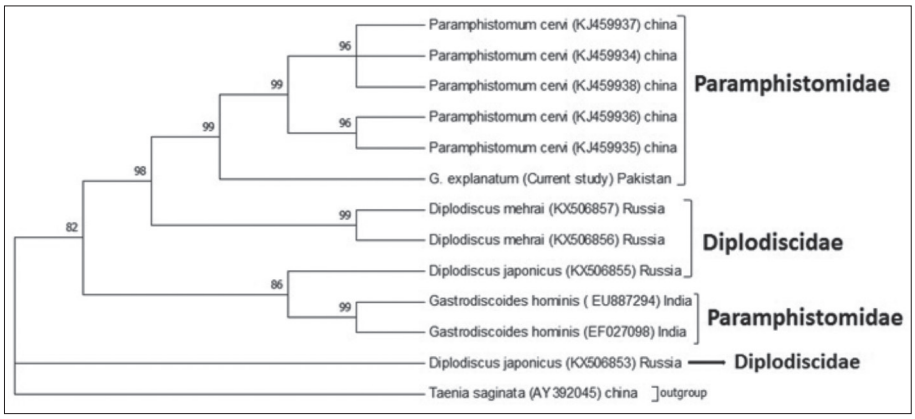


Fig 2. Molecular phylogenetic analysis of ITS-I gene of *G. explanatum* by maximum likelihood method

(EF027098), (EU887294) of 26bp at different positions from India but showed major differences with the same species of Columbia. The main types of mutations in the current study were A↔G and T↔C transition at 6 different positions and G↔A transition occured two times in the region with *Paramphistomum cervi* (KJ459934) of China. In addition, C↔T, A↔G, G↔A, T↔C, transition and A↔T, G↔T transversion mutation was found in various positions with *Watsonius watsoni* (KC763806) of the same country. Phylogenetic tree for ITSI revealed that the current species of *G. explanatum* formed a sub clade with *Paramphistomum cervi* (KJ459936) a rumen parasite of China with 99% similarity. Two species of *Gastrodiscoides hominis* (EU887294) from India also make a sub clade with 86% similarity index. Comparison with other paraphyletic clades as follows *Diplodiscus mehari* (KX506857) and *Diplodiscus japonicus* KX506855 of Russia with 82% bootstrap values *(Fig. 2)*. *Taenia saginata* (AY392045) of China considered as an out group.

Nucleotide blast search revealed that 40 sequences had 98% to 99% identity with the present sequence of 5.8S rDNA. The complete 5.8S region of the current species demonstrated no intra-specific variation and the Pakistani *G. explanatum* was genetically identical to the two species of *Paramphistomum cervi* (KJ459938) and *Dicrocoelium chinensis* (KF734784) from China. Interspecific single nucleotide polymorphisms (SNPs) have been identified between the sequences of genus *Centrocestus*, *Diplodiscus*, *Gynichthys, Acanthostomum, Chiorchis, Cryptocotyle, Siphomutabilus*. The *Apophallus* species from Hungary showed variation at positions 69,109,120 respectively. Nonetheless, genus *Homalometron* and *Thysanopharynx* sequence exhibit 3 fixed variable sites with present sequence. Russian *Clonorchis sinensis* showed variation at 4 positions with current species. Phylogenetic analysis for 5.8S in *Fig. 3* supports the blast results, by showing similarity with *Paramphistomum cervi* (KJ459938) of China with high bootstrap value (99%). The scrutiny involved 11 nucleotide sequences. All gaps and missing data were removed. The computed phylogram revealed 4 major clades. The *G. explanatum* formed a tight cluster with

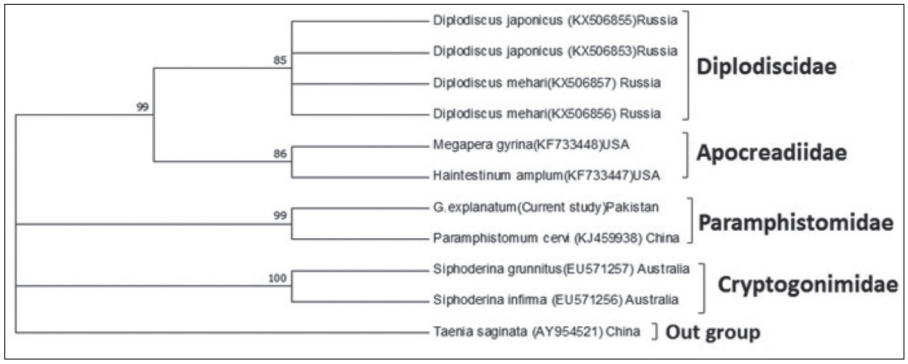
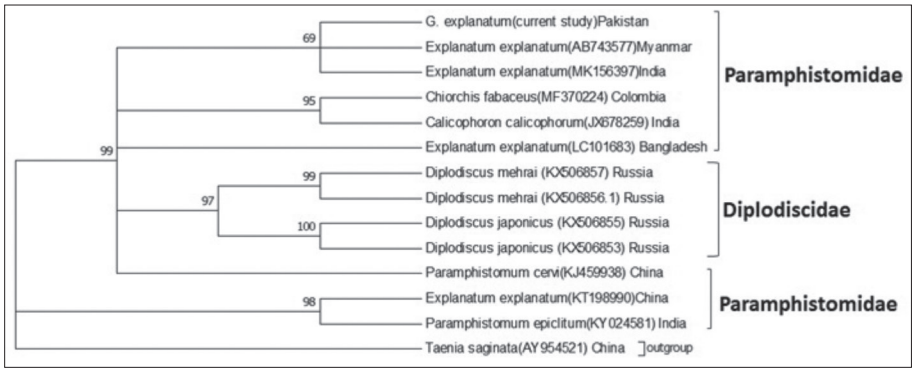


Fig 3. Molecular phylogenetic analysis of rDNA 5.8S gene of *G. explanatum* by maximum likelihood method

Fig 4. Molecular phylogeny based on rDNA ITS-2 sequence data of *G. explanatum*



family Paramphistomidae, Diplodiscidae, Apcreadiidae, and Cryptogonimidae, respectively. *Taenia saginata* (AY954521) from China showed a separate clade as out group.

The ITS2 sequences were checked for intraspecific variations and found identical. All the sequences were identified correctly and showed 96-100% similarity with reference sequences of *G. explanatum* from Myanmar, India, Bangladesh and China. The total sequence length was 282 bp and compared with the reference sequences for interspecific variations. The sequence was identical with Myanmar (AB743577) and showed difference of 1 bp (0.34%) at position of 253rd (C>A) with Bangladesh (LC101683), 1 bp (0.34%) difference with India (KF564869) at position 198 (C>T) and differ from China (KT198990) by 2 bp (0.69%) at 71st (T>C) and 218th (C>T) positions. However, the results differ from Indian isolates (KC503920, JX678250) by 7 bp (2.44%) and 13 bp (4.54%) respectively. The identified isolates within various taxa of Paramphistomidae showed variation from 10-16 bp. ITS2 tree indicated that identified isolates form a sub-clad with isolates of Paramphistomidae and Diplodiscidae, Gastrothylacidae families *(Fig. 4)*. However, the *Taenia saginata* (AY954521) diverged into separate clade as out group.

DISCUSSION

The current study sequenced the ITSI-5.8S-ITS2 of ribosomal DNA. The ITS2 sequences analysis confirmed the *G. explanatum* identity. The identification of this parasite has been previously confirmed in our neighboring countries i.e. Myanmar [8], Iran [14], India [15], Bangladesh and Nepal [16]. A study from Pakistan [11] identified the ITS2 region of *G. explanatum* as well from Rawalpindi, Punjab province.

The 5.8S showed 100% identity with number of trematodes species which indicates that this small region is highly conserved and not a reliable marker for species-level identification. However, ITS1 region of Paramphistomidae is usually characterized to infer the intraspecific variation among species [17]. Our results manifested high similarity index between *G. explanatum* of current study, and Chinese *Paramphistomum cervi* (KJ459936), and showed differences with each other at only 8 nucleotides. ITS1 regions of three opisthorchid liver fluke species *C. sinensis, O. viverrini, O. felineus* revealed high degree of interspecific sequence divergence and small amount of intraspecific variation in 42 individuals from eight different geographical localities that gives strong arguments that these species shared same ancestors and spread through the movement of infected host across different countries [18]. Zheng et al.[6] reported that no genetic difference is detected in 5.8S within species and only a small amount of intraspecific variation 0.04% is found in ITS-I region of *P. cervi* of China. This high level of similarity determined that these species are present in geographically linked countries and may share evolutionary history [19].

In addition, another digenean *Paragonimus westermani* showed valuable amount of intra individual differences as well as with other species of the same genus in amplified ITS1 region. The reason behind these variations is the varying number of repeat sequences in some species of digenean [20]. The homogenization of these repeat sequences of ribosomal

DNA is due to concerted evolution [21]. These repeat sequences are not operating uniformly in all genera of parasite species [20]. So this statement in turn supports the fact that no such type of successive repeats sequences was observed in our current ITS1region of *G. explanatum* from Pakistan. Different categories of ITS1 variation have been observed in various studies on trematodes DNA. This difference in degree of change in ribosomal DNA tandem array is probably due to the cluster segregation during replication that also enables to maintain homogeneity in their sequences [22].

The molecular analysis of ITS2 rDNA is very important for intraspecific and interspecific variation. The documented study showed that ITS-2 region of *G. explanatum* is highly conserved and hardly ever shows the intraspecific variation. But it differs interspecifically to other members of family i.e. from *Paramphistomum leydeni* at 7 nucleotide sites [8,11]. The current sequence ITS2 of 286 bp was genetically identical to *G. explanatum* of Myanmar (AB743577) and a difference of single nucleotide with Bangladesh (LC101683), and single nucleotide difference reported with Myanmar [11]. Interspecific variations were one to two nucleotides with India (KF564869) and China (KT198990) respectively. These minor differences of nucleotides may indicate that the haplotypes of these countries are closely related. The literature data shows that the interspecific variations of ITS2 of trematode are in range of 0.3 to 21%, which is very wide [9]. In contrary, it is believed that generally the ITS2 region shows very slow rate of evolution and in some cases demonstrate a complete absence of intraspecific and interspecific nucleotide variations [9,23]. But still, the ITS1 and ITS2 regions are the most frequently used markers for studying of population genetic and applications of evolutionary biology in digeneans parasites [24]. So the ITS2 is considered a highly significant genetic marker for the study of intraspecific as well as interspecific variation of species. Because the differences of even one nucleotide change can be used as an effective genetic marker for distinguishing the closely related species of digeneans [25].

The Phylogenetic tree is very useful in providing the hypothesis about the clade of various species and link between different species [26]. The Phylogenetic tree of our study showed that *G. explanatum* of our region forms clad with India, Myanmar, Bangladesh and China, confirming their closeness. Similarly, study reported that the haplotypes of *G. explanatum* found in India showed same clade with isolates from Bangladesh and Nepal [27]. Our systematic analysis of the ITSI and 5.8S revealed the closeness of current species of *G. explanatum* with *Paramphistomum cervi* (KJ459936) of China. Genetic pair wise distance between current species of *G. explanatum* also confirmed their close affiliation with *P. cervi*. These results may explain that as these countries are geographically similar with mostly identical culture, and have movement of the ruminant among these countries and might be shared through migration of animals [28].

Although the goal of the present work is to be stressed that morphological discrimination does not provide adequate information about amphistomes identity, however the molecular data is needed to justify their accurate taxonomic structure. In conclusion the appropriate identification of flukes will help to minimize anthelmintic resistance. The novel sequence data will help in formulation of early diagnostic tools, effective drugs and specific vaccinations against these amphistomes. Furthermore, detail genetic studies on mitochondrial (cytochrome c oxidase subunit 1) regions of *G. explanatum* are required to resolve many ambiguities in the literature regarding this species.

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