Genetic Characterization of *Gigantocotyle explanatum* Based on First Internal Transcribed Spacer (ITS-I) of Ribosomal DNA from Buffaloes of Peshawar, Pakistan.

By

Sana Ullah

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad, Pakistan

2019

Genetic Characterization of *Gigantocotyle explanatum* Based on First Internal Transcribed Spacer (ITS-I) of Ribosomal DNA from Buffaloes of Peshawar, Pakistan.

A dissertation submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

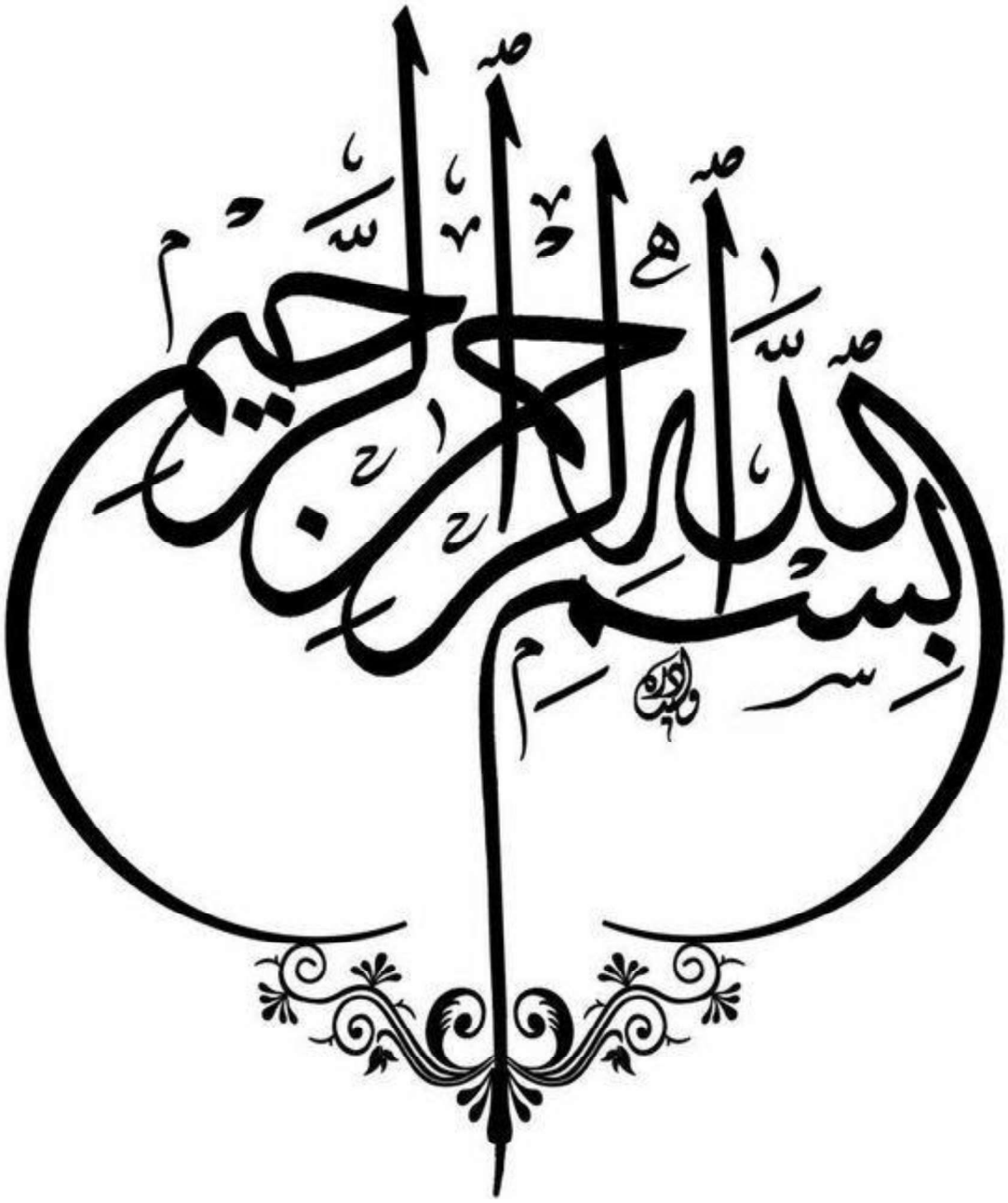
In Parasitology

By
Sana Ullah

Department of Animal sciences
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, Pakistan

2019

“In the name of ALLAH, the most Beneficent, the most Merciful”



DECLARATION

I hereby declare that the material contained in this thesis "Genetic Characterization of *Gigantocotyle explanatum* Based on First Internal Transcribed Spacer (ITS-I) of Ribosomal DNA from Buffaloes of Peshawar, Pakistan" is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Sana Ullah

February 2019

Dedicated

To My

beloved Irfan

ullah , Anwar ul

Haq , and other

family members

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviations	Full form
mm	Milli-meter
N	North pole
E	Equator
µl	Micro litter
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TE buffer	Tris EDTA buffer
KP	Khyber Pakhtunkhwa
PCR	Polymerase Chain Reaction
GEU	Gigantocotyle explanatum Universal
F Primer	Forward Primer
R Primer	Reverse Primer
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide Triphosphate
$MgCl_2$	Magnesium chloride
kb	Kilobit
TBE buffer	Tris/Borate/EDTA buffer
UV	Ultra Violet
USA	United States of America
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetics Analysis
FASTA	FAST-ALL

NCBI	National Center for Biotechnology Information
Ver	Version
bp	Base Pair
A	Adenine
G	Guanine
C	Cytosine
T	Thymine
U	Uracil
ΔG	Change of energy
kcal/mol	kilocalorie mole

ACKNOWLEDGEMENTS

All the praises for Allah, the lord of the heavens and the earth. The lord and cherisher of all the worlds. To Him be glory throughout the heavens and the earth, and He is exalted in power, full of wisdom. All the praises for Almighty Allah, who created us Muslim and gave us the power of knowledge to differentiate the right and wrong. Many thanks to Him as He blessed us with the Holy Prophet Hazrat Muhammad (Peace be upon him) for whom the whole universe is created. Many thanks to Prophet Muhammad (Peace be upon him) who enabled us to worship only one Allah and brought us out of darkness and enlightened the way of Heaven. I would like to thank my supervisor **Dr. Kiran Afshan** Assistant Professor Department Animal Science, Quaid-i-Azam University Islamabad for her affection, continuous support, guidance, and encouragement throughout my research work. She not only helped me in achieving my research goals but also taught me scientific writing, independent thinking and strong research ethics. I once again thank her for always being so nice with me.

I wish to express my sincere thanks to **Chairman, Dr. Sajid Malik Associate Professor** Department of Animal Sciences, Quaid-i-Azam University Islamabad for providing necessary research facilities. I would further like to thank to another faculty member, **Dr. Sabika Firasat** Assistant Professor Department of Animal Science, Quaid-i-Azam University Islamabad for being a source of inspiration and enlightenment for me.

I would like to acknowledge my family for all their moral support and undying encouragement; specially my mother for his continuous prayer and affections, my brothers and beloved sister for their love, support, encouragement, and hope. Most importantly, I would like to thank my beloved brother, **Mr.Irfan Ullah** for his love, help and support at every stage in my career. Whatever I am today is because of him.

And finally, I would like to acknowledge all my fellow lab-mates and colleagues **Raza Ullah, Muhammad Asim Khan, Arshad, Saqib Khan, Raeesa Tehreem, Samreen Akhtar, Dil Nawaz, Imad, Maria Komal, Dur-e-Shawar, Noushi, Humair Hayat, Sabah, Atif and Waheed Ahmad** for their cooperation.

Sana Ullah

ABSTRACT

Background: Paramphistomiasis is a gastrointestinal platyhelminth infection that presents high risk for livestock sectors in terms of morbidity, reduced milk production and meat. Therefore, characterization of these parasites on molecular level is preliminary to understand their successful propagation and relationship with host, in the form of resistance. This approach has proven a powerful tool to find out the taxonomic level of specie responsible for disease that may provide a foundation for the development of vaccines and drugs. ITS-I rDNA spacer region are used for characterization of digenean parasite at different systematic levels and find out the genetic differences within species and with other trematodes from different geographical regions.

Objective: The specific objective of the present study was to genetically characterize the *Gigantocotyle explanatum* liver fluke based on rDNA (ITS1) from Peshawar, Pakistan**.**

Methodology: Adult flukes of *G. explanatum* were collected from the bile ducts of infected buffaloes and were preserved in 70 percent ethanol. Genomic DNA from all specimens was successfully extracted through chloroform-phenol method and Polymerase Chain Reaction was done to amplify the required region. After confirmation of amplified product by Gel electrophoresis, it was sequenced. Similarity index was checked with other reference sequences by using BLAST and CLUSTALW. The phylogenetic analysis and genetic distance were computed in MEGA 7 by Maximum Likelihood Method.

Results: The total amplified sequenced length was of 1286 bp, and for intraspecific and interspecific genetic variation of 400 bp were analyzed**.** Current species of *G. explanatum* from Pakistan varied of 12 bp and formed a sub-clade with *Paramphistomum cervi* (KJ459934), a rumen parasite of China with 92% similarity, and *Watsonius watsoni* (KC763806) also shared 72 % similarity. Two species of *Gastrodiscoides hominis* (EU887294) from India also make a sub-clade with 78% similarity index. Comparison with other paraphyletic clades as follow *Diplodiscus mehrai* (KX506857) and *Diplodiscus japonicus* (KX506855) from Russia with 78% bootstrap values. *Chiorchis* (MF370224) of

Columbia make distinct position with 78% identity along with *G. explanatum* species. Genetic pair distance of current species was calculated 0.0000 to 0.03093 with the species of Paramphistomidae and 1.21492 with Cladorchiidae.

Conclusion: In summary our current results showed no intraspecific variation in the ITS1 region of *G. explanatum* from Peshawar, Pakistan and varied 12 bp from *Paramphistomum cervi* (KJ459934) rumen parasite of China. Phylogenetic analysis reveals high rate of variation with other species found in western countries like *Gastrodiscoides hominis* (MF370224) of Columbia. This characterization will be helpful to formulate a clear difference among digenean trematodes at their molecular level. The current molecular data will set bases and contribute in further studies on different paramphistomes, particularly for *G. explanatum* .

INTRODUCTION

Globally, livestock sector plays most important role for a great number of poor rural household communities because people utilize them not only as a source of income but also as a vital source of food, dairy product and fuel. According to a recent survey fast progress in the livestock production is remarkable. It is estimated, that by 2020, this sector will produce more than 50% of the agricultural output (Ahuja & Redmond, 2004). In developing countries sustainable use and growth is very difficult to achieve under current economic and conservation policies, because not much interest is shown on enhancing agricultural resources and food security (Weeks, 1999).

It has been estimated that global agricultural production will be essential to increase the production of food for an extra three billion people in next 50 years (Herrero & Thrnton, 2013). In Pakistan, agriculture is the second largest sector and has great contribution in economic development. Our 21% GDP is dependent on agriculture and contribute to over 45% of the total labor force (Pakistan Economic Survey, 2010). Almost 62 percent of total rural population is directly or indirectly connected with this sector for their survival and livelihood. Only livestock contribute about 11.4 % of total national GDP. There are 29.6 million cattle, 27.3 million buffaloes, 26.5 million sheep, 53.8 million goats and 0.9 million camels in the national herd (Shabbir, 2011). Human population is increasing day by day so it is mandatory to have sustainable agricultural and livestock production to achieve the needs according to global development. Brown (1981) has pointed out the pressure on world resources with ratio of human population growth and global development. These major issues should be immediately solved according to economic development approaches (Shabir, 2011). It is predicted that the animal production will have to increase to fulfill the requirements of sharply increasing global population. To overcome this challenge production efficiency is needed to be increased (Thornton, 2010). In the ice-free terrestrial area of the planet 30% is reserved for livestock (Steinfeld *et al*., 2006).

According to the Animal Health Yearbook of Food and Agricultural Organization of the United Nations (1984) the economy of livestock sector and food production is drastically decreasing because of diseases. In developing countries this information is not acknowledged and is given less importance. In this regard a mixed infection including various species (trematodes, nematodes and protozoans) are becoming the rule in all countries and mostly make it very difficult to assign their losses. Remarkable approach has been made in the previous few years in normalizing mortalities from parasitic infections in less developed countries (Dargie, 1987).

Significant measures should be taken to meet these challenges by fully upgraded animal health management, that has vital role in livestock sustainable efficiency. In this spectrum, parasitic helminth diseases of large and small ruminants are a major hurdle for effective livestock output, globally. Different helminth infections create negative impact on majority of grazing ruminants through less food intake, alteration in their growth rate, body weight, body composition, wool growth, fertility and milk yield (Fitzpatrick, 2013). The influence of helminth on bovine animal efficiency is well understood but agricultural output is also related to many other factors like diseases, management, local boundaries and limitations (Wilson, 2011). Most of the animal infections are strongly linked with weather and ecological parameters such as the developmental stages and mortality rates of the free-living phases of helminths and the population behavior of the intermediate host snails are drastically affected by environment and this favors the seasonal pattern of infection in moderate areas (Van Dijk *et al.*, 2010).

Globally buffaloes are divided into two major groups, the Asian buffaloes (*Bubalus bubalis*) and African buffaloes. The Asian buffalo which is the other name of domesticated wild Indian buffalo is also known as *Bubalus ami*. *B. bubalis* is broadly spread in most of Asia and has been introduced to China, South America, Europe, near east, former Soviet Union and the Caribbean (Annon, 2003). Research based on the importance of parasitic helminth infection on economy reveals the impact on the key performance indicators like weight gain, milk production and

reproductive ability of the animals and the losses are transformed into an economic output (Charlier *et al*, 2014).

1.1 Paramphistomiasis

The gastro-intestinal parasitic infections caused by platyhelminth (digenean trematodes) of the family Paramphistomidae are called as Paramphistomosis or Paramphistomiasis. These worms complete their life cycle stages with two hosts, fresh water snail act as an intermediate host while the common domestic bovine serves as a final or definitive host. More than seventy species of amphistomes have been identified so far, among them immature juvenile cause very severe mortality in large ruminants (Mazahery & Razmyar, 1994; Sey, 1991).With the help of acetabulum, *Explanatum* amphistomes attach themselves to the epithelium and make "granulomatous nodules" intruded by different inflammatory cells (Haque *et al*., 2011).

Initially the disease was believed to be the infection of tropical and subtropical areas (Taylor *et al.*, 2007). But current studies have revealed their occurrence in temperate areas, as well (Nikander & Saari, 2007). They are parasites of rumen and reticulum of sheep, cattle, goats and water buffaloes. In early stage of life, they stay in small intestine and then start to migrate towards rumen by abomasum (Sanabria & Romero, 2008). In tropical countries, this disease is very common in water buffaloes. The distribution of the infection in some geographical localities is about 80 to 90% and their intensity in individual animals reach tens of thousands of worms (Ambu, 1989). The prominent features of family Paramphistomidae are absence of interior oral sucker and the position of acetabulum on posterior region of the body in adult flukes and cercariae as well (Jones, 2005). The distinguish members are *Calicophoron calicophorum, Paramphistomum cervi, Gigantocotyle symmeri* and *Explanatum explanatum.*

1.2 General structure

Amphistomes are pear like flukes, having circular body in transverse section and belong to the family Paramphistomatidae and order Digenea. They are categorized by two special suckers, out of which one is for attachment and the other act as mouth. In the start of the diverged caecum (alimentary canal) oral sucker is located with muscular pharynx while at the posterior region, acetabulum is present, that is a well-developed structure.

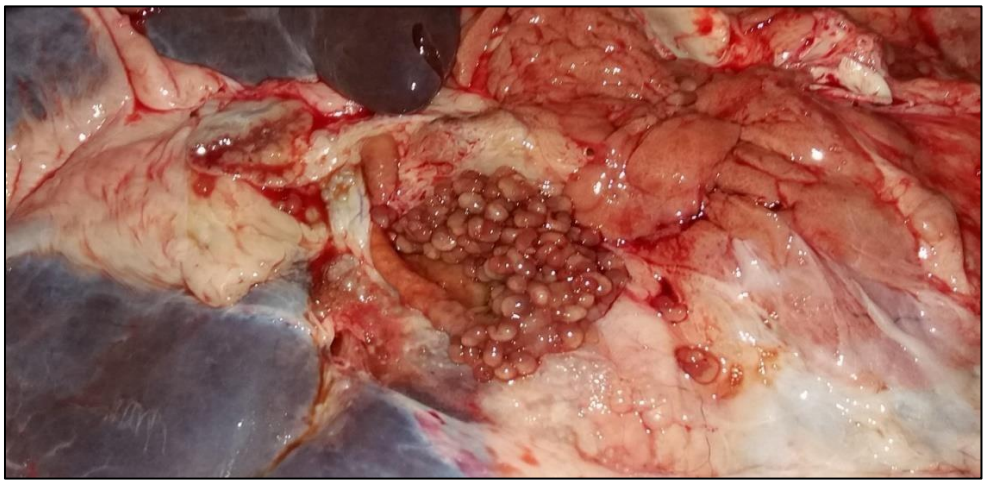


Figure: 1.1 G. *explanatum* in the liver of buffalo

G. explanatum flukes appear brownish and have slightly curved bodies when observed in fresh specimen. In the posterior most region dorsal and ventral edges are abruptly curved but on other hand it is evenly rounded posteriorly. Testes are smooth, rounded, diagonal and little pointed. One of the peculiar sphincters is present at the anterior region that links it with central layer of circular muscles. Both male and female reproductive organs are present in the same individual (Hermaphrodite), on the ventral side of the body genital pore is present and is used for common opening for both sexes. The internal body organs of the flukes are surrounded by parenchyma cells.

Few normal size acetabula are present along with pharynx without diverticula. Usually caeca are laterally located with sinuous. *Explanatum* body is pointed on ventral side and size is about 10mm in length having large esophagus without posterior sphincter. The seminal vesicle of the fluke is thin walled; less developed curve pars musculosa and medium pars prostatica are also present. Genitalium of *Explanatum* opens to median sagittal that opens at the side of pharynx (Mazahery & Razmyar, 1994).

1.3 Classification

Different authors classified *Explanatum* in various ways according to their morphological structures. Fukui (1929) considered it as a member of sub-genus Paramphistomum. Based on indirectly attached testes and absence of their last genital sucker, it was classified in a different ways. Later Nfismark (1937) isolated the genus Gigantocotyle as species which were previously described as a Paramphistomum with a unique acetabulum and assigned it into two species P. (Explanatum). In Northern Rhodesia now, Zambia Yeh reported *G. lerouxi* as a new species (Eduardo, 1983).

Kingdom: Animalia

Phylum: Platyhelminthes

Super Class: Trematoda

Class: Digenea

Order: Paramphistomoidea

Family: Paramphistomidae

Genus: *Gigantocotyle*

Species: *G. explanatum*

1.4 Life cycle of *G. explanatum*

Biology of *G. explanatum* is same as for *Fasciola hepatica* (Dunn *et al.*, 1985). *Bulinus* genera containing snails, Indoplerbus,and and Lymnaea act as an intermediate host. Under favorable condition metacercaria when ingested goes to the abomasum and intestine where excystment occurs and the juvenile stage attach themselves with the intestinal mucosa that creates serious problems in the host body. When the amphistomes attain definite size, they tend to move to bile duct along with rumen and finally produce amphistomiasis (Cheema *et al.,* 1997).

Explanatum eggs are then released in the host digestive tract and expelled from the body with faeces of host. After 17 days at 28^{o} C the miracidia are hatched and enter into the body of snail (Lengy, 1960). After penetration of miracidia in the snail shell, it develops ciliated layer and formation of sporocysts occurs. Miracidia develops into rediae about one to two weeks having developed ceca and pharynx with high phagocytic ability (Horak.,1971). In addition, a young rediae formation also occurs that may become responsible for the generation of juvenile rediae that will produce cercariae. The time span of cercariae emergence is approximately 10 days, inside the snail. Cercariae develops two unique pigmented eye spots and this stage is unique to cross the excretory vessels (Durie, 1956).

When cercaria leaves the snail body and gain reach in to water, they attach themselves with suitable vegetation where it begins the formation of cyst producing metacercaria, the infective stage for definite host when ingested. This whole time period is about 20 minutes (Horak, 1971).

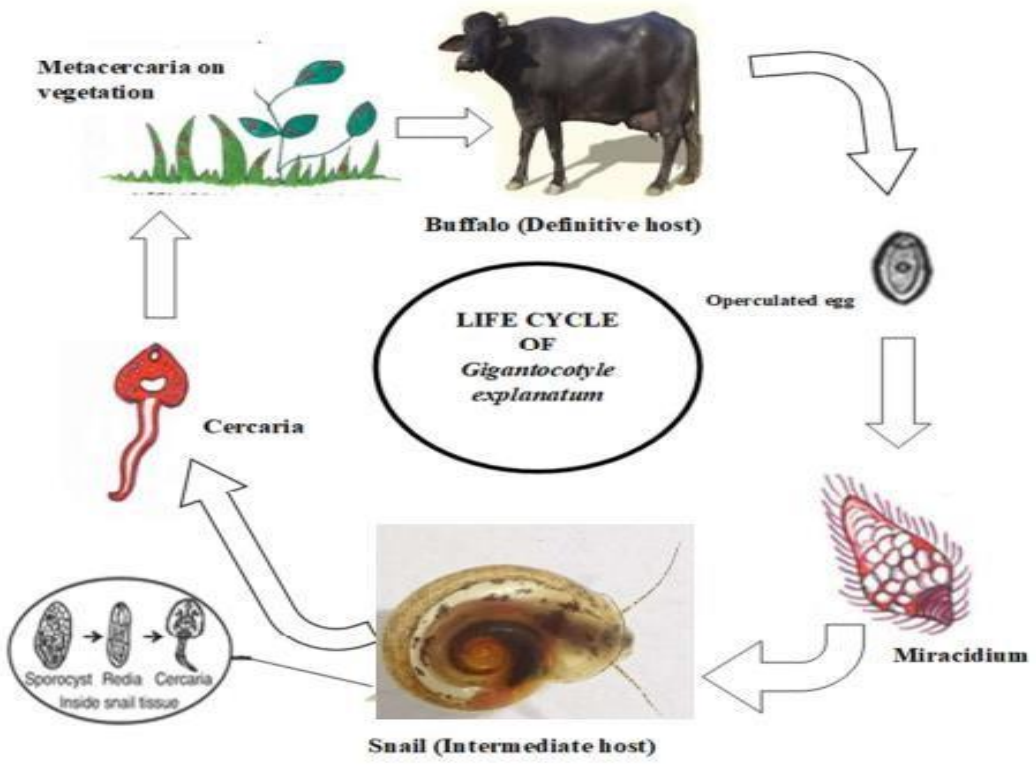


Figure 1.2: Life cycle of *G. explanatum*

The infective metacercariae survive under favorable condition for almost 29 days (Horak, 1962). The wall of the cyst dissolves in the medium of host intestine and the fresh young trematode becomes free that may migrate towards liver and finally enter the bile duct (Haque *et al.*, 2011).

1.5 Global Prevalence

Different studies provide estimate that more than 500 million ruminant animals throughout the world are subjected to risk because of different parasitic infections. Mortality rate due to juvenile paramphistomes is increasing rapidly and may reach up to 90% in farming livestock (Juyal & Hassan, 2003). The distribution of helminth diseases across the world gives knowledge about the successful evolving strategies and technical measures required to control these infections (Baghel, 2016)

Paramphistomes are distributed throughout the world but their prevalance is very high in tropicropical and subtropical areas specialy in Asian and African countries and also in Russia. *E. explanatum, P. cervi, Gastrothylax crumenifer, Cotylophoron cotylophoron, Fischoederius elongtsus* and *Gastrothylax cobboldia* are found in India, Ceylon and China (Boray at al., 1959). The pattern of distribution of *G. explanatum* in various countries may be because of different environmental conditions for Molluscus intermediate host (Sey & Eslami, 1982). In Vietnam the prevalence rate of *G. explanatum* was reported as 12.5% (Nguyen *et al*.,1997). In Barisal total amphistomiasis was recorded in 31.5 percent of buffaloes (Ahmedullah *et al*,. 2007). Distribution rate in Asia is still 3060 percent in some special regions (Raza *et al*., 2009).

1.6 Prevalence in Pakistan

Overall distribution of helminth disease in ruminants has been estimated as 25.1 to 92 percent in various regions of Pakistan (Iqbal et al., 1993). Total occurrence of Paramphistomum diseases was reported as 8.75 percent in Swat and Charsadda from the start of November 2011 to April 2012. As regard month wise prevalence was high 2.5 % in the month of march. Sex wise prevalence showed 10 times high infection rate in males then in females (Gul-Enayab, *et al.*, 2017). In district Jatoi, *G. explanatum* prevalence was reported as 7.82 % while mixed infection was near 9.34% (Gul-e-Nayab *et al*., 2017). Overall prevalence of *G. explanatum* in Pakistan is still in infancy. In buffaloes the prevalence of amphistomes is relatively high 75.07% as compared to cattle, which is 50.7% (Cheema *et al*., 1997).

Environmental factors also alter the distribution of paramphistomiasis in Pakistan. Different studies revealed that temperature has a major impact in disease propagation as it affects the parasite and snail metabolic process. It can affect the reproductive stages of parasite inside intermediate host, resulting in limited chances of survival and propagation of parasitic infection. The highest rate of prevalence is reported in the month of August that is about 52% in buffaloes and 43% in cattle

respectively. According to a study lowest prevalence was observed in the month of February, as March and December (16% each) in cattle least, prevalence (1.5%) was reported in the month of February (Khan & Maqbool, 2012).

1.7 Pathology and Clinical Aspects

The major clinical features of paramphistomiasis are mainly eating disorders, diarrhoea, water loss from the body, polydipsia and pale color mucous membrane. Pathology depends on the intensity of infection as early infection do not be able to create main problem but acute phase necrosis, erosion, and haemorrhages in the mucosa of infected intestine. These puncture regions create intestinal pain, reduced hunger desire and even leakage of blood plasma albumin from the gut that ultimately cause reduction of albumin.

The most prominent pathological aspects of this disease are protein reduction from the area of rumen and gut. This low level of plasma protein concentration is responsible for the formation of small watery fluid (oedema), accumulation of ascitic acid (Ascites), lungs oedema and hydropericardium (Chauhan, 2014). Paramphistomiasis in buffaloes alters blood volume in such a way that RBCs, hemoglobin, and total cell volume is significantly reduced when compared with healthy individual that indicates anemia.

1.8 Current investigation

Use of DNA based studies for formulation of phylogeny and specific molecular characterization in population of digenean trematodes has become a recent approach throughout the world, because species morphology alone has not been useful enough in precise differentiation between species. By availing recent molecular tools i.e. PCR and sequencing it has become quite accurate and reliable to solve the

different classification issues ,using the ribosomal DNA in general (Blair *et al*., 1996; Hust *et al*., 2004).

The use of ITS1 rDNA spacer region has proven to be of great importance in genetic characterization of digenean parasite at different systematic levels. These 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. This simultaneous utilization of ITS1 makes it important in parasitic molecular characterization. The most peculiar cell organelle ribosome plays an important role in protein formation and gene expression as well (Hinrich *et al*., 1999).

The ribosomal DNA unit of eukaryotes is composed of three distinct regions i.e., 18S, 5.8S and 28S separated by repeated transcribed spacers. First internal transcribed spacer (ITS-1) are positioned in between 18S and 5.8S region and second internal transcribed spacers (ITS-2) is present between 5.8S and 28S region while in upstream position external transcribed spacer (ETS) is located. The distinct features of rDNA make it ideal for parasite identification and phylogenetic analysis (Elder & Turner, 2018). Internal transcribes spacer (ITS1) has been recognized as a precise proof of evolution in studies of many groups of parasites i.e.(Van Herwer *et al*., 1998) trematodes (Hinrich & Englisch, 1999; Hung *et al*., 1999). Conserved genes present in the eukaryotic cells exhibits high nucleotides similarity even in far most taxonomic taxa that makes these non-coding internal transcribed spacers regions ITS1 and ITS2 suitable for specie specific diagnosis (Hillis, 1999). Coding regions which are highly conserved are used to infer the major higher level of classifications while on the other hand the noncoding spacers regions are utilized for lower level of phylogenetic studies due to higher evolutionary pressures (Chen *et al*., 2004; Nolan & Cribb, 2005).

Variability in the DNA molecules is the only evolutionary force that helps a species in their adaptation to continuously varying climatic conditions. Different facts about the dispersion of these genetic differences among same species and with other

population of parasites will provide precise raw materials to find out the most complex host-parasitic adaption and their successful interaction with each other. Different spacers regions or mitochondrial genome located in the whole genome of individual acts as a required specific target sites for the understanding of genetic differences within a population. Both these regions have accumulated different types of mutation over the time, but most important thing is the rate by which these changes occur. Consequently, the nuclear sequences show variability less in species then between species, therefore in molecular studies these are considered as excellent markers for identification and characterization of a species (Hoste *et al,*. 1998) (Newton *et al.*, 1998) (Chilton *et al.*, 2001).

Objective

- Molecular characterization of *G. explanatum* based on ribosomal internal transcribed spacer (ITS-1) from the buffaloes of Peshawar KP.

- Determination of the genetic diversity of *G. explanatum* by phylogenetic analysis.

MATERIALS AND METHODS

The current research was carried out in the Laboratory of Parasitology, Department of Animal sciences, Quaid-i-Azam University Islamabad, Pakistan.

2.1 Sampling sites

Sampling was carried out in various abattoirs of Peshawar Khyber Pakhtunkhwa to observe the liver and bile ducts of buffaloes for the collections of *Gigantocotyle explanatum* species. Buffaloes from different parts of the province are brought here for slaughtering. Large numbers of areas have been covered through these abattoirs. Geographically Khyber Pakhtunkhwa is divided in to two basic regions, mountainous zones to the north, and plains or slightly mountains to the south, with latitude of 34.95º N and longitude of 72.33º E. The total area of the province is 128,961 km². Climatic condition of KP is extremely variable. In the northern region of the province has very cold and high snowy winters with heavy rainfall but summer is enjoyable, while on the other hand the southern part has moderate climate, average rainfall and comparatively hotter summers. The moon soon period of the region starts in the middle of June and lasts in the mid of September.

2.2 Sample Collection

At least 8 visits per month were made to local buffalo slaughter house at Peshawar from February to October 2018. Adult liver flukes Amphistomes (*G. explanatum*) were collected from the bile ducts of infected buffaloes by forceps soon after their slaughtering. Total 501 buffaloes were observed. A total of 45 Infected animals were selected for genetic analysis. After carefully collection the worms were washed by PBS solution and kept in 70% ethanol for preservation. Then for further analysis they were carried to the Parasitology Lab, Quaid-i-Azam University Islamabad. Buffaloes included in the current study were slaughtered for food and protein demand of the public.

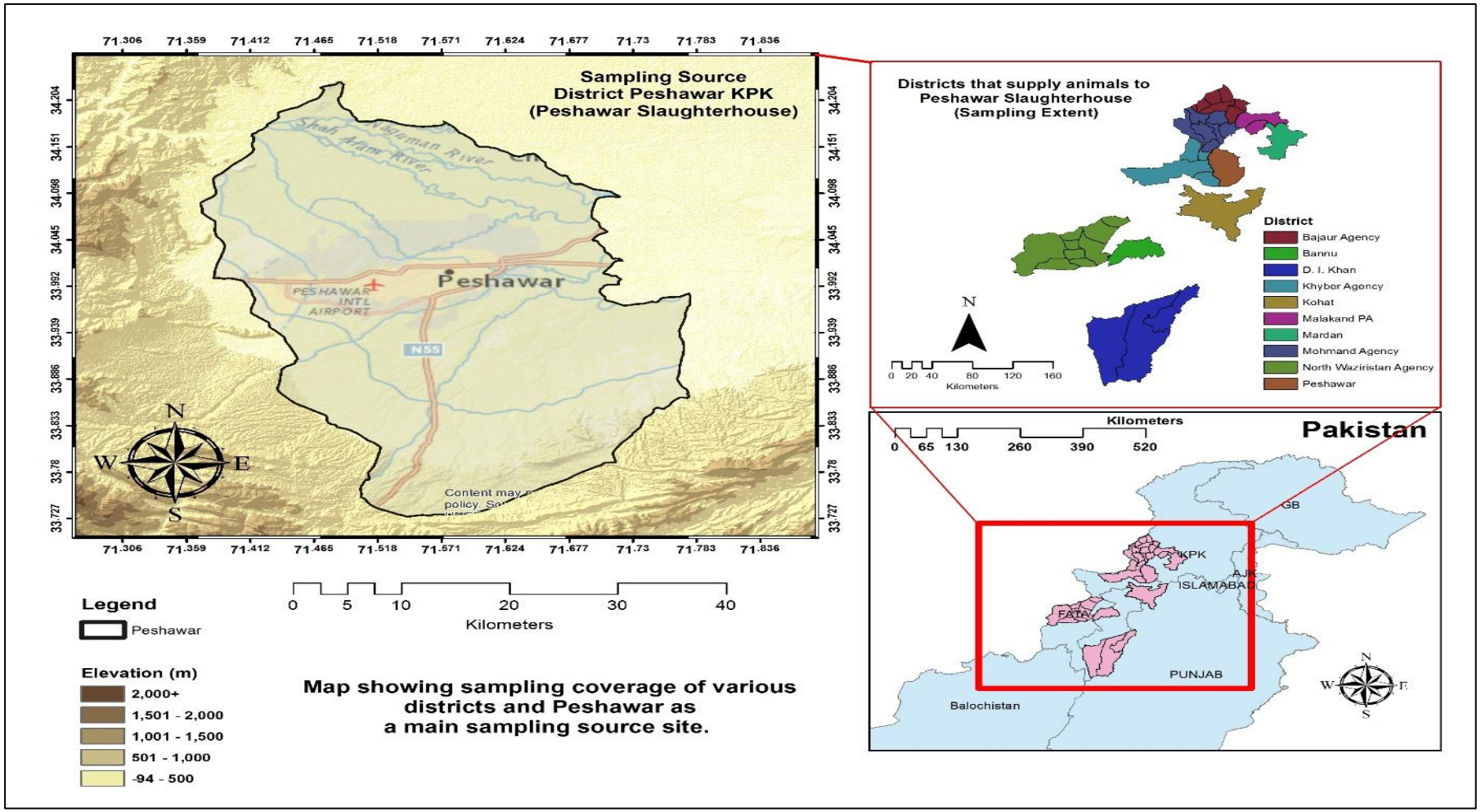


Figure 2.1: Map of Pakistan showing different sampling areas of Province Khyber Pukhtunkhwa

2.3 Genomic DNA Extraction

Genomic DNA of *G. explanatum* was isolated through consecutive three-day protocol (Chloroform-Phenol) method described by Barker (1998) with little bit required modifications. The adult worms which were preserved in 70% ethanol were crushed crushed in a tissue grinder with the addition of 400 µl of solution A, Inverted the tubes 4-5 times and then left for incubation for 30 minutes. After incubation they were centrifuged at 13000 rpm for 2 mints, discarded the supernatant, resuspend and shifted to new labelled Eppendorf tube. Add 400 µl of solution A in pellet mixed by tapping and again centrifuged at 13000 rpm for 2 minutes, discard the supernatant. Incubated the tube overnight at 37ºC with the addition of 400ul of solution B,25 µl of 20% SDS and 10 µl of proteinase-k, invert 5- 10 times.

Continued the step on next day by adding 400µl of phenol and centrifuged for 10 minutes at 13000 rpm. Discarded the supernatant and add 500 µl of solution C+D in equal amount. Solution D should be made freshly by mixing chloroform and Isoamyl alcohol at the ratio of 24 µl and 1 µl respectively. After Discarded the supernatant, invert the tube several times and then Centrifuged for 10 minutes at 13000 rpm. Transferred the upper layer to new Eppendorf tube add 500µl of solution D, tape the tube several times and centrifuged at 13000 rpm for 10 minutes. Again, Transfer the upper layer to new tube add 60 µl of sodium acetate and 500 µl of chilled isopropanol, DNA threads appeared. Again, centrifuged at 13000 rpm for 10 minutes and refrigerated the tube for 40 minutes. After refrigeration discard the supernatant add 200 µl of 70% ethanol to DNA pellet and tapped. Centrifuged at 13000 rpm for 7 minutes, discarded the ethanol and leaved the tube to dry for 10 minutes. Added 80-150 µl of TE buffer and incubate at 37º Cover night in incubator.

Sharply following the next day, the gel was run at 120 V on Gel Electrophoresis by loading 3µl of DNA sample and 3µl of loading dye, for 30 minutes. Confirmed the bands by computer connected Geldoc system with UV light source and saved the image.

2.4 Primer designing

Universal forward primer BD1-ITS1-F (GTCGTAACAAGGTTTCCGTA) and universal reverse primer ITS1-R (TATGCTTAAATTCAGCGGGT) used for the amplification process.

Table 2.1: Primers used for amplification and sequencing

Primer Name	5´-3´Sequence	Bps	Tm	Product size	Annealing temperature
Dend-ITS1-F	GTCGTAACAAGGTTTCCGTA	20	58	1200+ additional	61.5˚C
Dend-ITS1-R	TATGCTTAAATTCAGCGGGT	20	52		

2.5 Amplification of ITS-1 gene of *G.explanatum*

Polymerase chain reaction (Mullis, 1990) was used to amplify the DNA sequence. Total volume of 25 µl PCR mixture is composed of, dNTPs (2.5 µl), $Mgcl_2$ (2 µl), Taq polymerase (0.3 µl), each forward and reverse primer (0.5 µl), PCR buffer (2.5 µl), PCR water (14.7 µl) and DNA (2 µl). Carefully mixed all the above chemicals and centrifuged at 2000 rpm for 20 second. Thermo-cycler condition was maintained at 95ºC for 45 seconds follow by 35 cycles of 95 ºC for 45 seconds,61ºC for 45 seconds and 72º C for 90 seconds with a final extension process at 72 Cº for 10 minutes.

Table 2.2: Thermal cycler conditions for PCR

Steps	Temperature	Time
Initial	95ºc	45 Sec
Denaturation	94ºc	45 Sec
Annealing	61ºc	45 Sec
Extension	72ºc	90 Sec
Final extension	72ºc	10 min

2.6 Agarose Gel Electrophoresis

Amplified product of ITS-1 was checked by loading 5 µl of the PCR product with 3 µl of tracking dye (0.25% bromophenol Blue and 40% sucrose) in wells of gel which were already placed in gel tank (CS-Cleaver Scientific Limited). Performed Electrophoresis in a horizontal electrophoresis containing 1X TBE Buffer, maintained the voltage at 120 for 30 minutes.

After the completion of electrophoresis, by using gel documents system observed the result which is performed by keeping the gel under the UV trans-illuminator (Biometra, Gottigen, Germany). Visualized the band and capture the image and saved for further analysis. The voltage for this process was 120 while the time was 25-35 minutes. After the completion of electrophoresis, the results were observed by using gel documentation system. The bands were visualized, and the images were captured by using the Digital Camera EDAS290 (Kodak, New York, USA).

2.7 PCR purification and sequencing

The resultant PCR product were purified using Wizrep purification mini Kit.After electrophoresis and visualization of bands the PCR product were sent to Sequencing Services (Macrogen) Korea. 4 samples sequenced in both directions, 6 only forward and 6 in reverse as well. Chromas software were used to check the results of sequencing. By using FASTA format manually find out the differences among all results. Downloaded Multiple Sequence Alignment tool, CLUSTLW for the specific identification of intraspecific variations with in identified sequences and interspecific differences with different trematodes sequence of the same family and other as well.

2.8 Phylogenetic tree Analysis

By using software MEGA 7 latest version a phylogenetic tree was formulated. Selected Maximum likelihood method of MEGA 7. From NCBI GenBank the available reference sequences were obtained and made pairwise comparison by Kimura 2-model and kept bootstrap replicates 1000 for each dataset. The estimation of evolutionary divergence done to find out the variation between sequences. A total 43 nucleotide sequences along with identified sequence of *G.explanatum* were run in Poisson model for comparison.

RESULTS

In the current research study, a total of 45 infected buffaloes from Peshawar, Pakistan were included. The rDNA (ITS-1) fragments of all species were successfully amplified and sequenced. Gel electrophoresis picture of (ITS-1) amplified PCR product compared with the molecular weight marker of 1286 bp (Fig 3.1). Sequences were edited, and partial region was selected for genetic analysis. Kept equal base pair of all the reference sequences of different families. The sequence of *G. explanatum* was aligned in BLAST. Total length of amplified ITS-1 region was 400bp (Fig 3.2). The current sequence showed 98 % identity with *Paramphistomum cervi* (KJ459934) of China and 94% with that of *Watsonius watsoni* (KC763806) of the same country. The similarity index is as follow, *Gastrodiscoides hominis*(EU887294) 93% of India, *Homalometron armatum* (KT823419) 86% of USA, *Chiorchis fabaceus* (MF370224) 85% of Columbia respectively. In addition, the present ITS-1 region of *G. explanatum* exhibit 81% with the species of *Diplodiscus japonicus* (KX506855) from Russia. The sequence identity of *G. explanatum* with other trematodes species in BLAST are listed in table 3.1 and similarity is presented in Fig 3.3.

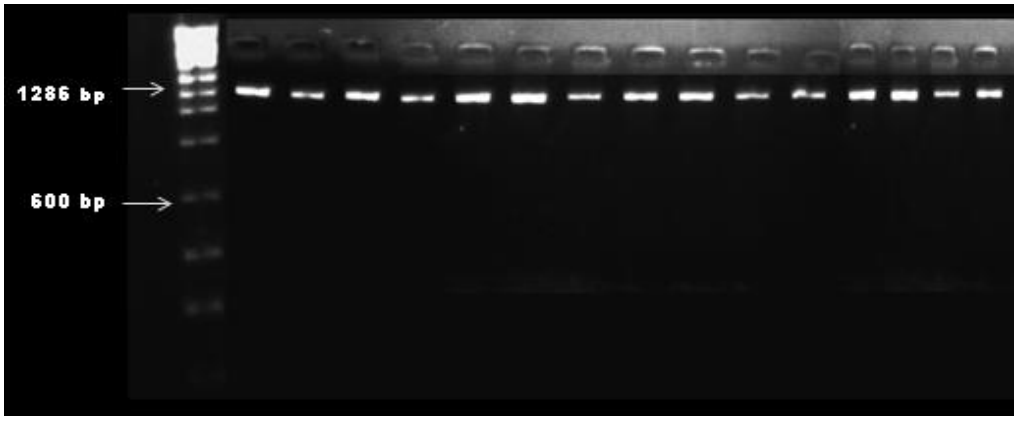


Figure 3.1: Gel electrophoresis picture of (ITS-1) amplified PCR product compared with the molecular weight marker of 1286 bp.

Table 3.1 BLAST similarity identity of *G. explanatum* with reference species.

Species Name	Isolate	Host	Country	Phylum	Class	Family	% Identity	Accession no
Paramphistomum cervi	PCB	Sheep	China	Platyhelminthes	Trematodes	Paramphistomoidae	98%	KJ459936
Paramphistomum cervi	PCA	Sheep	China	Platyhelminthes	Trematodes	Paramphistomoidae	98%	KJ459935
Paramphistomum cervi	PCC	Sheep	China	Platyhelminthes	Trematodes	Paramphistomoidae	97%	KJ459937
Paramphistomum cervi	PCD	Sheep	China	Platyhelminthes	Trematodes	Paramphistomoidae	97%	KJ459938
Paramphistomum cervi	PCE	Sheep	China	Platyhelminthes	Trematodes	Paramphistomoidae	97%	KJ459934
Watsonius watsoni	NM	*Macaca fascicularis*	China	Platyhelminthes	Trematodes	Paramphistomoidae	94%	KC763806
Gastrodiscoides hominis	NM	*Sus scrofa domestica*	India	Platyhelminthes	Trematodes	Paramphistomoidae	93%	EU887294
Gastrodiscoides hominis	NM	*Sus scrofa domestica*	India	Platyhelminthes	Trematodes	Paramphistomoidae	93%	EF027098
Homalometron frocioneae	ON16-2	*Fundulus diaphanus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	86%	KT823419
Homalometron armatum	sbP	*Planorbella trivolvis"*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	KC710975
Homalometron armatum	spB	*Lepomis microlophus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	KC710976
Homalometron robisoni	2	*Fundulus diaphanus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	86%	KT823418
Homalometron pallidum	1	*Fundulus heteroclitus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	86%	HM038043
Homalometron palmeri	4	*Pogonias cromis*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	JX400858
Homalometron palmeri	1	*Micropogonias undulatus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	JX400850
Homalometron palmeri	5	*Fundulus grandis*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	JX400859
Homalometron manteri	1	*Leiostomus xanthurus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	JX400851
Homalometron armatum	spA	*Campeloma sp*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	KC710978
Homalometron armatum	spA	*Aplodinotus grunniens*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	KC710977
Homalometron elongatum	1	*Gerres cinereus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	HM038037
Crassicutis archosargi	NM	*Archosargus probatocephalus*	USA	Platyhelminthes	Trematodes	Apocreadioidae	87%	EU170369
Chiorchis fabaceus	CO-Car	*Trichechus manatus manatus*	Colombia	Platyhelminthes	Trematodes	Paramphistomoidae	85%	MF370224
Diplodiscus japonicus	Djap-4	NM	Russia	Platyhelminthes	Trematodes	Paramphistomoidae	80%	KX506855
Diplodiscus japonicus	Djap-3	NM	Russia	Platyhelminthes	Trematodes	Paramphistomoidae	80%	KX506854
Diplodiscus japonicus	Djap-2	NM	Russia	Platyhelminthes	Trematodes	Paramphistomoidae	80%	KX506853
Diplodiscus mehrai	Dm-2	NM	Russia	Platyhelminthes	Trematodes	Paramphistomoidae	81%	KX506857

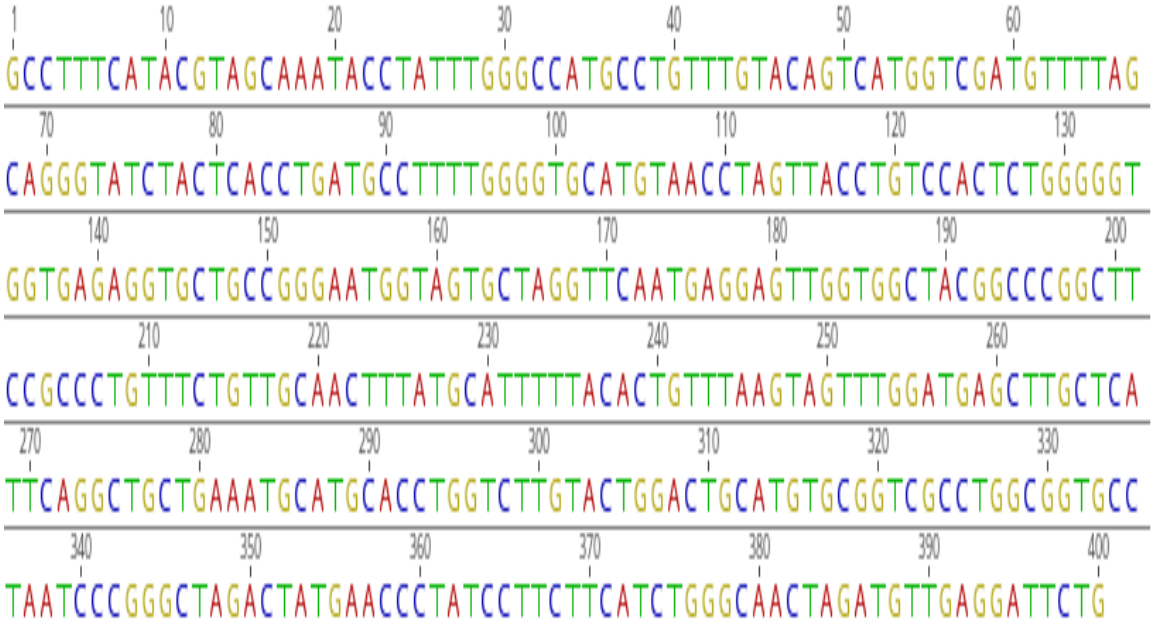


Figure 3.2: Identified sequence of ITS 1 region of *G .explanatum* from infected buffaloes of Khyber Pakhtunkhwa Pakistan.

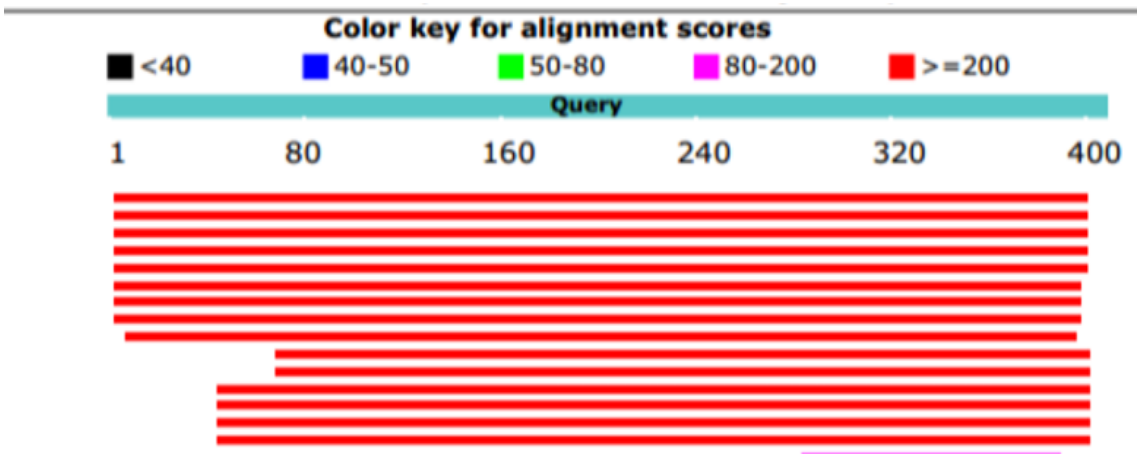


Figure 3.3: BLAST similarity Index

3.1 Intraspecific and interspecific variation

The present amplified sequences of G. *explanatum* were aligned in CLUSTALW to identify the intraspecific with in the species and interspecific

variation with other digenean trematodes. Reference sequences of Paramphistomidae amphistomes and other families were included to infer the genetic variations in their respective positions.

No intra-specific variation is observed among the present species. ITS-1 of current parasite show maximum similarity with *Paramphistomum cervi* of China and varied with other digenean. The present results showed 92% similarity with *Paramphistomum Cervi* of China (KJ459934) with 12 nucleotide variations at positions 14(A>G), 24(T>A), 36(G>T), 46(A>T), 112(A>G), 151(G>A), 153(G>A), 181(T>C), 183(G>T)and 383(A>G) respectively. *G. explanatum* on the other hand showed 21bp differences with *Watsonius watsoni* (KC763806) of China at following positions 10(A>T), 11(C>T), 46(A>T), 112(A>G), 151(G>A), 153(G>A), 168(G>A), 179(A>G), 182(T>A), 183(G>T), 189(T>C), 197(G>T), 200(T>C), 213(C>T), 259(A>G), 283(A>C), 302(T>C), 348(A>G), 253(A>G), 360(C>T), 379(C>T), 383(T>C), 384(A>G), 292(A>G). A consistent sequence variation was observed with the two species of *Gastrodiscoides hominis*(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 position and G↔A transition occurs 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. These mutations were found at 36 different polymorphic sites. The nucleotide diversity within the ITS-1 fragment is spread not in equal amount and the highly polymorphic positions were 14,24,36,42,182 and 183 bp respectively. Comparison of ITS-1 with other available reference sequences of trematode species shown table (3.2.1 to 3.2.3).

Table 3.2.1 Polymorphic sites identified in (ITS-1) from 1 to 150 bp sequences amplified from *G. explanatum* and potential matches with GenBank sequences.

Species	Accession no	Country	0 3	O 5	1 0	1 1	1 2	1 4	2 4	3 6	3 7	4 2	4 6	5 0	5 8	6 1	6 2	6 3	6 5	6 7	6 9	8 1	8 2	8 5	8 7	9 3	9 4	9 5	1 0 4	1 0 8	1 1 0	1 1 1	1 1 2	1 1 4	1 2 8	2 3 5	3 3 0	3 3 1	3 4 7	4 4 8	4 4 9	4 5 0
Gigantocotyle explanatum	Current study	Pakistan	C	T	A	C	C	A	T	G	C	T	A	T	G	G	T	T	T	G	A	C	A	T	A	T	T	T	T	A	C	T	A	T	C	C	G	G	T	G	A	G	G	C	C
Paramphistomum cervi	KJ459934	China	.	.	.	.	.	G	C	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459935	China	.	.	.	.	.	G	.	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459936	China	.	.	.	.	.	G	.	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459937	China	.	.	.	.	.	G	C	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459938	China	.	.	.	.	.	G	C	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Watsonius watsoni	KC763806	China	T	G	T	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	EU887294	India	.	.	T	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	EF027098	India	.	.	T	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	MF370224	Colombia	.	.	.	.	T	.	.	.	T	.	.	C	.	T	.	G	.	A	G	.	G	.	.	.	A	C	G	.	T	C	.	.	T	T	.	.	C	.	.	A	A	T	G

Table 3.2.2 Polymorphic sites identified in (ITS-1) from 151 to 243 bp sequences amplified from *G. explanatum* and potential matches with GenBank sequences

Species	Accession no	Country	1 5 1	1 5 2	1 5 3	1 5 5	1 5 7	1 5 8	1 6 8	1 6 9	1 7 1	1 7 2	1 7 4	1 7 5	1 7 8	1 7 9	1 8 0	1 8 1	1 8 2	1 8 3	1 8 9	1 9 1	1 9 2	1 9 3	1 9 4	1 9 5	1 9 6	1 9 7	2 0 0	2 0 8	2 1 2	2 1 3	2 1 8	2 1 9	2 2 0	2 2 1	2 2 3	2 2 5	2 2 7	2 2 8	2 2 9	2 3 0	3 3 1	3 3 9	4 3
Gigantocotyle explanatum	Current study	Pakistan	G	G	G	A	G	G	G	G	C	C	T	T	G	A	G	T	T	G	T	A	C	G	C	T	T	T	C	G	C	A	C	C	A	T	G	G	A	T	C	T
Paramphistomum cervi	KJ459934	China	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459935	China	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459936	China	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459937	China	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Paramphistomum cervi	KJ459938	China	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Watsonius watsoni	KC763806	China	A	.	A	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	EU887294	India	.	.	A	.	A	.	A	.	.	.	.	.	.	.	G	.	C	A	G	C	.	.	T	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	EF027098	India	.	.	A	.	A	.	A	.	.	.	.	.	.	.	G	.	C	A	G	C	.	.	T	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastrodiscoides hominis	MF370224	Colombia	.	T	C	.	_	_	A	.	.	.	.	.	.	.	G	A	C	.	.	.	G	T		.	C	C	.	.	.	_	_	_	.	_	A	.	T	G	.	.	.

Table 3.2.3 Polymorphic sites identified in (ITS-1) from 244 to 399 bp sequences amplified from *G. explanatum* and potential matches with GenBank

Species	Accession no	Country	248	255	258	259	261	262	263	264	266	267	268	273	275	283	294	298	299	302	306	337	343	348	353	354	360	361	363	364	365	367	368	369	379	380	381	383	384	389	392	395	398	399
Gigantocotyle explanatum	Current study	Pakistan	T	G	G	A	C	T	T	G	T	C	A	G	C	A	T	C	T	T	G	A	G	A	A	T	C	C	T	C	C	T	C	C	C	A	A	T	A	C	A	A	C	T
Paramphistomum cervi	KJ459934	China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Paramphistomum cervi	KJ459935	China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Paramphistomum cervi	KJ459936	China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Paramphistomum cervi	KJ459937	China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Paramphistomum cervi	KJ459938	China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Watsonius watsoni	KC763806	China	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	G	.	.	T	.	.	.	.	.	.	.	T	.	.	C	G	.	G	.	.	.
Gastrodiscoides hominis	EU887294	India	.	.	.	G	.	.	.	.	C	.	.	.	.	C	.	.	.	C	.	.	.	G	G	.	T	.	.	.	.	.	.	.	.	.	.	C	G	.	G	.	T	G
Gastrodiscoides hominis	EF027098	India	.	.	.	G	.	.	.	.	C	.	.	.	.	C	.	.	.	C	.	.	.	G	G	.	T	.	.	.	.	.	.	.	.	.	.	C	G	.	G	.	T	G
Gastrodiscoides hominis	MF370224	Colombia	.	A	.	G	.	.	.	.	C	T	G	.	_	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	T	G	.	.	G	.	.	.	.	.

3.2 Phylogenetic Analysis

Phylogenetic tree was formulated by comparing 45 current sequences of *G. explanatum* flukes with 16 closely related digenean trematodes species. All sequences of ITS-1 were entered in the MEGA 7 (Latest version) for construction of phylogenetic relationship using maximum parsimony (MP) and distance methods. The Kimura 2-parameter model was best fitted to current selected sequences (Kumar *et al*., 2016). Branches of the trees are considered in percentage along with taxa clustered. Trees of heuristic were calculated by automatically by choosing Neighbor-join and genetic pairwise are estimated by selecting Maximum Composite Likelihood method. All extra gaps and missing documents were removed. A total of 397 positions were included in the final databases. Calculated percentage of each clade shown next in the clustered branches. Precise topology was observed with the help of all bootstrapping replicates of phylogenetic tree by keeping their values higher than 50%. By using Neighbor-Join algorithm matrix of pairwise distance calculated with the help of Maximum Composite Likelihood (MCL) and after the topology of with higher like hood value was selected.

Sequences of *G. explanatum* are still not reported so ITS-1 of their close relatives was compared for phylogenetic analysis. Different digeneans of nearly divided geographical regions were taken with special preference for construction of phylogenetic tree.

Our results indicate that the current species of *G. explanatum* from Pakistan formed a sub clade with Paramphistomum *cervi* (KJ459934), a rumen parasite of China with 92% similarity. While *Watsonius watsoni* (KC763806) of the same country also shared maximum similarity. Two species of *Gastrodiscoides hominis* (EU887294) from India also make a sub clade with 78% similarity index. Comparison with other paraphyletic clades as follow *Diplodiscus mehrai* (KX506857) and *Diplodiscus japonicus* (KX506855) of Russia with 78% bootstrap values. *Chiorchis* (MF370224) of Columbia make distinct position with 78% identity along with *G.*

explanatum species. In addition, two out group Cestodes; *Taenia solium (*EU747673) of Mexico and *Taenia saginata* (AY392045) of China showed minimum similarity 73% with current under study species of *G. explanatum*.

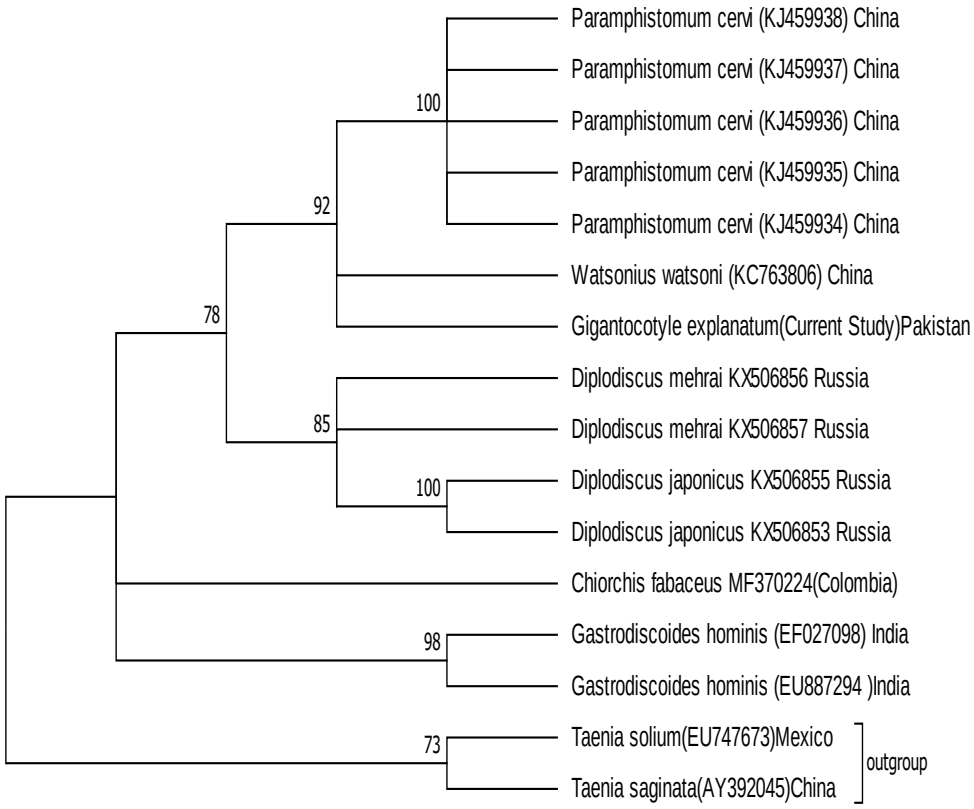


Figure 3.4: Molecular phylogenetic analysis by Maximum Likelihood method

The pairwise genetic distance of current specimen with other 11 species reveals the similarity of nucleotides. Our species closeness with *Paramphistomum cervi* in the phylogenetic tree verified by a small genetic distance in the range of 0.00 to 0.03093. High range of difference were also observed between our current *G. explanatum* from Pakistan and *Chiorchis fabaceus* (MF370224) of Colombia which is 0.03093 to1.21492. High value of mentioned out group was also generated. The sequence divergence with other trematodes shown in table 3.3.

Table 3.3 Estimates of evolutionary divergence between *G. explanatum* and other reference sequences

S.No	Species	Country	Accession no	1	2	3	4	5	6	7	8	9	10	11	12
1	*Gigantocotyle explanatum*	China	Current study												
2	*Paramphistomum cervi*	China	KJ459938	0.03093											
3	*Paramphistomum cervi*	China	KJ459937	0.03093	0.00000										
4	*Paramphistomum cervi*	China	KJ459936	0.03093	0.00000	0.00000									
5	*Paramphistomum cervi*	China	KJ459935	0.03093	0.00000	0.00000	0.00000								
6	*Paramphistomum cervi*	China	KJ459934	0.03093	0.00000	0.00000	0.00000	0.00000							
7	*Watsonius watsoni*	China	KC763806	0.06913	0.06068	0.06068	0.06068	0.06068	0.06068						
8	*Diplodiscus japonicus*	Russia	KX506855	1.03804	1.07899	1.07899	1.07899	1.07899	1.07899	1.08885	1.24356	1.24356			
9	*Diplodiscus japonicus*	Russia	KX506853	1.03804	1.07899	1.07899	1.07899	1.07899	1.07899	1.08885	1.24356	1.24356	0.00000		
10	*Diplodiscus mehrai*	Russia	KX506856	0.94295	0.98893	0.98893	0.98893	0.98893	0.98893	0.98850	1.29883	1.29883	0.04415	0.04415	
11	*Diplodiscus mehrai*	Russia	KX506857	0.94295	0.98893	0.98893	0.98893	0.98893	0.98893	0.98850	1.29883	1.29883	0.04415	0.04415	0.00000
12	*Chiorchis fabaceus*	Columbia	MF370224	1.21492	1.27561	1.27561	1.27561	1.27561	1.27561	1.31036	1.12873	1.12873	1.54378	1.54378	1.49677

DISCUSSION

So far identification and characterization of different amphistomes were based only on morphology which makes it difficult to precisely characterize the different life stages of flukes. But still morphological identification provide foundation for different digenean characterization (Nasmark, 1937; Eduardo, 1982). Amphistomes have been given a very little attention with respect to utilization of molecular tools to verify their morphological identification at different systematic level (Sanabria *et al*., 2011). Molecular analysis of the ribosomal DNA genes is considered a sound strategy to characterize digenean on molecular level because of their highly conserve nature across the Paramphistomidae family (Ma *et al*., 2015). PCR based studies by using ITS regions have proved a reliable tool for genetical characterization of different species having similar features along with phylogenetic relationship of many trematodes (Park, 2007).

The current study was designed to characterize the Pakistani species of *G. explanatum* based on internal transcribe spacer (ITS1) of ribosomal DNA. ITS1 region of Paramphistomidae is usually characterized to infer the intraspecific variation among species (Herwerden *at al*., 1998) But interestingly in present study no such polymorphism was observed within species. Our results manifested high similarity index between *G. explanatum* of current study, and Chinese *Paramphistomum cervi* (KJ459938), and showed differences with each other at only 12 nucleotides. This high level of similarity determined that these species are present in geographically linked countries and may share evolutionary history (Blair *et al*.,1996). On the other hand, our result depicts that a great extent of variation exists between Paramphistomidae in this region as compared to geographically distant regions like eastern European that may be due to the high evolutionary pressure on the ITS1 ribosomal region (Herwerden *et al*., 1998).

The genetic analysis of ITS-1 is very important to infer the intraspecific and interspecific variations. Itagaki et al., 2005 documented no intraspecific variations in

the ITS-1 sequences of *Fasciola hepatica* and *Fasciola gigantica* species in Korea. They also showed that ITS-1 sequence is identical with Japanize *Fasciola* isolates and gave strong arguments that these species shared same ancestors and spread through the movement of infected host across both countries.

In addition, another digenean *P. westermini* showed valuable amount of intra individual differences in the amplified ITS-1 region and other species of the same genus as well, and the reasons behind these variations is the varying number of repeat sequences in some species of digenean (Herwerden *et al*., 1998). The homogenization of these repeat sequences of ribosomal DNA is due to concerted evolution (Dover, 1982). Tang *et al*., 1996 provide information that these repeat sequences are not operating uniformly in all genera of trematodes species, this statement in turn supports the fact that in our current ITS-1 region of *G. explanatum* from Pakistan that no such type of successive repeats sequences was observed. Different categories of ITS1 variation have been observed in various studies on trematodes DNA. This different degree of change in ribosomal DNA tandem array is probably due to the clusters segregation during replication that also enables to maintain homogeneity in their sequences (Kane *et al.*, 1996).

The present study showed high range of variation in the ITS-1 region of *G. explanatum* with Columbian *Gastrodiscoides hominis* which is due to high evolutionary pressure in different geographically separated areas (Tatonova *et al*., 2012). A comparative study on the ITS-1 region of *Clonorchis sinensis* species from China and Kora with the nearby Russia also revealed significant amount of polymorphism and this variation is the only possible reason for their evolutionary adaptation throughout the world (Subbotin *et al*., 2011). With great interest studies suggested about the polymorphism in ITS-1 region have some evolutionary and adaptive importance for particular parasites but still this region is in infancy stage and induction of new data is the need to understand the parasite's adaptive strategies (Biémont & Vieira, 2006).

The phylogenetic tree provides potential information about the distribution pattern and clade of different species, provide a linkage with other species as well (Laidemitt *et al*., 2017). Our systematic analysis showed the closeness of current species of *G. explanatum* with *Watsonius watsoni* (KC763806) of china with 92 percent similarity. With above similarity, we can predict that the movements of infected host and similar cultural practices are the possible cause of parasite transmission. This mechanism of transmission is also elaborated by Mohanta *et al.* by studying *Fasciola* species in various regions of Bangladesh (Mohanta *et al*., 2014).

This study is the first attempt to genetically characterize *G. explanatum* species of Pakistan. *E. explanatum* is a major parasite of wild animals the ancestor of currently domestic buffaloes may be introduced in the domestic venues because of divergent evolution from various geographical areas also with the occurrence of other events in living domestic cattle, this observation suggested existence of at least four independent sub population of *E. explanatum* (Singh, 1958).

Our results showed insertion mutations at various positions with *Gastrodiscoides hominis* (MF370224) of Columbia that reveals the high range of variability in geographically isolated countries. Studied on the ITS-1 region of Clonorchis species of northern and southern china species showed such type of variations and suggested that these polymorphism are very useful in liver flukes evolutionary adaptations in various parts of the world (Tatonova *et al*., 2012).

Genetic pair wise distance between current species of *G. explanatum* also confined their close affiliation with *Paramphistomum cervi* (459938), In genetic divergence small value of compared individual are very close with each other. Out groups containing high distance indicates maximum range of variability with present species. The genetic distance with *P. cervi* was recorded in the range of 0.0000 to 0.03093. Maximum value was observed with *Chiorchis fabaceus* (MF370224) of Columbia which is 0.0000 to 1.21492, showed a considerable amount of variation in the ITS-1 region of both species. The species clade having genetic distance <1.3%

(0.013) are positioned in the same clade while >6.5% (0.065) are located different clades (Laidemitt *et al.,* 2017).

Conclusion and Recommendations

In summary, the present study provides a genetic spectrum of *G. explanatum* based on ITS1 in buffaloes of Peshawar, Pakistan and infer their phylogenetic relationship with other species in different geographical regions. No intraspecific variations were observed in current amplified region. The current species showed strong genetic affiliation with *Paramphistomum cervi* a rumen parasite of China.

Following are the recommendations which has been proposed on the basis of the results of the current study

- Further Complete molecular characterization of *G.explanatum* based on ITS1 from other areas of the country that may be helpful to formulate a precise phylogenetic relationship.
- The 5´variable end of ITS1 may be used as a molecular identification marker for species level of classification and conserve 3´end region will provide molecular data for higher level of classification.
- This bimodal nature of ITS1 will provide a valuable genetic information about digenean life cycles.
- In future these finding will help in the development of vaccines against amphistomes species.
- The current molecular data will set bases and contribute in further studies on different paramphistomes particularly for *G. explanatum* .

REFERENCES

Ahmedullah, F., Akbor, M., Haider, M. G., Hossain, M. M., Khan, M. A. H. N. A., Hossain, M. I., & Shanta, I. S. (2007). Pathological investigation of liver of the slaughtered buffaloes in Barisal district. *Bangladesh Journal of Veterinary Medicine*, 81-85.

Ahuja, V., & Redmond, E. (2004). Livestock services and the poor. *Tropical Animal Health and Production*, *36*(3), 247-268.

Ambu, S. (1978). survey of Amphistomes of cattle and buffaloes slaughtered at the Shah Alam abattoir, Selangor, Malaysia. *Southeast Asian journal of tropical medicine and public health*.

Annon. (2003). *Department of Animal husbandary, Dairying, Fisheries, Ministry of Agriculture, Government of India*.

Barker. (1998). Phenol-Chloroform Isoamyl Alcohol (PCI) DNA Extraction.

Biémont, C., & Vieira, C. (2006). News & views feature Junk DNA as an evolutionary force. *Nature*, *443*(October), 521–524.

Blair, D., Campos, A., Cummings, M. P., & Laclette, J. P. (1996). Evolutionary biology of parasitic platyhelminths: The role of molecular phylogenetics. *Parasitology Today*, *12*(2), 66–71.

Boray, J. C., & America, I. N. (1959). Studies on intestinal amphistomosis in cattle by J. C. Boray, D.V.M., (1950), 282–287.

Charlier, J., van der Voort, M., Kenyon, F., Skuce, P., & Vercruysse, J. (2014). Chasing helminths and their economic impact on farmed ruminants. *Trends in parasitology*, *30*(7), 361-367.

Chauhan, V., (2014). *Studies On Prevalence, Haemato–Biochemical And Histopathological Aspects Of Amphistomosis In Slaughtered Buffaloes* (Doctoral Dissertation, Aau, Anand).

Cheema, A. H., Khan, M. Q., & Chishti, M. A. (1997). Prevalence and Pathology of

Ampidstomosis in Buffaloes and Cattle in Pakistan. *Pakistan Veterinary Journal*, *17*(l), 13–17.

Chen, C. A., Chang, C. C., Wei, N. V., Chen, C. H., Lein, Y. T., Lin, H. E., … Wallace, C. C. (2004). Secondary structure and phylogenetic utility of the ribosomal internal transcribed spacer 2 (ITS2) in scleractinian corals. *Zoological Studies*, *43*(4), 759–771.

Chilton, N. B., Newton, L. A., Beveridge, I., & Gasser, R. B. (2001). Evolutionary relationships of trichostrongyloid nematodes (Strongylida) inferred from ribosomal DNA sequence data. *Molecular Phylogenetics and Evolution*, *19*(3), 367–386.

Dargie, J. D. (1987). The impact on production and mechanisms of pathogenesis of trematode infections in cattle and sheep. *International Journal for Parasitology*, *17*(2), 453–463.

David M. Hillis, and M. T. D. (1999). Ribosomal DNA: Molecular Evolution and Phylogenetic Inference. *The Quarterly Review of Biology*, *66*.

Dixit, A. K., Das, G., & Baghel, R. P. S. (2017). Gastrointestinal helminthosis: prevalence and associated determinants in goats of Jabalpur, India. *Journal of parasitic diseases*, *41*(2), 414-416.

Dover, G. (1982). Molecular drive: a cohesive mode of species evolution. *Nature*, *299*(5879), 111.

Dunn, T.S., Nizami, W.A. & Hanna, R. E. . (1985). Studies on the ultrastructure and histochemistry of the lymph system in three species of amphistome (Trematoda: Digenea) *Gigantocotyle explanatum, Gastrothylax crumenifer* and *Srivastavaia indica* from the Indian Water Buffalo *Bubalus bubalis*. *Journal of Helminthology*, (59), 1–18.

Durie, P. H. (1956). The Paramphistomes (Trematoda) of Australian Ruminants. III.The life history of *Calicophoron calicophorum* (Fischoeder) Nasmark., (4), 152–157.

Eduardo, S. L. (1983). The taxonomy of the family Paramphistomidae Fischoeder,

1901 with special reference to the morphology of species occurring in ruminants.

III. Revision of the genus Calicophoron Näsmark, 1937. *Systematic Parasitology*, *5*(1), 25–79.

Eduardo, S. L. (1982). Techniques for examining paramphistomes. *Journal of Helminthology*, *56*, 117.

Fitzpatrick, J. L. (2013). Global food security: The impact of veterinary parasites and parasitologists. *Veterinary Parasitology*, *195*(3–4), 233–248.

Gul-E-Nayab, Ijaz Muhammad, Sadaf Niaz, Z. A. and S. A. K. (2017). Prevalence of gastrointestinal parasite, Paramphistomum in domestic animals (Cows and Buffaloes) of district Swat and Charsadda, KP, Pakistan. *Journal of Entamology Studies*, *5*(3), 907–911.

Haque, M., Mohan, C., & Ahmad, I. (2011). Natural trematode infection in liver of water buffalo (*Bubalus bubalis):* Histopathological investigation.J Parasit Dis (Jan-June 2011) 35(1):50–53

Herrero, M., & Thornton, P. K. (2013). Livestock and global change: Emerging issues for sustainable food systems. *Proceedings of the National Academy of Sciences*, *110*(52)

Hillis, D. M., & Davis, S. K. (1986). Evolution of Ribosomal DNA: Fifty Million Years of Recorded History in the Frog Genus rana. *Evolution*, *40*(6), 1275.

Horak, I. G. (1971). Paramphistomiasis of Domestic Ruminants. *Advances in Parasitology*, *9*(C), 33–72.

Horak, I. G. (1962). Studies on Paramphistomiasis III: a method of testing the viability of paramphistome metacercariae. *J. Vet. Res*, *29*, 197–202.

Hoste, H., Chilton, N. B., Beveridge, I., & Gasser, R. B. (1998). A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of Trichostrongylus (Nematoda: Trichostrongylidae). *International Journal for Parasitology*, *28*(8), 1251–1260.

Hung, G.C., Gasser, R.B., Beveridge, I., Chilton, N.. (1999). Species-specific amplification by PCR of ribosomal DNA from some equine stronggyles. *Parasitology*, *119*, 68–80.

Hust, J., Frydenberg, J., Sauriau, P. G., Le Gall, P., Mouritsen, K. N., & Jensen, K. T. (2004). Use of ITS rDNA for discriminating of larval stages of two microphallid (Digenea) species using *Hydrobia ulvae* (Pennant, 1777) and Corophium volutator (Pallas, 1766) as intermediate hosts. *Parasitology Research*, *93*(4), 304–310.

Iqbal, Z., M. Akhtar, M. N. K. and M. R. (1993). Prevalence and economic significance of haemonchosis in sheep and goats slaughtered at Faisalabad abattoir. *Pakistan J. Agri. Sci*, *30*, 51–53.

Itagaki T, Kikawa M, Terasaki K, Shibahara T, (2005) Fukuda K. Molecular characterization of parthenogenic Fasciola sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. Journal of veterinary medical science.;67(11):1115-8.

Jones, AN Jones, A, Bray, RA Gibson, DI. Superfamily Paramphistomoidea Fischoeder, 1901 (Eds) Keys to the Trematoda. Wallingford, CABI Publishing, 221–327, 2005.

Juyal, P. D., K. Kasur, S. S. Hassan, K. P. (2003). Epidemiological status of paramphistomiasis in domestic ruminants in Punjab. *J. Parasit*, 231–235.

Kaewkes, S. (2003). Taxonomy and biology of liver flukes. *Acta Tropica*, *88*(3), 177–186. https://doi.org/10.1016/j.actatropica.2003.05.001

Kane, R. A., Ridgers, I. L., Johnston, D. A., & Rollinson, D. (1996). Repetitive sequences within the first internal transcribed spacer of ribosomal DNA in schistosomes contain a Chi-like site. *Molecular and Biochemical Parasitology*, *75*(2), 265–269.

Khan, U. J., & Maqbool, A. (2012). Prevalence of paramphistomosis in relation to meteorological factors. *Pakistan Journal of Zoology*, *44*(3), 823–828.

Laidemitt, M. R., Zawadzki, E. T., Brant, S. V., Mutuku, M. W., Mkoji, G. M., & Loker, E. S. (2017). Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants. *Parasitology*, *144*(2), 131–147.

Lengy, J. (1960). Study on Paramphistomum microbothrium Fischoeder 1901 a rumen parasite of cattle in Israel. *Bull. Res. Coun. Israel*, (9B), 71–130.

Ma, J., He, J. J., Liu, G. H., Zhou, D. H., Liu, J. Z., Liu, Y., & Zhu, X. Q. (2015). Mitochondrial and nuclear ribosomal DNA dataset supports that *Paramphistomum leydeni* (Trematoda: Digenea) is a distinct rumen fluke species. *Parasites and Vectors*, *8*(1), 1–9.

Mazahery, Y., & Razmyar, J. (1994). *Explanatum explanatum* (Creplin, 1847), *55*, 149–153.

Mohanta, U. K., Ichikawa-Seki, M., Shoriki, T., Katakura, K. and Itagaki, T. (2014). Characteristics and molecular phylogeny of Fasciola flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitol. Res.*, *113*, 2493–2501.

Mullis K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American.*, *262*(4), 56–61. 64–5.

Nasmark, K. E. (1937). A revision of the trematode family Paramphistomidae.

Zoologiska Bidrag Fran Uppsala, *16*, 301–365.

Newton, L. A., Chilton, N. B., Beveridge, I., Hoste, H., Nansen, P., & Gasser, R. B. (1998). Genetic markers for strongylid nematodes of livestock defined by PCRbased restriction analysis of spacer rDNA. *Acta Tropica*, *69*(1), 1–15.

Nguyen, V.K., Hoang. D.N., Jacquiet, P. H., & Dorchies, P. H. (1997). Prevalence of *Gigantocotyle explanatum* in Vietnamese buffaloes. A histopathological study [mastcells] [1997], *10*, 789–792.

Nikander, S., & Saari, S. (2007). Notable seasonal variation observed in the morphology of the reindeer rumen fluke (*Paramphistomum leydeni*) in Finland. *Rangifer*, *27*(1932), 47–57.

Nolan, M. J., & Cribb, T. H. (2005). The Use and Implications of Ribosomal DNA Sequencing for the Discrimination of Digenean Species.

Park, G. M. (2007). Genetic comparison of liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini,* based on rDNA and mtDNA gene sequences.

Parasitology Research, *100*(2), 351–357.

Raza, M. A., Murtaza, S., Bachaya, H. A., & Hussain, A. (2009). Prevalence of *Paramphistomum cervi* in ruminants slaughtered in district Muzaffar Garh. *Pakistan Veterinary Journal*, *29*(4), 214–215.

Sanabria, R. E. F., & Romero, J. R. (2008). Review Article Review and update of paramphistomosis, 64–68.

Sanabria, R., Moré, G., & Romero, J. (2011). Veterinary Parasitology Molecular characterization of the ITS-2 fragment of *Paramphistomum leydeni* (Trematoda : Paramphistomidae). *Veterinary Parasitology*, *177*(1–2), 182–185.

Schulenburg, J. H. G., Englisch, U., & Wägele, J. W. (1999). Evolution of ITS1 rDNA in the Digenea (Platyhelminthes: Trematoda): 3′ end sequence conservation and its phylogenetic utility. *Journal of Molecular Evolution*, *48*(1), 2-12.

Sey, O., & Eslami, A. (1982). Review of amphistomes (Trematoda: Paramphistomata) of Iranian domestic ruminants. *Parasit. Hung.*, *14*, 61–66.

Sey, O. (1991). *CRC handbook of the zoology of amphistomes.*

Shabbir, R. (2011). Sustainable Growth in livestock sector : A case study of Pakistan June 2011.

Singh, K. S. (1958). Trematoda : Paramphistomidae from India Author (s): Kunwar Suresh Singh Published by : The American Society of Parasitologists Linked references are available on JSTOR for this article : *The Journal of Parasitology*, *44*(2), 210–221.

Steinfeld, H., Gerber, P., Wassenaar, T. D., Castel, V., Rosales, M., Rosales, M., & de Haan, C. (2006). *Livestock's long shadow: environmental issues and options*. Food & Agriculture Org..

Subbotin, S. A., Deimi, A. M., Zheng, J., & Chizhov, V. N. (2011). Length variation and repetitive sequences of Internal Transcribed Spacer of ribosomal RNA gene, diagnostics and relationships of populations of potato rot nematode, *Ditylenchus destructor* Thorne, 1945 (Tylenchida: Anguinidae). *Nematology*, *13*(7), 773–785.

Tang, J., Toe, L., Back, C., & Unnasch, T. (1996). Intra-specific heterogeneity of the rDNA internal transcribed spacer in the Simulium. *Molecular Biology and Evolution*, *13*(1), 244–252.

Tatonova, Y. V., Chelomina, G. N., & Besprosvannykh, V. V. (2012). Genetic diversity of nuclear ITS1-5.8S-ITS2 rDNA sequence in *Clonorchis sinensis* Cobbold, 1875 (Trematoda: Opisthorchidae) from the Russian Far East. *Parasitology International*, *61*(4), 664–674.

Taylor, M. A., Coop, R. L., & Wall, R. L. (2007). *Veterinary Parasitology.*

Thornton, P. K. (2010). Livestock production: Recent trends, future prospects. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1554), 2853–2867.

Van Dijk, J., Sargison, N. D., Kenyon, F., & Skuce, P. J. (2010). Climate change and infectious disease: Helminthological challenges to farmed ruminants in temperate regions. *Animal*, *4*(3), 377–392.

Van Herwerden, L., Blair, D., & Agatsuma, T. (1998). Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology*, *116*(4), 311–317.

Williams, S. M., Debry, R. W., & Feder, J. L. (2009). Society of Systematic Biologists A Commentary on the Use of Ribosomal DNA in Systematic Studies, *37*(1), 60–62.

Weeks, J. (1999). CDPR Discussion Paper 0899 Economic Policy for Agriculture : A Guide for FAO Professionals By. *Russell The Journal Of The Bertrand Russell Archives*, *44*(0), 1–59.

Wilson, P. (2011). Decomposing variation in dairy profitability: The impact of output, inputs, prices, labour and management. *Journal of Agricultural Science*, *149*(4), 507–517.

Zhu, X., Chilton, N. B., Jacobs, D. E., Boes, J., & Gasser, R. B. (1999). Characterisation of Ascaris from human and pig hosts by nuclear ribosomal DNA sequences. *International Journal for Parasitology*, *29*(3), 469-478.

Annexure

Solutions	Compositions
Solution A	0.32 M sucrose, 5 mM MgCl2, 10 mM Tris hydroxy (methyl) aminomethane (pH 7.5), 1% v/v Triton X-100.
Solution B	10 mM Tris hydroxy (methyl) aminomethane (pH 8.0), 400 Mm NaCl, 2 mM EDTA (pH 7.5).
Solution C	Saturated phenol.
Solution D	Chloroform (24 mL) : Iso-amyl alcohol (1 mL)
10X TBE	0.89 M Tris hydroxyl(methyl)aminomethane, EDTA (pH 8.3), 0.025 M Borate
20% SDS	10 g Sodium dodecyl Sulphate in 50 mL water
Tris-EDTA(TE) Buffer	1 mM EDTA, 10 mM Tris hydroxyl(methyl amino) methane, (pH 8.0)
Bromophenol blue	0.25% Bromophenol blue, 40% Sucrose, dissolved in 100 mL distilled water
Ethidium bromide	2.5 mM ethidium bromide (Sigma-Aldrich, St Loui MO, USA)
Sodium Acetate	3 M sodium acetate
70% Ethanol	Absolute alcohol (70 mL): distilled water (30 mL)
Proteinase K (20X)	(EMD Chemicals, San diego, CA USA)
Iso-propanol	2-propanol (Merck, Germany)
Tamoxifen	99.9% (Sigma-Aldrich)
4-OH tamoxifen	99.9% (Cayaman chemical Company)
Triethylammonium phosphate	HPLC grade (Sigma-Aldrich)
Methanol	HPLC grade (Merck)
Acetonitrile	HPLC grade (Merck)

Hexane	HPLC grade, 60% n-hexane
HPLC water	Ultra-pure water

Genetic characterization of Gigantocotyle explanatum based on first internal transcribed spacer (ITS-I) of ribosomal DNA from buffaloes of Peshawar, Pakistan

ORIGINALITY REPORT

9%	6%	5%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	lup.lub.lu.se Internet Source	1%
2	Submitted to Higher Education Commission Pakistan Student Paper	1%
3	geneprimp.jgi-psf.org Internet Source	1%
4	Dargie, J.D.. "The impact on production and mechanisms of pathogenesis of trematode infections in cattle and sheep", International Journal for Parasitology, 198702 Publication	1%
5	pneumocystis.uc.edu Internet Source	<1%
6	Submitted to University of KwaZulu-Natal Student Paper	<1%
7	S. L. Eduardo. "The taxonomy of the family	