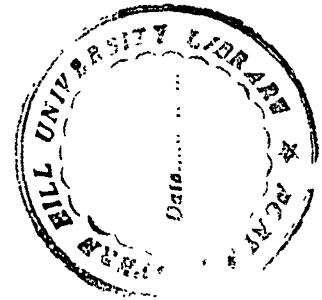
STUDIES ON THE STATUS AND MOLECULAR CHARACTERIZATION OF PARAGONIMIASIS AND OTHER CRUSTACEA-BORNE TREMATODE ZOONOSES IN NORTHEAST INDIA

ABSTRACT



by

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY

of

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ABSTRACT

The present work incorporates a study on the status and molecular characterization of paragonimiasis and other Crustacea-borne trematode zoonoses in Northeast India. The study aimed at examining the snail and crustacean species (the first and second intermediate hosts of the causative agent of infection) prevalent in Northeast India to recover the larval stages, if any, of the lung fluke *Paragonimus* and to identify the exact species prevailing and responsible for the disease. By using PCR-based molecular techniques the study aimed to identify the species of the parasite collected and to generate specific molecular markers with vital usage towards correct diagnostics.

• Status and prevalence of paragonimiasis in the region

- The crab hosts were surveyed from rural localities and countryside where eating of crabs is a common food practice among the natives of the region. Emphasis was given to procure those species which are commonly used in local traditional cuisine. Naturally infected freshwater crabs were mostly collected from mountain streams of the suspected foci of infection. Metacercariae were isolated from the muscles of the crustacean host by artificial gastric juice digestion technique. Of the 3 genera of crabs surveyed from various localities in Arunachal Pradesh, Assam, Manipur and Mizoram, only one, i.e., *Barytelphusa* was found to be harboring metacercarial cysts. The crabs collected from all the localities excepting those in Arunachal Pradesh, did not harbor any metacercarial infection; both the collection sites in Arunachal Pradesh were revealed to be positive for this infection. In the Kharshang site *Barytelphusa* (*M*) *lugubris lugubris* was found to be positive with a prevalence of 26%, the intensity of infection being in the range of 9-68 (mean intensity=36). In Miao region, the other sub species namely *B.* (*M.*) *l. mansoniana* revealed a much higher prevalence (87%) and intensity of metacercarial infection (ranging between 3-175, mean intensity=38).

- Snail species were also collected from selected localities wherever the crab hosts were suspected to be positive for metacercarial infections. Hundreds of specimens of 4 snail species (representing 4 genera with one species each) were examined for the recovery of intramolluscan stages, if any, of *Paragonimus*. Of the snail species surveyed, only one species viz. *Brotia costula* was found harbouring the sporocyst, redia and cercaria larval stages.

- Freshly recovered metacercariae from crab hosts and intramolluscan larval stages- sporocyst, redia and cercariae, were processed using standard protocols for light and electron microscopy. The newly excysted metacercaria has an elongate body (815.91µm x 492.79µm) in size; the ventral sucker situated somewhat pre-equitorally, is larger than the oral; the intestinal caeca are long and extend up to the posterior end of the body; the conspicuous excretory bladder extends medially in the intercaecal space. The SEM observations revealed that the encysted metacercaria is oval in shape and has smooth surface. The whole body surface of the excysted metacercaria is covered with numerous single-pointed and thorn-like tegumentary spines; those covering the anterior part of the body are bigger in size and show a gradual reduction in size towards the posterior end. On the basis of morphology and surface fine topography features of the excysted larval stage, the metacercariae were identified as those of *Paragonimus* species.

The cercariae recovered from the snail hosts were always of amphistome, leptocercous type. Microcercous type of cercariae (i.e., having a small stumpy tail) that are typically characteristic of *Paragonimus* spp were never encountered in the collections made during the present study. The larval stages recovered, not being representative of *Paragonimus,* were, therefore, not processed for further study towards molecular characterization.

- **Molecular characterization of *Paragonimus* and other trematodes**

  - The identification of closely related species based on morphological characters can be difficult, particularly so in the case of soft-bodied digenean trematodes. For most of the trematode-borne zoonoses the parasite eggs voided in exudates of the host are the only stages available for diagnostic purpose. Besides *Paragonimus,* infection of *Fasciola gigantica* and *Fasciolopsis buski,* both of which are putatively zoonotic species, have been reported in the northeastern region. The main objective of the present study was to provide molecular characterization of the parasite so as to supplement morphological criteria and develop species-specific molecular markers.

For the purpose of molecular characterization metacercariae of *Paragonimus* sp collected from the crab host *Barytelphusa lugubris* from Miao region in Arunachal Pradesh were used; live adult *F. buski* and *F. gigantica* were obtained from bovine hosts at local abattoirs. Eggs were obtained from mature adult flukes by squeezing between two glass slides; eggs recovered from each single specimen were also processed separately. DNA was extracted from metacercaria and eggs in Whatman's FTA card and from lysed individual adult worms by standard ethanol precipitation technique. The rDNA region spanning the ITS1 and ITS2 was amplified from the metacercarial, egg, larval and/or adult DNA by PCR. The primers generally used were designed based on the conserved sequences of *Schistosoma* spp, which are considered to be the universal primers for trematode species. The PCR amplification was performed following the standard protocol with minor modifications. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit, and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei, Bangalore and The Centre for Genomic Applications (TCGA), New Delhi, India and submitted to GenBank. No intra-specific variations in length or composition of the sequence were

observed, and the ITS sequences of egg, metacercaria and adult origin were found to be identical in length as well as composition. Sequence analysis was carried out using various bioinformatics tool e.g., BLAST, ClustalW, MEGA, mFOLD, Bayesian analysis phylogeny etc.

- ***Paragonimus* sp.:** The PCR amplified products of ITS2 of rDNA were successfully obtained and were compared with other sequences of trematode species from Genbank. The fragments of amplified DNA were estimated to be ~500bp long. Sequence analysis of the ITS2 PCR products revealed that the alignments of the rDNA region spanning the ITS2 were 496bp for forward primer and 494bp for reverse primer, respectively. The actual length of ITS2 was estimated to be 287bp. The Blast hit results showed that the query ITS2 *Paragonimus* metacercariae sequence is more similar to the sequence of the species *Paragonimus westerrmani*, *Paragonimus mexicanus*, *Paragonimus siamensis*, *Paragonimus sismensis*, *Paragonimus miyazakii*, *Euparagonimus cenocopiosus*. Comparing with the known sequences of the other lung fluke species, the present study revealed that the sequence of ITS2 (plus flanking regions) shows close resemblance with *Paragonimus westermani*, the expectation value (e-value) being most significant revealing absolute match. Phylogenetic analyses using the various distance methods and character state method like Maximum Parsimony show that the topology is similar among the trees obtained. A bootstrap value of > 70% among the trees obtained. Sequence analysis of ITS2 region of rDNA of metacercariae isolates of Miao showed that the species prevailing in the said location is *Paragonimus westermani* and not *P. heterotremus* as reported by earlier workers.

Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *Paragonimus*, so as to provide additional information for correct identification of the species prevalent in the region. The secondary structure analysis of the

same data also confirmed the results mentioned for primary sequence analysis. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Three similar topologies for seven species of the genus *Paragonimus* were obtained on the basis of traditional primary sequence analyses using MEGA and a Bayesian analysis of the combined data. The latter approach allowed to include both primary sequence and RNA molecular morphometrics; each data partition was allowed to have a different evolution rate. *Paragonimus westermani* was found to group with *P. siamensis* of Thailand; this was best supported by both the molecular morphometrics and combined analyses. *P. heterotremus*, *P. proliferus*, *P. skrjabini*, *P. bangkokensis* and *P. harinasutai* formed a separate clade in the molecular phylogenies, and were reciprocally monophyletic with respect to other species. The observed similarities at the secondary structural level are further reflected at the energy level. Only difference in their topology is due to differences in nucleotide sequences. These secondary structure predictions indicate that the domains basepair to form a core region central to several stem features implying that conservedness is more important for the proper rRNA folding pattern. Moreover the observed phylogenetic trend was identified with respect to the target accessibility sites for different isolates. The orders of preference were interior loop, bulge loop, multiple branch loop, hairpin loop and exterior loop in all the isolates.

***Fasciolopsis buski*:** With regard to *F. buski*, for which only 18S rDNA sequences were available so far, ITS regions were sequenced for the first time in the present study. The nucleotide sequences obtained for ITS1 & ITS2 of rDNA (of both adult and egg origin), were compared with sequences of other trematode species obtained from Genbank. The fragments of amplified DNA were estimated to be 480-550bp long. Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481bp and 498bp; 559bp and 548bp for ITS1, forward and reverse sequences,

respectively in adult and egg. The Blast hit results showed that the query ITS2 *Fasciolopsis buski* sequence is more similar to the sequence of the species *Fasciola gigantica, Echinostoma revolutum, Isthmiophora melis, Echinostoma sp, Paryphostomum radiatum, Echinostoma trivolvis, Echinostoma paraenei, Fasciola sp, Fasciola hepatica* and *Isthmiophora hortensis.* Phylogenetic trees were obtained by comparing the sequences of *F. buski* and available ITS (1&2) sequences for other digenean trematodes including fasciolid species. Boot strapping of the sequences with Neighbour-Joining revealed significant support (100%) for the clade containing *F. buski*, *F. hepatica*, *F. gigantica* and *Fascioloides magna* indicating reliable grouping among different members of fasciolids.

- ***Fasciola gigantica*:** The PCR-amplified products were successfully obtained and were compared with sequences of other fasciolid species. The fragments of amplified DNA were estimated to be 480-550bp long. For comparative purpose, the ITS2 sequences of fasciolids from various geographical regions were obtained from GenBank. The Blast hit results showed that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp., *F. hepatica* and *F. gigantica* besides *Fasciolopsis buski* and *Fascioloides magna* (both belonging to the same family, i.e., Fasciolidae). Primary sequence analysis of *Fasciola* spp, revealed a close relationship between the query sequence (from NE India) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia. ITS2 sequence of the Indian isolate revealed closest similarity with isolate from China with significant bootstrap value revealing that the species prevailing in the region is *Fasciola gigantica.* Sequence of another Indian isolate, designated as *F. gigantica* (Accession-EF198867) from IVRI, Bareilly, showed absolute match with *F. hepatica.* Hence on the basis of molecular similarity this isolate should be identified as *F. hepatica* and not *F. gigantica.*

Secondary structure analysis of data confirmed the results mentioned for primary sequence analysis. Five predicted RNA secondary structures were reconstructed from the unique sequences with highest negative free energy of *F. gigantica* to provide the basic information for phylogenetic analysis. The ITS2 plus flanking regions of nuclear region ranged from 720bp in *F. gigantica* India to a minimum length of 361bp in *F. gigantica* China. *F. gigantica* isolates from India and China show overall similarity in the ITS2 rRNA folding and have identical secondary structure. Secondary structures of remaining species are somewhat variant. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences.

- **Design of genus/species-specific primers**
  - To establish a more direct PCR procedure for species discrimination and identification, the genus/species-specific primers were designed using Primer3, a widely used program for designing PCR primers to target unique rDNA region spanning ITS2 for all the three trematodes viz. *Paragonimus westermani, Fasciolopsis buski* and *Fasciola gigantica.* Sequence analysis of the ITS2 PCR products revealed no stage-specific or intra-specific variations in length or composition. Multiple sequence alignment was done for all the three sequences using ClustalW programme. The *P. westermani*-specific (PwAR1), *F. buski*-specific (FbMR1) and *F. gigantica*-specific (FgMR1) primers were designed to target the 3'- terminal position of the ITS2 sequences, and the specificity of these primers was evaluated by PCR using primer 3S. As was expected, the primer set 3S-PwAR1 amplified a PCR product only from *P. westermani* DNA, 3S-FbMR1 amplified a PCR product only from *F. buski* DNA and 3S-FgMR1 amplified a PCR product only from *F. gigantica.* Primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample. These PCR

products were sequenced using the corresponding specific primer and were confirmed to be the ITS2 region of rDNA from the respective species.

Sequences deposited in GenBank

i) **DQ351841-** *Fasciolopsis buski* adult 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ii) **DQ351842-** *Fasciolopsis buski* egg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

iii) **DQ351843-** *Fasciolopsis buski* adult internal transcribed spacer 1, partial sequence.

iv) **DQ351844-** *Fasciolopsis buski* egg internal transcribed spacer 1, partial sequence.

v) **DQ351845-** *Paragonimus westermani* metacercariae 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vi) **EF027103-** *Fasciola gigantica* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vii) **EF027104-** *Fasciola gigantica* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

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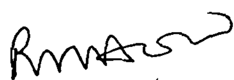
I, **Pramod Kumar Prasad**, hereby declare that the subject matter of the thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University / Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

Pramod Kumar Prasad
(Candidate)



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CONTENTS

Acknowledgement i-iii

Preface iv-vi

CHAPTER- 1: Status and prevalence of paragonimiasis in the region 1-19

Introduction 2

Materials and methods 5

- Survey of intermediate hosts
- Parasite material
- Light microscopy
- Scanning electron microscopy
- Analysis of prevalence data

Results 11

- Metacercarial infection in crab hosts in suspected foci
  - Status and prevalence
  - Morphology of metacercaria
- Intra-molluscan stages in suspected foci

Discussion 18

CHAPTER- 2: Molecular characterization of *Paragonimus* and other trematodes 20-94

Introduction 21

Materials and methods 25

- Collection of parasite material 25
- DNA isolation 25-26
- DNA amplification and sequencing 27
- Nucleotide sequence analysis 28
- Molecular Phylogenetic analysis 29
- Prediction of ITS2 RNA secondary structures and analysis 30

- Genus-specific primer design 31

Results 32-89

- PCR amplification and analysis
  - *Paragonimus* sp. 32-49
  - *Fasciolopsis buski* 50-74
  - *Fasciola gigantica* 75-86
- Amplification of specific primers by PCR 87-89

Discussion 90-94

CONCLUSION 95-96

SUMMARY 97-104

REFERENCES 105-120

Bio-data

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Doing research and earning a degree is not just a simple race but it's a heptathlon wherein you have to go through various trials and tribulations before you touch the finishing line. It is an exhilarating experience after touching the ribbon on completing a race and in the race of life nothing can be demarcated as being purely of sports or academic, the same experience applies to all acts of life As in sports we need a coach who hones the skill, brings forth the hidden talent, guides and takes care of the minutest aspects leading to the road to perfection, in research we have guides who perform the same encore and well who else but Prof. Veena Tandon and Prof. Anupam Chatterjee, who represents the highest echelons of this fast dwindling fraternity of sincere, enthusiastic and energetic supervisors, are role model for me as a coach-cum-guide in my brief career of research of around five years. Their constructive criticism, valuable comments, constant encouragement, and sometimes, soothing scolding, are some of the memories to be cherished the whole life. I cannot express my gratitude and acknowledgement in words since they are not part of vocabulary of any world renowned dictionaries, so I would like to be a bit moderate and pay them the highest tributes and regards and wish them all the very best for all their future endeavors I hope this is not the end but just the beginning of a lifelong collaboration and I would like to be a part of their brilliant team of researchers forever

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encouragements, inspiration and at times sacrifices that has made this work successful and also to all my family members and relatives for their well wishes and keeping me morally upright.

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Preface

Trematodes are flukes belonging to the phylum Platyhelminthes and about 70 species of food-borne trematodes are known to infect humans. Thus, their infections are classified as zoonoses because they are viewed as animal diseases transmitted to humans. Some infections appear to be rare, while others are common and cause serious disease. Food-borne fluke infections or trematodiases are emerging as a major public health problem worldwide with over 40 million people affected and over 10% of the world population at risk of infection. The major concentration of these infections is in Southeast Asian and Western Pacific Regions, where the epidemiological factors (including the prevalent socio-cultural food habits) are conducive for transmission of these infections. The preponderance of these infections is usually in food deficit poor communities that lack access to proper sanitary infrastructure. These parasites originate an unacceptable burden of sufference and mortality among animals and humans and also cause serious damage to aquaculture, which is a valuable source of food and employment in developing countries.

Food-borne trematodes present particular epidemiological and clinical features that should be carefully considered for the development of appropriate preventive and control measures. Transmission to humans is almost entirely caused by consumption of food containing infective metacercarial stages of the parasite. The distribution of these infections is highly focal, depending on the food habits of people and on the presence of susceptible snails, the latter serving as the first intermediate host in the multi-host life cycle of the pathogen. Trematode life cycle may implicate more than one intermediate host, with snails the gastropod molluscs being the first intermediate host, the 2nd intermediate host can be a crustacean, insect or mollusc. FBTs also present low host specificity, and a high number of definitive reservoir hosts can contribute to the contamination of water and snails, making it extremely difficult to control this aspect of the parasite cycle. From a clinical point of view, trematode eggs frequently cannot be

detected in the stool or sputum of suspected cases and the disease presents a specific symptomatology, leading to frequent misdiagnoses.

Among helminth parasites, the lung fluke *Paragonimus* gains considerable importance from veterinary and medical points of view because of its diversified effect on its host. The cost of the parasite in terms of misery and economic losses to human society is inaccessible. It is estimated that about one million people in Asia are infected with *P. westermani* through eating raw crustaceans containing metacercariae or by ingesting uncooked meat of paratenic hosts such as pigs. The crustacean that serve as the second intermediate host for *Paragonimus* spp may also harbour the infective metacercariae of other trematodes like *Fasciolopsis buski* and *Fasciola giga*ntica. In India the *Paragonimus* infection is sporadically reported from tiger, dog, cat, mongoose and humans. In recent years, a sizeable population was found infected in Manipur where crustaceans are consumed as routine food for protein source. Though the fluke is known to parasitize a wide range of mammalian hosts, the status of its prevalence, its host range (intermediate and final) and its possible reservoir host in nature from where human beings are getting the infections is not known, particularly in Northeast India, which is regarded as the endemic focus for human paragonimiasis. Even there is no record of the exact species of the adult worm recovered from pigs in Assam.

In crab muscle different types of metacercariae are available which could be either *Paragonimus spp*, *Fasciolopsis buski* or *Fasciola gigantica.* No attempt has been made so far to characterize the prevalent parasite species at the molecular level, as also to discriminate between the Crustacea-borne infective stages of these parasites, which pose a potential zoonotic threat in the northeastern region. Reproducible and sensitive diagnostic techniques are required for accurate genus and species discrimination and identification of the parasite. DNA techniques utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA have been employed to resolve taxonomic issues related to various helminthic parasites. These methods could be used for epidemiological investigations of the prevalence of the metacercariae in the crab host

and for controlling lung fluke disease and other trematode zoonoses. As the crustaceans are potential intermediate host for and harbour metacercariae of more than one trematode, identification and establishment of discriminatory molecular markers for the various metacercarial types occurring in them is essential.

The proposed study, therefore, aimed at examining the snail and crustacean species prevalent in Northeast India to recover the larval stages, if any, of *Paragonimus* and to identify the exact species prevailing and responsible for the disease. By using PCR-based molecular techniques we intended to identify the species of the parasite collected from muscles of the locally edible crabs and to generate specific molecular markers with vital usage towards correct diagnostics.

The study on completion would provide definite information about the prevalence and endemism of paragonimiasis in NE India. Identification of the plausible molluscan and crustacean intermediate hosts would provide an insight into the epidemiology and transmission dynamics of paragonimiasis and other Crustacea-borne trematode zoonoses in the region. Since *Paragonimus* is a zoonotic species transmittable between animals and humans and vice versa, causing not only bronchial haemoptysis, pneumothorax and secondary bacterial infections but also damaging several vital organs including the brain tissues, the study would have significance in the following directions as it would -

a) establish intermediate host(s) implicated in propagation of life cycle of *Paragonimus* species in Northeast India,
b) help in identifying the exact species of *Paragonimus* prevailing in the region, and
c) establish molecular methods for accurate discrimination between metacercariae of the various trematode species plausibly occurring in Crustacea.

Finally the investigation would explain locality-wise possible health hazard caused by the parasite and prompt the way for its prevention and control.

Chapter - 1

Status and prevalence of paragonimiasis in the region

INTRODUCTION

Trematodiases are the zoonoses that are caused by trematodes or flukes (Platyhelminthes: Trematoda: Digenea). The lung flukes of the genus *Paragonimus* have been known as one of the most important zoonotic parasites causing paragonimiasis, also known as endemic haemoptysis, in man. Zoonosis is a disease or infection naturally transmitted between vertebrate animals and man, depending on life cycle of parasite, the kind of hosts and geographical distribution of species (WHO, 1995). Zoonotic infections can be transmissed directly from environment when infective stages of parasite contaminate water or food. Source in humans come from foodstuffs of animal origin (eating meat, fish, crabs, shrimp, molluscs raw, undercooked, smoked, pickled or dried).

According to World Health Organisation, people currently infected with food-borne trematodes alone exceed 41 million worldwide; people at risk including those in developed countries are around 750 million. It is estimated that over 20 million people are infected worldwide due to several species of *Paragonimus* (Toscano et al., 1995). Over 40 species are known to infect the lung of different mammalian hosts throughout the world (Bunnag and Harinasuta, 1985) and approximately 15 species are known to infect humans. The parasite can migrate to several other vital tissues including brain (Kusner and King, 1993). The best-known species is *P. westermani* (Kerbert, 1878) Braun, 1899 - a human parasite that can undergo development in as many as 16 different snail species and 50 crustacean species. Beside *P. westermani*, *P. pulmonalis* (Baelz, 1880) Miyazaki, 1978; *P. ohirai* Miyazaki, 1939; *P. iloktsunensis* Chen, 1940; *P. skrjabini* Chen, 1959; *P. miyazaki* Kamo, 1961 and *P. heterotremus* Chen and Hsia, 1964 - all reported to be occurring in Asia; *P. africanus* and *P. uterobilateralis* Voelker and Vogel, 1965 in Africa; and *P. mexicanus* Miyazaki and Ishii, 1968 in America are considered pathogenic to man; while *P. westermani* is distributed mostly in Asia, *P heterotremus* is the predominant causative agent of paragonimiasis in Thailand (Blair et al., 1999a, b).

Adult species of *Paragonimus*, generally found in the lungs of their mammalian hosts, have been reported from Pakistan, East to South-eastern Russia, China, South Srilanka, Indonesia, Papua New Guinea, The Great Lakes region of Canada south of Brazil, Peru and West, Central and South Africa (Sogandares-Bernal and Seed, 1973; Zhong et al., 1981; Lamothe-Argumedo, 1985; Choi, 1990; Xu, 1991; Nwokolo, 1991; Shim et al., 1991; Im et al., 1993; Hinz, 1996; Tomimura et al., 1989). Paragonimiasis, which is quite prevalent in China, Japan, Korea, Philippines and Near-East Countries, has been sporadically recorded from tiger, dog, cat and mongoose from several parts of India (Gulati, 1926; Dutta and Gupta, 1978; Baruah et al., 1985; Gaur et al., 1980; Arora and Das, 1988; Razaque et al., 1991; Pythal et al., 1993a, b).

In the context of India, Chandler and Read (1961) indicated Bengal, Assam and some other parts of the country as endemic foci of human paragonimiasis. In recent years this infection has been reported in a sizeable human population of Manipur, a north-eastern state of the country (Razaque et al., 1991; Singh et al., 1993; Singh and Singh, 1997). Though the fluke is known to parasitize a wide range of mammalian hosts representing as many as eleven families, the status of its prevalence and host range in India is not well documented. Very recently in Manipur and Arunachal Pradesh (Northeast India), the suspected foci of human infection where consumption of crustacean intermediate hosts is of regular practice, the Chinese species, *P. hueitungensis* and *P. heterotremus*, respectively were identified as etiological agents of paragonimiasis (Singh, 2002; Singh, 2003; Narain et al., 2003). However, no or scanty information is available about the prevalence of the parasite among its molluscan and crustacean intermediate hosts as even in the suspected foci of human infection.

Morphology of the encysted and excysted metacercariae, which occur as the infective stage in the muscle tissue of the crustacean second intermediate host, has been conventionally used in identification of species of *Paragonimus*. The external appearance of the newly excysted metacercariae has also been studied for various species of the genus *Paragonimus* using scanning electron microscopy (Higo and Ishii, 1984, 1987;

Tongu et al., 1985, 1987, 1995; Kim et al., 1987; Sugiyama et al., 1990). During an exploratory survey of edible crab species, undertaken to ascertain the prevalence of crustacea-borne trematodiasis in the region, stream crabs from Miao, Changlang District of Arunachal Pradesh were found to be heavily infected with metacercariae of *Paragonimus* species. The present study was aimed at studying in depth the prevalence pattern of *Paragonimus* among potential intermediate hosts in the region and identifying the *Paragonimus* species implicated in infection in the region using surface fine topography of the metacercariae.

MATERIALS AND METHODS

1. Survey of intermediate hosts

(i) **Crabs:** The infective stage of the parasite, the metacercaria occurs in the muscles of crabs or crayfishes which serve as the 2nd intermediate host; the infection in the final host is contracted by eating inadequately cooked infected crustaceans. The crab hosts (Plate 1) were surveyed from rural localities and countryside where eating of crabs is a common food practice among the natives of the region. Emphasis was given to procure those species which are commonly used in local traditional cuisine. A total of 562 crustacean hosts representing 3 genera and 4 species were examined for the recovery of metacercarial infection (Table 1). Naturally infected freshwater crabs were mostly collected from mountain streams of the suspected foci of infection. The collection sites are depicted in the maps of the region (Figure 1).

(ii) **Snails:** The gastropod mollusks are the usual first intermediate hosts in the life cycle of most trematodes which harbor in their soft tissues, the pre metacercarial developmental stages like the sporocyst and redia, with the latter giving rise to many cercariae that emerge from the snail and invade the second intermediate host. In view of this, snail species (Plate 2) were also collected from selected localities wherever the crab hosts were suspected to be positive for metacercarial infections. Hundreds of specimens of snail species representing 4 genera with one species each were examined for the recovery of intramolluscan stages of digenetic trematodes (Table 2).

Plate 1

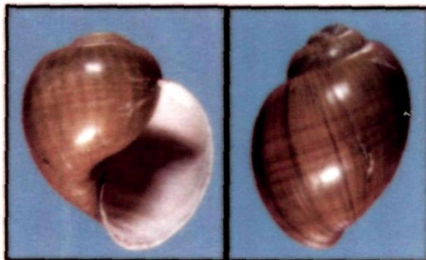
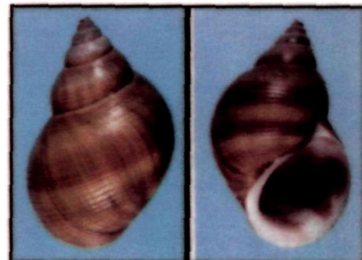
***Barytelphusa (M) lugubris mansoniana* (Henderson, 1893)**

***Barytelphusa (Maydelliathelphusa) lugubris lugubris* (Wood-Mason, 1871)**

***Sartoriana spinigera* (Wood-Manson, 1891)**

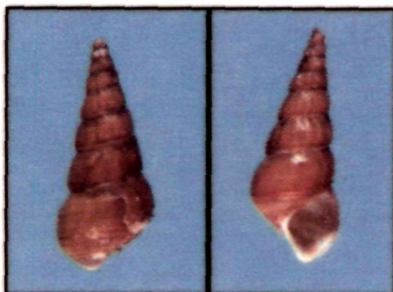
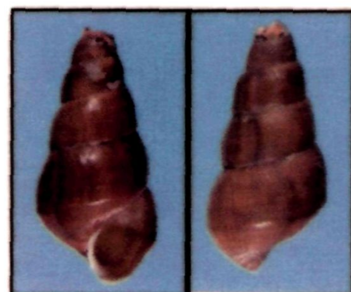
***Lobothelphusa woodmansoni* (Rathbun, 1905)**

Plate 2

***Pila theobaldi* (Hanley)**

***Paludomus (Paludomus) conica* (Gray)**

***Melanoides tuberculata* (Mueller)**

***Brotia (Antimelania) costula* (Rafinesque)**

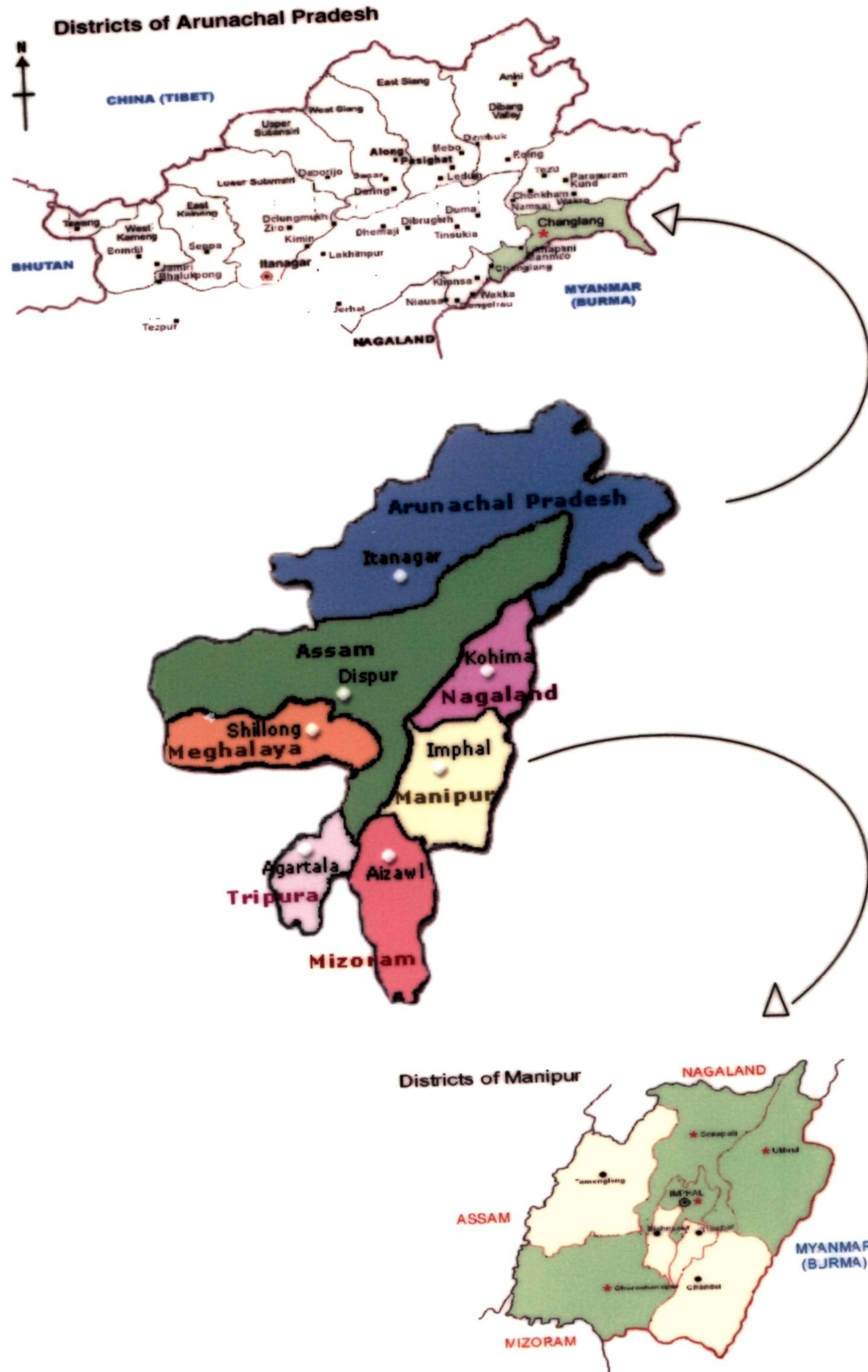


Figure 1. Northeastern states of India: (a) Arunachal Pradesh (b) Manipur

Table 1. Localities surveyed for collection of crabs

Sl. No.	Species	Locality (Collection sites)	Nos. examined
1	*Barytelphusa (Maydelliathelphusa) lugubris lugubris* (Wood-Mason, 1871)	Kharshang (Arunachal)	250
2	*Barytelphusa (M) lugubris mansoniana* (Henderson, 1893)	Miao (Arunachal)	78
3	*Lobothelphusa woodmansoni* (Rathbun, 1905)	Gossaigaon (Assam) Bishenpur (Manipur)	38 14
4	*Sartoriana spinigera (Wood-Manson, 1891)*	Gossaigaon (Assam) Aizawl (Mizoram) Imphal (Manipur) Itanagar (Arunachal) Miao (Arunachal)	22 56 22 44 38

Table 2. Localities surveyed for collection of snails

Sl. No.	Species	Locality (Collection sites)	Nos. examined
1	*Pila theobaldi* (Hanley)	Gossaigaon (Assam) Itanagar (Arunachal)	>500
2	*Paludomus* (Paludomus) *conica* (Gray)	Gossaigaon (Assam) Aizawl (Mizoram) Imphal (Manipur) Itanagar (Arunachal) Miao (Arunachal)	>200
3	*Brotia* (Antimelania) *costula* (Rafinesque)	Kharshang (Arunachal) Miao (Arunachal)	>500
4	*Melanoides tuberculata* (Mueller)	Gossaigaon (Assam) Bishenpur (Manipur) Imphal (Manipur) Miao (Arunachal)	>200

2. Parasite material

i) From Crabs:

Metacercariae were isolated from the muscles of the crustacean host by digestion technique. The crabs were cut into small pieces with the help of scissors, minced and digested by overnight incubation at 37°C in the artificial gastric juice.

[**Composition:**

HCL Conc. (35 – 37%) - 7-10ml

Distilled water - 1000ml

Pepsin (1:10000) - 6g]

The digested materials were filtered through mesh wire sieves and the filterable sediments were washed repeatedly with tap water in order to get a clearer supernatant. The sediments were examined for metacercariae under a dissecting stereoscopic microscope.

ii) From Snails:

Intra-molluscan stages were recovered by teasing the gut content in a petridish and examined under dissecting stereoscopic microscope. A few specimens were duly processed for whole mount preparation and subsequent light microscopy (LM) observations.

3. Light microscopy (LM)

Freshly recovered metacercariae from crab hosts and intramolluscan larval stages- sporocyst, redia and cercariae, were washed in 0.7% saline solution and narcotized with few drops of 70% ethyl alcohol. They were gently flattened between a slide and a cover glass, fixed in70% ethyl alcohol and stained with Borax carmine or Meyer's carmalum, followed by dehydration through usual dehydration media i.e. ascending grades of ethyl alcohol, clearing in Methyl benzoate and mounting in Canada balsam using standard protocols. All the prepared permanent slides were observed and studied under Wild M5APO stereo microscope, vision analyzer, Zeiss image

analyser, Leica S6D trinocular stereo zoom microscope and Leitz Ortholux-2 research microscope. Measurements of the specimens were taken using stage and ocular micrometers and/or morphometric software in the image analyser.

4. Scanning electron microscopy (SEM)

Live specimens recovered were washed thoroughly in 0.7% saline solution and were fixed in 10% cold neutral buffer formalin at 4^{0}C for 12-18 h. Following fixation, the specimens were washed in phosphate buffer and dehydrated with ascending grades of alcohol and ethanol-amyl acetate mixture to pure amyl acetate. After their final treatment in dry amyl acetate, the specimens were critical-point dried using liquid carbon dioxide. In lieu of critical-point drying the specimens after washing in phosphate buffer were dehydrated in ascending grades of acetone, treated with tetramethyl silane [TMS-$(CCH3)_4Si$, boiling point 26.3°C, surface tension 10.3 dynes/cm at 20°C] for 10 minutes and dried off TMS at 25° C (Dey et al., 1989; Roy et al., 1991). The dried samples were metal coated with gold in a fine coat ion sputter JFC-1100(JEOL). Observations were made with the scanning electron microscope JSM 35CF (JEOL) and LEO 435 VP SEM at electron-accelerating voltages ranging between 10-20 kV.

5. Analysis of prevalence data

The following parameters were used to analyze the data following Margolis et al. (1982):

i. **Prevalence** – Number of individuals of a host species infected with a particular parasite species divided by the number of host individuals examined x 100 (= percentage of infected hosts of each species).

ii. **Intensity**- number of individuals of a particular parasite species in each infected host in a sample.

iii. **Mean intensity**- mean number of individuals of a particular parasite species divided by the number of host species examined in a sample.

RESULTS

I. Metacercarial infection in crab host in suspected foci

(i) Status and Prevalence: 3 genera of crabs surveyed from various localities in Arunachal Pradesh, Assam, Manipur and Mizoram. Only one i.e. *Barytelphusa* was found to be harbouring metacercarial cyst. The details of crab hosts examined from the various localities and the status of infection among them are depicted in Table 3. The crabs collected from all the localities excepting those in Arunachal Pradesh did not harbor any metacercarial infection. However, both the collection sites in Arunachal Pradesh were revealed to be positive for this infection. In the Kharshang site *Barytelphusa* (*M*) *lugubris lugubris* was found to be positive with a prevalence of 26%, the intensity of infection being in the range of 9-68 (Mean Intensity=36). In Miao region, the other sub species namely *B.* (*M.*) *l. mansoniana* revealed a much higher prevalence of infection i.e. 87%, the intensity of metacercarial infection ranged between 3-175 (Mean Intensity=38).

(ii) Morphology of metacercaria: The newly excysted metacercaria has an elongate body (815.91µm x 492.79µm) in size; the ventral sucker situated somewhat pre-equitorally, is larger than the oral; the intestinal caeca are long and extend up to the posterior end of the body; the conspicuous excretory bladder extends medially in the intercaecal space (Figure 2a-e). The SEM observations revealed that the encysted metacercaria is oval in shape and has smooth surface. The whole body surface of the excysted metacercaria is covered with numerous single-pointed and thorn-like tegumentary spines; those covering the anterior part of the body are bigger in size and show a gradual reduction in size towards the posterior end. The tegument in the circum-oral region has a dense aggregation of small spines that are arranged in several circular rows. A few dome-shaped papillae abound on the rim of the oral sucker and adjacent area in a random

fashion. The tegument of the ventral sucker and its surrounding region and that of the general body surface also has a dense spination. The surface fine topography in the area reveals the presence of many papillate protuberances, but the latter are randomly distributed and do not exhibit a definite pattern of distribution and numbers. (Figure 3a-k).

Table 3. Prevalence/Intensity of metacercarial infection in crab hosts in suspected foci.

Host	Locality (Collection sites)	Nos. infected / Nos. Examined	Prevalence (%)	Intensity of infection (Mean)
Barytelphusa (M.) lugubris lugubris	Kharshang (Arunachal Pradesh)	65/250	26%	9-68 (36)
B. (M.) l. mansoniana (Henderson, 1893)	Miao (Arunachal Pradesh)	68/78	87%	3-175 (38)
Lobothelphusa woodmansoni (Rathbun, 1905)	Gossaigaon (Assam) Bishenpur (Manipur)	0/38 0/14	- -	- -
Sartoriana spinigera (Wood-Manson, 1891)	Gossaigaon (Assam) Aizawl (Mizoram) Imphal (Manipur) Itanagar (Arunachal) Miao (Arunachal)	0/22 0/56 0/22 0/44 0/38	- - - - -	- - - - -

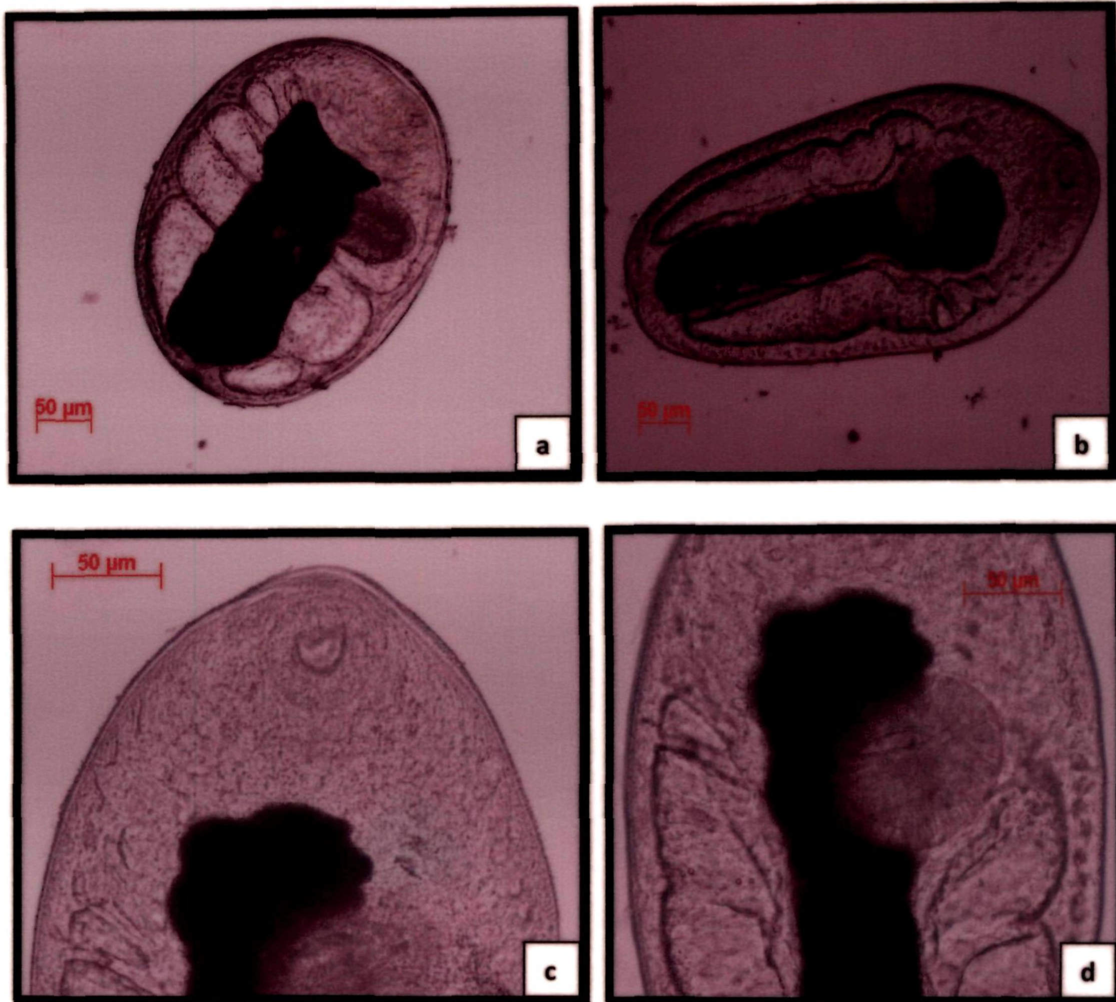


Figure 2a-d. Light microscopic view of metacercaria: whole mounts, stained preparations. (Scale bar = 50 µm)

a) Encysted metacercaria

b) Excysted metacercaria

c, d) Oral and ventral sucker, respectively, of excysted metacercaria

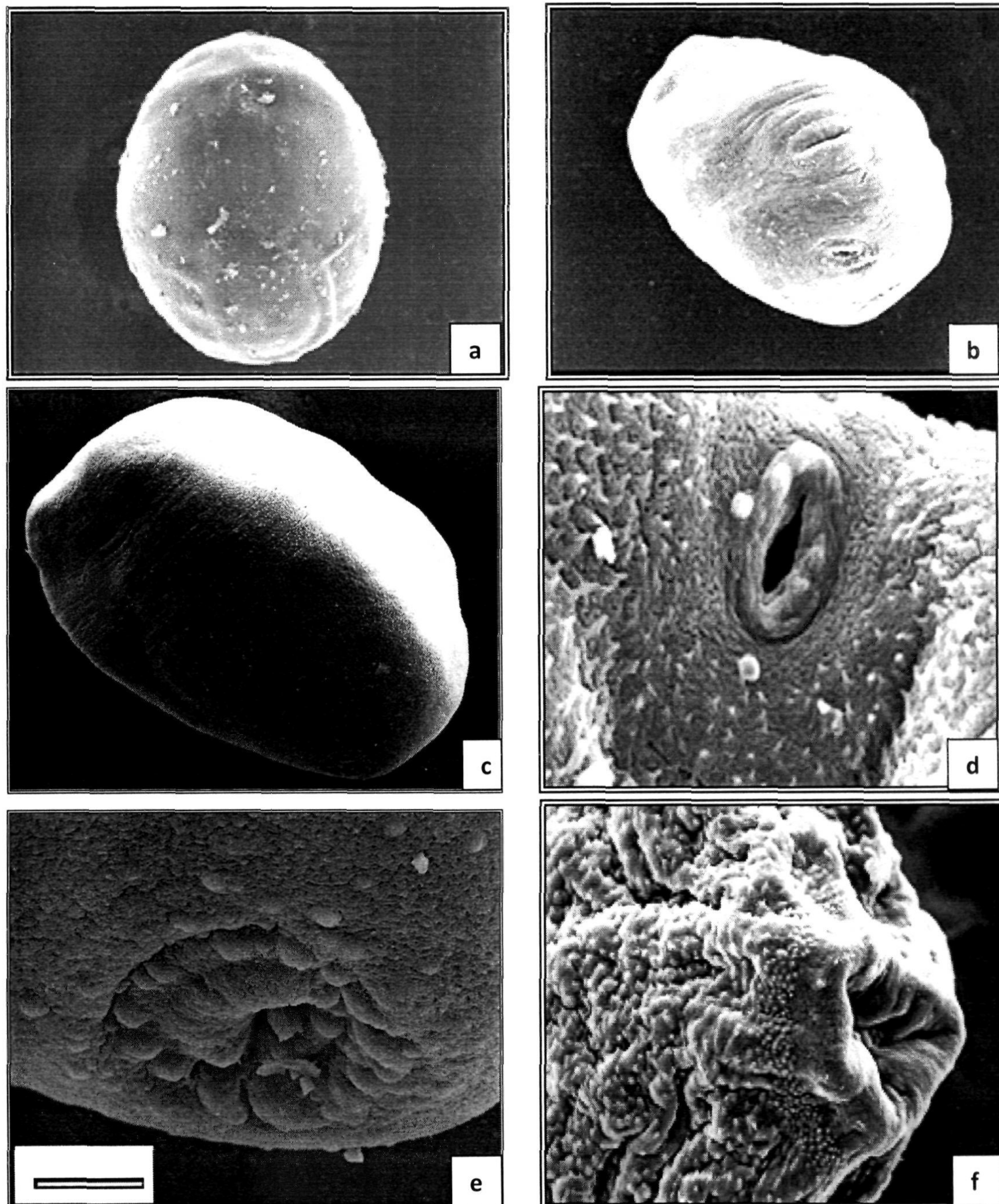


Figure 3a-f. Scanning electron micrographs of recovered metacercariae.

a, b, c) Encysted and excysted metacercaria (Scale bar = 100μm)

d, e) Oral sucker region. Note the presence of a few domed papillae on and around the rim of the sucker. (Scale bar= 10 μm)

f) Another view of the oral end, depicting spination pattern on circum oral tegument. Several rows of minute spines arranged in circular fashion are conspicuous. (Scale bar= 10 μm)

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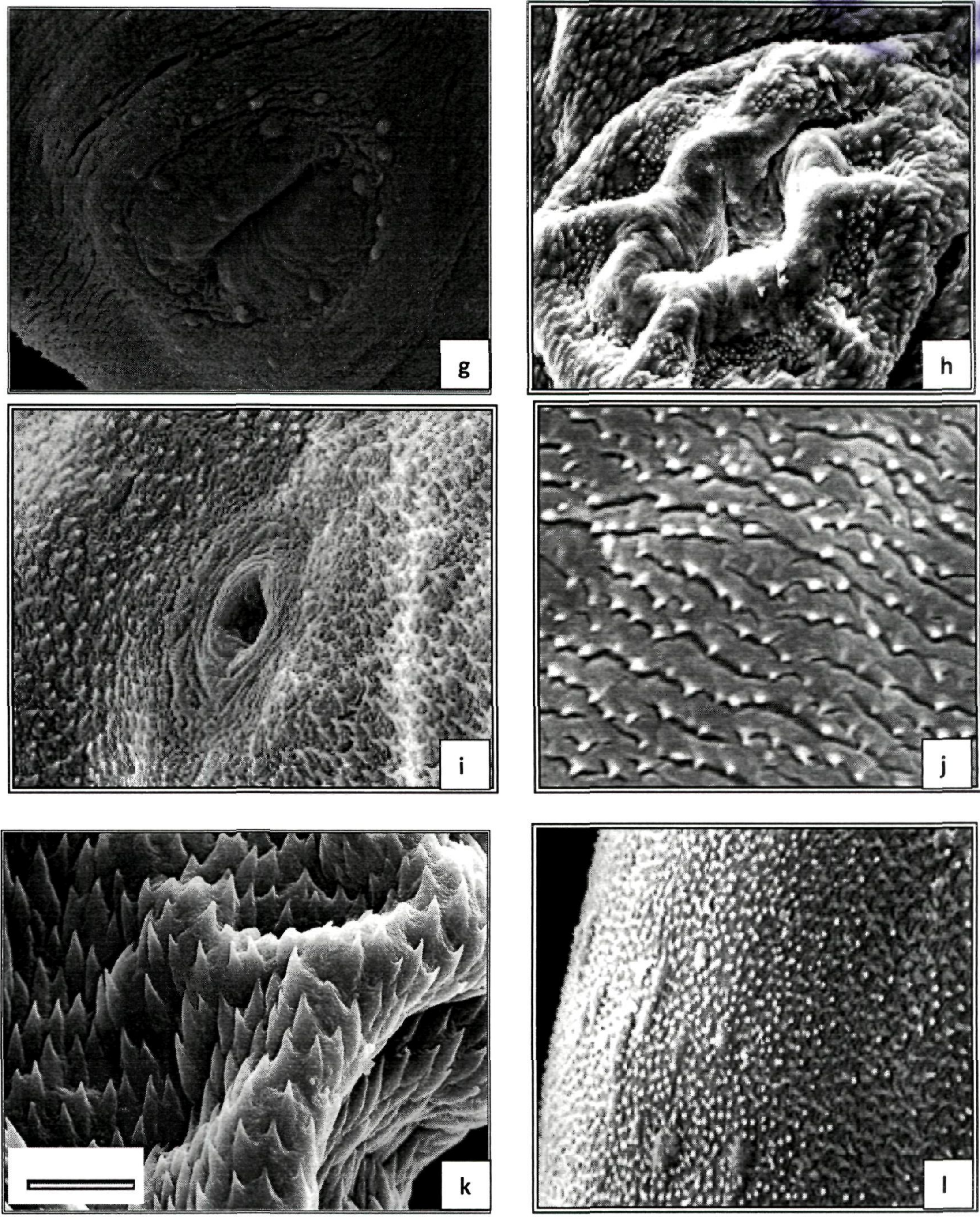


Figure 3g-l. Scanning electron micrographs of recovered metacercariae.
(Scale bar= 10 μm)

g) Ventral sucker with randomly distributed papillate protuberances.

h) Ventral sucker region in another specimen.

i) Spination in anterior region of body.

J, k) Single-pointed, backwardly directed spines in the mid-body region.

l) Smaller spines in the posterior part of the body.

II. Intramolluscan stages in suspected foci

4 species of snails from the same localities that were surveyed for collection of crabs were examined for recovery of intramolluscan stages (Table 4). Of these, while the species of *Pila, Paludomous* and *Melanoides* were found negative for any infection, it was only *Brotia* (*A.*) *costula* from which sporocyst, redia and cercaria stages were recovered. Of more than 500 snails both from Kharshang and Miao regions- the localities same as those of the crabs infected with metacercariae- about 10% harboured the various stages of digenean larvae (Figure 4). The cercariae recovered from these snails were of two types:

(i) typical leptocercous, with long slender tail and opthalmate (having a pair of eye spots), and

(ii) typical distome amphistomid, having the ventral sucker at the base of the fore body.

The microcercous cercariae were not found to be present in any of the infected snails.

Table 4. Prevalence of cercarial infection in Snail hosts in suspected foci.

Snail Hosts	Locality (Collection sites)	Nos. examined	Nos. infected (%)
Pila theobaldi (Hanley)	Gossaigaon (Assam) Itanagar (Arunachal)	>500	-
Paludomus (Paludomus) *conica* (Gray)	Gossaigaon (Assam) Aizawl (Mizoram) Imphal (Manipur) Itanagar (Arunachal) Miao (Arunachal)	>200	-
Brotia (Antimelania) *costula* (Rafinesque)	Kharshang (Arunachal) Miao (Arunachal)	>500	~50 (10%)
Melanoides tuberculata (Mueller)	Gossaigaon (Assam) Bishenpur (Manipur) Imphal (Manipur) Miao (Arunachal)	>200	-

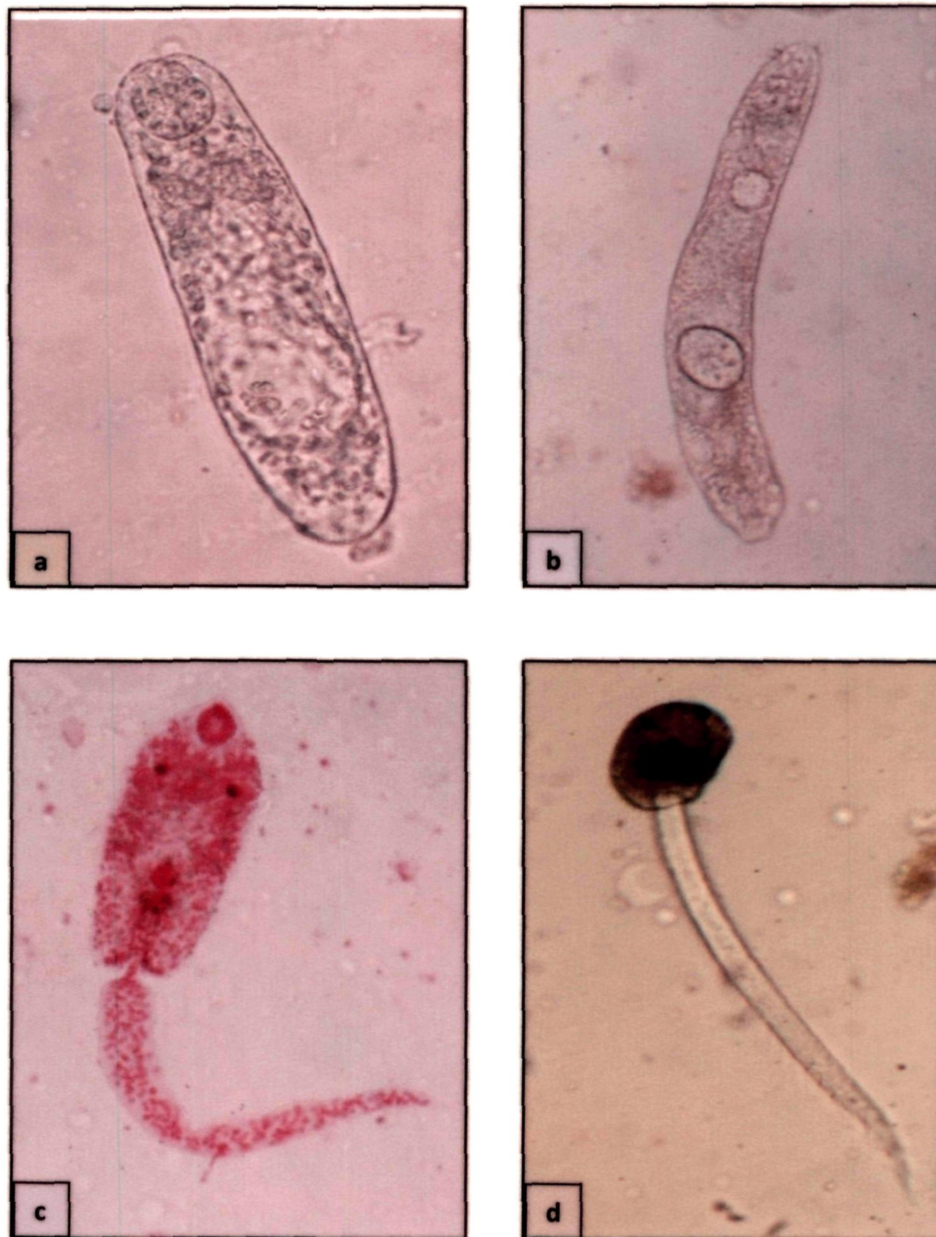


Figure 4. Intra-molluscan stages recovered from infected snails

(a) Sporocyst
(b) Redia
(c) Opthalmate leptocercous cercaria
(d) Amphistome cercaria

DISCUSSION

In the present study, of the various crab species examined only *Barytelphusa lugubris lugubris* and *B.* (*M.*) *l. mansoniana*, both collected from locales in Arunachal Pradesh were found to be naturally infected with *Paragonimus* metacercariae. The other crab species examined did not reveal any infection with *Paragonimus* metacercariae. In earlier studies, another crab species *Potamiscus manipurensis* was reported as the natural host for the lung fluke infection in Senapati District of Manipur (Singh and Singh, 1997). Narain et al. (2003) had reported the occurrence of another *Paragonimus* species *heterotremus* in *B. lugubris* crabs from Changlang District of Arunachal Pradesh, perhaps the same locality as of the present study. These authors also reported *Sartoriana spinigera* crabs to be free of any metacercarial infection. The present study also corrabortes that the crab species of the genus *Barytelphusa* are the second intermediate host for *Paragonimus* in the studied sites in Arunachal Pradesh.

Surface fine topography of encysted and newly excysted metacercariae has been described in respect of several species of *Paragonimus* e.g. *P. skrjabini, P. iloktsuensis, P. ohirai, P. pulmonalis, P. westermani* (diploid type), *P. miyazaki* (triploid types of *P. westermani*), *P. mexicanus, P. heterotremus* and *P. westermani* (He Yixun et al., 1982; Li et al., 1987; Higo and Ishi, 1987; Aji et al., 1984; Tongu et al., 1987; Sugiyama et al., 1990; Jiang and Xia, 1993; Sugiyama et al., 2001). Characters such as the number and distribution pattern of tegumental papillae around the oral and ventral suckers of the newly excysted metacercariae have been used as the morphological taxonomic tools for differentiating the various species of *Paragonimus*. For example, the number and size of the domed papillae in metacercariae of *Paragonimus* spp in Japan seems to vary with the species (Higo and Ishii, 1984, 1987), though geographical differences do not supposedly exist with regard to the morphology of the excysted metacercariae of *P. westermani*, in which the number of papillae ranges between 5 and 13 (Sugiyama et al., 2001). However, these morphological characters are prone to variations and thus not absolutely reliable.

The metacercariae under the present study revealed a ventral sucker larger than the oral unlike *P. heterotremus* (in which the oral sucker is larger than the ventral), a species that has earlier been reported from the same region in Northeast India (Narain et al., 2003). The surface fine topography, including the number and distribution of papillae and spination pattern of the present material suggests more closeness and resemblance with *P. westermani*. In the latter species, though Sugiyama et al. (2001) reported the occurrence of dome-shaped papillae as evenly distributed over the whole body and in circular fashion around the suckers, as per the observations the papillae were fewer in number and revealed to be randomly scattered across the general body surface. Thus on the basis of surface of surface fine topography features of the excysted metacercaria it may be concluded that *Paragonimus* species occurring in Kharshang and Miao regions of Arunachal Pradesh is indeed *P. westermani.*

Regarding the intramolluscan stages, the cercariae were recovered from only one of the four species of snails surveyed and examined. However, the cercariae recovered were all amphistome, leptocercous type. The cercaria stage of *Paragonimus* species is typically microcercous type, characterized by a tiny stumpy tail (Dawes, 1946). However, in none of the infected snails were the microcercous cercariae ever recovered. Hence, it may be assumed that the snail species examined during the present study are not the potential first intermediate host for *Paragonimus* in the study area. A more exhaustive exploration of snail host species from the localities where crabs reveal the prevalence of *Paragonimus* metacercariae needs to be undertaken to ascertain the mollusc species implicated in the life cycle of the parasite.

Chapter - 2

Molecular characterization of *Paragonimus* and other trematodes

INTRODUCTION

The identification of closely related species based on morphological characters alone can be difficult. This is particularly the case of soft-bodied animals such as digenean trematodes. The taxonomy of trematodes has been based mainly on morphological data complemented with ecological, cytological, and pathological results as well as clinical manifestations. Morphological differences found on stained and mounted adult specimens have been widely used to discriminate between platyhelminth species (Miyazaki, 1974). It is possible to distinguish between the adult trematodes but much variation exists. Differentiating between two species is not possible on the basis of clinical, pathological or immunological findings and their eggs are morphologically very similar (Lotfy and Hillyer, 2003). Consequently, where two species occur concurrently or in overlapping geographical distribution, it is not possible to be certain as to which species is responsible for the disease.

The giant intestinal fluke, *Fasciolopsis buski* (Trematoda: Fasciolidae), is widely distributed in India and neighboring countries of the continent in South and Southeast Asia (Roy and Tandon, 2003). The fluke is the etiological agent of the disease commonly known as fasciolopsosis. The infection occurs by ingestion of raw aquatic vegetation or food plants that are contaminated with the infective encysted larvae, the metacercariae. In endemic zones pigs, dogs and rabbits act as reservoir of infection. In India, the parasite has been reported from different states including those in the Northeast. Variations in the morphology of the fluke have been observed when collected from different geographical regions (Roy and Tandon, 1993).

The trematode flukes of the genus *Fasciola* (the sheep liver fluke) are parasites of herbivores and infect humans accidentally causing fascioliasis worldwide. Fascioliasis is an important human and animal disease caused by *Fasciola hepatica* and *Fasciola gigantica*. In Asia and Africa, the distribution of *F. hepatica* and *F. gigantic* overlaps in given areas. This makes it difficult to identify the particular species involved (Ashrafi et

al., 2006; Le et al., 2007; Periago et al., 2008). The parasite is very cosmopolitan in distribution being found throughout all regions of the world, both temperate and tropical. *F. hepatica* is the causative agent of fascioliasis or 'liver rot' in ruminants, where it may be an important pathogen. Human infections with *F. hepatica* are found in areas where sheep and cattle are raised, and where humans consume raw watercress, including Europe, the Middle East and Asia (Mas-Coma et al., 1999; Mas-Coma and Bargues, 1997). Infection with *F. gigantica* on the other hand is found more commonly in tropical regions of the world, areas affected including Africa, Asia, many Pacific islands including Hawaii (where it has been reported in man), the Middle East and Southern Europe and the south of the USA (Hammond, 1974). Both fasciolids follow a similar two-host life cycle, in which freshwater snails of the family Lymnaeidae act as intermediate or vector host and a broad spectrum of mammals including humans, mainly herbivorous large-sized species, act as definitive host (Mas-Coma et al., 2005).

However, recent advances in molecular biology, in particular the amplification of specific DNA regions via the polymerase chain reaction (PCR) and improved sequencing techniques, have been employed to resolve taxonomic issues related to various helminth parasites by comparing their DNA. The ribosomal DNA cluster (rDNA), which codes for structural components of ribosomes, is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Davis, 1988; Hillis & Dixon, 1991). It enhances PCR amplification because many templates are available for initial priming and allows primers to be designed to anneal the known conserved regions to amplify across unknown variable regions. The nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2), which occur between the 18S, 5.8S and 28S coding regions (Figure 5), have proven useful for diagnostic purposes at the level of species. This cluster of genes (and the spacers between them) is repeated in tandem hundreds of times, usually in a single chromosome. Large, small and 5.8S subunits combine after removal of spacers, to produce the RNA skeleton/framework of the ribosome.

DNA techniques utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA have been employed to resolve taxonomic issues related to various helminth parasites (Blair et al., 1996). The second internal transcribed spacer (ITS2) of rDNA have been proven to be particularly valuable in this context, especially for the species studies of the genus *Paragonimus* and many other helminth species and their phylogenetic relationships (Morgan and Blair, 1995; Jousson et al., 1998; Blair et al., 1999a; Leon-Regagnon et al., 1999; Iwagami et al., 2000; Blair, 2000; McManus et al., 2000; Tkach et al., 2000; Kostadinova et al., 2003; Scholz et al., 2004; McManus et al., 2004; Agatsuma et al., 2004; Huang et al., 2004; Vilas et al., 2005; Rinaldi et al., 2005) *Fasciola* spp and isolates of *Fascioloides magna* from different geographical regions were discriminated on the basis of ITS sequences (Adlard et al., 1993). Studies on phylogeny and/or intraspecific variation in *Paragonimus* species have also been done using ITS2 region in recent years (Blair et al., 1996, 1997; Herwerden et al., 1999; Maleewong, 2003; Cui et al., 2003; Park et al., 2003; Chen et al., 2004; Le et al., 2006; Doanh et al., 2007; Devi et al., 2007) and the usefulness of the method for species discrimination has also been demonstrated in nematodes (Campbell et al., 1994; Hoste et al., 1995; Samson-Himmelstjerna et al., 1997; Aboobaker and Blaxter, 2004; Ghedin et al., 2004; Roy et al., 2006). ITS2 sequences allow quick and accurate identification of genetically distinct but morphologically similar species (Nolan and Cribb, 2005).

Phylogenetic relationships of the various *Paragonimus* species have also recently been studied using genetic markers in the ITS2 region of rDNA (Blair et al., 1997, Blair et al., 1999b, Iwagami et al., 2000). In these studies, the ITS2 rDNA sequences for the analysis were generated from adult DNA using PCR primers that were designed based on the conserved 5.8S and 28S regions flanking the ITS2. Using these PCR-primers, the sequences of the amplified products from metacercariae of *P westermani* and *P. miyazakii* were analysed and were found identical in length and composition to those reported for the adults of the respective species and two species of *Paragonimus* could be unequivocally discriminated by PCR using species-specific primers from the DNA prepared from metacercariae (Blair et al., 1996, Sugiyama et al., 2002).

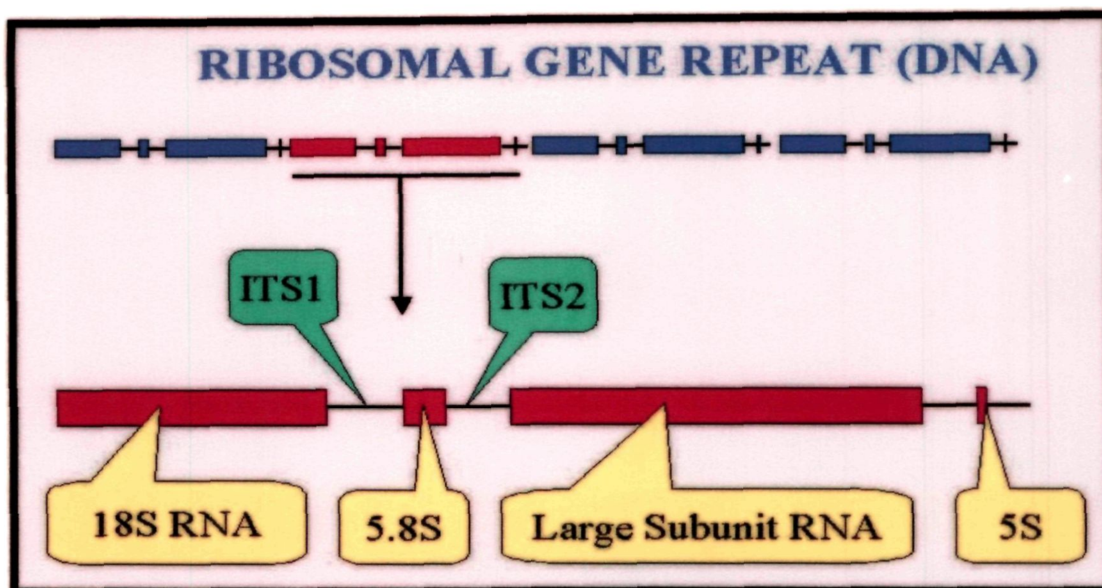


Figure 5. Ribosomal DNA gene cluster

(www.rzuser.uni-heidelberg.de)

The main objective of the present study was to establish a molecular method to differentiate between different species of *Paragonimus* from the metacercariae, which are the infective stage of the pathogens causing human paragonimiasis and also to discriminate between other plausible (crab-borne) trematode infections such as those of *Fasciolopsis* and *Fasciola.*

MATERIALS AND METHODS

Parasite materials

Metacercariae:

The trematode metacercariae of *Paragonimus* were collected from the freshwater crabs of mountain streams of the suspected focal area.

Adult flukes and eggs:

Live adult *Fasciolopsis buski* were obtained from the intestine of freshly slaughtered pig, *Sus scrofa domestica* at local abattoirs. The worms recovered from these hosts represented the geographical isolates from Assam region of Northeast India. *Fasciola* worms were obtained in live form from hepatic biliary ducts of freshly slaughtered cow, *Bos indicus*; the worms recovered from bovine hosts represented the geographical isolates from Assam, Northeast India and morphologically resembled *Fasciola gigantica* (deposition number of paratypes at Zoological Survey of India, Kolkata = W7787/1). Eggs were obtained from mature adult flukes by squeezing between two glass slides; eggs recovered were also processed separately.

DNA isolation

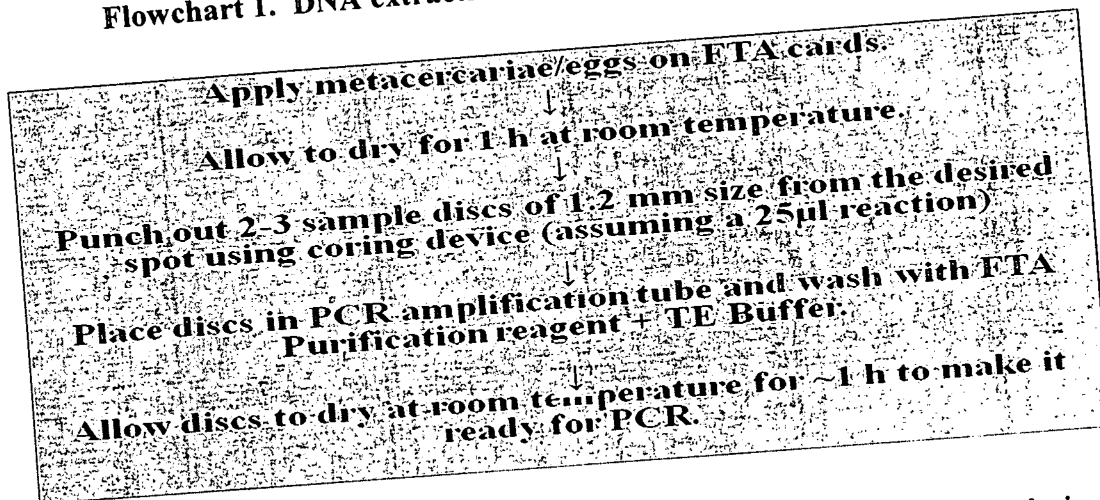
Eggs & Metacercariae:

DNA was extracted in FTA card by using Whatman's FTA Purification Reagent as detailed below (Flowchart 1).

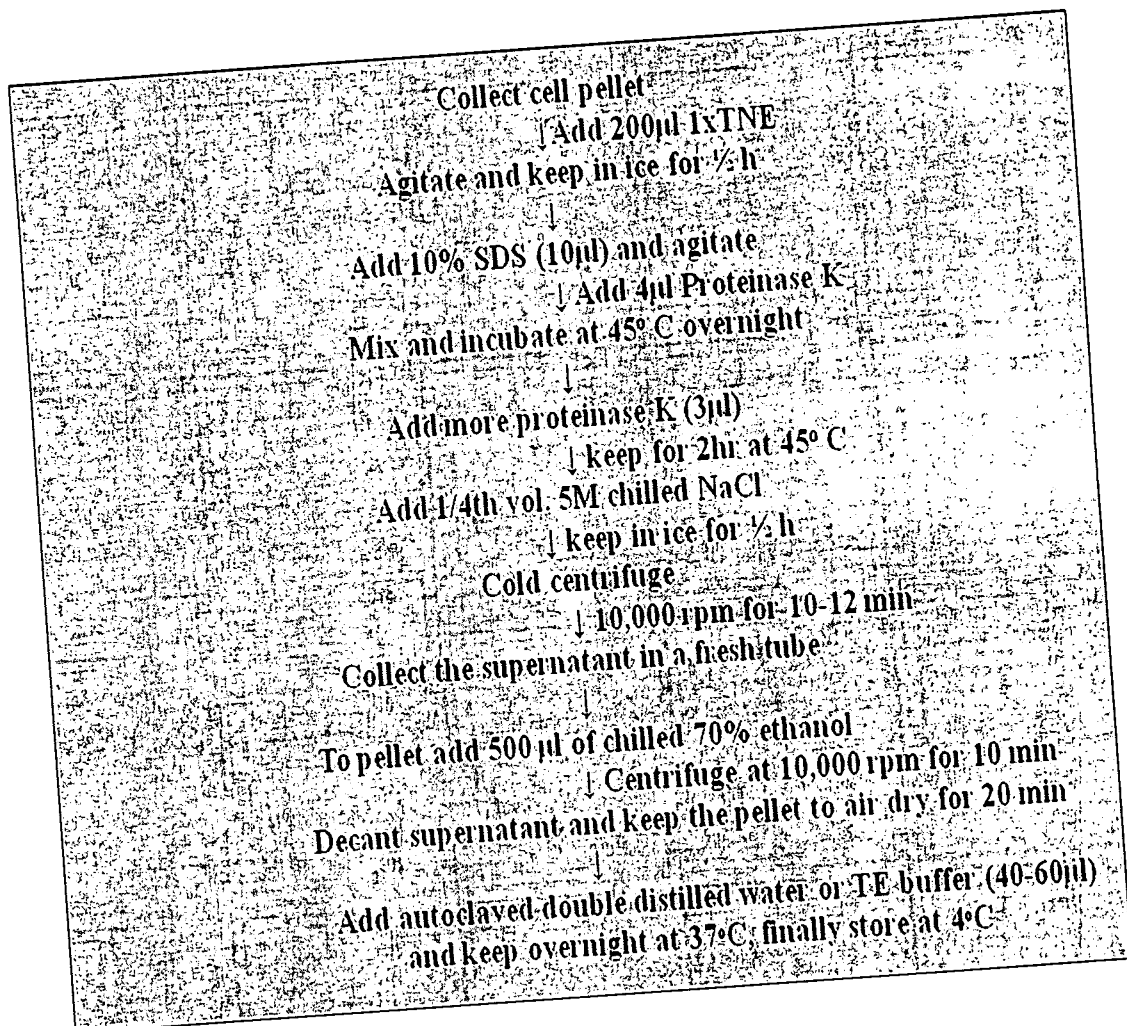
Adults:

DNA was extracted from lysed individual worms by standard ethanol precipitation technique (Sambrooke et al., 1989) as detailed below (Flowchart 2).

Flowchart 1. DNA extraction - Whatman's FTA card technique



Flowchart 2. DNA extraction from adult fluke - Ethanol precipitation technique.



DNA amplification and sequencing

Use of known primers

The rDNA region spanning the ITS1 and ITS2 was amplified from the egg, larval and/or adult DNA by PCR. The primers generally used were designed based on the conserved sequences of *Schistosoma* spp and are considered to be the universal primers for trematode species (Bowles et al., 1995).
The primers used were:

i) ITS2 region:
   3S (forward): 5'GGTACCGGTGGATCACTCGGCTCGTG-3'
   A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'

ii) ITS1 region:
   BD1 (forward): 5'GTCGTAACAAGGTTTCCGTA-3'
   4S (reverse): 5'TCTAGATGCGTTCGAA (G/A) TGTCGATG-3'

The PCR amplification was performed following the standard protocol (White, 1993) with minor modifications. The PCR cocktail (final reaction volume 25μl) was amplified with the following conditions for ITS1- initial denaturation at 94°C for 5 min, then 35 cycles including denaturation at 94°C for 60 sec, annealing at 54°C for 50 sec, extension at 72°C for 80 sec, followed by final extension for 7 min at 72°C. For amplification of ITS2 region, the conditions were as follows - initial denaturation at 94°C for 5 min, then 26 cycles including denaturation at 94°C for 30 sec, annealing at 55°C for 38 sec, extension at 72°C for 42 sec, followed by final extension for 10 min at 72°C.

The resultant PCR products were separated by electrophoresis through 1.5% (w/v) agarose gel in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed. The known size fragments of 100 bp ladder in agarose gel were used as marker. For DNA sequencing, the PCR products were purified

using Genei Quick PCR purification Kit, and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei, Bangalore and The Centre for Genomic Applications (TCGA), New Delhi, India and submitted to GenBank.

Nucleotide sequence analysis

The DNA sequences (of the PCR-amplified product) were put to further analysis with the usage of bioinfomatic tools including similarity search using **BLAST** (Basic Local Alignment Search Tool) provided at the URL http://www.ncbi.nlm.nih.gov/blast, and phylogenetic prediction using **Clustalw** provided at the URL http://www.ebi.ac.uk/clustalw for query DNA sequence.

[Similarity Searches (BLAST)

The General approach involves the use of a set of algorithms such as the BLAST programs to compare a query sequence to all the sequences in a specified database. Comparisons are made in a pair wise fashion. Each comparison is given a score reflecting the degree of similarity between the query and the sequence being compared. The higher the score, the greater is the degree of similarity. The similarity is measured and shown by aligning two sequences. Alignments can be global or local (algorithm specific).

Blastn is the nucleotide – nucleotide blast in which the query nucleotide sequence is searched for match against the database stored nucleotide sequence and the hits (matches) are obtained as result along with the distribution information and the alignments. The BLAST nucleotide algorithm finds similar sequences by generating an indexed table or dictionary of short subsequences called words for both the query and the database.

Phylogenetic relationships, are in general patterns of shared history between biological replicators, such as species or genes. The aim of *phylogenetic inference* is to propose a well-corroborated hypothesis of this shared history, but this is to some extent inseparable from the pattern of evolutionary change in the data (the characters) used to erect the hypotheses.

ClustalW is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent

sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylograms. Multiple alignments of DNA sequences are important tools in studying sequences. The basic information they provide is identification of conserved sequence regions. Sequences can be aligned across their entire length (global alignment) or only in certain regions (local alignment).

A cladogram is simple tree depicting only relationships between terminal nodes. A branching diagram (tree) is assumed to be an estimate of a phylogeny.

A phylogram has additional information in that edge lengths are drawn proportional to some attribute such as amount of change. A branching diagram (tree) assumed to be an estimate of a phylogeny; usually distinguished from a cladogram in that the branch lengths are proportional to the amount of inferred evolutionary change.

Sequence alignment is a way of arranging the primary sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that residues with identical or similar characters are aligned in successive columns.

Pair- wise sequence alignment methods are used to find the best-matching piece-wise (local) or global alignments of two query sequences. They are efficient to calculate and are often used for methods that do not require extreme precision (such as searching a database for sequences with high homology to a query).]

Molecular phylogenetic analysis

Molecular phylogenetics attempts to determine the rates and patterns of change occurring in DNA and proteins and to reconstruct the evolutionary history of genes and organisms. Initially, the sequences were aligned using ClustalW multiple alignment (Thompson et al. 1994) with the default gap and extension penalties used by this program. For interpreting the results obtained, different tree building models were considered to entertain possible explanations. Phylogenetic reconstructions were done using the MEGA 4.0 package (Tamura et al., 2007). Only unique sequences were used in tree construction. ITS sequences were entered in the MEGA for construction of the

phylogenetic trees using **Maximum Parsimony** and distance methods namely the **Neighbor-Joining, UPGMA** and **Minimum Evolution**. Branch support was given using 1000 bootstrap replicates in MEGA (Hillis and Bull 1993).

Phylogenetic analysis was also carried out using the Bayesian approach with combined datasets using Mrbayes 3.1 (Huelsenbeck and Ronquist 2001), wherein each data partition is allowed to have a different evolution rate.

[Steps of Phylogenetic Analysis
Alignment - building the data model and extracting a dataset.

- **Tree building.**
- **Tree evaluation.**

Tree Building Methods

- **Distance Methods:**
  - UPGMA, Neighbor Joining, Minimum Evolution
  - Requires distance measures between sequences
  - Suitable for continuous characters
- **Character State Methods**
  - Maximum Parsimony

Tree Evaluation
Bootstrapping: A statistical parameter to gauge the viability of a phylogenetic tree. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping.]

Predicted ITS2 RNA secondary structures and analyses

Most phylogenetic studies using current methods have focused on primary DNA sequence information. However, RNA secondary structures are particularly useful in systematics because they include characteristics, not found in the primary sequence, that give 'morphological' information (Caetano-Anolles, 2002). The novel approach of molecular morphometrics that relies both on traditional morphological comparison and on molecular sequence comparison by measuring the structural parameters of the ITS2 secondary structure homologies (geometrical features, bond energies, base composition etc.) is recently being used to study the phylogenetic relationships of various species (Billoud et al., 2000). This method allows one to take into account the regions where

multiple alignments are barely reliable because of a large number of insertions and deletions. This method is based on the assumption that secondary structure can be phylogenetically as significant as primary sequence. It is well known that rRNA is highly conserved throughout evolution. Thus, the secondary-structure elements of the RNA molecule, i.e., the helices, loops, bulges, and separating single-stranded portions, can be considered phylogenetic characters (Zwieb et al., 1981, Schultz et al., 2005, Grajales et al., 2007).

Secondary structures of ITS2 sequences of various *Paragonimus* and other fasciolid species were reconstructed by aligning their sequences using Bioedit (Hall, 1999). The acquired structures with restrictions and constrains were submitted in MFOLD (Zuker, 2003). RNA was folded at a fixed temperature of 37° C, and the structure choosen from different output files was the desired 6-helicoidal ring or the one with the highest negative free energy if various similar structures were obtained.

Design of genus/species specific primers and amplification

To establish a more direct PCR procedure for species discrimination and identification, the specific primers were designed using Primer3, a widely used program for designing PCR primers (Rozen and Skaletsky, 2000) to target unique regions of the ITS2 sequence of each species. Each specific primer was examined for genus-specific amplification with the 3S primer under the PCR conditions as described above. The primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample.

RESULTS

• *Paragonimus* sp.: PCR amplification of ITS region and its analysis

The PCR amplified products of ITS2 of rDNA were successfully obtained using the primers as mentioned in material and methods (Figure 6).

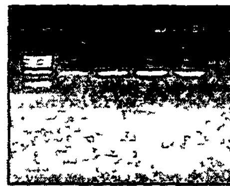


Figure 6. PCR Product of *Paragonimus* metacercariae in FTA card using primer 3S-A28 for ITS2

The nucleotide sequences obtained are shown below as raw sequences along with their depiction in bar diagrams (Figures 7, 8) and electropherogram (Figure 9).

ITS2 *Paragonimus* metacercariae (Forward sequence)

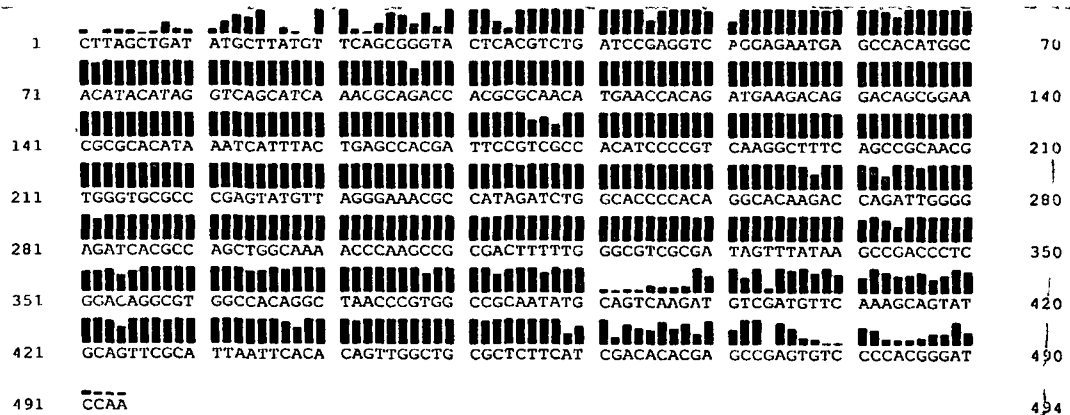
TGTCGATGACGAGCGCAGCCACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACATCGA
CATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTT
ATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCA
ATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCC
ACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCGACGGAATCGTGGCTCAGTAAATGATTT
ATGTGCGCGTTCCGCTGACCTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTTGATG
CTGACCTATGTATGTGCCATGTGGCTCATTCTCCTGACCTCGGATCAGACGTGAGTACCCGCTG
AACTTAAGCATATCACTAAGCGGAGGGAAAAGAAACTAACCCCGGATCCCAAA

ITS2 *Paragonimus* metacercariae (Reverse sequence)

CTTAGCTGATATGCTTATGTTCAGCGGGTACTCACGTCTGATCCGAGGTCAGGAGAATGAGCC
ACATGGCACATACATAGGTCAGCATCAAACGCAGACCACGCGCAACATGAACCACAGATGAA
GACAGGACAGCGGAACGCGCACATAAATCATTTACTGAGCCACGATTCCGTCGCCACATCCCC
GTCAAGGCTTTCAGCCGCAACGTGGGTGCGCCCGAGTATGTTAGGGAAACGCCATAGATCTG
GCACCCCACAGGCACAAGACCAGATTGGGGAGATCACGCCAGCTGGCAAAACCCAAGCCGCG
ACTTTTTGGGCGTCGCGATAGTTTATAAGCCGACCCTCGGACAGGCGTGGCCACAGGCTAACC
CGTGGCCGCAATATGCAGTCAAGATGTCGATGTTCAAAGCAGTATGCAGTTCGCATTAATTCA
CACAGTTGGCTGCGCTCTTCATCGACACACGAGCCGAGTGTCCCCACGGGATCCAA

**TGTCGATGACGAGCGCAGCCACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAAC
ATCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCG
AGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAG
CTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTT
CCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGC
GACGGAATCGTGGCTCAGTAAATGATTTATGTGCGCGTTCCGCTGACCTGTCTTCATC
TGTGGTTCATGTTGCGCGTGGTCTGCGTTTGATGCTGACCTATGTATGTGCCATGTGG
CTCATTCTCCTGACCTCGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTA
AGCGGAGGGAAAAGAAACTAACCCCGGATCCCAAA**

Figure 7. ***Paragonimus*** **sp. ITS2: Forward sequence plus flanking sequences**



**CTTAGCTGATATGCTTATGTTCAGCGGGTACTCACGTCTGATCCGAGGTCAGGAGAATGA
GCCACATGGCACATACATAGGTCAGCATCAAACGCAGACCACGCGCAACATGAACCACA
GATGAAGACAGGACAGCGGAACGCGCACATAAATCATTTACTGAGCCACGATTCCGTCGC
CACATCCCCGTCAAGGCTTTCAGCCGCAACGTGGGTGCGCCCGAGTATGTTAGGGAAAC
GCCATAGATCTGGCACCCCACAGGCACAAGACCAGATTGGGGAGATCACGCCAGCTGGC
AAAACCCAAGCCGCGACTTTTTGGGCGTCGCGATAGTTTATAAGCCGACCCTCGGACAG
GCGTGGCCACAGGCTAACCCGTGGCCGCAATATGCAGTCAAGATGTCGATGTTCAAAGC
AGTATGCAGTTCGCATTAATTCACACAGTTGGCTGCGCTCTTCATCGACACACGAGCCGA
GTGTCCCCACGGGATCCAA**

Figure 8. ***Paragonimus*** **sp. ITS2: Reverse sequence plus flanking sequences**

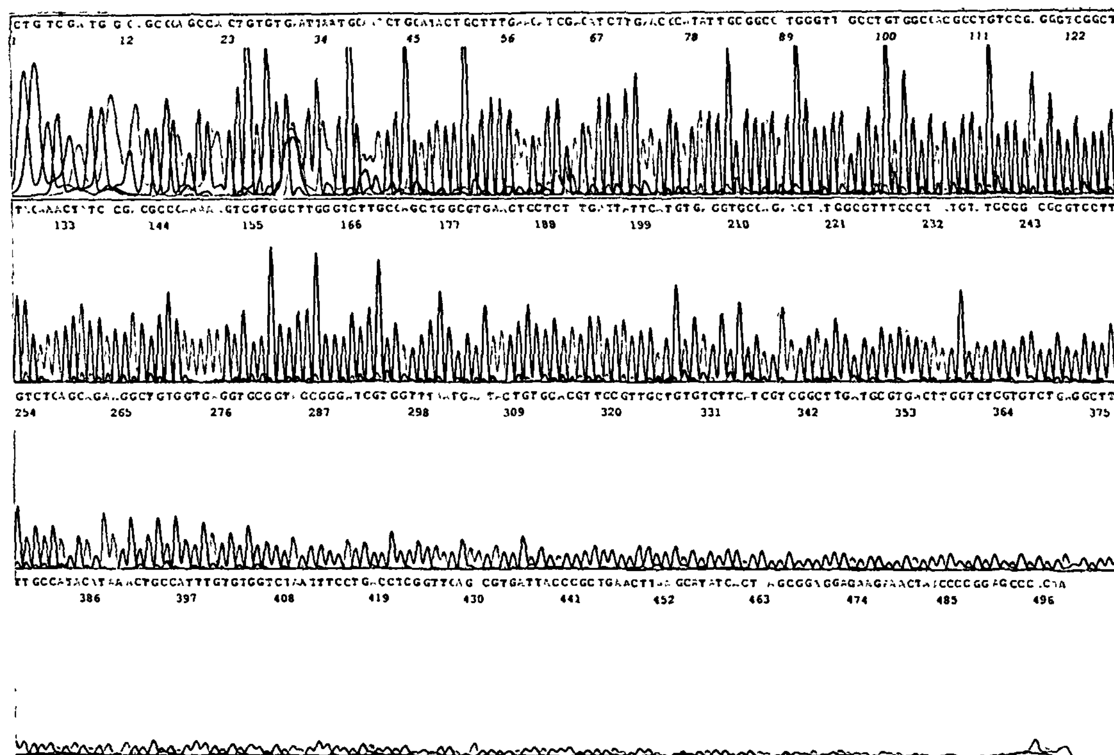


Figure 9. Electropherogram data showing peaks obtained by different nucleotides

Sequences were compared with other sequences of trematode species from Genbank. The fragments of amplified DNA were estimated to be ~500bp long. Sequence analysis of the ITS2 PCR products revealed that the alignments of the rDNA region spanning the ITS2 were 496bp for forward primer and 494bp for reverse primer, respectively. The actual length of ITS2 was estimated to be 287bp. No intra-specific variations in length or composition of the sequence were observed and all the ITS2 sequences of the metacercariae were found to be identical in all the samples.

The results of similarity search for the query sequence ITS2, forward and reverse were obtained using BLAST (Figures 10, 12). The top 15-20 best hits (having the maximum e-value) were used for the purpose of phylogenetic predictions using Clustalw bioinformatic tools. The resultant cladograms and phylograms along with respective gene identification number (gi) are depicted in Figures 11a & b and 13a & b.

ITS2 (Forward sequence)

Distribution of 148 Blast Hits on the Query Sequence

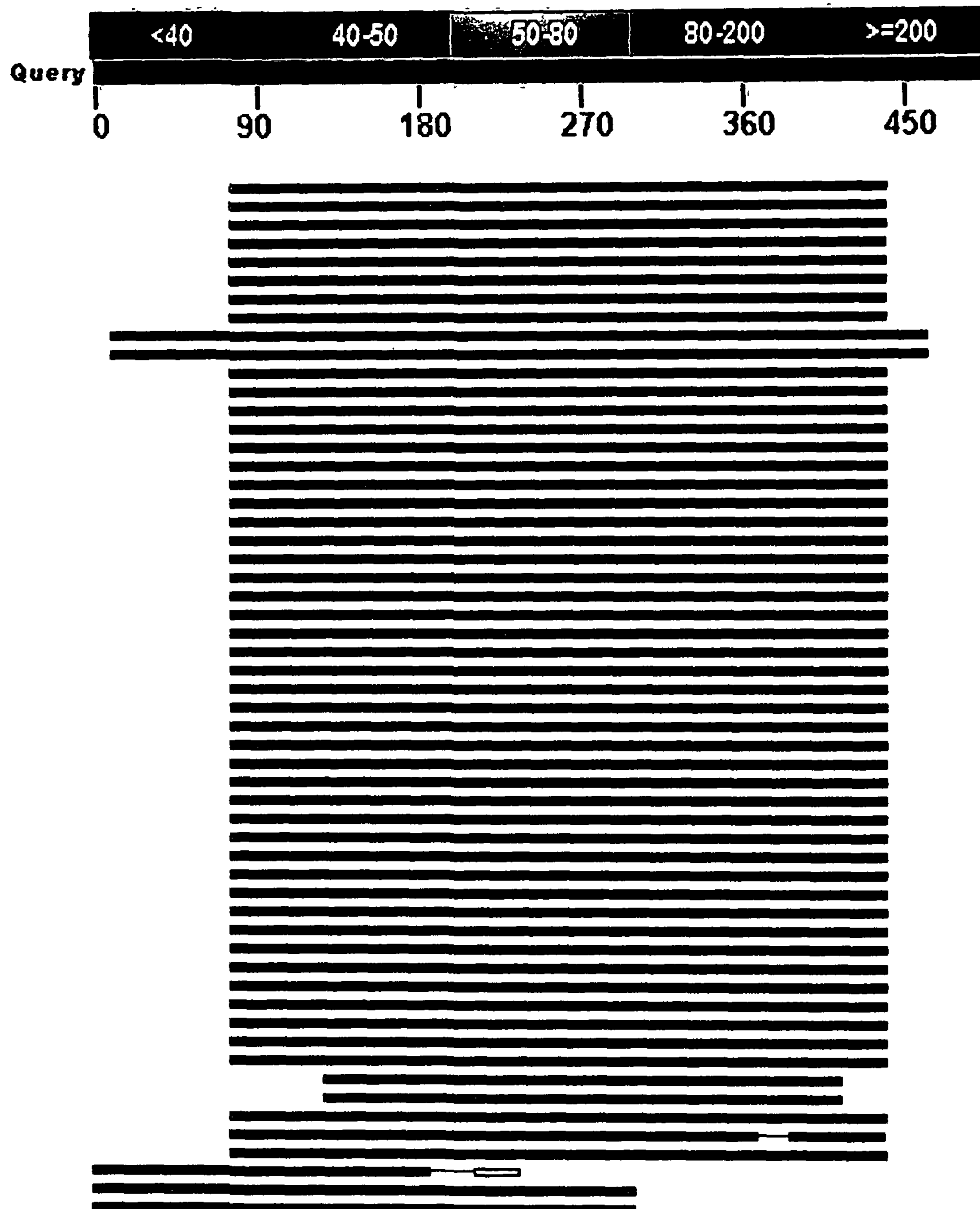
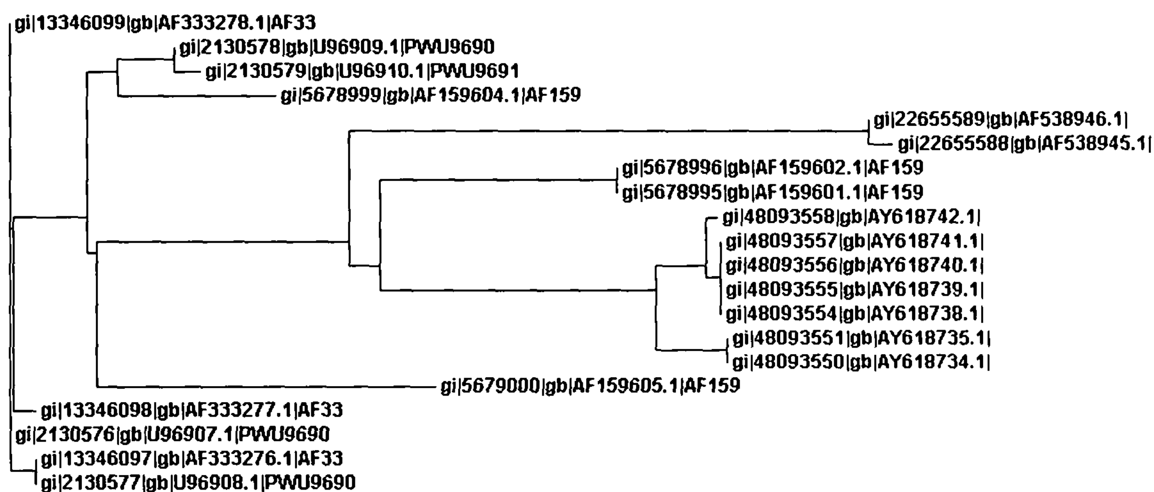


Figure 10. Blast hit result of ITS2 forward sequence of *Paragonimus* sp.

(a) *Phylogram*



(b) *Cladogram*

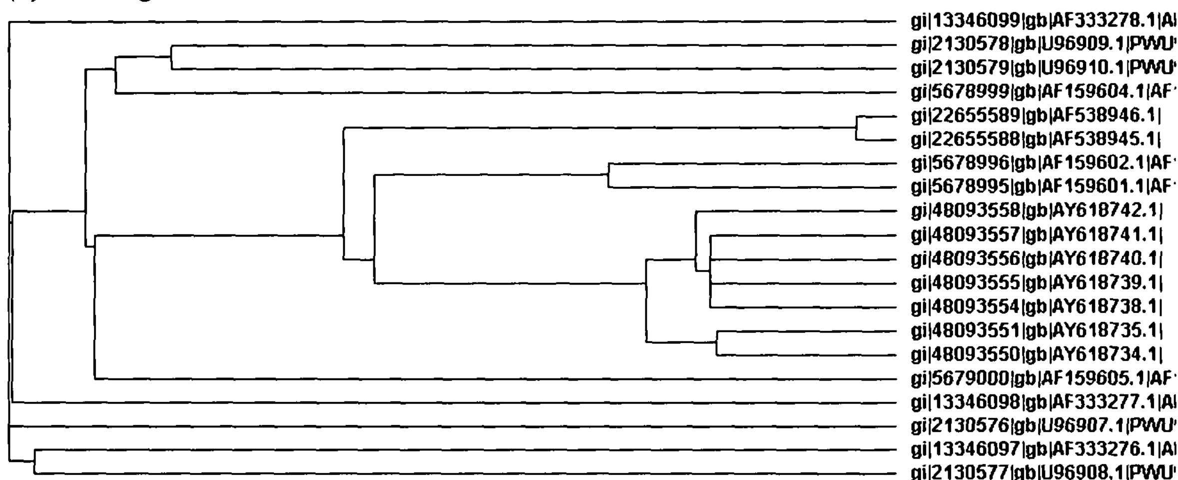


Figure 11a & b. Phylogram and cladogram using ITS2 (Forward) sequence along with respective gene identification numbers.

- gi|13346099|gb|AF333278.1|AF333278 *Paragonimus westermani* ITS 2, partial sequence
- gi|2130578|gb|U96909.1|PWU96909 *Paragonimus westermani* ITS 2, complete sequence
- gi|2130576|gb|U96907.1|PWU96907 *Paragonimus westermani* ITS 2, complete sequence
- gi|13346098|gb|AF333277.1|AF333277 *Paragonimus westermani* ITS 2, partial sequence
- gi|13346097|gb|AF333276.1|AF333276 *Paragonimus westermani* ITS 2, partial sequence

- gi|2130579|gb|U96910.1|PWU96910 *Paragonimus westermani* ITS 2, complete sequence
- gi|2130577|gb|U96908.1|PWU96908 *Paragonimus westermani* ITS 2, complete sequence
- gi|5678999|gb|AF159604.1|AF159604 *Paragonimus westermani* strain Thailand ITS 2, complete sequence
- gi|22655589|gb|AF538946.1| *Paragonimus mexicanus* isolate G ITS 2, partial sequence
- gi|22655588|gb|AF538945.1| *Paragonimus mexicanus* isolate E ITS 2, partial sequence
- gi|5679000|gb|AF159605.1|AF159605 *Paragonimus siamensis* ITS 2, complete sequence
- gi|5678996|gb|AF159602.1|AF159602 *Euparagonimus cenocopiosus* strain Langu ITS 2, complete sequence
- gi|5678995|gb|AF159601.1|AF159601 *Euparagonimus cenocopiosus* strain Nanjing ITS 2, complete sequence
- gi|48093558|gb|AY618742.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093551|gb|AY618735.1| *Paragonimus skrjabini* ITS 2, complete sequence
- gi|48093550|gb|AY618734.1| *Paragonimus skrjabini* ITS 2, complete sequence
- gi|48093557|gb|AY618741.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093556|gb|AY618740.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093555|gb|AY618739 1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093554|gb|AY618738.1| *Paragonimus miyazakii* ITS 2, complete sequence

The Blast hit results show that the query - ITS2 *Paragonimus metacercariae* Forward sequence is more similar to the sequence of the species *Paragonimus westerrmani, Paragonimus mexicanus, Paragonimus siamensis, Paragonimus sismensis, Paragonimus miyazakii, Euparagonimus cenocopiosus.*

From the phylogram and cladogram obtained using the tool ClustalW, sequences of distinct clusters of the species *Paragonimus westermani, Paragonimus mexicanus, Paragonimus siamensis, Paragonimus sismensis, Paragonimus miyazakii, Euparagonimus cenocopiosus* are revealed.

ITS2 (Reverse sequence)

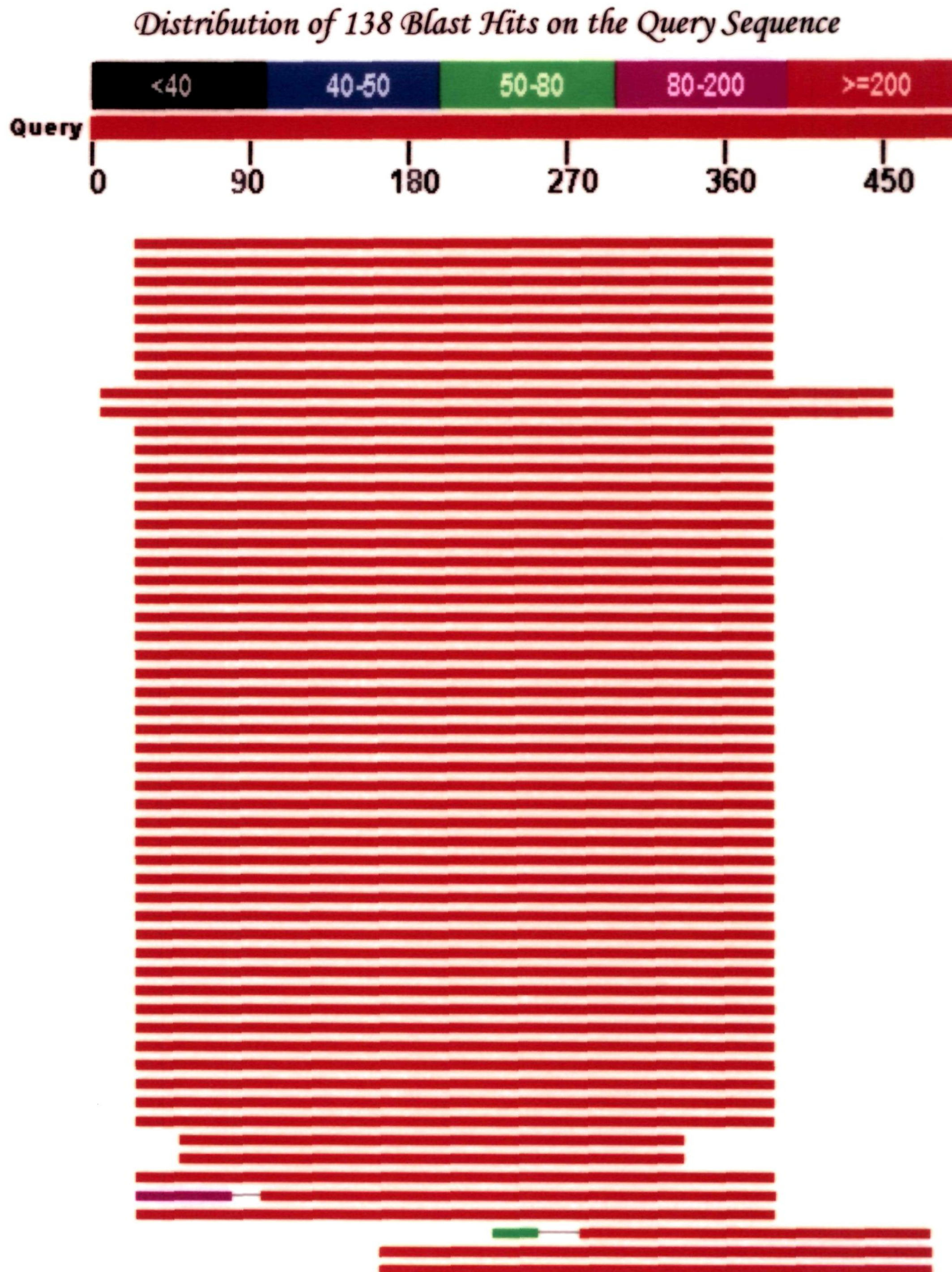
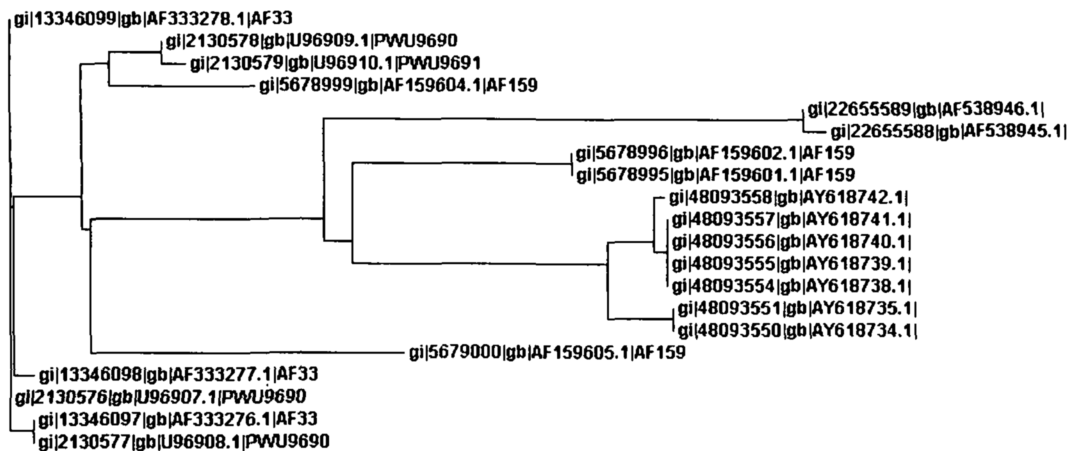


Figure 12. Blast hit result of ITS2 reverse sequence of *Paragonimus* sp.

(a) Phylogram



(b) Cladogram

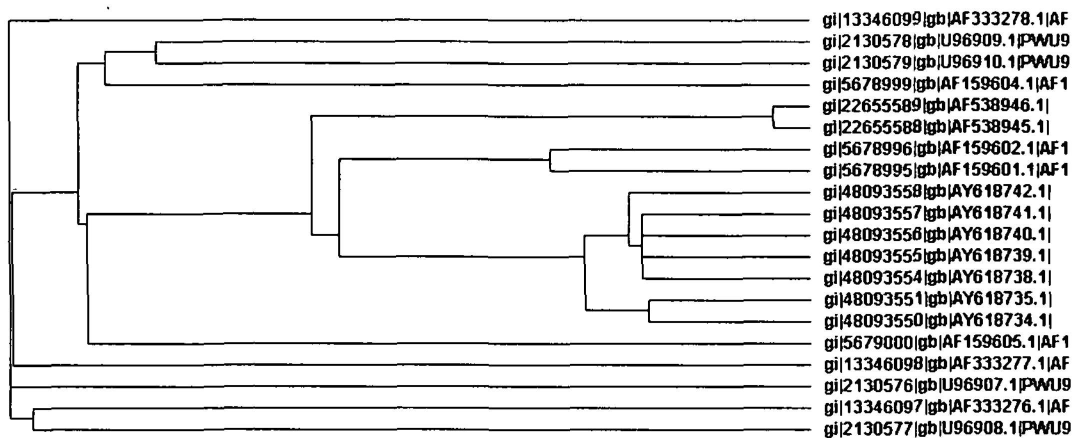


Figure 13a & b. Phylogram and cladogram using ITS2 (Reverse) sequence along with respective gene identification numbers.

- gi|13346099|gb|AF333278.1|AF333278 *Paragonimus westermani* internal transcribed spacer 2, partial sequence
- gi|2130578|gb|U96909.1|PWU96909 *Paragonimus westermani* internal transcribed spacer 2, complete sequence
- gi|2130576|gb|U96907.1|PWU96907 *Paragonimus westermani* internal transcribed spacer 2, complete sequence
- gi|13346098|gb|AF333277.1|AF333277 *Paragonimus westermani* internal transcribed spacer 2, partial sequence

- gi|13346097|gb|AF333276.1|AF333276 *Paragonimus westermani* internal transcribed spacer 2, partial sequence
- gi|2130579|gb|U96910.1|PWU96910 *Paragonimus westermani* internal transcribed spacer 2, complete sequence
- gi|2130577|gb|U96908.1|PWU96908 *Paragonimus westermani* internal transcribed spacer 2, complete sequence
- gi|5678999|gb|AF159604.1|AF159604 *Paragonimus westermani* strain Thailand internal transcribed spacer 2, complete sequence
- gi|22655589|gb|AF538946.1| *Paragonimus mexicanus* isolate G internal transcribed spacer 2, partial sequence
- gi|5679000|gb|AF159605.1|AF159605 *Paragonimus siamensis* internal transcribed spacer 2, complete sequence
- gi|5678996|gb|AF159602.1|AF159602 *Euparagonimus cenocopiosus* strain Langu internal transcribed spacer 2, complete sequence
- gi|48093558|gb|AY618742.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093551|gb|AY618735.1| *Paragonimus skrjabini* ITS 2, complete sequence
- gi|48093550|gb|AY618734.1| *Paragonimus skrjabini* ITS 2, complete sequence
- gi|48093557|gb|AY618741.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093556|gb|AY618740.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093555|gb|AY618739.1| *Paragonimus miyazakii* ITS 2, complete sequence
- gi|48093554|gb|AY618738.1| *Paragonimus miyazakii* ITS 2, complete sequence

The Blast hit results with the query - ITS2 *Paragonimus metacercariae* Reverse sequence show the same similarity as that of the forward sequence.

From the phylogram and cladogram obtained using the tool ClustalW, sequences of distinct clusters of the species *Paragonimus westermani*, *Paragonimus mexicanus*, *Paragonimus siamensis*, *Paragonimus skrjabini*, *Paragonimus miyazaki*, *Euparagonimus cenocopiosus* are revealed.

Construction of phylogenetic trees

Phylogenetic trees were obtained by comparing the ITS2 sequences of *Paragonimus* species from different geographical isolates (Table 5). Phylogenetic analyses using the various distance methods like Neighbor-joining, Minimum evolution and UPGMA showed that the topology is similar among the trees obtained. Due to similar topology only one example each of the trees constructed using NJ and character state method are shown in Figures 14 and 15. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping.

Species	GenBank Accession No.	Sequence length (bp)	Classification
Paragonimus westermani India: Northeast (A)	DQ351845	496	Digenea: Paragonimidae
Paragonimus westermani India: Arunachal Pradesh	DQ836246	511	Digenea: Paragonimidae
Paragonimus westermani Thailand: Saraburi	AB354214	463	Digenea: Paragonimidae
Paragonimus siamensis Thailand: Prachin Buri	AB354222	463	Digenea: Paragonimidae
Paragonimus proliferus China: Yunnan Province	EU401805	463	Digenea: Paragonimidae
Paragonimus skrjabini India: Manipur	AB325516	463	Digenea: Paragonimidae
Paragonimus heterotremus Thailand: Saraburi	AB354221	461	Digenea: Paragonimidae
Paragonimus bangkokensis Thailand: Surat Thani	AB248091	463	Digenea: Paragonimidae
Paragonimus harinasutai Thailand:Saraburi	AB354219	463	Digenea: Paragonimidae
Paragonimus heterotremus India: Manipur	AB308378	461	Digenea: Paragonimidae

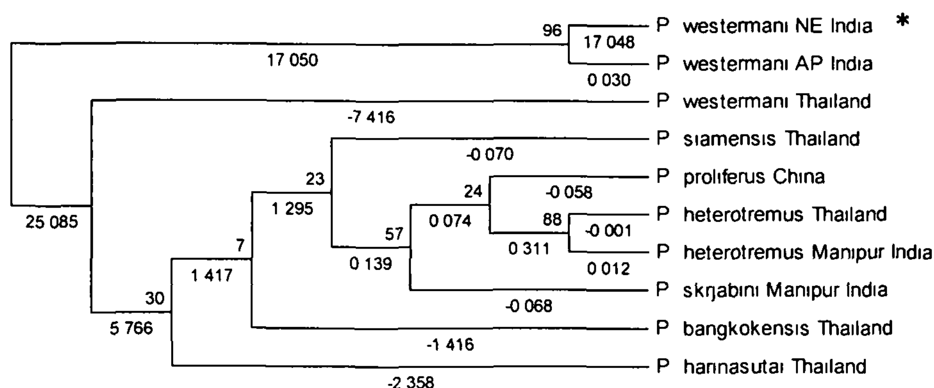


Figure 14. Neighbor- Joining Tree of ITS2 sequences of various *Paragonimus* species (* query sequence)

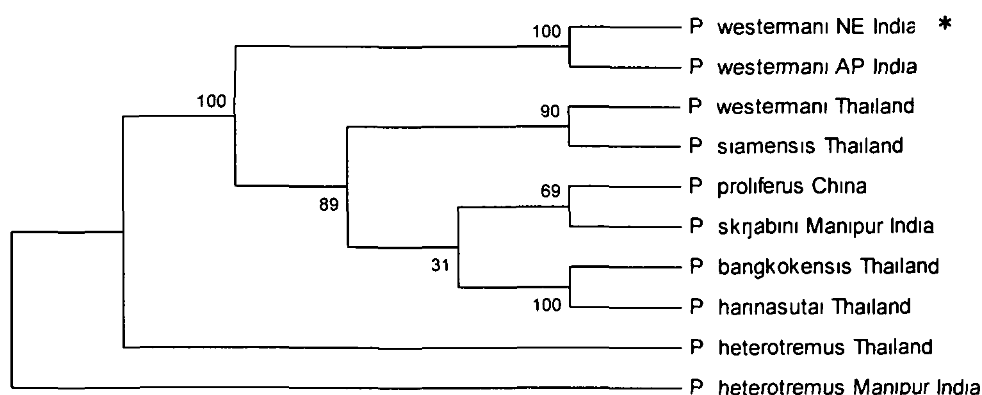


Figure 15. Maximum Parsimony Tree of ITS2 sequences of various *Paragonimus* species (* query sequence)

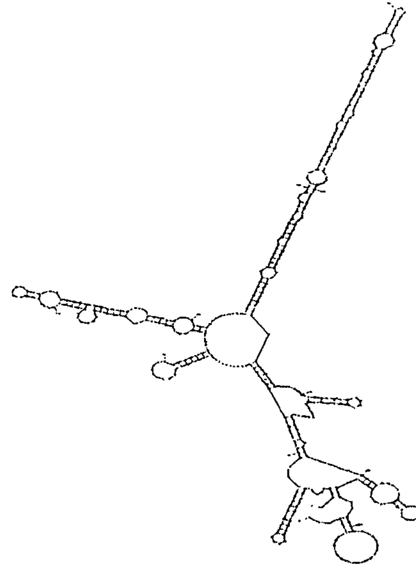
Secondary structure analysis

Initially, ten predicted RNA secondary structures were reconstructed to provide the basic information for phylogenetic analyses; they accorded with the 6 helicoidal ring model (Figure 16 a-j). The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. The secondary structural features of ITS2 regions as shown in the figures were analysed based on conserved stems and loops, which in order of preference were interior loop, bulge loop, multiple branch loop, hairpin loop and exterior loop in all the isolates. We obtained three similar topologies for seven species of the genus *Paragonimus* on the basis of traditional primary sequence analyses using MEGA and a Bayesian analysis of

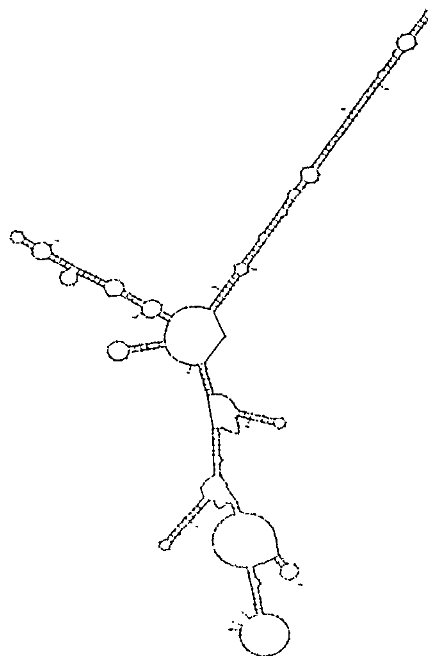
the combined data (Figure 17). The latter approach allowed us to include both primary sequence and RNA molecular morphometrics; each data partition was allowed to have a different evolution rate. *Paragonimus westermani* was found to group with *P. siamensis* of Thailand; this was best supported by both the molecular morphometrics and combined analyses. *P. heterotremus*, *P. proliferus*, *P. skrjabini*, *P. bangkokensis* and *P. harinasutai* formed a separate clade in the molecular phylogenies, and were reciprocally monophyletic with respect to other species.

Paragonimus westermani was found to group with *P. siamensis* of Thailand and show an overall similarity in the ITS2 rRNA folding and have identical secondary structures, which in remaining five isolates show some variation. The observed similarities at the secondary structural level are further reflected at the energy level (-ΔG). The difference in their topology, however, is due to differences in nucleotide sequence lengths. These secondary structure predictions indicate that the domains basepair to form a core region central to several stem features implying that conservedness is more important for the proper rRNA folding pattern. Moreover, the observed phylogenetic trend was identified with respect to the target accessibility sites for the seven different isolates. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences.

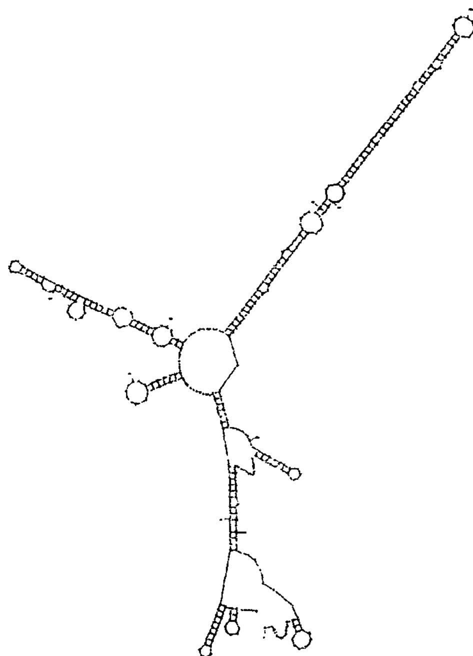
Figure 16 (a-j): Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD: Isolates from India and neighboring countries.



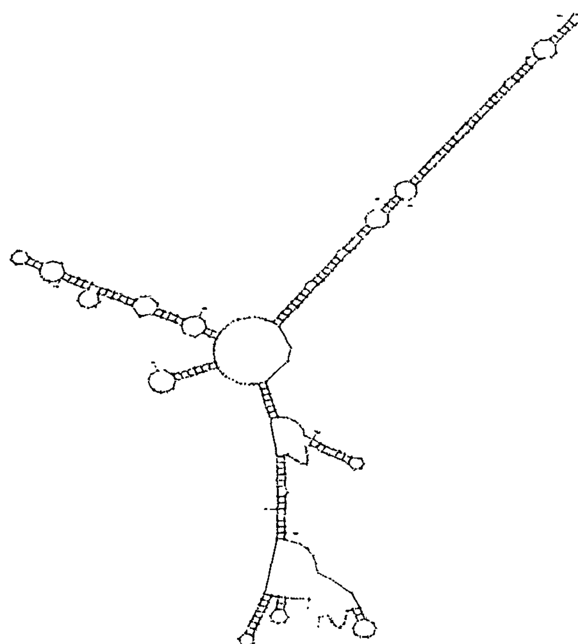
(a) dG= -151.78 *P. westermani* India: Northeast



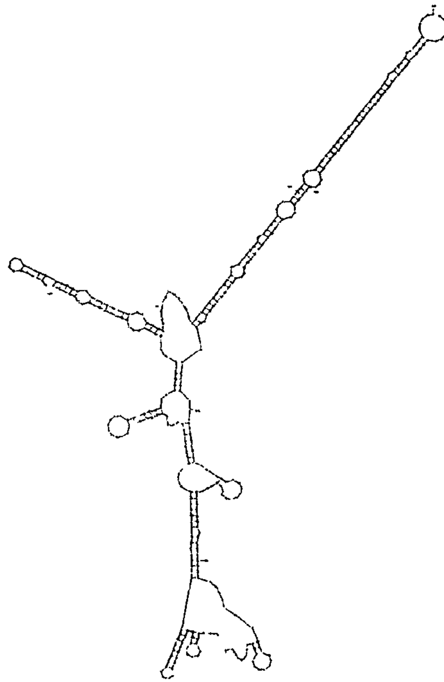
(b) dG= -147.588 *P. westermani* India: Arunachal Pradesh



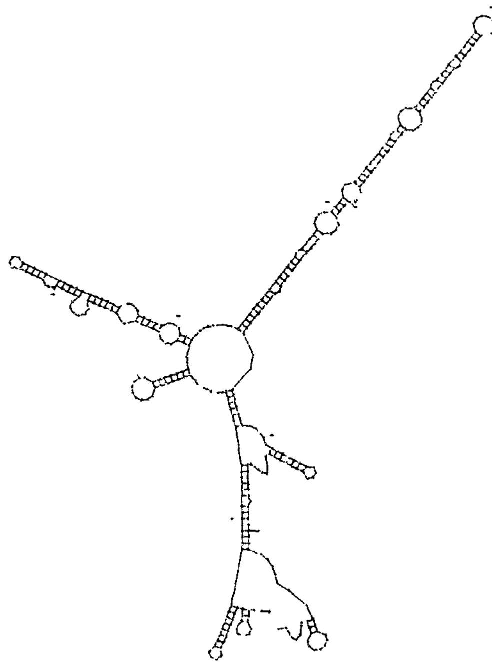
(c) dG= -149.95 *P. heterotremus* India: Manipur



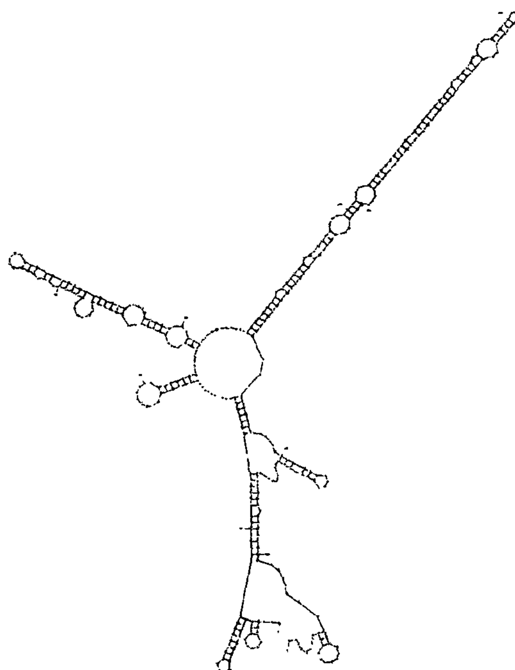
(d) dG= -162.34 *P. skrjabini* India: Manipur



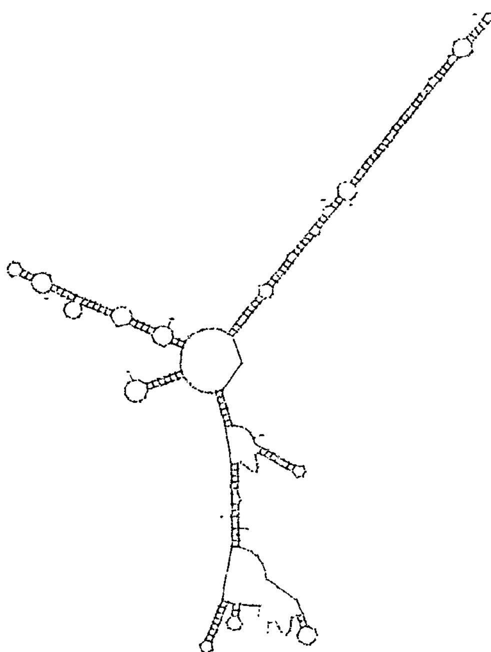
(e) dG= -149.11 *P. siamensis* Thailand: Prachin Buri



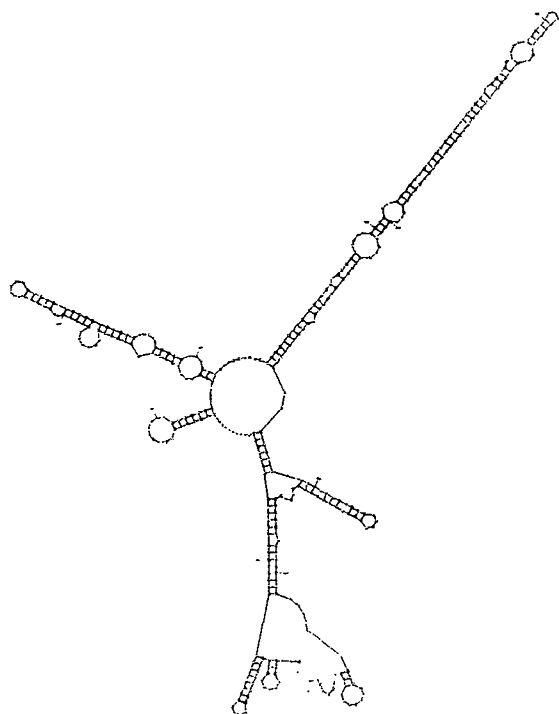
(f) dG= -155.85 *P. proliferusi* China: Yunnan Province



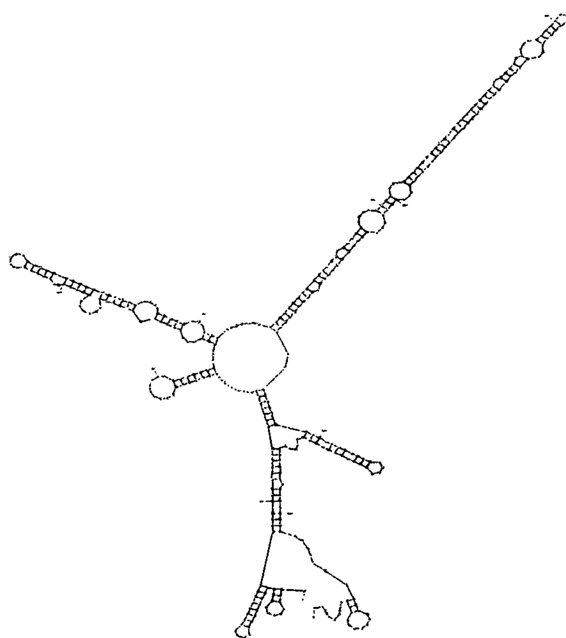
(g) dG= -151.55 *P. heterotremus* Thailand: Sasaburi



(h) dG= -144.44 *P. westermani* Thailand: Saraburi



(i) dG= -158.28 *P. bangkokensis* Thailand: Surat



(j) dG= -158.28 *P. harinasutai* Thailand: Saraburi

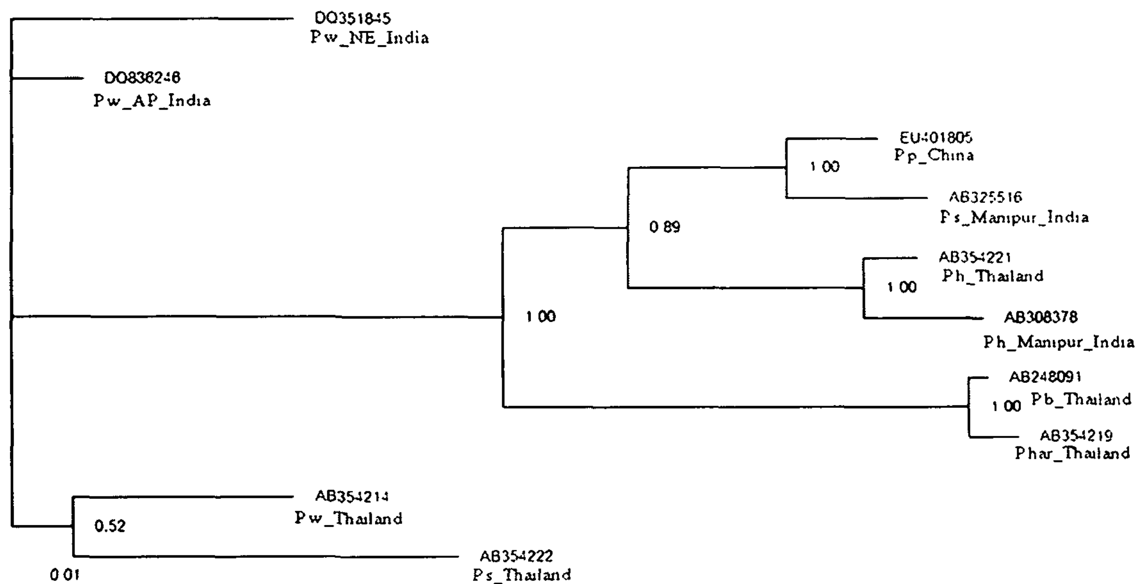


Figure 17. Phylogenetic relationships between members of family Paragonimidae. This tree shows hypothetical Bayesian analysis phylogeny based on the secondary structure alignment data of the ITS2 region. The numbers are equivalent to bootstrap percentages.

- ***Fasciolopsis buski*: PCR amplification of ITS regions and its analysis**

The PCR amplified products were successfully obtained using the primers as mentioned above and are depicted in Figures 18 & 19.

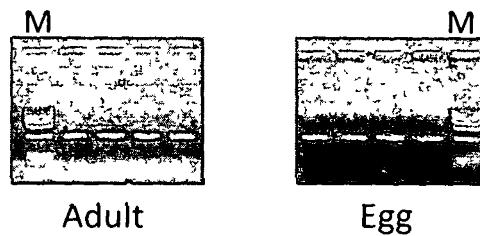


Figure 18. PCR products of *Fasciolopsis buski* using primer set 3S-A28- ITS2

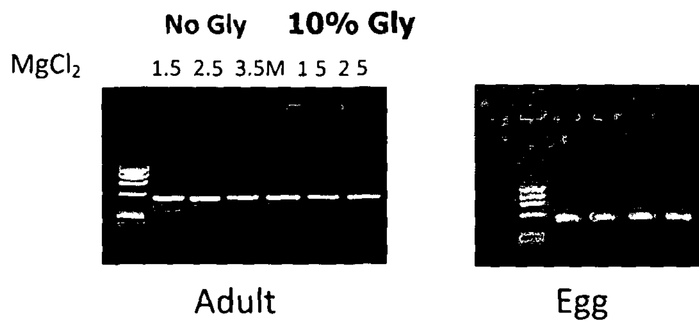


Figure 19. PCR product of *F buski* using primer set BD1-4S- ITS1

The nucleotide sequences obtained are shown below as raw sequences along with their depiction in bar diagrams (Figures 20 and 21).

ITS2 *Fasciolopsis buski* Adult (Forward sequence)

TGTCGATGAGGAGCGCAGCCACTGTGTGAATTAATGCAAACTGCATACTGCTTTGA
ACATCGACATCTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTG
TCCGAGGGTCGGCTTACAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCT
TGCCAGCTGGCGTGAACTCCTCTATGATTATTCATGTGAGGTGCCAGAACTATGGC
GTTTCCCTAATGTATCCGGACGCGTCCTTGTCTCAGCAGAAGGCTGTGGTGAGGT
GCGGTAGCGGGATCGTGGTTTAATGAATACTGTGCACGTTCCGTTGCTGTGTCTTC
ATCGTCGGCTTGATGCGTGACTTGGTCTCGTGTCTGAGGCTTTTGCCATACATAGA
CTGCCATTTGTGTGGTCTAATTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGA
ACTTAAGCATATCACTAAGCGGAGGAAAAGAACAAA

ITS2 *Fasciolopsis buski* Adult (Reverse sequence)

*GTTAGCTGATATGCTTATGTTCAGCGGGTAATCACGTCTGAACCGAGGTCAGGAAA
TTAGACCACACAAATGGCAGTCTATGTATGGCAAAAGCCTCAGACACGAGACCAAG
TCACGCATCAAGCCGACGATGAAGACACAGCAACGGAACGTGCACAGTATTCATTA
AACCACGATCCCGCTACCGCACCTCACCACAGCCTTCTGCTGAGACAAGGACGCG
TCCGGATACATTAGGGAAACGCCATAGTTCTGGCACCTCACATGAATAATCATAGA
GGAGTTCACGCCAGCTGGCAAGACCCAAGCCACGACTTTTTGGGCGTCGTGATAG
TTTGTAAGCCGACCCTCGGACAGGCGTGGCCACAGGCTAACCCATGGCCGCAATA
TGCGTTCAAGATGTCGATGTTCAAAGCAGTATGCAGTTTGCATTAATTCACACAGTT
GGCTGCGCTCTTCATCGACACACGAGCCGAGAGAACTACTCCGGATCCA*

ITS2 *Fasciolopsis buski* Egg (Forward sequence)

*CTGTCGATGAGGAGCGCAGCCACTGTGTGAATTAATGCAAACTGCATACTGCTTTG
AACATCGACATCTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCT
GTCCGAGGGTCGGCTTACAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTC
TTGCCAGCTGGCGTGAACTCCTCTATGATTATTCATGTGAGGTGCCAGAACTATGG
CGTTTCCCTAATGTATCCGGACGCGTCCTTGTCTCAGCAGAAGGCTGTGGTGAGG
TGCGGTAGCGGGATCGTGGTTTAATGAATACTGTGCACGTTCCGTTGCTGTGTCTT
CATCGTCGGCTTGATGCGTGACTTGGTCTCGTGTCTGAGGCTTTTGCCATACATAA
ACTGCCATTTGTGTGGTCTAATTTCCTGACCTCGGTTCAGACGTGATTACCCGCTG
AACTTAAGCATATCACTAAGCGGAGGAGAAGAAACTAACCCCGGAGCCCACAA*

ITS2 *Fasciolopsis buski* Egg (Reverse sequence)

*CTTAGCTGAGATGCTTATGTTCGGCGGGTAATCCCGTCTGAACCGAGGTCGGGAA
ATTAGACCCAACAAATGGCAGTCTATGTATGGCAAAAGCCTCAGACACGAGACCAA
GTCACGCATCAAGCCGACGATGAAGACACAGCAACGGAACGTGCACAGTATTCAT
TAAACCACGATCCCGCTACCGCACCTCACCACAGCCTTCTGCTGAGACAAGGACG
CGTCCGGATACATTAGGGAAACGCCATAGTTCTGGCACCTCACATGAATAATCATA
GAGGAGTTCACGCCAGCTGGCAAGACCCAAGCCACGACTTTTTGGGCGTCGTGAT
AGTTTGTAAGCCGACCCTCGGACAGGCGTGGCCACAGGCTAACCCATGGCCGCAA
TATGCGTTCAAGATGTCGATGTTCAAAGCAGTATGCAGTTTGCATTAATTCACACAG
TTGGCTGCGCTCTTCATCGACACACGAGGCGAGTGATCCCCCCCGGAGCCACACG*

ITS1 *Fasciolopsis buski* Adult (Forward sequence)

*CTGCTGACTGCGGAGGATCATTACCGTAATCCTATTCATACACAAGAGGTGAAACG
TTGTGACCGTCATGTCCAACGATACAAATTTGCGGACGGCTATGCCTGGCTCTTTG
AGGCCACAGCATATCCGATTATGACGGGGTGCCTACCTGTGTGATCCTCTGATGGT
ATGCATGCGTCCTTTGGGTCGTATGTCCAAGCCAGGAGAACAGGCTGTACTGCCG
TGACTGGTAGTGCTAGGCTTAAAGAGGAGATTTGAGCTACGGCCCTGCTCCCGCC
CTATGAACTGTTTCCTATATTACACTGTTCAAGTGGTATTGATTGGGTTCGCCCATT
CTTTGCCATTGCCCTCGCATGCACCTGGTCCTTGTGGCCGGACTGCACGTACGTC
GCCCGGCGGTGCCTATCCCGGGTAGGACTGATAACCTGGTCTTTGACCATTTGCA
CAACTCTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACT
GTGTGAATTAATGCAAACTGCATACTGCTTTGAACATCGACCTGGACAACGTATCTG
AA*

ITS1 *Fasciolopsis buski* Egg (Forward sequence)

*GTGACTGCGGAGGATCATTACCGTAATCCTATCTCATACACAAGAGGTGAAACGTT
GTGACCGTCATGTCCAACGATACAAATTTGCGGACGGCTATGCCTGGCTCTTTGAG
GCCACAGCATATCCGATTATGACGGGGTGCCTACCTGTGTGATCCTCTGATGGTAT
GCATGCGTCCTTTGGGTCGTATGTCCAAGCCAGGAGAACAGGCTGTACTGCCGTG
ACTGGTAGTGCTAGGCTTAAAGAGGAGATTTGAGCTACGGCCCTGCTCCCGCCCT
ATGAACTGTTTCCTATATTACACTGTTCAAGTGGTATTGATTGGGTTCGCCCATTCTT
TGCCATTGCCCTCGCATGCACCTGGTCCTTGTGGCCGGACTGCACGTACGTCGCC
CGGCGGTGCCTATCCCGGGTAGGACTGATAACCTGGTCTTTGACCATTTGCACAA
CTCTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTG
TGAATTAATGCAAACTGCATACTGCTTTGAACATCGACATTTCGCAA*

The results of similarity search for the query sequence were obtained using BLAST (Figures 22, 24, 26, 28, 30, 32). The top 15-20 best hits (having the maximum e-value) were used for the purpose of phylogenetic predictions using ClustalW bioinformatic tools. The resultant cladograms and phylograms along with respective gene identification number (gi) is depicted in Figures 23a & b; 25a&b, 27a&b, 29a&b, 31a&b, 33a & b.

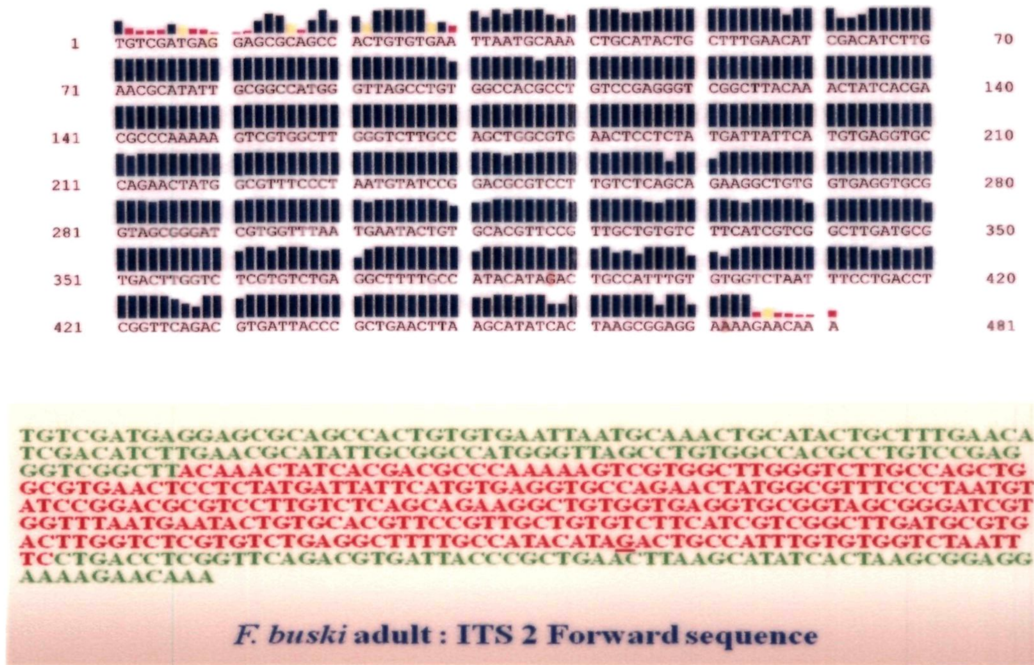


Figure 20. ***Fasciolopsis buski*** **adult ITS2: Forward sequence plus flanking sequences**

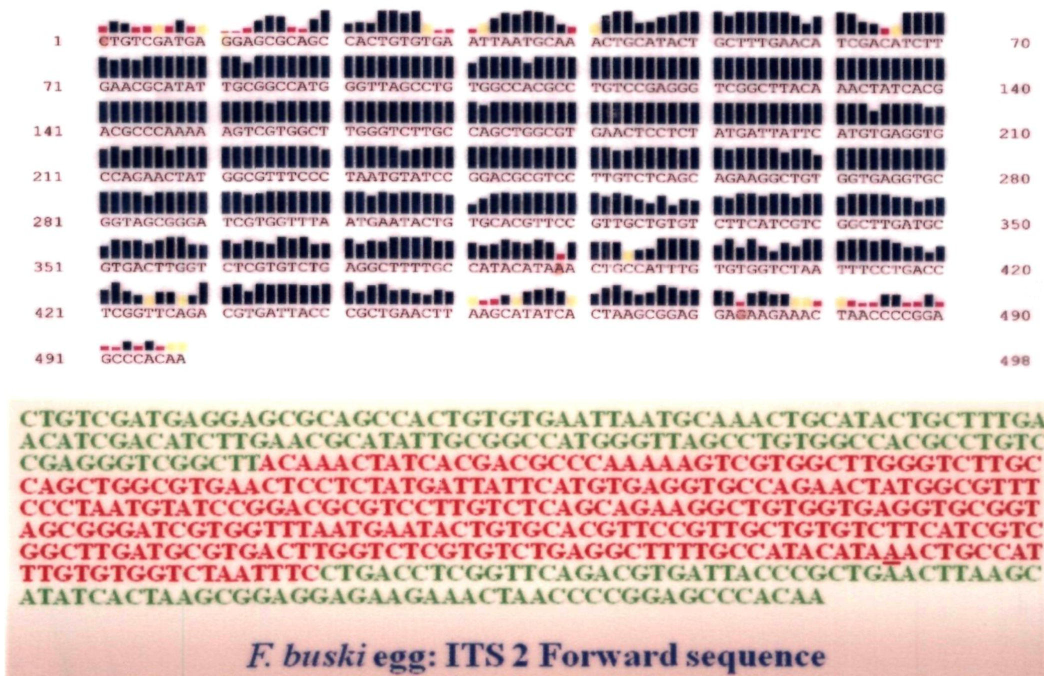


Figure 21. ***Fasciolopsis buski*** **egg ITS2: Forward sequence plus flanking sequences**

ITS2 Adult (Forward sequence)

Distribution of 149 Blast Hits on the Query Sequence

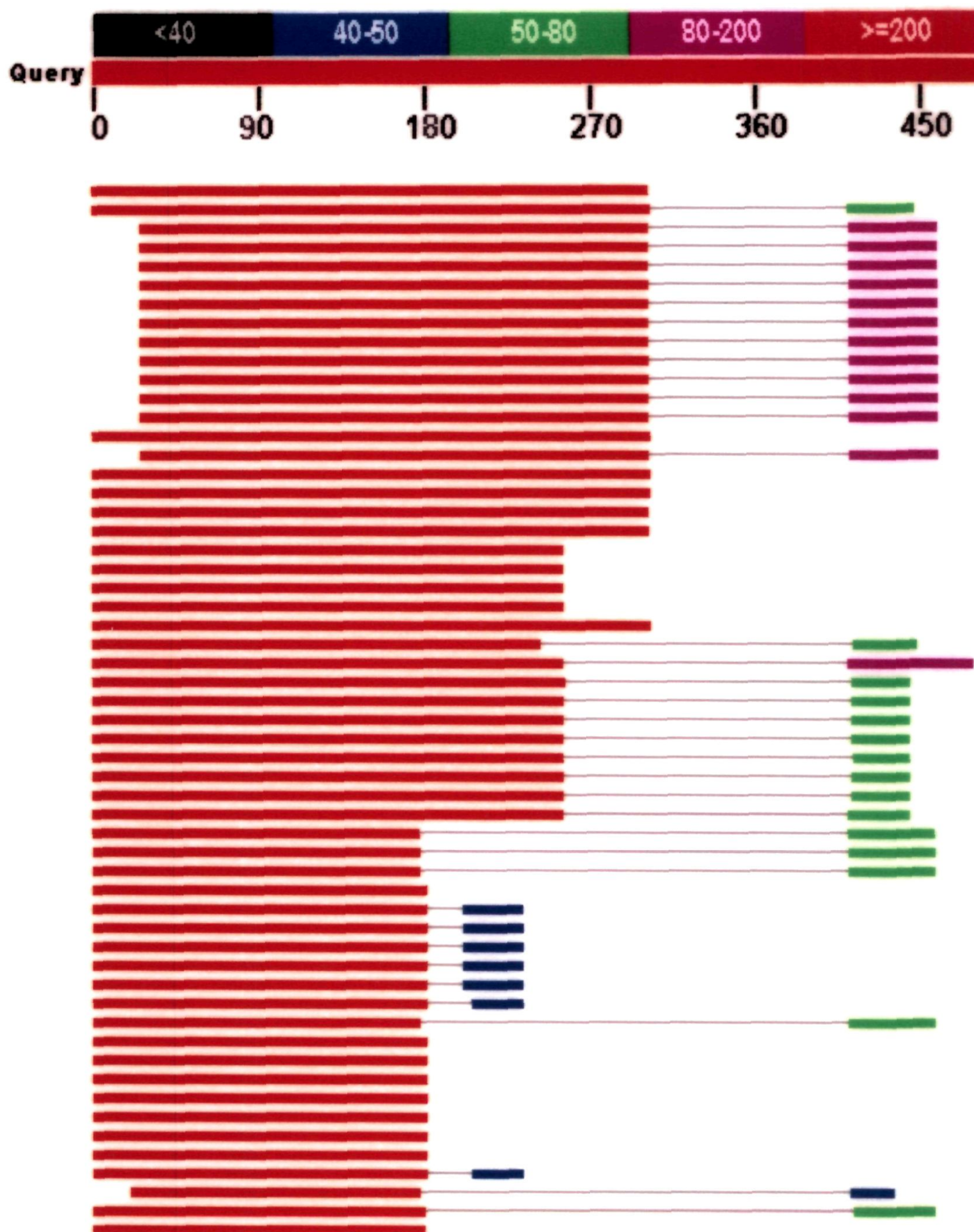
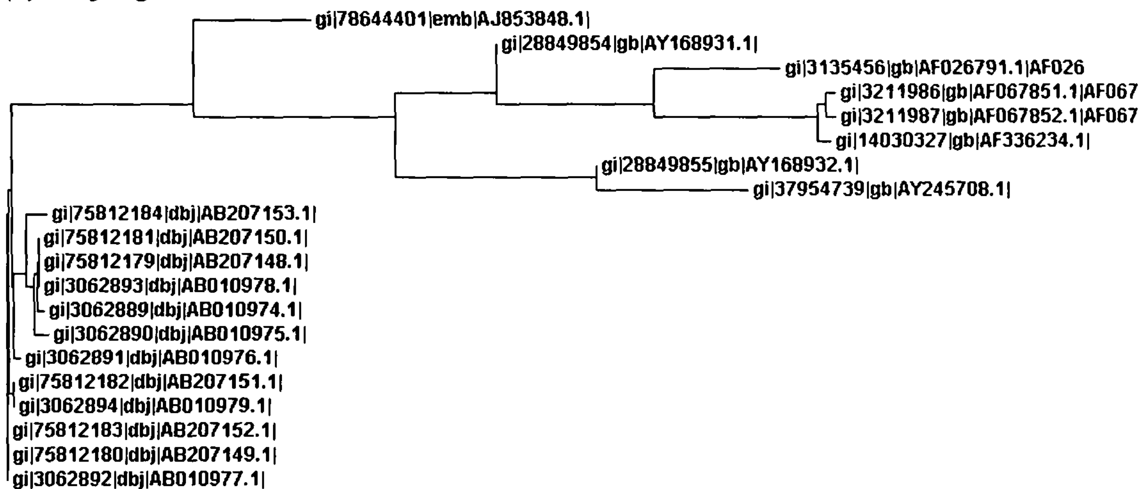


Figure 22. Blast hit result of ITS2 forward sequence (adult) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

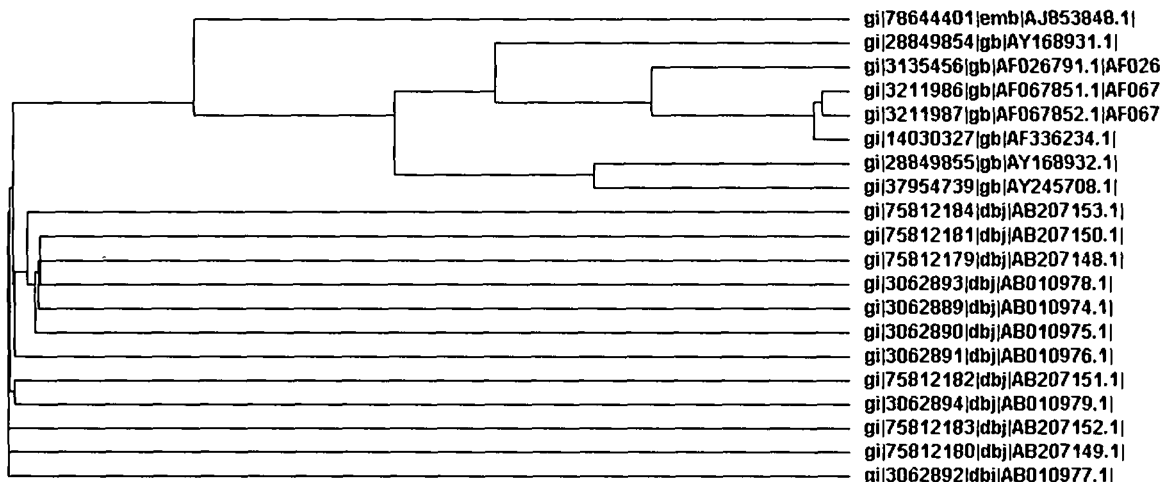


Figure 23a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso
- gi|28849854|gb|AY168931.1| *Echinoparyphium recurvatum* isolate W18 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|75812184|dbj|AB207153.1| *Fasciola sp.* Hiroshima-18 gene for ITS2
- gi|75812182|dbj|AB207151.1| *Fasciola sp* Saitama-10 gene for ITS2

- gi|75812181|dbj|AB207150.1| *Fasciola sp.* Hokkaido-1 gene for ITS2
- gi|3062893|dbj|AB010978.1| *Fasciola sp.* genes for 18S rRNA, 5.8S rRNA and ITS 2, partial sequence
- gi|3062892|dbj|AB010977 1| *Fasciola gigantica* genes for 18S rRNA, 5 8S rRNA and ITS2; partial sequence
- gi|3062891|dbj|AB010976.1| *Fasciola gigantica* genes for 18S rRNA, 5.8S rRNA and ITS2, partial sequence
- gi|3062889|dbj|AB010974.1| *Fasciola hepatica* genes for 18S rRNA, 5.8S rRNA and ITS2, partial sequence
- gi|28849855|gb|AY168932.1| *Isthmiophora melis* ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|3062890|dbj|AB010975.1| *Fasciola gigantica* genes for 18S rRNA, 5.8S rRNA and ITS2, partial sequence
- gi|3135456|gb|AF026791.1|AF026791 *Echinostoma sp.* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5 8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence
- gi|3211986|gb|AF067851.1|AF067851 *Echinostoma trivolvis* strain 11B ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|3211987|gb|AF067852.1|AF067852 *Echinostoma trivolvis* strain 12B ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030327|gb|AF336234.1| *Echinostoma paraensei* strain BH Laboratory ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence

The Blast hit results show that the query - ITS2 *Fasciolopsis buski* Adult Forward sequence is more similar to the sequence of the species *Fasciola gigantica, Echinostoma revolutum Isthmiophora melis, Echinostomasp, Paryphostomum radiatum, Echinostoma trivolvis, Echinostoma paraenei, Fasciola sp, Fasciola hepatica* and *Isthmiophora hortensis.*

From the phylogram and cladogram obtained using the tool ClustalW, sequences of two distinct clusters are revealed. One cluster is of *Fasciola sp* and other cluster consisting of genera *Echinostoma, Paryphostomum,* and *Isthmiophora.*

ITS2 Adult (Reverse sequence)

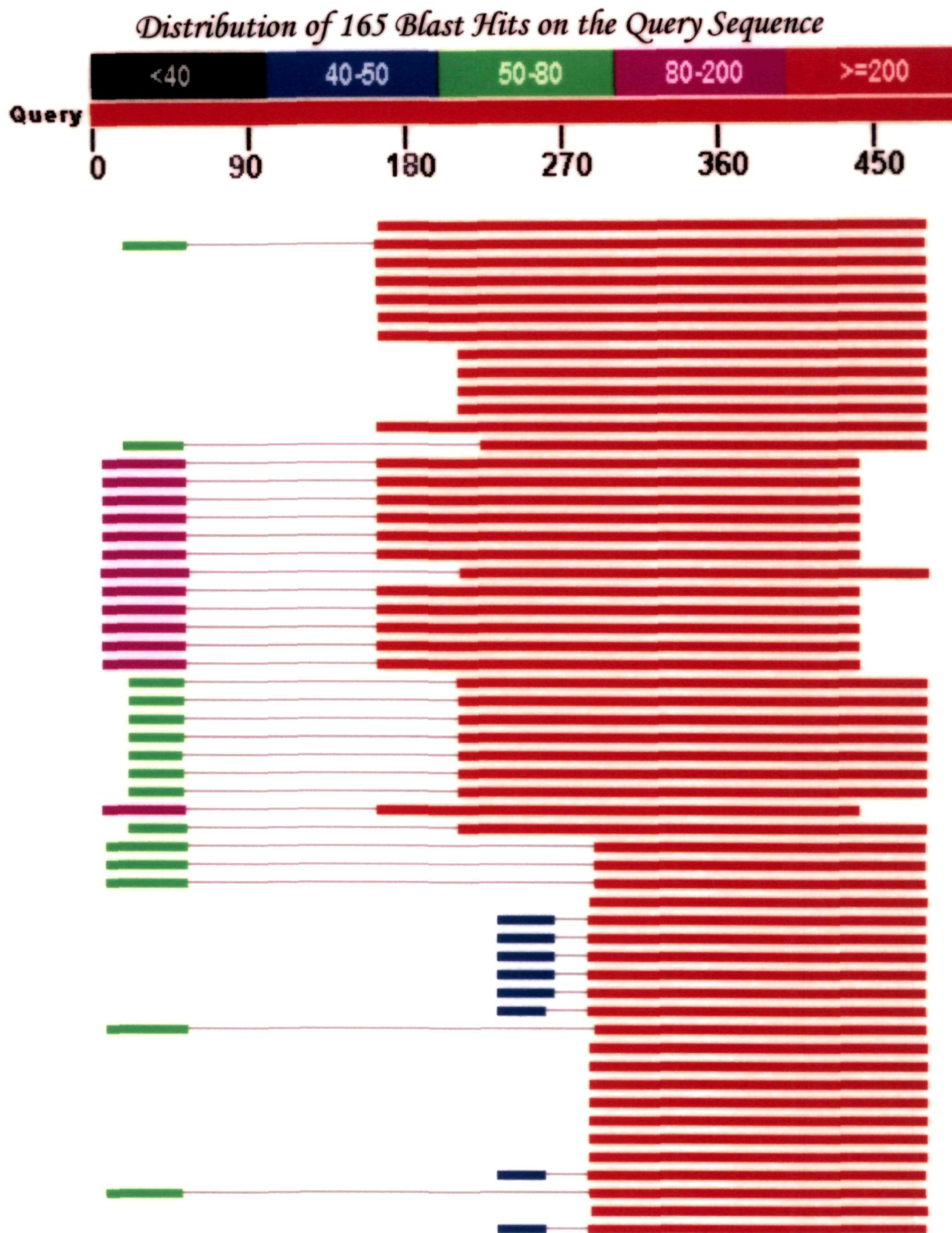
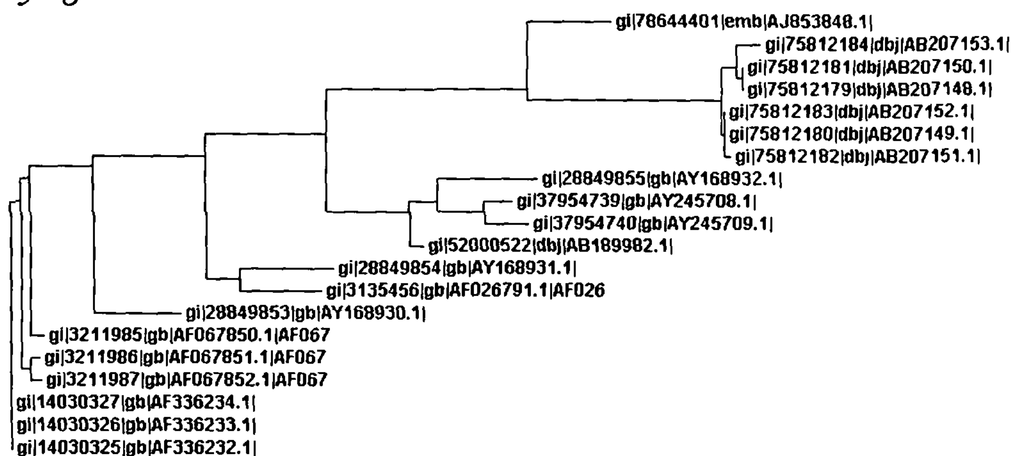


Figure 24. Blast hit result of ITS2 reverse sequence (adult) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

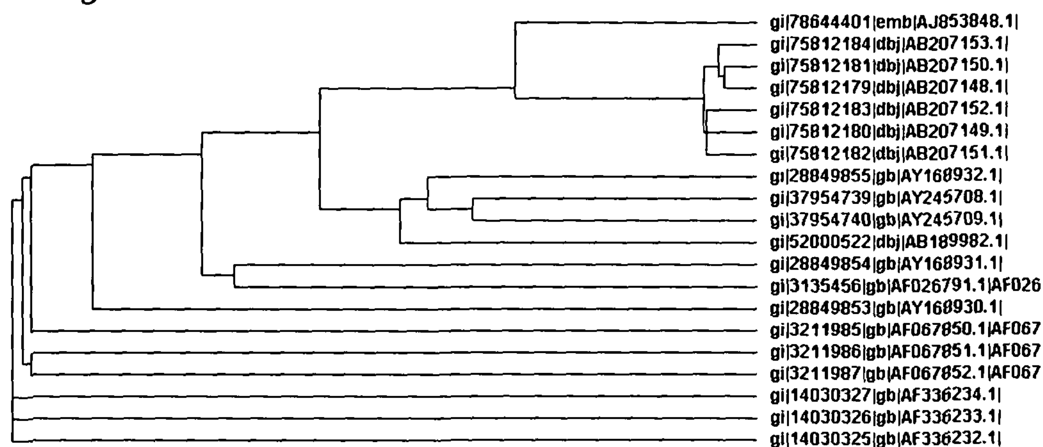


Figure 25a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso

- gi|28849854|gb|AY168931.1| *Echinoparyphium recurvatum* isolate W18 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene

- gi|28849855|gb|AY168932.1| *Isthmiophora melis* ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

- gi|75812184|dbj|AB207153.1| *Fasciola sp.* Hiroshima-18 gene for ITS2

- gi|3135456|gb|AF026791 1|AF026791 *Echinostoma sp* 18S ribosomal RNA gene, partial sequence, ITS 1 and 5 8S ribosomal RNA gene, complete sequence, and ITS 2, partial sequence
- gi|37954739|gb|AY245708 1| *Paryphostomum radiatum* 18S ribosomal RNA gene, partial sequence, ITS 1 and 5 8S ribosomal RNA gene, complete sequence, and ITS 2, partial sequence
- gi|3211986|gb|AF067851 1|AF067851 *Echinostoma trivolvis* strain 11B ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030327|gb|AF336234 1| *Echinostoma paraensei* strain BH Laboratory ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030326|gb|AF336233 1| *Echinostoma paraensei* strain RJ Laboratory ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030325|gb|AF336232 1| *Echinostoma paraensei* strain Sumidouro ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|3211985|gb|AF067850 1|AF067850 *Echinostoma revolutum* ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|37954740|gb|AY245709 1| *Petasiger phalacrocoracis* 18S ribosomal RNA gene, partial sequence, ITS 1 and 5 8S ribosomal RNA gene, complete sequence, and ITS 2, partial sequence
- gi|75812183|dbj|AB207152 1| *Fasciola sp* 25 TI-2005 gene for ITS2
- gi|75812182|dbj|AB207151 1| *Fasciola sp* Saitama-10 gene for ITS2
- gi|75812181|dbj|AB207150 1| *Fasciola sp* Hokkaido-1 gene for ITS2
- gi|75812180|dbj|AB207149 1| *Fasciola gigantica* gene for ITS2
- gi|52000522|dbj|AB189982 1| *Isthmiophora hortensis* genes for 18S rRNA, ITS1, 5 8S rRNA, ITS2, 28S rRNA, partial and complete sequence

The Blast hit resultsof reverse sequence show the same similarity as that of forward sequence.

From the phylogram and cladogram revealed sequences of two distinct clusters. One cluster is of *Fasciola sp* and other cluster consisting of genus *Echinostoma* In between these two clusters there found genera *Paryphostomum, Petasiger* and *Isthmiophora*

ITS2 Egg (Forward sequence)

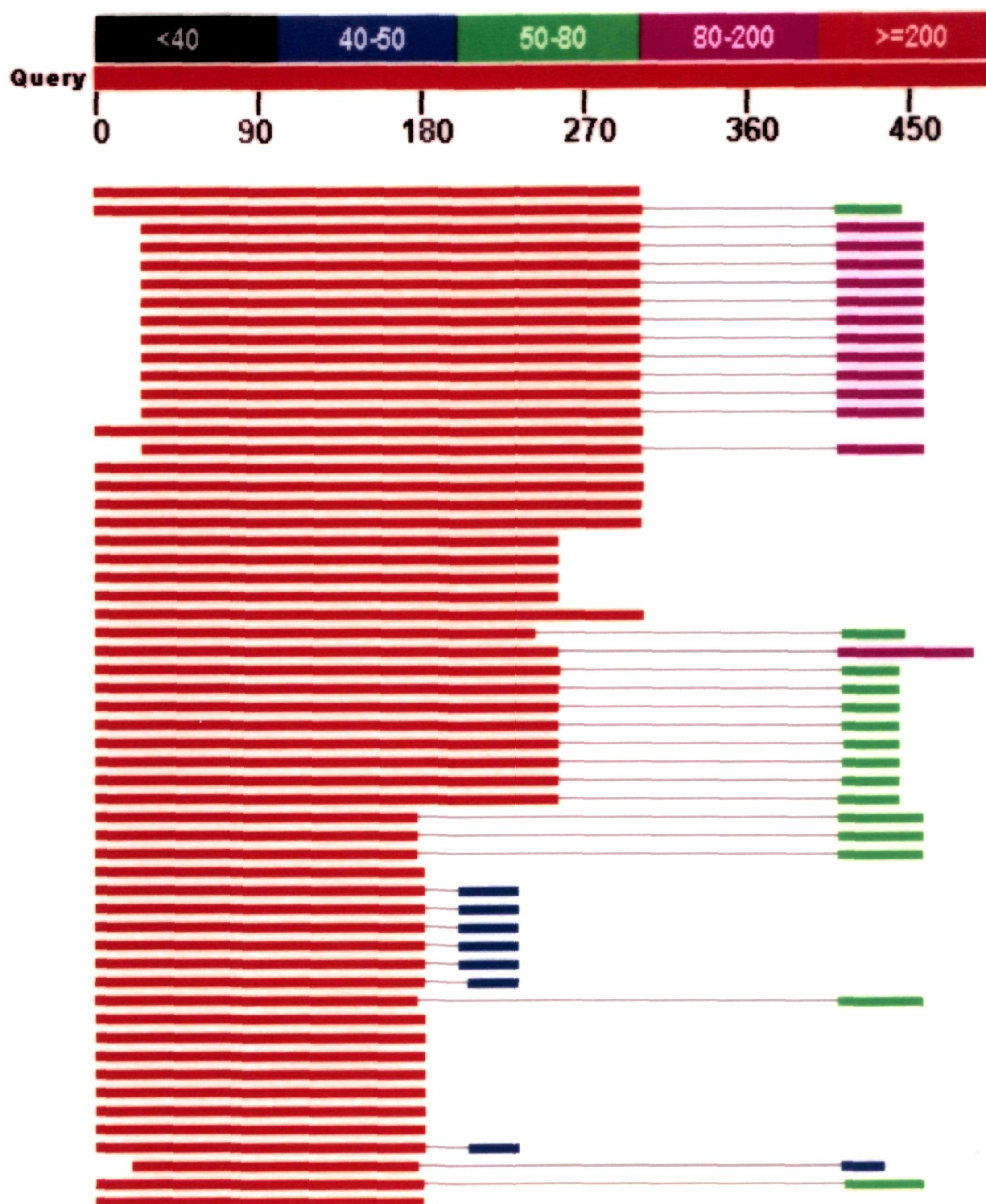
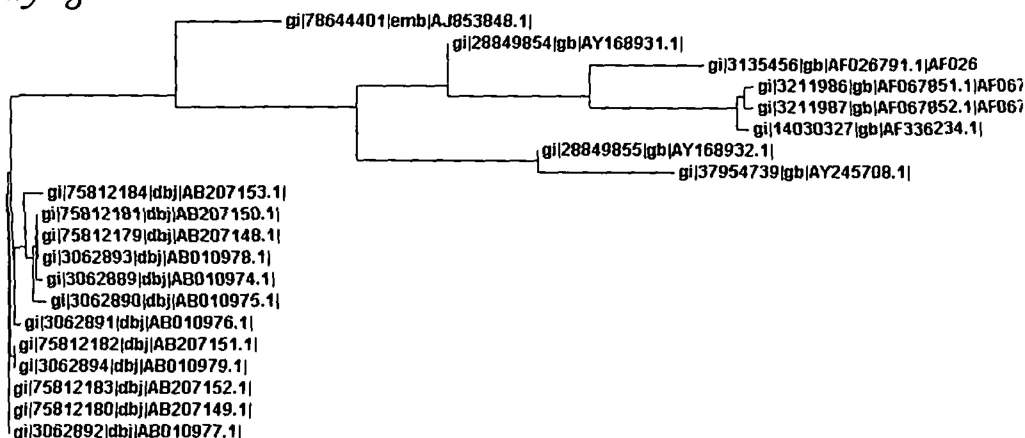


Figure 26. Blast hit result of ITS2 forward sequence (egg) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

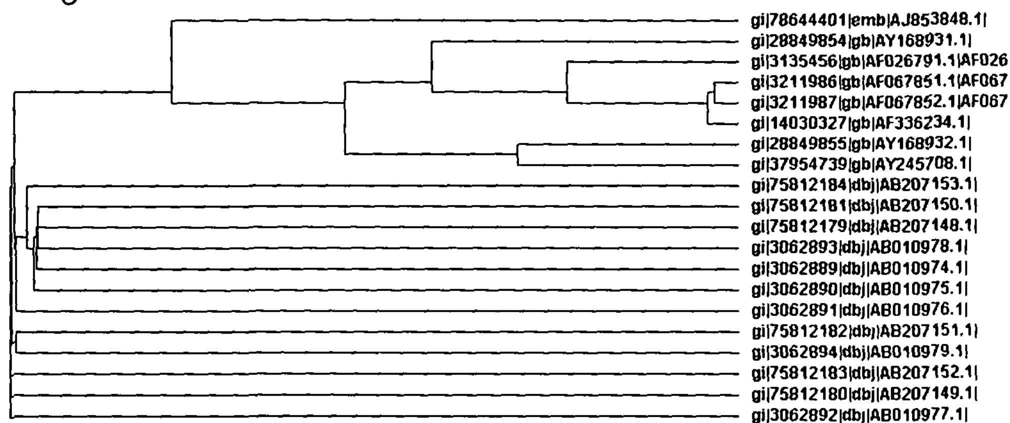


Figure 27a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso
- gi|28849854|gb|AY168931.1| *Echinoparyphium recurvatum* isolate W18 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|75812184|dbj|AB207153.1| *Fasciola sp.* Hiroshima-18 gene for ITS2
- gi|75812182|dbj|AB207151.1| *Fasciola sp.* Saitama-10 gene for ITS2
- gi|75812180|dbj|AB207149.1| *Fasciola gigantica* gene for ITS2
- gi|75812179|dbj|AB207148 1| *Fasciola hepatica* gene for ITS2

- gi|3062894|dbj|AB010979.1| *Fasciola sp.* genes for 18S rRNA, 5.8S rRNA and ITS 2, partial sequence
- gi|3062893|dbj|AB010978.1| *Fasciola sp.* genes for 18S rRNA, 5.8S rRNA and ITS 2, partial sequence
- gi|3062892|dbj|AB010977.1| *Fasciola gigantica* genes for 18S rRNA, 5.8S rRNA and ITS2, partial sequence
- gi|3062889|dbj|AB010974.1| *Fasciola hepatica* genes for 18S rRNA, 5.8S rRNA and ITS2, partial sequence
- gi|28849855|gb|AY168932.1| *Isthmiophora melis* ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|3062890|dbj|AB010975.1| *Fasciola gigantica* genes for 18S rRNA, 5.8S rRNA and internal transcribed spacer 2, partial sequence
- gi|3135456|gb|AF026791.1|AF026791 *Echinostoma sp.* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence
- gi|37954739|gb|AY245708.1| *Paryphostomum radiatum* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence
- gi|3211986|gb|AF067851.1|AF067851 *Echinostoma trivolvis* strain 11B ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030327|gb|AF336234.1| *Echinostoma paraensei* strain BH Laboratory ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence

The Blast hit results show that the query - ITS2 *Fasciolopsis buski* egg forward sequence is more similar to the sequence of the species *Fasciola gigantica, Echinostoma revolutum Isthmiophora melis, Echinostoma sp, Paryphostomum radiatum, Echinostoma trivolvis, Echinostoma paraenei, Fasciola sp, Fasciola hepatica* and *Isthmiophora hortensis.*

From the phylogram and cladogram obtained using the tool ClustalW, sequences of two distinct clusters are revealed. One cluster is of *Fasciola sp* and other cluster consisting of genera *Echinostoma, Paryphostomum* and *Isthmiophora*

ITS2 Egg (Reverse sequence)

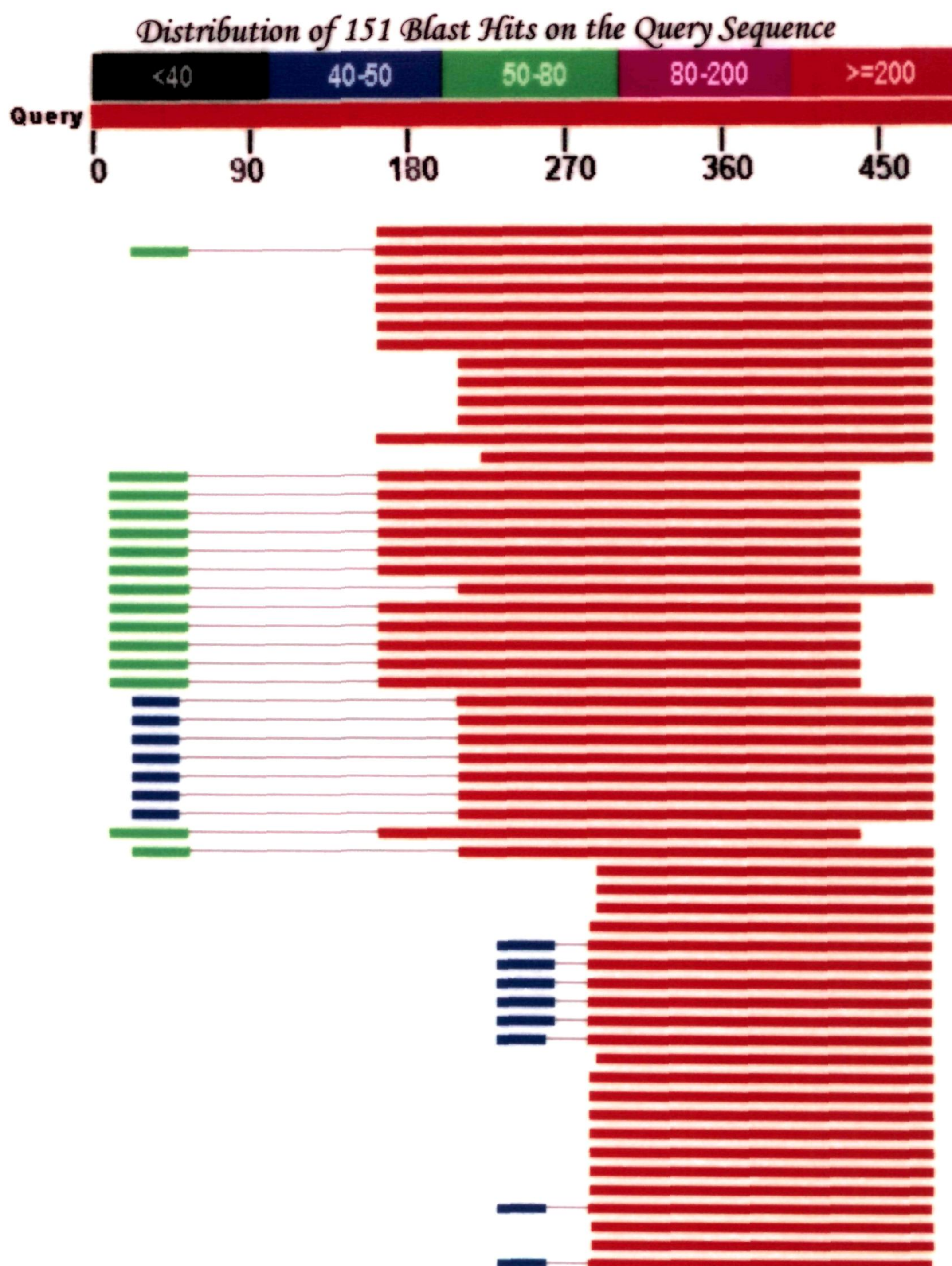
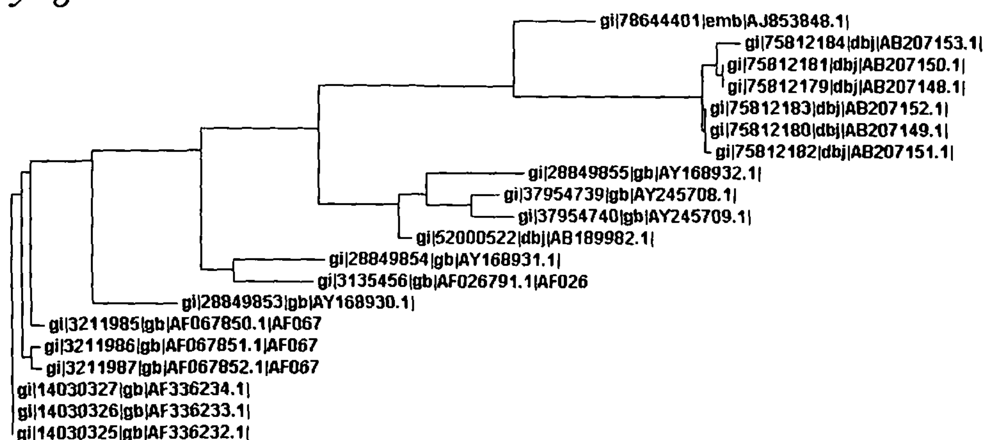


Figure 28. Blast hit result of ITS2 reverse sequence (egg) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

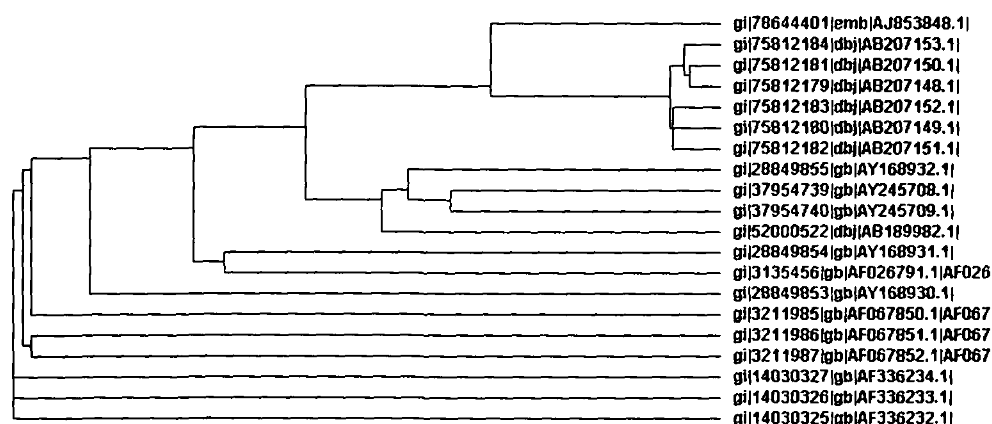


Figure 29a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso
- gi|28849854|gb|AY168931.1| *Echinoparyphium recurvatum* isolate W18 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene
- gi|28849855|gb|AY168932.1| *Isthmiophora melis* ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|3135456|gb|AF026791.1|AF026791 *Echinostoma sp.* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence

- gi|37954739|gb|AY245708.1| *Paryphostomum radiatum* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5.8S ribosomal RNA gene, complete sequence, and ITS 2, partial sequence

- gi|3211986|gb|AF067851.1|AF067851 *Echinostoma trivolvis* strain 11B ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence

- gi|14030327|gb|AF336234.1| *Echinostoma paraensei* strain BH Laboratory ITS 1, 5.8S ribosomal RNA gene, and ITS 2

- gi|14030325|gb|AF336232.1| *Echinostoma paraensei* strain Sumidouro ITS 1, 5.8S ribosomal RNA gene, and ITS 2

- gi|3211985|gb|AF067850.1|AF067850 *Echinostoma revolutum* ITS 1, 5.8S ribosomal RNA gene, and ITS 2

- gi|37954740|gb|AY245709.1| *Petasiger phalacrocoracis* 18S ribosomal RNA gene, partial sequence; ITS 1 and 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence

- gi|28849853|gb|AY168930.1| *Echinostoma revolutum* isolate J5 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

- gi|75812184|dbj|AB207153.1| *Fasciola sp.* Hiroshima-18 gene for ITS2

- gi|75812183|dbj|AB207152.1| *Fasciola sp* 25 TI-2005 gene for ITS2

- gi|75812182|dbj|AB207151.1| *Fasciola sp.* Saitama-10 gene for ITS2
- gi|75812181|dbj|AB207150.1| *Fasciola sp.* Hokkaido-1 gene for ITS2

- gi|75812180|dbj|AB207149.1| *Fasciola gigantica* gene for ITS2

- gi|75812179|dbj|AB207148.1| *Fasciola hepatica* gene for ITS2

- gi|52000522|dbj|AB189982.1| *Isthmiophora hortensis* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence

The Blast hit results showed that the reverse sequence show the same similarity as that of forward sequence.

From the chylogram and cladogram obtained using the tool ClustalW, sequences of two distinct clusters are revealed. One cluster is of *Fasciola sp* and other cluster consisting of genera *Echinostoma, Paryphostomum,* and *Isthmiophora*

ITS1 Adult (Forward sequence)

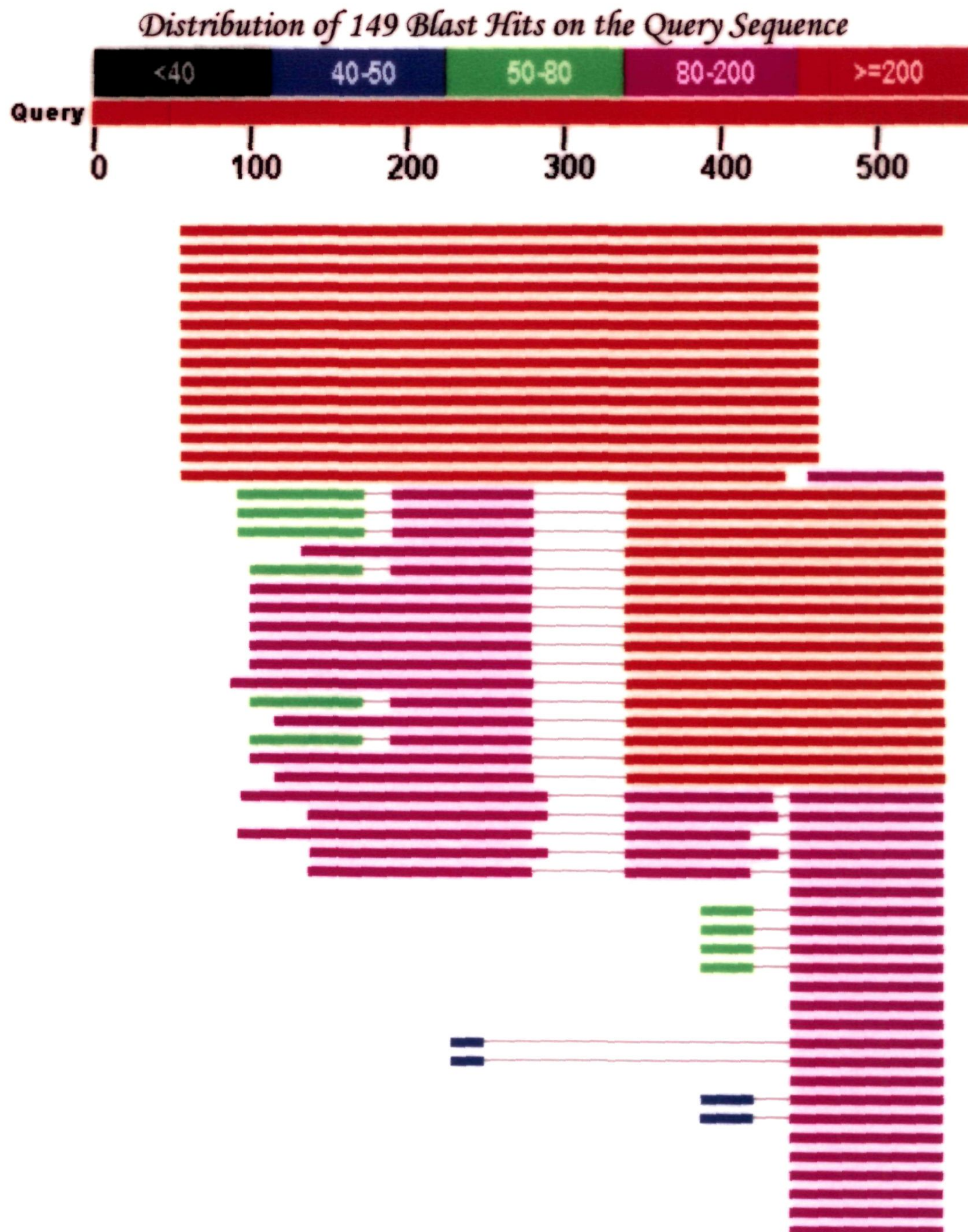
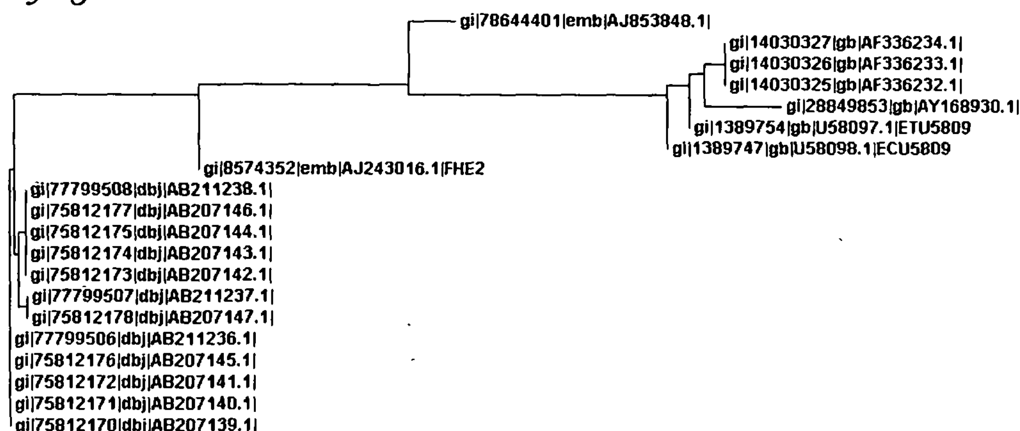


Figure 30. Blast hit result of ITS1 forward sequence (adult) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

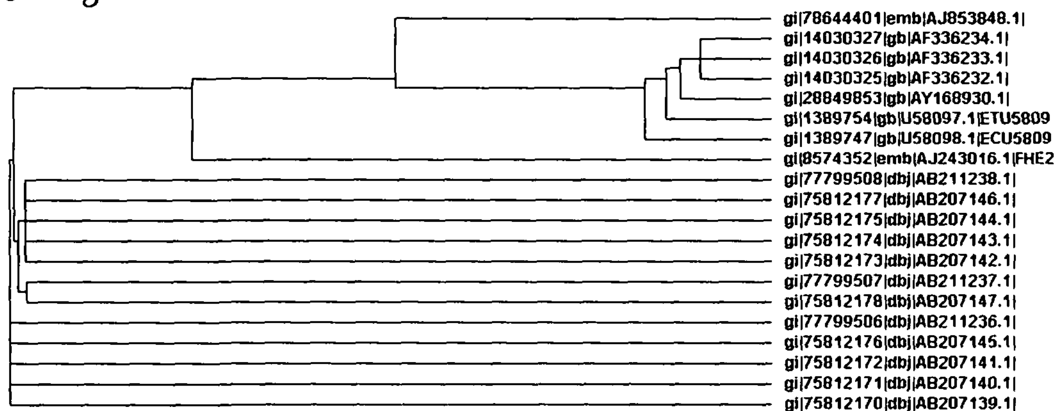


Figure 31 a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso
- gi|77799508|dbj|AB211238.1| *Fasciola sp.* HS-5 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, isolate:HS#5
- gi|75812177|dbj|AB207146.1| *Fasciola sp.* Saitama-10 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, strain:Japanese
- gi|75812175|dbj|AB207144.1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, country:Thailand: Bangkok
- gi|75812174|dbj|AB207143.1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Indonesia

- gi|75812173|dbj|AB207142.1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Zambia: Lusaka
- gi|77799507|dbj|AB211237.1| *Fasciola sp.* HS-3 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, isolate
- gi|75812176|dbj|AB207145.1| *Fasciola sp.* Hokkaido-1 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, strain:Japanese
- gi|75812172|dbj|AB207141.1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Ireland: Ireland
- gi|75812171|dbj|AB207140.1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Australia
- gi|75812170|dbj|AB207139.1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Uruguay: Montevideo
- gi|8574352|emb|AJ243016.1|FHE243016 *Fasciola hepatica* ITS 1 (ITS1)
- gi|14030325|gb|AF336232.1| *Echinostoma paraensei* strain Sumidouro ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|28849853|gb|AY168930.1| *Echinostoma revolutum* isolate J5 ITS 1, ; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|1389754|gb|U58097.1|ETU58097 *Echinostoma trivolvis* 18S and 28S ribosomal RNA genes, partial sequence, and 5.8S ribosomal RNA gene and ITS 1 and 2, complete sequence
- gi|1389747|gb|U58098.1|ECU58098 *Echinostoma caproni* 18S and 28S ribosomal RNA genes, partial sequence, and 5.8S ribosomal RNA gene and ITS 1 and 2, complete sequence

The Blast hit results show that the query – ITS1 *Fasciolopsis buski* adult forward sequence is more similar to the sequence of the species *Fasciola gigantica, Fasciola sp, Fasciola hepatica, Echinostoma paraenei, Echinostoma revolutum, Echinostoma trivolvis, and Echinostoma caproni.*

From the phylogram and cladogram obtained using the tool ClustalW, sequences of two distinct clusters are revealed. One cluster is of genus *Fasciola* and other cluster consisting of genus *Echinostoma.*

ITS1 Egg (Forward sequence)

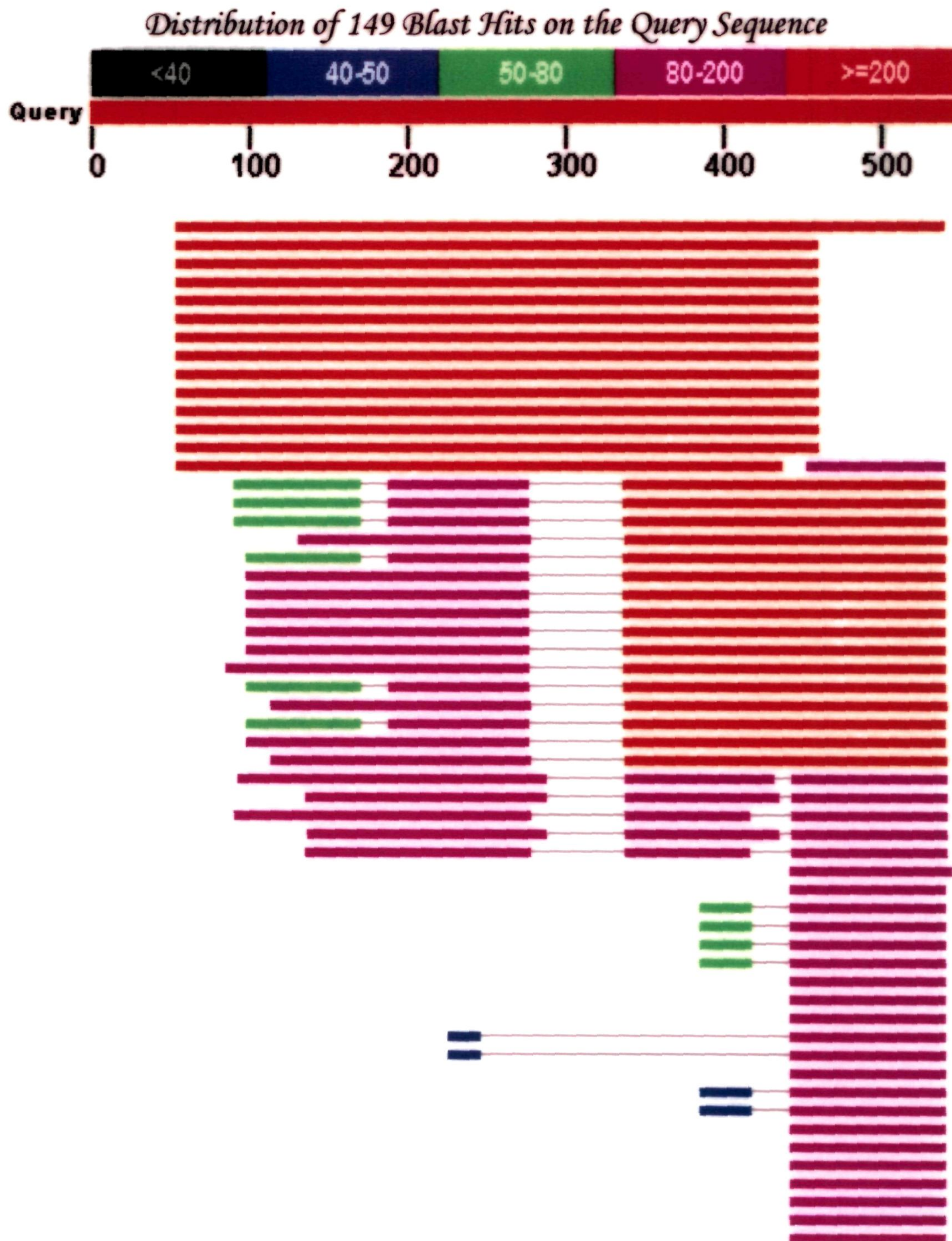
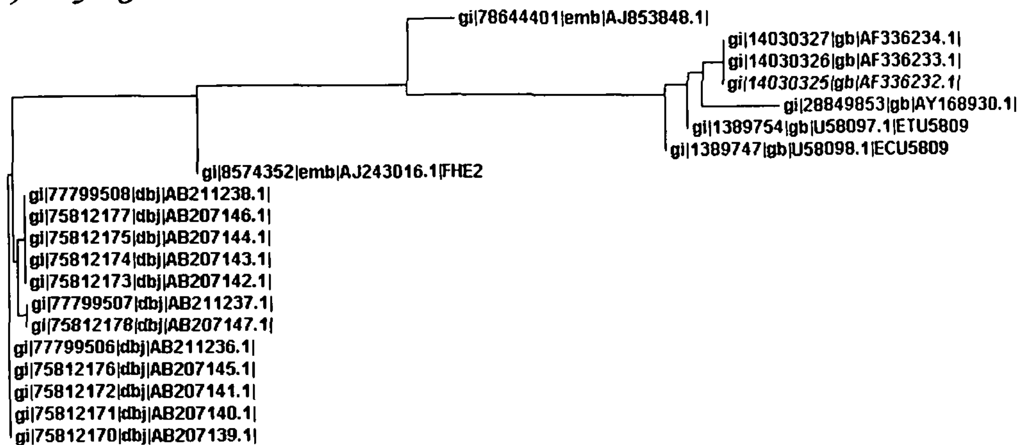


Figure 32. Blast hit result of ITS1 forward sequence (egg) of *Fasciolopsis buski*

(a) Phylogram



(b) Cladogram

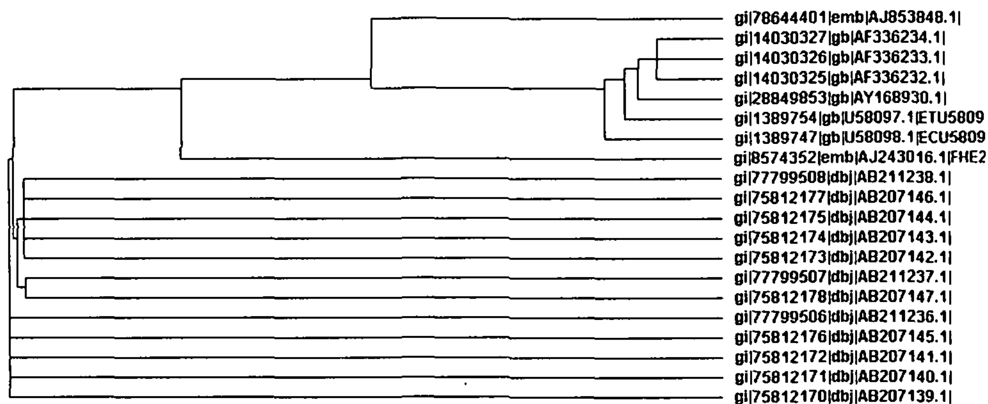


Figure 33 a & b. Phylogram and cladogram along with respective gene identification numbers.

- gi|78644401|emb|AJ853848.1| *Fasciola gigantica* ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso

- gi|77799508|dbj|AB211238.1| *Fasciola sp.* HS-5 genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, isolate:HS#5

- gi|75812177|dbj|AB207146.1| *Fasciola sp. Saitama-10 genes for 18S rRNA, ITS1, 5.8S* rRNA, partial and complete sequence, strain:Japanese (F. gigantica type)

- gi|75812175|dbj|AB207144.1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Thailand: Bangkok

- gi|75812174|dbj|AB207143.1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5.8S rRNA, partial and complete sequence, country:Indonesia

- gi|75812173|dbj|AB207142 1| *Fasciola gigantica* genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, country Zambia Lusaka
- gi|75812178|dbj|AB207147 1| *Fasciola sp* Hiroshima-18 genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, strain Japanese (Heterotype)
- gi|75812172|dbj|AB207141 1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, country Ireland Ireland
- gi|75812171|dbj|AB207140 1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, country Australia
- gi|75812170|dbj|AB207139 1| *Fasciola hepatica* genes for 18S rRNA, ITS1, 5 8S rRNA, partial and complete sequence, country Uruguay Montevideo
- gi|14030327|gb|AF336234 1| *Echinostoma paraensei* strain BH Laboratory ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030326|gb|AF336233 1| *Echinostoma paraensei* strain RJ Laboratory ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|14030325|gb|AF336232 1| *Echinostoma paraensei* strain Sumidouro ITS 1, 5 8S ribosomal RNA gene, and ITS 2, complete sequence
- gi|28849853|gb|AY168930 1| *Echinostoma revolutum* isolate J5 ITS 1, partial sequence, 5 8S ribosomal RNA gene and ITS 2, complete sequence, and 28S ribosomal RNA gene, partial sequence
- gi|1389754|gb|U58097 1|ETU58097 *Echinostoma trivolvis* 18S and 28S ribosomal RNA genes, partial sequence, and 5 8S ribosomal RNA gene and ITS 1 and 2, complete sequence
- gi|1389747|gb|U58098 1|ECU58098 *Echinostoma caproni* 18S and 28S ribosomal RNA genes, partial sequence, and 5 8S ribosomal RNA gene and ITS 1 and 2, complete sequence

The Blast hit results show that the query – ITS1 *Fasciolopsis buski* Egg Forward sequence is more similar to the sequence of the species *Fasciola gigantica, Fasciola sp, Fasciola hepatica, Echinostoma paraenei, Echinostoma revolutum, Echinostoma trivolvis, and Echinostoma caproni*

From the Phylogram and cladogram obtained using the tool ClustalW, sequences of two distinct clusters are revealed. One cluster is of genus *Fasciola* and other cluster consisting of genus *Echinostoma*

The nucleotide sequences were obtained for ITS1 & ITS2 of rDNA adult and egg stages of *F. buski* and were compared with sequences of other trematode species obtained from Genbank. The fragments of amplified DNA were estimated to be 480-550bp long. Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481bp and 498bp for forward primer and 559bp and 548bp for forward primer of ITS1, respectively in adult and egg. No intra-specific variations in length or composition of the sequence were observed, and the ITS sequences of both adult and egg origin were found to be identical in length as well as composition (Figure 34).

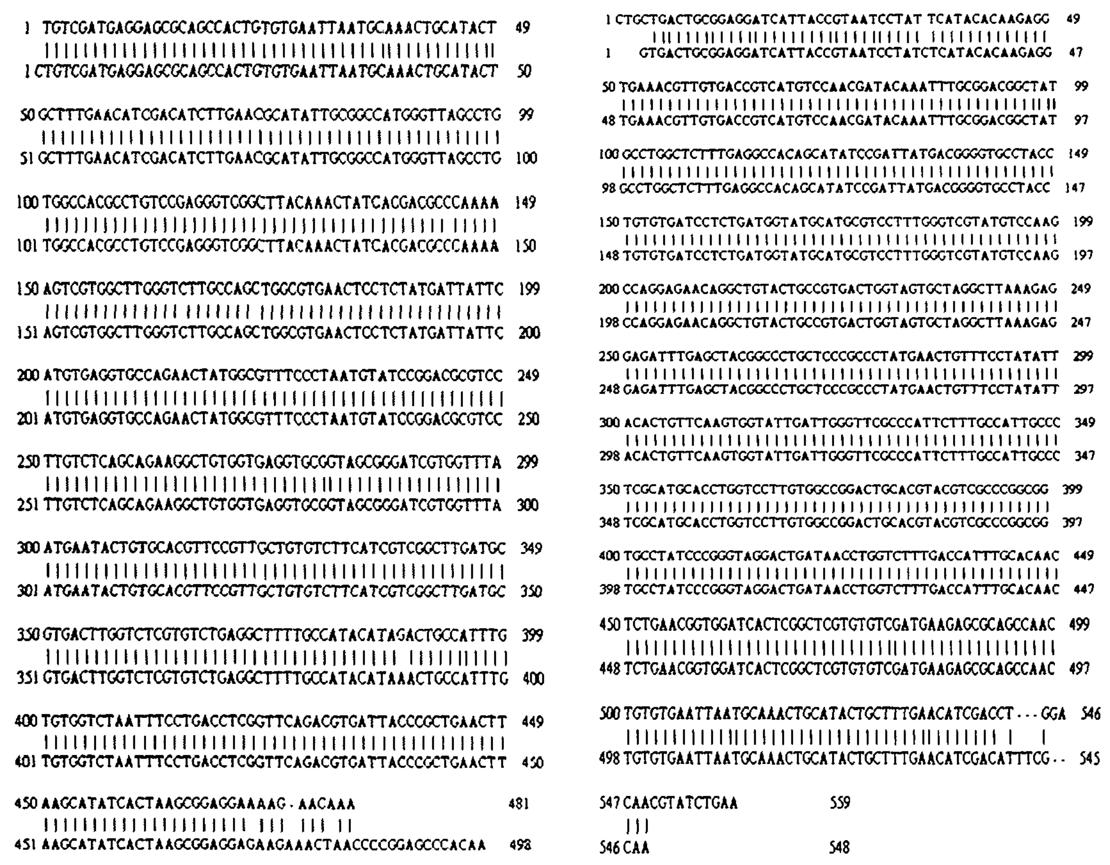


Figure 34. Pair-wise alignment of ITS sequences of Egg and Adult stages

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F. buski* and available ITS (I & II) sequences for other digenean trematodes including fasciolid species. Phylogenetic analyses using the various distance methods and character state method like Maximum Parsimony show that the topology is similar among the trees obtained (Figures 35-38). Boot strapping of the sequences with Neighbour- Joining revealed significant support (100%) for the clade containing *F. buski*, *F. hepatica*, *F. gigantica* and *Fascioloides magna*. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids.

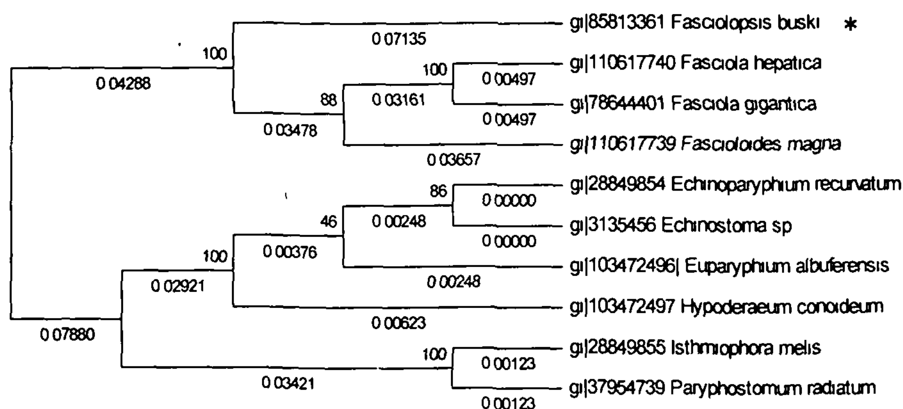


Figure 35. Neighbor-Joining tree for the query (*) ITS2 sequence

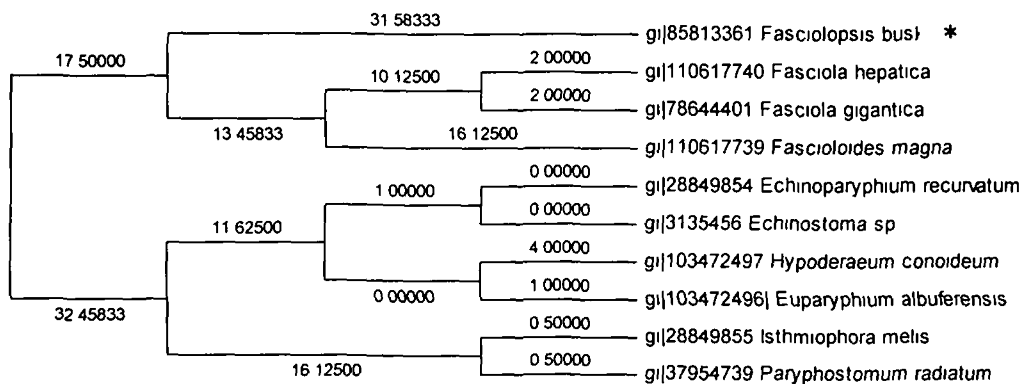


Figure 36. Maximum Parsimony tree for query (*) ITS2 sequence

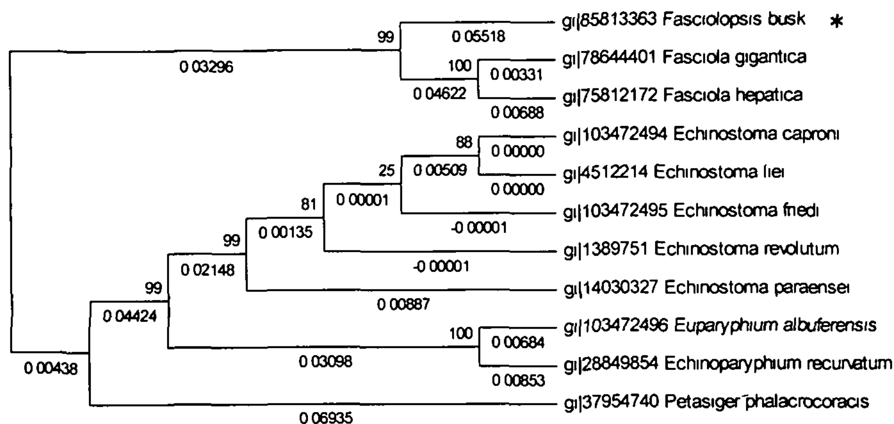
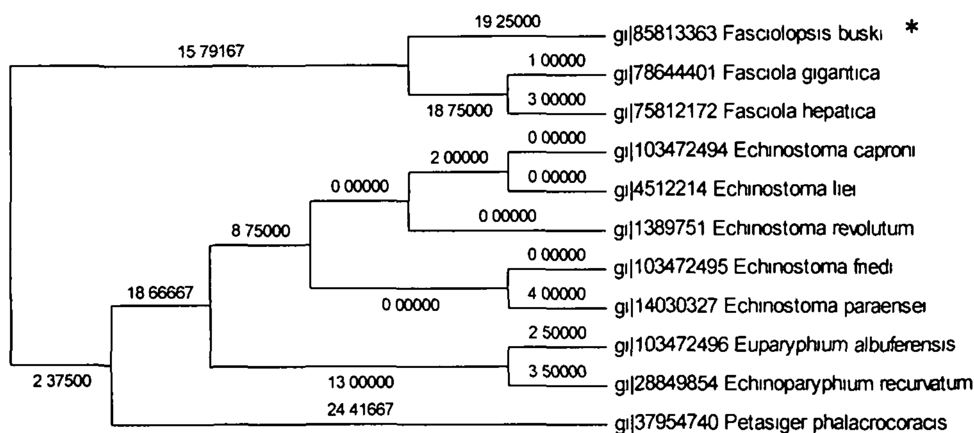


Figure 37. Neighbor Joining tree for query (*) ITS1 sequence



Figere 38. Maximum Parsimony tree for query (*) ITS1 sequence

- ***Fasciola gigantica*: PCR amplification of ITS regions and its analysis**

The PCR-amplified products were successfully obtained using the primers as mentioned above and are depicted in Figure 39.

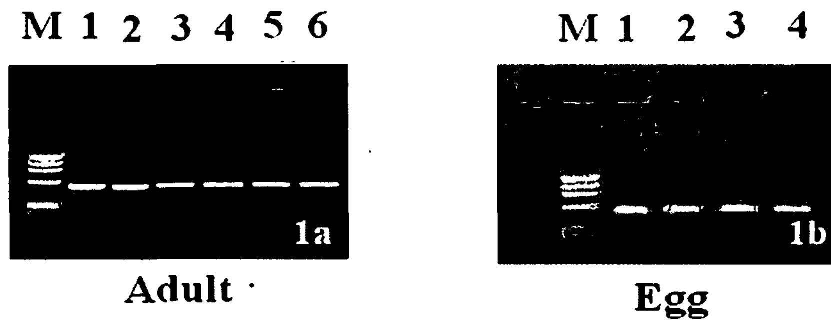


Figure 39. PCR product of *Fasciola gigantica* using primer set 3S-A28 for ITS2

ITS2 *Fasciola gigantica* Adult (Forward sequence)

*GGTCGAGTGAGCGTCGGCTTGTGGTGCATTTTAGCAACTCGCATACTGCTTTGAACATCG
ACATTTTGAACGCATCTTGAGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCG
GCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGAT
CTCCTCTATGAGTAATCATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTATCCGGAT
GCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGGTGCAGTGGCGGAATCGTGGTTTAATAA
TCGGGTTGGTACTCAGTTGTCAGTGTGTTCGGCGATCCCCTAGTCGGCACACTCATGATTT
CTGGGATAATTCCATACCAGGCACGTTCCGTTACTGTTACTTTGTCATTGGTTTGATGCTGA
ACTTGGTCATGTGTCTGATGCTATTTCATATAACGACGGTACCCTTCGTGGTCTGTCTTCCT
GACCTCGGTTCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGAAAAG
AAAACTAACAAGGATCCCACGGACGAGAGATAAACAAGGATCCACGCGCCGAGGGGATA
CACGGAATCCACGCCCCCCATTCATTCTCCGATTTCACCGCCCCGAGGGCTCCATTGTATT
CACGGCCCCGGGGGCTCGCTGGGATACCAGAGCCCAGACACGGTTGGTTTTCCT*

ITS1 *Fasciola gigantica* Adult (Forward sequence)

*GTTAGCTACTTACACAAGCGATACACGTGTGACCGTCATGTCATGCGATAAAAATTTGCGG
ACGGCTATGCCTGGCTCATTGAGGTCACAGCATATCCGATCACTGATGGGGTGCCTACCT
GTATGATACTCCGATGGTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACG
GGTTGTACTGCCATGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGCCCTG
CTCCCGCCCTATGAACTGTTTCATTACTACAATTACACTGTTAAAGTGGTATTGAATGGCTT
GCCATTCTTTGCCATTGCCCTCGCATGCACCCGGTCCTTGTGGCTGGACTGCACGTACGT
CGCCCGGCGGTGCCTATCCCGGGTTGGACTGATAACCTGGTCTTTGACCATACGTACAAC
TCTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATT
AATGCAAACTGCATACTGCTTTGAACATCGACATTTCGAACGCATCTAAAAAAA*

ITS2 Adult (Forward sequence)

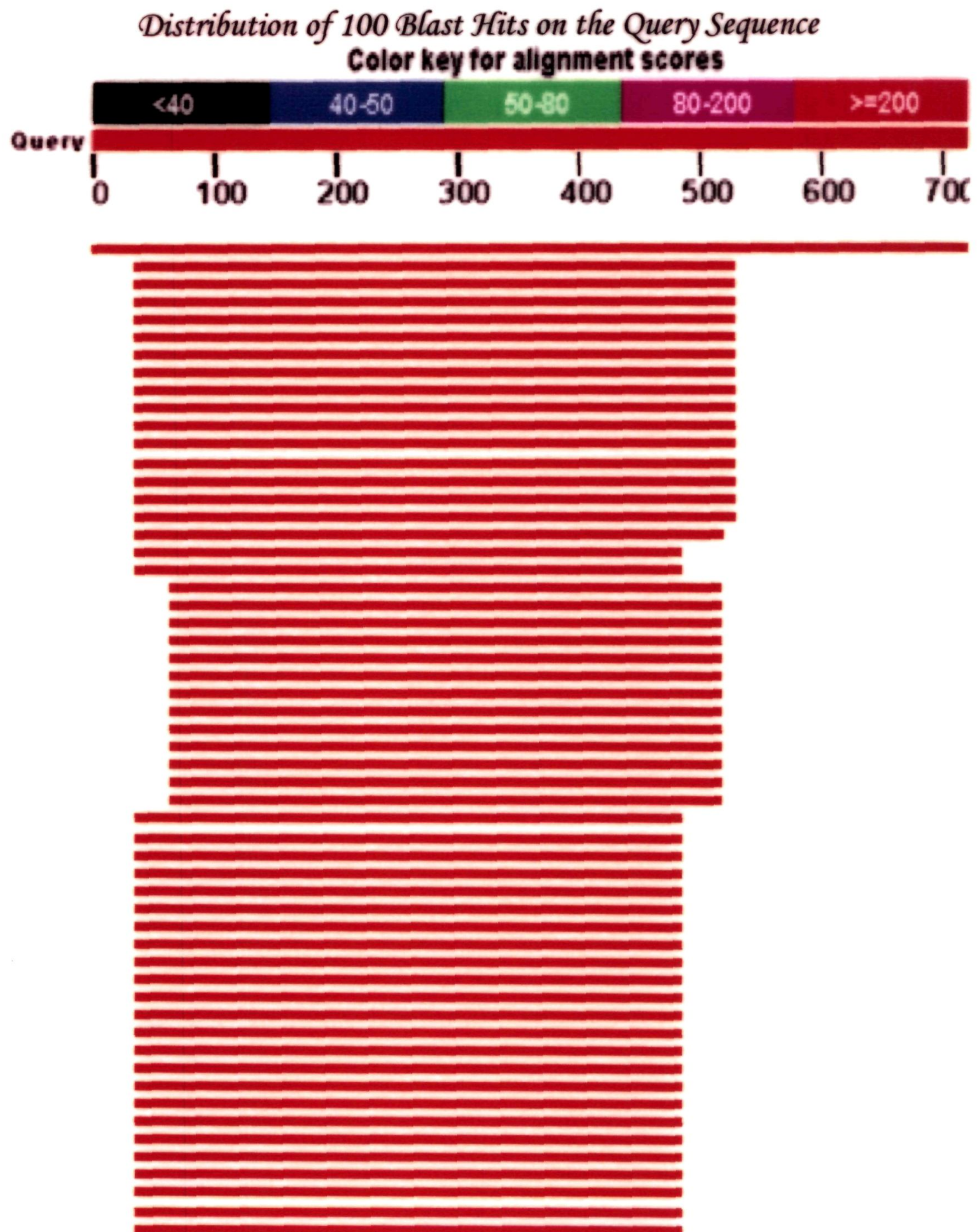


Figure 40. Blast hit result of ITS2 forward sequence (adult) of *Fasciola gigantica*

The results of similarity search for the query sequence were obtained using BLAST (Figures 40 and 41). The top 15-20 best hits (having the maximum e-value) were used for the purpose of phylogenetic predictions.

- gi|118153758|gb|EF027103.1| Fasciola gigantica 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|75812180|dbj|AB207149.1| Fasciola gigantica gene for ITS2
- gi|3062892|dbj|AB010977.1| Fasciola gigantica genes for 18S rRNA, 5.8S rRNA and internal transcribed spacer 2, partial sequence
- gi|75812182|dbj|AB207151.1| [75812182] Fasciola sp. Saitama-10 gene for ITS2
- gi|3062894|dbj|AB010979.1| Fasciola sp. genes for 18S rRNA, 5.8S rRNA and internal transcribed spacer 2, partial sequence
- gi|3062891|dbj|AB010976.1| Fasciola gigantica genes for 18S rRNA, 5.8S rRNA and internal transcribed spacer 2, partial sequence
- gi|75812184|dbj|AB207153.1| Fasciola sp. Hiroshima-18 gene for ITS2
- gi|222355880|gb|FJ593632.1| Fasciola hepatica isolate Firat 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|217323616|gb|FJ467927.1| Fasciola hepatica isolate Malatya 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|215788932|gb|FJ459806.1| Fasciola hepatica isolate Samsun 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
- gi|75812181|dbj|AB207150.1| Fasciola sp. Hokkaido-1 gene for ITS2

ITS1 Adult (Forward sequence)

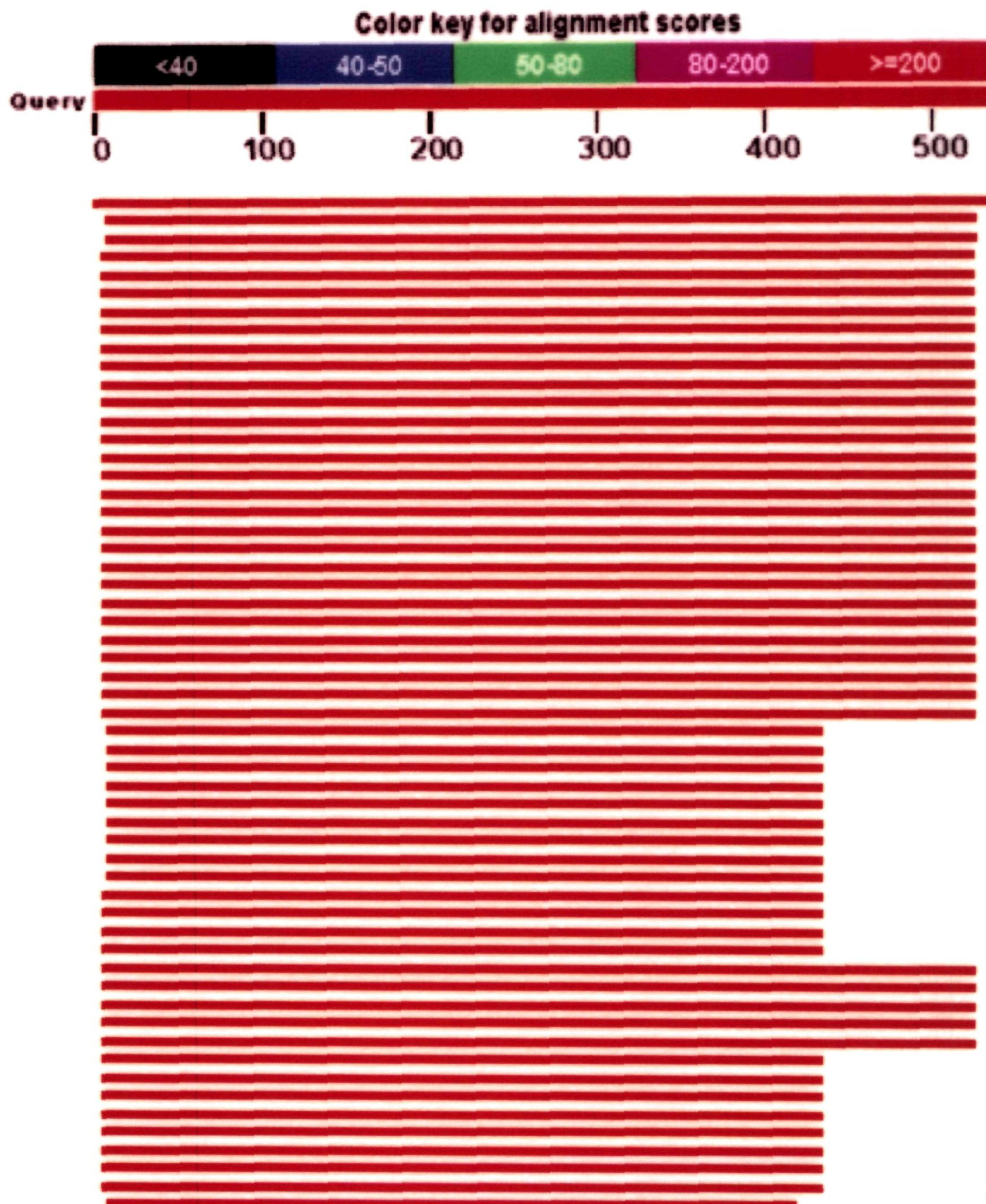


Figure 41. Blast hit result of ITS1 forward sequence (adult) of *Fasciola gigantica*

- gi|118153763|gb|EF027104.1|Fasciola gigantica 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
- gi|218304275|emb|AJ853848.2|Fasciola gigantica ITS1, 5.8S rRNA gene and ITS2, country Burkina Faso, Bobo Dioulasso
- gi|172046504|emb|AM900371.1|Fasciola gigantica ITS1, 5.8S rRNA gene and ITS2, isolate FgCAY1
- gi|172046503|emb|AM900370.1|Fasciola hepatica ITS1, 5.8S rRNA gene and ITS2, isolate FhCTO6
- gi|157954352|emb|AM709649.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhRp2
- gi|157954350|emb|AM709647.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhOa3
- gi|157954351|emb|AM709648.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhOa6
- gi|157954349|emb|AM709646.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhEc5
- gi|157954348|emb|AM709645.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhDd3
- gi|157954347|emb|AM709644.1|Fasciola hepatica partial ITS1, 5.8S rRNA gene and partial ITS2, isolate FhCe3

The nucleotide sequences were obtained for ITS rDNA of *Fasciola gigantica* and were compared with sequences of other fasciolid species obtained from Genbank by ClustalW tree building method. The fragments of amplified DNA were estimated to be 480-550bp long. No intra-specific variations in length or composition of the sequence were observed, and the ITS sequences of both adult and egg origin were found to be identical in length as well as composition. For comparative purpose, the ITS2 sequences of fasciolids from various geographical regions were obtained from GenBank (Table 6). The Blast hit results show that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp., *F hepatica* and *F. gigantica* besides *Fasciolopsis buski* and *Fascioloides magna* (both belonging to the same family, i.e., Fasciolidae).

Table 6. Species of Fasciolidae and their various geographical isolates used in this study with the respective GenBank accession numbers for corresponding ITS2 sequences. A= Sequence generated as part of the present study.

Species	GenBank Accession No.	Sequence length (bp)	Classification
Fasciola gigantica India (A)	EF027103	606	Digenea: Fasciolidae
Fasciola sp. Japan: Kochi	AB207152	505	Digenea: Fasciolidae
Fasciola gigantica Indonesia	AB010977	505	Digenea: Fasciolidae
Fasciola sp. Japan	AB010979	505	Digenea: Fasciolidae
Fasciola gigantica Zambia	AB010976	505	Digenea: Fasciolidae
Fasciola hepatica Uruguay	AB207148	506	Digenea: Fasciolidae
Fasciola gigantica Burkina Faso	AJ853848	588	Digenea: Fasciolidae
Fasciola gigantica China	AJ557569	361	Digenea: Fasciolidae
Fasciola gigantica Kenya	EF612484	364	Digenea: Fasciolidae
Fascioloides magna Austria	DQ683545	538	Digenea: Fasciolidae

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F. gigantica* and available ITS (I & II) sequences for other fasciolid species (Figure 42- 43 & 45 - 46). Phylogenetic analyses using the various distance methods and character method like Maximum Parsimony showed that the topology is similar among the trees obtained. Boot strapping of the sequences with Neighbour- Joining revealed significant support for the clade containing *F. hepatica*, *F. gigantica, F. buski* and *F. magna*. The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids. The phylogenetic trees constructed based upon the ITS (1 & 2) sequences by multiple tree building methods in MEGA revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia.

Figure 42. Neighbor-Joining tree for ITS2 sequence of fasciolid species (* query)

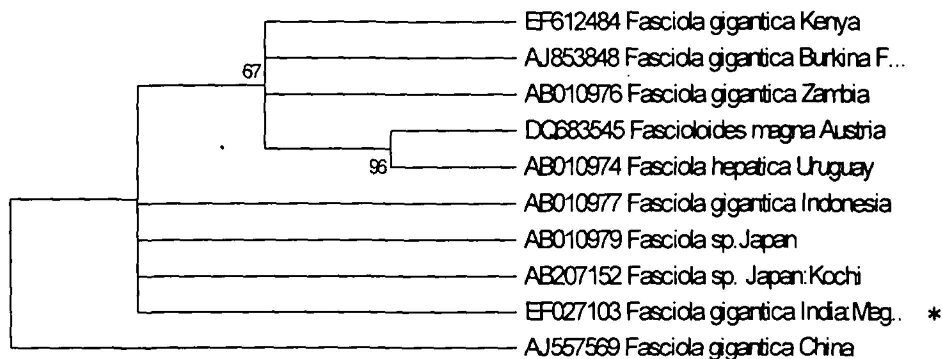


Figure 43. Maximum Parsimony tree for ITS2 sequence of fasciolid species (*query)

While comparing the ITS1 sequences through BLAST search (ClustalW alignment), the sequence of another Indian isolate designated as *F. gigantica* (Accession- EF198867) from Indian Veterinary Research Institute, Bareilly showed almost absolute match with *F. hepatica* (Figure 44).

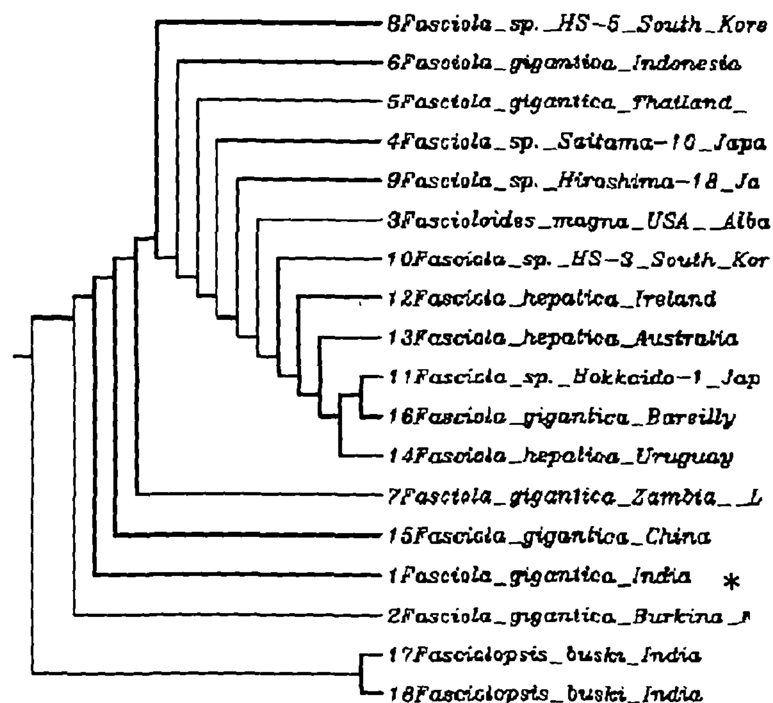


Figure 44. Clustal W tree of ITS1 sequence for fasciolid species (*query sequence)

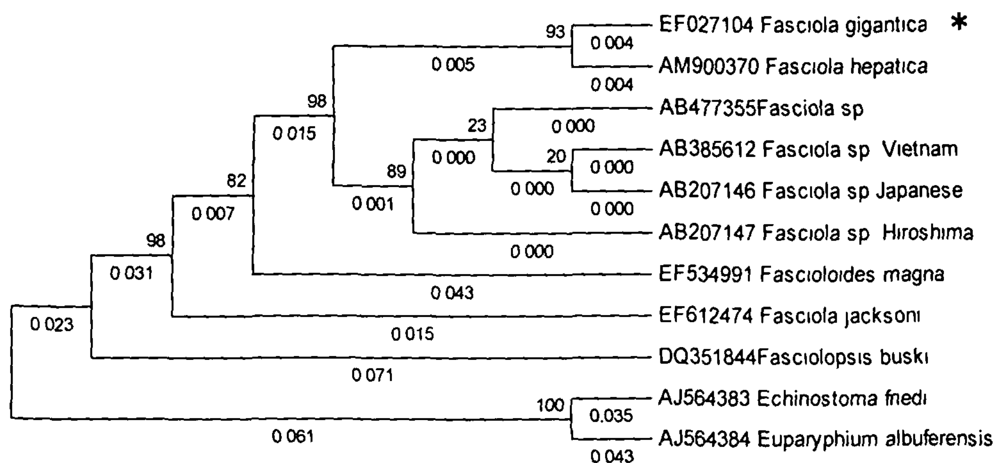


Figure 45. Neighbor - Joining tree for the (*query) ITS1 sequence

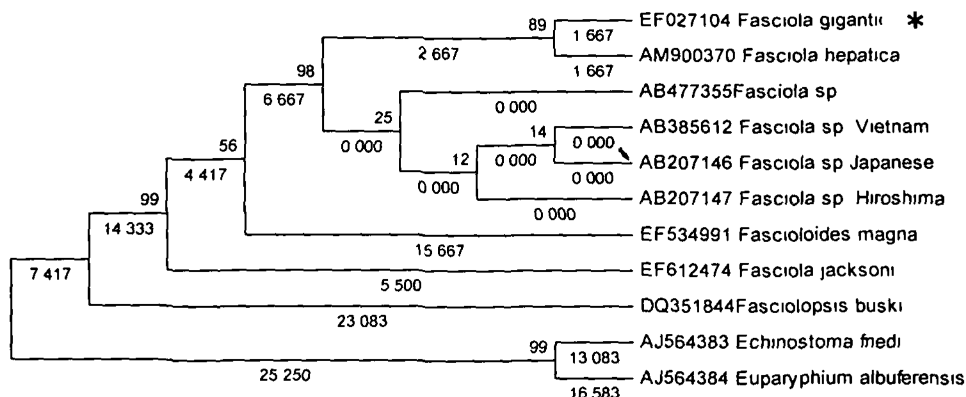
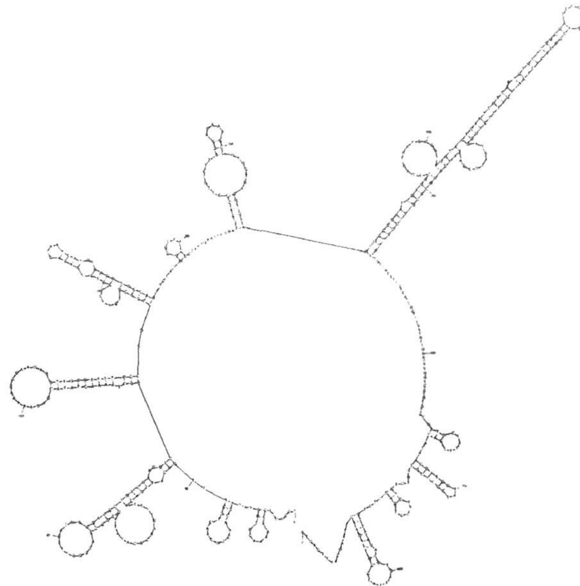


Figure 46. Maximum Parsimony tree for the (*query) ITS1 sequence

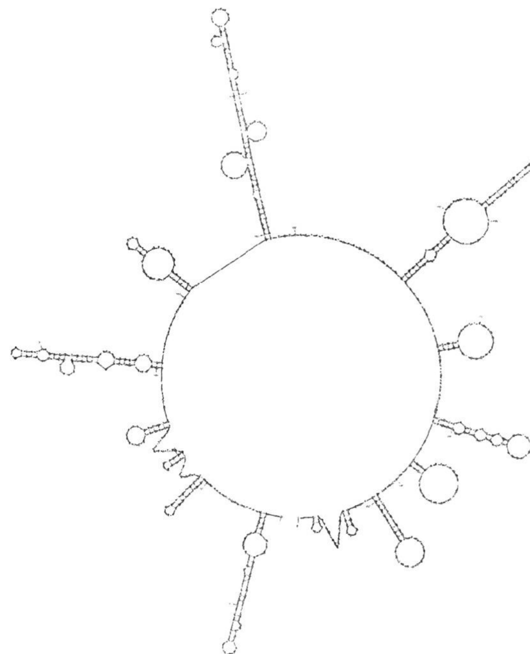
Secondary structure analysis

Five predicted RNA secondary structures were reconstructed from the unique sequences with highest negative free energy of *F. gigantica* to provide the basic information for phylogenetic analysis (Figure 47a-e). The ITS2 plus flanking regions of nuclear region ranged from 720bp in *F. gigantica* India to a minimum length of 361bp in *F. gigantica* China. The secondary structural features of ITS2 regions as shown in the figure were analysed based on conserved stems and loops. *F. gigantica* isolates from India and China show overall similarity in the ITS2 rRNA folding and have identical secondary structure. Secondary structures of remaining species are somewhat variant. The observed similarities at the secondary structural level are further reflected at the energy level. Only difference in their topology is due to differences in nucleotide sequences. Moreover the observed phylogenetic trend was identified with respect to the target accessibility sites for the five different isolates. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences (Figure 48). ITS2 sequence of the Indian isolate revealed closest similarity with the isolate from China with significant bootstrap value.

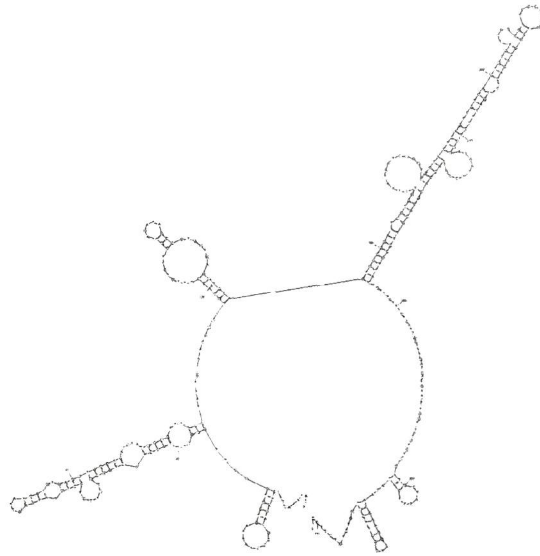
Figure 47a-e: Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD.



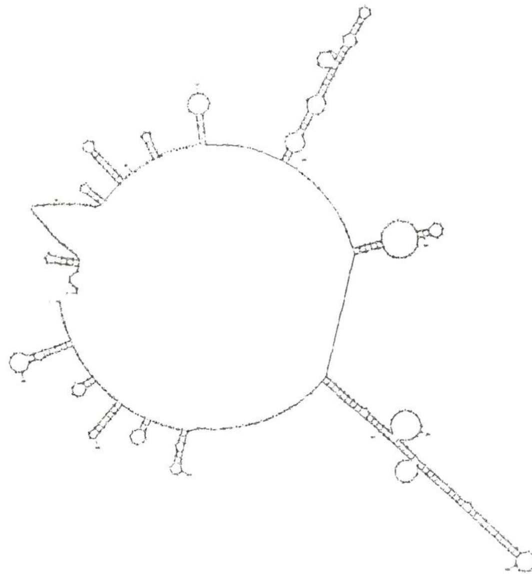
(a) dG= -52.19 *F. gigantica* Egypt



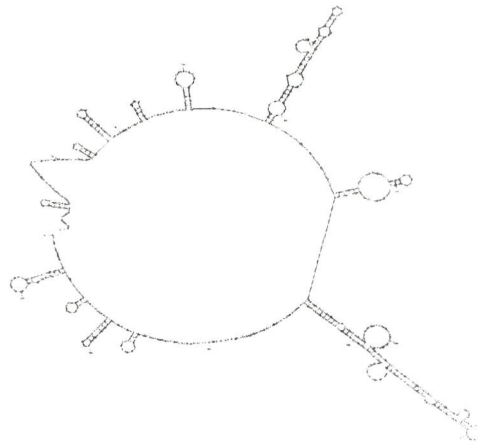
(b) dG= -86.27 *F. gigantica* India



(c) dG= -38.87 *F. gigantica* China

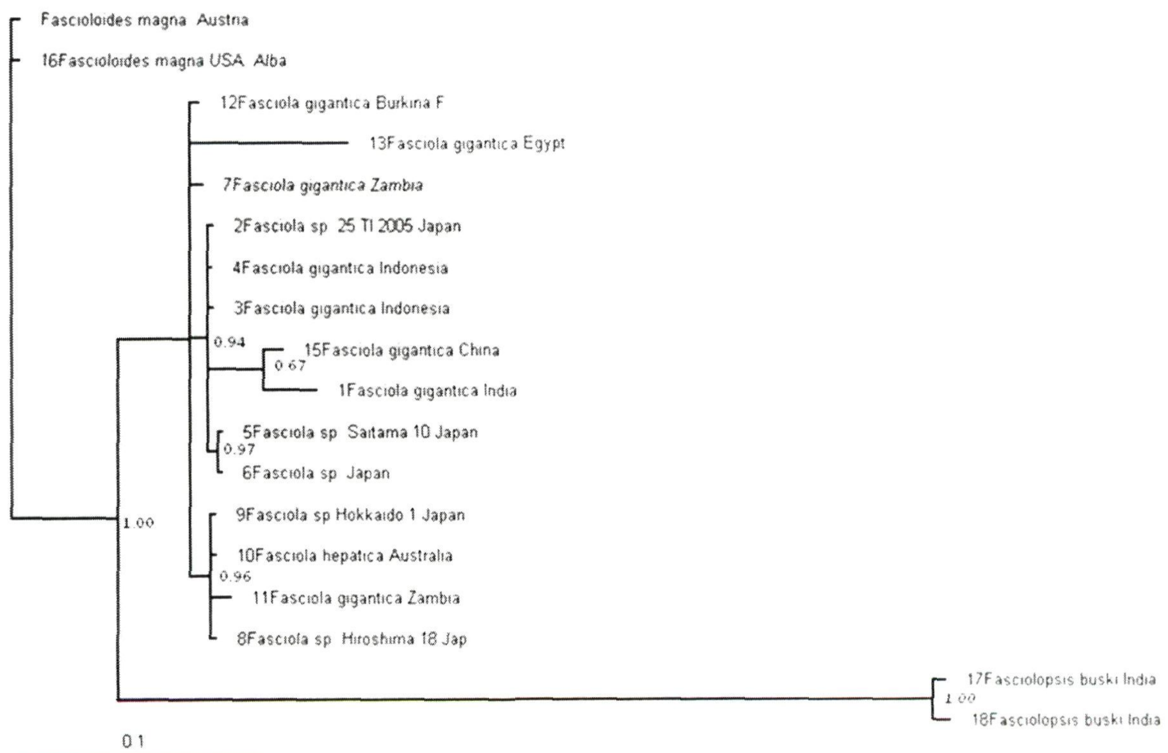


(d) dG= -61.25 *F. gigantica* Zambia



(e) dG= -58.45 *F. gigantica* Indonesia

Figure 48. Hypothetical Bayesian analysis phylogeny based on the secondary structure alignment data of the ITS2 region of members of family Fasciolidae. The numbers are equivalent to bootstrap percentages.



Design of genus/species specific primers and amplification by PCR

In the first step, we amplified the rDNA region spanning ITS2 from metacercarial DNA using primer set 3S-A28. Agarose gel electrophoresis showed that the generated ITS PCR products were about 500bp in size (including the primer annealing regions) for all the three trematodes. Multiple sequence alignment was done for all the three sequences using ClustalW programme (Figure 49). Sequence analysis of the ITS2 PCR products of *Fasciolopsis buski* (DQ351841), *Paragonimus* metacercariae (DQ351845) and *Fasciola gigantica* (EF027103) revealed no intra-specific variations in length or composition. The ITS sequences of different life cycle stages egg, metacercaria and adult were found to be identical in length as well as composition.

The *P. westermani*-specific, *F. buski*-specific and *F.gigantica*-specific primers (Table 7) were designed to target the 3'-terminal position of the ITS2 sequences. The species specificity of these primers was evaluated by PCR using primer 3S (Figure 50a, b, c). As was expected, the primer set 3S-PwAR1 amplified a PCR product only from *P. westermani* DNA, 3S-FbMR1 amplified a PCR product only from *F. buski* DNA and 3S-FgMR1 amplified a PCR product only from *F. gigantica*. Primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample. These PCR products were sequenced using the corresponding specific primer and were confirmed to be the ITS2 region of rDNA from the respective species.

Table 7. Details of the designed specific primers

Parasite and Specific primer	**Primer Sequence**	**Length (bp)**	**Accession Number**	**Tm**	**GC %**
P.westermani (PwAR1)	ATAGATCTGGCACCCCACAG	20	DQ351845	59.95	55
F.buski (FbMR1)	TTAAACCACGATCCCGCTAC	20	DQ351842	59.96	50
F.gigantica (FgMR1)	CCAAGTTCAGCATCAAACCA	20	EF027103	59.96	45

```
ITS2_Fasciolopsis_buski_Adult_      TGTCGATGAGGAGCGCAGCCACTGTGTGAATTAATGCAAACTGCATACTG
ITS2_Fasciola_gigantica_Adult_      GGTCGA--GTGAGCGTCGGCTTGTGGTGCATTTTAGCAACTCGCATACTG
ITS2_Paragonimus_westermani_me      TGTCGATGACGAGCGCAGCCACTGTGTGAATTAATGCGAACTGCATACTG
                                     *****    *****  * *     *** ***   ** *   ********

ITS2_Fasciolopsis_buski_Adult_      CTTTGAACATCGACATCTTGAACGCATATTGCGGCCATGGGTTAGCCTGT
ITS2_Fasciola_gigantica_Adult_      CTTTGAACATCGACATTTTGAACGCATCTTGAGGCCATGGGTTAGCCTGT
ITS2_Paragonimus_westermani_me      CTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGT
                                    **************** ********** *** ***** ************

ITS2_Fasciolopsis_buski_Adult_      GGCCACGCCTGTCCGAGGGTCGGCTTACAAACTATCACGACGCCCAAAAA
ITS2_Fasciola_gigantica_Adult_      GGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAA
ITS2_Paragonimus_westermani_me      GGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAA
                                    **************************** ******** ************

ITS2_Fasciolopsis_buski_Adult_      GTCGTGGCTTGGGTCTTGCCAGCTGGCGTGAACTCCTCTATGA----TTA
ITS2_Fasciola_gigantica_Adult_      GTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCTCTATGA----GTA
ITS2_Paragonimus_westermani_me      GTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTG
                                    **** ********* **************** **** * **       *

ITS2_Fasciolopsis_buski_Adult_      TTCATGTGAGGTGCCAGAACTATGGCGTTTCCCTAATGTATCCGGACGCG
ITS2_Fasciola_gigantica_Adult_      ATCATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTATCCGGATGCA
ITS2_Paragonimus_westermani_me      TGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCA
                                      * **** ********* *****************  **  ***  **

ITS2_Fasciolopsis_buski_Adult_      TCCTTGTCTCAGCAGAAGGCTGTGGTGAGG-TGCGGTAGCGGGATCGTGG
ITS2_Fasciola_gigantica_Adult_      CCCTTGTCTTGGCAGAAAGCCGTGGTGAGG-TGCAGTGGCGGAATCGTGG
ITS2_Paragonimus_westermani_me      CCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCGACGGAATCGTGG
                                     **  **    ** *** **  **  * ** **  *   *** *******

ITS2_Fasciolopsis_buski_Adult_      TTTAATGA------------------------------------------
ITS2_Fasciola_gigantica_Adult_      TTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTCGGCGATCCCCTA
ITS2_Paragonimus_westermani_me      CTCAGTAAATGA--------------------------------------
                                     * * * *

ITS2_Fasciolopsis_buski_Adult_      --------------------------------ATACTGTGCACGTTCCGT
ITS2_Fasciola_gigantica_Adult_      GTCGGCACACTCATGATTTCTGGGATAATTCCATACCAGGCACGTTCCGT
ITS2_Paragonimus_westermani_me      --------------------------------TTTATGTGCGCGTTCCGC
                                                                     *     ** *******

ITS2_Fasciolopsis_buski_Adult_      TGCTGTGTCTTCATCGTCGGCTTGATGCGTGACTTGGTCTCGTGTCTGAG
ITS2_Fasciola_gigantica_Adult_      TACTGTTACTTTGTCATTGGTTTGATGCTGAACTTGGTCATGTGTCTGAT
ITS2_Paragonimus_westermani_me      TGACCTGTCTTCATCTGTGGTTC-ATGTTGCGCGTGGTC-TGCGTTTGAT
                                    *    *  ***  **   ** *  ***     * *****  * ** ***

ITS2_Fasciolopsis_buski_Adult_      GCTTTTGCCATACATAGACTGCCATTTGTGTGGTCTAATTTCCTGACCTC
ITS2_Fasciola_gigantica_Adult_      GCTATT-TCATATAACGACGGTACCCTTCGTGGTCTGTCTTCCTGACCTC
ITS2_Paragonimus_westermani_me      GCTGAC-CTATGTATGTGCCA-------TGTGGCTCATTCTCCTGACCTC
                                    ***      **  *    *          ****       **********

ITS2_Fasciolopsis_buski_Adult_      GGTTCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGA
ITS2_Fasciola_gigantica_Adult_      GGTTCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGA
ITS2_Paragonimus_westermani_me      GGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGG
                                    ** ********** ***********************************

ITS2_Fasciolopsis_buski_Adult_      AAAGAACAAA----------------------------------------
ITS2_Fasciola_gigantica_Adult_      AAAGAAAACTAACAAGGATCCCACGGACGAGAGATAAACAAGGATCCACG
ITS2_Paragonimus_westermani_me      AAAAGAAACTAACCCCGGATCCCAAA------------------------
                                    ***  * *

ITS2_Fasciolopsis_buski_Adult_      --------------------------------------------------
ITS2_Fasciola_gigantica_Adult_      CGCCGAGGGGATACACGGAATCCACGCCCCCCATTCATTCTCCGATTTCA
ITS2_Paragonimus_westermani_me      --------------------------------------------------

ITS2_Fasciolopsis_buski_Adult_      --------------------------------------------------
ITS2_Fasciola_gigantica_Adult_      CCGCCCCGAGGGCTCCATTGTATTCACGGCCCCGGGGGCTCGCTGGGATA
ITS2_Paragonimus_westermani_me      --------------------------------------------------

ITS2_Fasciolopsis_buski_Adult_      ----------------------------
ITS2_Fasciola_gigantica_Adult_      CCAGAGCCCAGACACGGTTGGTTTTCCT
ITS2_Paragonimus_westermani_me      ----------------------------
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Figure 49. Multiple sequence alignments of ITS2 sequence and flanking regions of *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica*.

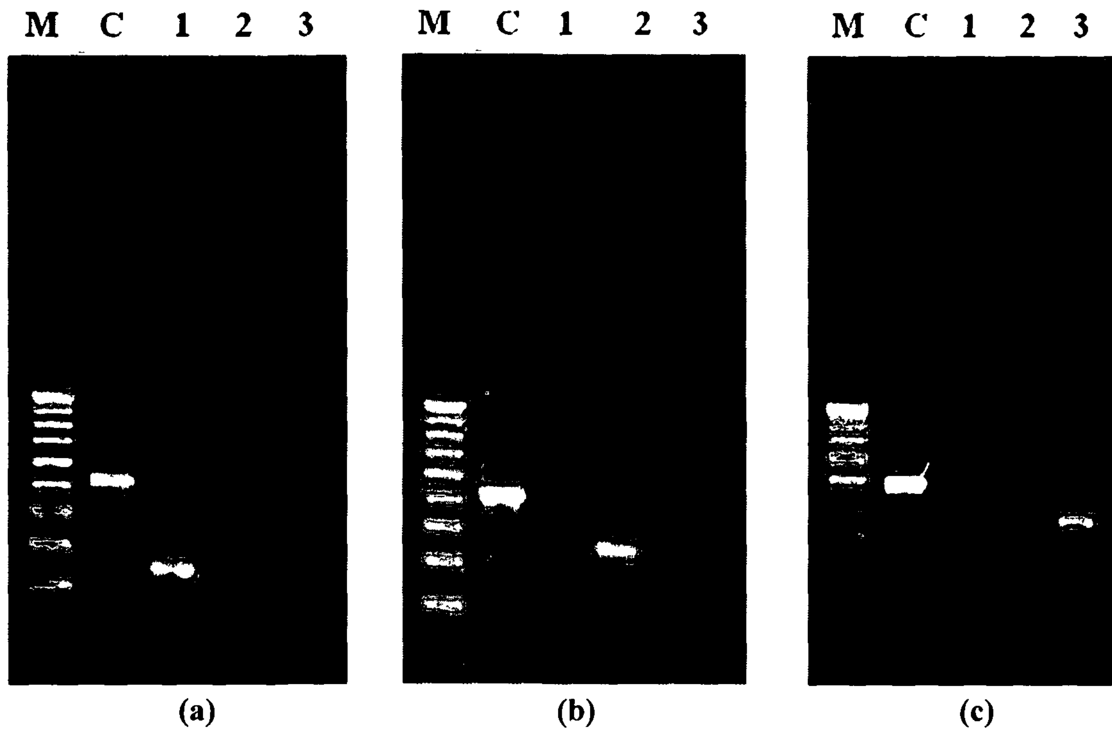

Figure 50a-c. Results of PCR amplification of DNA using designed primers.

(a) *Paragonimus westermani*
(b) *Fasciolopsis buski*
(c) *Fasciola gigantica*
M= 100-bp DNA ladder marker
C = Control primers 3S-A28
1 = *P. westermani* specific 3S-PwAR
2 = *F. buski* specific 3S-FbMR
3 = *F. gigantica* specific 3S-FgMR

DISCUSSION

Morphological differences found in adult specimens have been widely used to discriminate between platyhelminth species (Miyazaki, 1974). However, traditional diagnostic techniques in parasitology are now complemented by a variety of molecular tools to help in resolving the taxonomic issues associated with describing new species or strains on the basis of phenotypic characteristics (Thompson et al., 2004). PCR-based techniques utilizing the ITS sequences have proven to be a reliable tool to identify the various lung fluke species and their phylogenetic relationships (Blair et al., 1999; Iwagami et al., 2000). The taxonomy of *Paragonimus* species has traditionally relied on the morphology of metacercariae and adult worms, and about 50 species of this genus have been described based on the morphology (reviewed by Blair et al., 1999b). However, some of them are indistinguishable from each other in morphology and Blair et al. (1997, 1999b) put several species as the synonym of others. Recently, mainly based on molecular phylogenetic analyses, the *Paragonimus* species were divided into five major groups: *P. westermani, P. ohirai, P. mexicanus, P. heterotremus* and *P. skrjabini* (Blair et al., 2005). In sequence analysis of the rDNA ITS2 comparing with the known sequences of the other lung fluke species, the present study revealed that the sequences of *ITS2 (plus flanking regions) show close resemblance with the sequences of Paragonimus westermani,* both of metacercarial and adult origins. The results corroborate that the ITS2 sequences are not stage specific, and are conserved through different stages of the development of the fluke (Sugiyama et al., 2002). In phylogenetic analysis, as a general rule, if the bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, then the topology at that branch is considered "correct". The results of the present study showed a bootstrap value to be >70% among the trees obtained and the ITS2 sequence resembled *Paragonimus westermani.* Thus, on the basis of absolute matching of ITS2 sequence that could be used as one of the species markers and supplemented by surface fine topography features, it can be concluded that *Paragonimus* species prevailing in Kharshang and Miao regions of Arunachal Pradesh is indeed *P. westermani* and not *P. heterotremus* as reported by earlier workers (Narain et al., 2003).

In a new approach of molecular morphometrics, the measurable structural parameters of the molecules are directly used as specific characters to construct a phylogenetic tree. These structures are inferred from the sequence of the nucleotides, often using energy minimization (Zuker 1994). Molecular morphometrics appears to be complimentary to classical primary sequence analysis in phylogenetic studies as it takes into consideration only the size variations of homologous structural segments and this choice implies that the overall architecture of the molecule remains same among the observed taxa. This method helps in taking into account the regions where multiple alignments are barely reliable because of large number of insertion/deletion operations. Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *Paragonimus*, which provided us with the additional information for correct identification of the species prevalent in the region. In the present study the secondary structure analysis of the same data also confirmed the results mentioned for primary sequence analysis. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. We obtained three similar topologies for seven species of the genus *Paragonimus* on the basis of traditional primary sequence analyses using MEGA and a Bayesian analysis of the combined data. The latter approach allowed us to include both primary sequence and RNA molecular morphometrics; each data partition was allowed to have a different evolution rate. *Paragonimus westermani* was found to group with *P. siamensis* of Thailand; this was best supported by both the molecular morphometrics and combined analyses. *P. heterotremus*, *P. proliferus*, *P. skrjabini*, *P. bangkokensis* and *P. harinasutai* formed a separate clade in the molecular phylogenies, and were reciprocally monophyletic with respect to other species. Differences in their topology are only due to the fact that there are variable lengths of the sequences. However, there are difficulties in defining a distance between two related structures with variable topologies (Shapiro, 1998). Nevertheless, because there were inconsistencies in the placement of a few *Paragonimus* species, this study needs to be extended, in order to gain a better understanding of the systematics of this group as well as the evolution of their predicted ITS2 RNA secondary structures.

Besides *Paragonimus* spp., other trematode-borne zoonoses are known to occur in the region, e.g. *Fasciolopsis buski* and *Fasciola gigantica* to name a few. In the present study, these trematodes being among the most prevalent, were analysed for their ITS sequences with a view to finding out and delop molecular markers that could be used as species-specific identification tool. Lack of data on genotypic diversity of fasciolid flukes in India makes it difficult to perform fine-scale phylogeographic analysis of populations and does not allow the origin of regional populations to be umambiguously determined. Like most metazoan parasites, the taxonomy of *Fasciolopsis* and *Fasciola* has been based mainly on morphological data complemented with ecological, cytological and pathological results as well as clinical manifestations. It is possible to distinguish between adult *F. hepatica* and *F. gigantica* on the basis of morphology, but much variation exists. Differentiating between two species is not possible on the basis of clinical, pathological, or immunological findings and their eggs are morphologically very similar (Lotfy and Hillyer, 2003). Consequently, where both species occur concurrently or in overlapping geographical distribution, it is not possible to be certain as to which species is responsible for the disease. The low number of records of infection with *F. gigantica* may well be due to the lack of good tools to distinguish this species from *F. hepatica* (Marcilla et al., 2002).

In respect of *F. buski*, the 18S rRNA sequence is known (Accession number L06668: Blair and Barker, 1993) but the sequences from ITS markers are not described so far. In search for molecular markers for this species, we characterized the ITS region of rDNA. The ITS sequences of *F. buski* showed close resemblance with the members of the families Fasciolidae and Echinostomatidae (Echinostomatinae). From the phylogenetic trees constructed, sequences of two distinct clusters are revealed, one of *Fasciola* spp and the other comprising the genera *Echinostoma, Paryphostomum* and *Isthmiophora*. The results of the present study showed that the bootstrap values is almost 100% among the trees obtained and the ITS sequences of *F. buski* resemble those of other members of Fasciolidae. The sequences of the PCR products from adult and egg stages of the fluke were also found to be identical in length and composition and showed no genetic variability in the worms collected from pigs of the Assam region. Compared

to ITS1, the ITS2 sequences in the present study showed a higher bootstrap value of 100%, confirming that it is a highly conserved monophyletic group. This is in accordance with other studies on trematodes that indicate sequences in the ITS1 might be less conserved then those in the ITS2 region (Luton et al., 1992).

The comparison of ITS sequences from worms of different hosts and of different countries indicates that there exists a high species-specific homogeneity. In closely related fasciolids *Fasciola hepatica* and *F. gigantica* intraspecific nucleotide sequence divergence for ITS2 among the isolates from Mexico was found to be negligible or nil (Adlard et al., 1993). Likewise, no variation was observed between most of the populations of liver fluke species from different regions in East Europe, Central Asia and Caucasus; while only one specimen of *F. hepatica* from Armenia showed 0.3% variation, three out of 11 populations of *F. gigantica* differed at four nucleotide transitions only (Semyenova et al., 2005). In the present study, primary sequence analysis revealed a close relationship between the query sequence (*F. gigantica* from Northeastern region of India, i.e., generated as part of the present study) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia (Adlard et al., 1993). Phylogenetic trees constructed showed that the groups of multiple closely related genotypes of *F. gigantica* from Asia are broadly sympatric. Such a pattern is expected for species with high gene flow whose populations have not been sundered by long term biogeographic barriers (Avise, 2000). The sequence of another Indian isolate, designated as *F. gigantica* (Accession-EF198867) from Indian Veterinary Research Institute, Bareilly, showed almost absolute match with *F. hepatica*. Hence on the basis of molecular similarity this particular isolate should be identified as *F. hepatica* and not *F. gigantica*. The results also corroborate that the *Fasciola* species prevalent in Assam, Northeast India is in fact *F. gigantica* and not *F. hepatica*, which otherwise is the most common liver fluke throughout the globe. Several patterns of predicted secondary structures of RNA were also constructed from unique ITS sequences from different geographical isolates of *Fasciola gigantica*. The secondary structure analysis of the data also confirmed the results mentioned for primary sequence analysis. Lack of data on genotypic diversity of *Fasciola* species in Africa and India does not allow the origin of regional populations to be unambiguously determined. Further

studies with additional molecular markers are needed to determine the population structure and divergence between the two closely related species *gigantica* and *hepatica* of the genus *Fasciola*.

Studies on phylogeny and/or intraspecific variation in species of *Paragonimus* have been done using ITS2 region in recent years (Maleewong, 2003; Cui et al., 2003; Park et al., 2003; Chen et al., 2004; Le et al., 2006; Doanh et al., 2007; Devi et al., 2007) and the usefulness of the method for species discrimination has also been demonstrated in nematodes (Campbell et al., 1994; Hoste et al., 1995; Samson-Himmelstjerna et al., 1997; Aboobaker and Blaxter, 2004; Ghedin et al., 2004). Species-specific molecular markers in respect of *P. miyazaki* are also available (Sugiyama et al., 2002). In the present study, *P. westermani*-specific, *F. buski*-specific and *F. gigantica*-specific primers were designed to target the 3'-terminal position of the ITS2 sequences, and the species specificity of these primers was evaluated by PCR using primer 3S. Primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample. As was expected, the primer set named 3S-PwAR1 amplified a PCR product (250bp) only from *P. westermani* DNA, 3S-FbMR1 amplified (280bp) only from *F. buski* DNA and 3S-FgMR1 amplified (350bp) only from *F. gigantica*. These PCR products were sequenced using the corresponding specific primer and were confirmed to be the ITS2 region of rDNA from the respective species. Thus, the three primer sets appear to be reliable diagnostic molecular markers to identify the pathogen species implicated in infection.

The findings of the present study, as has already been demonstrated for other parasitic helminths, confirm that ITS can serve as effective genetic marker for molecular identification. To ascertain intra-specific strain variations, if any, and to determine the population structure, different geographical isolates of all the three parasite species from the region need to be studied with the use of additional molecular markers.

CONCLUSION

In several mountainous regions of Northeast India, suspected foci of *Paragonimus* infection have been identified in the recent past two decades. There are many localities in Arunachal Pradesh, Assam, Manipur and Mizoram states where crabs are a common food item consumed by the native populations. In the present study, of all these areas surveyed, two places i.e. Miao and Kharshang in Arunachal Pradesh emerged as the newer sites of *Paragonimus* infection in the region and the crab species *Barytelphusa (M) lugubris lugubris* and *B. (M.) l. mansoniana,* the second intermediate hosts, naturally infected with metacercarial infective stage of the parasite. On the basis of morphological and surface topographical features, the species implicated in the infection seems to be *P. westermani*, a species that is widely distributed in the neighbouring countries, which have similar socio-cultural practices and prevailing environmental/climatic conditions. **The molecular criteria, based on PCR-amplification of rDNA ITS2 regions and supplementing the morphology-based identification also strongly suggest that the *Paragonimus* species in Miao region of Arunachal Pradesh is indeed *P. westermani*.**

Besides *Paragonimus*, in molecular characterization of the other two commonly prevalent trematode species, viz. *Fasciola gigantica* and *Fasciolopsis buski*, the ITS sequences proved to be useful tool for species identification and determining phylogenetic relationships. **The present study confirmed that the *Fasciola* species prevalent in the region is infact *Fasciola gigantica.*** However, to ascertain intra-specific strain variations, if any, and to determine genetic variability among their populations, different geographical isolates of the parasites from the region need to be studied with the use of additional molecular markers.

From the amplified sequences of ITS2 of all the three parasites under the present study, specific primers were designed in respect of each species. When tested, each of these primer sets amplified the PCR product exclusively corresponding to the respective species of its origin and did not show any cross amplifications. **Thus, the designed primers PwAR1, FbMR1 and FgMR1 seem to be specific molecular signatures that can be used in discriminating *P. westermani, F. gigantica* and *F. buski* from one another.**

SUMMARY

The present work incorporates a study on the status and molecular characterization of paragonimiasis and other Crustacea-borne trematode zoonoses in Northeast India. The study aimed at examining the snail and crustacean species (the first and second intermediate hosts of the causative agent of infection) prevalent in Northeast India to recover the larval stages, if any, of the lung fluke *Paragonimus* and to identify the exact species prevailing and responsible for the disease. By using PCR-based molecular techniques the study aimed to identify the species of the parasite collected and to generate specific molecular markers with vital usage towards correct diagnostics.

Status and prevalence of paragonimiasis in the region

- The crab hosts were surveyed from rural localities and countryside where eating of crabs is a common food practice among the natives of the region. Emphasis was given to procure those species which are commonly used in local traditional cuisine. Naturally infected freshwater crabs were mostly collected from mountain streams of the suspected foci of infection. Metacercariae were isolated from the muscles of the crustacean host by artificial gastric juice digestion technique. Of the 3 genera of crabs surveyed from various localities in Arunachal Pradesh, Assam, Manipur and Mizoram, only one, i.e., *Barytelphusa* was found to be harboring metacercarial cysts. The crabs collected from all the localities excepting those in Arunachal Pradesh, did not harbor any metacercarial infection; both the collection sites in Arunachal Pradesh were revealed to be positive for this infection. In the Kharshang site *Barytelphusa (M) lugubris lugubris* was found to be positive with a prevalence of 26%, the intensity of infection being in the range of 9-68 (mean intensity=36). In Miao region, the other sub species namely *B. (M.) l. mansoniana* revealed a much higher prevalence (87%) and intensity of metacercarial infection (ranging between 3-175, mean intensity=38).

- Snail species were also collected from selected localities wherever the crab hosts were suspected to be positive for metacercarial infections. Hundreds of specimens of 4 snail species (representing 4 genera with one species each) were examined for the recovery of intramolluscan stages, if any, of *Paragonimus*. Of the snail species surveyed, only one species viz. *Brotia costula* was found harbouring the sporocyst, redia and cercaria larval stages.

- Freshly recovered metacercariae from crab hosts and intramolluscan larval stages-sporocyst, redia and cercariae, were processed using standard protocols for light and electron microscopy. The newly excysted metacercaria has an elongate body (815.91μm x 492.79μm) in size; the ventral sucker situated somewhat pre-equitorally, is larger than the oral; the intestinal caeca are long and extend up to the posterior end of the body; the conspicuous excretory bladder extends medially in the intercaecal space. The SEM observations revealed that the encysted metacercaria is oval in shape and has smooth surface. The whole body surface of the excysted metacercaria is covered with numerous single-pointed and thorn-like tegumentary spines; those covering the anterior part of the body are bigger in size and show a gradual reduction in size towards the posterior end. On the basis of morphology and surface fine topography features of the excysted larval stage, the metacercariae were identified as those of *Paragonimus* species.

The cercariae recovered from the snail hosts were always of amphistome, leptocercous type. Microcercous type of cercariae (i.e., having a small stumpy tail) that are typically characteristic of *Paragonimus* spp were never encountered in the collections made during the present study. The larval stages recovered, not being representative of *Paragonimus,* were, therefore, not processed for further study towards molecular characterization.

- **Molecular characterization of *Paragonimus* and other trematodes**

  - The identification of closely related species based on morphological characters can be difficult, particularly so in the case of soft-bodied digenean trematodes. For most of the trematode-borne zoonoses the parasite eggs voided in exudates of the host are the only stages available for diagnostic purpose. Besides *Paragonimus,* infection of *Fasciola gigantica* and *Fasciolopsis buski,* both of which are putatively zoonotic species, have been reported in the northeastern region. The main objective of the present study was to provide molecular characterization of the parasite so as to supplement morphological criteria and develop species-specific molecular markers.

For the purpose of molecular characterization metacercariae of *Paragonimus* sp collected from the crab host *Barytelphusa lugubris* from Miao region in Arunachal Pradesh were used; live adult *F. buski* and *F. gigantica* were obtained from bovine hosts at local abattoirs. Eggs were obtained from mature adult flukes by squeezing between two glass slides; eggs recovered from each single specimen were also processed separately. DNA was extracted from metacercaria and eggs in Whatman's FTA card and from lysed individual adult worms by standard ethanol precipitation technique. The rDNA region spanning the ITS1 and ITS2 was amplified from the metacercarial, egg, larval and/or adult DNA by PCR. The primers generally used were designed based on the conserved sequences of *Schistosoma* spp, which are considered to be the universal primers for trematode species. The PCR amplification was performed following the standard protocol with minor modifications. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit, and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei, Bangalore and The Centre for Genomic Applications (TCGA), New Delhi, India and submitted to GenBank. No intra-specific variations in length or composition of the sequence were observed, and the ITS sequences of

egg, metacercaria and adult origin were found to be identical in length as well as composition. Sequence analysis was carried out using various bioinformatics tool e.g., BLAST, ClustalW, MEGA, mFOLD, Bayesian analysis phylogeny etc.

- ***Paragonimus* sp.:** The PCR amplified products of ITS2 of rDNA were successfully obtained and were compared with other sequences of trematode species from Genbank. The fragments of amplified DNA were estimated to be ~500bp long. Sequence analysis of the ITS2 PCR products revealed that the alignments of the rDNA region spanning the ITS2 were 496bp for forward primer and 494bp for reverse primer, respectively. The actual length of ITS2 was estimated to be 287bp. The Blast hit results showed that the query ITS2 *Paragonimus* metacercariae sequence is more similar to the sequence of the species *Paragonimus westerrmani*, *Paragonimus mexicanus*, *Paragonimus siamensis*, *Paragonimus sismensis*, *Paragonimus miyazakii*, *Euparagonimus cenocopiosus*. Comparing with the known sequences of the other lung fluke species, the present study revealed that the sequence of ITS2 (plus flanking regions) shows close resemblance with *Paragonimus westermani*, the expectation value (e-value) being most significant revealing absolute match. Phylogenetic analyses using the various distance methods and character state method like Maximum Parsimony show that the topology is similar among the trees obtained. A bootstrap value of > 70% among the trees obtained. Sequence analysis of ITS2 region of rDNA of metacercariae isolates of Miao showed that the species prevailing in the said location is *Paragonimus westermani* and not *P. heterotremus* as reported by earlier workers.

Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *Paragonimus*, so as to provide additional information for correct identification of the species prevalent in the region. The secondary structure analysis of the same data also confirmed the results mentioned for primary sequence analysis. The topology

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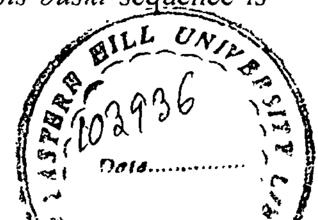
based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Three similar topologies for seven species of the genus *Paragonimus* were obtained on the basis of traditional primary sequence analyses using MEGA and a Bayesian analysis of the combined data. The latter approach allowed to include both primary sequence and RNA molecular morphometrics; each data partition was allowed to have a different evolution rate. *Paragonimus westermani* was found to group with *P. siamensis* of Thailand; this was best supported by both the molecular morphometrics and combined analyses. *P. heterotremus*, *P. proliferus*, *P. skrjabini*, *P. bangkokensis* and *P. harinasutai* formed a separate clade in the molecular phylogenies, and were reciprocally monophyletic with respect to other species. The observed similarities at the secondary structural level are further reflected at the energy level. Only difference in their topology is due to differences in nucleotide sequences. These secondary structure predictions indicate that the domains basepair to form a core region central to several stem features implying that conservedness is more important for the proper rRNA folding pattern. Moreover the observed phylogenetic trend was identified with respect to the target accessibility sites for different isolates. The orders of preference were interior loop, bulge loop, multiple branch loop, hairpin loop and exterior loop in all the isolates.

- ***Fasciolopsis buski***: With regard to *F. buski*, for which only 18S rDNA sequences were available so far, ITS regions were sequenced for the first time in the present study. The nucleotide sequences obtained for ITS1 & ITS2 of rDNA (of both adult and egg origin), were compared with sequences of other trematode species obtained from Genbank. The fragments of amplified DNA were estimated to be 480-550bp long. Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481bp and 498bp; 559bp and 548bp for ITS1, forward and reverse sequences, respectively in adult and egg. The Blast hit results showed that the query ITS2 *Fasciolopsis buski* sequence is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more similar to the sequence of the species *Fasciola gigantica, Echinostoma revolutum, Isthmiophora melis, Echinostoma sp, Paryphostomum radiatum, Echinostoma trivolvis, Echinostoma paraenei, Fasciola sp, Fasciola hepatica* and *Isthmiophora hortensis*. Phylogenetic trees were obtained by comparing the sequences of *F. buski* and available ITS (1&2) sequences for other digenean trematodes including fasciolid species. Boot strapping of the sequences with Neighbour-Joining revealed significant support (100%) for the clade containing *F. buski, F. hepatica, F. gigantica* and *Fascioloides magna* indicating reliable grouping among different members of fasciolids.

- ***Fasciola gigantica*:** The PCR-amplified products were successfully obtained and were compared with sequences of other fasciolid species. The fragments of amplified DNA were estimated to be 480-550bp long. For comparative purpose, the ITS2 sequences of fasciolids from various geographical regions were obtained from GenBank. The Blast hit results showed that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp., *F. hepatica* and *F. gigantica* besides *Fasciolopsis buski* and *Fascioloides magna* (both belonging to the same family, i.e., Fasciolidae). Primary sequence analysis of *Fasciola* spp, revealed a close relationship between the query sequence (from NE India) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia. ITS2 sequence of the Indian isolate revealed closest similarity with isolate from China with significant bootstrap value revealing that the species prevailing in the region is *Fasciola gigantica.* Sequence of another Indian isolate, designated as *F. gigantica* (Accession- EF198867) from IVRI, Bareilly, showed absolute match with *F. hepatica*. Hence on the basis of molecular similarity this isolate should be identified as *F. hepatica* and not *F. gigantica.*

Secondary structure analysis of data confirmed the results mentioned for primary sequence analysis. Five predicted RNA secondary structures were reconstructed from the unique sequences with highest negative free energy of *F.*

gigantica to provide the basic information for phylogenetic analysis. The ITS2 plus flanking regions of nuclear region ranged from 720bp in *F. gigantica* India to a minimum length of 361bp in *F. gigantica* China. *F. gigantica* isolates from India and China show overall similarity in the ITS2 rRNA folding and have identical secondary structure. Secondary structures of remaining species are somewhat variant. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences.

- **Design of genus/species-specific primers**
  - To establish a more direct PCR procedure for species discrimination and identification, the genus/species-specific primers were designed using Primer3, a widely used program for designing PCR primers to target unique rDNA region spanning ITS2 for all the three trematodes viz. *Paragonimus westermani, Fasciolopsis buski* and *Fasciola gigantica.* Sequence analysis of the ITS2 PCR products revealed no stage-specific or intra-specific variations in length or composition. Multiple sequence alignment was done for all the three sequences using ClustalW programme. The *P. westermani*-specific (PwAR1), *F. buski*-specific (FbMR1) and *F. gigantica*-specific (FgMR1) primers were designed to target the 3'- terminal position of the ITS2 sequences, and the specificity of these primers was evaluated by PCR using primer 3S. As was expected, the primer set 3S-PwAR1 amplified a PCR product only from *P. westermani* DNA, 3S-FbMR1 amplified a PCR product only from *F. buski* DNA and 3S-FgMR1 amplified a PCR product only from *F. gigantica.* Primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample. These PCR products were sequenced using the corresponding specific primer and were confirmed to be the ITS2 region of rDNA from the respective species.

Sequences deposited in GenBank

i) **DQ351841-** *Fasciolopsis buski* adult 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ii) **DQ351842-** *Fasciolopsis buski* egg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

iii) **DQ351843-** *Fasciolopsis buski* adult internal transcribed spacer 1, partial sequence.

iv) **DQ351844-** *Fasciolopsis buski* egg internal transcribed spacer 1, partial sequence.

v) **DQ351845-** *Paragonimus westermani* metacercariae 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vi) **EF027103-** *Fasciola gigantica* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vii) **EF027104-** *Fasciola gigantica* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

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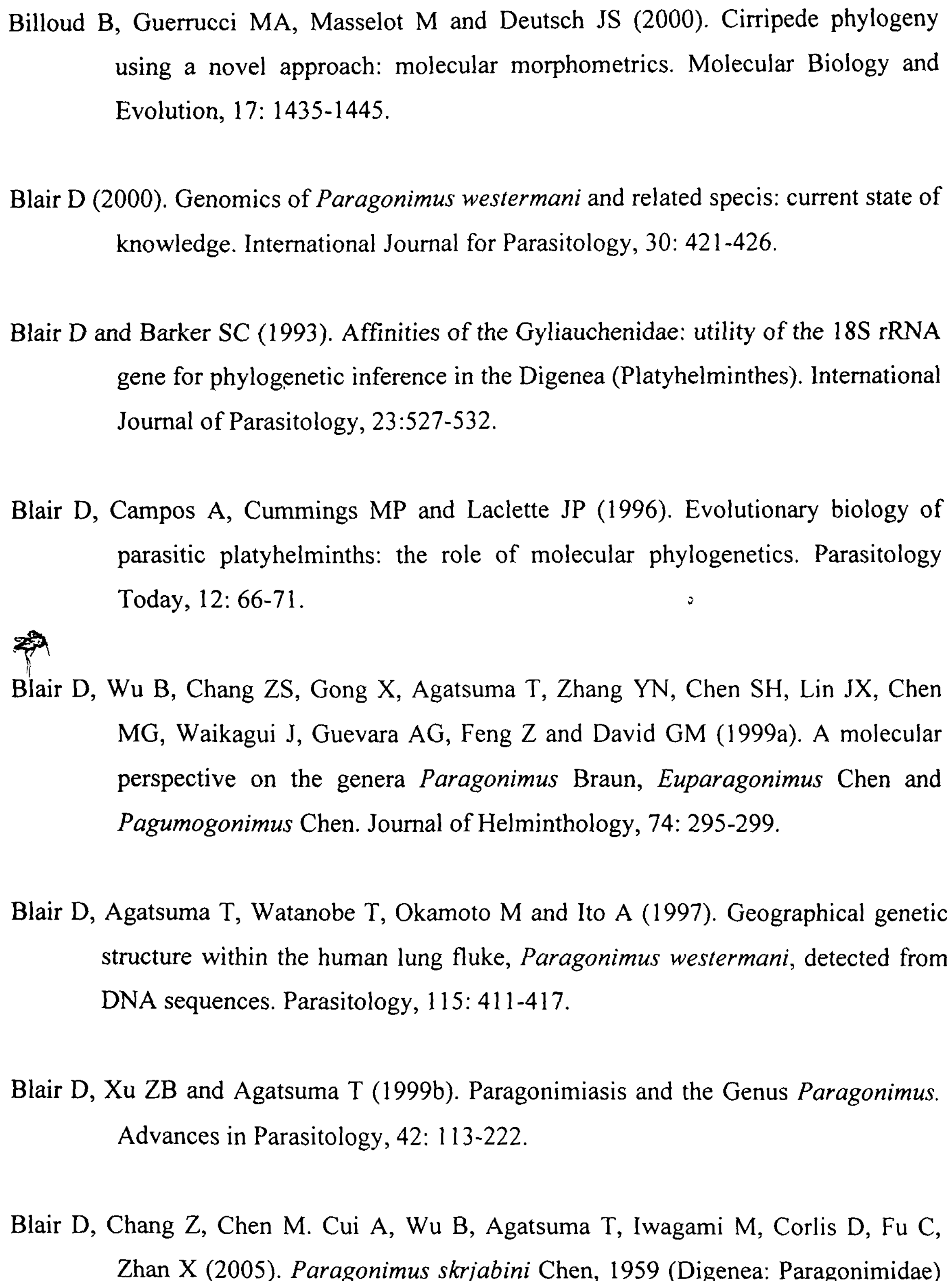

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BIO-DATA

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Achievements:

- Nominated as one of the **Genomic Pioneers** for contribution to the field of Genomics and was given this recognition during HUGO's International Human Genome Meeting 2008, September 27-30, Hyderabad.
- Awarded **Senior Research Fellowship (SRF)** under the subject code GENBIO-13 of CSIR w.e.f. 1 April 2008.

- Received **Prof. M.B. Mirza Medal** for the best publications of research work done in India at 19th National Congress of Parasitology from 26-28th October, 2007.
- Received Late. Prof. Sohan Lal Mishra **Gold Medal award for best poster presentation** at University of Allahabad during 'International Transfer of Technology Initiative Workshop on Parasitology & Genotoxicity in Sustainable Aquaculture' from 14-16th September, 2005.
- Secured **1st class 9th rank** in the M.Sc Zoology Exam conducted by NEHU, 2002.
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- Received college award from St. Anthony's College, Shillong for the **highest marks in zoology** secured by an Honours student in the degree examination of 2000.

Research Publications:

- Tandon V., Imkongwapang R. and **Prasad P. K.** (2005) On two new species of the trematode genera, Batrachotrema Dollfus et Williams, 1966 and *Opisthioparorchis* Wang, 1980 (Batrachotrematidae), with a report of a Chinese species of *Opisthioparorchis* from anuran amphibian hosts in India. **Zoos' Print Journal** Vol.20, No. 6 pp: 1883-1887.
- **Prasad P. K.,** Tandon V., Chatterjee A. and Bandyopadhyay S. (2007) PCR-based determination of internal transcribed spacer (ITS) regions of ribosomal DNA of giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899. **Parasitology Research** 101: 1581-1587.
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- **Prasad P. K.,** Tandon V., Biswal D. K., Goswami L. M. and Chatterjee A. (2009) Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae). **Bioinformation** 3(7): 314-321.
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Workshop/Training Course/Seminar/Conference attended:

- **'17th National Congress of Parasitology'** held at Regional Medical Research Center, Dibrugarh, 24th-26th October 2005, sponsored by Indian Society for Parasitology.

- **'International transfer of technology initiative workshop on Parasitology & Genotoxicity in sustainable Aquaculture'** held at Allahabad University, 14-16th, September, 2005, Sponsored by DBT, CSIR, ICAR, DST & UGC.
- **'3rd Global Meet on Parasitic Diseases'** held at Bangalore University, Bangalore, 12th-16th January 2004, organized by the Bangalore University, Indian Society for Parasitology and Society for Applied Genetics, Bangalore University.
- Training course on **'DNA Based Diagnostics'** Organized by Department of Animal Biotechnology, College of Veterinary Sciences, CCS Haryana Agricultural University, Hissar from 7-28th November, 2005.
- Short term course on **'Electron Microscopy'** conducted by the Electron Microscope Division of NEHU, Shillong from 22-30th September, 2003.
- Training course on **'Computer Basics & Application in Bioinformatics (Internet & Databases)'**, conducted by the Bio-informatics Centre, NEHU, Shillong, 18-20th April, 2005 funded by DBT, Ministry of Science & Technology, Govt. of India.
- **11th International Congress of Parasitology**, 6th-11th August 2006, held at Glasgow, Scotland, UK
- **18th National Congress of Parasitology** held at Indian Institute of Chemical Biology, Kolkata, 22- 24th November, 2006.
- Workshop on **'Instrumentation and Maintenance of Sophisticated Analytical Instruments'** Sophisticated Analytical Instrument Facility, NEHU, Shillong from 12-13th April, 2007.
- **International Symposium on Chromosomes to Genome** held at Centre for Cellular and Molecular Biology, Hyderabad, 3-5th July 2007.
- Workshop on **'Molecular Sequence Data and use in Diagnosis, Molecular Taxonomy, Molecular Epidemiology and Population Genetics'** University of Peradeniya, Sri Lanka, 20-24th July, 2007.
- **2nd International Symposium of Infectious Diseases and Health Sciences**, held at University of Peradeniya, Peradeniya, Sri Lanka, 26-27th July, 2007.
- **19th National Congress of Parasitology and International Symposium on Parasitic Diseases of Animals and Man** held at Department of Zoology, Andhra University, Visakhapatnam, 26-28th October, 2007.
- 77th Annual Session of NASI and Symposium on **'Novel Approaches for Food and Nutritional Security'** held at Central Food Technological Research Institute, Mysore, 6-8th December, 2007.
- Training Programme on **'Technology Led Entrepreneurship'** of HRDG, CSIR conducted by the Faculty of Indian Institute of Management, Bangalore at Indian Institute of Chemical Technology, Hyderabad, 2-27th June, 2008.
- **7th International Conference on Bioinformatics**, National Yang Ming University, Taipei, Taiwan, 20-22nd October, 2008.
- **IUBMB-FAOBMB-APBionet Workshop on Education in Bioinformatics and Computational Biology (WEBCB),** National Yang Ming University, Taipei, Taiwan, 24th October, 2008.
- "Molecular identification of the Indian liver fluke, *Fasciola gigantica* based on the ribosomal internal transcribed spacer region (rDNA, ITS2). **20th National Congress of Parasitology,** North-Eastern Hill University, Shillong, 3-5th November, 2008.

Conference Presentations:

Oral:

- "PCR-based determination of second internal transcribed spacer (ITS2) region of ribosomal DNA of giant intestinal fluke *Fasciolopsis buski*: egg and adult stages"- by **P. K. Prasad**, V. Tandon, A. Chatterjee and S. Bandyopadhyay. **17th National Congress of Parasitology** held at Regional Medical Research Center, Dibrugarh, 24 - 26th October 2005.
- "PCR-Based Determination of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, Northeast India"- by **P. K. Prasad**, V. Tandon and A. Chatterjee. **Regional Symposium on Research thrust in animal sciences in N.E. Region- An appraisal**, held at Department of Zoology, NEHU, Shillong, 24 -25th March 2006.
- "PCR-Based Determination of internal transcribed spacer (ITS) region of ribosomal DNA of giant intestinal fluke *Fasciolopsis buski*."- by **P. K. Prasad**, V. Tandon, A. Chatterjee and S. Bandyopadhyay. **11th International Congress of Parasitology**, 6th-11th August 2006, held at Glasgow, Scotland, UK.
- "Molecular Characterization of the liver fluke *Fasciola* sp. from India on the basis of DNA sequences of ribosomal Internal transcribed spacer (ITS) region."- by **P.K. Prasad**, V. Tandon, A. Chatterjee and L.M. Goswami. **18th National Congress of Parasitology** held at Indian Institute of Chemical Biology, Kolkata, 22- 24th November, 2006.
- "Molecular phylogenetic analysis of the giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899 from India by ITS ribosomal DNA sequences."- by **P. K. Prasad,** V. Tandon and A. Chatterjee. **2nd International Symposium of Infectious Diseases and Health Sciences,** 26-27th July, 2007 held at University of Peradeniya, Sri Lanka.
- "Molecular phylogenic location of the liver fluke, *Fasciola gigantica* (Cobbold, 1855) based on the ribosomal internal transcribed spacer regions."- **P. K. Prasad[1]**, V. Tandon[1], A. Chatterjee[2] and D. K. Biswal[3]. **77th Annual Session of NASI and Symposium on Novel Approaches for Food and Nutritional Security** held at Central Food Technological Research Institute, Mysore, 6 -8th December, 2007.
- "A combined morphological and molecular approach to identification of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, Northeast India."- Tandon V[1], **Prasad PK[1]**, Chatterjee A[2] and Bhutia PT[3]. **19th National Congress of Parasitology and International Symposium on Parasitic Diseases of Animals and Man** held at Department of Zoology, Andhra University, Visakhapatnam, 26-28th October, 2007.
- "Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae)."- **Prasad PK,** Tandon V, Biswal DK, Goswami LM and Chatterjee A. 7th International Conference on Bioinformatics, National Yang Ming University, Taipei, Taiwan, 20-23rd October, 2008.
- "Molecular identification of the Indian liver fluke, *Fasciola gigantica* based on the ribosomal internal transcribed spacer region (rDNA, ITS2). **20th National Congress of Parasitology**, North-Eastern Hill University, Shillong, 3-5th November, 2008.

Poster:

- "Surface fine topography of metacercaria of *Paragonimus sp.* From edible crabs in Arunachal Pradesh."- by V. Tandon, **P. K. Prasad** and P. T. Bhutia. **'International transfer of technology initiative workshop on Parasitology & Genotoxicity in sustainable Aquaculture'** held at Allahabad University, 14-16th, September, 2005.
- "Surface fine topography and PCR-based determination of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, North east India."- by V. Tandon, **P. K. Prasad** and P. T. Bhutia. **11th International Congress of Parasitology**, 6th-11th August 2006 held at Glasgow, Scotland, UK.
- "Comparison of the second internal transcribed spacer (ribosomal DNA) between two isolates of Fasciolidae (Trematoda: Digenea)"- by **P. K. Prasad**, V. Tandon, A. Chatterjee and L. M. Goswami. **'International Symposium on Chromosomes to Genome'** held at Centre for Cellular and Molecular Biology, Hyderabad, 3-5th July 2007.
- "In silico molecular phylogenic location of the Indian liver fluke, *Fasciola gigantica* (Cobbold, 1855) based on the ribosomal internal transcribed spacer regions"- **P. K. Prasad**[1], V. Tandon[1], D. K. Biswal[2], L. M. Goswami[1] and A. Chatterjee[3]. **77th Annual Session of NASI and Symposium on Novel Approaches for Food and Nutritional Security** held at Central Food Technological Research Institute, Mysore, 6-8th December, 2007.

Sequences submitted to Genbank and accession numbers received:

- **DQ351841-** *Fasciolopsis buski* adult 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **DQ351842-** *Fasciolopsis buski* egg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **DQ351843-** *Fasciolopsis buski* adult internal transcribed spacer 1, partial sequence.
- **DQ351844-** *Fasciolopsis buski* egg internal transcribed spacer 1, partial sequence.
- **DQ351845-** *Paragonimus westermani* metacercariae 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **EF027094** - *Fasciolopsis buski* cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
- **EF027103-** *Fasciola gigantica* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **EF027104-***Fasciola gigantica* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
- **EF027095-** *Gastrodiscoides hominis* cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.

- **EF027096-** *Gastrodiscoides hominis* adult 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **EF027097-** *Gastrodiscoides hominis* egg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **EF027098-** *Gastrodiscoides hominis* adult 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
- **EF027099-** *Paragonimus macrorchis* cytochrome oxidase subunit I-like (COI) gene, partial sequence; mitochondrial.
- **EF027100-** *Artyfechinostomum sufrartyfex* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
- **EF027101-** *Artyfechinostomum sufrartyfex* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2,partial sequence.
- **EF027102-** *Artyfechinostomum sufrartyfex* cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
- **EU887293-** *Gastrodiscoides hominis* cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial.
- **EU887294-** *Gastrodiscoides hominis* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.
- **EU887295-** *Gastrodiscoides hominis* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ON TWO NEW SPECIES OF THE TREMATODE GENERA, *OPISTHIOPARORCHIS* WANG, 1980 AND *BATRACHOTREMA* DOLLFUS AND WILLIAMS, 1966 (BATRACHOTREMATIDAE), WITH A REPORT OF A CHINESE SPECIES OF *OPISTHIOPARORCHIS* FROM ANURAN AMPHIBIAN HOSTS IN INDIA

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web supplement

ABSTRACT

*An examination of small samples of three species of anuran frogs (*Amolops afghanus, Rana liebigii *and* R. khare*) originating in Nagaland (northeastern India) revealed three species of trematodes, two of them being new to science. These include* Batrachotrema nagalandensis *sp. nov. (type host* A. afghanus*),* Opisthioparorchis indica *sp. nov. (type host* A. afghanus*) and* O. yunnanse *Li, 1996. This is the first record of their respective genera in India. The distinguishing features of* B. nagalandensis *sp. nov. are - testes located in the middle third of the body, and long cirrus sac extending posteriorly beyond the ventral sucker.* O. indica *sp. nov. is distinguished by having oral and ventral suckers of almost equal size, intestinal caeca extending beyond the ventral sucker up to the anterior border of testes, the genital pore at the level of the oral sucker and vitellaria confluencing medially in the pre and post testicular regions. Kohima (Nagaland) and* Rana liebigii *form a new locality and new host record, respectively, for* O. yunnanse.

KEYWORDS

Amolops afghanus, Batrachotrema nagalandensis *sp. nov., India, Nagaland,* Opisthioparorchis indica *sp. nov.,* Opisthioparorchis yunnanse, Rana khare, Rana liebigii, *Trematode*

During an exploratory survey of the helminth parasite fauna of anuran Amphibia in Nagaland (India) several digenetic trematode species were encountered. Two of these forms, on study, were found to be new to science and belong each to the genera *Batrachotrema* Dollfus and Williams, 1966 and *Opisthioparorchis* Wang, 1980. While the former genus has hitherto been reported from Africa (Dollfus & Williams, 1966), China (Wang, 1981; Zhang & Sha, 1985; Liang & Ke, 1988) and Vietnam (Moravec & Sey, 1989) the latter is represented so far in ranid frogs in China. The present communication deals with the description of a new species of each genus, and also forms the first record of the occurrence of these genera in the Indian subcontinent. The occurrence of *O. yunnanse* Li, 1996 is also reported from *Rana liebigii* as a new record.

MATERIALS AND METHODS

Eight specimens (range 1-4) of *Batrachotrema* were collected from the intestine of five *A. afghanus* and only one specimen, from *Rana khare* at Mokokchung in Nagaland. Twenty-one specimens of a form belonging to *Opisthioparorchis* were recovered from the intestine of three *Amolops afghanus* from Mokokchung, the maximum number of this parasite in a single host being 18. Two specimens, also belonging to the same genus but representing another species were collected from one *Rana liebigii* from Kohima.

Whole mount preparations of the flukes were made following standard procedure, using Mayer's carmalum or borax carmine as stains. For scanning electron microscopy (SEM) the specimens fixed in 4% cold neutral buffered formalin were processed and treated with tetramethylsilane as per the method described by Roy and Tandon (1991), metal coated and viewed under a JSM-35 CF (Jeol) scanning electron microscope at accelerating electron voltage ranging between 10-15kV.

Family Batrachotrematidae
Genus *Batrachotrema* Dollfus and Williams, 1966

***Batrachotrema nagalandensis* sp. nov.**
(Figs. 1-3; Images 1-2*)

Material examined

Holotype: W 8338/1; from the intestine of *Amolops afghanus* (Günther), Mokokchung, Nagaland (25°-27°4'N & 93°2'-95°15'E).
Paratypes: W 8339/1, W 8340/1; from the intestine of *Amolops afghanus* (Günther) and *Rana khare* (Kiyasetuo & Khare), Mokokchung.
Deposited at the Zoological Survey of India, Kolkata.

Etymology

Named after Nagaland state, India.

Specific diagnosis

Body unspined, fusiform, testes located in middle third of body; cirrus sac long, extending beyond ventral sucker posteriorly, ovary rounded.

Diagnosis

(Based on measurements of 5 mature specimens and SEM observations on 1 specimen.). Body elongated or some what spindle shaped, broadest at shoulder, tapering and terminating bluntly towards anterior and posterior regions; surface devoid of spines. Oral sucker subterminal. Ventral sucker pre-

* Also see SEM Images in the web supplement at www.zoosprint.org

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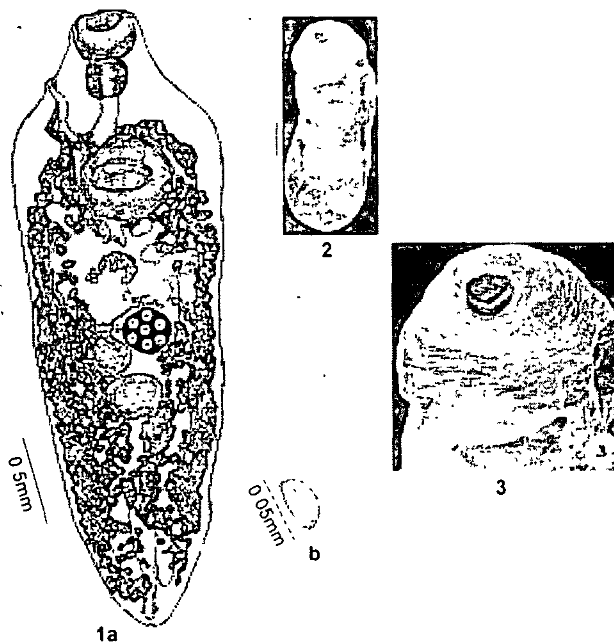


Figures 1-3. **Batrachotrema nagalandensis** *sp. nov.*
1a - Whole mount, dorsal view; 1b - Egg; 2-3 - Scanning electron micrographs: 2 - Full worm, ventral view (scale 100mm); 3 - A magnified view of anterior end, showing the oral end and ventral sucker (scale 100mm).
[See Figures 2 & 3 on the web as Images 1 & 2]

Table 1. Measurements (in mm) of **Batrachotrema nagalandensis** *sp. nov.*

Characters	Range (Mean)	± S.D.
Length of Body	2 38-3 89 (2 93)	0 49
Maximum width of body (at level of ventral sucker)	0 80-1 27 (1 01)	0 202
Oral sucker		
Length	0 17-0 27 (0 21)	0 043
Breadth	0 26-0 37 (0 33)	0 034
Ventral Sucker		
Length	0 30-0 49 (0 42)	0 07
Breadth	0 39-0 56 (0 47)	0 07
Pharynx		
Length	0 17-0 25 (0 22)	0 032
Breadth	0 17-0 31 (0 20)	0 036
Length of Oesophagus	0 24-0 46 (0 41)	0 023
Distance of intestinal caeca from hind end	0 07-0 25 (0 22)	0 037
Intestinal bifurcation from anterior end	0 46-0 75 (0 64)	0 01
Testis I		
Length	0 20-0 22 (0 22)	0 017
Breadth	0 22-0 34 (0 27)	0 035
Testis II		
Length	0 24-0 46 (0 31)	0 077
Breadth	0 19-0 36 (0 31)	0 058
Ovary		
Length	0 22-0 29 (0 26)	0 012
Breadth	0 19-0 28 (0 24)	0 022
Extent of Vitellaria	1 89-3 05 (2 37)	0 419
Eggs	0 03-0 04 x 0 02-0 023 (0 036 x 0 022)	0 002

equatorial, located in broader part of body, three times larger than oral sucker, covering half of body in width, strongly muscular. Prepharynx indistinct; pharynx muscular; oesophagus of moderate length or short; intestinal caeca reaching near posterior end. Testes rounded or oval, lying in tandem in middle third of body; anterior testis at level of ovary, slightly smaller than posterior; cirrus sac long, extending posteriorly beyond ventral sucker, lying on its left side, extending anteriorly up to and ending in level with pharynx. Ovary rounded, pretesticular, postacetabular; oviduct, Mehlis' gland complex in region anterior to ovary; uterine coils mainly limited in ovarian and postacetabular area, overlapping intestinal caeca. Genital pore at left side of body, marginal or sub marginal, male and female pores opening into genital atrium. Vitellaria follicular, extending from level of intestinal bifurcation up to posterior most part of body, overlapping intestinal caeca, also scattered in intercaecal fields. Eggs numerous, large, oval. The measurements of this form are given in Table 1.

Discussion

In having a pretesticular ovary, which is nearer to the anterior testis than the ventral sucker and the genital pore lying laterally to the median line in level with the pharynx, the present form bears a close morphological resemblance to the genus *Batrachotrema*. Dollfus and Williams (1966) proposed a new family Batrachotrematidae for the genus. Yamaguti (1971) retained the family as proposed by Dollfus and Williams and Wang (1980) also accepted its validity. However, Prudhoe and Bray (1982), on the basis of its close morphological relationship with the members of Opecoeliidae, placed the genus under the latter family.

The genus *Batrachotrema* so far includes five species: *B. petropedetis* Dollfus and Williams, 1966 from *Petropedetes natator* in Sierra Leone, Africa; *B pseudobagri* Wang, 1981 (from *Pseudobagrus fulvidraconis* and *Pseudogastromyzon zebroidus* in Fujian Province, China); *B. yaanensis* Zhang and Sha, 1985 (from *Rana phrynoides* in Yaan, Sichuan, China); *B opistosacca* Liang and Ke, 1988 (from *Rana spinosa* in Meixian, Guangdong, China); *B vietnamensis* Moravec and Sey, 1989 (from *Rana kuhlii* in Hanoi Vietnam).

A comparison of the present form with the type species reveals several differences between the two. In the present form the testes lie in the middle third of the body and the cirrus sac is long extending posteriorly beyond the ventral sucker In the type species the testes are located much posteriorly and the claviform cirrus sac is quite small extending posteriorly only up to the anterior margin of the ventral sucker. Both *B petropedetis* and *B opistosacca* have an elongated oval body shape and have intestinal caeca extending posteriad a little beyond the testes. In having a fusiform body, the present species resembles *P pseudobagri*, a species described from piscine hosts, but distinctly differs from it in having a larger body size, a rounded ovary (lobate in *P pseudobagri*), and a long cirrus sac that extends posteriad much beyond the ventral sucker In possessing an unspined body, testes situated in the middle third of the body and immediately pretesticular ovary that is

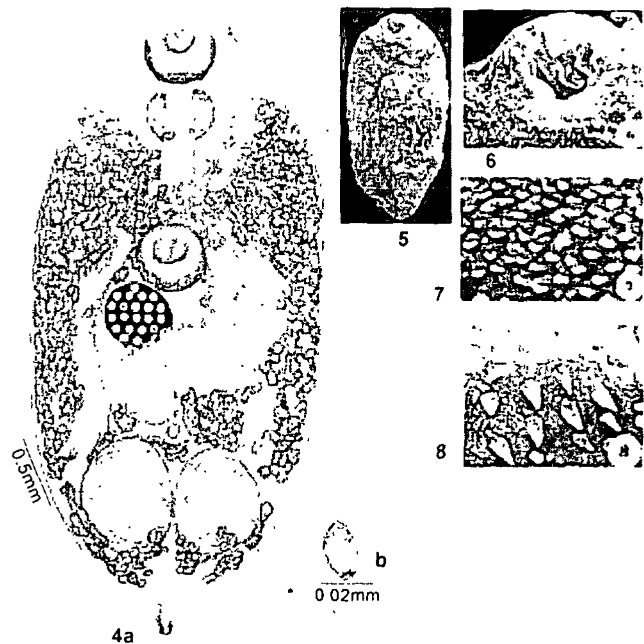
not separated from testes by uterine coils. the present form comes close to *B. petropedetis*, *B pseudobagri* and *B. opistosacca* and stands apart from *B yaanensis* and *B vietnamensis*. In view of the apparent morphological differences and also different amphibian host species the present form is considered a new species under the genus *Batrachotrema*. It is for the first time that a representative of the genus is being reported from the Indian subcontinent.

Family Batrachotrematidae
Genus *Opisthioparorchis* Wang. 1980
***Opisthioparorchis indica* sp. nov.**
(Figs. 4-8; Images 3-6")

Material examined
Holotype: W 8341/1; from the intestine of *Amolops afghanus* (Günther), Mokokchung. Nagaland (25°-27°4'N & 93°2'-95°15'E)
Paratypes: W 8342/1, 8343/1; same as holotype.
Deposited at the Zoological Survey of India. Kolkata.

Etymology
Named after the country.

Specific Diagnosis
Intestinal caeca extending much beyond ventral sucker up to anterior level of testes. ventral sucker almost equal to oral sucker in size, genital pore in level with anterior margin of oral sucker, vitellaria extending beyond testes posteriorly, confluent medially in pre-and posttesticular regions.



Figures 4-8. **Opisthioparorchis indica *sp. nov.***
4a. Whole mount, ventral view, b. Egg; 5-8. Scanning electron micrographs: 5 - Whole worm, ventral view (scale 100mm); 6 - Oral end, magnified view (scale 10mm); 7 - A magnified view of the tegumental spination in the mid ventral region (scale 10mm); 8 - Tegument of the posterior part of the body depicting conical spines (scale 10mm) [See Figures 5-8 on the web as Images 3-6]

Diagnosis
(Based on measurements of 10 mature flukes and SEM observations on 2 specimens.). Body elongate or oval with rounded anterior end. rounded or slightly conical posterior end. spinose. Oral sucker subterminal. spherical. Ventral sucker pre-equatorial or equatorial almost equal to oral sucker in size. Prepharynx indistinct: pharynx muscular: oesophagus of moderate length; intestinal caeca extending in postacetabular region up to level of anterior margin of testes. Testes round or oval, lying symmetrically juxtaposed in posterior region of body: cirrus sac well developed. elongated. somewhat S-shaped, recurved. lying on left side of body. extending from near junction of oesophagus and intestinal caeca up to anterior margin of oral sucker. Ovary rounded. pretesticular. postacetabular; receptaculum seminis conspicuous. lying posterior to ovary; uterus pretesticular, occupying major area between ovary and testes, extending anteriad partially overlapping intestinal caeca. Genital pore marginal on left at level of anterior margin of oral sucker. Vitellaria in varying follicular size, extending in lateral fields from level of pharynx posteriorly up to post-testicular region, becoming confluent medially in intercaecal. pretesticular and post testicular fields. Eggs numerous. ovoid. operculate.

SEM revealed the surface fine topography of the fluke. The body surface is studded throughout with dense spination except for the anterior circum-oral region, which appears devoid of spines. Whereas the spines covering the tegument in most parts of the body are scale like with rounded tips. those abounding in the posterior region are conical. with broad base and pointedly tapering distal end. The non-spinous tegument of the anterior region presents a spongy texture. The measurements of this form are given in Table II.

Discussion
In having a well-developed cirrus sac and marginal or submarginal genital pore located in the region of the oral sucker or pharynx. the present form belongs to the family Batrachotrematidae.

The genus *Opisthioparorchis* was created by Wang (1980) for an intestinal fluke of *Rana spinosa*. which was characterized by having juxtaposed testes located at the posterior end of the body and intestinal caeca extending up to just near the anterior border of testes. At present the genus includes six species. all described from China: *O. ranae* Wang. 1980 (type species) from *Rana spinosa* in Fujian Province South; *O. pleurogenitus* Wang. 1980 from the same host and locality as those of the type species: *O. boheansis* Wang. 1980 from *Staurois wuyiensis* also from Fujian: *O. megaloonis* Liang and Ke. 1988 and *O. meixianensis* Liang and Ke. 1988. both from *R. spinosa* in Changsha (Meixian. Guangdong Province) and *O yunnanse* Li. 1996 also from *R. spinosa* from Yunnan Province.

In having the vitellaria extending in the posttesticular region.

Table 2. Measurements (in mm) of Opisthioparorchis indica *sp. nov.*

Characters	Range (Mean)	± S.D.
Length of Body	1.80-2.33 (2.05)	0.18
Maximum width of body (at level of ventral sucker)	0.89-1.08 (0.97)	0.08
Oral sucker:		
Length	0.21-0.26 (0.24)	0.02
Breadth	0.23-0.30 (0.26)	0.026
Ventral Sucker:		
Length	0.21-0.25 (0.23)	0.014
Breadth	0.21-0.27 (0.24)	0.018
Pharynx:		
Length	0.19-0.23 (0.21)	0.018
Breadth	0.2-0.24 (0.22)	0.016
Length of Oesophagus	0.14-0.23 (0.18)	0.03
Distance of intestinal caeca from hind end	0.58-0.77 (0.68)	0.06
Intestinal bifurcation from anterior end	0.53-0.65 (0.61)	0.04
Testis (right):		
Length	0.33-0.46 (0.39)	0.04
Breadth	0 26-0.33 (0.29)	0.023
Testis (left):		
Length	0.33-0.41 (0.38)	0.03
Breadth	0.25-0 32 (0.28)	0.029
Cirrus sac Length	0 52-0.66 (0.57)	0.05
Ovary:		
Length	0.17-0.25 (0.22)	0.027
Breadth	0.17-0.23 (0.20)	0.022
Receptaculum seminis:		
Length	0.36-0.53 (0.44)	0.07
Breadth	0.03-0.065 (0.047)	0.013
Eggs	0.027-0.037 x 0.011-0.018 (0.031 x 0.015)	0.002

the present form resembles the type and other species but stands apart from *O. pleurogenitus* and *O. yunnanse*, in both of which the vitelline follicles are distributed only in the pretesticular region and the testes are the posterior most structures in location in the fluke body. It also differs from all those species having posttesticular vitellaria in several characters; the latter species. have a much smaller body size. the ventral sucker is smaller than the oral and unlike the present form, the vitellaria do not become confluent medially in the pretesticular, inter-intestinal and posttesticular regions.

In view of the conspicuous differences of the present form with the hitherto known species of *Opisthioparorchis*, it is proposed to consider this as a new species named *O. indica*.

Opisthioparorchis yunnanse Li, 1996
(Fig. 9)

Material examined
From the intestine of *Rana liebigii* (Günther). Kohima (Nagaland, 25^0-27^{0}4'N, India).

Diagnosis
(Whole mount). Body elongate, somewhat narrow anteriorly. broader posteriorly. with bluntly rounded ends. spinose. Oral sucker subterminal, almost twice as large as ventral sucker.

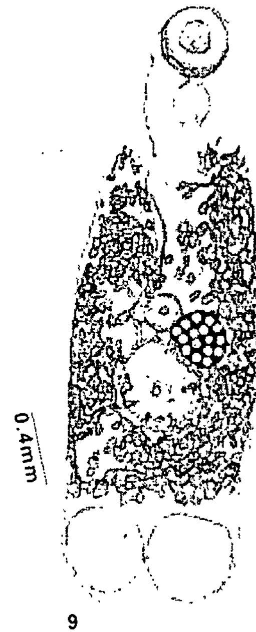


Figure 9. Opisthioparorchis yunnanse *Li, 1996 Whole mount, ventral view*

Table 3. Measurements (in mm) of Opisthioparorchis yunnanse

Characters	Measurement
Length of Body	2.5
Maximum width of body (at level of ventral sucker)	1 94
Oral sucker:	
Length	0.28
Breadth	0.29
Ventral Sucker:	
Length	0 08
Breadth	0.08
Pharynx	
Length	0.18
Breadth	0.20
Length of Oesophagus	0.09
Distance of intestinal caeca from hind end	0.58
Intestinal bifurcation from anterior end	0.6
Testis (right):	
Length	0.37
Breadth	0.35
Testis (left):	
Length	0.38
Breadth	0.31
Cirrus sac Length	0.97
Ovary:	
Length	0.23
Breadth	0.23
Receptaculum seminis:LengthBreadth	0.420.047
Eggs	0.041 x 0.021

latter equatorial in position. Prepharynx short. pharynx muscular: oesophagus short; intestinal caeca extending posterial up to a little in front of testes. Testes round or oval. juxtaposed near posterior extremity of body; cirrus sac club-shaped, elongated, extending from a little in front of ventral

sucker anteriad up to level of oral sucker. Ovary rounded, just by side or slightly posterior to ventral sucker; uterus mainly in near post ovarian region, extending as ascending narrow tube anteriad. Genital pore marginal, on left at level of oral sucker. Vitellaria extending extensively from level of oesophagus posteriad up to just in front of testes. Eggs elliptical, operculate. The measurements of this form are given in Table III.

Remarks

The whole mount preparation of one specimen collected from *Rana liebigii*, though apparently resembled *Opisthioparorchis* species, turned out to be different from the new species of the genus described above. In its general morphology (elongated body, oral sucker larger than the ventral sucker- almost double the size, vitellaria limited to only pretesticular zone and long club-shaped cirrus sac), the present specimens stands close to *O. yunnanse* Li, 1996, which was originally described from *Rana spinosa* from Yunnan Province, China. However, the only conspicuous deviation it shows from this species is the length of the oesophagus; in *O. yunnanse* the oesophagus is quite long, though short in the present form. However, considering that just one character i.e. length of the oesophagus, is not enough to erect a new species, the present form is considered representing *O. yunnanse*.

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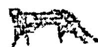

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ANT DIVERSITY IN SOME SELECTED LOCALITIES OF SATTUR TALUK, VIRUDHUNAGAR DISTRICT, TAMIL NADU

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Social insects in general, and ants in particular have achieved unprecedented ecological success and dominance in tropical ecosystem (Gadagkar *et al.*, 1993). Social way of life adapted by all ant species brings with it both benefits and cost (Brain, 1975). Being such dominant and successful component of ecosystems, it is likely that ants would turn out to be good indicators of the well being of the ecosystem (Daniels, 1991). However, a literature survey revealed that only limited studies are available on diversity of ants. This may be due to lack of appropriate methodologies of tropical insect communities, especially ants (Gadagkar *et al.*, 1990). The present study was carried out in an attempt to understand and measure the status of ant species diversity in some selected localities of Sattur taluk (9°10'N & 77°52'E; 61.19m), Virudhunagar district, Tamil Nadu, in riverine, cultivated and industrial areas.

The study was carried out for seven months from August 2001 to February 2002. The ants were collected from three selected localities: cultivated areas (paddy, cotton, brinjal, ladies finger, *Cassia fistula*, banana, coconut, guava, palm etc), riverine areas (vegetation found along the banks of Arjuna and Vaiparu rivers), and industrial areas (match factories, fire works and printing industries). The average rainfall during the period was 75.84mm in Sattur taluk. Maximum and minimum temperatures were 35°C and 26°C, respectively. All the collected specimens were preserved in 70% alcohol. Ants were separated and sent to the Center for Ecological Science at Indian Institute of Science, Bangalore for identification and confirmation of ant specimens, with the help of Dr. R. Gadagkar, Director, CES. Relative abundance of ant species were calculated using the following formula (Micheal, 1986).

$$\text{Relative abundance} = \frac{\text{Total number of individuals of the species}}{\text{Total number of individuals of all species}} \times 100$$

A total of 25 species of ants belonging to 14 genera distributed in six subfamilies were recorded (Table 1). The subfamilies included Formicinae (9 species) followed by Myrmicinae (8 species), Pseudomyrmicinae (4 species), Ponerinae (2 species), Dorylinae / Dolichoderinae (1 species). The study revealed that more number of ant species were recorded in the riverine (24 species) and cultivated areas (20 species).

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ORIGINAL PAPER

PCR-based determination of internal transcribed spacer (ITS) regions of ribosomal DNA of giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899

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Abstract *Fasciolopsis buski*, the zoonotic intestinal flukes of pigs in South and Southeast Asia, is commonly prevalent in regions across Northeast India. Populations of the fluke collected from different parts of the region exhibit variations in morphology. The main objective of our study was to provide molecular characterization of the parasite so as to supplement morphological criteria, using ribosomal DNA cluster (rDNA), which is flanked by more conserved internal transcribed spacer (ITS) regions. We describe herein the ITS sequences of the parasite collected from swine hosts of Assam region. The ITS sequences of both egg and adult origins were found to be identical in length and composition. Phylogenetically, *F. buski* resembles closely the other members of family Fasciolidae, showing significant expectation value in the alignment. The results corroborate that the ITS sequences are not stage specific and are conserved through different stages of development of the fluke, and thus could be used as species markers.

Nucleotide sequence data reported in this paper have been submitted to the Genbank data with the accession numbers DQ351841–DQ351844.

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Introduction

The giant intestinal fluke, *Fasciolopsis buski* (Trematoda: Fasciolidae), is widely distributed in India and neighboring countries of the continent in South and Southeast Asia (Roy and Tandon 2003). The fluke is the etiological agent of the disease commonly known as fasciolopsosis. The infection occurs by ingestion of raw aquatic vegetation or food plants that are contaminated with the infective encysted larvae, the metacercariae. In endemic zones, pigs, dogs, and rabbits act as reservoir of infection. In India, the parasite has been reported from different states including those in the Northeast. Variations in the morphology of the fluke have been observed when collected from different geographical regions (Roy and Tandon 1993).

Identification of closely related species based on morphological characters alone can be difficult. This is particularly so in the case of soft-bodied animals parasites such as digenean trematodes. However, recent advances in molecular biology, in particular the amplification of specific DNA regions via the polymerase chain reaction (PCR) and improved sequencing techniques, have been employed to resolve taxonomic issues related to various helminth parasites by comparing their DNA, utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA in particular (Blair et al. 1996). The rDNA cluster, which codes for structural components of ribosomes, is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon 1991). It enhances PCR amplification because many templates are available for initial

priming and allows primers to be designed to anneal the known conserved regions to amplify across unknown variable regions. In context of metazoan parasites, the nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2), which occur between the 18S, 5.8S, and 28S coding regions, have proven useful for diagnostic purposes at the level of species (Morgan and Blair 1995; Leon-Regagnon et al. 1999; Tkach et al. 2000; Kostadinova et al. 2003; Scholz et al. 2004; Nolan and Cribb 2005). These sequences are often assumed to be homogenized within individuals and populations of the same species by concerted evolution (Dover 1982; Hillis and Davis 1988). ITS2 sequences have been shown to be a sensitive marker at the species level in trematodes, whereas the sequences in the ITS1 might be less conserved than those in the ITS2 (Luton et al. 1992). Among the fasciolid digeneans, isolates of *Fasciola* spp. and of *Fascioloides magna* from different geographical regions have been discriminated on the basis of ITS sequences (Adlard et al. 1993). Studies on phylogeny and/or intraspecific variations in *Paragonimus* species have recently been done using second ITS (ITS2) sequences (Blair et al. 1997, 1999; Iwagami et al. 2000). The usefulness of the ITS2 region for species discrimination has also been demonstrated in nematodes, for example, *Strongylus*, *Trichostrongylus* species, larvae of *Dictyocaulus viviparus* and *D. eckerti* (Campbell et al. 1994; Hoste et al. 1995; Samson-Himmelstjerna et al. 1997).

In the present study, we amplified the ITS regions of *Fasciolopsis* (DNA from egg and adult stages) and assessed their potential for discriminating between species and genera within the group. Our main objectives were to determine whether the sequences are stage specific and conserved or not and to find out the species-specific molecular markers by amplifying the ITS (I and II) regions of the parasite DNA both from adult and egg stages.

Materials and methods

Parasite material and DNA isolation

Live adult *F. buski* were obtained from the intestine of freshly slaughtered pig, *Sus scrofa domestica* at local abattoirs. The worms recovered from these hosts represented the geographical isolates from Assam region of Northeast India. Eggs were obtained from mature adult flukes by squeezing between two glass slides. For the purpose of DNA extraction, adult flukes collected from different host animals were processed singly; eggs recovered from each of these specimens were also processed separately. The adult flukes were first immersed in digestion extraction buffer [containing 1% sodium dodecyl sulfate (SDS), 25 mg Proteinase K] at 37°C for overnight.

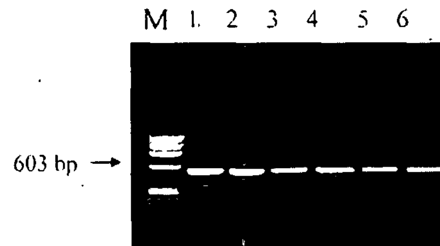


Fig. 1 Agarose gel stained with ethidium bromide showing the PCR products of *Fasciolopsis buski* genomic DNA from adult fluke using the primer set 3S-A28 for ITS2 (*lanes 1–6*). *M* Molecular weight marker (Ø× 174 DNA/HaeIII Digest)

DNA was then extracted from lysed individual worms by standard ethanol precipitation technique (Sambrooke et al. 1989) and also extracted on FTA cards using Whatman's FTA Purification Reagent. The FTA cards were allowed to dry for 1 h at room temperature before punching; two to three sample discs of 1.2-mm size were taken from the desired spot using coring device assuming a 25-μl reaction and placed in PCR amplification tube. The discs, then washed with FTA Purification reagent and TE Buffer, were allowed to dry at room temperature for 1 h to make them ready for PCR. DNA from the eggs was extracted only with the FTA card technique.

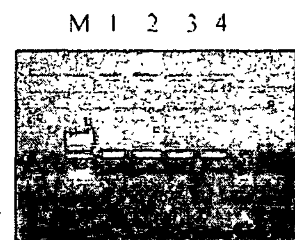
DNA amplification, sequencing, and its analysis

The rDNA region spanning the ITS regions was amplified from DNA obtained from the egg and adult stages of the fluke by PCR. We used the universal primers based on conserved ITS sequences of *Schistosoma* species following (Bowles et al. 1995) as detailed below:

1. ITS2 region-3S (forward), 5′GGTACCGGTGGATC ACTCGGCTCGTG-3′ and A28 (reverse), 5′-GGGATC CTGGTTAGTTTCTTTTCCTCCGC-3′
2. ITS1 region-BD1 (forward), 5′GTCGTAACAAGG TTTCCGTA-3′ and 4S (reverse), 5′TCTAGATGCG TTCGAA (G/A) TGTCGATG-3′

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications in 100 mM Tris HCl (pH 9.0), 500 mM KCl, 1.5 mM $MgCl_2$,

Fig. 2 Agarose gel stained with ethidium bromide showing the PCR products of *Fasciolopsis buski* genomic DNA from adult fluke using the primer set BD1-4S for ITS1 (*lanes 1–4*). *M* Molecular weight marker (Ø× 174 DNA/HaeIII Digest)



```
1   TGTCGATGAGGAGCGCAGCCACTGTGTGAATTAATGCAAACTGCATACT    49
    |||||||||||||||||||||||||||||||||||||||||||||||||
1  CTGTCGATGAGGAGCGCAGCCACTGTGTGAATTAATGCAAACTGCATACT    50

50  GCTTTGAACATCGACATCTTGAACGCATATTGCGGCCATGGTTAGCCTG    99
    |||||||||||||||||||||||||||||||||||||||||||||||||
51  GCTTTGAACATCGACATCTTGAACGCATATTGCGGCCATGGTTAGCCTG    100

100 TGGCCACGCCTGTCCGAGGGTCGGCTTACAAACTATCACGACGCCCAAAA   149
    |||||||||||||||||||||||||||||||||||||||||||| |||||
101 TGGCCACGCCTGTCCGAGGGTCGGCTTACAAACTATCACGACGCCCAAAA   150

150 AGTCGTGGCTTGGGTCTTGCCAGCTGGCGTGAACTCCTCTATGATTATTC   199
    ||||||||||||||| |||||||| |||||||||||||||||||||||||
151 AGTCGTGGCTTGGGTCTTGCCAGCTGGCGTGAACTCCTCTATGATTATTC   200

200 ATGTGAGGTGCCAGAACTATGGCGTTTCCCTAATGTATCCGGACGCGTCC   249
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 ATGTGAGGTGCCAGAACTATGGCGTTTCCCTAATGTATCCGGACGCGTCC   250

250 TTGTCTCAGCAGAAGGCTGTGGTGAGGTGCGGTAGCGGGATCGTGGTTTA   299
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TTGTCTCAGCAGAAGGCTGTGGTGAGGTGCGGTAGCGGGATCGTGGTTTA   300

300 ATGAATACTGTGCACGTTCCGTTGCTGTGTCTTCATCGTCGGCTTGATGC   349
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 ATGAATACTGTGCACGTTCCGTTGCTGTGTCTTCATCGTCGGCTTGATGC   350

350 GTGACTTGGTCTCGTGTCTGAGGCTTTTGCCATACATAGACTGCCATTTG   399
    ||||||||||||||||||||||||||||||||||||||.|||||||||||
351 GTGACTTGGTCTCGTGTCTGAGGCTTTTGCCATACATAAACTGCCATTTG   400

400 TGTGGTCTAATTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTT   449
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 TGTGGTCTAATTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTT   450

450 AAGCATATCACTAAGCGGAGGAAAAG - AACAAA                  481
    ||||||||||||||||||||||.|||  |||.||
451 AAGCATATCACTAAGCGGAGGAGAAGAAACTAACCCCGGAGCCCACAA     498
```

Fig. 3 Pair-wise alignment of ITS2 sequence of egg and adult stages of *Fasciolopsis buski*

and 0.2 mM deoxynucleotide triphosphates each of dATP, dGTP, dCTP, dTTP, 0.25 mM of each primer, and 2.5 units of Taq polymerase (Bangalore Genei Pvt., India). The DNA was preheated at 94°C for 5 min and added to each PCR reaction. The PCR cocktail (final reaction volume 25 μl) was amplified with the following conditions: 26 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 38 s and extention at 72°C for 42 s followed by a final extention at 72°C for 10 min. The resultant PCR products were separated by electrophoresis through 1.6% (*w/v*) agarose gels in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed. The known size fragments of Phi X 174 DNA/HaeIII Digest in agarose gel were used as marker. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei.

The DNA sequences were put to further analysis by using various Bioinformatics tools including similarity search basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast) and phylogenetic prediction by ClustalW (http://www.ebi.ac.uk/clustalw) for each query DNA sequence.

Molecular phylogenetic analysis

Phylogenetic tree-building methods presume particular evolutionary models. Therefore, while interpreting the results obtained, we considered different tree building models to entertain possible explanations. Only unique sequences were used in tree construction. ITS sequences were entered in the MEGA for construction of the phylogenetic trees using maximum parsimony and distance methods namely the neighbor-joining, UPGMA and minimum evolution. The distance methods were used so as to augment maximum parsimony because they are less likely to give errors when trees contain long branches (Blair and Barker 1993).

```
1   CTGCTGACTGCGGAGGATCATTACCGTAATCCTAT-TCATACACAAGAGG   49
        .|||||||||||||||||||||||||||||||| |||||||||||||
1       GTGACTGCGGAGGATCATTACCGTAATCCTATCTCATACACAAGAGG   47

50  TGAAACGTTGTGACCGTCATGTCCAACGATACAAATTTGCGGACGGCTAT   99
    ||||||||||||||||||||||||||||||||||||||||||||||||||
48  TGAAACGTTGTGACCGTCATGTCCAACGATACAAATTTGCGGACGGCTAT   97

100 GCCTGGCTCTTTGAGGCCACAGCATATCCGATTATGACGGGGTGCCTACC   149
    ||||||||||||||||||||||||||||||||||||||||||||||||||
98  GCCTGGCTCTTTGAGGCCACAGCATATCCGATTATGACGGGGTGCCTACC   147

150 TGTGTGATCCTCTGATGGTATGCATGCGTCCTTTGGGTCGTATGTCCAAG   199
    ||||||||||||||||||||||||||||||||||||||||||||||||||
148 TGTGTGATCCTCTGATGGTATGCATGCGTCCTTTGGGTCGTATGTCCAAG   197

200 CCAGGAGAACAGGCTGTACTGCCGTGACTGGTAGTGCTAGGCTTAAAGAG   249
    ||||||||||||||||||||||||||||||||||||||||||||||||||
198 CCAGGAGAACAGGCTGTACTGCCGTGACTGGTAGTGCTAGGCTTAAAGAG   247

250 GAGATTTGAGCTACGGCCCTGCTCCCGCCCTATGAACTGTTTCCTATATT   299
    ||||||||||||||||||||||||||||||||||||||||||||||||||
248 GAGATTTGAGCTACGGCCCTGCTCCCGCCCTATGAACTGTTTCCTATATT   297

300 ACACTGTTCAAGTGGTATTGATTGGGTTCGCCCATTCTTTGCCATTGCCC   349
    ||||||||||||||||||||||||||||||||||||||||||||||||||
298 ACACTGTTCAAGTGGTATTGATTGGGTTCGCCCATTCTTTGCCATTGCCC   347

350 TCGCATGCACCTGGTCCTTGTGGCCGGACTGCACGTACGTCGCCCGGCGG   399
    ||||||||||||||||||||||||||||||||||||||||||||||||||
348 TCGCATGCACCTGGTCCTTGTGGCCGGACTGCACGTACGTCGCCCGGCGG   397

400 TGCCTATCCCGGGTAGGACTGATAACCTGGTCTTTGACCATTTGCACAAC   449
    ||||||||||||||||||||||||||||||||||||||||||||||||||
398 TGCCTATCCCGGGTAGGACTGATAACCTGGTCTTTGACCATTTGCACAAC   447

450 TCTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAAC   499
    ||||||||||||||||||||||||||||||||||||||||||||||||||
448 TCTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAAC   497

500 TGTGTGAATTAATGCAAACTGCATACTGCTTTGAACATCGACCT---GGA   546
    ||||||||||||||||||||||||||||||||||||||||||||  |   |
498 TGTGTGAATTAATGCAAACTGCATACTGCTTTGAACATCGACATTTCG--   545

547 CAACGTATCTGAA                                        559
    |||
546 CAA                                                  548
```

Fig. 4 Pair-wise alignment of ITS1 sequence of egg and adult stages of *Fasciolopsis buski*

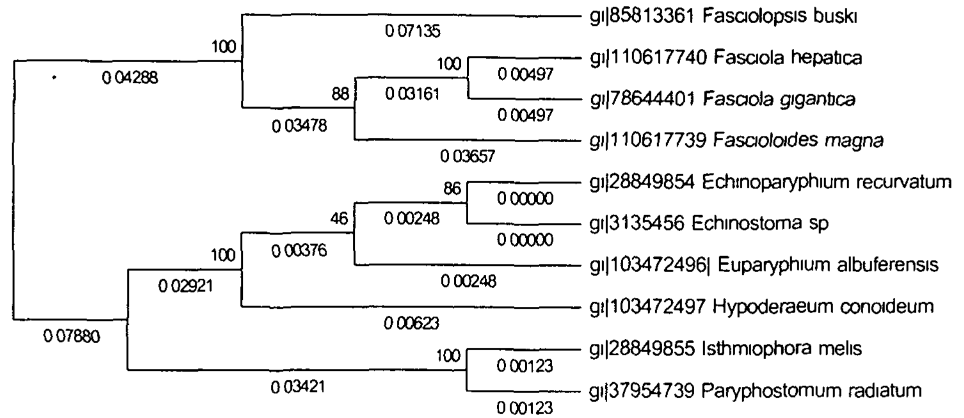


Fig. 5 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS2 data by neighbor-joining method showing bootstrap values

Results

PCR amplification of ITS regions and its analysis

The PCR-amplified products were successfully obtained using the primers as mentioned above and are depicted in Figs 1 and 2 The nucleotide sequences were obtained for ITS1 and ITS2 of rDNA adult and egg stages of *F buski* and were compared with sequences of other trematode species obtained from GenBank The fragments of amplified DNA were estimated to be 480–550 bp long Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481 and 498 bp for forward primer and 559 and 548 bp for forward primer of ITS1, respectively, in adult and egg No intra-specific variations in length or composition of the sequence were observed, and the ITS sequences of both adult and egg origin were found to be identical in length and composition (Figs 3, 4)

The BLAST hit results show that the query-ITS2 *F buski* forward and reverse sequences are more similar to the sequences of the species *Fasciola gigantica*, *Echinostoma revolutum*, *Isthmiophora melis*, *Echinostoma* sp , *Paryphostomum radiatum*, *Echinostoma trivolvis*, *Echinostoma paraenei*, *Fasciola* sp , *Fasciola hepatica*, and *Petasiger phalacrocorasis* obtained from the nucleotide sequence databases of NCBI These results also showed that the query-ITS1 *F buski* forward sequence is more similar to the sequence of the species *F gigantica*, *Fasciola* sp , *F hepatica*, *E paraenei*, *E revolutum*, *E trivolvis*, and *Echinostoma caproni*

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F buski* and available ITS (I and II) sequences for other digenean trematodes including fasciolid species Phylogenetic analyses using the various distance methods and character state method like maximum parsimony show that the topology is similar among the trees obtained (Figs 5, 6, 7, and 8) Boot strapping of the sequences with neighbor-joining revealed significant support (100%) for the clade containing *F buski*, *F hepatica*, *F gigantica*, and *F magna* The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids

Discussion

Morphological differences found in adult specimens have been widely used to discriminate between platyhelminth

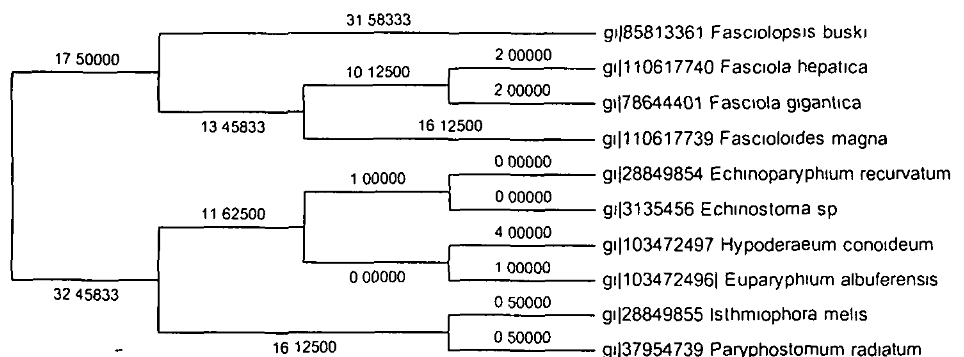


Fig. 6 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS2 data by maximum parsimony method

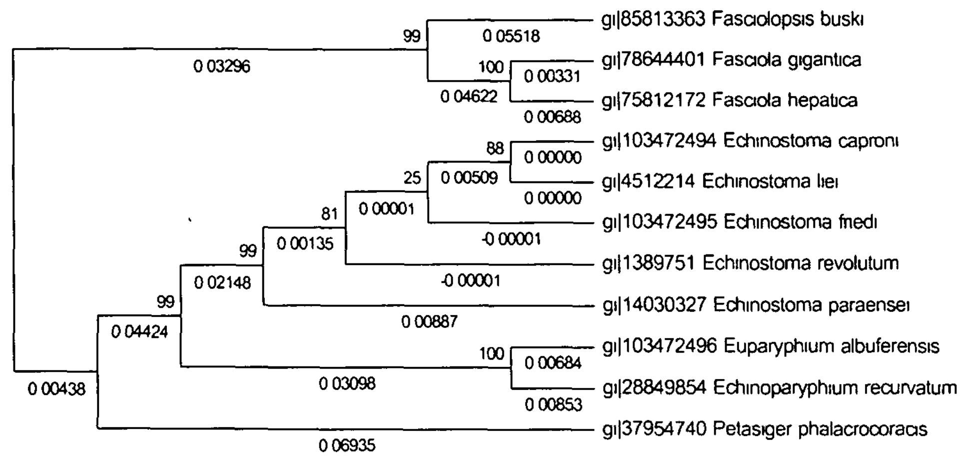


Fig. 7 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS1 data by neighbor-joining method

species (Miyazaki 1974). However, traditional diagnostic techniques in parasitology are now complemented by a variety of molecular tools to help in resolving the taxonomic issues associated with describing new species or strains on the basis of phenotypic characteristics (Thompson et al. 2004). Lack of data on genotypic diversity of fasciolid flukes in India makes it difficult to perform fine-scale phylogeographic analysis of populations and does not allow the origin of regional populations to be umambiguously determined. The taxonomy of *F. buski* has been based mainly on morphological data complemented with ecological, cytological, and pathological results as well as clinical manifestations. PCR-based techniques utilizing the ITS sequences have proven to be a reliable tool to identify the various lung fluke species and their phylogenetic relationships (Blair et al. 1999, Iwagami et al. 2000). In respect of *F. buski*, the 18S rRNA sequence is known (Blair and Barker 1993, accession number L06668) but the sequences from ITS markers are not described so far. In search for molecular markers for this species, we characterized the ITS region of rDNA.

The sequences showed close resemblance with the members of families Fasciolidae and Echinostomatidae (Echinostomatinae). From the phylogenetic trees constructed, sequences of two distinct clusters are revealed, one of *Fasciola* spp. and the other comprising the genera *Echinostoma*, *Paryphostomum*, and *Isthmiophora*. Our results showed that the bootstrap values is almost 100% among the trees obtained, and the ITS sequences of *F. buski* resemble other members of Fasciolidae. In closely related fasciolids, *F. hepatica* and *F. gigantica*, intraspecific nucleotide sequence divergence for ITS2 among the isolates from Mexico was found to be negligible or nil (Adlard et al. 1993). Likewise, no variation was observed between most of the populations of liver fluke species from different regions in East Europe, Central Asia, and Caucasus, while only one specimen of *F. hepatica* from Armenia showed 0.3% variation, three out of 11 populations of *F. gigantica* differed at four nucleotide transitions only (Semyenova et al. 2005).

The evolutionary distance between organisms is indicated by horizontal branch lengths, which reflect the number of nucleotide substitutions per site along the branch from the node to the end points. Compared to ITS1, the ITS2 sequences in our study showed a higher bootstrap value of 100% confirming that it is a highly conserved monophyletic

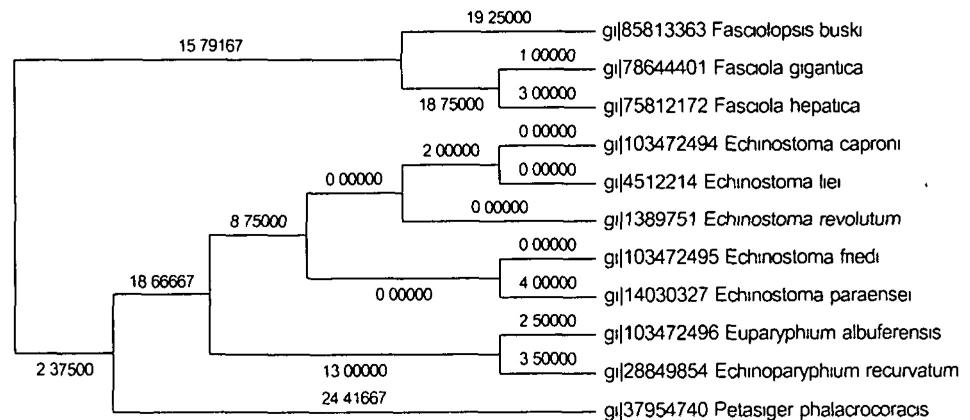


Fig. 8 Phylogenetic tree depicting relationship among *Fasciolopsis buski* and other fasciolid and echinostomatid species as inferred from ITS1 data by maximum parsimony method

group This is in accordance with other studies on trematodes, which indicate that sequences in the ITS1 might be less conserved then those in the ITS2 region (Luton et al 1992)

The sequences of the PCR products from adult and egg stages of the fluke in the present study were found to be identical in length and composition and showed no genetic variability in the worms collected from pigs of the Assam region These findings indicate that the different life cycle stages do not alter the applicability of the method and corroborate that the ITS sequences are not stage specific and are conserved through different stages of the development of the fluke (Sugiyama et al 2002)

In conclusion, as has already been demonstrated for other parasitic helminths, ITS can serve as an effective genetic marker for molecular identification However, to ascertain intra-specific strain variations, if any, and to determine the population structure, different geographical isolates of *F buski* from the region need to be studied with the use of additional molecular markers

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ORIGINAL PAPER

Surface fine topography and PCR-based determination of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, Northeast India

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Abstract In several mountainous regions of Northeastern India, foci of *Paragonimus* infection reportedly involving species that are known to prevail in China have been identified. The present study was undertaken to demonstrate the surface fine topography and sequence analysis of the ribosomal deoxyribonucleic acid (rDNA; second internal transcribed spacer, ITS2) of the metacercarial stages of the lung fluke collected from a mountain stream of the area (Miao, Changlang District in Arunachal Pradesh). The encysted metacercariae were oval in shape and had a smooth surface. The newly excysted metacercaria had a ventral sucker larger than the oral; the body surface was covered with numerous single-pointed and thorn-like tegumentary spines, of which those on the anterior part of the body were bigger in size and showed a gradual reduction in length and number towards the posterior end; dome-shaped papillae in variable numbers were seen around the rim of the oral sucker and were sparsely distributed all over the body surface. The polymerase chain reaction-amplified rDNA ITS2 sequences of the metacercariae were aligned with known sequences for the various species of *Paragonimus*, and the expectation value was found to be most significant with *P. westermani*, revealing an absolute match. The surface topography including the number and distribution of papillae and spination patterns and the ITS2 sequences of the metacercariae strongly suggest that the *Paragonimus* species, prevalent in the region of India, is in fact *P. westermani*.

The nucleotide sequence data reported in this paper have been submitted to the Genbank data with the accession number DQ351845.

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Introduction

The lung flukes of the genus *Paragonimus* have been known as one of the most important zoonotic parasites causing paragonimiasis, also known as endemic haemoptysis, in man. It is estimated that more than 20 million people are infected worldwide because of several species of *Paragonimus* (review, Toscano et al. 1995). More than 40 species are known to infect the lung of different mammalian hosts throughout the world (Bunnag and Harinasuta 1985), and approximately 15 species are known to infect humans. The parasite can migrate to several other vital tissues including the brain (Kusner and King 1993). The best-known species is *P. westermani* (Kerbert 1878) Braun 1899—a human parasite that can undergo development in as many as 16 different snail species and 50 crustacean species. Beside *P. westermani*, *P. pulmonalis* (Baelz 1880) Miyazaki 1978; *P. ohirai* Miyazaki 1939; *P. iloktsunensis* Chen 1940; *P. skrjabini* Chen 1959; *P. miyazaki* Kamo et al. 1961 and *P. heterotremus* Chen and Hsia 1964, all reported to be occurring in Asia, *P. africanus* and *P. uterobilateralis* Voelker and Vogel 1965 in Africa and *P. mexicanus* Miyazaki and Ishii 1968 in America are considered pathogenic to man. While *P. westermani* is distributed mostly in Asia, *P. heterotremus* is the predominant causative agent of paragonimiasis in Thailand (Blair et al. 1999a, b).

In the context of India, Chandler and Read (1961) indicated Bengal, Assam and some other parts of the country as endemic foci of human paragonimiasis In recent years, this infection has been reported in a sizeable human population of Manipur, a northeastern state of the country (Razaque et al 1991, Singh et al 1993) Although the fluke is known to parasitize a wide range of mammalian hosts representing as many as 11 families, the status of its prevalence and host range in India is not well documented Very recently, in Manipur and Arunachal Pradesh (Northeast India), the suspected foci of human infection where consumption of crustacean intermediate hosts is of regular practice, the Chinese species, *P hueitungensis* and *P heterotremus*, respectively, were identified as etiological agents of paragonimiasis (Singh 2002, Narain et al 2003) However, no or scanty information is available about the prevalence of the parasite among its molluscan and crustacean intermediate hosts as even in the suspected foci of human infection

Morphology of the encysted and excysted metacercariae, which occur as the infective stage in the muscle tissue of the crustacean second intermediate host, has been conventionally used in the identification of the species of *Paragonimus* The external appearance of the newly excysted metacercariae has also been studied for various species of the genus *Paragonimus* using scanning electron microscopy (Higo and Ishii 1984, 1987, Tongu et al 1985, 1987, 1995, Sugiyama et al 1990)

The identification of closely related species based on morphological characters can be difficult This is particularly the case of soft-bodied animals such as digenean trematodes However, recent advances in molecular biology, in particular the amplification of specific deoxyribonucleic acid (DNA) regions via the polymerase chain reaction (PCR) and improved sequencing techniques, have been employed to resolve taxonomic issues related to various helminth parasites by comparing their DNA The ribosomal DNA cluster (rDNA), which codes for structural components of ribosomes, is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon 1991) PCR-based techniques utilizing the rDNA second internal transcribed spacer (ITS2) sequences, which occur between the 5 8S and 28S coding regions, have proven to be a reliable tool to identify the helminth species and their phylogenetic relationships (Morgan and Blair 1995, Blair et al 1999a,b, Leon-Regagnon et al 1999, Iwagami et al 2000, Tkach et al 2000, Kostadinova et al 2003, Scholz et al 2004) The nuclear ribosomal DNA ITS2 sequences, which occur between the 5 8S and 28S coding regions, have proven useful for diagnostic purposes at the level of species *Fasciola* spp and isolates of *Fascioloides magna* from different geographical regions were discriminated on the basis of ITS sequences (Adlard et al 1993) Studies on phylogeny and/or intra-specific variation in *Paragonimus* species have also been done using ITS2 region in recent years (Blair et al 1996, 1997), and the usefulness of the method for species discrimination has also been demonstrated in nematodes (Campbell et al 1994, Hoste et al 1995, Samson-Himmelstjerna et al 1997)

During an exploratory survey of edible crab species, undertaken to ascertain the prevalence of crustacea-borne trematodiasis in the region, stream crabs from Miao, Changlang District, of Arunachal Pradesh were found to be heavily infected with metacercariae of *Paragonimus* species The present study was aimed at identifying the *Paragonimus* species implicated in infection in the region using surface fine topography of the metacercariae and molecular markers as the identifying tools

Materials and methods

Parasite material

Naturally infected freshwater edible crabs (*Barytelphusa lugubris*) were collected from a mountain stream of the suspected focal area Miao, Changlang District, in Arunachal Pradesh (altitude=213 m above sea level, longitude= 96°15′N and latitude=27°30′E) Metacercariae were isolated from the muscles of the crustacean host by digestion technique The crabs were cut into small pieces with the help of scissors, minced and digested by overnight incubation at 37°C in the artificial gastric juice The digested materials were filtered through mesh wire sieves, and the filterable sediments were washed repeatedly with tap water to get a clearer supernatant The sediments were examined for *Paragonimus* metacercariae under a dissecting stereoscopic microscope A few specimens were duly processed for whole-mount preparation and subsequent light microscopy observations

Scanning electron microscopy

The isolated metacercariae were fixed in 10% neutral-buffered formalin at 4°C for 24 h, washed in phosphate-buffered saline and dehydrated with ascending grades of acetone to pure dried acetone The specimens were then treated with Tetra methyl Silane (boiling point 23 3°C, surface tension 10 2 dynes/cm at 20°C) following Roy and Tandon (1991) The dried specimens mounted on brass stubs were coated with a thin (300 Å) layer of gold vapour The gold-coated specimens were observed using LEO 435 VP scanning electron microscope at electron-accelerating voltages ranging between 10 and 20 kV

DNA isolation

The 70% alcohol-fixed metacercariae were further processed for DNA extraction and PCR amplification. For the purpose of extraction, metacercariae recovered from one single host were pooled together, DNA was extracted from metacercariae in the FTA card by using Whatman's FTA Purification Reagent and amplified by PCR. After applying the tissue homogenate on FTA cards, the latter were allowed to dry for 1 h at room temperature before punching. Two to three sample discs of 1.2 mm size were taken from the desired spot using a coring device assuming a 25-μl reaction volume and placed in a PCR amplification tube. The discs were then washed with FTA purification reagent and Tris–ethylenediamine tetraacetic acid (EDTA) buffer and allowed to dry at room temperature for 1 h to make them ready for PCR.

DNA amplification and sequencing

The rDNA region spanning the ITS2 was amplified from metacercarial DNA by PCR. As primers, we used the following:

1. 3S (forward): 5′-GGTACCGGTGGATCACTCGGCT CGTG-3′
2. A28 (reverse): 5′-GGGATCCTGGTTAGTTTCTTTT CCTCCGC-3′

which were designed based on the conserved sequences of the 5.8S and 28S genes of *Schistosoma* species (Bowles et al. 1995). The PCR amplification was performed following the standard protocol (White 1993) with minor modifications in 100 mM Tris HCl (pH 9.0), 500 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM deoxynucleotide triphosphates each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate, 0.25 mM of each primer and 2.5 U of *Taq* polymerase (Bangalore Genei, India). DNA was preheated at 94°C for 5 min and added to each PCR reaction. The PCR cocktail (final reaction volume, 25 μl) was amplified with the following conditions: 26 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 38 s and extention at 72°C for 42 s followed by a final extention at 72°C for 10 min. The resultant PCR products were separated by electrophoresis through 1.6% (w/v) agarose gels in Tris–acetate–EDTA buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed. The known size fragments of Phi X 174 DNA/*Hae*III Digest in agarose gel were used as marker.

For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit and sequenced in both directions using PCR primers A28 and 3S on an automated sequencer.

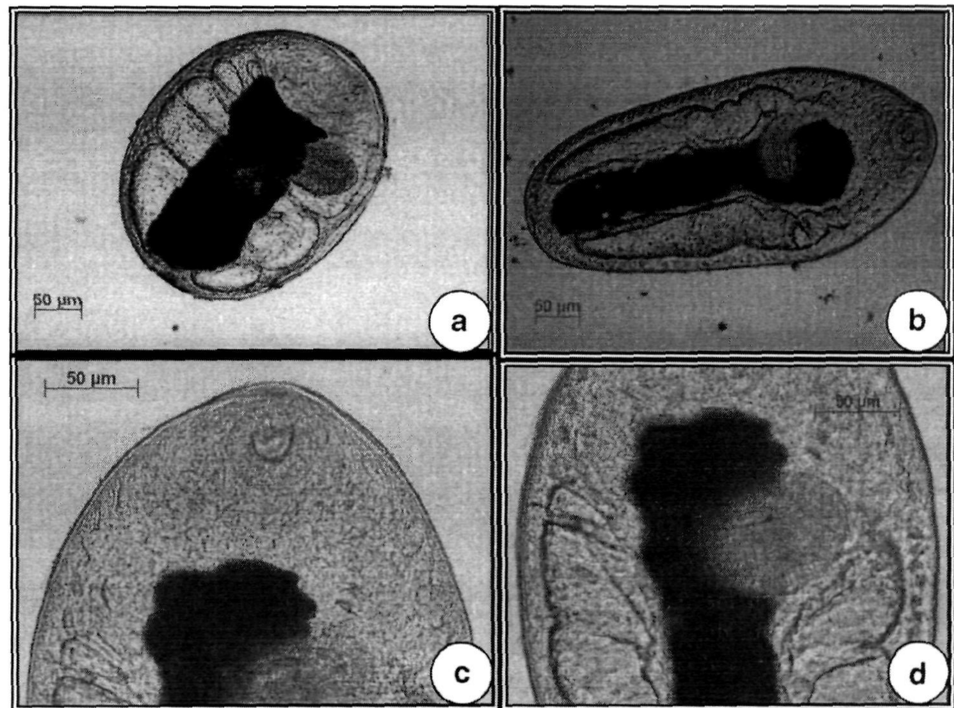

Fig. 1 Whole mounts, stained preparations, LM view. **a** Encysted metacercaria (10×). **b** Excysted metacercaria (10×). **c–d** Oral and ventral sucker, respectively, of excysted metacercaria (20×)

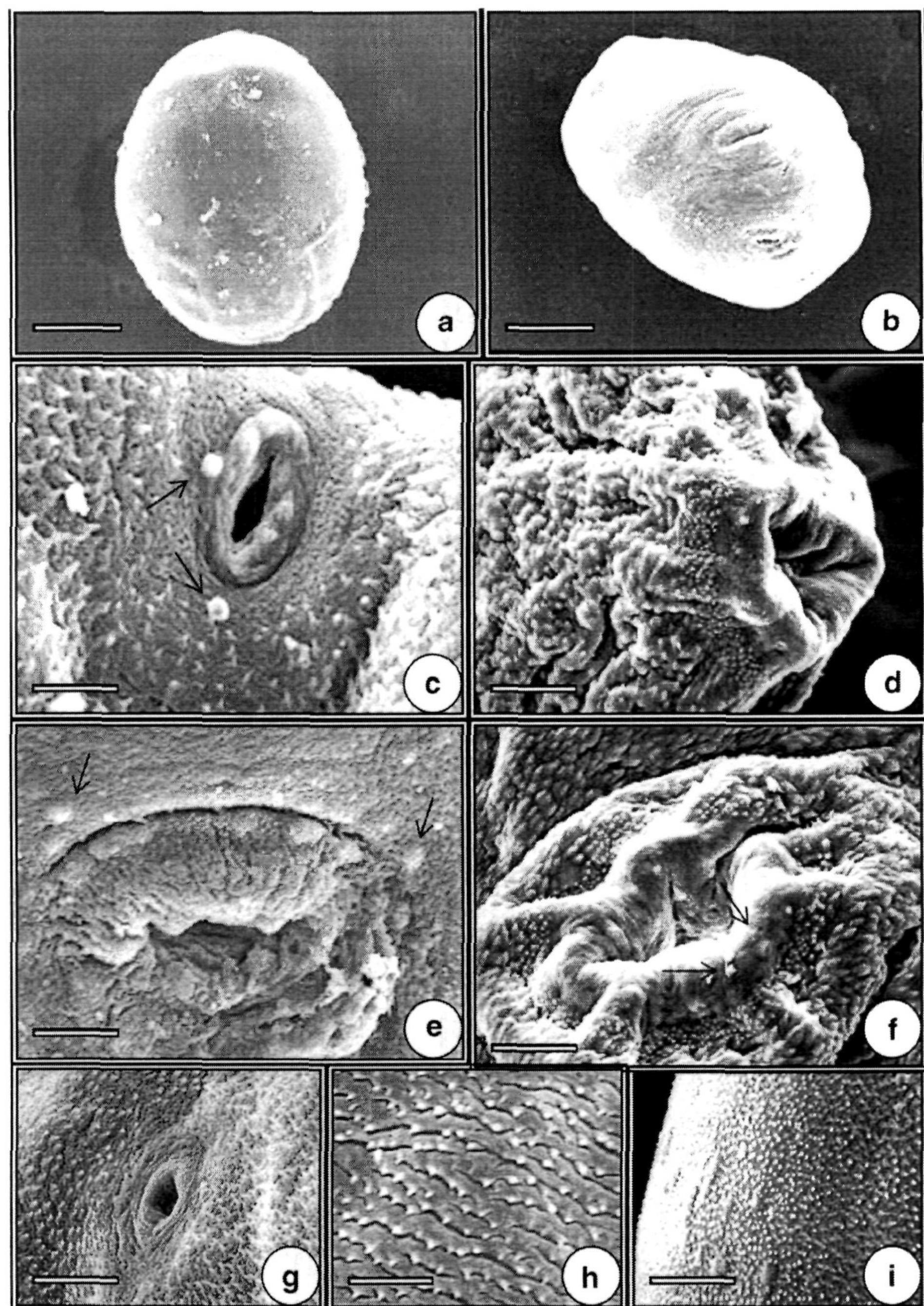

Fig. 2 SEM views. **a, b** Encysted and excysted metacercaria (scale bar=100 μm). **c–i** Scale bar= 10 μm **c** Oral sucker region. Note the presence of a few domed papillae on and around the rim of the sucker. Frontal view. **d** Another view of the oral end, depicting spination pattern on circum oral tegument. Several rows of minute spines arranged in circular fashion are conspicuous. **e** Ventral sucker region. A few randomly distributed papillate protuberances are present. **f** Ventral sucker region in another specimen. **g** Spination in anterior region of body. **h** Single-pointed, backwardly directed spines in the mid-body region. **i** Smaller spines in the posterior part of the body

Molecular phylogenetic analysis using bioinfomatic tools

The DNA sequences were put to further analysis with the usage of bioinfomatics tools including a similarity search using Basic Local Alignment Search Tool (BLAST) provided at http://www.ncbi.nlm.nih.gov/blast and phylogentic prediction using ClustalW provided at http://www.ebi.ac.uk/clustalw for DNA sequence query. Phylogenetic tree-building methods presume particular evolutionary models. Therefore, while interpreting the results obtained, different tree building models were considered to entertain possible explanations. Only unique sequences were used in tree construction. ITS sequences arranged with the MEGA format were entered in the MEGA for construction of the phylogenetic trees that were inferred using distance methods like neighbor joining, minimum evolution, un-

weighted pair group method with arithmetic mean and character state method like maximum parsimony Test of phylogenetic accuracy was done by bootstrap for the neighbor-joining and minimum evolution trees

Results

Morphology

The newly excysted *metacercaria has an elongate body* (815 91 × 492 79 μm) in size, the ventral sucker situated somewhat pre-equitorally, is larger than the oral, the intestinal caeca are long and extend up to the posterior end of the body, the conspicuous excretory bladder extends medially in the intercaecal space (Fig 1a–d) The scanning electron microscopy observations revealed that the encysted metacercaria is oval in shape and has a smooth surface The whole body surface of the excysted metacercaria is covered with numerous single-pointed and thorn-like tegumentary spines, those covering the anterior part of the body are bigger in size and show a gradual reduction in size towards the posterior end The tegument in the circum-oral region has a dense aggregation of small spines that are arranged in several circular rows A few dome-shaped papillae abound *on the rim of the oral sucker and the adjacent area in a random fashion* The tegument of the ventral sucker and its surrounding region and that of the general body surface also has a dense spination The surface fine topography in the area reveals the presence of many papillate protuberances, but the latter are randomly distributed and do not exhibit a definite pattern of distribution and numbers (Fig 2a–i)

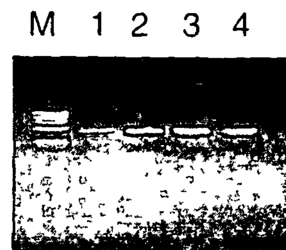

Fig. 3 PCR Product of *Paragonimus* metacercariae in FTA card using primer set 3S-A28

PCR amplification of ITS region and its analysis

The PCR amplified products of ITS2 of rDNA were successfully obtained using the primers as mentioned above (Fig 3) The nucleotide sequences obtained were compared with other sequences of trematode species from Genbank The fragments of amplified DNA were estimated to be

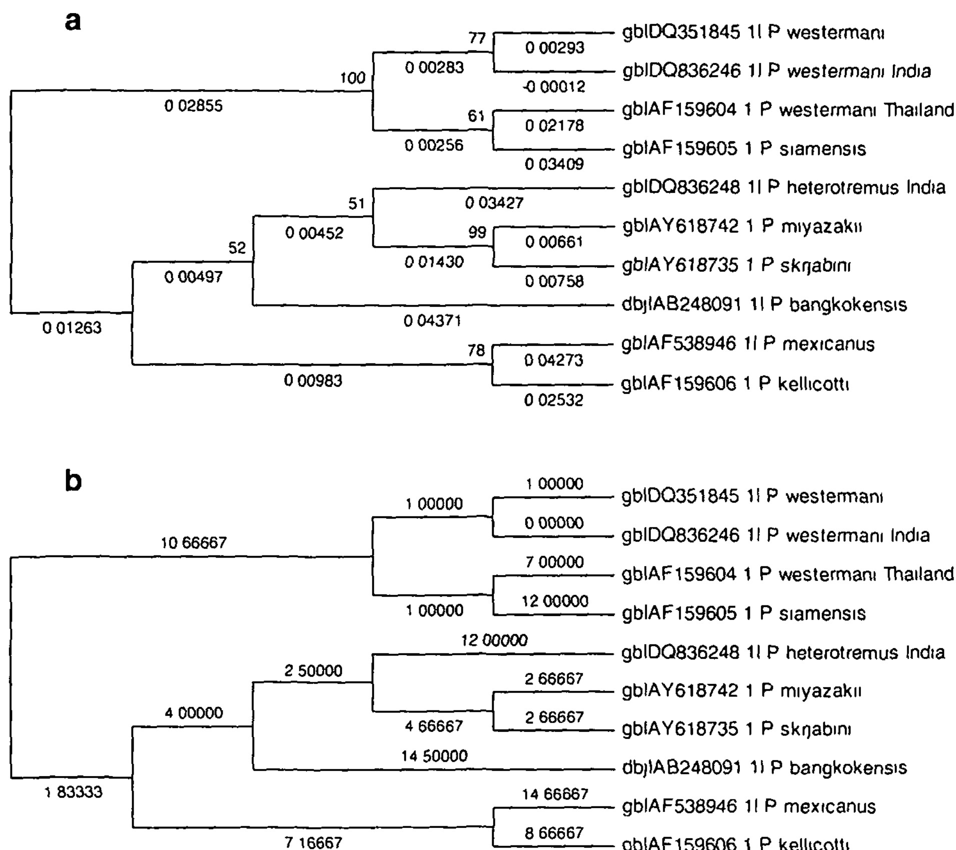

Fig. 4 a, b Phylogenetic trees depicting relationship among *Paragonimus* spp from different geographical isolates inferred from ITS2 data Distance values are shown in all the different trees obtained and bootstrap values are shown for Neighbor Joining tree

~500 bp long. Sequence analysis of the ITS2 PCR products revealed that the alignments of the rDNA region spanning the ITS2 were 496 bp for the forward primer and 494 bp for the reverse primer, respectively. No intra-specific variations in length or composition of the sequence were observed, and all the ITS2 sequences of the metacercariae were found to be identical in all the samples.

The BLAST hit results show that the query ITS2 *Paragonimus* metacercariae forward and reverse sequences are closer and more similar to the sequences of the species *P. westermani*, *P. mexicanus*, *P. siamensis*, *P. miyazakii* and *Euparagonimus cenocopiosus* with maximum similarity being with *P. westermani*.

Construction of phylogenetic trees

Phylogenetic trees were obtained by comparing the ITS2 sequences of *Paragonimus* species from different geographical isolates. Phylogenetic analyses using the various distance methods and character state method like maximum parsimony show that the topology is similar among the trees obtained (Fig. 4a,b). The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping.

Discussion

Surface fine topography of encysted and newly excysted metacercariae has been described in respect of several species of *Paragonimus*, e.g. *P. skrjabini*, *P. iloktsuensis*, *P. ohirai*, *P. pulmonalis*, *P. westermani* (diploid type), *P. miyazaki* (triploid types of *P. westermani*), *P. mexicanus*, *P. heterotremus* and *P. westermani* (Miyazaki 1974; He et al. 1982; Li et al. 1987; Higo and Ishii 1987; Aji et al. 1984; Tongu et al. 1987; Sugiyama et al. 1990; Jiang and Xia 1993; Sugiyama et al. 2001). Characters such as the number and distribution pattern of tegumental papillae around the oral and ventral suckers of the newly excysted metacercariae have been used as the morphological taxonomic tools for differentiating the various species of *Paragonimus*. For example, the number and size of the domed papillae in metacercariae of *Paragonimus* spp. in Japan seems to vary with the species (Higo and Ishii 1984, 1987), although geographical differences do not supposedly exist with regards to the morphology of the excysted metacercariae of *P. westermani*, in which the number of papillae ranges between 5 and 13 (Sugiyama et al. 2001). However, these morphological characters are prone to variations and thus not absolutely reliable.

The metacercariae under the present study revealed a ventral sucker larger than the oral unlike *P. heterotremus* (in which the oral sucker is larger than the ventral), a species that has earlier been reported from the same region in Northeast (Narain et al. 2003). The surface fine topography, including the number and distribution of papillae and spination pattern of our material, suggests more closeness and resemblance with *P. westermani*. In the latter species, although Sugiyama et al. (2001) reported the occurrence of dome-shaped papillae as evenly distributed over the whole body and in circular fashion around the suckers, as per our observations, the papillae were fewer in number and revealed to be randomly scattered across the general body surface.

In the sequence analysis of the rDNA ITS2 comparing with the known sequences of the other lung fluke species, the present study revealed that the sequence of ITS2 (plus flanking regions) show close resemblance with the sequences of *P. westermani*, both of metacercarial and adult origins. The results corroborate that the ITS2 sequences are not stage specific and are conserved through different stages of the development of the fluke (Sugiyama et al. 2002). In phylogenetic analysis, as a general rule, if the bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, then the topology at that branch is considered 'correct.' Our results showed a bootstrap value to be greater than 70% among the trees obtained, and the ITS2 sequence resembled *P. westermani*. Thus, on the basis of surface fine topography features and supplemented by absolute matching of ITS2 sequence that could be used as one of the species markers, it can be concluded that *Paragonimus* species prevailing in Miao region of Arunachal Pradesh is indeed *P. westermani* and not *P. heterotremus* as reported by earlier workers.

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ORIGINAL PAPER

Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions

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Abstract The species of liver flukes of the genus *Fasciola* (Platyhelminthes: Digenea: Fasciolidae) are obligate parasitic trematodes residing in the large biliary ducts of herbivorous mammals. While *Fasciola hepatica* has a cosmopolitan distribution, the other major species, i.e., *Fasciola gigantica* is reportedly prevalent in the tropical and subtropical regions of Africa and Asia. To determine the phylogenic location of *Fasciola* sp. of Assam (India) origin based on rDNA molecular data, ribosomal ITS regions were sequenced and compared with other species of trematodes in the family Fasciolidae. NCBI databases were used for sequence homology analysis using BLAST and ClustalW programs. The phylogenetic trees constructed based upon the ITS (1 and 2) sequences revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia, the isolate from China with significant bootstrap values being the closest. Using the novel approach of molecular morphometrics that is based on ITS2 secondary structure homologies, phylogenetic relationships of the various isolates of fasciolid species have also been discussed. While comparing ITS1, the sequence of another Indian isolate designated as *F. gigantica* (accession no. EF198867) showed almost absolute match with *F. hepatica*. Hence, this particular isolate should be identified as *F. hepatica* and not *F. gigantica*.

The nucleotide sequence data reported in this paper have been submitted to the GenBank data with the accession numbers EF027103 and EF027104.

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Introduction

The trematode flukes of the genus *Fasciola* (the sheep liver fluke) are parasites of herbivores and infect humans accidentally causing fascioliasis worldwide. The parasite is very cosmopolitan in distribution being found throughout all regions of the world, both temperate and tropical. *Fasciola hepatica* is the causative agent of fascioliasis or 'liver rot' in ruminants where it may be an important pathogen. Human infections with *F. hepatica* are found in areas where sheep and cattle are raised and where humans consume raw watercress, including Europe, the Middle East, and Asia (Mas-Coma et al. 1999). Infection with *Fasciola gigantica*, on the other hand, is found more commonly in tropical regions of the world, areas affected including Africa, Asia, many Pacific islands including Hawaii (where it has been reported in man), the Middle East, Southern Europe, and the south of the USA (Hammond 1974). Both fasciolids follow a similar two-host life cycle in which freshwater snails of the family Lymnaeidae act as intermediate or vector host and a broad spectrum of mammals including humans, mainly herbivorous large-sized species, act as definitive host (Mas-Coma et al. 2005).

Identification of closely related species based on morphological characters alone can be difficult. The rDNA,

which codes for structural components of ribosomes, is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon 1991). The nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2), which occur between the 18S, 5.8S, and 28S coding regions, have proven useful for diagnostic purposes at the level of species (Morgan and Blair 1995, Leon-Regagnon et al. 1999, Tkach et al. 2000, Kostadinova et al. 2003, Scholz et al. 2004, Prasad et al. 2007 and Tandon et al. 2007). *Fasciola* spp. and isolates of *Fascioloides magna*, another member of the same family, from different geographical regions have been discriminated on the basis of ITS sequences (Adlard et al. 1993). The ITS2 sequences have also been used to characterize the liver flukes from mainland China, which include *F. hepatica*, *F. gigantica*, and an intermediate genotype, including polymorphism among ITS2 copies within the same fluke individual (Huang et al. 2004). ITS1, however, has been used for only relatively few numbers of species, though the ITS1 studies confirm the results and conclusions previously reached with ITS2 (Mas-Coma et al. 2001).

Most phylogenetic studies using current methods have focused on primary DNA sequence information. However, RNA secondary structures are particularly useful in systematics because they include characteristics not found in the primary sequence that give 'morphological' information (Caetano-Anolles 2002). The novel approach of molecular morphometrics that relies both on traditional morphological comparison and on molecular sequence comparison by measuring the structural parameters of the ITS2 secondary structure homologies (geometrical features, bond energies, base composition, etc.) is recently being used to study the phylogenetic relationships of various species (Billoud et al. 2000). This method allows one to take into account the regions where multiple alignments are barely reliable because of a large number of insertions and deletions. This method is based on the assumption that secondary structure can be phylogenetically as significant as primary sequence. It is well-known that rRNA is highly conserved throughout evolution. Thus, the secondary structure elements of the RNA molecule, i.e., the helices, loops, bulges, and separating single-stranded portions, can be considered phylogenetic characters (Zwieb et al. 1981, Schultz et al. 2005, Grajales et al. 2007).

In the present study, our main objective was to describe the molecular identification of the Indian liver fluke (the Assam isolates) on the basis of ITS sequences of the parasite collected from ruminant hosts. We also aimed at determining the species prevalent in the northeastern region of the country by comparing these sequences by primary sequence analysis and molecular morphometrics data.

Materials and methods

Parasite material and DNA isolation

Adult *Fasciola* were obtained in live form from hepatic biliary ducts of freshly slaughtered cow, *Bos indicus*. The worms recovered from these hosts represented the geographical isolates from Assam, Northeast India and morphologically resembled *F. gigantica* (deposition number of paratypes at Zoological Survey of India, Kolkata=W7787/1). Eggs were obtained from mature adult flukes by squeezing between two glass slides. For the purpose of DNA extraction, adult flukes were processed singly, eggs recovered from each of these specimens were also processed separately. The adult flukes were first immersed in digestion extraction buffer (containing 1% SDS, 25 mg Proteinase K) at 37°C overnight. DNA was then extracted from lysed individual worms by standard ethanol precipitation technique (Sambrooke et al. 1989) and also extracted on FTA cards using Whatman's FTA Purification Reagent as described elsewhere (Prasad et al. 2007). DNA from the eggs was extracted only with the FTA card technique.

DNA amplification, sequencing, and its analysis

The rDNA region spanning the ITS regions was amplified from DNA obtained from the fluke by polymerase chain reaction (PCR). We used the universal primers considered to be the general primers for trematodes and are designed based on conserved ITS sequences of *Schistosoma* species following Bowles et al. (1995) as detailed below:

1. ITS2 region—3S (forward) 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′ and A28 (reverse) 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′
2. ITS1 region—BD1 (forward) 5′-GTCGTAACAAGGTTTCCGTA-3′ and 4S (reverse) 5′-TCTAGATGCGTTCGAA (G/A) TGTCGATG-3′

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications as described elsewhere (Prasad et al. 2007, Tandon et al. 2007). The resultant PCR products were separated by electrophoresis through 1.6% (*w/v*) agarose gels in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and then photographed. The known size fragments of Phi X 174 DNA/*Hae*III digest in agarose gel were used as marker. For DNA sequencing, the PCR products were purified using the Genei Quick PCR purification kit and sequenced in both directions using PCR primers on an automated sequencer by the DNA sequencing services of TCGA, New Delhi and Bangalore Genei.

The DNA sequences were put to further analysis by using various bioinformatics tools including similarity

search BLAST (http://www.ncbi.nlm.nih.gov/blast) and phylogenetic prediction by ClustalW (http://www.cbi.ac.uk/clustalw) for each query DNA sequence.

Molecular phylogenetic analysis

Initially, the sequences were aligned using ClustalW multiple alignment (Thompson et al. 1994) with the default gap and extension penalties used by this program. Phylogenetic tree-building methods presume particular evolutionary models. Therefore, while interpreting the results obtained, we considered different tree-building models to entertain possible explanations. Only unique sequences were used in tree construction. ITS sequences were entered in the MEGA for construction of the phylogenetic trees using maximum parsimony and distance methods, namely, neighbor-joining, UPGMA, and minimum evolution. Branch support was given using 1,000 bootstrap replicates in MEGA (Hillis and Bull 1993).

Phylogenetic analysis was also carried out using the Bayesian approach with combined datasets using Mrbayes 3.1 (Huelsenbeck and Ronquist 2001), wherein each data partition is allowed to have a different evolution rate.

Predicted ITS2 RNA secondary structures and analyses

Secondary structures of ITS2 sequences of various fasciolid species were reconstructed by aligning their sequences using BioEdit (Hall 1999). The acquired structures with restrictions and constrains were submitted in MFOLD (Zuker 2003). RNA was folded at a fixed temperature of 37°C, and the structure chosen from different output files was the desired six-helicoidal ring or the one with the highest negative free energy if various similar structures were obtained.

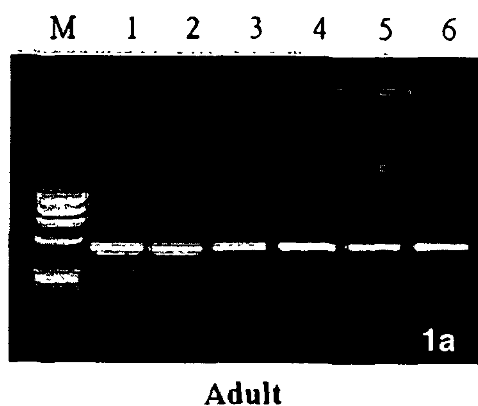

Fig. 1 PCR products of *Fasciola* sp. adult DNA using primer set 3S–A28 for ITS2, marker Phi X 174 DNA/*Hae*III digest

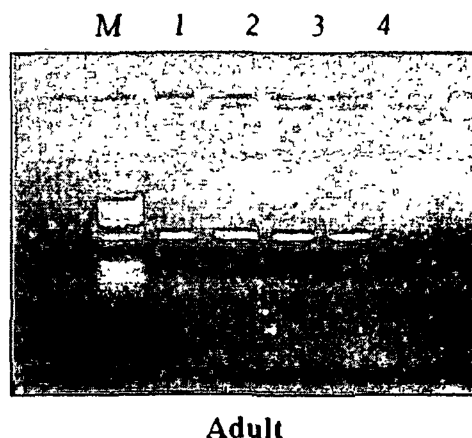

Fig. 2 PCR product of *Fasciola* sp. adult DNA using primer set BD1-4S for ITS1, marker Phi X 174 DNA/*Hae*III digest

Results

PCR amplification of ITS regions and its analysis

The PCR-amplified products were successfully obtained using the primers as mentioned above and are depicted in Figs. 1 and 2. The nucleotide sequences were obtained for ITS1 and ITS2 of rDNA of *F. gigantica* and were compared with sequences of other fasciolid species obtained from GenBank by the ClustalW tree-building method (Fig. 3). The fragments of amplified DNA were estimated to be 480–550 bp long. No intraspecific variations in length or composition of the sequence were observed, and the ITS sequences of both adult and egg origin were found to be identical in length as well as composition. For comparative purpose, the ITS2 sequences of fasciolids from

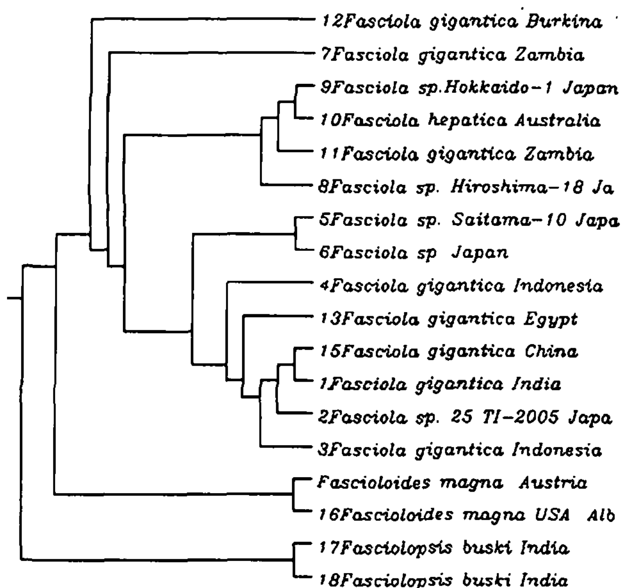

Fig. 3 ClustalW tree for ITS2 sequence

Table 1 Fasciolidae species and their various geographical isolates used in this study with the respective GenBank accession numbers for the corresponding ITS2 sequences

Species	GenBank accession no	Sequence length (bp)	Classification
Fasciola gigantica India (B)	EF027103	720	Digenea Fasciolidae
F sp 25 TI 2005 Japan	AB207152	505	Digenea Fasciolidae
F gigantica Indonesia (E)	AB207149	505	Digenea Fasciolidae
F gigantica Indonesia	AB010977	505	Digenea Fasciolidae
F sp Saitama 10 Japan	AB207151	505	Digenea Fasciolidae
F sp Japan	AB010979	505	Digenea Fasciolidae
F gigantica Zambia	AB010976	505	Digenea Fasciolidae
F sp Hiroshima 18 Japan	AB207153	506	Digenea Fasciolidae
F sp Hokkaido 1 Japan	AB207150	506	Digenea Fasciolidae
F hepatica Australia	AB207148	506	Digenea Fasciolidae
F gigantica Zambia (D)	AB010975	505	Digenea Fasciolidae
F gigantica Burkina Faso	AJ853848	950	Digenea Fasciolidae
F gigantica Egypt (A)	DQ383512	509	Digenea Fasciolidae
Fasciola gigantica China (C)	AJ557569	361	Digenea Fasciolidae
Fascioloides magna Austria	DQ683545	538	Digenea Fasciolidae
F magna USA Albany	EF051080	2920	Digenea Fasciolidae
Fasciolopsis buski India	DQ351842	498	Digenea Fasciolidae
F buski India	DQ351841	481	Digenea Fasciolidae

B sequence generated as part of the present study *A C D* and *E* sequences from different geographical isolates of *F gigantica* used for the construction of secondary structures

various geographical regions were obtained from GenBank (Table 1) The BLAST hit results show that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp, *F hepatica* and *F gigantica* besides *Fasciolopsis buski* and *F magna* (both belonging to the same family, i e, Fasciolidae)

Phylogenetic trees

Phylogenetic trees were obtained by comparing the sequences of *F gigantica* and available ITS (1 and 2) sequences for other fasciolid species (Figs 4 and 5) Phylogenetic analyses using the various distance methods and character method like maximum parsimony showed that the topology is similar among the trees obtained Bootstrapping of the sequences with neighbor-joining revealed significant support for the clade containing *F hepatica*, *F gigantica*, *F buski*, and *F magna* The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids The phylogenetic trees constructed based upon the ITS (1 and 2) sequences by multiple tree-building methods in MEGA revealed a close relationship with isolates of *F gigantica* from China, Indonesia, Japan, Egypt, and Zambia While comparing the ITS1 sequences through BLAST search (ClustalW alignment), the sequence of another Indian isolate designated as *F gigantica* (accession no EF198867) from

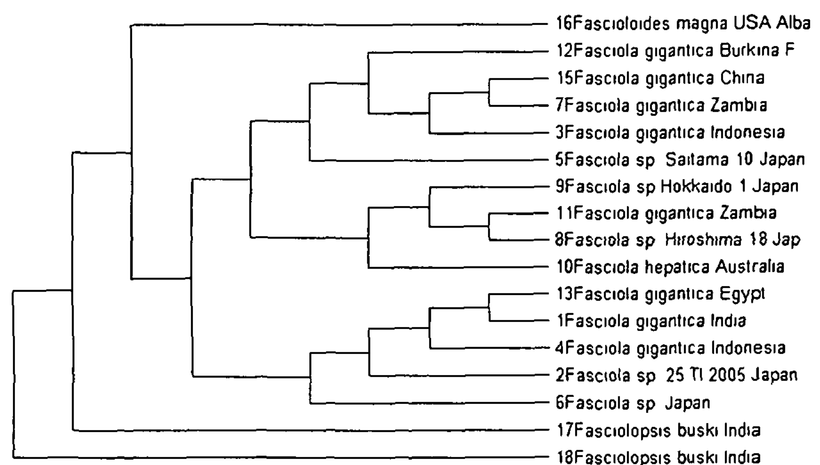

Fig. 4 Maximum parsimony tree of ITS2 sequence

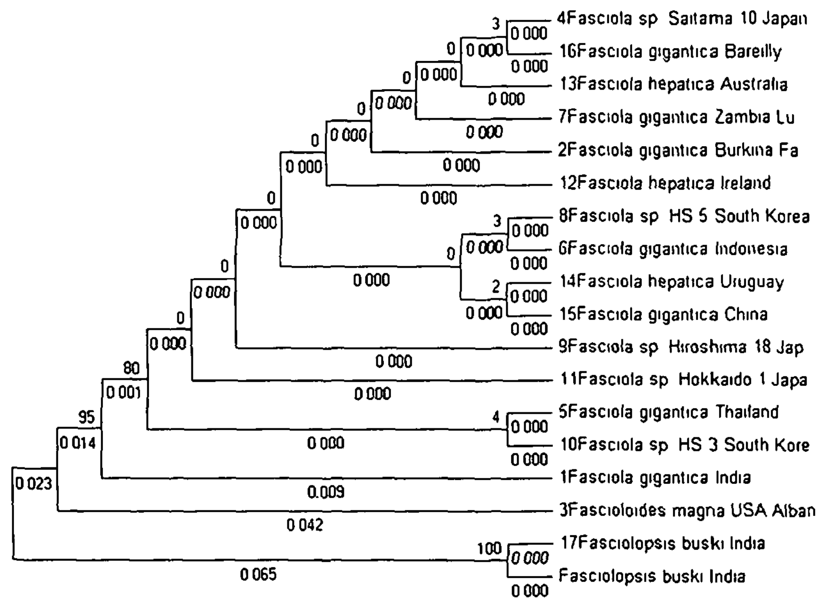

Fig. 5 Neighbor-joining tree of ITS1 sequence showing bootstrap values and distance

the Indian Veterinary Research Institute, Bareilly showed almost absolute match with *F. hepatica*.

Secondary structure analysis

Five predicted RNA secondary structures were reconstructed from the unique sequences with the highest negative free energy of *F. gigantica* to provide the basic information for phylogenetic analysis (Figs. 6, 7, 8, 9, and 10). The ITS2 plus flanking regions of the nuclear region ranged from 720 bp in *F. gigantica* India to a minimum length of 361 bp in *F. gigantica* China. The secondary structural features of

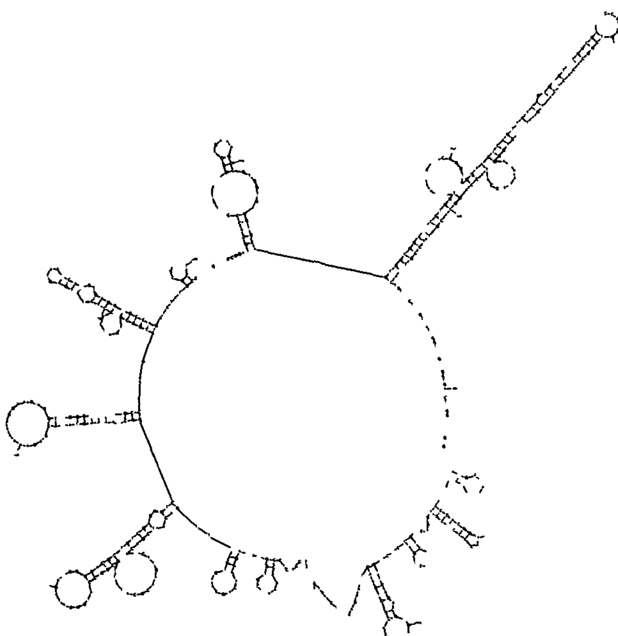

Fig. 6 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

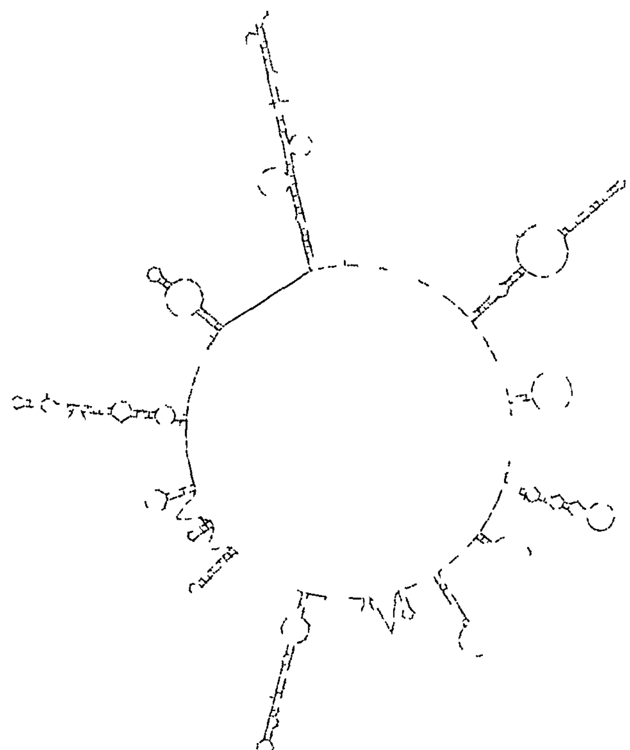

Fig. 7 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

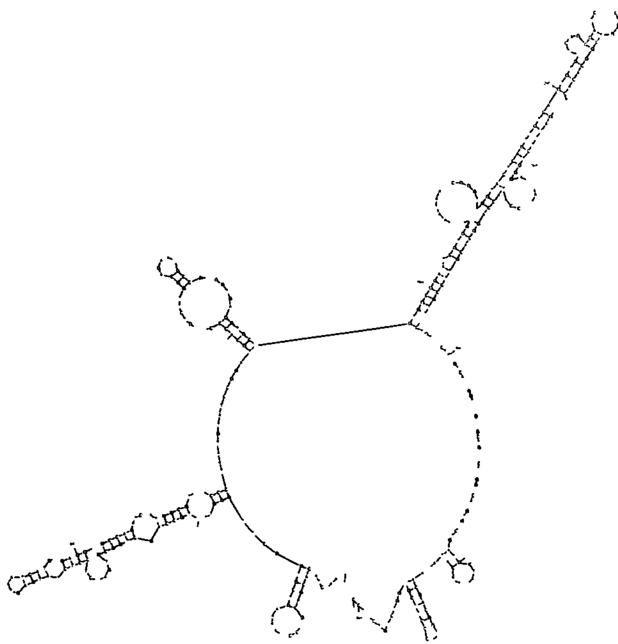

Fig. 8 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

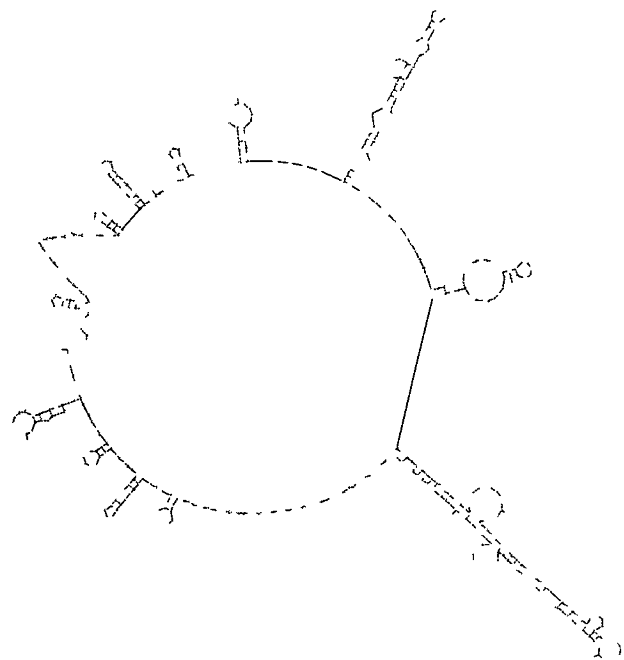

Fig 10 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

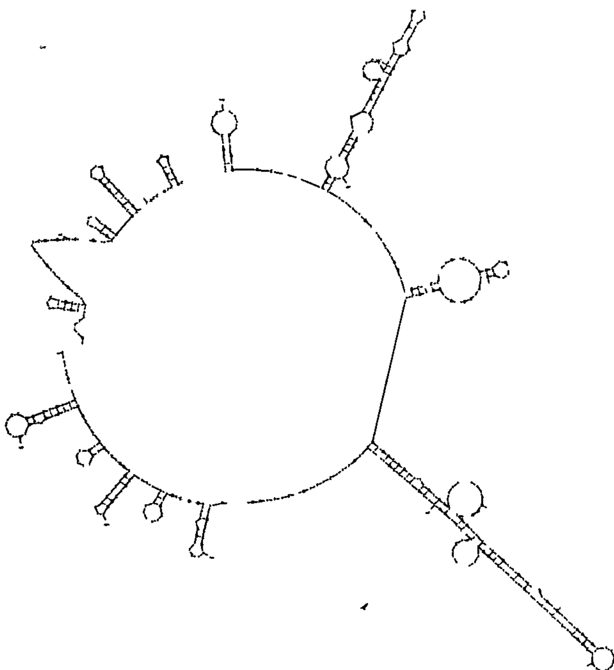

Fig. 9 Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

ITS2 regions as shown in the figures were analyzed based on conserved stems and loops, which, in order of preference, were interior loop, bulge loop, multiple branch loop, hairpin loop, and exterior loop in all the isolates *F gigantica* isolates from India and China show an overall similarity in ITS2 rRNA folding and have identical secondary structures, which, in the remaining three isolates, show some variant The observed similarities at the secondary structural level are further reflected at the energy level ($-\Delta G$) The difference in their topology, however, is due to the differences in nucleotide sequence lengths These secondary structure predictions indicate that the domains base pair to form a core region central to several stem features imply that conservedness is more important for proper rRNA folding pattern Moreover, the observed phylogenetic trend was identified with respect to the target accessibility sites for the five different isolates The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences (Fig 11) The ITS2 sequence of the Indian isolate revealed closest similarity with the isolate from China with significant bootstrap value

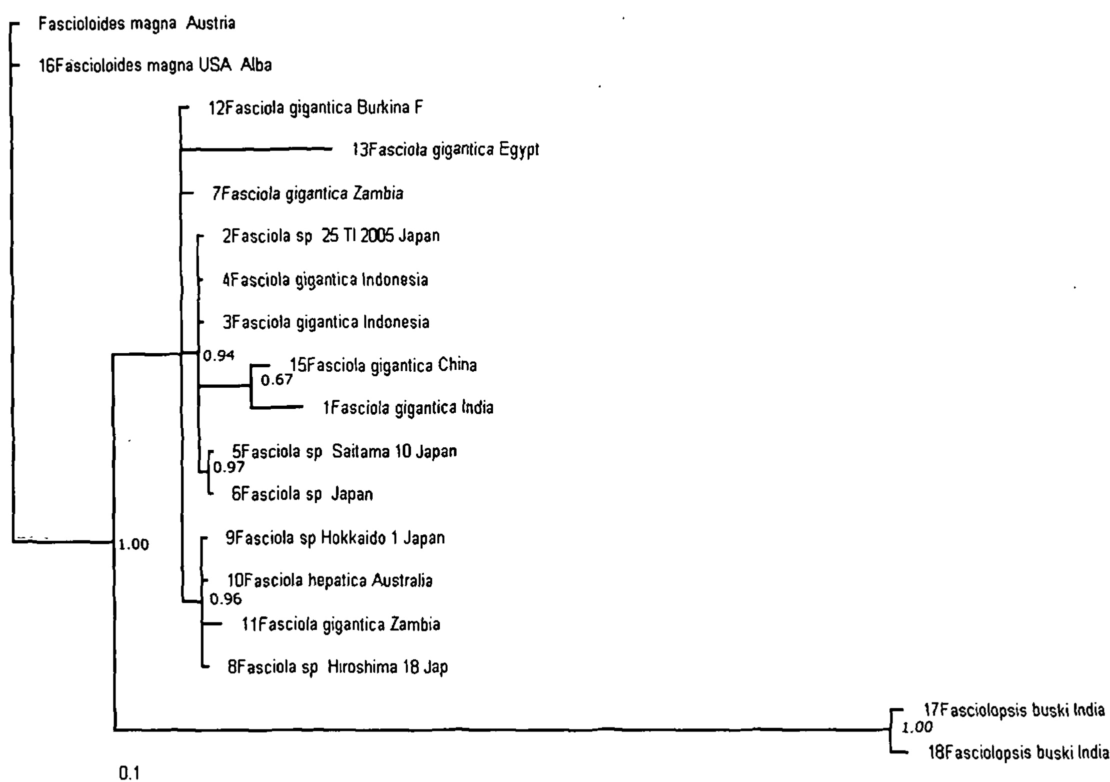

Fig. 11 Phylogenetic relationships between members of family Fasciolidae. This tree shows hypothetical Bayesian analysis phylogeny based on the secondary structure alignment data of the ITS2 region. The numbers are equivalent to bootstrap percentages

Discussion

The taxonomy of *Fasciola* spp. has been based mainly on morphological data complemented with ecological, cytological, and pathological results as well as clinical manifestations. Morphological differences found on stained and mounted adult specimens have been widely used to discriminate between platyhelminth species (Miyazaki 1974). It is possible to distinguish between adult *F. hepatica* and *F. gigantica* on the basis of morphology, but much variation exists. Differentiating between two species is not possible on the basis of clinical, pathological, or immunological findings and their eggs are morphologically very similar (Lotfy and Hillyer 2003). Consequently, where both species occur concurrently or in overlapping geographical distribution, it is not possible to be certain as to which species is responsible for the disease. The low number of records of infection with *F. gigantica* may well be due to the lack of good tools to distinguish this species from *F. hepatica* (Marcilla et al. 2002).

The comparison of ITS sequences from worms of different hosts and of different countries indicates that there exists a high species-specific homogeneity. In the present study, primary sequence analysis revealed a close relationship between the query sequence (from Northeastern region of India) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia (Adlard et al. 1993). The phylogenetic trees constructed showed that the groups of multiple closely related genotypes of *F. gigantica* from Asia are broadly sympatric. Such a pattern is expected for species with high gene flow whose populations have not been sundered by long-term biogeographic barriers (Avise 2000). The sequence of another Indian isolate, designated as *F. gigantica* (accession no. EF198867) from the Indian Veterinary Research Institute, Bareilly showed almost absolute match with *F. hepatica*. Hence, on the basis of molecular similarity, this particular isolate should be identified as *F. hepatica* and not *F. gigantica*.

In this study, we present a new approach of molecular morphometrics in which the measurable structural parameters of the molecules are directly used as specific characters to construct a phylogenetic tree. These structures are inferred from the sequence of the nucleotides, often using energy minimization (Zuker 1994). Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *F. gigantica*, which provided us with the additional information for the correct identification of the species prevalent in the region. Molecular morphometrics appears to be complimentary to classical primary sequence analysis in

phylogenetic studies as it takes into consideration only the size variations of homologous structural segments, and this choice implies that the overall architecture of the molecule remains the same among the observed taxa This method helps in taking into account the regions where multiple alignments are barely reliable because of large number of insertion/deletion operations In the present study, the secondary structure analysis of the same data also confirmed the results mentioned for primary sequence analysis Differences in their topology are only due to the fact that there are variable lengths of the sequences However, there are difficulties in defining a distance between two related structures with variable topologies (Shapiro 1998) Nevertheless, because there were inconsistencies in the placement of a few *Fasciola* species, this study needs to be extended in order to gain a better understanding of the systematics of this group as well as the evolution of their predicted ITS2 RNA secondary structures

The results also corroborate that the *Fasciola* species prevalent in Assam, Northeast India is in fact *F gigantica* and not *F hepatica*, which otherwise is the most common liver fluke throughout the globe Lack of data on genotypic diversity of *Fasciola* species in Africa and India does not allow the origin of regional populations to be unambiguously determined Further studies with additional molecular markers are needed to determine the population structure and divergence between the two closely related species of this genus

Acknowledgements This study was carried out under the 'All India Co-ordinated Project on Capacity Building in Taxonomy Research on Helminths," sanctioned to VT by the Ministry of Environment and Forests, GOI, DBT Project to VT and AC, DSA (UGC-SAP) in Zoology and UGC's UPE (Biosciences) program in the School of Life Sciences at NEHU Shillong

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ORIGINAL PAPER

Molecular characterization of *Gastrodiscoides hominis* (Platyhelminthes: Trematoda: Digenea) inferred from ITS rDNA sequence analysis

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Abstract *Gastrodiscoides hominis* (Digenea: Paramphistomata: Gastrodiscidae) is an amphistomid intestinal fluke of pigs causing gastrodiscoidiosis. With the use of molecular tools assisting the conventional diagnostic procedures, we aimed at finding out molecular characterization of *G. hominis* using PCR amplifications of rDNA ITS (1, 2) sequences. The sequences obtained (GenBank accession numbers EF027096, EF027097, EF027098, EU887294, and EU887295) were compared with available sequences of other digenean parasites, particularly those having a zoonotic potential in the northeastern region of India. The BLAST search revealed a close similarity with members of the family Paramphistomidae, showing maximum similarity with the amphistome, *Homalogaster paloniae* (subfamily Paramphistominae). Based on various tree construction methods, phylogeny of *G. hominis* is discussed.

Introduction

Gastrodiscoides hominis, a digenean trematode, is commonly found in cecum and colon regions of pig and human where pig is a normal host species (Ahluwalia 1960; Kumar 1980, 1999) and is the only amphistomid parasite of man (Mas-Coma et al. 2005). Apart from pig, some other animals are also found to be infected such as Napu mouse deer (*Tragulus napu*), common field rat (*Rattus brevicaudatus*), rhesus monkey (*Macaca mulatta*), and some other species of monkey (Buckley 1964; Fox and Hall 1970; Herman 1967). Easwaran et al. (2003) reported a wild pig also infected by this parasite in Thekaddy forest, South India. *G. hominis* has been reported from American muskrat (*Ondatra zibethica*) in Volga Delta, Russia (Ivanov and Semenova 2000). Human infection was reported in a 7-year-old girl in Nigeria, diagnosed by eggs in stools (Dada-Adegbola et al. 2004). The exact life cycle is unknown but probably similar as in other species of Gastrodiscidae involving aquatic vegetations as the second intermediate environment that is used for the encystment of the metacercarial infective stage (Zablotski 1964; Dutt and Srivastava 1972; Mas-Coma et al. 2007). Gastrodiscoidiosis has symptoms similar to diarrhea and, if untreated, might kill the patient, mostly children (Kumar 1980). Eggs are voided in feces, but species identification and diagnosis of infection from eggs alone may be problematic, since most amphistomid eggs have similar morphology.

G. hominis has a wide distribution throughout India including the states of Assam, Bengal, Bihar, Uttar Pradesh, Madhya Pradesh and Orissa (Shrivastav and Shah 1970; Murty and Reddy 1980). Apart from India, it is widely distributed in countries like Pakistan, Burma, Thailand, Vietnam, Philippines, China, Kazakhstan, and Russia (Ahuwalia 1960; Buckley 1939; Kumar 1980; Harinasuta et al. 1987; Yu and Mott 1994; Ivanov and Semenova 2000). Buckley (1964) reported high prevalence of *Gastrodiscoides* in humans, mostly children (around 41%), in Kamrup district of Assam, India. In a later study carried out in Meghalaya (India), *G. hominis* was shown to have seasonal prevalence (Roy and Tandon 1992).

The application of DNA methods to studies on the systematics and population genetics of platyhelminth parasites has become widespread in recent years, since identification based on morphology is not always unequivocal. With the use of PCR and sequencing approaches, taxonomic issues can be resolved quite accurately by comparing the DNA of the taxa, utilizing genetic markers in nuclear ribosomal DNA in particular (Blair et al. 1996; Hust et al. 2004). The ribosomal

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non-coding DNA internal transcribed spacer regions (ITS1 and ITS2) can be utilized for species level diagnosis, as these are the most conserved genes in eukaryotic cells showing an extreme sequence similarity even between distant phylogenetic taxa (Hillis and Dixon 1991) ITS sequences were found to be useful in characterizing the species of *Fasciolopsis* and *Paragonimus* that occur as potential zoonoses in the northeastern regions of India (Prasad et al 2007, Tandon et al 2007) With regard to amphistomid trematodes, the published information about ITS2 sequences is so far limited to only a few species, namely *Calicophoron calicophorum*, *C daubneyi*, *C microbothrioides*, *Orthocoelium streptocoelium* and *Homalogaster paloniae* (Itagaki et al 2003)

As the eggs pose a greater challenge in species level diagnosis than the much larger adults, the present study aimed at amplifying the ITS1 and ITS2 regions of (rDNA derived from egg and adult) *G hominis*, yet another trematode parasite of zoonotic implications in the region, the information emerging from the study would be useful in differentiating this species from other trematode-borne potential zoonoses viz *Paragonimus westermani*, *Fasciolopsis buski*, *Fasciola gigantica*, *Clonorchis sinensis* and *Opisthorchis viverrini* for all of which ITS (1 and 2) regions have been characterized and will be further used for assessing the extent of genetic diversity, if any, of the parasite

Materials and methods

Parasite material and DNA isolation

Live adult *G hominis* flukes were obtained from the intestine of freshly slaughtered pigs of Assam and Meghalaya origin, *Sus scrofa domestica*, at local slaughter houses To isolate DNA, five adult worms each collected from different host animals of the two geographical locations were processed singly, they were first immersed in digestion extraction buffer (containing 1% SDS 25 mg Proteinase K) at 37°C overnight DNA was then extracted from lysed individual worms by standard phenol chloroform technique (Sambrook et al 1989) Eggs were taken out from the mature adult fluke by squeezing between two slides and placed on the FTA cards (Whatman Biosciences Ltd) The cards were dried for 1 h, punched, and two sample discs were taken from each card for one PCR reaction Sample discs were washed with Whatman's FTA purification reagent and TE buffer, and allowed to dry for 1 h at room temperature, after which they were ready for PCR

DNA amplification, sequencing, and its analysis

The rDNA region spanning the ITS regions was amplified from genomic DNA obtained from the adult or egg stage by PCR We used the universal primers based on conserved ITS sequences of *Schistosoma* species (Bowles et al 1995) as detailed below

1. ITS1 region—BD1 (forward) 5′ GTCGTAACAAG GTTTCCGTA-3′ and 4S (reverse) 5′-TCTAGATGCG TTCGAA (G/A) TGTCGATG-3′
2. ITS2 region—3S (forward) 5′-GGTACCGGTGGATC ACTCGGCTCGTG-3 and A28 (reverse) 5′-GGGATC CTGGTTAGTTTCTTTTCCTCCGC-3′

The PCR amplification was performed following the standard protocol (White 1993) with minor modifications as described elsewhere (Prasad et al 2007, Tandon et al

```
Adult  51 TACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGGTTTT 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Egg    49 TACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGGTTTT  98

Adult 101 CCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCC 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Egg    99 CCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCC 148

Adult 151 AAAAAGTCGTGGCTTGGAATCTGCCAGCTGGCGTGATTTCCTCTGTGGTT 200
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Egg   149 AAAAAGTCGTGGCTTGGAATCTGCCAGCTGGCGTGATTTCCTCTGTGGTT 198

Adult 201 CGCCACGTGAGGTGCCAGATCTATGGCGTTTTCCTAATGTCTCCGGACAC 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Egg   199 CGCCACGTGAGGTGCCAGATCTATGGCGTTTTCCTAATGTCTCCGGACAC 248

Adult 251 AACCGCGTCTTGCTGGTAGCGCAGACGAGGGTGTGGCGGTAGAGTCGT-G 299
          |||||||||||||||||||||||||||||||||||||||||||||||| |
Egg   249 AACCGCGTCTTGCTGGTAGCGCAGACGAGGGTGTGGCGGTAGAGTCGTGG 298

Adult 300 CTCAATGTAATGTATGTGGTATCACGCTCTTCTGTTGTGCCTTTGTTAGT 349
          |||||||||||||||||||||.||||||||.|||||||||||||||||||
Egg   299 CTCAATGTAATGTATGTGGTAGCACGCTCTGCTGTTGTGCCTTTGTTAGT 348
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Fig 1 Pair-wise alignment of ITS2 sequence of egg and adult stages of *G hominis*

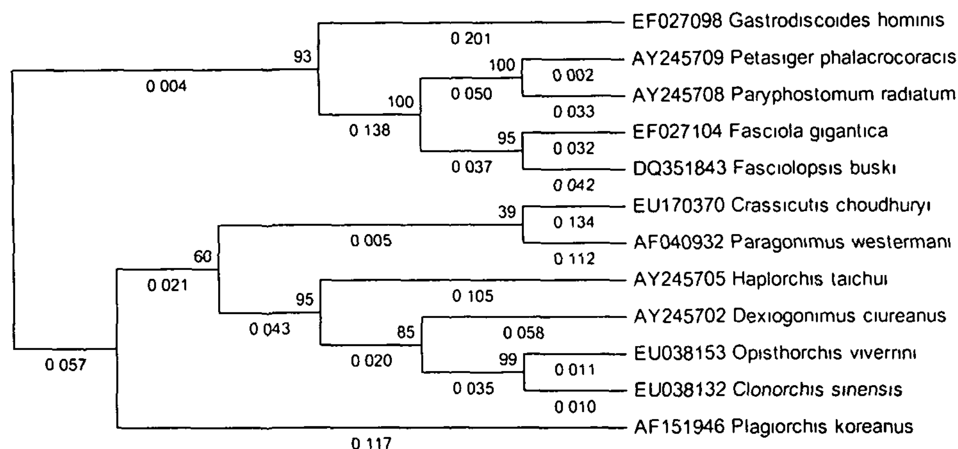

Fig. 2 Neighbor-Joining tree of ITS1 sequences of *G hominis* and other zoonotic trematodes showing bootstrap values and distance

2007) The PCR cocktail (final reaction volume 25 µl) was amplified with the following conditions for ITS1—initial denaturation at 94°C for 5 min, then 35 cycles including denaturation at 94°C for 60 s, annealing at 54°C for 50 s, extension at 72°C for 80 s, followed by final extension for 7 min at 72°C For amplification of ITS2 region, the conditions were as follows—initial denaturation at 94°C for 5 min, then 26 cycles including denaturation at 94°C for 30 s, annealing at 55°C for 38 s, extension at 72°C for 42 s, followed by final extension for 10 min at 72°C The resultant PCR products were separated by electrophoresis through 1 5% (*w/v*) agarose gel in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and then photographed The known size fragments of 100 bp ladder in agarose gel were used as marker For DNA sequencing, the PCR products were purified using Genei Quick PCR purification kit, sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of The Centre for Genomic Applications, New Delhi, India, and submitted to GenBank under the accession numbers EF027096 (ITS2, adult), EF027097 (ITS2, egg), EF027098 (ITS1, adult), EU887294 (ITS1, adult), and EU887295 (ITS2, adult)

The ITS1 and ITS2 sequences were compared using nucleotide BLAST (blastn) with default settings (URL http //www ncbi nlm nih gov/blast), and phylogenetic prediction was done by CLUSTALW (URL http //www ebi ac uk/clustalw) for each query DNA sequence Phylogenetic reconstructions were done using the MEGA 4 0 package (Tamura et al 2007) ITS sequences were entered in MEGA for construction of the phylogenetic trees using maximum parsimony and distance methods namely the Neighbor-Joining, UPGMA, and Minimum Evolution

Results

The ITS1 and ITS2 regions of *G hominis* DNA of adult fluke and egg origin were successfully amplified by using the abovementioned trematode primers The size of the

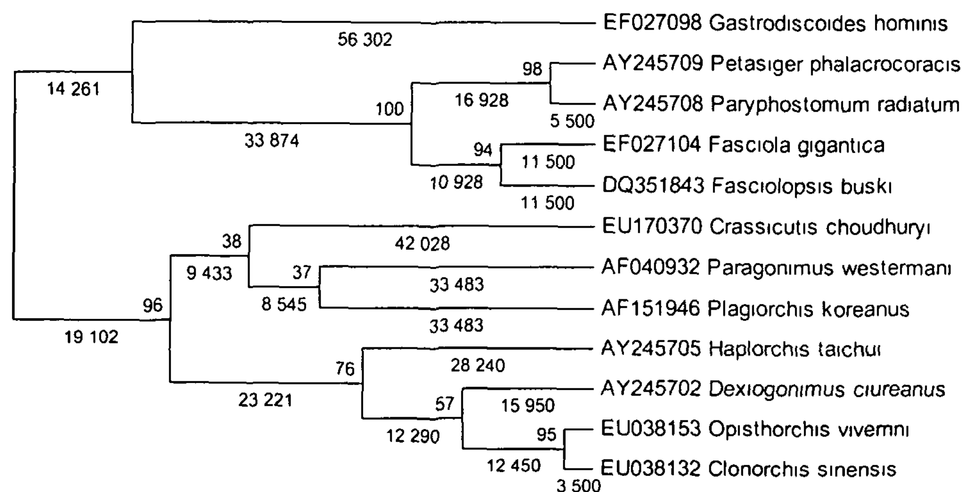

Fig. 3 Maximum parsimony tree of ITS1 sequences of *G hominis* and other zoonotic trematodes showing bootstrap values and distance

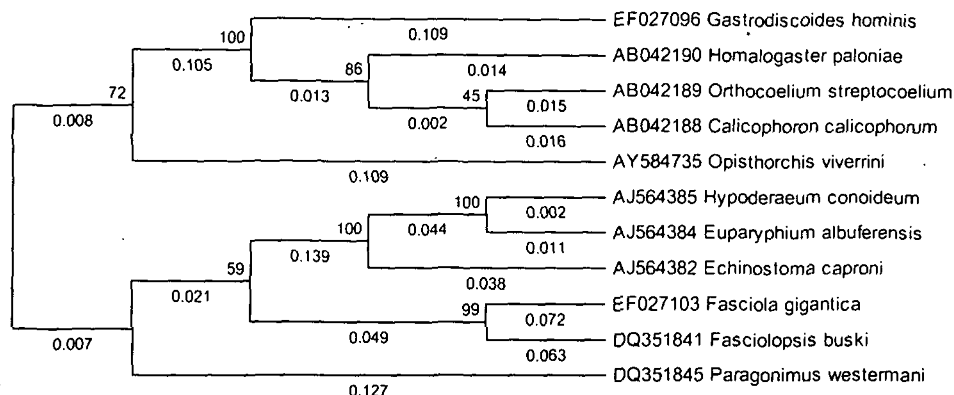

Fig. 4 Neighbor-Joining tree of ITS2 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap value and distance

amplified DNA from the adult parasite was 841 bp for ITS1 and 494 bp for ITS2 with flanking regions. The ITS2 sequences of the egg and adult stage DNA of the parasite were aligned and found to be absolutely similar in both (Fig. 1), thus revealing no stage-specific difference.

The sequences were aligned in BLAST. Sequences generated from both the geographical isolates (of Assam and Meghalaya origin) were found to be identical. Phylogenetic analyses using the various distance methods and character state method like maximum parsimony show that the topology is similar among the trees obtained. Since the ITS1 sequences of paramphistomid trematodes are not available in the GenBank database so far, the sequences available for other digenean parasites were used for alignment. Phylogenetic trees constructed showed that the query ITS1 *G. hominis* forward and reverse sequences stand close to and are similar in descending order to the sequences of *Petasiger phalacrocoracis*, *Paryphostomum radiatum*, *Fasciola gigantica*, *Fasciolopsis buski*, *Crassicutis choudhuryi*, *Paragonimus westermani*, *Haplorchis taichui*, *Dexiogonimus ciureanus*, *Opisthorchis viverrini*, *Clonorchis sinensis* and *Plagiorchis koreanus* (Figs. 2 and 3). The ITS2 sequences of *G. hominis* were aligned with those available for other members of the family Paramphistomidae, Echinostomata, and also of other families having zoonotic potential in the region and neighboring countries. The phylogenetic trees based on ITS2 sequences showed close resemblance with *Homalogaster paloniae*, *Orthocoelium streptocoelium*, *Calicophoron calicophorum*, *Opisthorchis viverrini*, *Hypoderaeum conoideum*, *Euparyphium albuferensis*, *Echinostoma caproni*, *Fasciola gigantica*, *Fasciolopsis buski* and *Paragonimus westermani* (Figs. 4 and 5), maximum similarity being shown with *H. paloniae* with significant bootstrap value. The values 70% and above in the bootstrap test of phylogenetic accuracy indicated reliable grouping among different members of Paramphistomidae.

Discussion

Morphological differences found in adult specimens have been widely used for platyhelminth discrimination (Miyazaki 1974). However, traditional diagnostic techniques are now being complemented by a variety of molecular tools to help in resolving the taxonomic issues associated with describing new species or strains on the basis of phenotypic characteristics (Thompson et al. 2004). PCR-

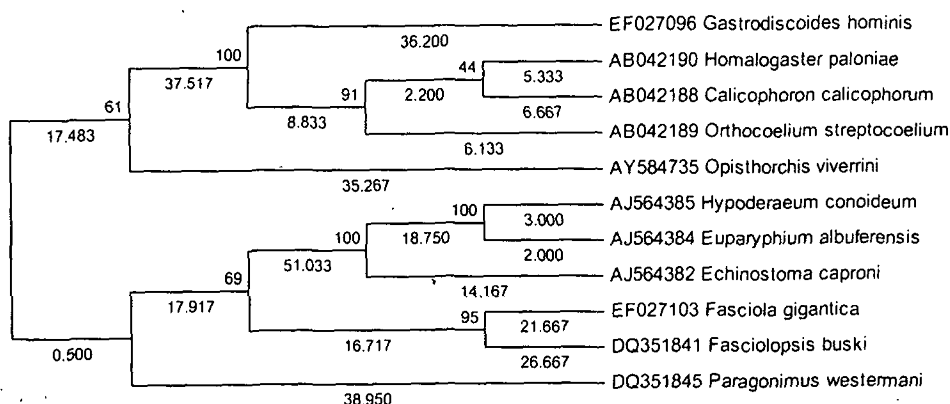

Fig. 5 Maximum parsimony tree of ITS2 sequences of *G. hominis* and other zoonotic trematodes showing bootstrap value and distance

based techniques using the ITS sequences have been proven to be a reliable tool to identify the various lung fluke species and their phylogenetic relationships (Blair et al 1999, Iwagami et al 2000, Tandon et al 2007) In the phylogenetic analysis of the ITS1 sequence and comparison with the available sequences of other Digenea, *G hominis* shows close resemblance with members of Paramphistomidae and Fasciolidae due to lack of sequences for amphistomes In case of ITS2 sequences, *G hominis* stood close to the members of families Paramphistomidae and Echinostomatidae Based upon the trees constructed, two distinct clusters are revealed one of Paramphistomidae and other for Echinostomatidae The ITS2 sequences in the present study showed a higher bootstrap value confirming that it is a highly conserved monophyletic group compared to ITS1, the results being in accordance with other studies on trematodes (Luton et al 1992, Prasad et al 2007)

The present study herewith provides the first molecular characterization of *G hominis* using ITS1 and ITS2 sequences However, to ascertain intra-specific strain variations, if any, and to determine the population structure and genetic variability, different geographical isolates of *G hominis* from the region need to be studied with the use of additional molecular markers

Acknowledgements This study was carried out under the Department of Biotechnology-sanctioned project to VT & AC and partly supported by DSA (UGC-SAP) program in the Department of Zoology and UPE-Biosciences program in the School of Life Sciences at NEHU, Shillong We are also thankful to the Coordinator Bioinformatics Centre NEHU, for allowing access to its facility

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www.bioinformation.net **Hypothesis**

Use of sequence motifs as barcodes and secondary structures of Internal Transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae)

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Abstract:

Most phylogenetic studies using current methods have focused on primary DNA sequence information However, RNA secondary structures are particularly useful in systematics because they include characteristics that give "morphological" information which is not found in the primary sequence Also DNA sequence motifs from the internal transcribed spacer (ITS) of the nuclear rRNA repeat are useful for identification of trematodes The species of liver flukes of the genus *Fasciola* (Platyhelminthes Digenea Fasciolidae) are obligate parasitic trematodes residing in the large biliary ducts of herbivorous mammals While *Fasciola hepatica* has a cosmopolitan distribution, the other major species, i e , *F gigantica* is reportedly prevalent in the tropical and subtropical regions of Africa and Asia To determine the *Fasciola* sp of Assam (India) origin based on rDNA molecular data, ribosomal ITS2 region was sequenced (EF027103) and analysed NCBI databases were used for sequence homology analysis and the phylogenetic trees were constructed based upon the ITS2 using MEGA and a Bayesian analysis of the combined data The latter approach allowed us to include both primary sequence and RNA molecular morphometrics and revealed a close relationship with isolates of *F gigantica* from China, Indonesia and Japan, the isolate from China with significant bootstrap values being the closest ITS2 sequence motifs allowed an accurate in silico distinction of liver flukes The data indicate that ITS2 motifs (≤ 50 bp in size) can be considered promising tool for trematode species identification Using the novel approach of molecular morphometrics that is based on ITS2 secondary structure homologies, phylogenetic relationships of the various isolates of fasciolid species have been discussed

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, secondary structure, internal transcribed spacer, bar coding, motifs

Background:

The identification of closely related species based on morphological characters can be difficult This is particularly the case for soft-bodied animals such as digenean trematodes PCR-based techniques utilizing the rDNA ITS2 sequences, which occur between the 5 8S and 28S coding regions, have proven to be a reliable tool to identify the helminth species and their phylogenetic relationships [1] The nuclear ribosomal DNA second internal transcribed spacer (ITS2) sequences, which occur between the 5 8S and 28S coding regions, have proven useful for diagnostic purposes at the level of species *Fasciola* spp and isolates of *Fascioloides magna*, another member of the same family, from different geographical regions have been discriminated on the basis of ITS sequences [2] The ITS2 sequences have also been used to characterize the liver flukes from mainland China, which include *F hepatica*, *F gigantica* and an intermediate genotype, including polymorphism among ITS2 copies within the same fluke individual [3] The emergence of sequence-based identification with a BLAST similarity search connected to public databases has resolved several experimental and taxonomic constraints In addition, BLAST outcomes give no information about species delimitation for closely related species The identification of unknown ITS sequences based on these approaches therefore needs to be supported by phylogenetic analysis [4]

The trematode flukes of the genus *Fasciola* (the sheep liver fluke) are parasites of herbivores and infect humans accidentally causing fascioliasis worldwide The parasite is very cosmopolitan in distribution being found throughout all regions of the world, both temperate and tropical *F hepatica* is the causative agent of fascioliasis or 'liver rot' in ruminants, where it may be an important pathogen Human infections with *F hepatica* are found in areas where sheep and cattle are raised, and where humans consume raw watercress, including Europe the Middle East and Asia [5] Infection with *F gigantica* on the other hand is found more commonly in tropical regions of the world, areas affected including Africa, Asia, many Pacific islands including Hawaii (where it has been reported in man), the Middle East and Southern Europe and the south of the USA [6] Both fasciolids follow a similar two-host life cycle, in which freshwater snails of the family Lymnaeidae act as intermediate or vector hosts and a broad spectrum of mammals including humans, mainly herbivorous large-sized species, act as definitive hosts [7].

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Most phylogenetic studies using current methods have focused on primary DNA sequence information However, RNA secondary structures are particularly useful in systematics because they include characteristics, not found in the primary sequence, that give 'morphological' information [8] The novel approach of molecular morphometrics that relies both on traditional morphological comparison and on molecular sequence comparison by measuring the structural parameters of the ITS2 secondary structure homologies (geometrical features, bond energies, base composition etc) is recently being used to study the phylogenetic relationships of various species [9] This method allows one to take into account the regions where multiple alignments are barely reliable because of a large number of insertions and deletions This method is based on the assumption that secondary structure can be phylogenetically as significant as primary sequence It is well known that rRNA is highly conserved throughout evolution Thus, the secondary-structure elements of the RNA molecule, i e , the helices, loops, bulges, and separating single-stranded portions, can be considered phylogenetic characters [10, 11, 12]

At the same time, the ITS offers sequence motifs that are useful for the development of DNA barcoding This is a technique that uses short DNA sequences from a standardized region of the genome as a diagonistic "biomarker" for species identification [13, 14] as testified to by the creation of the consortium for the Barcode of life (http //www barcoding si edu/) Different species have different barcodes, which allow them to be used to (i) identify specimens, (ii) discover possible new species, and (iii) make taxonomy more effective for science and society In the present study, our main objective was to identify the Indian liver fluke (the Assam isolates) collected from ruminant hosts by the design and testing of ITS sequence motifs for oligonucleotide bar codes We also aimed at determining the species prevalent in the northeastern region of the country, by comparing these sequences by primary sequence analysis and molecular morphometrics data

Methodology:

Parasite material and DNA isolation

Adult *Fasciola* were obtained in live form from hepatic biliary ducts of freshly slaughtered cow, *Bos indicus* The worms recovered from these hosts represented the geographical isolates from Assam, Northeast India and morphologically resembled *Fasciola gigantica* (deposition number of paratypes at Zoological Survey of India, Kolkata = W7787/1) Eggs were obtained from mature adult flukes by squeezing between two glass slides For the purpose of DNA extraction, adult flukes were processed singly, eggs recovered from each of these specimens were also processed separately The adult flukes were first immersed in digestion extraction buffer (containing 1% SDS, 25 mg Proteinase K) at 37° C overnight DNA was then extracted from lysed individual worms by standard ethanol precipitation technique [15] and also extracted on FTA cards using Whatman's FTA Purification Reagent as described elsewhere [16] DNA from the eggs was extracted only with the FTA card technique

DNA amplification, Sequencing and its Analysis

The rDNA region spanning the ITS2 region was amplified from DNA obtained from the fluke by PCR We used the universal primers, considered to be the general primers for trematodes and are designed based on conserved ITS sequences of *Schistosoma* species following [17] as given in **Figure 1** The PCR amplification was performed following the standard protocol [18] with minor modifications The resultant PCR products were separated by electrophoresis through 1 6% (w/v) agarose gels in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed The known size fragments of Phi X 174 DNA/ Hae III Digest in agarose gel were used as marker For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit, and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of TCGA, New Delhi and Bangalore Genei Ltd The DNA sequences were put to further analysis by using various Bioinformatics tools including similarity search BLAST (URL http //www ncbi nlm nih gov/blast) and phylogenetic prediction by CLUSTALW (URL http // www ebi ac uk/clustalw) for each query DNA sequence

Molecular Phylogenetic Analysis

Initially, the sequences were aligned using ClustalW multiple alignment [19] with the default gap and extension penalties used by this program Phylogenetic tree-building methods presume particular evolutionary models Therefore, while interpreting the results obtained, we considered different tree building models to entertain possible explanations Only unique sequences were used in tree construction ITS sequences were entered in the MEGA for construction of the phylogenetic trees using Maximum Parsimony and distance methods namely the Neighbor-Joining, UPGMA and Minimum Evolution Branch support was given using 1000 bootstrap replicates in MEGA [20]

ITS2 region- 3S (forward): 5'-GGTACCGGTGGATCACTCGGCTCGTG-3'
A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'

Figure 1: Primer design for ITS2

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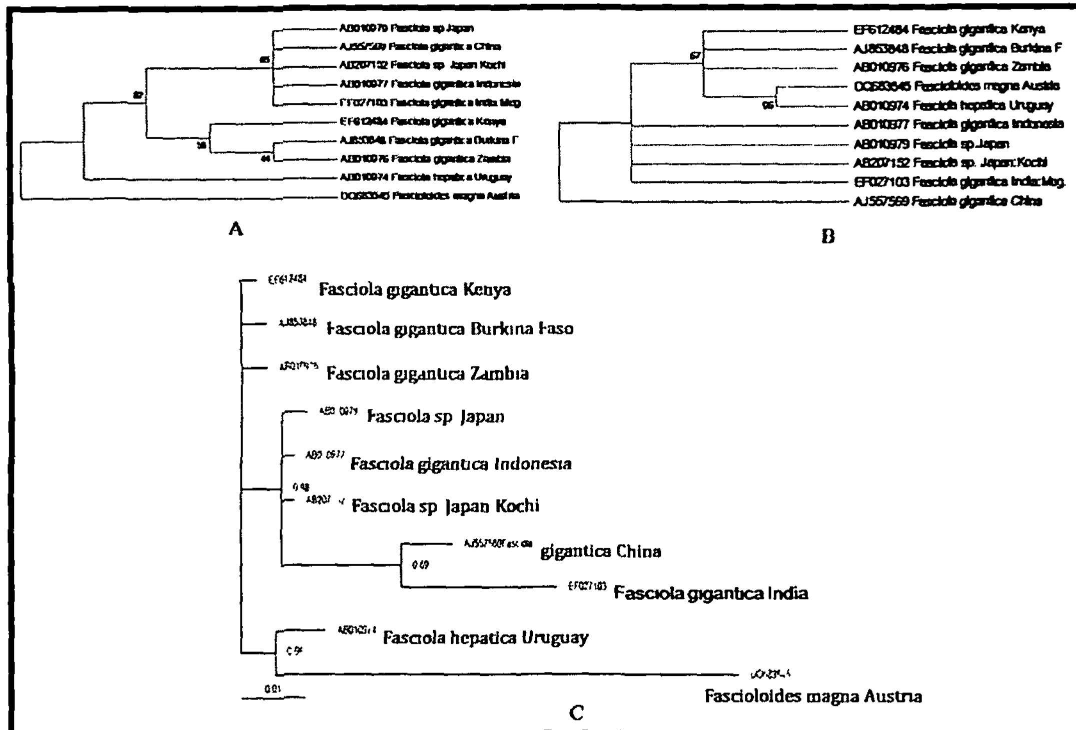

Figure 2: Phylogenetic trees of ITS2 sequences of fasciolid species showing bootstrap values (A) Neighbor Joining, (B) Maximum Parsimony, (C) Hypothetical Bayesian analysis

Bayesian phylogenetic analysis

DNA sequences were aligned using ClustalX 2 0 7 [21] The interleaved NEXUS file was edited manually in order for it to be recognized by Mr Bayes V3 1 2 programme Phylogenetic analysis was carried out using the Bayesian approach with combined datasets using Mrbayes 3 1 [22] wherein each data partition is allowed to have a different evolution rate The model of evolution and prior settings for individual loci was set to the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with four incrementally heated chains that were combinatorial run for 40,000 generations The convergence of MCMCMC was then monitored by examining the value of the marginal likelihood through generations Coalescence of substitution rate and rate model parameters were also examined Average standard deviation of split frequencies was checked and the generations were kept on adding until the standard deviation value was below 0 01 The values slightly differed because of stochastic effects The sample of substitution model parameters and samples of trees and branch lengths were summarized by the 'sump burnin" and "sumt burnin" commands respectively The values in the following commands were adjusted as per the 25% of our samples The cladogram with the posterior probabilities for each split and a phylogram with mean branch lengths were generated and subsequently read by the tree drawing program Tree view V1 6 6 [23]

Motif identification, testing and validation

The ITS sequence motifs were identified from aligned sequences of the data set for the species using PRATT software (http //genoweb1 irisa fr/Serveur-GPO/outils_acces php3?id_syndic=70) The minimum percentage of sequences to match (C %) parameter was adjusted to report pattern matching at 100% of the sequence input The motifs were expressed using the DNA alphabet (A, T, C, G) in PROSITE language The validation of the motifs was performed for each species using a 'PATTERN MATCHING' Web application (http //genoweb univ-rennes1 fr/Serveur-GPO/outils_acces php3?id_syndic=186) We considered that a motif was highly specific to a *Fasciola* species if it matched most or all the ITS sequences of this species but no other ITS of any other trematodes species

Evaluation of the ITS motif specificity through BLAST analysis

The *Fasciola* ITS sequence motifs (patterns 1-15) were subjected to BLAST algorithm against the non-redundant GenBank database of the National Center for Biotechnology Information (nr at NCBI) The BLAST output were then analyzed to find only the exact or perfect pairwise matches showing significantly high scores in terms of percent identity and low expect (E) values for each species

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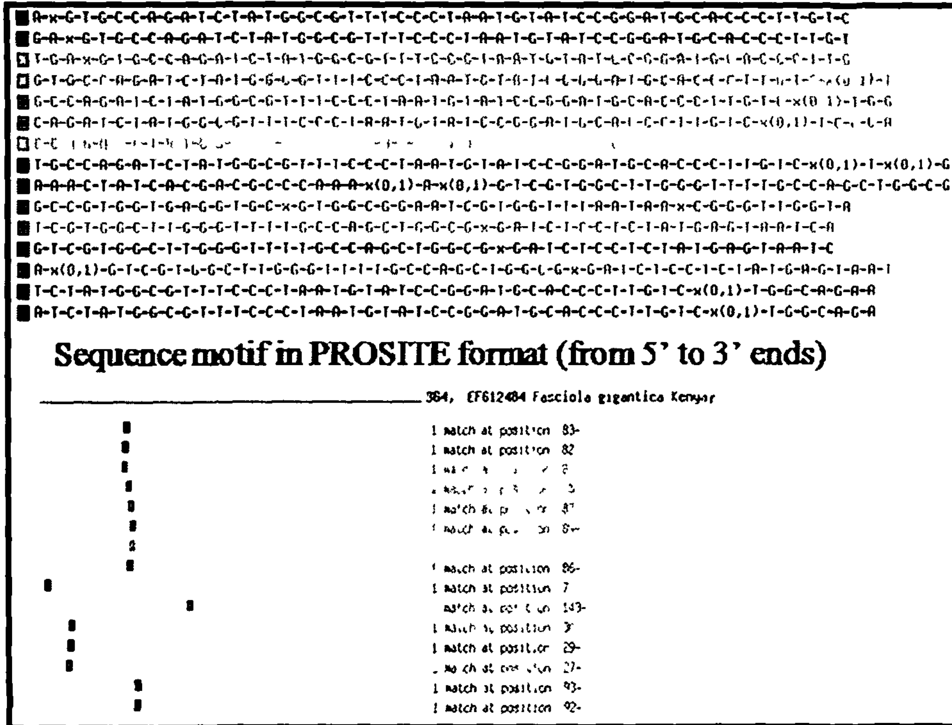

Figure 3: Sample of sequence motif in PROSITE format (from 5' to 3' ends) and pattern matching of ITS motifs of different geographical isolates of fasciolid species

Predicted ITS2 RNA secondary structures and analyses
Secondary structures of ITS2 sequences of various fasciolid species were reconstructed by aligning their sequences using Bioedit [24] The acquired structures with restrictions and constrains were submitted in MFOLD [25] RNA was folded at a fixed temperature of 37° C, and the structure chosen from different output files was the desired 6-helicoidal ring or the one with the highest negative free energy if various similar structures were obtained

Results:

PCR amplification of ITS regions and its analysis
The PCR-amplified products were successfully obtained using the primers as mentioned above The nucleotide sequences were obtained for ITS2 of rDNA and were compared with sequences of other fasciolid species obtained from Genbank The fragments of amplified DNA were estimated to be 480-550bp long For comparative purpose, the ITS2 sequences of fasciolids from various geographical regions were obtained from GenBank (Table 1) The Blast hit results show that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp, *F hepatica* and *F gigantica* besides *Fascioloides magna* (belongs to the same family, i e, Fasciolidae)

Phylogenetic trees
Phylogenetic trees were obtained by comparing the sequences of *F gigantica* and available ITS sequences for other fasciolid species Phylogenetic analyses using the various distance methods and character method like Maximum Parsimony showed that the topology is similar among the trees obtained with significant bootstrap support for the clades The values of 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of fasciolids The phylogenetic trees constructed in MEGA revealed a close relationship with isolates of *F gigantica* from China, Indonesia and Japan (**Figure 2**)

***In silico* identification of *Fasciola* sp. based on pattern matching ITS motifs**
A total of 15 ITS2 PROSITE motifs were tested by BLAST analysis against the generalized GenBank database at NCBI (**Figure 3**). The *Fasciola* motifs exhibited exact or perfect matches with *Fasciola* from different geographical isolates with significant E-value scores Eight (pattern 1-8) out of 15 *Fasciola* motifs exactly matched the sequences of *Fasciola* isolates from GenBank (best hits = more than 100, 100% of identity E-values = $2e^{16}$ to $1e^{17}$) Five (pattern 11-15) showed (best hits = more than 100 95% identity and significant E-value ranging from $5e^{14}$ to $1e^{14}$) The remaining two motifs (pattern 9-10) exhibited more than 100 best hits with 92% identity and an E-value score ranging from $4e^{11}$ to $1e^{11}$ (**Tables 2 & 3 in supplementary material**)

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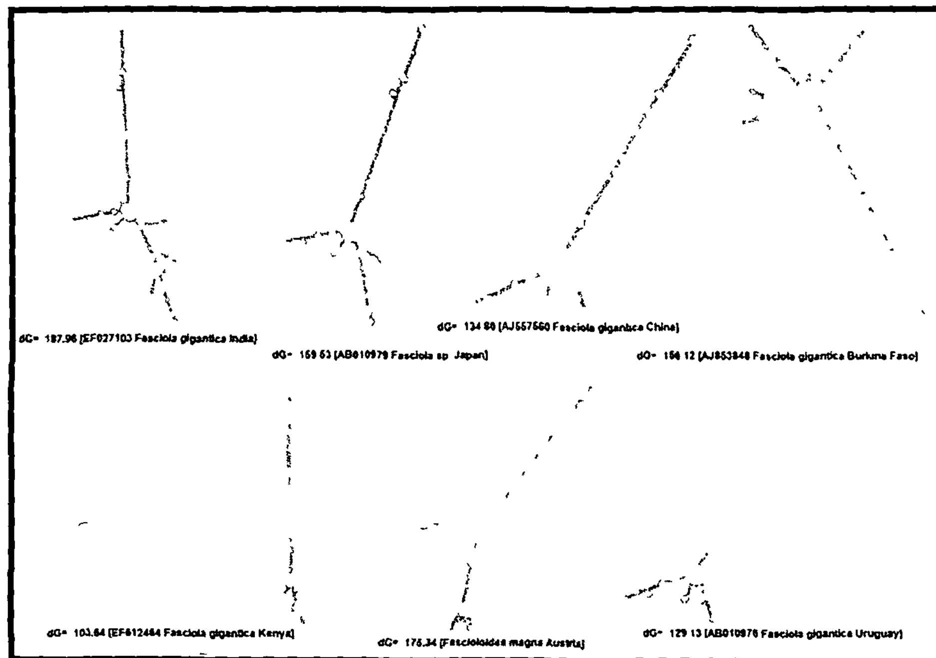

Figures 4: Predicted ITS2 RNA secondary structures and their structure formation enthalpies according to MFOLD

Secondary structure analysis

Six predicted RNA secondary structures were reconstructed from the unique sequences with highest negative free energy of *F gigantica* to provide the basic information for phylogenetic analysis (**Figure 4**) The ITS2 plus flanking regions of nuclear region ranged from 606bp in *F gigantica* India to a minimum length of 361bp in *F gigantica* China The secondary structural features of ITS2 regions as shown in the figure were analysed based on conserved stems and loops *F* sp Japan, *F gigantica* Indonesia, *F gigantica* Zambia and *F hepatica* Uruguay exhibited same type of secondary structures *F gigantica* isolates from India and China show overall similarity in the ITS2 rRNA folding and have identical secondary structure Secondary structures of the remaining species are weakly variable The observed similarities at the secondary structural level are further reflected at the energy level The only difference in their topology is due to differences in nucleotide sequences These secondary structure predictions indicate that the domains basepair to form a core region central to several stem features implying that conservedness is more important for the proper rRNA folding pattern Moreover the observed phylogenetic trend was identified with respect to the target accessibility sites for the seven different isolates The orders of preference were interior loop, bulge loop, multiple branch loop, hairpin loop and exterior loop in all the isolates The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences (**Figure 4**) ITS2 sequence of the Indian isolate revealed closest similarity with the Chinese isolate (*Fasciola gigantica*) with significant bootstrap value

Discussion:

Species identification and evolutionary inference generally use molecular and phylogenetic approaches on the ITS as target genomic loci But taxonomy of *Fasciola* spp has been based mainly on morphological data complemented with ecological, cytological and pathological results as well as clinical manifestations Morphological differences found on stained and mounted adult specimens have been widely used to discriminate between platyhelminth species It is possible to distinguish between adult *F hepatica* and *F gigantica* on the basis of morphology, but much variation exists Differentiating between two species is not possible on the basis of clinical, pathological, or immunological findings and their eggs are morphologically very similar [26] Consequently, where both species occur concurrently or in overlapping geographical distribution, it is not possible to be certain as to which species is responsible for the disease The low number of records of infection with *F gigantica* may well be due to the lack of good tools to distinguish this species from *F hepatica* [27]

The comparison of ITS sequences from worms of different hosts and of different countries indicates that there exists a high species-specific homogeneity In the present study, primary sequence analysis revealed a close relationship between the query sequence (from Northeastern region of India) and isolates of *F gigantica* from China, Indonesia and Japan Phylogenetic trees constructed showed that the groups of multiple closely related genotypes of *F gigantica* from Asia are broadly sympatric Such a pattern

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is expected for species with high gene flow whose populations have not been sundered by long term biogeographic barriers

We present here a new approach of molecular morphometrics, in which the measurable structural parameters of the molecules are directly used as specific characters to construct a phylogenetic tree These structures are inferred from the sequence of the nucleotides, often using energy minimization [28] Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *F gigantica*, which provided us with the additional information for correct identification of the species prevalent in the region Molecular morphometrics appears to be complimentary to classical primary sequence analysis in phylogenetic studies as it takes into consideration only the size variations of homologous structural segments and this choice implies that the overall architecture of the molecule remains same among the observed taxa This method helps in taking into account the regions where multiple alignments are barely reliable because of large number of insertion/deletion operations In the present study the secondary structure analysis of the same data also confirmed the results mentioned for primary sequence analysis Differences in their topology are only due to the fact that there are variable lengths of the sequences However, there are difficulties in defining a distance between two related structures with variable topologies [29] Nevertheless, because there were inconsistencies in the placement of a few *Fasciola* species, this study needs to be extended, in order to gain a better understanding of the systematics of this group as well as the evolution of their predicted ITS2 RNA secondary structures

Conclusion:

The present in silico identification of the *Fasciola* spp with ITS sequence motifs and secondary structure is consistent with investigations made using traditional approaches (i e, by morphology) The results also corroborate that the *Fasciola* species prevalent in Assam, Northeast India is in fact *F gigantica* and not *F hepatica*, which otherwise is the most common liver fluke throughout the globe Lack of data on genotypic diversity of *Fasciola* species in Africa and India does not allow the origin of regional populations to be unambiguously determined Further studies with additional molecular markers and bar coding will contribute towards determining the population structure and divergence between the two closely related species of this genus

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www.bioinformation.net **Hypothesis**

Supplementary material

Table 1: Fasciolidae species and their various geographical isolates used in this study with the respective GenBank accession numbers for corresponding ITS2 sequences A= Sequence generated as part of the present study

Species	GenBank accession	Sequence length (bp)	Classification
Fasciola gigantica India (A)	EF027103	606	*Digenea Fasciolidae*
Fasciola sp Japan Kochi	AB207152	505	*Digenea Fasciolidae*
Fasciola gigantica Indonesia	AB010977	505	*Digenea Fasciolidae*
Fasciola sp Japan	AB010979	505	*Digenea Fasciolidae*
Fasciola gigantica Zambia	AB010976	505	*Digenea Fasciolidae*
Fasciola hepatica Uruguay	AB207148	506	*Digenea Fasciolidae*
Fasciola gigantica Burkina Faso	AJ853848	588	*Digenea Fasciolidae*
Fasciola gigantica China	AJ557569	361	*Digenea Fasciolidae*
Fasciola gigantica Kenya	EF612484	364	*Digenea Fasciolidae*
Fascioloides magna Austria	DQ683545	538	*Digenea Fasciolidae*

Table 2: BLAST outputs of *Fasciola* ITS sequence motifs against NCBI GenBank database (nr db)

Fasciola species motif patterns	Length (bp)	No. of best Hits	Identity (%)	E-value
>Pattern 1 A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C	50	> than 100	100	$1e^{17}$
>Pattern 2 G-A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T	50	> than 100	100	$2e^{16}$
>Pattern 3 T-G-A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G	50	> than 100	100	$2e^{16}$
>Pattern 4 G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T	50	> than 100	100	$1e^{17}$
>Pattern 5 G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G	50	> than 100	100	$2e^{16}$
>Pattern 6 C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G-C-A	50	> than 100	100	$3e^{15}$
>Pattern 7 C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G-C	50	> than 100	100	$3e^{15}$

Table 3: BLAST outputs of Fasciola ITS sequence motifs against NCBI GenBank database (nr db)

Fasciola species motif patterns	Length (bp)	No. of best Hits	Identity (%)	E-value
>Pattern 1 A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C	50	> than 100	100	$1e^{17}$
>Pattern 2 G-A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T	50	> than 100	100	$2e^{16}$
>Pattern 3 T-G-A-x-G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G	50	> than 100	100	$2e^{16}$
>Pattern 4 G-T-G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T	50	> than 100	100	$1e^{17}$
>Pattern 5 G-C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G	50	> than 100	100	$2e^{16}$
>Pattern 6 C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G-C-A	50	> than 100	100	$3e^{15}$
>Pattern 7 C-C-A-G-A-T-C-T-A-T-G-G-C-G-T-T-T-C-C-C-T-A-A-T-G-T-A-T-C-C-G-G-A-T-G-C-A-C-C-C-T-T-G-T-C-x(0,1)-T-G-G-C	50	> than 100	100	$3e^{15}$

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