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Review

A review on the molecular characterization of digenean parasites using molecular markers with special reference to ITS region

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Summary

The rDNA region of eukaryotes has the immense potential to resolve the evolutionary and phylogeny problems using molecular markers. As evident from the present review, ITS region data is considered for interpretation of inter and intra-specific variations of 136 studies of 33 families including 78 genus and 114 species affecting individuals worldwide. Along with ITS-1 and ITS-2 region in 29 studies 18S region, in 38 studies 28S region and in 43 studies mitochondrial genes (COI and NDI) were also analyzed. Three new genera (*Allobilharzia* gen. nov., *Caulanus* gen. nov., and *Latuterus* gen. nov.) and 49 new species were discovered. Only 47 studies expressed variations at intra-specific and inter-specific level in complete ITS region, ITS-1 and ITS-2 rDNA sequences due to differences in nucleotide positions. According to the findings ITS region is more reliable and precise marker for demarcation and identification of species in combination of other DNA markers. Major studies were involved around the parasites of families Fasciolidae, Schistosomatidae, Opisthorchidae, Paragonimidae and Paramphistomidae, Clinostomidae, Diplostomidae, Haploporidae, among others infecting humans, farm animals, birds, fishes, reptiles and amphibians on the clinical basis. In future, molecular and bioinformatics aspects based on genetic variations will lead to explore the untouched areas of trematodes.

Keywords: Internal transcribe spacer; Cytochrome c oxidase I; Inter and Intra-specific variations; phylogeny; Fasciolidae; Schistosomtidae; trematodes

Introduction

The ribosomes, intracellular and molecular machines, found in all living organisms play major role in protein synthesis and gene expression. The ribosomal RNA (rRNA) gene and spacer regions extensively provide the phylogenetic information in prokaryotes and eukaryotes, collectively known as ribosomal DNA (rDNA), composed of coding regions (18S, 5.8S and 28S) and non-coding region of 2 internal transcribed spacers (ITS-1, ITS-2) and one non-transcribed spacer (NTS) (Wei *et al*., 2006) (Fig. 1).

The coding regions are highly conserved sequences in living creatures and infer the phylogenetic relationship between major phyla, while the non-coding regions are highly variable and have great potential to study the relationships among closely related genera or species due to faster evolutionary rate (Chen *et al*., 2004; Nolan & Cribb, 2005). The ITS regions are extensively used as molecular markers for taxonomy and phylogenetic analyses (Porter & Collins, 1991). The preference of ITS over other non-coding regions are: (1) multiple copies of rRNA genes; (2) suitable for PCR amplification with several universal primers for different organisms; (3) average sequence length for sequencing; and (4) high degree of variations at the generic and species level due to frequent nucleotide polymorphisms or insertions/deletions in sequences (Poczai & Hyvönen, 2010; Calonje *et al*., 2009).

The digeneans form the group of flatworms and are most common and abundant among parasitic worms. They parasitize all the classes of vertebrates and inhabit nearly every body organ. Their life cycle involves at least two hosts, although few species include a second and even a third intermediate host. Thus, the complexity is reflected in involvement of many representatives in devel-

opment, at both permanent and temporary levels. They cause serious and fatal diseases in many animals including humans (Bunkley-Williams & Williams, 1994). The class Digenea comprises some 25 superfamilies, 148 families of about 2800 genera and ~18,000 species (Bray *et al*., 2008). In this review, ITS region will be emphasized due to high degree of inter-specific and low level of intra-specific genetic variations among populations from different hosts and geographical localities at generic and species level.

2. Allocreadiidae (Looss, 1902)

The members of the family are found in the digestive system of teleosts and occasionally snakes, salamanders and frogs. The important species are *Creptotrematina dissimilis*, *Bunoderella metteri*, *Crepidostomum metoecus* and *Creptotrema creptotrema*. Curran *et al*. (2006) morphologically and genetically characterized *Polylekithum* sp. from catfish from USA using 3' end of 18S, ITS-1,

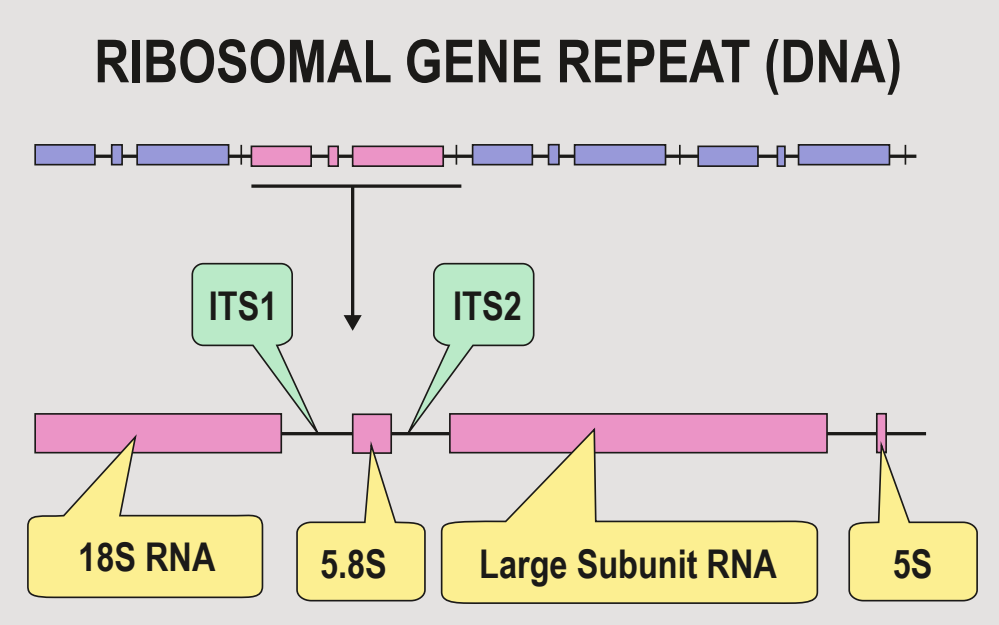

Fig.1. Ribosomal subunits and internal transcribed spacer region

Molecular studies of ITS region in different digeneans

In previous literature Nolan and Cribb (2005) reported several studies of different ITS region sequences in digeneans till 2004. The current review accentuates the studies done 2004 onwards. The classification of digeneans is based on the works of Gibson *et al*. (2002); Jones *et al*. (2005), Bray *et al*. (2008).

1. Acanthocolpidae (Lühe, 1906)

The members of the family are found in marine teleosts and occasionally sea snakes and important species are *Venusicola inusitatus*, *Acaenodera placophora*, *Manteria costalimai* and *Pleorchis sciaenae* etc.

Barnett *et al*. (2010) characterized morphologically and genetically two new *Stephanostomum* resembling cercariae, *Cercaria capricornia* VII and *C. capricornia* VIII from gastropods from Australia using LSU and ITS-2 rDNA sequences. No intra-specific variation was not noticed for these ceracariae. ITS-2 secondary structure predictions for two cercariae varied by two compensatory base changes (CBCs) and nine hemi-CBCs.

Curran & Pulis (2014) examined morpholigcally and molecularly *Pseudolepidapedon balistis* from triggerfish from Gulf of Mexico using 18S, ITS-1, 5.8S and ITS-2 and 28S (D1-D3 domain) rDNA sequences. The phylogenetic analysis corroborated that *P. balistis* belongs to family acanthocolpidae.

5.8S, ITS-2 and 5' end of 28S (D1-D3) rDNA sequences. The sequence alignment between *P. ictaluri* and *P. catahoulensis* showed 27 different sites (1.1 % of all 2,443 positions) and recommended *P. catahoulensis* as a new species. ITS-2 region is better marker for easy distinction of *Polylekithum* sp. due to short length and highly variable.

Petkevičiūte *et al*. (2012) analysed chromosomes and rDNA sequences (ITS2 and partial 28S) of larva of *Cercariaeum crassum* from bivalve *Pisidium amnicum* to describe the systematic, probable adult form and estimation of intra- and interspeicfic variability. The phylogenetic analysis clustered *C. crassum* into one clade with *Allocreadium* spp. and was nearly sister clade to *A. isoporum*. The intra-generic variation for ITS2 and 28S was 2.67 % and 1.16 %, respectively and constant according to the expected level of difference.

Curran *et al*. (2012) established the phylogenetic relationship of *Creptotrema funduli* with other allocreadiids using rDNA consists of 3' end of 18S, ITS region (ITS1, 5.8S and ITS2), 5' end of 28S gene and variable domains (D1-D3). According to results *Creptotrema* was closely related to *Megalogonia* and 3 neo-tropical genera (*Wallinia*, *Creptotrematina* and *Auriculostoma*).

3. Apocreadiidae (Skrjabin, 1942)

The members of the family inhabit the intestine of marine, brackish and fresh water fishes and comprised of about 80 species. The

important species are *Laureriella lateripora, Homalometron elongatum, Globoporum morone* and *Choanodera caulolatili*.
Razo-Mendivil *et al*. (2010) examined morphologically and molecularly *Crassicutis cichlasomae* from cichlids using nuclear ITS1 and mitochondrial COI gene sequences to establish phylogenetic relationships between the cryptic species complex. The genetic variations among seven cryptic species of *C. cichlasomae* were 1.0 – 5.2 % for ITS1 and 7.2 – 30 % for COI. The phylogenetic data was not sufficient to establish the strong correlation and host species or geographic distribution among cryptic species.
Parker *et al*. (2010) differentiated the *Homalometron* sp. from *Eucinostomus currani* from Costa Rica and Nicaragua, morphologically and molecularly using 3'end of 18S, 5'end of 28S, ITS-1, 5.8S and ITS-2 sequences. rDNA analysis between *H. elongatum* and *H. lesliorum* n.sp. disclosed 1 variable base in 18S, 12 bases in ITS-1, 10 bases in ITS-2 and 11 bases in 28S. *H. elongatum* and *H. lesliorum* n.sp. showed genetic dissimilarity of 1.4 % of aligned bases.
Curran *et al*. (2013) distinguished two cryptic species of genus *Homalometron* from USA, using partial 18S, ITS-1, 5.8S, ITS-2 and partial 28S gene sequences. Both the cryptic species (adult and metacercariae forms) resemble *Homalometron armatum* and rDNA disclosed significant genetic variations.

4. Brachycladiidae (Odhner, 1905)

The members of the family parasitize marine mammals and the main species are *Nasitrema dalli*, *Odhneriella rossica, Hunterotrema macrosoma* and *Synthesium tursionis*.
Mateu *et al*. (2011) identified and genetically distinguished brachycladiid species (*Oschmarinella rochebruni* and *Brachycladium atlanticum*) parasitizing cetaceans from Western Mediterranean using ITS-2 rDNA sequences. The molecular data affirmed that samples of *B. atlanticum* were conspecific in spite of host species.
Marigo *et al*. (2013) genetically distinguished the host cetacean species (*Pontoporia blainvillei*) infected by *Synthesium pontoporiae* from Brazil and Argentina using ITS-1, ITS-2 rDNA and mitochondrial ND3 and COI gene sequences. Any intra-specific differences were not observed among ITS-1 and ITS-2 reigon of *S. pontoporiae*. In *S. pontoporiae*, the mitochondrial COI and ND3 sequences proposed the wanted population structure and population expansion.

5. Bucephalidae (Poche, 1907)

The family was earlier known as gasterostomatidae (Poche, 1907) and the members infect marine, brackish and freshwater fishes. The main species are *Bucephalus fimbriatum, Alicicornis carangis, Prosorhynchoides ovatus* and *Dollfustrema vaneyi*.
In the study Chen *et al*. (2007) distinguished morphologically similar bucephalids (*Dollfustrema vaneyi* and *D. hefeiensis*) from intestine, gills or ceca from fishes using ITS-1, 5.8S, ITS-2 rDNA sequences and established phylogenetic relationship. The authors found 21 haplotypes of *D. vaneyi* and 16 haplotypes of *D. hefeiensis,* respectively and similar nucleotide variations between species which suggested that these species expressed different intra-relationships as shown by clades and phylogentic tree analysis.
Pina *et al*. (2009) described the life cycle pattern of *Bucephalus minimus* (adult from *Dicentrarchus labrax*, encysted metacercariae from *Mugil cephalus* and cercaria from *Cerastoderma edule*) from Portugal using ITS1 rDNA sequences along with morphological and histopathological investigations. All the three stages of *B. minimus* expressed 100 % homology and belonged to *B. minimus*.
Eydal *et al*. (2011) reported the morphometric and molecular studies of all the life stages (cercariae, sporocysts, metacercariae and adults) of *Prosorhynchoides borealis* from *Lophius piscatorius* in Icelandic waters using SSU rDNA, ITS-1, 5.8S and ITS-2 region sequences. rDNA sequences analyses disclosed 100 % similarity among cercariae, metacercariae and adults of *P. borealis* and corroborated the findings from morphological data.
Bott *et al*. (2013) investigated morphologically and molecularly the four species of *Plectropomus* (*P. areolatus*, *P. laevis*, *P. leopardus* and *P. maculatus*), three new species of *Neidhartia* and five new species of *Prosorhynchus* from Great Barrier Reef (Australia) using ITS-2 region sequences. Morphologically similar four species of *Prosorhynchus* (*P. freitasi*, *P. heronensis* n. sp., *P. munozae* n. sp., *P. plectropomi* n.sp.) were differentiated on the basis of ITS-2 rRNA sequences. The inter-specific variations in ITS-2 region were 4 – 16 % for *Neidhartia* sp. and 3 – 21 % for *Prosorhynchus* sp. The inter-generic difference was 17 – 21 % and no intra-specific variation was noticed in multiple sequence replicates of six species ITS-2 rRNA.

6. Choanocotylidae (Jue Sue & Platt, 1998)

The members of this family are found in Australian freshwater turtles and the main species are *Choanocotyle platti* and *Choanocotyle juesuei*.
Tkach & Synder (2007) morphologically and molecularly distinguished the new species of *Choanocotyle platti* sp. nov. from turtle from Australia using 18S, ITS-1, 5.8S, ITS-2 and partial 28S rDNA sequences. The sequence comparison of *C. nematoides*, *C. hobbsi* and *C. platti* sp. nov. confirmed *C.platti* sp. nov. as a new species.

7. Clinostomidae (Lühe, 1901)

The members of this family reside in oral cavity or pharynx of birds, reptiles and occasionally mammals (man). The species of the family are *Clinostomum complanatum*, *Clinostomatopsis sorbens* and *Clinostomoides brieni*.
Gustinelli *et al*. (2010) first time involved the morphological and molecular approach to identify the adult stage of *Clinostomum cutaneum* from Kenya using 18S, ITS-1, 5.8S, ITS-2 and partial 28S sequences. The molecular data corroborated the similarity between adult and metacercaria of *C. cutaneum* from bird and fish, respectively along with sequences of other *Clinostomum* sp.
Caffara *et al*. (2011) morphologically and genetically identified *Clinostomum complanatum* and *C. marginatum* using the 18S, ITS and mitochondrial COI sequences. The average variability was 7.3 % in ITS and 19.4 % in COI sequences of both species and these molecular results affirmed the morphological findings.

Caffara *et al*. (2014) first time reported the metacercarial stage of *Clinostomum phalacrocoracis* from wild cichlids of Israel on the basis of morphological and molecular analysis using ITS rDNA and mitochondrial COI gene sequences. The ITS sequences analysis revealed 100 % similarity with *C. phalacrocoracis,* 98.8 % with *C. cutaneum,* 97.2 % with *C. complanatum,* 94 % with *C. marginatum* and 92.6 % with *C. tataxumui.*

8. Collyriclidae (Ward, 1917)

The adult members inhabit in the skin or intestinal wall of birds and mammals. The two species of the family are *Collyriclum faba* and *Collyricloides massanae.*
Heneberg *et al*. (2014) checked the variability of infra-population for five DNA loci of *Collyriclum faba* from barn swallow in Central Europe using ITS-1, 5.8S, 28S, mitochondrial COI, tRNA-Thr and partial NDI gene sequences. ITS-1, ITS-2, 5.8S and 28S rDNA showed 100 % homology in four loci. The study first time reported the presence of high variable number of repetitive chi-like sequences in ITS-1 locus of *C. faba* from multiple hosts and from different geographical sites along with ITS-1 variability within 48bp repetitive sequences (chi-like sequences).

9. Cryptogonimidae (Ward, 1917)

The members of this family reside in intestine or pyloric caeca of marine and fresh teleosts, reptiles and rarely amphibians. The main species are *Retrobulla angelae*, *Polyorchitrema piscicola*, *Iheringtrama iheringi* and *Mitotrema anthostomatum*.
Miller & Cribb (2007) reported three new species belonging to two new genera (*Caulanus* gen. nov. and *Latuterus* gen. nov.) from reef fish (*Lutjanus bohar*) based on morphological and molecular approach using 28S, ITS-1 and ITS-2 sequences. According to ITS region analysis, *C. thomasi* differed in 5.0 % of sequences from *L. tkachi* and in 5.2 % of sequences from *L. maldivensis*, while *L. tkachi* and *L. maldivensis* differed by 0.6 % of sequences. In ITS-1 region *C. thomasi* varied in 6.3 % of sequences from *L. tkachi* and in 6.7 % of sequences from *L. maldivensis*, whereas *L. tkachi* and *L. maldivensis* differed by 0.4 % of sequences. 5.8S region was similar in all the species. The ITS-2 reigon of *C. thomasi* varied in 6.6 % of sequences from *L. tkachi* and *L. maldivensis* while *L. tkachi* and *L. maldivensis* differed by 1.4 % of sequences.
Miller & Cribb (2008) morphologically and molecularly described the eight of nine new species of *Siphoderina* from five species of Lutjanidae (*Lutjanus adetii*, *L.argentimaculatus*, *L.carponotatus*, *L. fulviflamma* and *L. russelli*), one species of Haemulidae (*Plectorhinchus gibbosus*) and two species of metacercariae from *Atherinomorus capricornensis* from GBR in Western Australia using large subunit (LSU), 5.8S, ITS-1 and ITS-2 rDNA sequences. The molecular analysis strongly corroborated the integrity of *Siphoderina* and reported it to be sister taxa to *Beluesca*. Any intra-specific variation was not examined between taxa along with rDNA sequences. According to the pattern of infection of *Siphoderina* to lutjanids and haemulids co-volutionary divergence with host switching was also suggested.

10. Dicrocoeliidae (Looss, 1899)

The genera inhabit bile ducts and gall bladder of domestic and wild ruminants. The important species of this family are *Dicrocoelium dendriticum*, *Controrchis biliophilus*, *Megacetabulum microrchum* and *Lyperosomum longicauda*.
Maurelli *et al*. (2007) selected isolates of *Dicrocoelium dendriticum* from sheep and cattle in Southern Italy and *D. hospes* from *Bos indicus* from Senegal to molecularly identify using 28S and ITS-2, 5.8S region. Any intra-specific differences were not observed for 28S rDNA of *D. dendriticum* while variations were noticed in *D. hospes.* For *D. dendriticum* intra- and inter-population differences were detected whilst 100 % homology for *D. hospes* was observed in ITS-2+ region. The ITS-2+ of *D. dendriticum* and *D. hospes* varied in 33 sites for inter-specific variations. In this study, ITS-2+ region variability of 8.2 – 8.5 % amid *D. dendriticum* and *D. hospes* used to differentiate the species.
Otranto *et al*. (2007) studied the morphological and molecular discrimination of *Dicrocoelium chinensis* and *D. Dendriticum* from sika deer, sheep and cattle from Austrlia, Germany and Italy by means of 18S and ITS-2 along with 5.8S and 28S rDNA sequences. Inter-specific variations were 0.14 % and 3.8 % of 18S and ITS-2, respectively between these two species. Findings from morphological and molecular analysis clearly distinguished the two species of *Dicrocoelium*.
Bian *et al*. (2013) reported the genetic differences in ITS-1, 5.8S and ITS-2 rDNA of *Dicrocoelium dendriticum* from sheep and goats from China. Intra-specific variations in ITS-1 and ITS-2 were 0 – 0.5 % and 0 – 1.3 %, respectively whereas inter-specific variations for ITS-2 region were 3.4 – 12.3 %. The ITS-2 region was proved as genetic marker to establish phylogenetic relationships of *Dicrocoelium* sp.

11. Diplostomidae (Poirier, 1886)

The members of this family parasitize birds and mammals and the main species are *Neodiplostomum attenuatum, Diplostomum spathaceum* and *Dolichorchis marahoueense*. Locke *et al*. (2010a) studied the barcode region of COI and ITS sequences to differentiate species of diplostomoids from fishes in St. Lawrence River, Canada. 47 species of diplostomoids were noticed and COI sequences analysis was supported by ITS region data. The great diversification was observed for metacercariae in different fishes through molecular study.
Locke *et al*. (2010b) distinguished *Diplostomum* sp. from 497 metacercarial specimens from fishes of St. Lawrence River using mitochondrial COI sequences and findings were confirmed with the help of ITS rDNA region. The ITS region of other species were identical to *D. pseudospathaceum* while ITS data were inadequate to express conspecificity. In general COI region was advanced than commonly used ITS region for species differentiation.
Locke *et al*. (2011) studied the distribution of adult and larval parasites *Apharyngostrigea cornu*, *Hysteromorpha triloba* and *Alaria mustelae* from fish, frogs, birds and mammals of North America using COI and ITS region sequences. The sequences of all adult parasites of definitive hosts matched with meta and meso-cercari-

ae of fish and frogs. ITS sequences of samples support the same species boundaries specified by COI divergence.

Haarder *et al*. (2013) investigated the snails from Copenhagen for infection of diplostomid using ITS rDNA region sequences. Out of three isolates, first isolate of *D. pseudopathaceum* showed 100 % homology with isolates from Finland, Germany and Poland; second isolate showed 100 % similarity with *D. mergi* from Finland and 99.3 % and 99.0 % similarity with *D. mergi* from Poland and Scotland, respectively; and third isolate showed high identity to *D. mergi* from Poland, Finland and Scotland with 99.5 %, 99.3 % and 99.0 %, respectively.

Georgieva *et al*. (2013) first time developed molecular database for species diversity of *Diplostomum* from snail, fish and gulls in Europe using mitochondrial COI, ITS-1, 5.8S and ITS-2 region sequences. The sequence analyses revealed 20 species and three complexes of molecularly diverse lineages of *D. mergi*, *D. baeri* and *D. huronense*.

Ndeda *et al*. (2013) established phylogenetic relationship for 21 diplostomatid metacercariae from Nile tilapia using 18S, ITS-1, 5.8S and ITS-2 region. The findings from ITS genes showed the close relationship of metacercariae of *D. mashonense* and *D. baeri*. 18S rDNA analysis mentioned the close relationship between *D. compactum*, *D. phoxini* and *D. spathaceum*. In this study the ribosomal genetic markers (18S and ITS rDNA) were efficient for inter-species phylogenetic relationships.

Athokpam & Tandon (2014) genetically characterized metacercaria of *Posthodiplostomum* sp. from India, using 18S, ITS-2, 28S rDNA region. The Indian isolate of *Posthodiplostomum* showed 97.5-99.7 % identity with Japanese isolate and secondary structure of ITS-2 rDNA expressed 7 transitions and 5 transversions at 12 nucleotide sites. The molecular data analyses supported the results from morphological findings.

12. Echinostomatidae (Looss, 1899)

The members of this family found in fish, reptiles, birds and mammals and the important species are *Prionosomoides scalaris*, *Singhia thapari*, *Pameileenia gambiensis* and *Petasiger exaeretus*.

Leung *et al*. (2009) established the molecular phylogeny of rediae of *Acanthoparyphium* sp. and *Curtuteria australis* from snails from New Zealand using mitochondrial 16S and ITS-1 rDNA sequences. The level of variance in clades of *Curtuteria* sp. was 0.0 % while for *Acanthoparyphium* sp. was 0.0 – 0.2 % for ITS-1 region. The 16S sequences of *Custuteria* metacercariae was matched with C. australis with >0.5 % divergence while *Acanthoparyphium* metacercariae sequences showed >0.6 % divergence.

Noikong *et al*. (2014) studied the identification and intra-specific genetic diversity of metacercariae from snails from Thailand using ITS-2 rRNA and mitochondrial NDI region. The findings pointed that major prevalent species were most closely asoociated to *Echinostoma revolutum*, *E. trivolvis*, *E. robustum*, *E. malayanum* and *Euparyphium albuferensis*.

13. Fasciolidae (Railliet, 1895)

The members of this family are recognized as liver flukes reside in liver, biliary duct and intestine of herbivorous mammals and the important genera are *Fasciola*, *Fascioloides*, *Fasciolopsis* and *Protofasciola*.

Huang *et al*. (2004) sequenced ITS-2 rDNA of *Fasciola* species from China and found no variation in length or composition from France, Sichuan and Guangxi region, while the sequence difference of 1.7 % (6/362) was observed. *Fasciola* from Sichuan as *F. hepatica*, Guangxi as *F. gigantica* and Heilongjiang reported as an intermediate genotype, as the ITS-2 sequences were unique. Out of six sequences, one sequence is identical to *F. hepatica* and rest of the five were almost identical to *F. gigantica* and nucleotides at five of the six polymorphic positions represent *F. gigantica*. The microheterogeneity may be due to polymorphism in ITS-2 sequences in *Fasciola sp.*

Semyenova *et al*. (2005) compared the ITS-2 sequences of *Fasciola hepatica* and *F. gigantica* from several hosts of different countries and detected only 4 polymorphic sites at 18 geographical regions of *F. hepatica.* The genotypes of *F. hepatica* from Asia and America expressed complete homology with European origin and also from Australia and New Zealand. In *F. hepatica* from Armenia, Uruguay and Mexico only one variation was noticed (transversion C-G, 0.3 % variation) at 217 nucleotide positionwhile in *F. gigantica* four nucleotide transitions (1.1 % variation, T-C at 334 nucleotide position) were observed from Turkmenistan, Tajikistan and Uzbekistan.

Itagaki *et al*. (2005a) considered ITS-1 and ITS-2, mitochondrial COI and NDI gene sequences to genetically characterize the parthenogenic *Fasciola* sp., from Japan. The sequences of ITS-1, partial 18S and 5.8S rDNA expressed 6 variable nucleotide positions. ITS-1 sequences of *F. hepatica* and *F. gigantica* showed variations at 6 positions. 3 different haplotypes of Japanese flukes (Fsp 1, Fsp 2 and Fsp ½) had identical nucleotide sequences to *F. hepatica* and *F. gigantica* and intermediate *Fasciola* sp., respectively. At 8 nucleotides positions, the sequences of ITS-2, partial 5.8S and 28S were variable among *Fasciola* sp. On the basis of nucleotides at 7 different sites Japanese flukes were categorized into 4 haplotypes (Fsp 1, Fsp 2, Fsp 2a and Fsp 1/2). Intra-specific variability of COI and NDI sequences was greater in *F. gigantica* than *F. hepatica*. All the four DNA markers resulted that Fsp 1 and Fsp 2 had identical nucleotide sequences closely resemble to *F. hepatica* and *F. gigantica,* respectively. The heterozygous Fsp ½ in rDNA and Fsp2 haplotype in mitochondrial DNA might be originated through inter-specific cross hybridization among paternal *F. hepatica* and maternal *F. gigantica*.

In 2005b, Itagaki *et al*. analyzed ITS-1 and mitochondrial NDI gene sequences to distinguish aspermic *Fasciola* sp. in Korea. On the basis of ITS-1 rDNA, Korean flukes were categorized into 3 haplotypes (Kor1, Kor2 and Kor1/2) representing identical nucleotides to *F. hepatica*, *F. gigantica* and intermediate form, respectively. Through NDI gene analysis three haplotypes were also reported as Kor1 of *F. hepatica* type and Kor2a and Kor2b of *F. gigantica* type. The heterozygous haplotype Kor1/2 has also been reported from Korean *Fasciola* sp. using D2 sequences of 28S rDNA, in Chinese flukes using ITS-2 and in Japanese flukes using ITS-1 and ITS-2. The Kor1 and Kor2a expressed 100 % homology to Fsp1 and Fsp2, respectively from Japan. The results of the study

firmly proposed that aspermic *Fasciola* sp. of Korea and Japan descended from the same ancestors and recently dispersed all over both the countries.

The study of Ashrafi *et al*. (2007) firstly reported that *Galba truncatula* serves as an intermediate host of *F. hepatica* in Iran. The ITS-2 sequences of liver fluke were identical to *F. hepatica* of Spain and Northern Bolivian Altiplano and of *Galba truncatula* identical to haplotype H-2 from Portugal, Spain, France and Netherlands. The molecular findings proposed that both may be involved in human fascioliasis in Gilan.

In 2007, Lin *et al*. used the PCR-SSCP technique along with DNA sequencing to characterize *Fasciola* sp. of several host and geographical locations of mainland China. Any variation in length and constitution of ITS-1 sequences from different specimens of *F. hepatica* and *F. gigantica* was not observed which differed at five nucleotide positions (1.2 %) while the intermediate *Fasciola* was unique due to two different ITS-1 sequences identical to *F. hepatica* and *F. gigantica*.

Alasaad *et al*. (2007) genetically characterized the complete ITS region sequences of *Fasciola* (*F. hepatica*, *F. gigantica* and intermediate *Fasciola*) specimens from 9 host and 19 geographical locations of Spain. Any nucleotide variation was not observed in the ITS-1 and 5.8S rDNA in *Fasciola* samples. Two different ITS-2 sequences of *Fasciola* specimens differed at one nucleotide (0.3 %, 1/362, sequence position; 868 bp) and revealed two genotypes among all the 25 samples. These findings provide the basis for population genetic studies of Spanish *F. hepatica*.

Prasad *et al*. (2007) molecularly characterized *Fasciolopsis buski* from swine host from India, considering ITS sequences of egg and adult stages, which were identical in length and composition. According to phylogenetic analysis *F. buski* closely related to other members of the family Fasciolidae. The findings suggested that ITS sequences were conserved through several developmental stages of the fluke and used as molecular marker to identify species.

In their study, Ali *et al*. (2008) genetically identified multiple specimens of *Fasciola* sp. from sheep and cattle from seven locations in Niger, using ITS-1 and ITS-2 sequences. Comparison of different *Fasciola* species sequences revealed that Niger samples represented only two species, *F. hepatica* and *F. gigantica* and this was the first report for genetical recognition of *Fasciola* sp.

Králová-Hromadová *et al*. (2008) deciphered the complete ribosomal and mitochondrial genes sequences of *Fasciolides magna* by means of 18S (small subunit), ITS-1, ITS-2, mitochondrial COI and NDI. The 18S and ITS region of *F. magna* was compared with *Fasciola hepatica* to reveal the inter-specific genetic variations. COI and NDI genes showed intra-specific sequence polymorphism in geographically isolated *F. magna* population.

Le *et al*. (2008) reported the hybrid/introgressed form of *Fasciola* sp. from cattle, buffaloes and also human patients from Vietnam, using ITS-2 region and two mitochondrial protein coding genes (COI and NDI). Sequences of ITS-2 belong to either *F. hepatica* or *F. gigantica*, and sequences of both species were present in the same individual and mitochondrial sequences in all samples showed similarity to *F. gigantica*.

In 2008, Prasad *et al*. verified the phylogenic location of *Fasciola* sp. from Indian origin on the basis of ITS sequences and compared with other members of Fasciolidae. The phylogenetic tree disclosed the close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia and according to bootstrap values the isolate from China is closest to Indian isolate. Any intra-specific variations in length and composition of sequences (adult and egg origin) were not observed and query ITS-2 sequences were more similar to various sequences of *F. hepatica*, *F. gigantica* as well as *Fasciolopsis buski* and *F. magna*.

Lotfy *et al*. (2008) studied the molecular phylogeny of seven species of the family using 28S, ITS-1, ITS-2 and mitochondrial NDI gene sequences to understand the evolutionary monophyletic relationships, origins, diversification and host shifting. The origin of this monophyletic family was from African elephants and later diversified in Eurasian herbivores and shared the common life cycle pattern.

Ribosomal ITS-2 region was sequenced and analysed by Prasad *et al*. (2009) for determination of Indian origin of *Fasciola* sp. The unique approach of molecular morphometrics based on ITS-2 secondary structure homology, deduced the close relationship with isolates of *F. gigantica* from China, Indonesia and Japan and Chinese isolate is closest due to considerable bootstrap values. The ITS-2 sequence motifs might be prognosticating tool for species identification.

Erensoy *et al*. (2009) used ITS-2 sequences, first time as molecular marker to characterize *Fasciola* sp. (*F. hepatica*, *F. gigantica* and intermediate *Fasciola*) from different locations of Turkey. No variations were observed in length or composition of ITS-2 region. The results concluded that *Fasciola* sp. from different geographical locations represented a single species of *F. hepatica*. The phylogenetic analyses deduced the close relationship among isolates of *F. hepatica*, *F. gigantica* and intermediate *Fasciola* sp.

Peng *et al*. (2009) characterized the aspermic *Fasciola* forms along with *F. hepatica* and *F. gigantica* from mainland China based on spermatogenesis and ITS-1 and mitochondrial NDI sequences. On the basis of ITS-1 region, three different genotypes were distinguished and represented by ITS1-Fh, ITS1-Fg and ITS1-Fh/Fg. ITS1-Fh and ITS1-Fg genotypes sequences were identical to *F. hepatica* and *F. gigantica*, respectively and differed at six variable nucleotide sites of ITS1 region (600 bp) and nucleotides of ITS1-Fh/Fg genotype overlie between two *Fasciola* sp. at six sites. The Fh-C4 haplotype was observed through NDI sequences in aspermic samples and *F. hepatica* denoting the possible existence of aspermic haplotype in China.

In the study of Ghavami *et al*. (2009), significant variations were observed in morphometric indices among *Fasciola* sp. from Iran and recognized species comprised of 31 % *F. hepatica* like, 7 % *F. gigantica* like and 62 % resembling intermediate forms. Based on ITS-2 RFLP and PCR sequence analysis of 535 amplified samples expressed no variation at species-specific nucleotide positions (230, 340 and 341). Comparison of ITS-2 sequences with BLAST GenBank database concluded that all parasites were *F. hepatica*.

Farjallah *et al*. (2009) first time genetically characterized the morphologically distinguished *Fasciola hepatica* from sheep and cattle from several sites of Tunisia and Algeria using ITS-1, 5.8S, ITS-2 and mitochondrial COI gene sequences. Evaluation of different

ITS and COI sequences of *Fasciola* sp. from GenBank verified that all examined specimens belong to *F. hepatica*. Out of 65 samples of *F. hepatica*, 58 isolates showed 100 % homology while 7 isolates exhibited variation at single nucleotide (C/T) in 859 bp site. According to results the variants of *F. hepatica* from Tunisia, Algeria and Spain might be common in origin due to movement of infected hosts across these countries.

ITS-2 and part of mitochondrial COI gene sequences were first time considered for genotypic status and hybridization/introgression of *Fasciola* sp. from goats and sheep in Vietnam by Nguyen *et al*. (2009). 48 ITS-2 sequence alignments pointed out that *F. hepatica* and *F. gigantica* varied at 7 positions while one of the sequences showed deletion (T at 327 bp site) in *F. gigantica* relative to *F. hepatica.* ITS-2 constitution was relatively similar to *F. hepatica* isolates from goats of Vietnam. Mitochondrial COI sequences analysis of Viatnamese goat-of-origin samples recommended the maternal linkage to *F. gigantica*.

Ichikawa & Itagaki 2010, established PCR-RFLP of ITS-1 region of different specimens of *Fasciola* sp. At six variable sites the amplicons with heterogeneous nucleotides provided bands of *F. hepatica* and *F. gigantica*. The results ascertained that PCR-RFLP technique was useful to differentiate ITS-1 region of *Fasciola* sp.

Bazsalovicsová *et al*. (2010) constructed the species-specific ITS-2 markers for characterization of *Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum* and *Paramphistomum cervi* from ruminants. Within geographically isolated populations intra-specific variation was very low in *F. hepatica and D. dendriticum* and even absent in *F. magna* and *P. Cervi.* The primers of ITS-2 region could be used for molecular characterization of morphologically indiscernible *F. hepatica, F. magna* and *P. Cervi* eggs.

In 2010, Rokni *et al*. genetically characterized adult *F. hepatica* isolates from different hosts of Iran with the help of sequencing of ITS-1 and RAPD-PCR. ITS-1 sequences of different isolates of *F. hepatica* expressed 100 % similarity and any significant intra-species variations were not observed.

Amor *et al*. (2011), first time genetically characterize the morphologically verified specimens of *Fasciola* sp., in *Equus caballus* from Tunisia, based on ITS-1, 5.8S and ITS-2 sequences. Sequences of Tunisian samples were identical to that of *F. hepatica* compared with sequences from GenBank. No nucleotide variations were monitored in ITS-1, 5.8S and ITS-2 sequences of all *Fasciola* samples although some ITS-2 region of *F. hepatica* from other locations expressed nucleotide difference at least at one site (substitution C/T at 859 bp). ITS-2 haplotype (FhITS-H1), most common in distribution, was the main halpotype indulged in the dispersal of *F. hepatica* from Spain, Iran, Japan, Korea, Vietnam, Egypt, Tunisia, Algeria and Niger.

With the use of molecular markers (ITS-1, 5.8S and ITS-2 region) Amor *et al*. (2011) differentiated the species and intermediate forms of *Fasciola* sp. in buffaloes and goats from Iran. The ITS sequences of the samples were identical to those of *F. hepatica, F. gigantica* differed at several variable nucleotide sites and nucleotide of intermediate forms overlapped all sites in both *Fasciola* sp. Any nucleotide variation was not observed in the sequences of ITS region of *F. hepatica* and *F. gigantica* from Tonekabon city and two ITS-2 haplotypes of *F. hepatica* from GenBank. The study of ITS region could allow the genetic categorization of *Fasciola* sp., and intermediate forms in endemic areas and might be applied for diagnosis.

Application of ITS-1 marker with the help of RFLP and RAPD-PCR methods, Shahbazi *et al*. (2011) estimated the diversity and several genotypes of *Fasciola hepatica,* and *F. gigantica* isolated from Iran. Any distinguishable variations were not detected among the size of PCR products of ITS-1 region. Two and three fragments of samples representing *F. hepatica* and *F. gigantica* were noticed, respectively, via PCR-RFLP techniques. The study might suggest the relative intra-species heterogeneity of isolated parasites in Iran but the authenticity of findings could be increased through inclusion of other animals (buffalo and goat) and more samples for controlling zoonotic diseases.

Study of Mahami-Oskouei *et al*. (2011) applied ITS-1 and ITS-2 sequences for molecular characterization through PCR-RFLP of *Fasciola* sp. samples from sheep and cattle from three locations in Iran. Two species of *Fasciola* identified as *F. hepatica* and *F. gigantica* were observed using new PCR-RFLP assay including Tsp509I restriction enzyme, rendered a cost effective and consistent method for molecular characterization.

Choe *et al*. (2011) characterized 19 *Fasciola* sp., samples from Korean native cattle using ITS-2 region as molecular marker. Out of 19 samples, 12 specimens were fully identical to *F. hepatica*, 5 samples identical to *F. gigantica* and last two samples expressed intermediate form of *F. hepatica* and *F. gigantica.* Any variation in length and composition of ITS-2 region was not observed in isolates of *F. hepatica* and *F. gigantica.* At 218 nucleotide site, five specimens revealed single base substitution (C>T) and outlined a separate branch under *F. gigantica* group with origin similar to Asian isolates.

According to Ai *et al*. (2011) phenotypic methods were not sufficient to characterize the different varieties of *Fasciola* sp. as compared to advantageous molecular tools. The authors reviewed several molecular techniques (Conventional PCR, multiplex PCR, specific PCR, PCR-RFLP, PCR-SSCP, RAPD-PCR, SRAP, DNA probe, TaqMan real time PCR, LAMP and PCR) currently applied to different areas of *Fasciola* biology such as epidemiology, genetic, detection and genotyping.

16 isolates of aspermic *Fasciola* sp. specimens were considered for morphological and genetical identification from cattle from Vietnam by Nguyen *et al*. (2012) using ITS-1, ITS-2 and partial mitochondrial COI and NDI gene sequences. Sequencing of ITS-1 and ITS-2 disclosed that 13 *Fasciola* sp. belong to *F. gigantica* while 3 species represented intermediate form of *F. hepatica* and *F. gigantica*. The mitochondrial COI and NDI sequences belong to *F. gigantica* proposing that maternal lineage of intermediate form was of *F. gigantica* type. Any intra-sequence variation was not observed.

Mohammad *et al*. (2012) molecularly categorized *Fasciola* sp., by means of ITS-1 region using PCR-RFLP technique. The spermic *Fasciola* sp. after digestion with restriction enzyme provided 6 fragments (360, 100 and 60 bp of *F. hepatica* and 360,170 and 60 bp of *F. gigantica*) while in aspermic *Fasciola* sp., 4 fragments (360, 170, 100 and 60 bp) were noticed. The findings of PCR-RFLP method were similar to that of sequence analysis.

In this study Dar *et al*. (2012) genetically characterized liver flukes from cattle, sheep and buffaloes from Egypt using ITS-1 with the help of PCR-RFLP technique. In all the samples from Egypt, 69.4 % and 30.6 % flukes were recognized as *F. hepatica* and *F. gigantica*, respectively and any intermediate species was not observed. In ITS-1 region only single nucleotide variation was noticed at 116 site between *F. gigantica* from Cameroon and Egypt.

Shu *et al*. (2012) first time investigated the taxonomic status of the *Fasciola* spp. in Yunnan province, mainland China using ITS-1 and ITS-2 rDNA sequences. Through molecular analysis two *Fasciola* spp., *F. hepatica* and *F. gigantica* were found in the province.

Chaichanasak *et al*. (2012) examined 147 liver flukes of cattle in Thailand on the basis of spermatogeneticity, ITS-1 and mitochondrial NDI sequences. ITS-1 PCR-RFLP pattern of 128 spermic flukes was similar to *F. gigantica* and rest 19 aspermic flukes showed RFLP pattern similar to *F. gigantica*. From 128 spermic flukes, 29 ND1 haplotypes (Fg-ND1-Thai) were recognized and unique to Thailand and from other countries recommend the introduction of ancestral haplotypes into Thailand. Out of 19 aspermic flukes the sequence of only one haplotype (Fg-ND1-Thai 1) was similar to aspermic Fasciola from Japan, Korea, China, Vietnam and Myanmar proposing that haplotypes were descendants with common origin and dispersed to these countries in recent past.

Heneberg (2013), established the phylogenetic relationships of *Fasciola jacksoni* along with members of Echinostomatoidea using 28S rDNA, ITS-1, ITS-2 and mitochondrial NDI gene sequences. *F. jacksoni* was more closely related to *Fascioloides magna* rather than other Fasciolids on the basis of ITS-1 sequences. The phylogenetic and morphological data proposed that *F. magna* and *F. jacksoni* created the sister clade to *F. hepatica* and *F. gigantica* complex along with reclassification of *F. jacksoni* as *Fascioloides jacksoni* comb. nov. The sequence analyses affirmed the monophyletic provenance of Fasciolidae family.

In the study, Farjallah *et al*. (2013) first time reported the genetic diversification of morphologically distinguished *Fasciola hepatica* from sheep and cattle from Sardinia using ITS-1, 5.8S, ITS-2, mitochondrial COI and NDI gene sequences. Sequences comparison of isolates and from GenBank ensured that all specimens were of *F. hepatica* (FhITS-H1 haplotype) and any variations were not noticed in ITS-1, 5.8S and ITS-2 rDNA. According to ND1 sequences the phylogenetic results expressed authentic grouping among haplotypes from Sardinia, mitochondrial lineage I and main N1 haplotype. The common haplotypes (FhCOI-H1 and FhCOI-H2) of *F. hepatica* from Sardinia matched with first lineage including main C1 haplotype from ealiear reported populations, belonged to phylogenetically different clade pointing the involvement of main C1 haplotype in the dispersion of *F. hepatica* in all continents.

Omar *et al*. (2013) reported the characterization of *Fasciola* sp., from cattle in Egypt on the basis of ITS-2 and mitochondrial COI sequences. Any variations were not observed in *Fasciola* samples and *F. hepatica* from other hosts of Egypt. The phylogenetic analyses disclosed that isolates of *Fasciola* represented single species of *F. hepatica.*

Liu *et al*. (2014) genetically compared *Fasciola* sp. (intermediate form) and *F. gigantica* with *F. hepatica* using ITS-1, ITS-2 sequences and complete mitochondrial genome (COI and ND4 gene). According to phylogenetic analyses of 12 protein coding mitochondrial genes, samples of *Fasciola* sp. were more similar to *F. gigantica* than *F. hepatica*.

In 2014, Phalee *et al*. examined the infection of *Fasciola gigantica* in cattle of Thailand and genetically characterized using ITS-2 region sequences. The infection of *F. gigantica* in cattle was affirmed through findings and ITS-2 region sequence verified as diagnostic marker for detection of *F. gigantica.*

On the basis of ITS-1 and mitochondrial NDI gene sequences Mohanta *et al*. (2014) differentiated the *Fasciola* sp. from cattle, buffaloes, sheep and goats from Bangladesh. With the help of PCR-RFLP techniques the aspermic flukes were categorized as Fg type in ITS-1, Fh/Fg type showing combination of ITS-1 of *F. hepatica* and *F. gigantica.* In NDI gene analyses all aspermic flukes exhibited Fsp-NDI-Bd11 haplotype similar to aspermic *Fasciola* sp. from Asian countries. Later on spermic flukes were distinguished as *F. gigantica* on the basis of spermatogenic status and Fg type in ITS-1.

Mufti *et al*. (2014) genetically differentiated *Fasciola* sp. from cattle and buffaloes of Pakistan using ITS-1 and ITS-2 sequences. The results confirmed the intermediate/hybrid *Fasciola* sp. from hosts, genetically similar due to equal number of base pairs in ITS region and resembling more with *F. gigantica*. The close relationship of *F. gigantica* with isolates from China, India, Iran and Vietnam were established through phylogenetic analyses. This study first time reported the hybrid/intermediate *Fasciola* sp. in Pakistan.

Shafiei *et al*. (2014) focused on the morphometric and genetic deviations of *Fasciola* sp. from different hosts from Iran considering ITS-1, ITS-2, mitochondrial NDI and COI gene sequences. Evaluation of ITS-1 and ITS-2 sequences confirmed six and seven single nucleotide substitutions leading to isolation of samples in two genotypes as *F. hepatica* and *F. gigantica.* Mitochondrial COI and NDI sequences expressed 42 and 48 variable sites in six haplotypes, respectively.

14. Gastrodiscidae (Monticelli, 1892)

The members of this family are intestinal parasites of mammals as well as human and the important species are *Gastrodiscoides aegyptiacus*, *Homalogaster paloniae*, *Pseudodiscus collinsi* and *Choerocotyle epuluensis*.

Goswami *et al*. (2009) first time characterized *Gastrodiscoides hominis* with the help of molecular techniques using ITS-1 and ITS-2 sequences. Using molecular phylogeny approach, the close similarity with paramphistomidae and maximum homology with amphistomes (*Homalogaster paloniae*) was observed.

15. Gastrothylacidae (Stiles & Goldberger, 1910)

The members of this family found in digestive tracts of vertebrates (fishes to mammals) and the important species are *Gastrothylax crumenifer*, *Fischoederius elongatus*, *Carmyerius spatiosus* and *Velasquezotrema brevisaccum*.

Ghatani *et al*. (2012) established the molecular phylogeny of five gastrothylacid species using ITS-2 and secondary structure predictions. Inter-specific variations were noticed among the five species through sequencing results, whereas secondary structures

analyses expressed structural similarities. The phylogenetic inferences showed that five species in the study creating a monophyletic group as compared to family paramphistomidae.

16. Gorgoderidae (Looss, 1899)

The members of this family inhabit amphibians, fishes and reptiles and the main species are *Phyllodistomum folium, Gorgotrema barbius, Amazonadistoma negrense* and *Phyllodistomoides duncani*.
Peribáñez *et al*. (2010) established phylogenetic relationship between adult and sporocyst of *Phyllodistomum folium* from fish and zebra mussel, respectively using ITS-1, 5.8S and ITS-2 region sequences. The molecular data revealed 100 % similarity between adult and sporocyst of *P. folium*. The *P. Folium* specimens from *Rutilus rutilus* genetically showed 1.6 % sequence variation.

17. Haploporidae (Nicoll, 1914)

The members of this family are ubiquitous and found in the alimentary canal of fishes and the main species are *Saccocoelium tensum*, *Haploporus benedeni*, *Dicrogaster fastigata* and *Lecithobotrys brisbanensis*.
Blasco-Costa *et al*. (2009) supported the taxonomical characterization of Haploporidae at the molecular level using partial 28S and complete ITS-2 rDNA sequences of ten species. Any intra-specific variations were not observed in all the samples of Haploporinae, while differences in inter-specific sequences was 2.1 – 10.9 % in ITS-2 and 0.9 – 4.8 % in 28S (*Dicrogaster* sp. and *Saccocoelium* sp.) rDNA. There was slight convergence in the inter-generic variability with species level data, 6.7 – 21.2 % and 4.6 – 11.4 % for *Dicrogaster* and *Saccocoelium*, respectively. The authors firmly proposed the close relationship between Atractotrematidae and Haploporidae.
Pulis & Overstreet (2013) studied the three genera (*Waretrema* sp., resurrected and new one) using ITS-1, ITS-2 and 28S gene sequences. The molecular analysis disclosed that *Spiritestis* and *Capitimitta* were not closely resembles due some morphological characters but form the sister clade to Haploporinae.
Pulis *et al*. (2013) reported the re-description of *Intromugil alachuaensis* n.sp. from *Mugil cephalus* from USA using partial 18S, complete ITS-1, 5.8S, ITS-2 and partial 28S rDNA sequences. The firstly reported sequence analyses between *I. mugilicolus* and *I. alachuaensis* n.sp. disclosed 110 pairwise variations and gaps and proposed *I. alachuaensis* n.sp. as new species.

18. Heterophyidae (Leiper, 1909)

The members of this family parasitize birds, mammals and freshwater siluroid fishes and the main species are *Alloheterophyes chini*, *Heterophyes nocens*, *Heterophyopsis continua* and *Protoheterophyes spuriocirrus*.
Miura *et al*. (2005) morphologically and genetically characterized *Cercaria batillariae* and undescribed philophthalmid from gastropod using ITS-1 and mitochondrial COI gene sequences. RFLP and sequence analyses of COI and ITS-1 region differentiated eigth cryptic species of *C. batillariae* and three cryptic species of undescribed philophthalmid species. Due to the absence of heterozygosity in ITS-1 region there was not any evidence of gene flow between different lineages.
Van *et al*. (2009) morphologically and molecularly distinguished the different stages (cercariae, metacercariae and adults) of *Haplorchis taichui* and *H. pumilio* from different hosts (snail, fish, dog, cat and human) from Vietnam and Thailand using ITS-2 rDNA sequences. This was the first molecular report to clearly differentiate the morphologically indistinguishable stages of *H. taichui* and *H. pumilio*.
Mehrdana *et al*. (2014) morphologically and molecularly differentiated the metacercariae of *Centrocestus* sp. from *Xiphophorus maculatus* using 5.8S, ITS-2 and 28S rDNA sequences. PCR amplification of rDNA region validated the infection of *Centrocestus* sp. in fish specimens.

19. Hirudinellidae (Dollfus, 1932)

The members of this family inhabit usually stomach of marine teleosts and the major species are *Lampritrema miescheri*, *Hirudinella ventricosa* and *Botulus microporous*.
Calhoun *et al*. (2013) identified four species of *Hirudinella* (*H. ventricosa*, *H. ahi* and two unidentified species) from Gulf of Mexico on the basis of 3' end of 18S, ITS-1, 5.8S, ITS-2 and 5' end of 28S (D1-D3 domain) region sequences. Through analyses of especially ITS region, it was observed that *Hirudinella* is not the monotypic genus. The phylogeny of suborder Hemiurata was steady with pervious phylogenies and the Hirudinellidae was derived group most closely related to Syncoeliidae.

20. Isoparorchiidae (Travassos, 1922)

The only species of the family inhabits swim bladder of fresh water fishes, is *Isoparorchis hypselobagri*.
Shimazu *et al*. (2014) re-described morphologically and molecularly the isolates of *Isoparorchis* sp. from Russia, Japan, Vietnam, Cambodia, Bangladesh, India and Australia using ITS-2 rDNA sequences. Any intra-specific differences were not noticed while inter-specific variations from 3-18 nucleotides in ITS-2 rDNA.

21. Lecithasteridae (Odhner, 1905)

The members of this family found in intestine of marine teleosts and the important species are *Lecithaster confusus*, *Lecithophyllum anteroporum*, *Monorchiaponurus hemirhamphi* and *Hysterolecitha rosea*.
Chambers & Cribb (2006) morphologically and molecularly characterized subfamily members of Quadrifoliovariinae (*Quadrifoliovarium*, *Bilacinia* and *Unilacinia* sp.) using ITS-2 and 28S region rDNA and established three new species *Q. macreia* n. sp., *Q. simplex* n. sp. and *Q. quattuordecim* n. sp. from the genus *Naso* from Indo-Pacific waters. The ITS-2 rDNA were similar in samples of *Q. pritchardae* and the phylogeny revealed that *U. asymmetrica* was the most basal taxon and *Q. simplex* n.sp. and *Q. quattuordecim* n. sp. were most derived species. The ITS-2 sequences were similar among samples of *Q. pritchardae* pointing the broad Indo-Pacific distribution.

Carreras-Aubets *et al*. (2011) morphologicaly and molecularly characterized *Aponurus laguncula* and a cryptic species *A. mulli* n.sp. from Spain using 28S (D1-D3) and ITS-2 rRNA gene sequences. The authors submitted the ITS-2 rRNA sequences of *A. mulli* n.sp.which would help in distinction of this cryptic species from *A. laguncula* species complex.

22. Macroderoididae (McMullen, 1937)

The members of this family inhabit freshwater fishes, crustaceans, leeches and marine fishes and the important species are *Alloglossidium kenti*, *Macroderoides spinifer*, *Rauschiella palmides* and *Gauhatiana batrachii*.

Razo-Mendivil *et al*. (2004) described two new species of *Glypthelmins* from anurans from Mexico using mitochondrial COI, ITS-2 and 28S (LSU) rDNA sequences. The intra-specific sequence variation was detected for COI (18.53 %), ITS-2 (5.44 %) and 28S (4.63 %). The sequence divergence of three isolates of *G. facioi* was 10.70 – 11.22 %, 0.48 – 0.97 % and 1.33 – 1.88 % for COI, ITS-2 and 28S, respectively. According to phylogenetic results the 3 isolates of *G. facioi* form the clade with isolate from Veracruz as a sister group to isolate from Costa Rica.

Tkach & Mills (2011) morphologically and genetically characterized four species *Alloglossidium fonti* sp. nov., *A. corti*, *A. geminum* and *A. kenti* using partial 18S, complete ITS and partial 28S rDNA sequences. Along with morphology the molecular results suggested the *A. fonti* as a new species. Any intra-specific variability was not observed except 1 bp variation in ITS-2 of *A. fonti*, 1 bp variation in ITS-1 of *A. corti* and 1 bp variation in 28S of *A. kenti* but showed the articulated inter-specific variations.

Tkach & Kinsella (2011) morphologically and molecularly identified the new species of *Macroderoides minutus* n. sp. using partial 18S, complete ITS and partial 28S region sequences. Any intra-specific variations were not noticed among species of *Macroderoides* except *M. minutus*. They also recommended that long fragments in ITS-1 of *Macroderoides* sp. are accountable for sequence length variations due to insertions via mutations. Two discrete clades of *Macroderoides* except *M. parvus* was revealed, one included (*M. spinifer* + *M. minutes* n.sp. + *M.* texanus) and other (*M. trilobatus* + *M. typicus* + *M.* flavus). The results first time disclosed the phylogenetic affinities and host shifting of *M. flavus* from holostean fishes to teleosts.

23. Microphallidae (Ward, 1901)

The members of this family found in the intestine of most vertebrates especially in birds and the main species are *Maritrema subdolum*, *Plenosoma minimum*, *Metamaritrema prolixum* and *Odhneria odhneri*.

Hust *et al*. (2004) identified and characterized the two congeneric microphallid species (*Maritrema subdolum* and microphallid sp. No. 15) from snail using ITS-1, 5.8S and ITS-2 rDNA sequences. The sequence divergence was 3.9 % for ITS-1, 2.1 % for ITS-2 and 2.9 % for overall sequences of the two species. Along with morphology the molecular data of cercariae of congeneric species suggested the two distinct species.

Warberg *et al*. (2005) investigated three closely related species (*Levinseniella* group) from snail and marine crustaceans using ITS-1 rDNA sequences. The worker observed the uniform patterns of repetitive 130 bp sequences in ITS-1 region in microphallid species.

Pina *et al*. (2011a) morphologically and molecularly characterized *Maritrema portucalensis* sp. nov. from crab using 18S, ITS-1 and 5.8S rDNA sequences. Sequence alignments of *M. portucalensis* sp. nov. disclosed 28 bp variations (3.8 % divergence) with *M. subdolum* and showed 100 % identity with microphallid sp. no. 15 cercaria from snail.

Al-kandari *et al*. (2011) first time genetically characterized the larval stages of *Maritrema eroliae* from gastropod from Kuwait using partial 28S, 18S, ITS-1 and ITS-2 rDNA sequences. The ITS-2 sequences comparison of all samples showed identity in length and composition whereas ITS-1 region showed 99.8 % homology with two nucleotide variation. ITS-1 region comparison disclosed that *M. eroliae* was closely identical to *Maritrema* sp. SP-2011 (10.9 % variation). The phylogenetic analyses of different sequences of *M.* cf. *eroliae* formed the clade with other microphallids.

Pina *et al*. (2011b) depicted the anatomy and topography of metaceracria of *Microphallus primas* from crabs. The morphological and molecular characterization of metacercaria was performed using 18S, ITS-1, 5.8S and 28S rDNA sequences. ITS-1 sequence analysis confirmed the two forms of isolated cysts (spherical and oval) belong to the same species. 28S rDNA sequence comparison of cysts with *M. primas* showed 100 % homology.

Presswell *et al*. (2014) morphologically and genetically characterized the two new species of microphallids (*Maritrema deblocki* n.sp. and *M. poulini* n. Sp.) from New Zealand using 28S (LSU) and ITS-1 rDNA region. Diversity in the *Maritrema* sp. was 2.6 – 17.1 % in ITS-1 rDNA. The lowest divergence was 2.6 % between *M. eroliae* and *M. novaezealandense* for ITS-1 region. ITS-1 region of *M. oocysta* was diverged 9.1 % and 8.8 % from *M. deblocki* and *M. poulini*, respectively. 3.9 % divergence in ITS-1 region was detected for *M. deblocki* and *M. poulini*. *M. deblocki* and *M. poulini* suggested as different species on the basis of genetic diversity among 28S and ITS-1 region. The sister species (*Maritrema deblocki* n.sp. and *M. poulini* n. Sp.) with *M. novaezealandense*, *M. heardi*, *M. eroliae* and *M. oocysta* formed the clade.

24. Opecoelidae (Ozaki, 1925)

The members of family inhabit marine and freshwater teleosts, occasionally amphibians and important species are *Genitocotyle mediterranea*, *Discoverytrema gibsoni*, *Dactylostomum gracile* and *Apertile holocentri*.

Born-Torrijos *et al*. (2012) reported the morphological and molecular discrimination of larval stages of *Macvicaria obovata* and *Cainocreadium labracis* from gastropods using ITS-1, 5.8S, ITS-2 and partial 28S (domain D1-D2) rDNA sequences. The comparative analysis of ITS rDNA sequences from larval stages and early reported sequences helped to describe the life cycle of *Macvicaria obovata* and the first time morphological identification of *Cainocreadium labracis*.

Andres *et al*. (2014) examined two opecoelids (*Helicometra fascia-*

ta and *Macvicaria crassigula*) from fishes using partial 18S, ITS-1, 5.8S, ITS-2 and partial 28S rDNA sequences. Any intra-specific variations were not noticed between two replicates of *M. crassigula* sensu stricto whereas 0.4 % variation was detected in case of *H. fasciata* in ITS-1. Finally, findings disclosed *H. manteri* sp. nov. as a new species.

25. Opisthorchiidae (Looss, 1899)

The members of this family reside in the liver, bile duct and gall bladder of birds, mammals and sometimes in digestive tract of reptiles and teleosts. The main species are *Clonorchis sinensis*, *Opisthorchis felineus*, *Evranorchis ophidiarum* and *Gomtia piscicola*.

Lee & Huh (2004) distinguished the isolates of *Clonorchis sinensis* from Korea and China using 18S, ITS-1, ITS-2 and mtDNA (COI) sequences. In 18S, ITS-1, ITS-2 and COI sequences of isolates of *C. sinensis*, very little intra-specific nucleotide substitution and few insertion and deletion in ITS-1 were noticed. The sequences of three isolates were highly conserved and indicated that Korean and Chinese isolates were similar at the DNA level.

Liu *et al*. (2007) studied the ITS-1 and ITS-2 rRNA sequences from ancient and modern eggs of *Clonorchis sinensis*. The findings showed that ITS-2 sequences of ancient sample were similar to of modern sample while ITS-1 was differed at 15 nucleotide sites. The ITS-1 sequence of ancient specimen differed from modern samples due to sequence divergence or polymorphism.

Kang *et al*. (2008) reported the molecular identification of flukes (*Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis*) using PCR-RFLP technique for 18S, ITS-1 and 5.8S rDNA region. Any intra-specific polymorphism or cross-reaction was not observed in the restriction pattern of ITS-1 region. The results were inadequate to differentiate for cladogenesis among species.

Skov *et al*. (2008) morphometrically and molecularly distinguished *Pseudamphistomum truncatum* metacercariae from fishes and adults from mink using ITS-2 rDNA sequence. The molecular identification of pseudamphistomosis in Northern Europe was the affirmed diagnosis of *Pseudamphistomum* sp. Sequences of parasite from roaches and mink confirmed it as opisthorchiid fluke and nucleotide similarity with *Clonorchis sinensis* and *Opisthorchis viverrini* from Asia might suggested the close relationship and rather latest dispersal.

Traub *et al*. (2009) developed PCR method for ITS-2 rDNA for adult flukes (*Opisthorchis viverrini*, *Clonorchis sinensis* and *Haplorchis taichui*) and eggs in Thailand. The PCR-RFLP technique allowed for the detection of *O. viverrini* and *C. Sinensis* infection in human.

Ai *et al*. (2010) investigated the sequence diversity in ITS-1, 5.8S, ITS-2 and mtDNA (COI, NDI) of metacercariae of *Metorchis orientalis* from China. The findings indicated that sequences of ITS, COI and NDI were moderately conserved among isolates of *M. orientalis* and fairly differed from other species.

Sun *et al*. (2011) devised the effective PCR-based method (MLPA assay) for the molecular characterization of *Clonorchis sinensis*, *Opisthorchis viverrini* and *O. felineus* with the use of ITS-1 region.

Huang *et al*. (2012) established the PCR assay to diagnose *Clonorchis sinensis* from fish, cat and human using ITS-1 and ITS-2 rDNA sequences. This assay was the confirmatory tool to assess the infection and molecular epidemiological examination of parasite.

Tatonova *et al*. (2012) reported the molecular identification and sequence difference in *Clonorchis sinensis* from Russia using ITS-1, 5.8S and ITS-2 region. The findings expressed the 100 % homology for all 5.8S and ITS-2 sequences while two levels of intra-specific differences were observed in complete ITS-1 region. This intra-genomic variation generated due to C/T polymorphism at single site. The phylogenetic analyses divided ITS-1 region in two different clusters with widespread ribotype and expressed the high ribotype diversity in China than Russia and Korea.

Brusentsov *et al*. (2013) first time surveyed the genetic variety of *Opisthorchis felineus* population from Eastern Europe, Northern Asia and Central Asia, using mitochondrial COI, COX3 and ITS-1 region sequences. This study reported the low genetic diversity of *O. felineus* throughout large geographic areas.

Sun *et al*. (2013) investigated the intra-species genetic variations of *Clonorchis sinensis* using multilocus analysis of eight genes (ITS-1, act, tub, ef-1a, cox1, cox3, nad4 and nad5) from China. The study suggested that ITS-1 was effective marker for detecting infection of *C. sinensis* throughout the world. The nuclear and mitochondrial phylogeny analyses formed three clusters, which showed low divergence in populations.

Xiao *et al*. (2013) investigated the genetic diversity of *Clonorchis sinensis* from different host (cat, dog, human and rabbit) from China by means of sequences of mitochondrial NAD2, NAD5 and ITS-1 region. Intra-specific variations were 0 – 1.7 %, 0 – 1.4 % and 0 – 0.9 % for ITS-1, NAD2 and NAD5, respectively. The results expressed the low-level intra-specific variations between rDNA and mtDNA sequences of *C. sinensis*.

26. Paragonimidae (Dollfus, 1939)

The members of this family inhabit the lungs of mammals and the main species are *Paragonimus westermani* and *Paragonimus skrjabini.*

Ming-gang *et al*. (2004) reported the variations in *Paragonimus skrjabini* populations from China using ITS-2 and mitochondrial COI gene sequences. The little variations were observed among the populations of *P. skrjabini* and some similarity between *P. miyazaki* of Japan and Fujian strains of *P. skrjabini* was also noticed. The populations in this study considered as different geographical strains of *P. skrjabini*.

Blair *et al*. (2005) distinguished morphometrically and molecularly the metacercariae of *Paragonimus skrjabini* and *P. miyazakii* from dogs or cats using ITS-2 and mitochondrial COI gene sequences. ITS-2 sequence analysis showed less variation within species complex (*P. skrjabini* and *P. westermani*) and small extent of intra-population polymorphism. The *P. skrjabini* species complex was monophyletic according to ITS-2 and COI gene analysis. *P. skrjabini* from China and *P. miyazakii* form Japan were phylogenetically very close, so both the species should be considered as subspecies *P. skrjabini miyazakii*.

Sugiyama *et al*. (2005) distinguished the metacercariae of *Paragonimus heterotremus* and *P. westermani* from Thailand using ITS-

2 rDNA sequences. On the basis of DNA analysis *P. heterotremus* and *P. westermani* were different.

Le *et al*. (2006) reported the molecular identification and phylogenetic relationship of *Paragonimus* samples (eggs, miracidia, metacercariae and adults) from several hosts from Vietnam using complete ITS-2 and partial mitochondrial COI sequences. The ITS-2 sequences showed homology in all the specimens. Phylogenetic analyses disclosed that all *P. heterotremus* specimens from Veitnam, Thailand and China created a distinct group. The molecular data confirmed all the specimens as *P. heterotremus* from Vietnam.

Zhou *et al*. (2008) established the phylogenetic relationships of metacercariae of *Paragonimus proliferus* from China using ITS-2 and mitochondrial COI genes. The findings expressed that ITS-2 and COI sequences of *P. proliferus* of China were 100 % similar to *P. hokuoensis* from Vietnam and also similar to *P. hokuoensis* from China.

Sugiyama *et al*. (2008) differentiated the metacercariae of *Paragonimus westermani* and *P. heterotremus* from crab in Thailand using ITS-2 rDNA with the help of PCR-RFLP technique.

Prasad *et al*. (2009) studied the ITS-2 sequence region of metacercariae of *Paragonimus* sp. from crab from India using molecular morphometric methods (ITS-2 secondary structure homologies and phylogenetic relationships). They found three similar topologies for seven species of *Paragonimus*. ITS-2 motifs can be utilized for species identification along with RNA secondary structures.

Fischer *et al*. (2011) genetically characterized metacercariae of *Paragonimus kellicotti* found in cray fish from USA using ITS-2 region, ITS-1, 5.8S and 28S rDNA sequences. The ITS-2 sequences from metacercariae of *P. kellicotti* showed 99 % similarity with GenBank sequences of *P. kellicotti* along with one bp deletion at site 55 and transversion at site 298. The study revealed that *P. kellicotti* is more closely related to *Paragonimus* sp. such as *P. mexicana* and *P. westermani*.

Intapan *et al*. (2012) first time reported the verification of human pulmonary paragonimiasis due to *Paragonimus pseudoheterotremus* (genetically identical to *P. heterotremus* and considered as sister species) using ITS-2 and mitochondrial COI gene sequences. ITS-2 and COI sequences form the eggs showed 98 – 100 % similarity with those of *P. pseudoheterotremus*. In *P. heterotremus* partial ITS-2 showed 99 – 100 % similarity while partial COI sequence showed 90 – 95 % similarity.

Sanpool *et al*. (2013) molecularly characterized the metacercariae of *Paragonimus heterotremus* and *P. pseudoheterotremus* from crabs from Thailand using ITS-2 and mitochondrial COI gene sequences. The ITS-2 sequence analysis revealed close identity among all specimens (99 – 100 %). Indian *P. heterotremus* formed the sister group with *P. pseudoheterotremus* and *P. heterotremus* from Thailand, Vietnam and China created a distinct clade. In the *P. heterotremus* complex from Thailand obvious genetic variation was observed.

27. Paramphistomidae (Fischoeder, 1901)

The members of the family were distinguished through the lack of oral sucker and position of acetabulum near to posterior end of body in adults and cercariae (Jones, 2005) and affect cattle and sheep. The important species are *Paramphistomum cervi*, *Calicophoron calicophorum*, *Gigantocotyle symmeri* and *Explanatum explanatum*.

Rinaldi *et al*. (2005) genetically identified *Calicophoron daubneyi* from different hosts from Italy using ITS-2, 5.8S and 28S region sequences with the help of PCR-RFLP. Any intra-specific variations were not detected in nucleotide constitution of ITS-2+ (100 % similarity) while inter-specific variations in ITS-2+ of *C. daubneyi* and *C. calicophorum* (97.2 % similarity) and *C. microbothrioides* (97.4 % similarity) were observed. Out of 16 sites possessing variable nucleotides, 15 showed single base substitution (point mutation) while one exhibited single base deletion.

In the study Lotfy *et al*. (2010) analyzed specimens of cercariae, rediae from snail and flukes from Kenya, Tanzania, Egypt and Nepal using ITS-2 as genetic marker. In the specimens, eight *Calicophoron microbothrium* species showed identity in ITS-2 sequences along with cercariae from *Bulinus forskalii*

Shylla *et al*. (2011) genetically identified *Homalogaster paloniae*, *Calicophororn calicophorum* and *Orthocoelium streptocoelium* of bovine hosts in India using ITS-2 rDNA sequences. The Indian isolates of these species expressed 99 % similarity to isolates from Japan. The species varied from each other in few nucleotides and revealed typical four helix ITS-2 secondary structure. Monophyly of the clade of Japanese and Indian paramphistomids is well affirmed.

Sanabria *et al*. (2011) firstly reported the molecular identification of *Paramphistomum leydeni* from different origin, definite hosts and compared to *Fasciola hepatica* and *Notocotilidae* cercariae samples using ITS-2, 5.8S and 28S partial ITS-2+ rDNA sequences. This PCR method could be valuable tool for analyzing specimens from Argentina and adjacent territories along with additional genus or species characterization.

Ichikawa *et al*. (2013) studied amphistomes from cattle and water buffaloes from Myanmar for morphological and molecular characterization using ITS-2, partial 5.8S and 28S rDNA sequences. ITS-2 sequences of 55 samples of *Explanatum explanatum* expressed similarity and varied at 7 nucleotide sites compared to *Paramphistomum leydeni*.

Zheng *et al*. (2014) considered 5 specimens of *Paramphistomum cervi* to establish phylogenetic relationships using 18S, ITS-1, 5.8S, ITS-2, 28S rDNA and IGS regions. No variations were detected in the length and composition of analyzed sequences. In the samples 5.8S and ITS-2 sequences showed 100 % similarity and IGS region expressed 53.7 – 99.8 % identities. The 18S rDNA analyses pointed the evolutionary relationship of *P. cervi* was close to other members of Paramphistomidae than Fasciolidae, Echinostomatidae, Dicrocoeliidae, Paragonimidae and Opisthorchiidae.

28. Pleurogenidae (Looss, 1899)

The members of this family found in amphibians, reptiles and occasionally in fish and mammals. The important species are *Langeronia macrocirra*, *Magniuterina leoi*, *Pleurolobatus lobatus* and *Pleurogenes claviger*.

Martínez-Salazar & León-Règanon (2010) morphologically and molecularly examined specimens of *Langeronia* sp. from Mexico

using ITS-2 and mitochondrial COI gene sequences. ITS-2 region is generally highly conserved so did not reveal any phylogenetic information in this study.

29. Psilostomidae (Looss, 1900)

The members of the family inhabit birds and amphibians and the important species are *Psilochasmus oxyurus*, *Psilotrema simillimum*, *Psilostomum brevicolle* and *Ribeiroia insignis*.
Wilson *et al*. (2005) studied the systematics and biogeography of *Ribeiroia* sp. from amphibians using the ITS-1 and ITS-2 region sequences. This study first time reported ITS sequence from *Cathaemasia* and *Ribeiroia* sp. Six different sites were observed in ITS-2 sequences of *R. ondatrae*, *R. marini* and *Cercaria lileta* pointing towards sequence variation among species. Phylogenetic relationships disclosed that *R. ondatrae, R. marini* and *C. lileta* create a monophyletic group.

30. Sanguinicolidae (von Graff, 1907)

The members of the family inhabit blood of fishes and important species are *Sanguinicola inermis*, *Parasanguinicola vastispina*, *Pearsonellum corventum* and *Deontacylix ovalis*.
Nolan & Cribb (2006) reported the molecular phylogeny of sanguinicolid blood flukes from several species of fishes using ITS-2 rDNA region for host/parasite/location combinations. Any intra-specific differences were not observed among replicate sequences while inter-specific variations were 0.3 – 12.7 % from 1 – 41 nucleotide sites.
Holzer *et al*. (2008) described the morphological and molecular aspects of *Cardicola aurata* n. sp. from the Spain along with prevalence and seasonality using partial 28S and ITS-2 rDNA sequences. The phylogenetic results suggested the close relationship between *Cardicola* and *Paradeontacylix* and *C. aurata* n.sp. was intermediary in position to these two genera. Molecular and morphological investigations on other members of *Cardicola* were required for phylogeny and evolution of flukes more precisely in this family.

31. Schistosomatidae (Stiles & Hassall, 1898)

The members of the family mature in blood vascular systems of hosts (birds, mammals and crocodiles) and generally are dioecious. The main species are *Schistosomatium pathlocopticum, Schistosoma mansoni*, *Orientobilharzia bomfordi* and *Heterobilharzia americana*.
Kolářová *et al*. (2006) morphologically and molecularly examined the schistosomes from *Cygnus Cygnus* using 18S, ITS-1, 5.8S and ITS-2 region sequences. Through findings new genus of *Allobilharzia visceralis* gen. nov., sp. nov. was discovered. Phylogenetic analyses of isolate from swan revealed the sister relationship to other bird schistosomes (*Trichobilharzia* etc.).
Rudolfová *et al*. (2007) reported the schistosomes infection in wildfowl from Czech and Poland using ITS-1, 5.8S, ITS-2 and 28S rDNA sequencing. The four species of schistosomes were identified, *Bilharzia polonica, Trichobilharzia regent, T. szidati* and undetermined schistosome.
Brant & Loker (2009), examined birds and snails from North America to deduce the phylogenetic relationship among schistosomes (*Trichobilharzia* sp.) on the basis of 18S, 28S, ITS-1, 5.8S, ITS-2 and mitochondrial COI gene sequences. The phylogenetic analyses rendered to identification of parasites, evolution, diversification and host-parasite relationships.
Aldhoun *et al*. (2009) described morphological and molecular characterization of cercariae from snails (*Lymnaea stagnalis*, *Radix auricularia*, *Valvata macrostoma*) from Finland using ITS rDNA sequences. Cercariae from *L. stagnalis* and *R. auricularia* belong to *Trichobilharzia szidati* and *T. franki*, respectively, affirmed the diversity of two species in Europe and other isolates from *V. macrostoma* corresponded to unknown schistosome.
Jouet *et al*. (2010) attempted to establish the phylogenetic position of *Trichobilharzia franki* from *Radix auriculaia* and *R. peregra* from France and Iceland, using 28S (D2 domain), ITS-1, ITS-2 rDNA and mitochondrial COI gene sequences. The authors showed the existence of two clades based on specificity of intermediate hosts (*R. auriculaia* and *R. peregra*) and the separation of species of *T. franki*.
Cipriani *et al*. (2011) examined snails for *Trichobilharzia franki* from Italy to identify the impact of human cercarial dermatitis using 18S, ITS-1 and ITS-2 rDNA sequences. In this study out of 125 snails, 12 were distinguished as *Radix auricularia* infected by *T. franki* with infection prevalence of 9.6 %.
Rizevsky *et al*. (2011) on the basis of ITS region analysis reported the systematic position and biodiversity of avian schistosomes from Belarus. Through sequence analysis of ITS region, 4 species of schistosomes were identified (*Trichobilharzia szidati*, *T. franki*, *Bilharzia polonica* and novel *Trichobilharzia* sp.).
Kolářová *et al*. (2013) reported schistosome infection in *Mergus serrator* from Iceland and characterized morphologically and molecularly using 28S (D2), ITS-1, ITS-2 rDNA and mitochondrial COI gene. The molecular results of D2-ITS-2 and COI region proved that flukes belong to *Trichobilharzia* sp. (*T. mergi* sp. n.). D2-ITS-2 sequences revealed the 100 % similarity between eggs and adults. According to ITS-1 region analysis, haplotypes in this study belonged to same species with only one difference noticed out of 948 bp.
Brant *et al*. (2013) identified the new genus, *Anserobilharzia brantae* n. comb. from geese from Europe and North America using morphological and molecular characterization through 28S, ITS and mitochondrial COI gene sequences.
Schuster *et al*. (2014) discovered new species, *Gigantobilharzia melanoides* from chickens and described its complete life cycle along with establishment of phylogenetic relationship using partial 18S, 28S and complete ITS-1, 5.8S and ITS-2 rDNA region. Through phylogenetic results *G. melanoides* is proved as distinct and new species.

32. Strigeidae (Railliet, 1919)

The members of the family inhabit birds and mammals and important species are *Parastrigea intermedia*, *Strigea falconis*, *Ophiosoma patagiatum* and *Cotylurus cornutus*.
Portier *et al*. (2012) first time molecularly described *Alaria alata* furcocercariae from two intermediate host (*Planorbis planorbis*

and *Anisus vortex*) from France using 18S , ITS-2 r DNA, mitrochondrial 16S and COI gene sequences. Through ITS-2 marker haplotypes were detected and confirmed by other DNA markers.
Hernández-Mena et *al*. (2014) reported *Parastrigea plataleae* n. sp. from spoonbill from Mexico and morphometrically and molecualry characterized using ITS rDNA and mitochondrial COI gene sequences. The molecular variations for *P. plataleae* n.sp., *P. cincta* and *P. diovadena* were 0.5 – 1.48 % for ITS and 9.31 – 11.47 % for COI. The phylogenetic analyses revealed that three species of *Parastrigea* showed reciprocal monophyly.

33. Zoogonidae (Odhner, 1902)

The members of the family inhabit teleosts and occasionally elasmobranch fishes and the main species are *Diphtherostomum brusinae*, *Zoogonoides viviparous*, *Neozoogonus californicus* and *Parvipyrum acanthuri*.
Francisco *et al*. (2010) first time morphologically and genetically distinguished *Diphtherostomum brusinae* from mollusc from Portugal using partial 18S and ITS-1 rDNA sequences. In the 18S region, any intra-specific polymorphism was not detected due to 100 % homology among all the samples. ITS-1 region analysis showed intra-specific variations and also fixed polymorphisms of *D. brusinae* metacercariae from multiple hosts.

Conclusion

The digenean genera affecting mainly the humans and livestock are best studied till date on the ground of immunology, epidemiology and other medical sides (Lockyer *et al*., 2003; Snyder, 2004). Recently, efforts have been made towards the understanding of origin, diversification and phylogenic interrelationships. There is a great urge to increase the knowledge on other families to generate data for better perceptive to resolve the evolutionary and taxonomical problems using different molecular markers.
To the best of our knowledge so far 136 studies of 33 digenean families, including 78 genus and 114 species (during 2004 – 2014), infecting hosts from countries in different geographical regions have been carried out. To update the information, the present review is being presented here. As observed, ITS-1 and ITS-2 of rDNA provided trustworthy markers for molecular systematics along with mitochondrial DNA (Huang *et al*., 2004; Razo-Mendivil *et al*., 2004; Miura *et al*., 2005; Maurelli *et al*., 2007; Al-Kandari *et al*., 2011; Caffara *et al*., 2011; Amor *et al*., 2011; Huang *et al*., 2012; Bott *et al*., 2013; Presswell *et al*., 2014). Only 47 studies mentioned the intra-specific (0.05 – 10.9 %) or inter-specific variations (0.01 – 16 %) and inter-generic variations of 2.67 – 21.0 %. of 47 studies, 11 expressing variations in ITS-1 region, 20 in ITS-2 region and 16 in both ITS-1 and ITS-2 region. Apart from ITS region, mitochondrial genes like COI, NDI (Caffara *et al*., 2014; Heneberg *et al*., 2014; Locke *et al*., 2011) etc. are also included to corroborate the findings. With the help of these studies, 3 new genera (*Caulanus* gen. nov., *Latuterus* gen. nov., (Miller & Cribb, 2007) and *Allobilharzia* gen. nov. (Kolářová *et al*., 2006) and 49 new species have been established.
Molecular studies of ITS rDNA region verified as promising genetic marker having broad range of application for phylogenetic analyses at generic and species level (Blair *et al*., 1999; Iwagami et al., 2000; Huang *et al*., 2004; Tandon *et al*., 2007; Goswami *et al*., 2009). According to Vilas *et al*. (2005) and Al-Kandari *et al*. (2011) ITS-1 is more variable than ITS-2 due to variable nucleotide repeats (Warberg *et al*., 2005 in microphallid species) and accretion of substitutions at higher rate (Morgan & Blair, 1995; van Herwerden *et al*., 1999; Tkach *et al*., 2000). Slow rate of concerted evolutionary processes (hybridization, incomplete lineage sorting and retention of ancestral polymorphism) can cause the sharing of various ITS regions among trematodes (Vilas *et al*., 2005) and reveal the possible existence of intermediate genotypes (Huang *et al*., 2004; Itagaki *et al*., 2005a; Itagaki *et al*., 2005b; Liu *et al*., 2014; Mufti *et al*., 2014). Sequence length variations, due to mutations (Tkach & Kinsella, 2011) or polymorphism (Liu *et al*. 2007) in ITS-1 region point towards the differentiation of species on the basis of genetic diversity (Presswell *et al*., 2014). Miura *et al*. (2005) reported the absence of heterozygosity in ITS-1 genes, which did not provide any support of gene flow among different lineages. Some workers suggested the ITS-2 as effective marker for species identification, phylogenetic relations and developmental stages as well as molecular epidemiology and infection control (Rinaldi *et al*., 2005; Curran *et al*., 2006; Bazsalovicsová *et al*., 2010; Lotfy *et al*., 2010; Shylla *et al*., 2011; Ghatani *et al*., 2012; Phalee *et al*., 2014; Bian *et al*., 2013; Portier *et al*., 2012). In helminth parasites, ITS pseudogenes and mitochondrial genes are present and identification of ITS region is not easy due to non-protein coding nature as compared to mitochondrial genes (Benasson *et al*., 2001). For the efficient marker, rapid rate of evolution is necessary and mtDNA of flukes evolved more swiftly than ITS region. In the small population size cryptic species can be easily distinguished by mtDNA than ITS of large population size (Vilas *et al*. 2005). The mitochondrial COI genes could be suitable and strong tool for the primary evaluation of diversity and host-parasite interaction than ITS sequences (Locke *et al*., 2010a; Locke *et al*., 2010b; Vilas *et al*., 2005).
However, we are of the opinion that the only ITS region is insufficient to determine the phylogenetic tree, genetic variations and species differentiation at different developmental stages of parasites in multiple hosts. Therefore, inclusion of other genes such as mitochondrial DNA, SSU (small ribosomal subunit), LSU (large ribosomal subunit), IGS (inter generic spacer) region and microsatellite etc. necessitate to study the different molecular systematic aspects.
In the different geographical regions of Asia, Africa and Europe etc. major studies were carried on the members of the family Fasciolidae, followed by Schistosomatidae, Opisthorchidae, Paragonimidae and Paramphistomidae. In Indian subcontinent major studies have been conducted only on the Fasciolidae, Paragonimidae and Paramphistomidae etc. family members (Prasad *et al*., 2009; Prasad *et al*., 2011; Mohanta *et al*., 2014; Shylla *et al*., 2011). More localities from South America, North America, Africa and other continents are therefore, to be explored for empathizing host-parasite relationship including the depiction of life cycle stages with their environment along with genotypic variations.
Most of the phylogenetic studies have been carried out on the basis of morphological, anatomical and morphometrical analysis of different families of trematodes. Till now, phylogenetic interrela-

tionships of several genera and species are still unresolved and hence there is great need to adjudicate evolutionary issues with the help of modern day molecular and bioinformatics approaches.

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