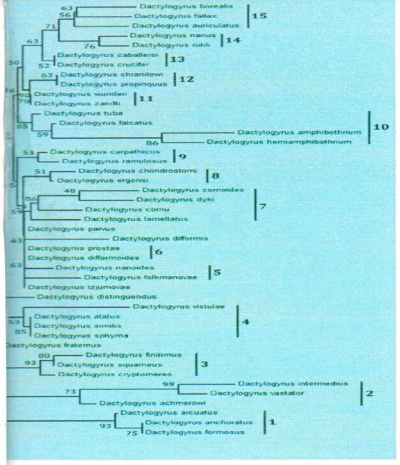
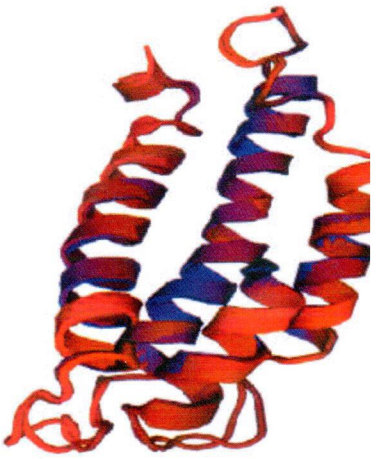




A

UGC-Major Research Project

[F.No.: **41-34/2012 (SR)] Final Report**

on

In-silico phylogenetic studies on some members of class Monogenea Carus, 1863

Submitted to

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

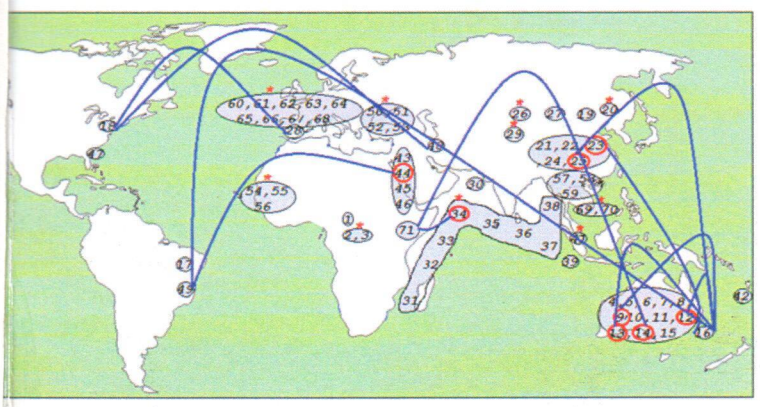
By

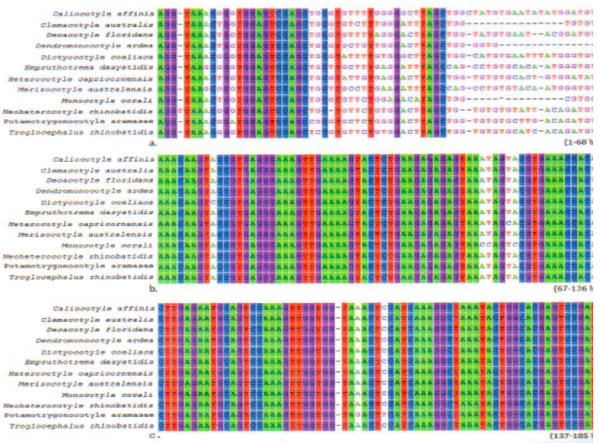
Dr. P. V. Arya, *M.Phil., Ph.D.*

Principal Investigator &Assistant Professor

Dept. of Zoology, Dyal Singh College (University of Delhi),

Lodhi Road, New Delhi-110003





दयाल सिंह कॉलेज
(दिल्ली विश्वविद्यालय)
लोधी रोड, नई दिल्ली – 110003
दूरभाष: 24367819, 24365948
फैक्स : 24365606

DYAL SINGH COLLEGE
(University of Delhi)
Lodi Road, New Delhi – 110003
Telephone : 24367819, 24365948
Fax: 24365606

Ref. No.:DSC/ADMN:.....

Date : 12.04.2016

Certificate of Originality

This is to certify that the Project Investigators and the Project Fellow of UGC-MRP having Project code **F.No.:41-34/2012(SR)** and title ***In silico* phylogenetic studies on some members of Class Monogenea Carus, 1863** awarded to **Dr. P. V. Arya, Dept. of Zoology, Dyal Singh College** College/Centre have carried out the research work submitted as Report to the University Grants Commissions, Delhi. The research work and the report are original. Any plagiarism dispute arising out of the project will be our responsibility.

Dr. P.V. Arya
Principal Investigator
Assistant Professor
Dept. of Zoology

Title : *In silico* phylogenetic studies on some members of Class Monogenea Carus, 1863
F.No.: 41-34/2012 (SR) **Funding Agency :** University Grants Commission.

INDEX

1. Introduction

There are more than 5000 known species of monogenea and all of them are reported as parasitic (de Meeus and Renaud, 2000). Monogeneans are highly host-specific aquatic ectoparasites, and exhibit important morphological variability in their attachment organ (opisthohaptor or haptor). This variability is often thought to be shaped by adaptive processes. However theoretically these species should have reflected the differences at the molecular level as well. Since the species appears to be morphologically different and hence either they are different at molecular level or they are passing through the process of speciation and all the final evolutionary developments have not finally occurred. If the morphological difference among species is reflected by their molecular divergence, and if the closest species are really different at the molecular level (*i.e.*, distinct species). The amount of interspecific differences relationship can be assessed with DNA sequence comparison, in particular by using the internal transcribed spacers (ITS) (Hillis *et al*., 1996). The ITS lies in the ribosomal DNA cluster between the 18S rRNA and 28S rRNA coding regions. The ITS is divided into two (ITS1 and ITS2), separated by the gene coding for 5.8S rRNA. The ribosomal coding regions are relatively slow-evolving and highly conserved, while the ITS are known to show a lot of variability (Hillis *et al*., 1996 and Hillis & Dixon, 1991) because of their faster evolving rate. The ITS have already been used for diagnostic purposes at the species level among the Digenea (Adlard *et al*., 1993) and the Nematoda (Newton *et al*., 1998). Evolutionary relationships of monogeneans have also been depicted through rDNA analysis (Baverstock *et al*., 1991; Cunningham *et al*., 1995 and Littlewood *et al*., 1997), and among-species differences in ITS have been assessed for monogeneans (Cunningham, 1997).

Indeed, as monogeneans are highly host specific, they have been suggested to show tight coevolutionary interactions with their hosts (Noble *et al*., 1989). This has been shown at the family level (Boeger and Kritsky, 1997), where cospeciation events are widespread, but remains to be investigated at a finer scale (genus or species level) where coevolution studies are scarce (Klassen and Beverley-Burton, 1987).

Recently many studies have brought a new dimension to relations among various members of Class Monogenea using various Phylogenetic tools. But the studies are mainly somewhat localized in nature and hence there is a great need to diversify (source data) and integration (result data) of studies as far as possible.

2. Historical Review

In past decades much importance has been given by the researchers to the morphological studies and identification of monogeneans. Studies related to population dynamics, host parasite relationship have also been very popular along with some selected control studies. A paradigm shift have also been observed of using molecular biology/bioinformatics tools in establishing phylogentic relationship among various taxa of monogeneans which previously exclusively relied upon morphological characters only. Some of the recent workers and their work related to phylogeny as well as phylogeny of monogeneans must include following work.

International status : Ali *et al*. (1991); Allard *et al*. (1992); Aquaro *et al*. (2009); Ax (1984); Banks *et al*. (2006); Barker (1994); Basaglia (1991); Baverstock *et al*. (1991); Bentz *et al*. (2001); Blair (1993); Boeger (1997); Boeger and Kritsky (2001); Boeger and Kritsky (1989); Boeger and Kritsky (1997); Bohning-Gaese *et al*. (2003); Booton *et al*. (1999); Boris *et al*. (2005); Bourdy *et al*. (2003); Bremer (1994); Briolay *et al*. (1998); Brooks and McLennan (1991, 1993 & 1996); Buchmann *et al*. (2009); Bychowsky (1961); Cable *et al*. (1999); Cantatore *et al*. (1994); Chakraborty *et al*. (2006); Champaign *et al*. (1997); Charleston (1998); Chilton *et al*. (1995); Chisholm *et al*. (2001); Cribb *et al*. (2002); Cunha *et al*. (2002); Cunningham (1997); Cunninghamn *et al*. (1995, 2000 & 2001); Day and Young (2004); De Meeus and Renaud (2002); Desdevises (2001); Desdevises *et al*. (2000, 2001 & 2002); Dieckmann and Doebeli (1999); Domingues (2009); Domingues and Marques (2009); Domingues and Boeger (2008); Dominigues *et al*. (2009); Durand *et al*. (2002); Gilles *et al*. (1998 & 2001); Gotelli (2000); Gotelli and Ellison (2002); Gotelli and Entsminger (2001); Guégan and Agnèse (1991); Hafner *et al*. (1994); Hall (1999); Hanel and Sturmbauer (2000); Hansen *et al*. (2006); Harris *et al*. (1999); Hernandez *et al*. (2009); Hey (2001); Hillis and Dixon (1991); Hillis *et al*. (1996); Hoberg (1986); Huelsenbeck *et al*. (2000); Huyse and Volckaert (2002a & 2002b); Jeanmougin *et al*. (1998); Jondelius and Thollesson (1993); Jousson *et al*. (1998 & 2000); Jovelin and Justine (2001); Justine (1991a, b & 2001); Kennedy and Bush (1992); Kontula and Väinölä (2001); Korbsrisate *et al*. (1991); Krasnov *et al*. (2005); Kritsky and Lim (1995); Kumar *et al*. (1993 & 2001); Larkin *et al*. (2007); Lawton (1999); Li (1997); Lim (1996); Lim *et al*. (2001); Littlewood *et al*. (1997, 1998 & 1999); Litvaitis and Rohde (1999); Maddison and Maddison (1992); Madlen *et al*. (1991); Malmberg (1998); Manly (1998); Matejusová *et al*. (2001); Meinilä *et al*.

(2002); Mendlova *et al*. (2009); Milinkovitch *et al*. (1993); Mollaret *et al*. (1997 & 2000); Morand *et al*. (1999 & 2002); Moravec (2001); Mouillot *et al*. (2003 & 2005); Nadler *et al*. (1990); Neefs *et al*. (1993); Newton *et al*. (1998); Nilsson *et al*. (2001); Noble *et al*. (1989); Olson and Littlewood (2002); Paterson and Gray (1997); Paterson and Poulin (1999); Paterson *et al*. (1993); Paterson and Banks (2001); Peres-Neto (2004); Perkins *et al*. (2009); Philippe (1993); Poisot *et al*. (2008); Posada and Crandall (1998); Poulin (1996, 2002 & 2004); Riutort *et al*. (1993); Rohde (1990, 1991 & 1996); Rohde *et al*. (1993 & 1994); Ronquist (1995 & 1997); Sasal *et al*. (1998); Šimková *et al*. (2001, 2003, 2004a, 2004b & 2006); Smith *et al*. (1986); Stevenson *et al*. (1995); Sunderland and Malmberg (1970); Sunderland *et al*. (2000 & 2001); Swofford (1990); Toft and Karter (1990); Tofts and Silvertown (2000); Tokeshi (1999); Valtonen *et al*. (1990); Verneau *et al*, (1997); Vickeryand Poulin (1998); Webb *et al*. (2002); Weither *et al*. (1998); Zardoya and Doadrio (1999); Zardoya *et al*. (1999); Zietara and Lumme (2002); Zietara *et al*. (2000 & 2002). Now a day a combination of studies is taken up to establish the phylogenetic relations among various taxa. This is especially essential to ensure actual position of the organism based of molecular data rather than purely morphological which could be by and large insufficient.

National Status : In past years, many investigators have been engaged in the morphological studies of monogeneans in India. But Phylogenetic studies have altogether remained unattended by many Indian workers. However, only very few studies have taken this aspect into account *viz*., Agrawal *et al*. (2006 & 2009); Arya (2009); Arya and Singh (2010a, b & c, 2011); Arya and Vinita (2011); Pandey *et al*. (2003); Ramasamy and Brennan (2000); Ramasamy *et al*. (1995); Sharma *et al*. (2009); Sharma *et al*. (2011); Singh and Arya (2002, 2003); Vinita *et al*. (2010) and Tripathi *et al*. (2009a, b, c & d). Chaudhary and Singh (2012a & b), Chaudhary *et al*. (2013) Arya & Singh (2015).

3. Objective

The main objective of the proposed research work was to perform systematic experimental and theoretical studies on phylogenetis relationship among the various members of Class-Monogenea till date of data (date upto which sequences was to be retrieved) using available sequences. During the proposed study it is expected-

⇒ *To develop database of sequences of Class Monogenea their host and other related information till date of data using available sequences*.

⇒ *To compare various available sequences of Class Monogenea till date of data using available sequences*.

⇒ *To propose evolutionary relationship for Class-Monogenea till date of data using available sequences*.

⇒ *To study phylogenetic relationship among members of Class-Monogenea till date of data using available sequences*.

⇒ *To validate relationship among various members, compare it with previously established relation and suggest modification if required till date of data using available sequences*.

To work towards clearing prevailing doubts regarding positions of various members of Class-Monogenea till date of data using available sequences.

4. Methodology

A. Genetic Database for Class Monogena: Details about various members and their taxonomic relations was retrieved from the available literature. Simultaneously a detailed account about the related hosts, habitat was also be developed and updated from the available literature. As the genetic database is updated and large numbers of new sequences are added regularly in order to have a justified approach a date as suitable may be taken into consideration for the retrieval of data (although no final date was decided and data was continued to be added in the interest of project).

B. Sequences of the Class Monogenea: All the available sequences representing class monogenea was retrieved from the genebank and tabled accordingly. Sequences so retrieved was arranged as per their taxonomic status, host, size, type *viz*., 18S rRNA; 28S rRNA; ITS-1 etc. Their nature *i.e*., partial or complete sequence.

C. Sequences Analysis for Class Monogenea: Initially sequence alignment was first performed with Clustal X-2.0.11 (Higgins and Sharp 1988 &1989; Higgins *et al*.,1992 & 1996; Jeanmougin *et al*., 1998; Larkin *et al*., 1992, 1996 & 2007 and Thompson *et al*., 1994 & 1997) and sequences editing using BioEdit (Hall, 1999) as implemented in the BioEdit program. The Phylogenetic tree were reconstructed using Neighbour-Joining (NJ) analysis and UPGMA using MEGA 4.0 (Tamura et al., 2007). Phylogenetic reconstructions (Phenograms) and validation (Lapointe, 1998) with a boostrap procedure (Felsenstein, 1985). Pairwise evolutionary distances calculated following suitable methods & softwares. Bootstrap values set as required (Felsentein, 1985). Application of commercial softwares especially developed for the purpose were also considered to enhance the quality and to treat bulky data.

D. Geo mapping : A new concept was introduced of using geographical distribution and relative manual mapping for studies as required.

Note- Additional method (as applicable) followed were explained in detail in respective publication (where ever applicable).

5. Results & Discussion -

The main objective of the proposed research work was to perform systematic experimental and theoretical studies on phylogenetic relationship among the various members of Class-Monogenea till date of data (date upto which sequences was retrieved) using available sequences. During the present study following time line was proposed and observed with due respect in the study-

Time Line of the Completed Project

	Activities	**Parts**					
		I	**II**	**III**	**IV**	**V**	**VI**
	Period →	**Work to be done during the period**					
1.	Literature survey (of the studies in the area)	Exhaustive literature survey of last 20 years & current studies in the proposed research area will be carried out					
2.	Purchase of equipments	Equipments purchased					
3.	Tools required	The needed softwares and tools arranged					
4.	Experimental work		Experimental work done using different & tools.				
5.	Computation and interpretation of data		Computation and interpretation of experimental data done using suitable programs and softwares				
6.	Communication of research papers			Research papers communicated to journals and seminars/conferences			
7.	Interaction with UGC			Regarding the progress of the project, etc.			
8.	Conferences / symposia/ seminars		Attended on the recent trends in the proposed research area and utilizated in the project				

The proposed objectives *vis-a-vis* results achieved are as under-

A. To develop database of sequences of Class Monogenea their host and other related information till date of data using available sequences- A sincere effort was attempted to develop database cum information resource on various members of Class -Monogena. The strategy followed was having two main steps- **(i). Identification of Data-** For this purpose all possible sources of online as well as offline data sources were explored. Class-Monogenea members were studied and information with reference to large number of members studied. The data to studied was used in the present study directly or indirectly. All the studied members are tabulated in the form of a table as appendix-I (page 1-31). Due consideration to 219 genus spread over 40 families of the class Monogenea was given during the present study. The taxonomic

status available at NCBI (www.ncbi.nlm.nih.gov/taxonomy) was followed. for family, genus and species level details.

(ii). Collection and storage of data- During the course of study huge data of diverse nature was collected and stored in the form of a structured database. The search can be continued due to ongoing research and day to day developments in the field of Monogenean study. All the members studied in the present work is tabulated in the form of Microsoft excel file name as **UGC-MRP.F.No.41-34(2012)_Database.xls** and relevant data related to each subject or member is compiled in the folder numbered with reference to respective members serial number. The database file contained 01 main sheet with 1676 active rows having 2058 F.No. (denoting record file serial number). In active 1676 rows 1674 active members studied. Information related to available sequence and literature is stored in the respective folder. The information so collected was subsequently used for further analysis. All such data along with key file in Microsoft excel format is written of the CD (enclosed with the report). The data collection was continued beyond the initially thought deadlines for the project in the interest of project. This strategy helped in extensive analysis and elimination of possible limitation in the present study. Only trouble resulted in delay and overburden on the team during analysis of newly generated data during the late phases. *One paper related to the concept, strategy and key issues in under preparation/communication stage*. *Any update regarding the paper will be communicated to the UGC in the near future*.

B. To compare various available sequences of Class Monogenea till date of data using available sequences- After initial screening of the available data in the database and consultation with experts an initial attempt was made on genus level studies. Three major genus *viz*., *Gyrodactylus* (Monogenea: Gyrodactylidae), *Dactylogyrus* (Class : Monogenea) and *Lamellodiscus* (Monogenea: Diplectanidae) using 28S ribosomal RNA and 18S ribosomal RNA were investigated. The findings were published in the form of three very important and much appreciated papers in referred journals *viz*.,

1. Fozail Ahmad, D. Singh & P.V. Arya (2015). *In silico* phylogenetic studies on some members of parasitic genus *Gyrodactylus* (Monogenea: Gyrodactylidae) for assessment of evolutionary relatedness inferred from 28S ribosomal RNA and geomapping the sample. International Journal of Recent Scientific Research; 6 (7) : 4970-4977. **[ISSN :0976-3031].**

2. Fozail Ahmad, D. Singh & P.V. Arya (2015). Comparative evaluation of speciation and zoogeographical distribution for *Lamellodiscus* (Monogenea: Diplectanidae) using 18S rRNA. International Journal of Innovation Science and Research (IJISR); 4 (6), 235-241. **[ISSN : 2319-9369].**

3. Fozail Ahmad, D. Singh & P.V. Arya (2015). *In-silico* phylogenetic study of *Dactylogyrus* (Class : Monogenea) using 18S rRNA with a note on zoogeographical investigations on the genus. International Journal of Biological and Biomedical Sciences; 4(8) : 055-058. [ISSN:2319-9806].

C. To propose evolutionary relationship for Class-Monogenea till date of data using available sequences- A large number of members were considered for in depth detail study on various possible parameters. But due to limited number of common sequences for the organisms under study only few members could be studied at *in-silico* level. In the process of the study of phylogenetic relationship among members of five family *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae was studied. A relatively new concept cm combination approach was followed in the present study. The approach was much appreciated as it involve practical ideology as well. In the process we identified the geographical location of various parasites their habitat and other details during the studied as retrieved from the literature. The finddings were mapped on the world map manually. Subsequently the closest neighbour was matched or aligned as observed from molecular data comparison in phylogenetic studies. This approach was followed in majority of papers published by the team and helped us in advocating our findings. The findings were published in the form of an important and much appreciated paper in referred journal *viz*.,

1. Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. [ISSN: 0972-0030].

D. To study phylogenetic relationship among members of Class-Monogenea till date of data using available sequences- The phylogenetic relationship among the members of Class-Monogenea was investigated for not only genus level but also on family levels. In addition multiple family study was also adopted for developing better understanding on the issue. In the process of the study of phylogenetic relationship in addition to study on the genus *Dactylogyrus* , *Gyrodactylus*, and *Lamellodiscus* family Ancyrocephalidae and Monocotylidae was also studied. The

findings were published in the form of two important and much appreciated papers in referred journals *viz*.,

1. Fozail Ahmad, D. Singh & P.V. Arya and HS Singh (2015). *In silico* phylogenetic study on Ancyrocephalidae (Class : Monogenea) using 28SrRNA extending geo-mapping in search of evolutionary cues. Biochemical and Cellular Archives; 15 (2) : 391-399. **[ISSN :0972-5075]. [NAAS Score : 3.77].**

2. Fozail Ahmad, D. Singh & P.V. Arya (2015). A combination study in some members of Monocotylidae (Monogenea) in molecular phylogeny employing 28SrRNA along with geographical distribution. International Journal of Science and Research (IJSR); 4(8): 1292-1298. **[ISSN: 2319-7064].**

3. Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. **[ISSN: 0972-0030].**

E. To validate relationship among various members, compare it with previously established relation and suggest modification if required till date of data using available sequences- A large number of members were considered for in depth detail study on various possible parameters. But due to limited number of common sequences for the organisms under study only few members could be studied at *in-silico* level. In the process of the study of phylogenetic relationship among members of Class-Monogenea five family *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae were studied. The findings were published in the form of important and much appreciated papers in referred journals *viz*.,

1. Fozail Ahmad, D. Singh & P.V. Arya and HS Singh (2015). *In silico* phylogenetic study on Ancyrocephalidae (Class : Monogenea) using 28SrRNA extending geo-mapping in search of evolutionary cues. Biochemical and Cellular Archives; 15 (2) : 391-399. **[ISSN :0972-5075]. [NAAS Score : 3.77].**

2. Fozail Ahmad, D. Singh & P.V. Arya (2015). A combination study in some members of Monocotylidae (Monogenea) in molecular phylogeny employing 28SrRNA along with geographical distribution. International Journal of Science and Research (IJSR); 4(8): 1292-1298. [ISSN: 2319-7064].

3. Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. [ISSN: 0972-0030].

F. To work towards clearing prevailing doubts regarding positions of various members of Class-Monogenea till date of data using available sequences- In another extension to the present study Cytochrome C oxidase-1 was selected for 16 species from four different families based upon the availability of particular type of protein sequences for sufficient number of species in a particular family, in order to carry out analytical studies. The Gyrodactylidae, Diplozoidae,

Diplectanidae and Dictylophoridae had 5, 2, 6 and 3 selected species respectively. Overall, four groups in the study provides a generalized evolutionary distinction of COX-I protein of Monogenean families in terms of sequence and structure. The four groups are highly diverging members of parasitic class, representing variability in conserved protein. Monogeneans can be evaluated on the basis of such analysis for their origin and evolution. This finding just gives an idea of evolutionary relatedness in all families/genus in term of COX-I protein changing over the period or may provide the beginning of evolution of class Monogenea. In an attempt to explore the concept of relatedness and global diversity evolution in 05 major families of these classes using various *in-silico* tools. Study involve investigations on 227 species using 28S rRNA data and its geomapping co relations *i.e*., on families *viz*., Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae, Monocotylidae, Polystomatidae. These findings provided a range of enumerations that how species went prevalent into specific geographical zones of the world and what was the amount of change that caused their migration to other corner of the globe. Monogeneans have versatile nature to switch from one place to another and rapidly change morphology and become adapted, suggesting that families are specific to their member species and allow evolving when exposed to suitable environmental conditions. Further based on global representation and species diversity eight minor families *viz*., Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae were selected for the further investigation. A systematic effort was made towards understanding diversity, distribution and milestone chronology of the family all families equally, by means of geographical distribution showing a lower degree of occurrence in a particular area. As per the high density of species in a specific area is concerned, it is the family Cichlidogyridae that strictly occur in South Africa, and with small number in Madagascar. We have mentioned in the previous work that richness of a particular member from a particular area (geographical area/location) is an indication of its origin. And definitely, taxonomic and phylogenetic status, from across the globe fall into the same geographical zone, confirming their classification into the updated record. More detail molecular investigation is required to establish relative evolutionary linkage/lineage of these families. The findings were published in the form of three very important and much appreciated papers in referred journals *viz*.,

1. Fozail Ahmad, & P.V. Arya, H.S. Singh (2015). COX-1 studies in evaluation and assessment of molecular diversity among Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae families (Class : Monogenea). International Journal of Innovation Science and Research (IJISR); 4(10) : 494-500. [ISSN : 2319-9369].

2. Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. [ISSN: 0972-0030].

3. Fozail Ahmad, C. Sharma , V.P. Aggarwal & P.V. Arya (2016). Revisiting diversity and geographical distribution of eight minor families *viz*., Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae of Class Monogenea. International Journal of Innovation Science and Research (IJISR) ; 5(1) : 608-610. [ISSN : 2319-9369].

The Year wise work done in the present study is as under

Year-1 : The process of appointment of the suitable & qualified (M.Sc. Bioinformatics with research experience in the related field) project staff was completed as soon as possible. Due to sudden change and developments Co-PI (Dr. V.P. Aggarwal) was inducted with due permission from UGC and charge of PI taken during the his absence period (on account of Duty Leave). Extensive literature survey (both physical as well as from online resources), identification of various potential sources of information relevant for the present work was done. Process for the purchase of necessary equipments was initiated (*as approved*) as per rules. All efforts were done to identify and establish contact with the various experts working in the same field. This was done so as to facilitate the smooth completion of the project. Data collection was also initiated from the available sources. NCBI database identified as main source for the present work. The data so collected was tabulated in Excel sheet (with backup in respective folders) for ready reference and analysis. The process of establishment of experimental design was initiated and tested upon many times on various dataset. Based on available expertise, literature survey methods to be adopted in the ongoing analysis were decided (with possibility of flexibility for any future modification). A skeleton of main database was designed and finalised considering Family, Genus and species and main component of the structure. The idea of exploring various workshop, seminar and conferences being organized in the related field and active participation for necessary inputs/updates for the ongoing work was well taken and followed. Periodic review of the work done *vis-a-vis* the proposed objectives of the project.

Year-2 : The original PI of the project (Dr. P.V. Arya) resumed (*after completion of Duty leave*) the project as PI and same was intimated to UGC for necessary record. Extensive literature survey continued (both physical as well as from online resources), identification of various potential sources of information relevant for the present work was done. Process initiated for the purchase of necessary equipments (*as approved*) was completed as per rules. All efforts were done to identify and establish contact with the various experts working in the same field. This was done so as to facilitate the smooth completion of the project. Data collection was also continued from the available sources including NCBI database which was identified as main source for the present work. The data continued to be tabulated in Excel sheet (with backup in respective folders using control number as Unit for the Species) for ready reference and analysis. As mentioned species was taken as main unit for the present work and all the possible efforts were made to ensure the collection of all possible data on the species level. The species were identified on control number and relevant information was stored on that control number (Numeric Value in ascending order) folder for ready reference. For extension of initial analysis some Genus were identified having less studied and much diversity in the form of molecular data richness viz., *Gyrodactylus* (Monogenea: Gyrodactylidae); *Lamellodiscus* (Monogenea: Diplectanidae) & *Dactylogyrus* (Monogenea : Dactylogridae). The extensive analysis carried out on these three genus resulted in producing three important papers in the current project *i.e.*,

Fozail Ahmad, D. Singh & P.V. Arya, 2015. In silico phylogenetic studies on some members of parasitic genus *Gyrodactylus* (Monogenea: Gyrodactylidae) for assessment of evolutionary relatedness inferred from 28S ribosomal RNA and geomapping the sample. International Journal of Recent Scientific Research; 6 (7): 4970-4977. **[ISSN :0976-3031].**

Fozail Ahmad, D. Singh & P.V. Arya, 2015. Comparative evaluation of speciation and zoogeographical distribution for *Lamellodiscus* (Monogenea: Diplectanidae) using 18S rRNA. International Journal of Innovation Science and Research (IJISR); 4 (6) : 235-241 **[ISSN : 2319-9369].**

Fozail Ahmad, D. Singh & P.V. Arya, 2015. *In-silico* phylogenetic study of *Dactylogyrus* (Class : Monogenea) using 18S rRNA with a note on zoogeographical investigations on the genus. International Journal of Biological and Biomedical Sciences; 4(8): 055-058. **[ISSN:2319-9806].**

Findings were an update on these genus some are well documented from the Indian region as well. The main problem faced during the work was limited numbers of contribution of molecular data for diversity from the Indian region. However during recent past same is being enriched from various centres across the country. The process of establishment of experimental design was initiated and tested upon many times on various dataset. Based on available expertise, literature survey methods being adopted in the analysis were updated (due to possibility of flexibility for any future modification as incorporated in the beginning). The skeleton of main database as designed previously and finalised considering Family, Genus and species and main component of the structure was further upgraded. Additional components were incorporated in the form of habitat (freshwater, marine etc), host (fish amphibia etc.), locality (region of the world) and type of sequence (protein, nucleotide, DNA, RNA, complete genome, 18SrRNA, 28SrRNA etc.) available. The related information was continuously updated in the respective folders as per assigned control numbers. Exploring the various workshop, seminar and conferences being organized in the related field and active participation for necessary inputs/updates for the ongoing work. Periodic review of the work done *vis-a-vis* the proposed objectives of the project. Mid-term meeting was attended and the work done so far was updated to the experts. The guidance provided in the mid-term meeting was utilised in further progressing of the project.

Year-3 : Extensive literature survey continued (both physical as well as from online resources), identification of various potential sources of information relevant for the present work was done. All efforts were done to identify and establish the contact with the various experts working in the same field. After initial success on the selected genus of class monogenea during the previous year and as per suggestion of collaborating experts as well as experts during mid-term review meeting the study was expanded on next higher level of family. The main families with majority of representation and molecular data richness was identified and explored for the present work *i.e.,* Ancyrocephalidae, Monocotylidae, Gyrodactylidae, Diplectenidae, Diplozoidae, Dictilophoridae, Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae. Initially findings on 02 main families *viz.,* Ancyrocephalidae, Monocotylidae were were published in the form of two important papers as listed below.

Fozail Ahmad, D. Singh & P.V. Arya and HS Singh, 2015. *In silico* phylogenetic study on Ancyrocephalidae (Class : Monogenea) using 28SrRNA extending geo-mapping in search of evolutionary cues. Biochemical and Cellular Archives; 15 (2): 391-399. **[ISSN :0972-5075].**

Fozail Ahmad, D. Singh & P.V. Arya, 2015. A combination study in some members of Monocotylidae (Monogenea) in molecular phylogeny employing 28SrRNA along with geographical distribution. International Journal of Science and Research (IJSR); 4(8): 1292-1298. **[ISSN: 2319-7064].**

Later on the idea of considering more than one family together was employed. Although it was a challenging task to handle such a huge data simultaneously. But the analysis was compiled in another paper using five different families viz., Monocotylidae,

Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae. The findings were published in a peer reviewed refereed Journal (details including ISSN number given) for 2016 issue.

Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz*., Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. **[ISSN: 0972-0030].**

So far as the analysis was mainly done using 28SrRNA or 18SrRNA data for all the earlier studies. The motivated team take another step incorporating another relatively conserved sequence of COX-1 into consideration for four different families *viz*., Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae. The findings were again accepted in the form of another research paper published during the year 2015.

Fozail Ahmad, & P.V. Arya, H.S. Singh 2015. COX-1 studies in evaluation and assessment of molecular diversity among Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae families (Class : Monogenea). International Journal of Innovation Science and Research (IJISR); 4(10): 494-500. **[ISSN : 2319-9369].**

Another paper dealing with eight minor families *viz*., Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae of Class Monogenea. was also published in 2016 issue of refereed Journal (details including ISSN number given).

Fozail Ahmad, C. Sharma , V.P. Aggarwal & P.V. Arya (2016). Revisiting diversity and geographical distribution of eight minor families *viz*., Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae of Class Monogenea. International Journal of Innovation Science and Research (IJISR) ; 5(1) : 608-610. [**ISSN : 2319-9369**].

Various workshop, seminar and conferences being organized in the related field were explored by active participation for necessary inputs/updates for the ongoing major research project. Periodic review of the work done *vis-a-vis* the proposed objectives of the project.

5.1 Limitations & Future Prospects

Present study was based on the *in-silico* approach only for majority of the studies. Although the incorporation of latest possible data, concept and tools during the study was definitely useful in the process of study. Incorporation of geo mapping was one such addition along with usage of multiple gene target approach like 18SrRNA and 28SrRNA as well as COX-1 in the study. The main limitation was limited availability of similar region molecular data on all the species under study. This limitation forced us to drop couple of important species during the present study. Any development in the form of addition of new data may be useful in future studies.

6. Summary

The approach of *in-silico* phylogenetic investigation was more or less successful in the study on the members of Class Monogenea. Incorporation of additional data from habitat, geographical distribution and host preferences enabled in giving better results. The concept of secondary structure of RNA and comparative energy level charts further helped in clearing the doubts and establishing firm relationships. In all 39 species of genus *Gyrodactylus* was studied and on the basis of 28SrRNA secondary structure 06 clades were formed. These clades were segregated on the basis of relative negative free energy (ΔG), interior loop, Hairpin loop, bulge loop and total number of loops. A global geo mapping of the members helped in better understanding of the global diversity relatedness and probable evolutionary trends. In genus *Lamellodiscus* (Monogenea: Diplectanidae), a total 28 species were investigated and 07 clades were formed. Due consideration was given to relative negative free energy (ΔG), interior loop, Hairpin loop, bulge loop and total number of loops. Again the concept of global geo mapping of the members helped in better understanding of the global diversity relatedness and probable evolutionary trends.

For genus *Dactylogyrus*, a total 45 species were investigated and 15 clades were formed. Due consideration was given to concept of global geo mapping of the members which helped in better understanding of the global diversity relatedness and probable evolutionary trends. While study the family, it was started with Ancyrocephalidae and a total 71 species of 12 genus were investigated. A total sum of 12 clades and many sister clades were formed based of previously established parameters. The family Ancyrocephalidae showed species richness due to having dual evolutionary features in the family. Phylogenetic study confirmed the monophyletic and paraphyletic feature which was further supported by secondary structure analyses of representative species. Cladistic analysis giving strong clues about ancient lineage, origin and range of similarity was comprehended by secondary structure of 28S rRNA. Species distribution strengthened intra genus relationship, divergence, and migration over period of times. In the phylogenetic tree, clustering and cladistic hypothesis was supported by zoogeographical (geo-mapping) distribution in different zones of the world.

In case of study on family M**onocotylidae** a total 39 species of 12 genus were selected and 07 clades were formed. Due consideration was given previously established parameters. The finding paved way to a hypothesis that host plays substantial role in the formation of new species especially for monogenetic parasites. Cladistic analysis giving strong clues about ancient lineage, origin and range of similarity was comprehended by secondary structure of 28S rRNA. Species distribution strengthened intra genus relationship, divergence, and migration over period of times. In the phylogenetic tree, clustering and cladistic hypothesis was supported by zoogeographical distribution of Monocotalidae in different regions of the world.

Cytochrome C oxidase-1 was also selected for 16 species from four different families based upon the availability of particular type of protein sequences for sufficient number of species in a particular family, in order to carry out analytical studies. All sequences had varying length, differ by one or two amino acids with no phylogenetic issue at all. The Gyrodactylidae, Diplozoidae, Diplectanidae and Dictylophoridae had 5, 2, 6 and 3 selected

species respectively. Overall, four groups in the study provides a generalized evolutionary distinction of COX-I protein of Monogenean families in terms of sequence and structure. The four groups are highly diverging members of parasitic class, representing variability in conserved protein. Monogeneans can be evaluated on the basis of such analysis for their origin and evolution. Further studies can be performed with more families/group in order to justify the ancestral lineage. This finding just gives an idea of evolutionary relatedness in all families/genus in term of COX-I protein changing over the period or may provide the beginning of evolution of class Monogenea.

In an attempt to explore the concept of relatedness and global diversity evolution in 05 major families of this class using various *in-silico* tools. Study involve investigations on 227 species using 28S rRNA data and its geomapping co relations i.e., Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae, Monocotylidae, Polystomatidae. This finding provides a range of enumerations that how species went prevalent into specific geographical zones of the world and what was the amount of change that caused their migration to other corner of the globe.

Based on global representation and species diversity eight minor families *viz.,* Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae were selected for the further investigation. A systematic effort was made towards understanding diversity, distribution and milestone chronology of the family all families equally, by means of geographical distribution showing a lower degree of occurrence in a particular area. As per the high density of species in a specific area is concerned, it is the family Cichlidogyridae that strictly occur in South Africa, and with small number in Madagascar. We have mentioned in the previous work that richness of a particular member from a particular area (geographical area/location) is an indication of its origin. And definitely, taxonomic and phylogenetic status,from across the globe fall into the same geographical zone, confirming their classification into the updated record. More detail molecular investigation is required to establish relative evolutionary linkage/lineage of these families. This study may give a motivation to take up detailed molecular investigation for establishing relative evolutionary tree for all the members in the class.

Monogeneans have versatile nature to switch from one place to another and rapidly change morphology and become adapted, suggesting that families are specific to their member species and allow evolving when exposed to suitable environmental conditions.

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Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1	Acanthocotylidae	*Acanthocotyle*	*A. sp. EMP-2009*
2			*A. borealis*
3			*A. brachyuropsi*
4			*A. elegans*
5			*A. greeni*
6			*A. monticellii*
7			*A. oligoterus*
8			*A. pacifica*
9			*A. patagonica*
10			*A. scobini*
11		*No genus found*	*Amphibdellatidae sp. EMP-2009*
12		*Bifurcohaptor*	*B. indicus*
13		*Bychowskyella*	*B. pseudobagri*
14		*Hamatopeduncularia*	*H. arii*
15			*H. thalassini*
16			*H. elongata*
17		*Cleidodiscus*	*C. capax*
18			*C. pricei*
19		*Cornudiscoides*	*C. longicirrus*
20			*C. proximus*
21		*Malayanodiscoides*	*M. indicus*
22		*Notopterodiscoides*	*N. notopterus*
23		*Pseudancylodiscoides*	*P. sp. HSY1*
24			*P. sp. HSY3*
25			*P. sp. HSY4*
26		*Quadriacanthus*	*Q. sp. 1 AS-2013*
27			*Q. kobiensis*
28		*Schilbetrema*	*S. sp. 1 AS-2013*
29		*Thaparocleidus*	*T. alatus*
30			*T. aori*
31			*T. asoti*
32			*T. caecus*
33			*T. campylopterocirrus*
34			*T. citreum*
35			*T. cochleavagina*
36			*T. combesi*
37			*T. crassipenis*
38			*T. durandi*
39			*T. indicus*
40			*T. infundibulovagina*
41			*T. komarudini*
42			*T. lebrunae*
43			*T. legendrei*
44			*T. levangi*
45			*T. magnicirrus*
46			*T. mutabilis*
47			*T. obscura*
48			*T. omegavagina*
49			*T. parvulus*
50			*T. rukyanii*
51			*T. siamensis*
52			*T. siluri*
53			*T. sinespinae*
54			*T. sudhakari*
55			*T. summagracilis*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
56			*T. susanae*
57			*T. tacitus*
58			*T. turbinatio*
59			*T. varicus*
60			*T. vistulensis*
61			*T. yogendraii*
62			*T. sp. 1 HS-2010*
63			*T. sp. 1 XW-2007*
64			*T. sp. 2 HS-2010*
65			*T. sp. 2 XW-2007*
66			*T. sp. BDY*
67			*T. sp. HSS-2011*
68			*T. sp. NY1*
69			*T. sp. NY2*
70		*Actinocleidus*	*Ancylodiscoides Yamaguti*
71			*Actinocleidus recurvatus*
72		*Ancyrocephalus*	*A. bassensis*
73			*A. cobitis*
74			*A. macrogaster*
75			*A. manilensis*
76			*A. parupenei*
77			*A. pauu*
78			*A. salinus*
79			*A. unicirrus*
80			*A. vesiculosus*
81			*A. visakhapatnamensis*
82			*A. platycephali*
83			*A. pseudorhombi*
84			*A. rarus*
85			*A. parvus*
86			*A. ornatus*
87			*A. atherinae*
88			*A. mogurndae*
89			*A. paradoxus*
90			*A. percae*
91		*Bravohollisia*	*B. geruti*
92			*B. kritskyi*
93			*B. magna*
94			*B. pomadasis*
95			*B. tecta*
96			*B. reticulata*
97			*B. gussevi*
98			*B. maculatus*
99			*B. parvianchoratus*
100			*B.rosetta*
101			*B.sp. 1 XW-2006*
102			*B.sp. Malaysia*
103		*Enterogyrus*	*E. coronatus*
104			*E. sp. 1 AS-2010*
105			*E. sp. 2 AS-2010*
106			*E. adelpha*
107			*E. ambassisi*
108			*E. amydrum*
109			*E. anecorhizion*
110			*E. anguiforme*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
111			*E. annulocirrus*
112			*E. aspistis*
113			*E. atlanticum*
114			*E. berenguelae*
115			*E. bychowskyi*
116			*E. carbuncularium*
117			*E. carbunculus*
118			*E. cardinale*
119			*E. chaoi*
120			*E. chrysotaeniae*
121			*E. cognatus*
122			*E. cribbi*
123			*E. cryptophallus*
124			*E. diplops*
125			*E. distinctum*
126			*E. dontykoleos*
127			*E. dunlapae*
128			*E. eukurodai*
129			*E. fajeravilae*
130			*E. fajeravilae*
131			*E. fatuum*
132			*E. ferocis*
133			*E. grandis*
134			*E. guangdongense*
135			*E. guangzhouense*
136			*E. hainanense*
137			*E. johni*
138		*Euryhaliotrema*	*E. kurodai*
139			*E. lisae*
140			*E. longibaculoides*
141			*E. longibaculum*
142			*E. lovejoyi*
143			*E. lutiani*
144			*E. lutjani*
145			*E. mehen*
146			*E. microphallus*
147			*E. monacanthus*
148			*E. monoporosum*
149			*E. nanaoense*
150			*E. paracanthi*
151			*E. paralonchuri*
152			*E. paululum*
153			*E. perezponcei*
154			*E. pirulum*
155			*E. potamocetes*
156			*E. ramulum*
157			*E. sagmatum*
158			*E. seyi*
159			*E. simplicis*
160			*E. spirotubiforum*
161			*E. spirulum*
162			*E. succedaneus*
163			*E. thatcheri*
164			*E. tormocleithrum*
165			*E. torquecirrus*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
166	Ancyrocephalidae		*E. triangulovagina*
167			*E. tubocirrus*
168			*E. xinyingense*
169			*E. youngi*
170			*E. zhangjianying*
171		*Eutrianchoratus*	*E. cleithrium*
172			*E. inequalis*
173		*Glyphidohaptor*	*G. plctcirra*
174		*Haliotrema*	*H. angelopterum*
175			*H. aurigae*
176			*H. bihamulatum*
177			*H. chrysotaeniae*
178			*H. cromileptis*
179			*H. ctenochaeti*
180			*H. digyroides*
181			*H. epinepheli*
182			*H. fleti*
183			*H. geminatohamula*
184			*H. grossecurvitubus*
185			*H. johnstoni*
186			*H. kurodai*
187			*H. leporinus*
188			*H. macasarensis*
189			*H. macracantha*
190			*H. nanaoensis*
191			*H. platycephali*
192			*H. pratasensis*
193			*H. scyphovagina*
194			*H. shenzhenensis*
195			*H. spirotubiforum*
196			*H. subancistroides*
197			*H. sp. 1 TY-2005*
198			*H. sp. 2 TY-2005*
199			*H. sp. HBDQY*
200			*H. sp. WXY-2005*
201			*H. sp. WXY-2007*
202			*H. sp. ZHDDa*
203			*H. sp. ZHDDb*
204		*Haliotrematoides*	*H. guttati*
205			*H. plectridium*
206			*H. spinatus*
207		*Heteronchocleidus*	*H. buschkieli*
208		*Lethrinitrema*	*L. zhanjiangense*
209			*L. dossenus*
210			*L. gibbus*
211			*L. nebulosum*
212			*L. chrysostomi*
213			*L. fleti*
214			*L. lethrini*
215			*L. grossecurvitubum*
216			*L. austrosinense*
217			*L. acuminatus*
218			*L. angustus*
219			*L. cephali*
220			*L. confusus*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
221		*Ligophorus*	*L. heteronchus*
222			*L. imitans*
223			*L. leporinus*
224			*L. llewellyni*
225			*L. macrocolpos*
226			*L. mediterraneus*
227			*L. minimus*
228			*L. mugilinus*
229			*L. pilengas*
230			*L. szidati*
231			*L. vanbenedenii*
232		*Mastacembelocleidus*	*M.sp. 1 AA-2014*
233			*M.heteranchorus*
234			*M.bam*
235			*M.sp. HS-2010*
236		*Metahaliotrema*	*M.filamentosum*
237			*M.kulkarnii*
238			*M.kulkarnii*
239			*M.geminatohamula*
240			*M.mizellei*
241		*Onchobdella*	*O. aframae*
242			*O. bopeleti*
243		*Onchocleidus*	*O. ferox*
244			*O. nactus*
245			***O. Mueller***
246			*O. sp. XJD-2004*
247		*Placodiscus*	*P. acanthopagri*
248		*Pseudohaliotrema*	*P. sphincteroporus*
249			*P. virgata*
250		*Sciadicleithrum*	*S. variabilum*
251		*Scutogyrus*	*S. bailloni*
252			*S. longicornis*
253			*S. minus*
254			*S. sp. 1 XW-2006*
255		*Tetrancistrum*	*T. fusiforme*
256			*T. longiphallus*
257			*T. longispicularis*
258			*T. lutiani*
259			*T. makau*
260			*T. nasonis*
261			*T. nebulosi*
262			*T. suezicus*
263			*T. oraminii*
264			*T. polymorphus*
265			*T. sigani*
266			*T. strophosolenum*
267			*T. nebulosi*
268			*T. sp.*
269		*Thylacicleidus*	*T. brunensis*
270			*T. latus*
271			*T. serendipitus*
272			*T. sp. Malaysia-AS-2002*
273			*T. acleithrium*
274			*T. grandis*
275			*T. gussevi*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
276		*Trianchoratus*	*T. leerium*
277			*T. longianchoratus*
278			*T. malayensis*
279			*T. ophicephali*
280			*T. pahangensis*
281			*T. parvulus*
282			*T. trichogasterium*
283			*T. sp. AC-2013*
284			*T. sp. HS-2010*
285		*Urocleidus*	*U. principalis*
286			*U. dispar*
287			*U. similis*
288	Anoplodiscidae	*Anoplodiscus*	*A. cirrusspiralis*
289	Bothitrematidae	*Bothitrema*	*B. bothi*
290			*B. rarus*
291	Calceostomatidae	*Calceostoma*	*C. glandulosum*
292		*unclassified Calceostomatidae*	*Calceostomatidae sp. EMP-2009*
293	Capsalidae	*Allobenedenia*	*A. petangulata*
294			*A. patagonica*
295			*A. sebastedi*
296			*A. convoluta*
297			*A. zanghi*
298			*A. epinepheli*
299		*Benedenia*	*B. acanthopagri*
300			*B. anticavaginata*
301			*B. epinepheli*
302			*B. hoshinai*
303			*B. lutjani*
304			*B. rohdei*
305			*B. sargocentron*
306			*B. sciaenae*
307			*B. sekii*
308			*B. seriolae*
309			*B. cf. seriolae FAS-2013*
310			*B. sp. DTJL*
311		*Benedeniella*	*B. incertae sedis*
312			*B. unnithani*
313			*B. congeri*
314			*B. macrocolpa*
315			*B. posterocolpa*
316		*Capsala*	*C. poeyi*
317			*C. albsmithni*
318			*C. ovalis*
319			*C. laevis*
320			*C. martinieri*
321			*C. onchidiocotyle*
322			*C. pricei*
323			*Ca. sp. 1 EMP-2009*
324			*C. sp. 2 EMP-2009*
325			*C. sp. C8*
326			*C. sp. C9*
327			*C.cornutus*
328			*C.hoffmannae*
329			*C.istiophori*
330			*C.marielenae*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
331		*Capsaloides*	*C.nairagi*
332			*C.perugiai*
333			*C.tetrapteri*
334			*C.sinuatus*
335			*C.cristatus*
336			*C.magnaspinosus*
337			*C.sp. 1 CY-2011*
338		*Dioncopseudobenedenia*	*D.macracantha*
339			*D.ancoralis*
340			*D.kala*
341		*Encotyllabe*	*E. antofagastensis*
342			*E. caballeroi*
343			*E. callaoensis*
344			*E. carangis*
345			*E. caranxi*
346			*E. cheilodactyli*
347			*E. chironemi*
348			*E. embiotocae*
349			*E. fotedari*
350			*E. kuwaitensis*
351			*E. lintoni*
352			*E. sp. 2 FAS-2013*
353			E. sp. 1 FAS-2013
354			*E. lutjani*
355			*E. masu*
356			*E. monticelli*
357			*E. nordmanni*
358			*E. pagelli*
359			*E. pagrosomi*
360			*E. paronae*
361			*E. pricei*
362			*E. punctatai*
363			*E. souzalimae*
364			*E. spari*
365			*E. vallei*
366			*E. xiamenensis*
367		*Entobdella*	*E. aegyptiacus*
368			*E. brattstroemi*
369			*E. brinkmanni*
370			*E. bumpusii*
371			*E. curvunca*
372			*E. diadema*
373			*E. guberleti*
374			*E. hippoglossi*
375			*E. pugetensis*
376			*E. rosaceus*
377			*E. soleae*
378			*E. squamula*
379			*E. stenolepis*
380			*E. vanbenedeni*
381			*E. sp. 1-AHC 28428-9*
382			*E. sp. 2-AHC 28430-1*
383		*Interniloculus*	*I. sebastidis*
384			*I. chilensis*
385			*L. guberleti*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
386		*Listrocephalos*	*L. whittingtoni*
387			*L. corona*
388			*L. kearni*
389		*Macrophyllida*	*M. antarctica*
390			*M. sp. EMP-2009*
391		*Mediavagina*	*M. forsteri*
392			*M. latridis*
393			*M. macropteri*
394			*M. sp. EMP-2009*
395		*Megalobenedenia*	*M. australis*
396			*M. derzhaveni*
397			*M. helicoleni*
398		*Menziesia*	*M. sp. sdwh030924*
399		*Nasicola*	*N. hogansi*
400			*N. brasileinsis*
401			*N. klawei*
402	Encotyllabe	*Neobenedenia*	*N. issabellae*
403			*N. pargueransis*
404			*N. paceficia*
405			*N. muelleri*
406			*N. manelai*
407			*N. longiprostata*
408			*N. girellae*
409			*N. melleni*
410			*N. sp. 1-AHC 28432-3*
411			*N. sp. 2-AHC 28434-5*
412			*N. sp. EMP-2009*
413			*N. sp. EMP-2010*
414			*N. sp. FAS-2013*
415			*N. sp. M07-2296-04*
416			*N. sp. OLH-2001*
417		*Neoentobdella*	*N. garneri*
418			*N. parvitesticulata*
419			*N. apiocolpos*
420			*N. australis*
421			*N. diadema*
422			*N. natans*
423			*N. taiwanensis*
424		*Nitzschia*	*N. sigmoidea*
425			*N. sturionis*
426		*Pseudonitzschia*	*P. uku*
427		*Tristoma*	*T. papillosum*
428			*T. adintegrum*
429			*T. adcoccineum*
430			*T. coccineum*
431			*T. integrum*
432			*T. sp. EMP-2009*
433			*T. antigoniae*
434			*T. ephydres*
435			*T. pseudomarginatus*
436			*T. pini*
437			*T. oncacanthus*
438			*T. goniistii*
439			*T. plumbea*
440			*T. plectropomi*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
441			*T. hobo*
442			*T. tubiporus*
443		*Trochopus*	*T. micracanthus*
444			*T. lineatus*
445			*T. heteracanthus*
446			*T. gaillimhe*
447			*T. diplacanthus*
448			*T. differens*
449			*T. brauni*
450			*T. plumbeus*
451			*T. marginatus*
452			*T. trituba*
453			*T. sprostoni*
454			*Capsalidae sp. 1 EMP-2009*
455			*Capsalidae sp. 2 EMP-2009*
456			*Capsalidae sp. 3 EMP-2009*
457			*Capsalidae sp. 4 EMP-2009*
458		*unclassified Capsalidae*	*Capsalidae sp. 5 EMP-2009*
459			*Trochopodinae sp. 1 EMP-2009*
460			*Trochopodinae sp. 2 EMP-2009*
461			*Trochopodinae sp. 3 EMP-2009*
462			*Trochopodinae sp. 4 EMP-2009*
463			*Trochopodinae sp. 5 EMP-2009*
464			*C. acerbus*
465			*C. aegypticus*
466			*C. agnesi*
467			*C. amphoratus*
468			*C. arthracanthus*
469			*C. bilongi*
470			*C. cirratus*
471			*C. cubitus*
472			*C. digitatus*
473			*C. douellouae*
474			*C. ergensi*
475			*C. falcifer*
476			*C. flexicolpos*
477	Cichlidogyridae	*Cichlidogyrus*	*C. gallus*
478			*C. halli*
479			*C. longicirrus*
480			*C. njinei*
481			*C. pouyaudi*
482			*C. sclerosus*
483			*C. thurstonae*
484			*C. tiberianus*
485			*C. tilapiae*
486			*C. yanni*
487			*C. sp. 1 AS-2010*
488			*C. sp. 1 XW-2006*
489			*C. sp. 2 AS-2010*
490			*C. sp. 2 XW-2006*
491			*C. sp. MLJ1*
492		*Acolpenteron*	*A. catostomi*
493			*A. ureterocetes*
494		*Caballeria*	*C. pedunculata*
495			*C. robusta*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
496		*Caballena*	*C. liewi*
497			*C. intermedius*
498		*Dactylogyroides*	*D. dorsalis*
499			*D. longicirrus*
500			*D. mahecoli*
501			*D. tripathii*
502		*Dactylogyrus*	*D. achmerowi*
503			*D. alatus*
504			*D. amphibothrium*
505			*D. anchoratus*
506			*D. arcuatus*
507			*D. auriculatus*
508			*D. borealis*
509			*D. caballeroi*
510			*D. carpathicus*
511			*D. catlaius*
512			*D. chondrostomi*
513			*D. chranilowi*
514			*D. cornoides*
515			*D. cornu*
516			*D. crivellius*
517			*D. crucifer*
518			*D. cryptomeres*
519			*D. ctenopharyngodonis*
520			*D. difformis*
521			*D. difformoides*
522			*D. distinguendus*
523			*D. dulkeiti*
524			*D. dyki*
525			*D. ergensi*
526			*D. eucalius*
527			*D. extensus*
528			*D. falcatus*
529			*D. falciformis*
530			*D. fallax*
531			*D. finitimus*
532			*D. folkmanovae*
533			*D. formosus*
534			*D. fraternus*
535			*D. gotoi*
536			*D. hemiamphibothrium*
537			*D. hypophalmichthys*
538			*D. inexpectatus*
539			*D. intermedius*
540			*D. inversus*
541			*D. izjumovae*
542			*D. kikuchii*
543			*D. labei*
544			*D. lamellatus*
545			*D. longiacus*
546			*D. malleus*
547			*D. minor*
548			*D. nanoides*
549			*D. nanus*
550			*D. parabramis*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
551	Dactylogyridae		*D. parvus*
552			*D. pekinensis*
553			*D. petenyi*
554			*D. petruschewskyi*
555			*D. propinquus*
556			*D. prostae*
557			*D. quanfami*
558			*D. ramulosus*
559			*D. rarissimus*
560			*D. rutili*
561			*D. similis*
562			*D. sphyrna*
563			*D. squameus*
564			*D. subtilis*
565			*D. tuba*
566			*D. vastator*
567			*D. vistulae*
568			*D. vranoviensis*
569			*D. wunderi*
570			*D. zandti*
571			*D. sp. 1 AC-2012*
572			*D. sp. 1 RRS-2013*
573			*D. sp. 2 AC-2012*
574			*D. sp. 2 RRS-2013*
575			*D. sp. 3 RRS-2013*
576			*D. sp. 4 RRS-2013*
577			*D. sp. 5 RRS-2013*
578			*D. sp. LY1*
579			*D. sp. YY*
580		*Neocalceostomoides*	*N. hamatum*
581			*N. brisbanensis*
582			*N. simplex*
583			*N. arii*
584			*N. spinivaginalis*
585		*Paradactylogyrus*	*P. catlaius*
586		*Protogyrodactylus*	*P. alatus*
587			*P. alienus*
588			*P. amacleithrium*
589			*P. bancrofti*
590			*P. chaetodontis*
591			*P. constrictus*
592			*P. delicatus*
593			*P. elegantis*
594			*P. elongatus*
595			*P. ethiopicus*
596			*P. fissilis*
597			*P. fredericae*
598			*P. gussevi*
599			*P. hainanensis*
600			*P. johnstonettiegsi*
601			*P. kritskyi*
602			*P. leptocirrus*
603			*P. marinoides*
604			*P. marinus*
605			*P. perforatus*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
606			*P. pricei*
607			*P. pyriformis*
608			*P. quadratus*
609			*P. scapulasser*
610			*P. solidus*
611			*P. sprostonae*
612			*P. youngi*
613			*P. zullini ritsky*
614			*P. sp. 1 WXY*
615			*P. sp. 1 XW-2006*
616			*P. sp. 2 WXY*
617		*Spicocleidus*	*S. sp. 1 AA-2014*
618			*S. namae*
619		*Xenentocleidus*	*X. xenentodoni*
620		*Dactylogyridae gen. FS-2009*	*Dactylogyridae sp. FS-2009*
621		*Dactylogyridae gen. YS-2008*	*Dactylogyridae sp. 1 YS-2008*
622			*Dactylogyridae sp. 2 YS-2008*
623			*Dactylogyridae sp. 3 YS-2008*
624			*Dactylogyridae sp. 4 YS-2008*
625		*unclassified Dactylogyridae*	*Dactylogyridae sp. EMP-2009*
626		*Acleotrema*	*A.girellae*
627			*A.spiculare*
628			*A.diplobulbus*
629			*A.flebelliforme*
630			*A.tamatavense*
631			*A.nenue*
632			*A.parastromatei*
633			*A.serrulopenis*
634			*A.sp.*
635		*Calydiscoides*	*C.terpsichore*
636			*C.scolopsidis*
637			*C.rohdei*
638			*C.nemipteris*
639			*C.monogrammae*
640			*C.limae*
641			*C.kemamanensis*
642			*C.japonicus*
643			*C.gussevi*
644			*C.flexuosus*
645			*C.euzeti*
646			*C.duplicostatus*
647			*C.difficilis*
648			*C.cymbidioides*
649			*C.conus*
650			*C.australis*
651			*C.indianus*
652			*C.sp. DJXY*
653			*C.sp. XBLJD*
654		*Diplectanocotyla*	*D. parva*
655			*D. megalopis*
656			*D. langkawiensis*
657			*D. gracilis*
658			*D. aculeatum*
659			*D. aequans*
660			*D. americanum*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
661			*D. amplidiscatum*
662			*D. banyulense*
663			*D. bilobatum*
664			*D. blairense*
665			*D. bocqueti*
666			*D. bychowskyi*
667			*D. cayennensis*
668			*D. cazauxi*
669			*D. chabaudi*
670			*D. collinsi*
671			*D. cupatum*
672			*D. decorum*
673			*D. dollfusi*
674			*D. elongatum*
675			*D. enyenihii*
676			*D. flagritubus*
677			*D. fluviatilus*
678			*D. fujianense*
679			*D. furcelamellosum*
680			*D. glandulosum*
681			*D. grassei*
682			*D. grouperi*
683			*D. hargisi*
684			*D. hilum*
685			*D. jaculator*
686			*D. jamestownense*
687			*D. jerbuae*
688			*D. kuhliae*
689			*D. labourgi*
690			*D. laubieri*
691			*D. longipenis*
692			*D. lutiani*
693			*D. maa*
694			*D. maculatum*
695		*Diplectanum*	*D. magnodiscatum*
696			*D. megacirrus*
697			*D. melvillei*
698			*D. minousi*
699			*D. minutum*
700			*D. monticellii*
701			*D. nagibinae*
702			*D. narimeen*
703			*D. oliveri*
704			*D. orissai*
705			*D. penangi*
706			*D. pescadae*
707			*D. pisciniarius*
708			*D. polynemus*
709			*D. psammopercis*
710			*D. puriense*
711			*D. robustitubum*
712			*D. sciaenae*
713			*D. secundum*
714			*D. setosum*
715			*D. simile*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
716			*D. spinosum*
717			*D. spirale*
718			*D. squamatum*
719			*D. stetoxus*
720			*D. summanae*
721			*D. sumpit*
722			*D. tangzhongzhangi*
723			*D. toxotes*
724			*D. trichocarpoides*
725			*D. uitoe*
726			*D. umbrinum*
727			*D. undulicirrosum*
728			*D. veropolynemi*
729			*D. wennigeri*
730			*D. aequans*
731			*D. penangi*
732			*D. umbrinum*
733			*D. veropolynemi*
734			*E. rarum*
735			*E. pudicum*
736			*E. plectropomi*
737		*Echinoplectanum*	*E. laeve*
738			*E. echinophallus*
739			*E. chauvetorum*
740			*E. leopardi*
741		*Furnestinia*	*F. echeneis*
742			*L. acanthopagri*
743			*L. baeri*
744			*L. bidens*
745			*L. butcheri*
746			*L. caballeroi*
747			*L. cirrusspiralis*
748			*L. confusus*
749			*L. corallinus*
750			*L. coronatus*
751			*L. crampus*
752			*L. dentexi*
753			*L. donatellae*
754			*L. drummondi*
755			*L. echeneis*
756			*L. elegans*
757			*L. epsilon*
758			*L. ergensi*
759			*L. erythrini*
760			*L. euzeti*
761	Diplectanidae		*L. falcus*
762			*L. flagellatus*
763			*L. fraternus*
764			*L. furcillatus*
765			*L. furcosus*
766			*L. gracilis*
767			*L. hilii*
768			*L. ignoratus*
769			*L. impervius*
770		*Lamellodiscus*	*L. indicus*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
771			*L. kechemirae*
772			*L. knoeffleri*
773			*L. magnicornis*
774			*L. major*
775			*L. mirandus*
776			*L. mormyri*
777			*L. neifari*
778			*L.niedashui*
779			*L. pagrosomi*
780			*L. parisi*
781			*L. parvicornis*
782			*L. rastellus*
783			*L. sanfilippoi*
784			*L. sarculus*
785			*L. sigilatus*
786			*L. spari*
787			*L. squamosus*
788			*L. takitai*
789			*L. theroni*
790			*L. toguebayei*
791			*L. tomentosus*
792			*L. triacies*
793			*L. tubulicornis*
794			*L. typicus*
795			*L. vaginalis*
796			*L. verberis*
797			*L. vicinus*
798			*L. virgula*
799		*Laticola*	*L. seabassi*
800			*L. dae*
801			*L. cyanus*
802			*L. latesi*
803			*L. lingaoensis*
804			*L. paralatesi*
805		*Lepidotrema*	*L. longipenis*
806		*Lobotrema*	*L. sciaenae*
807		*Murraytrema*	*M. bychowskyi*
808			*M. robustum*
809			*M. johniui*
810			*M. pricei*
811		*Murraytrematoides*	*M. lateolabracis*
812			*M. kuhliae*
813			*M. ditrematis*
814			*M. bychowskii*
815			*M. sp. LL-2012*
816		*Paradiplectanum*	*P. blairense*
817			*P. sillagonum*
818			*P.americanus*
819			*P.amplidiscatus*
820			*P.argus*
821			*P.auitoe*
822			*P.beverleyburtonae*
823			*P.bocquetae*
824			*P.bouaini*
825			*P.buitoe*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
826			*P.caballeroi*
827			*P.calathus*
828			*P.caledonicus*
829			*P.capurroi*
830			*P.chauveti*
831			*P.chinensis*
832			*P.coioidesis*
833			*P.cuitoe*
834			*P.cupatus*
835			*P.cyanopodus*
836			*P.cyathus*
837			*P.duitoe*
838			*P.enitsuji*
839			*P.epinepheli*
840			*P.euitoe*
841			*P.exoticus*
842			*P.fuitoe*
843			*P.guitoe*
844			*P.hargisi*
845			*P.hirundineus*
846			*P.huitoe*
847			*P.justinei*
848			*P.kritskyi*
849			*P.lantauensis*
850			*P.maaensis*
851			*P.magnisquamodiscum*
852			*P.malabaricus*
853			*P.manifestus*
854			*P.manipulus*
855		*Pseudorhabdosynochus*	*P.marcellus*
856			*P.maternus*
857			*P.melanesiensis*
858			*P.minutus*
859			*P.monaensis*
860			*P.podocyanus*
861			*P.querni*
862			*P.riouxi*
863			*P.serrani*
864			*P.shenzhenensis*
865			*P.sinediscus*
866			*P.sulamericanus*
867			*P.summanae*
868			*P.summanoides*
869			*P.vagampullum*
870			*P.venus*
871			*P.yucatanensis*
872			*P.coioidesis*
873			*P.cupatus*
874			*P.cyanopodus*
875			*P.epinepheli*
876			*P.grouperi*
877			*P.lantauensis*
878			*P.aff. lantauensis BTD-2009*
879			*P.aff. lantauensis BTD-2011*
880			*P.latesis*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
881			*P.melanesiensis*
882			*P.seabassi*
883			*P.shenzhenensis*
884			*P.summanoides*
885			*P.sp. 1 BTD-2009*
886			*P.sp. 1 BTD-2011*
887			*P.sp. 2 BTD-2009*
888			*P.sp. 2 BTD-2011*
889			*P.sp. 3 BTD-2009*
890			*P.sp. 4 BTD-2009*
891			*P.sp. 5 BTD-2009*
892			*P.sp. BTD-2009*
893		*Sinodiplectanotrema*	*S. argyromus*
894			*S. malayanum*
895			*S. sp. HGY-2007*
896		*Acanthoplacatus*	*A. adlardi*
897			*A. amplihamus*
898			*A. brauni*
899			*A. parvihamus*
900			*A. puelli*
901			*A. shieldsi*
902			*A. sigani*
903			*A. sp. WWAB-2002*
904		*Afrogyrodactylus*	*A. sp. IP-2012*
905		*Aglaiogyrodactylus*	*A. ctenistus*
906		*Diplogyrodactylus*	*D. martini*
907		*Fundulotrema*	*F. foxi*
908			*F. porterensis*
909			*F. prolongis*
910			*F. stableri*
911		*Gyrdicotylus*	*G. gallieni*
912			
913		*Gyrodactyloides*	*G. bychowskii*
914			*G. sp. IP-2012*
915		*Gyrocerviceanseris*	*G. passamaquoddyensis*
916			*G. aeglefini*
917			*G. aideni*
918			*G. alburnensis*
919			*G. alekosi*
920			*G. alexanderi*
921			*G. alexgusevi*
922			*G. anguillae*
923			*G. anisopharynx*
924			*G. aphyae*
925			*G. arcuatoides*
926			*G. arcuatus*
927			*G. barbi*
928			*G. bliccensis*
929			*G. brachymystacis*
930			*G. branchialis*
931			*G. branchicus*
932			*G. bullatarudis*
933			*G. cameroni*
934			*G. carassii*
935			*G. carolinae*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
936			*G. cernuae*
937			*G. chileani*
938			*G. cichlidarum*
939			*G. colemanensis*
940			*G. coriicepsi*
941			*G. corleonis*
942			*G. corydori*
943			*G. derjavini*
944			*G. derjavinoides*
945			*G. elegans*
946			*G. emembranatus*
947			*G. eos*
948			*G. ergensi*
949			*G. eyipayipi*
950			*G. flavescensis*
951			*G. flesi*
952			*G. fossilis*
953			*G. gasterostei*
954			*G. gobiensis*
955			*G. gobii*
956			*G. gondae*
957			*G. gracilihamatus*
958			*G. groenlandicus*
959			*G. gurleyi*
960			*G. harengi*
961			*G. hildae*
962			*G. hrabei*
963			*G. hronosus*
964			*G. jennyae*
965			*G. jiroveci*
966			*G. jussii*
967			*G. katharineri*
968			*G. kobayashii*
969			*G. laevis*
970			*G. laevisoides*
971			*G. lavareti*
972			*G. leptorhynchi*
973			*G. leucisci*
974			*G. lomi*
975			*G. cf. longidactylus*
976			*G. longipes*
977			*G. longiradix*
978			*G. longoacuminatus*
979			*G. lotae*
980			*G. lucii*
981			*G. luciopercae*
982			*G. macracanthus*
983			*G. macronychus*
984			*G. magnificus*
985			*G. malalai*
986			*G. mariannae*
987			*G. marinus*
988			*G. markakulensis*
989			*G. mediotorus*
990			*G. micropsi*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
991	Gyrodactylidae	*Gyrodactylus*	*G. cf. micropsi*
992			*G. cf. micropsi 1-TH-2003*
993			*G. cf. micropsi 2-TH-2003*
994			*G. misgurni*
995			*G. neili*
996			*G. neretum*
997			*G. cf. niger TH-2003*
998			*G. nigritae*
999			*G. nipponensis*
1000			*G. notatae*
1001			*G. nudifronsi*
1002			*G. orecchiae*
1003			*G. osmeri*
1004			*G. ostendicus*
1005			*G. ouluensis*
1006			*G. pannonicus*
1007			*G. papernai*
1008			*G. parvae*
1009			*G. percotti*
1010			*G. perlucidus*
1011			*G. pharyngicus*
1012			*G. phoxini*
1013			*G. pictae*
1014			*G. pleuronecti*
1015			*G. poeciliae*
1016			*G. pomeraniae*
1017			*G. pomeraniae x G. lavareti*
1018			*G. prostae*
1019			*G. pseudonemacheili*
1020			*G. pterygialis*
1021			*G. pungitii*
1022			*G. rarus*
1023			*G. rhodei*
1024			*G. robustus*
1025			*G. rogatensis*
1026			*G. rugiensis*
1027			*G. rugiensoides*
1028			*G. rutilensis*
1029			*G. rysavyi*
1030			*G. salaris*
1031			*G. salinae*
1032			*G. salmonis*
1033			*G. salvelini*
1034			*G. samirae*
1035			*G. sedelnikowi*
1036			*G. spathulatus*
1037			*G. sprostonae*
1038			*G. stephanus*
1039			*G. sturmbaueri*
1040			*G. superbus*
1041			*G. synodonti*
1042			*G. teuchis*
1043			*G. cf. teuchis*
1044			*G. thymalli*
1045			*G. thysi*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1046			*G. truttae*
1047			*G. turnbulli*
1048			*G. ulinganisus*
1049			*G. vimbi*
1050			*G. zimbae*
1051			*G. pannonicus*
1052			*G. sp. (Alburnus alburnus)*
1053			*G. sp. (Fundulus kansae) WWAB-2002*
1054			*G. sp. (Pimephales promelas) WWAB-2002*
1055			*G. sp. (Rhynichthis osculus) WWAB-2002*
1056			*G. sp. (Richardsonius balteatus) WWAB-2002*
1057			*G. sp. (Rutilus rutilus) Oulu type 1*
1058			*G. major*
1059			*G. bueni*
1060			*G. scleromystaci*
1061			*G. sp. 3 TH-2003*
1062			*G. sp. 6 TH-2003*
1063			*G. sp. DC-EC-058*
1064			*G. sp. DC-ON-002*
1065			*G. sp. DC-ON-004*
1066			*G. sp. DC2-01-01*
1067			*G. sp. ex Astyanax sp. WAB-2012*
1068			*G. sp. HH-2009b*
1069			*G. sp. HSS-2009*
1070			*G. sp. IP-2011-4*
1071			*G. sp. IP-2012a*
1072			*G. sp. IP-2012b*
1073			*G. sp. JW-47*
1074			*G. sp. JW-60*
1075			*G. sp. Ladoga*
1076			*G. sp. Ladoga x G. pannonicus*
1077			*G. sp. Poland-MZ-2003*
1078			*G. granoei*
1079			*G. sp. PY-2010b*
1080		*Ieredactylus*	*I. rivuli*
1081		*Laminiscus*	*L. gussevi*
1082		*Macrogyrodactylus*	*M. heterobranchii*
1083			*M. clarii*
1084			*M. clarii x M. heterobranchii*
1085			*M. congolensis*
1086			*M. heterobranchii*
1087			*M. karibae*
1088			*M. polypteri*
1089			*M. simentiensis*
1090			*M. sp. IP-2012*
1091		*Paragyrodactylus*	*P. variegatus*
1092		*Scleroductus*	*S. sp. ex Rhamdia sp. WAB-2012*
1093		*Swingleus*	*S. ancistrus*
1094	Hexabothriidae	*Branchotenthes*	*B. octohamatus*
1095		*Hexabothrium*	*H. musteli*
1096			*H. canicula*
1097			*H. akaroensis*
1098			*H. appendiculata*
1099			*H. appendiculatum*
1100		*Pseudohexabothrium*	*P. taeniurae*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1101		*Loimosina*	*L. sp. WAB-2014*
1102			*L. parawilsoni*
1103			*L. wilsoni*
1104	Microbothriidae	*Asthenocotyle*	*A. taranakiensis*
1105			*A. kaikourensis*
1106		*Dermophthirius*	*D. carcharhini*
1107			*D. carcharini*
1108			*D. maccallumi*
1109			*D. melanopteri*
1110			*D. penneri*
1111			*D. sp. EMP-2009*
1112			*D. sp. VG-2008*
1113		*Leptocotyle*	*L. minor*
1114		*Pseudoleptobothrium*	*P. aptychotremae*
1115			*P. sp. EMP-2009*
1116	Monocotylidae	*Calicotyle*	*C. affinis*
1117			*C.australis*
1118			*C.japonica*
1119			*C.kroyeri*
1120			*C.palombi*
1121			*C.stossichi*
1122			*C.urolophi*
1123			*C.sp. CWA1*
1124			*C.sp. EMP-2009*
1125			*C.sp. VG-2008*
1126		*Clemacotyle*	*C. australis*
1127		*Decacotyle*	*D. youngi*
1128			*D. octona*
1129			*D. elpora*
1130			*D. cairae*
1131			*D. floridana*
1132			*D. lymmae*
1133			*D. tetrakordyle*
1134		*Dendromonocotyle*	*D. ardea*
1135			*D. bradsmithi*
1136			*D. octodiscus*
1137		*Dictyocotyle*	*D. coeliaca*
1138		*Empruthotrema*	*E. torpedinis*
1139			*E. dasyatidis*
1140			*E. tasmaniensis*
1141			*E. stenophallus*
1142			*E. raiae*
1143			*E. kearni*
1144			*E. quindecima*
1145		*Heterocotyle*	*H. minima*
1146			*H. capricornensis*
1147		*Merizocotyle*	*M. australensis*
1148			*M. icopae*
1149			*M. sinensis*
1150			*M. urolophi*
1151		*Monocotyle*	*M. pricei*
1152			*M. diademalis*
1153			*M. corali*
1154			*M. helicophallus*
1155			*M. multiparous*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1156			*M. spiremae*
1157			*M. sp. Tunisia*
1158		*Neoheterocotyle*	*N. rhinobatidis*
1159			*N. rhinobatis*
1160			*N. rhynchobatis*
1161		*Potamotrygonocotyle*	*P. aramasae*
1162			*P. auriculocotyle*
1163			*P. chisholmae*
1164			*P. dromedarius*
1165			*P. quadracotyle*
1166			*P. rarum*
1167			*P. rionegrense*
1168			*P. septemcotyle*
1169			*P. tatianae*
1170			*P. tsalickisi*
1171			*P. umbella*
1172			*P. sp. KHFA-2009*
1173		*Triloculotrema*	*T. sp. Tunisia*
1174		*Troglocephalus*	*T. rhinobatidis*
1175	Neocalceostomatidae	*Neocalceostoma*	*N. elongatum*
1176			*N. sp.*
1177		*Thysanotohaptor*	*T. rex*
1178	Ooegyrodactylidae	*Phanerothecium*	*P. spinulatum*
1179			*P. sp. ex Rineloricaria lima WAB-2012*
1180	Pseudodactylogyridae	*Pseudodactylogyroides*	*P. apogonis*
1181		*Pseudodactylogyrus*	*P. anguillae*
1182			*P. bini*
1183			*P. haze*
1184			*P. microrchis*
1185			*P. sp. DTJL-2000*
1186			*P. sp. GZ-2012*
1187			*P. sp. UK*
1188			*P. sp. XHY*
1189	Pseudomurraytrematidae	*Pseudomurraytrema*	*P. ardens*
1190			*P. copulatum*
1191			*P. sp. USA*
1192	Sundanonchidae	*Sundanonchus*	*S. behuri*
1193			*S. micropeltis*
1194	Tetraonchidae	*Ergenstrema*	*E. labrosi*
1195			*E. mugilis*
1196		*Tetraonchus*	*T. monenteron*
1197	Udonellidae	*Udonella*	*U. australis*
1198			*U. caligorum*
1199			*U. fugu*
1200			*U. myliobati*
1201			*U. sp. 'Isolate Vancouver'*
1202			*U. sp. EMP-2009*
1203		*Euzetrema*	*E. knoepffleri*
1204		*Metacamopia*	*M. oligoplites*
1205	Axinidae	*Zeuxapta*	*Z. seriolae*
1206	Chauhaneidae	*Pseudochauhanea*	*P. macrorchis*
1207	Chimaericolidae	*Chimaericola*	*C. leptogaster*
1208		*Chalguacotyle*	*C. mugiloides*
1209			*C. aspinachorda*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1210	Diclidophoridae	*Choricotyle*	*C. caulolatili*
1211			*C. exilis*
1212			*C. louisianensis*
1213			*C. oregonensis*
1214			*C. prionoti*
1215			*C. chrysophrii*
1216			*C. anisotremi*
1217			*C. australiensis*
1218			*C. cf. chrysophryii*
1219		*Cyclocotyla*	*C. belones*
1220		*Diclidophora*	*D.luscae capelanii*
1221			*D. bellones*
1222			*D. coelorhynchi*
1223			*D. denticulata*
1224			*D. esmarkii*
1225			*D. luscae*
1226			*D. maccallumi*
1227			*D. merlangi*
1228			*D. minor*
1229			*D. minuti*
1230			*D. morrhuae*
1231			*D. pagelli*
1232			*D. palmata*
1233			*D. phycidis*
1234			*D. pollachii*
1235			*D. tubiformis*
1236		*Grubea*	*G. gracilis*
1237			*G. pneumatophori*
1238			*G. cochlear*
1239			*G. pnematophori*
1240			*G. australis*
1241		*Heterobothrium*	*H. bychowskyi*
1242			*H. elongatum*
1243			*H. lamothei*
1244			*H. lineatum*
1245			*H. okamotoi*
1246			*H. praeorchis*
1247			*H. shinagawai*
1248			*H. tetrodonis*
1249			*H. tonkinense*
1250			*H. torquigeneri*
1251			*H. yamagutii*
1252		*Neoheterobothrium*	*N. chilense*
1253			*N.cynoscioni*
1254			*N.hippoglossini*
1255			*N.affine*
1256			*N.hirame*
1257			*N.sp. SF*
1258			*N.sp. TY-2008*
1259			*N.insularis*
1260			*N.mcdonaldi*
1261			*N.paralichthyi*
1262			*N.syacii*
1263		*Paraeurysorchis*	*P. sarmientoi*
1264		*Parapedocotyle*	*P. prolatili*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1265		*Pedocotyle*	*P. annakohni*
1266			*P. bravoi*
1267			*P. minima*
1268			*P. morone*
1269		*Urocotyle*	*U. anellus*
1270			*U. nibae*
1271		*Diplozoon*	*D. bliccae*
1272			*D. homoion*
1273			*D. paradoxum*
1274			*D. sp.*
1275		*Eudiplozoon*	*E. nipponicum*
1276		*Inustiatus*	*I. aristichthysi*
1277			*I. inustiatus*
1278		*Paradiplozoon*	*P. rutili*
1279			*P. chazaricum*
1280			*P. bingolensis*
1281			*P. bliccae*
1282			*P. diplophyllorchidis*
1283	Diplozoidae		*P. hemiculteri*
1284			*P. homoion*
1285			*P. jiangxiensis*
1286			*P. megan*
1287			*P. nagibinae*
1288			*P. opsariichthydis*
1289			*P. parabramisi*
1290			*P. parapeleci*
1291			*P. pavlovskii*
1292			*P. sapae*
1293			*P. sp. BJVV-2012*
1294			*P. sp. BJVV-2013*
1295		*Sindiplozoon*	*S. ctenopharyngodoni*
1296	Discocotylidae	*Discocotyle*	*D. sagittata*
1297		*Gastrocotyle*	*G. buckleyi*
1298			*G. macedonica*
1299			*G. mozambiquensis*
1300			*G. japonica*
1301			*G. kurra*
1302			*G. indica*
1303	Gastrocotylidae		*G. hispida*
1304			*G. trachuri*
1305		*Pricea*	*P. minimae*
1306			*P. multae*
1307		*Pseudaxine*	*P. kurra*
1308			*P. bivaginalis*
1309			*P. trachuri*
1310		*Gotocotyla*	*G.queenslandici*
1311			*G.niphonii*
1312			*G.heapae*
1313			*G.elagatis*
1314	Gotocotylidae		*G.africanensis*
1315			*G.acanthura*
1316			*G.bivaginalis*
1317			*G.sawara*
1318			*G.secunda*
1319			*G.sp. JJ1*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1320	Heteraxinidae	*Cemocotyle*	*C. trachuri*
1321			*C. trachuri*
1322			*C. noveborancensis*
1323			*C. noveboracensis*
1324			*C. carangis*
1325			*C. boringuenensis*
1326	Hexostomatidae	*Hexostoma*	*H.thynni*
1327			
1328	Mazocraeidae	*Heteromazocraes*	*H. thrissoclissae*
1329			*H. phasae*
1330			*H. vicinus*
1331			*H. kazikodiensis*
1332			*H. hexacanthus*
1333			*H. dodecacantha*
1334			*H. coiliae*
1335			*H. sp. 1 BS-2013*
1336			*H. lingmueni*
1337			*H. sp. 2 BS-2013*
1338		*Kuhnia*	*K. otolithis*
1339			*K. guttatumai*
1340			*K. pricei*
1341			*K. arabica*
1342			*K. kanagurta*
1343			*K. microlepidotusi*
1344			*K. fruticosa*
1345			*K. scombercolias*
1346			*K. thunni*
1347			*K. indica*
1348			*K. pinnata*
1349			*K. sprostonae*
1350			*K. gooddingii*
1351			*K. frutescens*
1352			*K. scombri*
1353			*K. sp.*
1354		*Leptomazocraes*	*L. dussemerii*
1355			*L. trispina*
1356			*L. orientalis*
1357			*L. arabica*
1358		*Mazocraes*	*M. Sp.*
1359			*M.australis*
1360			*M.harengi*
1361			*M.longicauda*
1362			*M.mehrai*
1363			*M.pilchardi*
1364			*M.tadoore*
1365		*Neogrubea*	*N. seriolellae*
1366		*Mazocraeoides*	*M. dussumieri*
1367			*M. gonialosae*
1368		*Paramazocraes*	*P. thrissocles*
1369		*Unclassified Paramazocraes*	*P. sp. SB-2013*
1370		*Probursata*	*P. brasiliensis*
1371		*unclassified Mazocraeidae*	*Mazocraeidae gen. sp. 1 BS-2013*
1372		*Anchoromicrocotyle*	*A. guaymensis*
1373		*Atrispinum*	*A. acarne*
1374		*Bivagina*	*B. punctipinnis*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1375		*Bivagina*	*B. pagrosomi*
1376		*Cynoscionicola*	*C. pseudoheteracantha*
1377			*C. heterocantha*
1378			*C. branquialis*
1379		*Diplostamenides*	*D. sciaenae*
1380		*Kahawaia*	*K. truttae*
1381		*Metamicrocotyla*	*M.mugilis*
1382			*M.macracantha*
1383			*M.filiformis*
1384			*M.bora*
1385			*M.cephalus*
1386		*Microcotyle*	*M.Acanthurum*
1387			*M.Archosargi*
1388			*M.Argenticus*
1389			*M.Arripis*
1390			*M.Bassensis*
1391			*M.Bothi*
1392			*M.Brevis*
1393			*M.cantharivan*
1394			*M.Caudata*
1395			*M.Centrodonti*
1396			*M.Centropristis*
1397			*M.Constricta*
1398			*M.donavinivan*
1399			*M.Draconis*
1400			*M.Emmelichthyops*
1401			*M.erythrinivan*
1402			*M.Eueides*
1403			*M.Fistulariae*
1404			*M.Fusiformis*
1405			*M.Guanabarensis*
1406			*M.Gussevi*
1407			*M.Hainanensis*
1408			*M.Helotes*
1409			*M.Hemiatriospinalis*
1410			*M.Hiatulae*
1411			*M.Inglisi*
1412			*M.Jonii*
1413			*M.Korathai*
1414			*M.Lichiae*
1415			*M.Longirostri*
1416			*M.Macropharynx*
1417			*M.Madrasi*
1418			*M.Mouw*
1419			*M.Nemadactylus*
1420			*M.Oceanica*
1421			*M.Odacis*
1422			*M.Omani*
1423			*M.Otrynteri*
1424			*M.Peprili*
1425			*M.Polymixiae*
1426	Microcotylidae		*M.Polynemi*
1427			*M.Pomatomi*
1428			*M.pontica*
1429			*M.Poronoti*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1430			*M.Priacanthi*
1431			*M.Pseudopercis*
1432			*M.Rubrum*
1433			*M.Sebastis*
1434			*M.Sebastisci*
1435			*M.Seriolae*
1436			*M.Spinicirrus*
1437			*M.Stenotomi*
1438			*M.Tampicensis*
1439			*M.Tanago*
1440			*M.victoriae*
1441			*M.arripis*
1442			*M.bassensis*
1443			*M.erythrini*
1444			*M.mugilis*
1445			*M.pomatomi*
1446			*M.Sebastis*
1447		*Microcotyloides*	*M. incisa*
1448		*Neomicrocotyle*	*N. pacifica*
1449			*N. sp. DG-2013*
1450		*Pagellicotyle*	*P. mormyri*
1451		*Paramicrocotyle*	*P. sp. FAS-2014*
1452			*P. sillaginae*
1453			*P. sigani*
1454			*P. tubicirrus*
1455			*P. angifer*
1456			*P. sandarsae*
1457			*P. rhabdosargi*
1458			*P. queenslandensis*
1459			*P. maomao*
1460			*P. mamaevi*
1461			*P. madagascariensis*
1462			*P. kuhliae*
1463			*P. japonicus*
1464		*Polylabris*	*P. indica*
1465			*P. halichoeres*
1466			*P. girellae*
1467			*P. gerres*
1468			*P. diplodi*
1469			*P. carnarvonensis*
1470			*P. australiensis*
1471			*P. acanthogobii*
1472			*P. acanthopagri*
1473			*P. halichoeres*
1474			*P. heterodus*
1475			*P. sillaginae*
1476			*P. sp. JYW-2010*
1477		*Sciaenacotyle*	*S. sciaenicola*
1478		*Sparicotyle*	*S. chrysophryii*
1479		*unclassified Microcotylidae*	*Microcotylidae sp. M10*
1480			*Microcotylidae sp. M11*
1481			*Microcotylidae gen. sp. MAF-2012*
1482	Neothoracocotylidae	*Mexicotyle*	*M. sp. Brazil*
1483			*M. sp. DTJL-2000*
1484		*Paradewesia*	*P. sp. Brazil*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1485		*Paradewesia*	*P. sp. DTJL-2000*
1486	Octomacridae	*Octomacrum*	*O. europaeum*
1487			*O. lanceatum*
1488			*O. mexicanum*
1489			*O. microconfibula*
1490			*O. semotili*
1491			*O. spinum*
1492	Plectanocotylidae	*Plectanocotyle*	*P. gurnardi*
1493			*P. major*
1494			*P. sp.*
1495		*Plectanocotyloides*	*P. obscurum*
1496		*Concinnocotyla*	*C. australensis*
1497		*Diplorchis*	*D. ranae*
1498			*D. shilinensis*
1499		*Eupolystoma*	*E. alluaudi*
1500			*E. vanasi*
1501			*E. sp. DNA-179*
1502		*Madapolystoma*	*M.biritika*
1503			*M.sp. DNA-844*
1504			*M.sp. DNA-847*
1505			*M.sp. DNA-851*
1506			*M.sp. DNA-853*
1507			*M.sp. DNA-981*
1508			*M.sp. DNA-989*
1509			*M.sp. DNA-Mi18*
1510			*M.sp. DNA-Mi19*
1511			*M.sp. DNA-Mi292*
1512			*M.sp. DNA-Mi67*
1513			*M.sp. DNA-Mi878*
1514			*M.sp. DNA-Mi884*
1515			*M.sp. n. 1 PAB-2011*
1516			*M.sp. n. 2 PAB-2011*
1517			*M.sp. PAB-2011*
1518		*Metapolystoma*	*M.aff. brygoonis DNA-Mi407*
1519			*M.aff. brygoonis DNA-Mi461*
1520			*M.aff. brygoonis DNA-Mi476*
1521			*M.aff. brygoonis DNA-Mi863*
1522			*M.aff. brygoonis DNA-Mi864*
1523			*M.sp. DNA-Mi484*
1524			*M.sp. DNA-Mi866*
1525			*M.sp. DNA-Mi881*
1526			*M.sp. DNA-Mi70*
1527			*M.brygoonis*
1528			*M.cachani*
1529			*M.sp. DNA-990*
1530			*M.sp. DNA-991*
1531			*M.sp. DNA-Mi69*
1532			*M.sp. DNA-Mi71*
1533		*Nanopolystoma*	*N. sp. OV-2014*
1534		*Neodiplorchis*	*N. scaphiopi*
1535			*N.sp. H57*
1536			*N.sp. H80*
1537			*N.sp. H83*
1538			*N.spratti*
1539			*N.chelodinae*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1540	Polystomatidae	*Neopolystoma*	*N.euzeti*
1541			*N.fentoni*
1542			*N.liewi*
1543			*N.orbiculare*
1544			*N.palpebrae*
1545			*N.spratti*
1546			*N.sp. 1 OV-2011*
1547			*N.sp. 2 OV-2011*
1548			*N.sp. 3 OV-2011*
1549			*N.sp. 4 OV-2011*
1550			*N.sp. 5 OV-2011*
1551			*N.sp. 6 OV-2011*
1552			*N.sp. 7 OV-2011*
1553			*N.sp. 8 OV-2011*
1554			*N.sp. 9 OV-2011*
1555		*Parapolystoma*	*P. bulliense*
1556		*Polystoma*	*P. australis*
1557			*P. baeri*
1558			*P. carvirostris*
1559			*P. claudecombesi*
1560			*P. combesi*
1561			*P. cuvieri*
1562			*P. dawiekoki*
1563			*P. fuscus*
1564			*P. gallieni*
1565			*P. indicum*
1566			*P. integerrimum*
1567			*P. lopezromani*
1568			*P. mangenoti*
1569			*P. marmorati*
1570			*P. naevius*
1571			*P. nearcticum*
1572			*P. occipitalis*
1573			*P. pelobatis*
1574			*P. testimagna*
1575			*P. umthakathi*
1576			*P. sp. DNA-25*
1577			*P. sp. DNA-38*
1578			*P. sp. DNA-40*
1579			*P. sp. DNA-7*
1580			*P. sp. DNA-8*
1581			*P. sp. LXF-2008*
1582			*P. sp. MB-2009*
1583			*P. sp. Mi851*
1584			*P. sp. Mi852*
1585			*P. sp. OV-2005*
1586			*P. sp. OV-2007*
1587			*P. sp. TJ-2008*
1588		*Polystomoidella*	*P. sp. 1 OV-2011*
1589		*Polystomoides*	*P. asiaticus*
1590			*P. australiensis*
1591			*P. bourgati*
1592			*P. coronatum*
1593			*P. malayi*
1594			*P. oris*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1595		*Polystomoides*	*P. renschii*
1596			*P. siebenrockiella*
1597			*P. tunisiensis*
1598			*P. sp. 1 OV-2011*
1599			*P. sp. 2 OV-2011*
1600			*P. sp. 4 OV-2011*
1601		*Protopolystoma*	*P. occidentalis*
1602			*P. orientalis*
1603			*P. simplicis*
1604			*P. xenopodis*
1605			*P. sp. 003.48.1*
1606			*P. sp. 003.52.1*
1607			*P. sp. G12.13.1*
1608			*P. sp. KAP.1*
1609			*P. sp. Mab F.1*
1610			*P. sp. WULF.1*
1611		*Pseudodiplorchis*	*P. americanus*
1612		*Pseudopolystoma*	*P. dendriticum*
1613		*Sundapolystoma*	*S. chalconotae*
1614		*Wetapolystoma*	*W. almae*
1615		*Polystomatidae gen. PB-2010*	*P. gen. sp. PB-2010*
1616		*unclassified Polystomatidae*	*Polystomatidae sp. 1 OV-2011*
1617			*Polystomatidae sp. 2 OV-2011*
1618			*Polystomatidae sp. 3 OV-2011*
1619			*Polystomatidae sp. 4 OV-2011*
1620			*Polystomatidae sp. 5 OV-2011*
1621			*Polystomatidae sp. 6 OV-2011*
1622			*Polystomatidae sp. 7 OV-2011*
1623			*Polystomatidae sp. Eol92/Mi84*
1624			*Polystomatidae sp. Eos10/Mi707*
1625			*Polystomatidae sp. Eos10/Mi709*
1626			*Polystomatidae sp. Eos2/Mi687*
1627			*Polystomatidae sp. Eos23/Mi932*
1628			*Polystomatidae sp. Eos9/Mi702*
1629			*Polystomatidae sp. Eos9/Mi704*
1630			*Polystomatidae sp. Eos9/Mi705*
1631			*Polystomatidae sp. Mi125*
1632			*Polystomatidae sp. Mi126*
1633			*Polystomatidae sp. Mls18/MiAB12*
1634			*Polystomatidae sp. Mls18/MiAB13*
1635			*Polystomatidae sp. Mls28/MiAB10*
1636			*Polystomatidae sp. Mls4/Mi-719*
1637			*Polystomatidae sp. Mls6/Mi939*
1638			*Polystomatidae sp. Mls6/MiAB9*
1639			*Polystomatidae sp. P15Mb06*
1640			*Polystomatidae sp. PB-2010*
1641			*Polystomatidae sp. PL011126B1*
1642			*Polystomatidae sp. PL011126B2*
1643			*Polystomatidae sp. PL050114E*
1644			*Polystomatidae sp. PL050123J1*
1645			*Polystomatidae sp. PL050123J2*
1646			*Polystomatidae sp. PL060209G*
1647			*Polystomatidae sp. PL060210B*
1648			*Polystomatidae sp. PL060211I*
1649			*Polystomatidae sp. PL060214E*

Summary of members of Class Monogenea explored for the study

F.No.	Family	Genus	Species
1650			*Polystomatidae sp. PL060220K*
1651			*Polystomatidae sp. PL070221D*
1652			*Polystomatidae sp. PL080204A*
1653			*Polystomatidae sp. Pv2*
1654			*Polystomatidae sp. Tses10/Mi950*
1655			*Polystomatidae sp. Tses19/Mi894*
1656			*Polystomatidae sp. Tses19/Mi895*
1657			*Polystomatidae sp. Tses2/Mi744*
1658			*Polystomatidae sp. Tses2/Mi745*
1659			*Polystomatidae sp. Tses2/Mi746*
1660			*Polystomatidae sp. Tses46/Mi898*
1661			*Polystomatidae sp. Tses46/Mi899*
1662			*Polystomatidae sp. Tses46/Mi901*
1663			*Polystomatidae sp. Tses6/Mi751*
1664			*Polystomatidae sp. Tsss55/Mi922*
1665			*Polystomatidae sp. Tsss55/Mi925*
1666		*Bilaterocotyle*	*B. carangis*
1667			*B. madrasensis*
1668	Protomicrocotylidae	*Lethacotyle*	*L. fijiensis*
1669			*L. sp. n. DG-2013*
1670	Pyragraphoridae	*Pyragraphorus*	*P. caballeroi*
1671			*P. pyragraphorus*
1672			*P. hollisae*
1673	Sphyranuridae	*Sphyranura*	*S. oligorchis*

दयालसिंहकॉलेज
DYAL SINGH COLLEGE
(दिल्लीविश्वविद्यालय)
(University of Delhi)
लोधीरोड, नईदिल्ली110003 –
Lodi Road, New Delhi – 110003
दूरभाष/Telephone24367819 : , 24365948
फैक्स/Fax:- 24365606

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2. Fozail Ahmad, D. Singh & P.V. Arya (2015). Comparative evaluation of speciation and zoogeographical distribution for *Lamellodiscus* (Monogenea: Diplectanidae) using 18S rRNA. International Journal of Innovation Science and Research (IJISR); 4 (6), 235-241. **[ISSN : 2319-9369].**
3. Fozail Ahmad, D. Singh & P.V. Arya (2015). *In-silico* phylogenetic study of *Dactylogyrus* (Class : Monogenea) using 18S rRNA with a note on zoogeographical investigations on the genus. International Journal of Biological and Biomedical Sciences; 4(8) : 055-058. **[ISSN:2319-9806].**
4. Fozail Ahmad, D. Singh & P.V. Arya and HS Singh (2015). *In silico* phylogenetic study on Ancyrocephalidae (Class : Monogenea) using 28SrRNA extending geo-mapping in search of evolutionary cues. Biochemical and Cellular Archives; 15 (2) : 391-399. **[ISSN :0972-5075]. [NAAS Score : 3.77].**
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6. Fozail Ahmad, & P.V. Arya, H.S. Singh (2015). COX-1 studies in evaluation and assessment of molecular diversity among Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae families (Class : Monogenea). International Journal of Innovation Science and Research (IJISR); 4(10) : 494-500. [**ISSN : 2319-9369**].
7. Fozail Ahmad, D. Singh, P.V. Arya and H.S. Singh (2016). *In-silico* Phylogenetic tools employed on some members of five major families of Monogenea *viz.,* Monocotylidae, Ancylodiscoididae, Ancyrocephalidae, Cichlidogyridae and Polystomatidae for investigating their relatedness and global diversity distribution. Journal of Experimental Zoology, India; 19(1) : 505-513. **[ISSN: 0972-0030].**
8. Fozail Ahmad, C. Sharma , V.P. Aggarwal & P.V. Arya (2016). Revisiting diversity and geographical distribution of eight minor families *viz.,* Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae of Class Monogenea. International Journal of Innovation Science and Research (IJISR) ; 5(1) : 608-610. [**ISSN : 2319-9369**].

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RESEARCH ARTICLE

IN SILICO PHYLOGENETIC STUDIES ON SOME MEMBERS OF PARASITIC GENUS GYRODACTYLUS (MONOGENEA: GYRODACTYLIDAE) FOR ASSESSMENT OF EVOLUTIONARY RELATEDNESS INFERRED FROM 28S RIBOSOMAL RNA AND GEOMAPPING THE SAMPLE

Fozail Ahmad, Dharmendra Singh and Priya Vrat Arya*

Department of Zoology, Dyal Singh College, University of Delhi, Lodhi Road, New Delhi, 110003

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ABSTRACT

Present day biodiversity need to be explored though the clues of evolution and migration for understanding the ancient relationship/origins. Traditionally zoogeographical distribution was a handy tool for deriving evolutionary relationships. Presently molecular comparison among species by constructing phylogenetic tree using nucleic acid and protein sequences is widely used in exploring the same. Secondary structure of RNA (which accounts for negative free energy of molecule) has also been employed in relating two or more than two species in some studies. Construction of secondary structure from 28S rRNA data of few species of *Gyrodactylus* is employed in molecular comparison; evolution pattern and level of complexity developed by organisms itself. The analysis performed in this work reflect that a range of patterns of evolution in the secondary structure of rRNA (number and types of loops) can be set by exploiting one species of a cluster as common/representative species. Geo-mapping of the different species when compared with phylogenetic tree bring better understanding in probable evolution/migration patterns in their hosts.

INTRODUCTION

Addition to knowledge base in the form of new evidences present new avenues for the study of evolutionary aspects. Zoogeographical distribution of organisms pose a picture for their present as well as ancient history. Host specific parasite create much more clearer picture in terms of themselves along with their hosts. Monogenean parasites can be taken as one such tool for indirectly study their host zoogeographical diversity, distribution, migration and settlement over period of time. Monogenean genus *Gyrodactylus* is having greatest diversity with approximately 409 species recorded from 400 hosts [1]. This genus offers a broader range for evolution and ecology due to its versatile nature (reported from marine and freshwater and brackish habitats) having much occurrence from freshwater sources [2, 3]. On account of their exposure to various environments and switching from one to other host, they have noticeable variation in their genetic compositions, which is necessary for their survival in that particular environment [4]. Staying onto a host after switching from the previous environment; be it marine to freshwater they gradually tend to change their morphology and genetic composition [4,5,6]. Sometimes they exhibit a significant development in certain structures, if the host possesses hefty protective system [7].

The comparative studies primarily involve morphological features, habitat, mode of nutrition and adaptation and anatomical characters especially in case of parasitic organisms like monogeneans, whereas the molecular comparison shows the way more specific towards their evolution and evolutionary relationships[8], comparing the sequences of 28S rRNA and secondary structures and measuring their structural parameters (bond energy, base composition, geometrical features etc.) regarded as best suited methods [9]. As the rRNAs have been conserved throughout the evolution, bulges, loops, helices and separation of single strands are considered as the phylogenetic characters of secondary structure elements [10]. RNA secondary structure is substantially useful in terms of giving morphological information that cannot be inferred from primary structure (simple sequence) [9,11]. It is also worth mentioning that RNA contains sequence motifs that lead to the development of DNA markers or biomarkers for individual species [10,12]. In past, intensive phylogenetic analyses have been carried out on the various species of the genus *Gyrodactylus*, including species validation and evolutionary relationship whenever some new species were discovered[13]. Most of these analyses were performed through sequence (DNA/RNA) comparison and through construction of phylogenetic tree but a little attention were paid on the structural components of 28S rRNA molecules. Since data on 28S are available in National Center for Biotechnology

Corresponding author:* **Fozail Ahmad
Department of Zoology, Dyal Singh College, University of Delhi, Lodhi Road, New Delhi, 110003

Information (NCBI) and many other databases, it is worth analyzing the phylogenetic relationships and re-setting the evolutionary relations among species of the genus *Gyrodactylus*[14]. A general trend among Monogenean parasites is that morphologically, complexity level of species increases from simpler to more complex system with developing structures (capillaries, ducts, flame bulbs, haptor etc.)[15]. Also, closely related monogeneans parasitize the closely related host species[16]. Therefore, understanding the molecular trends and utilizing 28S RNA will be useful in correlating the hosts and their parasites as well as level of complexity and extent of parasitism can be easily known from 28S secondary structure of species[17].

In this paper, authors intend to employ molecular diversity of genus *Gyrodactylus* in evaluating relative relationship among global representatives and predicting probable host zoogeographical diversity, distribution, migration and settlement over period of time using the secondary structure of 28S rRNA of some species of *Gyrodactylus*.

species were confirmed from literature and other sources (Gyrodb, Encyclopedia of Life, World Register of Marine Species etc.).

Molecular Phylogenetic Analysis

Sequences for selected species (Table-1) were subjected to alignment using ClustalW (inbuilt in MEGA 6) for multiple sequence alignment (Thompson *et al*. 1994) with the default gap and extension penalties used by this tool. MEGA 6 was used for constructing the phylogenetic tree using neighbor joining (NJ) method, . The average pathway method was used to calculate the branch length depicted in the number of variations all over the sequences. Resultantly, the most parsimonious tree was chosen by the close-neighbor-interchange algorithm. A bootstrap procedure with 1000 replication was executed for assessing the robustness of the inferred phylogenetic tree. The constructed NJ tree consisted of 39 species was represented with six clades for further analysis (Figure 1).

Table1 List of species of the genus *Gyrodactylus,* corresponding source, host and accession id.

Sl.	Parasite	Host	Marine/Fresh	Country/Area	Accession ID	Reference
1.	*G. nudifronsi* Rokicka *et al*., 2009	*Gaudy notothen*	Freshwater	Antarctica	FJ009452	[18]
2.	*G. coriicepsi* Rokicka *et al*.,2009	*Gaudy notothen*	Freshwater	Antarctica	FJ009451	[19][18]
3.	*G. anguillae* Ergens, 1960	*Anguillae reinhardti*	Marine	Australia	AB063294	[20],[21]
4.	*G. corti* Mizelle & Kritsky, 1967	*Anarrhichthys ocellatus*	Marine	California	KJ095103	[22]
5.	*G. alburnensis* Prost 1972	*Phoxinus eos*	Marine	Canada	AY278032	[30]
6.	*G. brachymystacis* Ergens, 1978	*Salvelinus fontinalis*	Freshwater	Canada	GQ368237	[23],[24]
7.	*G. parvae* You, Easy & Cone, 2008	*Pseudorasboraparva*	Freshwater	Central China	EF450249	[25]
8.	*G. rivularae* Basilewsky, 1855	*Abbottina rivularis*	Marine	Central China	HM18588	[26]
9.	*G. sprostonae* Ling, 1962	*Carassius carassius*	Freshwater	China	AY278044	[27]
10.	*G. salmonis* Yin & Sproston, 1948	*Oncorhynchus clarki*	Marine	China	GQ368233	[28],[29]
11.	*G. pomeraniae* Jussi Kuusela, 2008	*Rutilus rutilus*	Freshwater	Finland	EF143069	[30]
12.	*G. ouluensis* Kuusela *et al*., 2008	*Rutilus rutilus*	Freshwater	Finland	AF484546	[30]
13.	*G. truttae* Mikailov, 1975	*Salmo trutta*	Freshwater	Germany	AJ132260	[31]
14.	*G. pannonicus* Molnar, 1968	*Barbus barbus*	Freshwater	Hungary	EU678645	[32]
15.	*G. gussevi* Ling Mo-en, 1962	*Heteropneusts fossilis*	Freshwater	India	KJ461316	[33]
16.	*G. colisai* Bloch & Schn.	*Colisa fasciatus*	Freshwater	India	GQ925912	[34]
17.	*G. derjavinoides* Malmberg, 1975	*Salmo trutta trutta*	Marine	Iran	DQ357215	[35]
18.	*G. neretum* Paladini *et al*., 2010	*Syngnathus scovelli*	Marine	Italy	FJ183748	[36]
19.	*G. corleonis* Paladini *et al*., 2010	*Syngnathus scovelli*	Freshwater	Italy	FJ183747	[22][36],[37]
20.	*G. kobayashii* Kobayashi J ,1988	*Carassius auratus*	Freshwater	Japan	KJ755086	[26]
21.	*G. zimbae* Vanhove *et al*., 2011	*Simochromis diagramma*	Freshwater	Lake Tanganyika	HQ214482	[38]
22.	*G. thysi* Vanhove *et al*., 2011	*Simochromis diagramma*	Freshwater	Lake Tanganyika	HQ214481	[39]
23.	*G. sturmbaueri* Vanhove *et al*., 2011	*Simochromis diagramma*	Freshwater	Lake Tanganyika	HQ214480	[39],[40]
24.	*G. chileani* Ziętara, *et al*., 2012	*Helcogrammoides chileani*	Marine	Mediterranean & N. Seas	JQ045347	[22]
25.	*G. gondae* Huyse *et al*., 2004	*Pomatoschistus minutes*	Marine	Mediterranean Sea	AF328866	[41]
26.	*G. aideni* Mullen *et al*., 2010	*Pseudopleuronectes americanus*	Marine	Canada (New Brunswick)	HM48128	[42]
27.	*G. gurleyi* Price, 1937	*Carassius auratus*	Marine	North America	KC922453	[43]
28.	*G. leptorhynchi* Cone *et al*., 2013	*Syngnathus leptorhynchus*	Marine	North America	JX110633	[37]
29.	*G. bullatarudis* Turnbull, 1956	*Poecilia reticulate*	Freshwater	Northern Trinidad	AY692024	[44],[45]
30.	*G. pictae* Cable 2005	*Poecilia reticulate*	Freshwater	Northern Trinidad	AY692023	[46]
31.	*G. papernai* Ergens & Bychowsky, 1967	*salmon Salmo*	Freshwater	Russia	AF484533	[47]
32.	*G. ergensi* Prikrylova, *et al*., 2009	*Oreochromis niloticus*	Freshwater	Senegal	FN394985	[48]
33.	*G. eyipayipi* Vaughan *et al*., 2010	*Syngnathus acus*	Marine	South Africa	FJ040184	[49]
34.	*G. robustus* Malmberg, 1957	*Platichthys flesus*	Marine	Sweden	AY278040	[18]
35.	*G. phoxini* von Nordmann, 1832	*Phoxinus phoxinus*	Freshwater	Sweden	AY278037	[50]
36.	*G. flesi* Malmberg, 1957	*Platichthys flesus*	Marine	Sweden	AY278039	[18],[51]
37.	*G. magnificus* Malmberg, 1957	*Phoxinus phoxinus*	Freshwater	Sweden	AY278035	[50]
38.	*G. salaris* Malmberg, 1957	*Salmo salar*	Freshwater	Sweden	EF464678	[52],[53]
39.	*G. ch. Teuchis* Lautraite *et al*.,1999	*Oncorhynchus mykiss*	Marine	North America	KM19223	[54]

MATERIAL AND METHODS

Selection of Species of genus Gyrodactylus

In all thirty nine species were selected considering global distribution representation (Table-1). Distribution and source of

Inferring Secondary Structure of 28SrRNAs

The formation of secondary structure is based upon the alignment score of the sequences of clades. Subsequently, the sequence with the highest score was subjected to Mfold (URL

http://mfold.rna.albany.edu) for constructing the secondary structure of 28S rRNA at a fixed temperature of 37^0 C and analyzed for loops, stems and bulges. Similarly, the procedure was repeated for all clades and as a result six RNA secondary structures were formed. In this way, every clade in the tree had been associated with its rRNA which averaged out the evolutionary commonalities between the species of a particular clade. This has made the cladistic analysis more precise than the traditional comparison of clades with bootstrap values.

Geo mapping

In order to understand the global scenario of the species relatedness and diversity all the selected species as per table-1 were marked on simple world map manually. Later on marked species were joined with reference to their respective clades for inferring molecular relatedness.

RESULTS

Construction of phylogenetic tree

After alignment and processing for phylogenetic tree as per selected methods tree with six clades was formed (Fig. 1).

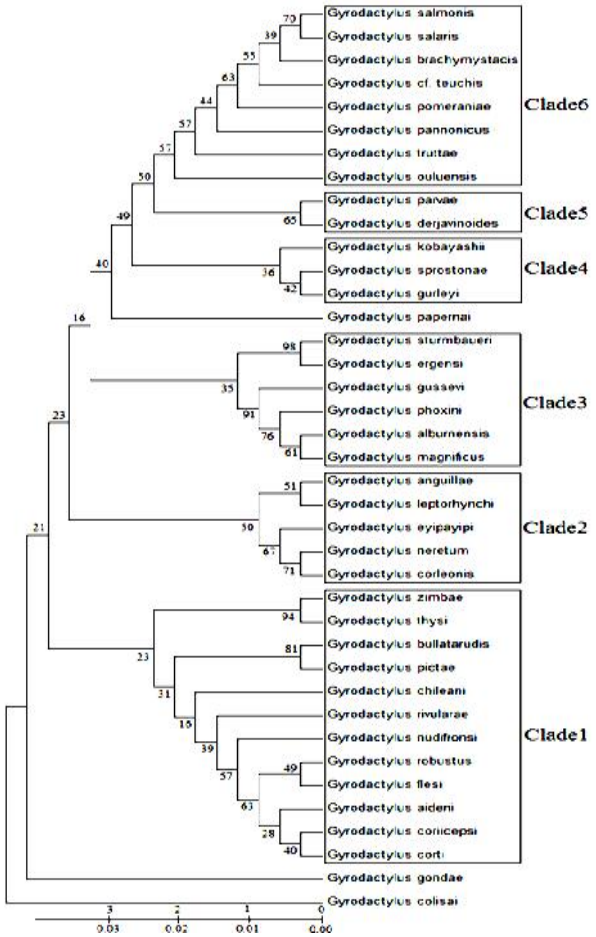

Figure 1 Phylogenetic tree (Neighbor joining) using 28S rRNA sequences for the 39 species of genus *Gyrodactylus*.

In the tree, Clade1, Clade2, Clade3, Clade4, Clade5 and Clade6 have 12, 5, 6, 3, 2 and 8 species respectively. Three species: *G. papernai, G. gondae* and *G. colisai* were kept out of the cluster since they didn't show the default/optimum evolutionary relatedness/relationship with any other species in the tree. We only aim to compare the groups of species in clades and not the individual ones, therefore these three species were left unmarked and hence were not considered in the analysis. In our analysis, out-group does not affect the in-group (cluster) which is the only concerned in constructing this phylogenetic tree. First cluster (Clade) had 12 species in which representative species *G. zimbae* formed a sister clade with *G. thysi* with 94% bootstrap value. This relationship showed that these species had the closely related origin. In the second sister clade of the same cluster *G. bullatardis* and *G. pictae* were related by 81% bootstrap value. The second clade had five species with sister clades and commonly linked by 50% bootstrap value. Among the sister clades, bootstrap value were considerably significant as they were linked by higher bootstrap values. The third cluster, although had 35% bootstrap value in common but sister clade in the cluster had highly significant bootstrap values. The fourth cluster with three species had 36% and 42% bootstrap value, does not represent significant evolutionary relationship. The fifth cluster comprising of two species had a 65% bootstrap value. The sixth and last cluster comprising of eight species formed seven sister clades with considerable bootstrap values among which the top most sister clade comprising of two species had the best bootstrap value of 77%.

Secondary structure analyses

Secondary structure (Fig. 2) generated by Mfold exhibited differences (Table-2) between clades using maximum negative free energy and pattern of loop and bulge formation. Secondary structure of *G. ergensi and G. sprostoni* (representative of clade3 and clade4) had highest (ΔG = -227.20 Kcal/mol) negative free energy (Fig. 2 c. and d.). *G. zimbae* (Clade1) had the second highest (ΔG= -226.70 Kcal/mol) negative free energy. *G. leptorhynchi* (Clade2), *G. derjivinoides* (Clade5), *G. branchymystacis* (Clade6), had ΔG = -198.80 Kcal/mol, ΔG = -196.00 Kcal/mol, ΔG = -206.10 Kcal/mol negative free energies respectively. The negative free energies except Clade2, Clade5 and Clade6 had a range from -226.70 to -227.20 Kcal/mol. Clades falling in this range were Clade1, Clade3, Clade4 and Clade5, confirmed the closer relatedness and evolution pattern. Clade1, Clade3 and Clade4 showed the closest evolutionary relatedness of these 28S RNAs with a difference of ΔG = -0.50 Kcal/mol negative free energy, proved to be of the same evolution pattern.

RNA in the folded form exhibit paired and unpaired (loops) bases. Qualitatively. The pattern of loops in secondary structure varied for all forms *i.e.,* interior loop, hairpin loop and bulge loop. Among all three types of loops, interior loops are more in number.Clade4 had the maximum number (45) of loops, where as Clade3 had the second most (42) loops in number. Clade1, Clade2, Clade5 and Clade6 had 39, 41, 41 and 41 loops respectively. Three Clades 2,5 and 6 are equal in number in loops, confirmed the similar stability which is also corroborated by the range of negative free energies of these Clades. They are falling in the range of -196.00 to -206.10 kcal/mol negative free energy.

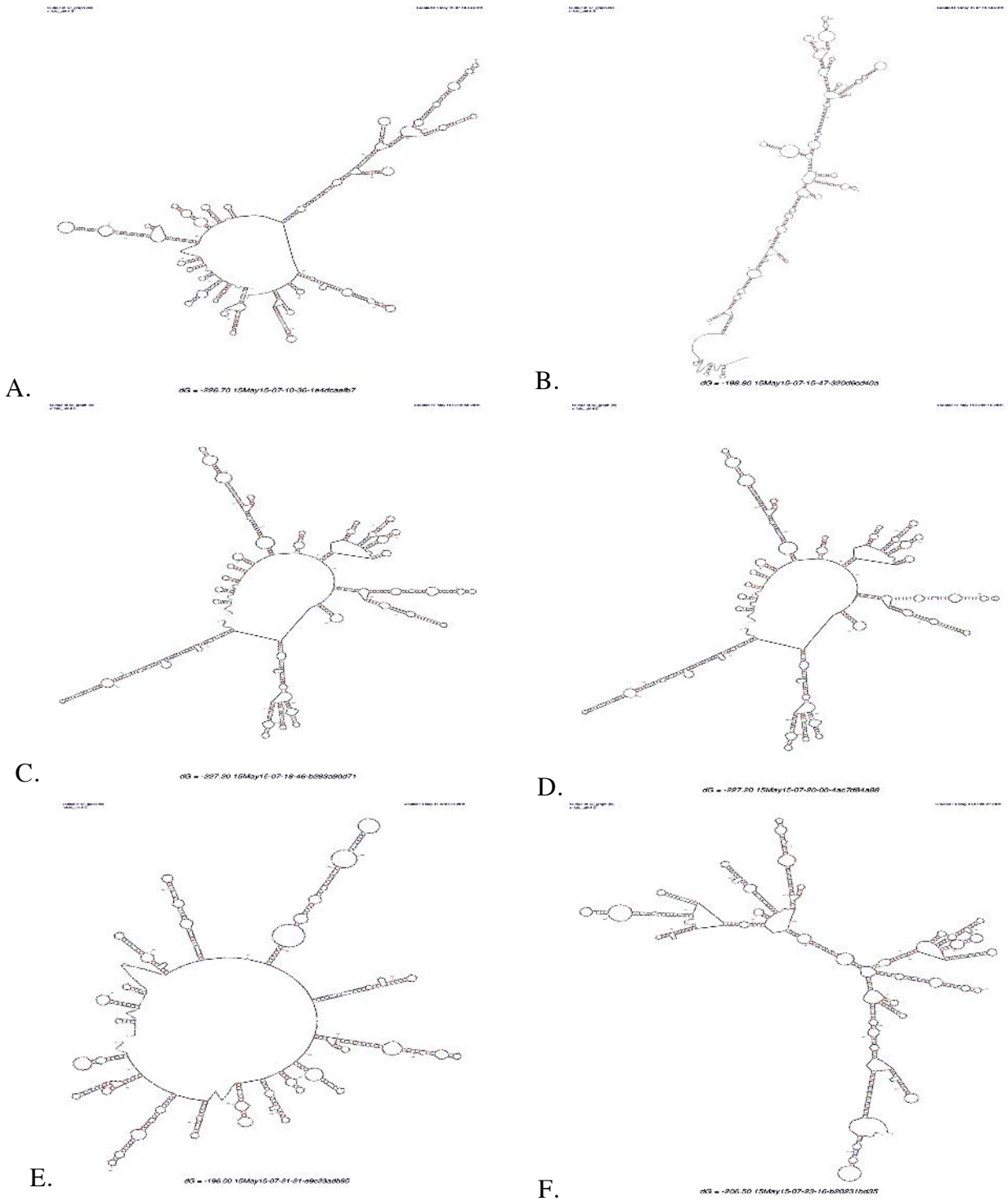

Figure 2 28S rRNA Secondary structure of **A.** *G. alburnensis*, **B.** *G. pictae*, **C.** *G. corti*, **D.** *G. stumbaeuri*, **E.** *G. corleonis*, **F.** *G. truttae*

Table 2 Clade details listed with representative species showing various parameters.

S. no.	Clade (Species)	Negative free energy (□ G)	Interior loop	Hairpin loop	Bulge loop	Total number of loops
1.	Clade1 (*G. zimbae*)	-226.70	15	19	5	39
2.	Clade2 (*G. leptorhynchi*)	-198.80	20	15	6	41
3.	Clade3 (*G. ergensi*)	-227.20	17	19	6	42
4.	Clade4 (*G. sprostoni*)	-227.20	19	19	7	45
5.	Clade5 (*G. derjavinoides*)	-196.00	17	18	6	41
6.	Clade6 (*G. branchymystatic*)	-206.10	20	16	5	41

Fig.3 Geo mapping of selected species of genus *Gyrodactylus* on physical map.

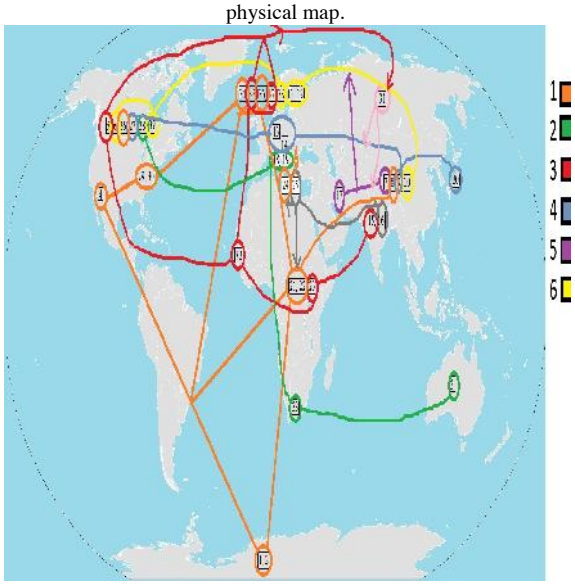

Fig.4 Geo mapping of selected species of genus *Gyrodactylus* and clade connectivity. Each number representing respective clade.

DISCUSSION

The phylogenetic tree from neighbor joining method showed that clades vary greatly in possessing the number of species which represents the variations among species of the genus *Gyrodactylus* [55] (figure-1). The species *G. closai* was the out-group in the tree as it has no bootstrap value[56]. The criteria of selecting an out-group depend upon the kind of analysis being performed[57]. The comparison between all six common RNA from each clade proves that all are genetically distinct[58,59]. RNA in the folded form showed paired and unpaired (loops) bases. Qualitatively, bases which are bonded, tend to stabilize RNA due to negative free energy whereas unpaired bases tend to destabilize the molecule due to positive free energy[60]. Quantitatively, loop that are more in number destabilize the secondary structure because they require more positive free energy[61]. Thus, clade3 and clade4 are the most stable and Clade5 is the least stable structure signifying that organisms belonging to the particular clade will be of equal stability in terms of negative free energy of RNA. The phylogenetic analysis was performed with the aim of finding the organism which could represent its clade, making comparative studies fast and easier whereas secondary structure analysis strengthens them[62]. From first to sixth cluster, each organism representing its own clade showed distinction in the term of number of neighbor organisms and 28S rRNA secondary structure. Although negative free energy and number of loops varied within all clades but a correlation between the two parameters have been established. Clade5 with a total of 39 loops (least in number) possessed second highest G (negative free energy) whereas Clade2, clade5 and clade6 with a total of 41 loops (all having the same number) possessed least negative free energy. Systematically, these groups should have higher ΔG than the presented ones because more loops require more G[63]. Clade4 and clade5 with maximum number of loops possessed the highest G. Comparatively, they don't coincide with other clades in number of loops and G because each group of organisms have their particular pattern of evolution of RNA[64]. The distinctions among clades were accounted due to the size of loops. Loops more in number but smaller in size are formed with less negative free energies whereas loops less in number but larger in size require more negative free energies[65]. Evidently, both, size and number of loops are accounted for estimating out the stability of a structure[66, 67]. The pattern of evolution of species is reflected by the development of loops and their sizes which in turn account for the overall stability of RNA. Evolution has always increased level of complexity which of course coincides with the necessities of situation[68]. RNA having more complex secondary structure presents with more loops and small sizes whereas molecule with lesser loops and large sizes shows lower level of complexity[69]. Same clade have the species which are more or less relatively close to each other in terms of geographical distribution or possibly connected through probable migration cycle (Fig. 3-4). Being able to survive in variety of habitats [2-4] this genus is ideal to study the variable habitat (fresh and marine) migration and settlements among their host.

CONCLUSION

The molecular comparison between large numbers of species has been possibly made easier and time required for such analysis is reduced by representing more than two evolutionarily related species with a common species. Through forming clades and clusters, grouped species will be further related in terms of negative free energy. This will not be limited up to individual evolution pattern of a species only but the entire group as a whole. The representing species of a cluster/clade will provide a range of evolution, stability (RNA structure) and complexity between other related groups. Same clade represents the commonly related species and indirectly host as well. Ideally reflecting the distribution (over a long period of time) and diversification of their host on zoogeographical scale.

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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF SPECIATION AND ZOOGEOGRAPHICAL DISTRIBUTION FOR *LAMELLODISCUS* (MONOGENEA: DIPLECTANIDAE) USING 18S rRNA

Fozail Ahmad, Singh, D. and *Arya, P. V.

Department of Zoology, Dyal Singh College (University of Delhi), Lodhi Road, New Delhi

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ABSTRACT

Zoogeographic distribution may present evolutionary cues for diversity and speciation. Evaluation of zoogeographical distribution together with molecular clues could insight into evolutionary history including probable origin as well. Genus *Lamellodiscus* (Monogenea: Diplectanidae) *may* offers great opportunity to analyze the inter-host specifity for understanding molecular conservation and phylogenetic relationship. Members of the genus were integrated in terms of zoographical distribution and diversity. Significant relatedness of species were shown and confirmed from across the globe, irrespective of distant evolutionary relationship. The evolving 18S rRNA structure confirmed the extent of speciation and demonstrated that anomaly in their evolution was accounted mainly due to separation of species into different geographical zones. Representative species of different clades were not well connected either geographically or cladistically but secondary structure proved that they evolved into different individuals/species thousand years ago and maintained the same pattern of origin. Molecular information of evolution pattern was stored and remain conserved in their ribosomal RNAs.

Key Words: Zoogeographic distribution, Speciation, *Lamellodiscus*, 18S rRNA

INTRODUCTION

Zoogeographic distribution may present evolutionary cue for diversity and speciation. Evaluation of zoogeographical distribution together with molecular clue may present evolutionary history including probable origin of the organisms. Monogenea is the class of parasitic Playtehelmenthis has approximately 35 families, 220 genus and 1850 species[1] with almost all members having a wide range of intra host specificity and representing great speciation events[2]. Some of the genera may have generalist species parasitizing several hosts[3]. One of the example is the genus *Lamellodiscus* in which a few species are found to infect up to six hosts[3,4] as the inter host specificity reflects a great evolution and significant zoogeographical distribution[5]. Addition to knowledge base in the form of new evidences may presents new avenues for the study of evolutionary aspects. Such as a picture of present and ancient history of organism can be possessed by Zoogeographical distribution[6]. Monogenean parasites have been taken as one such tool for indirectly study their host zoogeographical diversity, distribution, migration and settlement over period of time[7]. Monogenean genus *Lamellodiscus* is having greatest inter host diversity with a higher number of host[8,9]. This genus offers a broader range for evolution and ecology due to its versatile nature having much occurrence from one host to another and hence reflects a great distribution across the globe[9,10]. On account of their exposure to various environments and switching from one to other host, they have noticeable variation in their genetic compositions, which is necessary for their survival in the varying environment[7,11]. Staying onto a host after switching from the previous environment, they gradually tend to change their morphology and genetic composition but 18S rRNA stores and conserves those evolving information for thousands of year[7,12]. Comparison of 18S rRNA, secondary structures and measuring its structural parameters (bond energy, geometrical features, base composition etc.) is proved as the best methods to study molecular phylogeny and correlation with zoogeographical distribution[13, 14].

Bulges, loops, helices and separation of single strands are considered the phylogenetic characters of rRNA as they have been conserved throughout the evolution[15]. RNA secondary structure provides substantial information regarding evolutionary relationship that cannot be simply inferred from cladistic analyses using simple RNA sequences[15]. RNA also provides necessary information regarding the development of biomarker of individual species[15,16]. In past, intensive phylogenetic analyses have been carried out on the various species of the genus *Lamellodiscus,* including validation of species and evolutionary relatedness upon the discovery of novel species. For all, 28S or 18S rRNA have been employed and phylogenetic tree have been constructed[17]. Since data on both RNAs is available in National Center for Biotechnology Information (NCBI) and many other databases, it is worth analyzing the phylogenetic relationships and re-setting the evolutionary relations in context of zoogeographical distribution. A general trend among Monogenean parasites *Lamellodiscus* is that most of them occurred on one or more than two host and show a versatility and wide distribution, therefore, understanding the molecular trends and utilizing 18S rRNA would be useful in correlating the hosts and their

Corresponding author: Arya, P. V.
Department of Zoology, Dyal Singh College (University of Delhi), Lodhi Road, New Delhi

parasites as well as the speciation easily[3]. In present paper, authors employed molecular diversity of genus *Lamellodiscus* and evaluated relative relationship among global representatives for predict probable host zoogeographical diversity, distribution, migration and settlement over period of time using the secondary structure of 18S rRNA of some species of *Lamellodiscus*.

MATERIALS AND METHODS

Selection of Species of genus *Lamellodiscus*

In all 28 marine parasitic species were selected considering global distribution representatives (Table 1) and source of species, and distribution confirmed from authentic sources (i.e., Gyrodb, Encyclopedia of Life, World Register of Marine Species etc.).

Table 1. List of selected members of genus *Lamellodiscus*

Sl.	Parasite	Host	Country/Area	Accession ID	Reference
1.	*L. confuses* Linnaeus,1758	*Sarpa salpa*	Coast of Algeria	JF427643	[7]
2.	*L. donatellae* Aquaro, Riva and Galli, 2009	*Acanthopagrus bifasciatus*	Egypt	FN296214	[18]
3.	*L. impervious* Euzet, 1984	*Diplodus puntazzo*	France	AY038195	[19]
4.	*L. obeliae* Delaroche, 1809	*Pagellus centrodontus*	France	AJ276443	[20]
5.	*L. ignoratus* Desdevises *et al.*, 2002	*Diplodus sargus*	Golfe du Lion	AF294957	[21]
6.	*L. japonicas* Pillai and Pillai, 1976	*Acanthopagrus latus*	Japan	EU836236	[22]
7.	*L. hilii* Euzet, 1984	*Diplodus puntazzo*	Kerkennah Islands	AY038194	[23]
8.	*L. bidens* Euzet, 1984	*Diplodus puntazzo*	Kerkennah Islands	AY038188	[23]
9.	*L. diplodi* Faust, 1920	*Diplodus sargus*	Lybia	JF427654	[7]
10	*L. ergensi* Amine et Euzet, 2005	*Diplodus sargus*	Mediterranean Sea	AY038190	[24]
11.	*L. elegans* Desdevises *et al.*, 2002	*Diplodus sargus*	Mediterranean Sea	JF427636	[9]
12.	*L. abbreviatus* Sanfilippo, 1978	*Diplodus sargus*	Mediterranean Sea	JF427625	[24]
13.	*L. parisi* Oliver, 1969	*Sarpa sapta*	Mediterranean Sea	AY038198	[25]
14.	*L. mirandus* Euzet & Oliver, 1966	*Diplodus sargus*	Mediterranean Sea	AY038197	[25]
15.	*L. erythrini* Euzet & Oliver, 1966	*Pagellus erythrinus*	Mediterranean Sea	AJ276440	[26]
16.	*L. theroni* Euzet, 1984	*Diplodus puntazzo*	Mediterranean Sea	KC470297	[27]
17.	*L. verberis* Euzet & Oliver, 1967	*Lithognathus mormyrus*	Mediterranean Sea	AF294955	[28]
18.	*L. mormyri* Linnaeus, 1758	*Lithognathus mormyrus*	Mediterranean Sea	AF294954	[29]
19.	*L. baeri* Olive,1974	*Pagrus pagrus*	Mediterranean Sea	AY038187	[30]
20.	*L. pagrosomi* Murray, 1931	*Pagrus auratus*	New Zealand	EU836235	[31]
21.	*L. neifari* Amine Euzet, Kechemir-Issad, 2006	-	North Atlantic Ocean	AY038196	[7]
22.	*L. gracilis* Euzet and Oliver, 1966	-	North Atlantic Ocean	AY038193	[25]
23.	*L. furcosus* Euzet and Oliver, 1966	-	North Atlantic Ocean	AY038192	[25]
24.	*L. fraternus* Bychowsky, 1957	-	North Atlantic Ocean	AY038191	[25]
25.	*L. coronatus* Euzet & Oliver, 1966	-	North Atlantic Ocean	AY038189	[7][25]
26.	*L. virgule* Euzet & Oliver, 1967	-	North Atlantic Ocean	AJ276442	[25]
27.	*L. knoeffleri* Oliver, 1969	-	North Atlantic Ocean	AY038196	[25]
28.	*L. falcus* Amine *et al*, 2006	*Diplodus puntazzo*	Spanish Mediterranean	KC470294	[25]

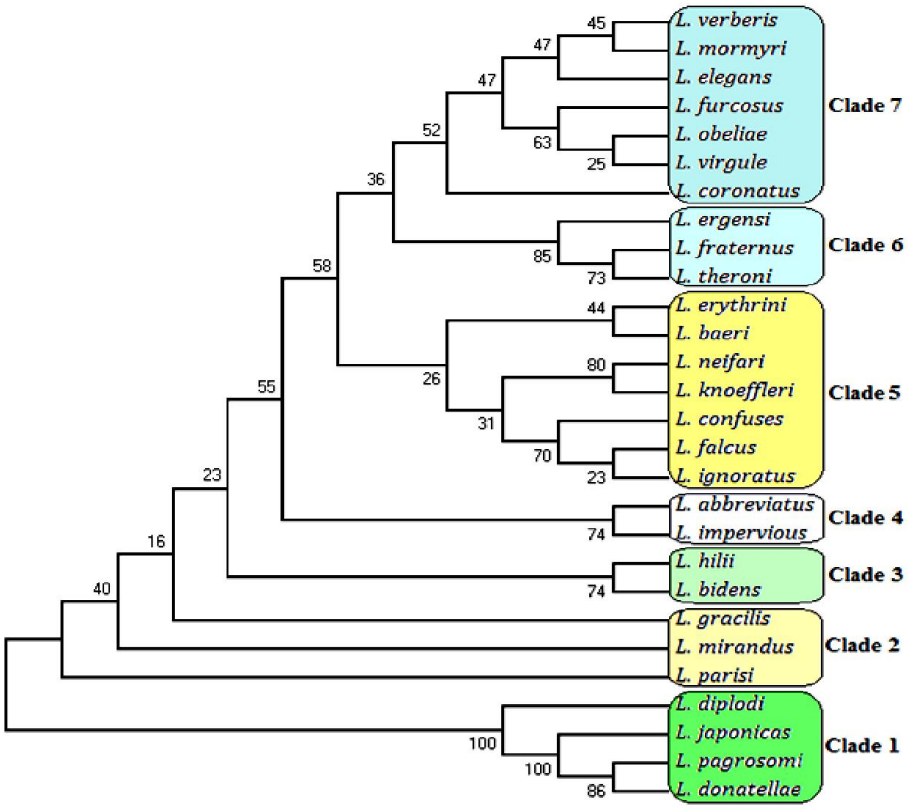

Fig. 1. Phylogenetic tree of 28 species of the genus *Lamellodiscus*, constructed using NJ method

Molecular Phylogenetic Analysis

Sequences for selected species (Table 1) were alignment using ClustalW program (inbuilt in MEGA 6) for multiple sequence alignment (Thompson *et al.* 1994) with the default gap and extension penalties used by this tool. MEGA 6 was used for constructing the phylogenetic tree by neighbor joining (NJ) method. The average pathway method calculated the branch length, depicted in the number of variations all over the sequences. Resultantly, the most parsimonious tree was chosen by the close-neighbor-interchange algorithm with a bootstrap procedure with 1000 replication for assessing the robustness of the constructed phylogenetic tree. The constructed NJ tree consisted of 28 species, represented with seven clades for further analysis (Figure 1).

Inferring Secondary Structure of 28SrRNAs

The formation of secondary structure is based upon the alignment score of the sequences of clades in the phylogenetic tree. In order to construct secondary structure of 18S rRNA, the sequence with the highest score from each clade was subjected to Mfold (URL http://mfold.rna.albany.edu) at a fixed temperature of 37^0 C and formed structure was analyzed for loops, bulges and stems. Similarly, the procedure was repeated for all clades and as a result seven RNA secondary structures were formed. In this way, every clade in the tree had been associated with its rRNA which averaged out the evolutionary commonalities between the species of a particular clade. This procedure made the cladistic analysis more precise than the traditional comparison of clades with bootstrap values only.

Geo mapping

In order to understand the global scenario of the species relatedness and diversity, all the selected species (Table 1) were marked on simple world map manually (Figure 3). Later on marked species were joined with reference to their respective clades for inferring molecular relatedness.

RESULTS AND DISCUSSION

Construction of Phylogenetic Tree

The multiple sequence alignment of 28 species by ClustalW was subjected to MEGA6 followed by the formation of seven clades (fig-1). Tree was presented with bootstrap values (1000 replicates) for every species. Each clade had two or more than two species showing an evolutionary relationship with each other. In the tree, Clade1, Clade2, Clade3, Clade4, Clade5, Clade6 and Clade7 had 4, 3, 2, 2, 7, 3, and 7 species respectively. The first clade in the tree with four species and two sister clades showed an average bootstrap value of 100 percent, representing the closest relatedness among all clusters. The second cluster with three species was given very poor bootstrap values (40 & 16 percent) and demonstrated that these species were distantly related and evolved at the beginning of their earlier speciation. The third and fourth clusters with only two species were given 74 percent bootstrap values equally. The bootstrap values above 70-75 percent are considered as significant and phylogenetically important.

The fifth cluster with seven species and four sister clades showed poor bootstrap values, in which only one sister clade with *L. neifari* and *L. knoeffleri* was given the best bootstrap value of 80 percent. Except the two species, all were distantly related and exhibited the earlier relatedness during speciation. The sixth cluster contended three species with average bootstrap values of 79 percent indicating close evolutionary relationship among species. The seventh cluster with seven species represented with poor bootstrap values. There were four sister clades in the cluster wherein only *L. furcosus* was connected by 63 percent bootstrap values with *L. virgulae* and *L. coronatus*. The poor bootstrap values shown by clades included clade2, clade5 and clade7. Only few species of these clades were presented by significant bootstrap values. The result presented also expresses that speciation event in the genus Lamellodiscus followed by a highly random consequence (the longer exposure to various environments and nutrition) due to which the conserved nucleic acid (18S rRNA) compositions became changed over the period of times.

Phylogenetic relationship among species and clades were shown to be intra-connected (Fig 1). All the seven clades in the tree did not show good evolutionary relationship but the secondary structure of the representative species were shown to be distinct in terms of free energy and formation of loops (Table 2). Few of them represented strong relationship like clade4, clade5 and clade6 in terms of their negative free energies (Fig 2). In the tree although they were clustered with different number of species though, in the study, our concerned was to find relatedness among species by accounting only single species as representative one. The negative free energy varied for all the clusters, demonstrating that a particular group of organism had gone through great speciation event [32]. The phylogenetic tree from neighbor joining method exhibited that all the seven clades vary in possessing the number of species, represented the variations among species of the genus *Lamellodiscus* (Figure 1).

Secondary Structure Analysis

The predicted 18S rRNA secondary structure by Mfold of representative species from seven clades showed the evolutionary distinction among species and cluster of species as a whole (Fig. 2). The secondary structure of the representative species also provided the stability of rRNA molecules in terms of negative free energy (ΔG). As mentioned earlier that the representative species were selected by multiple sequence alignment of species from each clade individually and the most conserved sequence of the species was chosen based on alignment score given by ClustalW. Formation of secondary structure is characterized by the formation of bulge loops, interior loops and hairpin loops conferred by negative free energy of RNA. Higher the negative free energy (ΔG), more stable the molecule. Negative free energy of clade1, calde2, clade3, clade4, clade5, clade6 and clade7 (rRNA from species) had been -212.40kcal/mol, -163.30kcal/mol, -167.80kcal/mol, -158.30kcal/mol, -155.40kcal/mol, 158.30kcal /mol and 172.10kcal/mol (Table 2). Except clade1, negative free energies of clade2 and clade3 are discrete by -4.5kcal/mol, representing that species from both groups had followed similar pattern of evolution. Anomaly to this finding can be accounted since varying number of different loops directly affects stability.

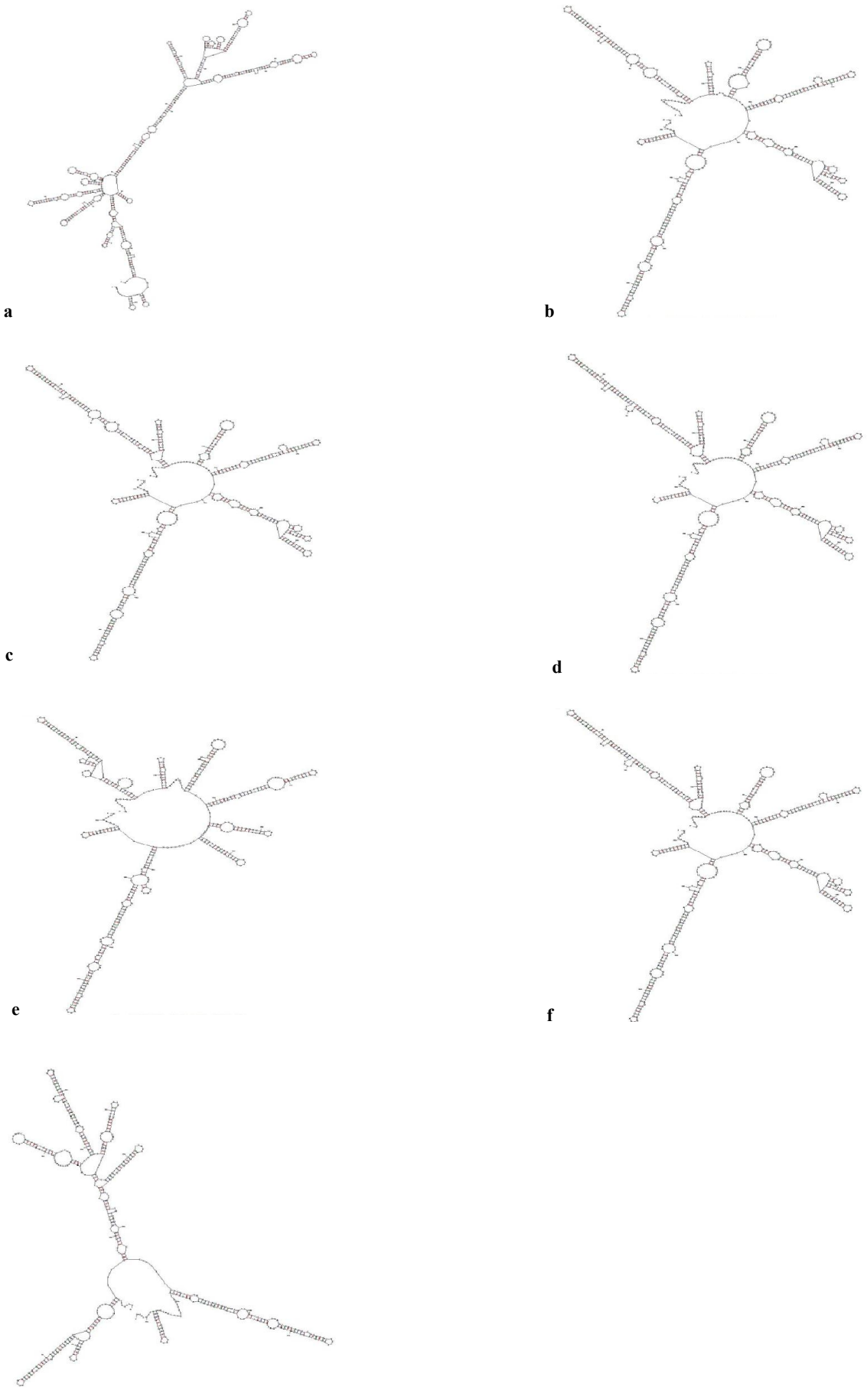

Fig. 2. Secondary structure of 7 representative 18S rRNAs from seven clades, a. *L. japonicas, b. L. mirandus, c. L. bidens, d. L. impervious, e. L. baeri, f. L. fraternus, L. mormyri*

Table 2. Clade details listed with representative species showing various parameters

Sl.	Clade (Species)	Negative free energy (ΔG) (kcal/mol)	Interior loop	Hairpin loop	Bulge loop	Total number of loops
1.	Clade1 (*L. japonicus*)	-212.40	16	13	4	33
2.	Clade2 (*L. mirandus*)	-163.30	12	8	5	25
3.	Clade3 (*L. bidens*)	-167.80	13	8	6	27
4.	Clade4 (*L. impervious*)	-158.30	12	8	6	26
5.	Clade5 (*L. baeri*)	-155.40	6	11	5	22
6.	Clade6 (*L. fraternus*)	-158.30	11	9	6	26
7.	Clade7 (*L. mormyri*)	-172.10	13	8	4	25

Third, fourth and fifth clades had an average negative free energy of 157.3kcal/mol (discrete by approximately ΔG =-2.0kcal/mol), shown to be correlating each other and representing evolutionary relatedness. The seventh clade, just like first one had different ΔG that did not match with other clad. Number of loops varied for the seven molecules (clade/representative species) in their secondary structure. Among all, interior loops are more in number except clade5 whose **ΔG** is least as well as total number of loops. Clade1 with greater negative free energy represented highest number (33) in all forms and total number of loops as well. Second highest number of loops (27) was represented by the clade3 that did not seem to coincide with its **ΔG** (-167.80kcal/mol) which should be, thermodynamically, second most of all. This happened mainly due to specific pattern and number of nitrogenous bases participated in forming loops. Clade2 (25) and clade3 (27) are varied by two loop hence their **ΔG** varied by -4.5 kcal/mol. They demonstrated that species from these two groups will be strongly related although their distribution may fall into different regions. It also showed that they remained conserved (18S rRNA) for a longer period of times. The same pattern and number of loops (26) formation and negative free (-158.30 kcal/mol) energy was represented by clade4 and clade 6.

Clade5 showed a drastic variation in number of its interior loops (6) and hence accounted by 22 loops in total. Surprisingly, its **ΔG** fell in range of clade5 and clade6, showing a unique pattern of loop formation. **ΔG** (-172.10 kcal/mol) and number of loops (25) of clade7 seemed to coincide well. The comparison between all seven ribosomal RNAs from each clade proved that all are genetically distinct. RNA in the folded form showed paired and unpaired (loops) bases[33]. Qualitatively, bases which are bonded tend to stabilize molecule due to higher negative free energy whereas unpaired bases tend to destabilize the molecule due to lesser negative free energy[34]. Quantitatively, loop that are more in number destabilize the secondary structure because they require more positive free energy[35]. Thus, clade1 and clade7 are the most stable and Clade5 is the least stable structure, signifying that organisms belonging to the particular clade will be of equal stability in terms of negative free energy of their RNA molecules. From first to seventh cluster, each organism representing its own cluster showed distinctions in the term of number of neighbor/sister clade organisms and 28S rRNA secondary structure. Although negative free energy and number of loops varied within all clades but a correlation between the two parameters have been established. Except clade1 and clade5, remaining five clades (clade2, clade3, clade4, clade6 and clade7) represented equal stability, conservation pattern and sympatric speciation events.

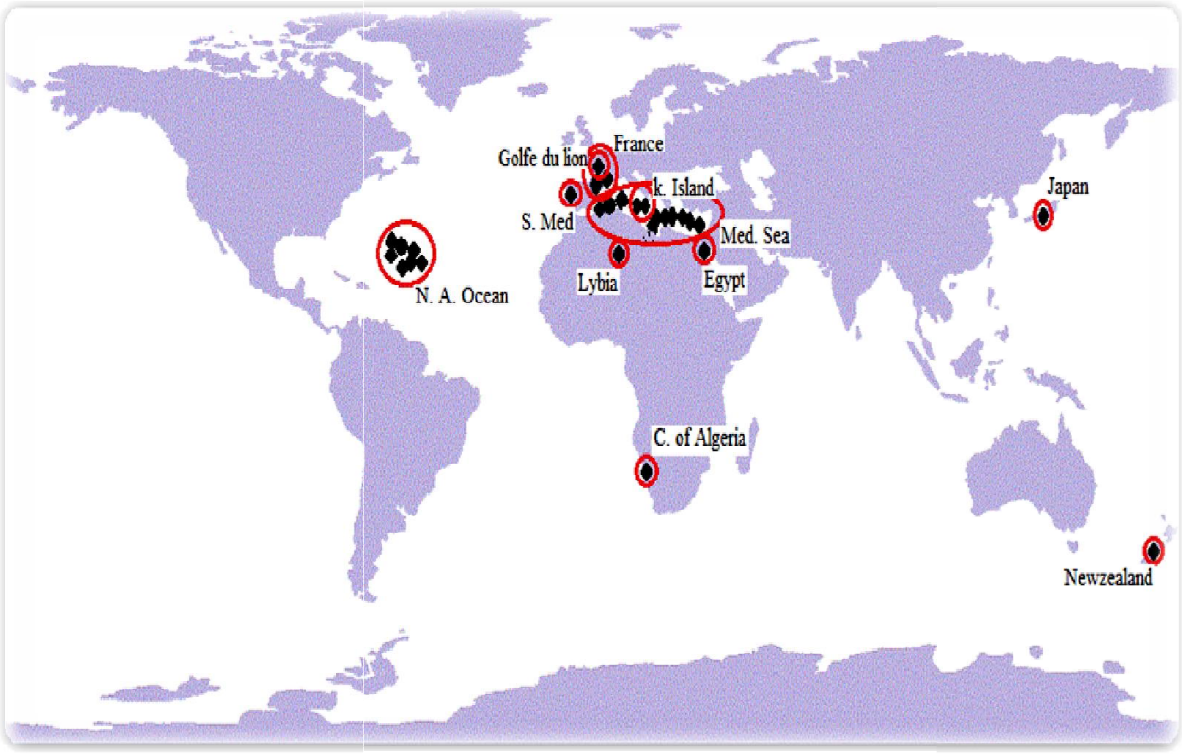

Fig.3. Geo mapping of selected species of *Lamellodiscus* distributed at 11 different geographical regions (A= Atlantic, C=Coast, K= Kerkennah, Med=Mediterranean, N=North, S= Spanish)

This was further strengthened by their, almost, equal number of loops. Clade1 and clade5 with their respective higher and lower number of loops and negative free energies, did not coincide with other clades in number of loops and ΔG because each group of organisms have their particular pattern of evolution of RNA. The distinctions among clades about ΔG were accounted due to the size of loops. Loops more in number but smaller in size are formed with less negative free energies whereas loops less in number but larger in size require more negative free energies. Evidently, both, size and number of loops are accounted for estimating out the stability of a molecule. The pattern of evolution and relatedness among species is reflected by the development of loops and their sizes which in turn account for the overall stability of RNA. Evolution has been raising the level of complexities which should be coincided with the necessities of situations. RNA having more complex secondary structure presents with more loops and small sizes whereas molecule with lesser loops and large sizes shows lower level of complexity.

Geo mapping

Once molecular pattern had confirmed, the different origin of species could be automatically correlated and expressed in terms of geographical distribution. The same clade has the species which are more or less relatively close to each other in terms of geographical distribution or possibly connected through probable migration cycle. Species from different geographical regions showed significant relatedness. Their evolving 18S rRNAs confirmed their speciation and indicated that anomaly in the evolution was accounted mainly due to separation of species into different geographical zones. Although, geographically and cladistically not much connected but they tend to represent the same origin pattern that a very long time ago they were evolved into different individuals. The information of being from the same pattern of evolution was stored and remains conserved in their ribosomal RNAs.

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Conflict of Interest

Authors do not have any conflict of interest.

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Research Article

IN-SILICO PHYLOGENETIC STUDY OF *DACTYLOGYRUS* (CLASS: MONOGENEA) USING 18S rRNA WITH A NOTE ON ZOOGEOGRAPHICAL INVESTIGATIONS ON THE GENUS

Fozail Ahmad, Singh, D. and *Arya, P. V.

Department of Zoology, Dyal Singh College, University of Delhi, Lodhi Road, New Delhi, 110003

Abstract

Attempts on finding relationships among different organisms remain an attractive branch. Incorporation of molecular understanding along speed of calculation from *in-silico* tools created altogether new area of research. Monogenean parasitic genus *Dactylogyrus* is fairly represented globally and their 18SrRNA sequences is well documented. Present study is an attempt to examine the phylogenetic relationship of selected members of the genus and zoogeographic mapping of the same for global view of the distribution, diversity and migration patterns during the ancient past.

Key words: Monogenea, geomapping, *Dactylogyrus,* Evolution.

INTRODUCTION

Monogenean parasites can be utilized for studying species evolutionary relationship, zoogeographical diversity, distribution, migration and settlement over period of time (Poulin, 2002 and Mendlová and Šimková, 2014). Monogenean genus *Dactylogyrus* is having largest number of species of about 900 in various databases, repository and literature (Gibson et al., 1996 and Simková *et al*., 2001). Among monogeneans, this genus volunteers a broader range for evolution, diversity and zoogeography due to its high host specificity (reported from marine and freshwater) having much occurrence on freshwater (Borji *et al*., 2012 and Mladineo *et al*., 2013). They don't switch from host to host rather reside on specific host (Borji *et al*., 2012). The comparative studies primarily involve morphological features, habitat, mode of nutrition and adaptation and anatomical characters especially in case of parasitic organisms like monogeneans, whereas the molecular comparison shows the way, more specific towards their evolution and evolutionary relationships (Huyse and Malmberg, 2004 and Crandall and Templeton, 1999). As the *Dactylogyrus* species are found to be conserved in terms of their host-parasite relationship, studying their geographical distribution with reference to cladistic analysis will be useful in comprehending out extent of specificity and strictness of individuals towards host. Zoogeographical distribution of individual represents its probable origin, as if species in a particular region might have migrated from some other region (Ashe *et al*., 1987 and Aitken *et al*., 2008). Migration have caused the individual to evolve for adaption in that particular environment but its genetic information have been conserved in the form of ribosomal RNA throughout generetions, witnessing the real origin of parasite (Ishikawa, 1977; Wang *et al*., 2015 and Rogers, 2007). Meanwhile, the evolutionary clue may be traced back for individual even showing distant relatedness with other discovered species. As far as the evolutionary relationship of species of the genus *Dactylogyrus* is concerned, it can be explored on the basis of cladistic relationship in a phylogenetic tree. Geographically, individuals reports fall into different zones but molecular (phylogenetic) insight reveals their hidden relations, determining origin and ancestral lineage (Safi *et al*., 2011). Moreover, extent of species due to geographical distribution can be understood. Even those of without host knowledge and probable origin can be determined through the same analysis. In past, intensive phylogenetic analyses have been carried out in the genus *Dactylogyrus,* including species validation and evolutionary relationship whenever some new species were discovered.

Corresponding author: Arya, P. V.
Department of Zoology, Dyal Singh College, University of Delhi, Lodhi Road, New Delhi, 110003

Present paper is an attempt to employ molecular diversity of genus *Gyrodactylus* in evaluating relative relationship among global representatives and predicting probable host zoogeographical diversity, distribution, migration and settlement using the secondary structure of 18S rRNA of some species of *Gyrodactylus*.

MATERIALS AND METHODS

Species Selection in the Genus *Dactylogyrus*

Species were selected on the basis of availability of 18SrRNA (in NCBI : National Center for Biotechnology Information)), specificity. In all, 54 species were selected and their validity, host and distribution were confirmed through various authentic sources like EOL (Encyclopedia of life), WorMs (World Register of Marine Species) and literatures.

Molecular Phylogenetic Analysis

Initially sequences for the selected species were subjected to alignment using ClustalW (inbuilt in MEGA 6) for multiple sequence alignment (Thompson *et al.* 1994) with the default gap and extension penalties used by this tool. MEGA 6 was again used for constructing the phylogenetic tree using neighbor joining (NJ) method. The average pathway method was used to calculate the branch length depicted in the number of variations all over the sequences. Subsequently, the most parsimonious tree was chosen by the close-neighbor-interchange algorithm. A bootstrap procedure with 1000 replications was executed for assessing the robustness of the inferred phylogenetic tree.

Geo mapping

In order to understand the global scenario of the species relatedness and diversity all the selected species (table-1) were marked on simple world map manually. Later on marked species were joined with reference to their respective clades for inferring molecular relatedness.

RESULTS AND DISCUSSION

The phylogenetic tree was formed with 14 clades, representing monophyletic and paraphyletic origin for selected species (Fig-1). Each clade in the tree did not show strong relationship with sister clades yet represented a range of

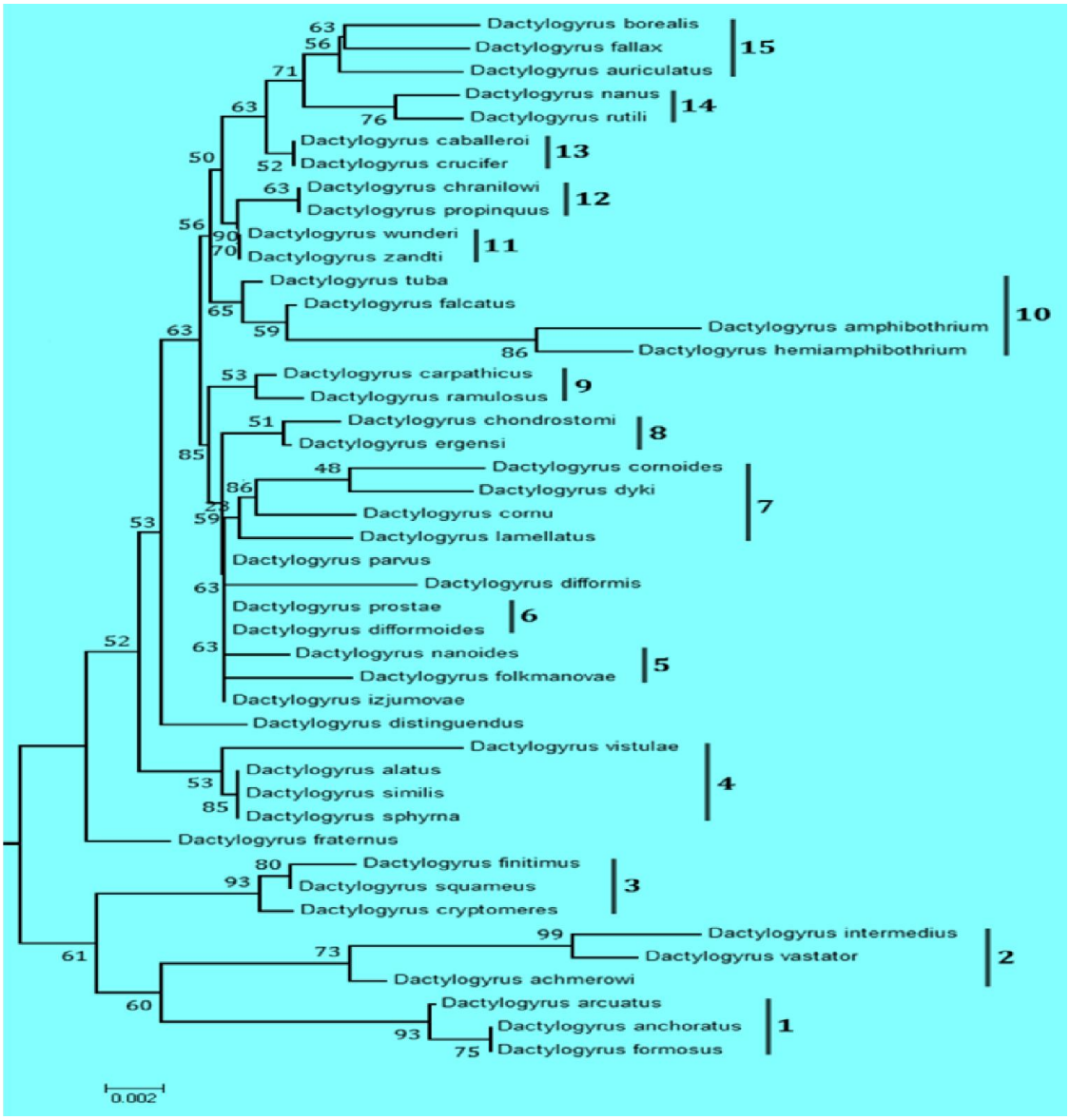

Fig. 1. *In-silico* phylogenetic tree (NJ method) of the genus *Dactylogyrus* for selected (54) species.

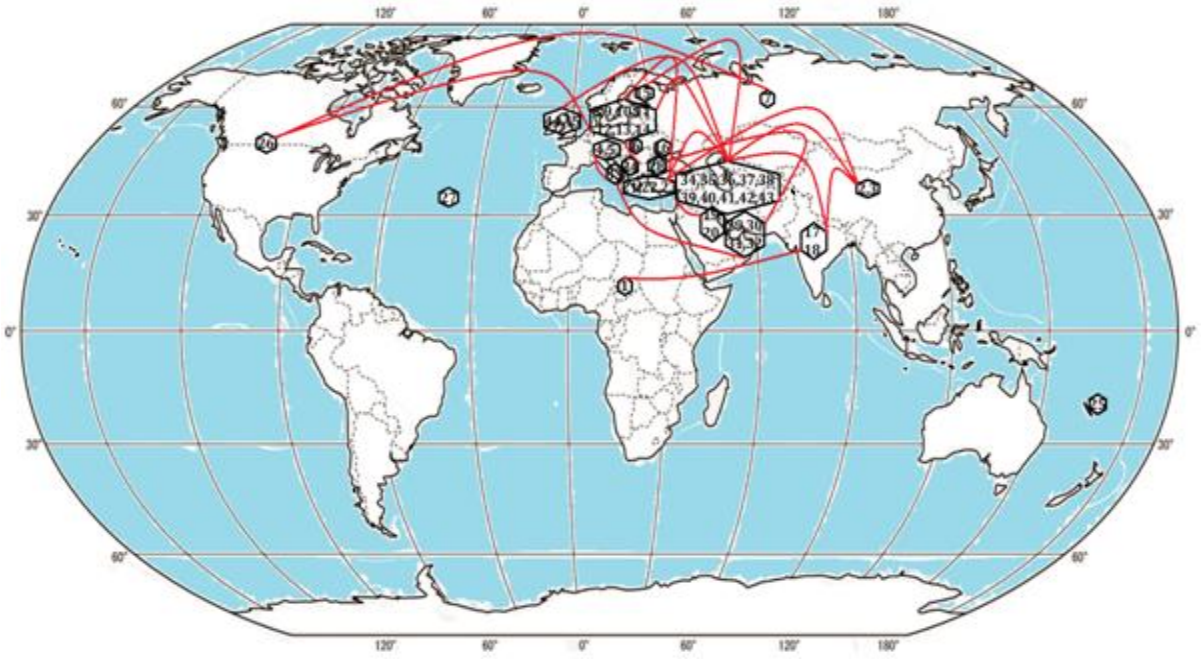

Fig. 2. Geomapping of selected members of genus *Dactylogyrus* reflecting related species based on clustering through phylogenetic tree.

Table 1. *Dactylogyrus* species their host, locality, habitat along with gene bank accession ID studied during present paper

Sl	*Species*	Host	Locality	Habitat	Accession ID
1.	*D. dyki* Ergens & Lucky 1959	*Barbus balcanicus*	Africa	M	EF582622
2.	*D. formosus* Kulwiec, 1927	*Carassius auratus*	Central China	M	KM525669
3.	*D. intermedius* Wang, 2008	*Carassius auratus*	Central China	M	AJ564139
4.	*D. chranilowi* Bychowsky, 1931	*Abramis ballerus*	Central Europe	F	AJ564117
5.	*D. cryptomeres* Bychowsky 1943	*Gobio gobio*	Central Europe	F	AJ564123
6.	*D. tuba* Linstow 1878	*Aspius aspius*	Czech Republic	F	AJ564158
7.	*D. hemiamphibothrium* Ergens, 1956	*Acerina cernua*	Eurasia	F	AJ564137
8.	*D. borealisi* Nybelin, 1937	-	Europe	F	AJ564113
9.	*D. caballeroi* Prost, 1960	*Siganus rivulatus*	Europe	F	AJ564114
10.	*D. chondrostomi* Malewitzkaja 1941	-	Europe	F	AJ564116
11.	*D. cornoides* Glaser & Gussev, 1971	*Vimba vimba*	Europe	F	AJ564118
12.	*D. falcatus* Wedl, 1857	-	Europe	F	AJ564130
13.	*D. nanoides* Gussev 1966	*Leuciscus cephalus*	Europe	F	AJ564144
14.	*D. vranoviensis* Ergens 1956	*Leuciscus cephalus*	Europe	F	AJ564162
15.	*D. nanus* Dogiel & Bychowsky 1934	*Rutilus rutilus*	Finland	F	AJ564145
16.	*D. achmerowi* Gussev, 1955	*Cyprinus carpio*	Hungary	F	AJ564108
17.	*D. lamellatus* Achmerow, 1952	*Ctenopharyngodon idella*	India	F	AJ564141
18.	*D. anchoratus* Dujardin 1845	*Cyprinus carpio*	India	F	AJ564111
19.	*D. fallax* Wagener, 1857	*Chalcalburnus mosseulensis*	Iraq	F	AJ564132
20.	*D. sphyrna* Diesing, 1850	*Alburnus caeruleus*	Iraq	F	AJ564155
21.	*D. ergensi* Molnar 1964	*Leuciscus cephalus*	Italy	F	AJ564128
22.	*D. similis* Wagener, 1909	*Rutilus rutilus*	Italy	M	KP202254
23.	*D. vastator* Nybelin, 1924	*Cyprinus carpio*	Italy	F	AJ564159
24.	*D. folkmanovae* Ergens 1956	*Leuciscus cephalus*	Macedonia	F	AJ564134
25.	*D. parvus* Wegener, 1910	*Cephalopholis urodeta*	New Caledonia	M	AJ564146
26.	*D. amphibothrium* Wegener, 1857	*Gymnocephalus cernuus*	North America	F	AJ564110
27.	*D. auriculatus* Nordmann, 1832	*Cyprinus carpio*	N. Atlantic Ocean	M	AJ564112
28.	*D. zandti* Bychowsky, 1933	*Abramis brama*	Poland	F	AJ564165
29.	*D. finitimus* Gussev 1966	*Carassius auratus*	Tehran	F	AJ564133
30.	*D. propinquus* Bychowsky, 1931	*Carassius auratus*	Tehran	M	AJ564147
31.	*D. ramulosus* Malewitzkaja, 1941	*Carassius auratus*	Tehran	F	AJ564150
32.	*D. wunderi* Bychowsky, 1931	*Carassius auratus*	Tehran	M	AJ564164
33.	*D. squameus* Gussev, 1985	*Pseudorasbora parva*	Italy	F	AJ564156
34.	*D. prostae* Molnar, 1964	*Squalius cephalus*	Turkey	F	AJ564148
35.	*D. rutili* Glaser 1965	*Rutilus rutilus*	Turkey	F	AJ564152
36.	*D. arcuatus* Yamaguti, 1942	*Gasterosteus aculeatus*	Turkey	M	KC876019
37.	*D. alatus* Wegener, 1909	*Chondrostoma regium*	Turkey	F	AJ564109
38.	*D. cornu* Linstow, 1878	*Vimba vimba tenella*	Turkey	M	AJ564119
39.	*D. crucifer* Wagener, 1857	*Rutilus rutilus*	Turkey	F	AJ564122
40.	*D. distinguendus* Nybelin 1937	*Abramis brama*	Turkey	F	AJ564125
41.	*D. fraternus* Wagener, 1909	*Alburnus alburnus*	Turkey	F	AJ564136
42.	*D. izjumovae* Gussev, 1966	*Scardinius erythrophthalmus*	Turkey	F	AJ564140
43.	*D. vistulae* Reda, 1987	*Chondrostoma regium*	Turkey	F	AJ564162
44.	*D. difformis* Wagener, 1857	*Scardinius erythropthalamus*	UK	M	AJ490160
45.	*D. difformoides* Glaser & Gussev 1971	*Scardinius erythropthalmus*	UK	M	AJ564124

(**M:** Marine; **F:** Freshwater; All the sequences for the present study were taken from NCBI database. Acknowledgement is due to all the contributors)

clue for diversity and similarity. Clade 1 had three species, *D. formosus, D. anchoratus* and *D. arcuatus* were distributed in three different geographical zones (Fig-2) being molecularly connected with each other. Bootstrap values for the clade is quite significant as 75% and 93%, demonstrating *D. arcuatus* would have been the ancestral species. Clade 2 had three species, *D. achmerowi, D. vastatori* and *D. intermedius* were distributed in nearby zones and represented better bootstrap values of 99% and 73%. This clade reflected a large variation in evolution and indicated by branch length of the phylogenetic tree. In its ancestors *D. achmerowi* variability is more than the descendent ones. The third clade had the same number of species as previous ones but comparatively lesser deviation in bootstrap values have been observed among ancestor and descendant. Geographically all the species in the clade were found to be distributed in different zones. Fourth clade contained four species whose ancestor *D. vistulae* and *D. alatus* fall in the same geographical place, whereas, others show a distant geographical relationship. The ancestral lineage was tagged with *D. vistulae.* Clade 5 & clade 6 contained two poorly connected species by both means of distributions molecular as well as geographical. Clade 7 represented a fine example of lineage hierarchy that having four species with significant bootstrap values confirmed a systematic evolution irrespective of their geographical distribution. Clade 8 & 9 both would have been highly diversified in terms of molecular conservation. Species from both clades reflected a poor bootstrap values, geographic scenario could not strengthen their molecular relatedness. Tenth clade with four species, two of them got to have 86% bootstrap value but meanwhile followed a distant route of evolution from other species in the cluster, coinciding distant geographical distribution.

Clade 11 was contented with four species, wherein *D. propinquus* & *D. wunderi* did not evolve and migrated to other place, but a large variation in molecular pattern was shown. Clade 12 found to be non significant in terms of molecular similarity whereas, clade 13 represented considerable relatedness between two species. The descendant of clade 14 had poor bootstrap values wherein, the ancestor species with significant relatedness with other clade in the tree as well as geomapping. In conclusion, species diversity in the genus *Dactylogyrus* could be simply understood by reconstructing the phylogenetic tree with reference to the geomapping (zoogeography). A coincidence between cladistic pattern and geomapping was established and confirmed. It was also demonstrated that only topology of a phylogenetic tree is not enough to infer the evolution pattern in a genus or even a family. On the contemporary, zoographical distribution strengthened the idea of ecological variances for species from the same genus. This study supports that species richness can be understood by knowing geographical distribution and species falling in the same clade does not mean to have been originated from the same place.

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IN SILICO PHYLOGENETIC STUDY ON ANCYROCEPHALIDAE (CLASS: MONOGENEA) USING 28 S RIBOSOMAL RNA EXTENDING GEO-MAPPING IN SEARCH OF EVOLUTIONARY CUES

Fozail Ahmad[1], D. Singh[1], P. V Arya[1]* and H. S. Singh[2]

[1]Department of Zoology, Dyal Singh College (University of Delhi), Lodhi Road, New Delhi - 110 003, India.
[2]Department of Zoology, Ch. C. S. University, Meerut - 250 004, India.
e-mail: zoology.dsc@gmail.com

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ABSTRACT : Class Monogenea attracted lot of attention for phylogenetic study in recent past. Family Ancyrocephalidae present a good material to employ *in silico* tools. Present study is an attempt to employ 28S rRNA information in predicting phylogeny of the different members. Another approach of integrating geo-mapping is also attempted for understanding diversity, distribution and relatedness among the various members of the family under the study.

Key words: Monogenea, Ancyrocephalidae, phylogeny 28SrRNA, geo-mapping.

INTRODUCTION

Monogenea is the class of parasitic Platyhelmenthes being extensively studied over the past many decades(Littlewood, 2007). Being a diversity rich class among lower parasite, there have been a chance to determine the ancient evolutionary clue(Chaudhary *et al*, 2013), with approximately 35 families, 220 genus and 1850 species (Poulin, 2002). Ancyrocephalidae, subjected to the present study, has 24 genus and 218 species (Gillardin *et al*, 2012) and represents two kinds of evolutionary descendents; monophyletic and paraphyletic descendents (Kritsky and Boeger, 1989). Monophyletic species- one that includes the most recent common ancestor of a group of organisms, and all of its descendents, whereas paraphyletic- includes the most recent common ancestor, but not all of its descendents (Hörandl and Stuessy, 2010). Diversity and molecular distinction are greatly raised by speciation of organism(Hunter, 2007), higher the level longer the time to diversification and *vice versa* (Rabosky, 2009). Simply lower organisms are needed lesser time to evolve and diversify at a faster rate (Mittelbach *et al*, 2007). Above all, unique genomic composition has significant effect over physiological behavior, determining the intrinsic tendency of organism (Milne *et al*, 2011). In ancient time, organism from the same family or genera were distributed in different geographical zones and exposed to varied environment and habitat that drastically modified genetic composition to the extent of speciation (Golestani *et al*, 2012). In order to evaluate the phylogenetic relationship among species of different genus and/or species from a particular family with their zoogeographic distribution, evaluation of zoogeographical distribution together with molecular clue may present evolutionary history including probable origin of the organisms (Lomolino and Brown, 2009). New evidences may present new avenues for the study of evolutionary aspects, such as a picture of present and ancient history of organism that can be possessed by Zoogeographical distribution (Boero and Bouillon, 1993). Monogeneans have been used as one such tool for indirect study of their host, distribution, migration, zoogeographical diversity and settlement over period of time. Reasons behind great speciation and high diversity in family Ancyrocephalidae is that species represent either monophyletic or paraphyletic pattern of evolution(Pariselle *et al*, 2011). In both of the cases, species descendent is shown from nearby ancestors that indicate close chance of speciation at any time(Struck *et al*, 2014). Also, it is said to follow a faster route of evolution(Teeling *et al*, 2002).

Molecular phylogeny and its correlation with zoogeographical distribution are to measure the structural parameters of 28S rRNA (Amit Roy, 2014)(Chaudhary and Singh, 2012). Phylogenetic characters of ribosomal RNA basically include loops, bulges, helices and separation of single strands since they have been conserved throughout the evolution (Mathews *et al*, 2010). The Secondary structure of ribosomal RNA also provides satisfying information about evolutionary relationship that cannot be simply inferred from phylogenetic tree analyses using simple RNA/DNA sequences (Fozail *et al*, 2015) (Chen *et al*, 1999). Information regarding development of biomarker can also be obtained from ribosomal RNA for each species (Adams *et al*, 2013). In past, exhaustive

phylogenetic analyses have been performed on various family of monogenea. With data available in National Center for Biotechnology Information (NCBI), it is worth analyzing the phylogenetic relationships and re-setting the evolutionary relations in context of zoogeographical distribution. A general trend among Monogenean parasites Ancyrocephalidae is that almost all of them exhibit monophyletic and paraphyletic feature of evolution, therefore, understanding the molecular trends and utilizing 28S rRNA would be useful in comprehending and tracking ancient lineage of this family.

MATERIALS AND METHODS

Selection of species and genus

A total of 71 species from 12 genus of the family Ancyrocephalidae were selected based on upon confirmation of geographical distribution and the availability of 28S rRNA data.

Molecular Phylogenetic Analysis

Sequences were aligned by ClustalW, analysis and optimization of MSA was performed in the same program. MEGA 6 was used for construction of phylogenetic tree using Neighbor joining method. Most parsimonious tree was obtained using the close-neighbor-interchange algorithm. Bootstrap procedure with 1000 replications for every species. The optimized phylogenetic tree was represented with 12 and many sister clades for further analysis.

Inferring Secondary Structure of 28SrRNAs

Every cluster in the tree was given with two or more than two sequences, so they were aligned using ClustalW multiple sequence program and the sequence with the highest alignment score was obtained for inferring secondary structure. Multiple sequence alignment of each cluster provided the most conserved sequence based on score generated by ClustalW. Secondary structure of RNA was predicted using Mfold (URL http://mfold.rna.albany.edu); at a fixed temperature of 37^0C. Formed structure was analyzed for unique structural patterns like bulges, stems, loops and negative free energy (ΔG). The procedure was repeated for all clusters that resulted in the formation of seven different structures of RNA molecule. Every cluster in the tree had been associated with its common ribosomal RNA that averaged out the evolutionary commonalities between the species of a particular cluster. This procedure facilitated the cladistic analysis more precise than the traditional comparison of clusters with bootstrap values only.

Geo mapping

For global scenario of the species relatedness and diversity, all the selected species as per table1 were marked on simple world map manually (Fig-5) and joined with reference to their respective cluster for inferring molecular relatedness.

RESULTS

Construction of Phylogenetic Tree

MEGA 6 constructed the phylogenetic tree are presented with bootstrap values (1000 replicates) forming 12 clusters, consisting of two or more than two species showing evolutionary cross relationship (fig-1).

Bootstrap values below 50% were removed from the tree. In the tree, Cluster A, B, C, D, E, F, G, H, I, J, K and L had 3, 3, 12, 3, 8, 5, 2, 10, 5, 2, 5 and 6 species respectively. Cluster A with three species exhibited strong (99%) bootstrap value and all species in the cluster belong to the same genus (*Enterogyrus*). This cluster showed the evolutionary distinction from other genus as represented in the crosses diagram with phylogenetic tree. Cluster B presented 99% bootstrap value but unlike A, clustered for two different genus (*Ancyrocephalus* & *Actinocleidus*), confirming their ancestral relationship and earlier divergence from each other. Cluster C was represented with two clades in which first clade expressed inter genus relationship between *Haliotrema* and *Pseudohaliotrema* whereas second clade expressed pure lineage of *Bravohalisa* with significant bootstrap values. Cluster D was shown with species from two genus (*Lethrinitrema* and *Haliotrema*). Cluster E was shown with pure lineage of *Haliotrema* with considerable bootstrap values of 60-70 %. Cluster F was represented with species from two genus (*Metahaliotrema* and *Haliotrema*) with significant bootstrap values of 80-90%. Cluster G showed strong relatedness between the members of *Scutogyrus* with significant bootstrap value of 99 %. Cluster H showed a unique pattern of cladistic relationship in which two clades were formed for the same genus, *Ligophorus*.

Both of the clades showed poor but considerable bootstrap values. Bootstrap for the second clade is 79% which is better than previous one with 62% only. The anomaly can be further accounted for the course of speciation and genus diversification. It has indicated that over the period of time cladistic relationship between members of the same the genus would follow a new route of lineage since mutation in genetic composition is more than enough to go through a different path of diversity.

Few of the species from the genus like *Onchobdella, Haliotrema* & *Ancyrocephalus* did not show cladistic relationship and hence were not counted as clade. For, four species of *Ligophorus* expressed strong values of

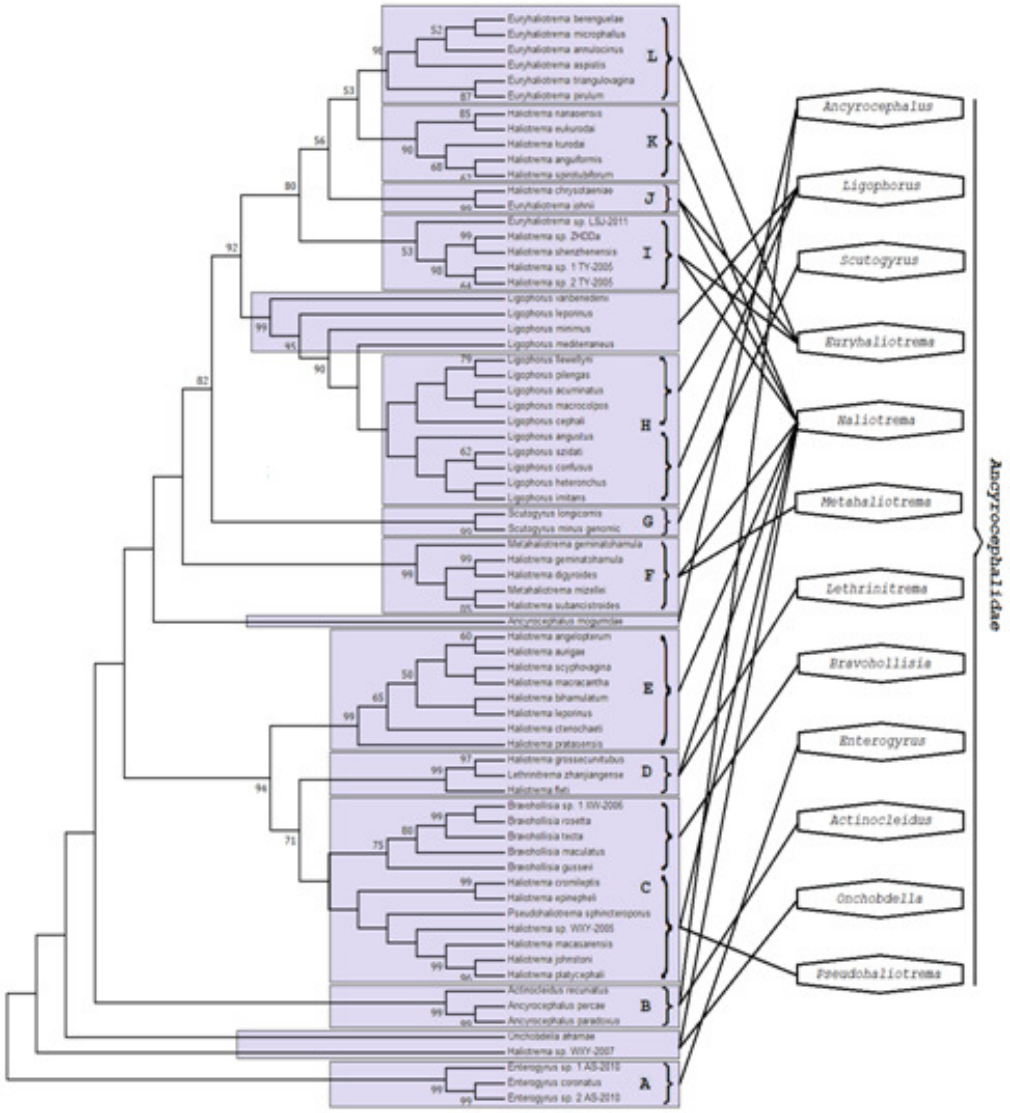

Fig. 1 : Phylogenetic tree of 71 species from family Ancyrocephalidae (Neighbor joining method).

90-99%, but fail to represent relatedness with others. Cluster I and J showed the inter genus relatedness *i.e.* species from two different genus whereas K and L showed pure lineage of the clusters with significant bootstrap values.

Secondary Structure Analysis

The inferred secondary structure of 28S rRNA by Mfold of representative species from 13 clusters exhibited the evolutionary distinction between species and clusters as well (Fig. 2) also provided the stability of molecules in terms of negative free energy (ΔG). Representative species were selected by multiple sequence alignment of species from each cluster, and the most conserved sequence of the species was considered based on alignment score given by ClustalW. Formation of secondary structure is characterized by bulge loops, interior loops and hairpin loops conferred by negative free energy of molecule. Higher the negative free energy (ΔG), more stable the molecule. Negative free energy of cluster A, B, C_1, C_2, D, E, F, G, H, I, J, K and L (rRNA from species) were predicted to be -242.70 kcal /mol, -243.10 kcal /mol, -219.40 kcal/mol, -222.20 kcal/mol, -208.60 kcal/mol, -231.40kcal/mol, -202.20Kcal/mol, -243.40kcal/mol, -284.40kcal/mol, 244.60kcal/mol, -218.20kcal/mol, -235.10kcal/mol and -220.70kcal/mol respectively (Fig-3). The negative free energies of clusters A, B, G and I fall in the range of -243 kcal /mol and discrete by -2.0 kcal / mol approximately, representing that species from these groups had followed similar pattern of evolution. Anomaly to this observation can be accounted as the varying number of different loops directly affects stability of molecule. Cluster C_1 and cluster J had an average negative free energy of -218.5 kcal/mol (discrete by approximately ΔG =-0.5 kcal/mol), shown to be correlating each other and representing evolutionary

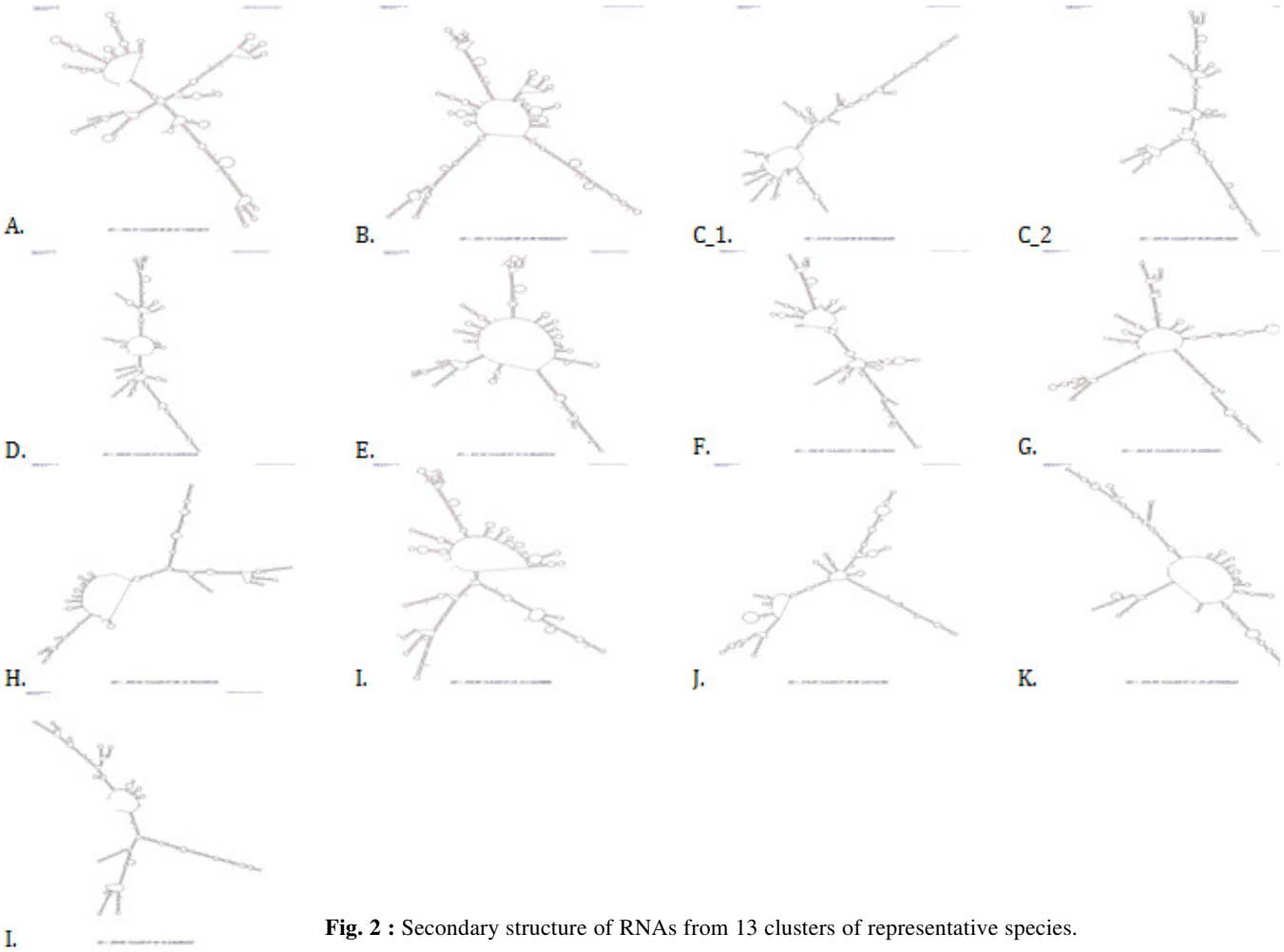

Fig. 2 : Secondary structure of RNAs from 13 clusters of representative species.

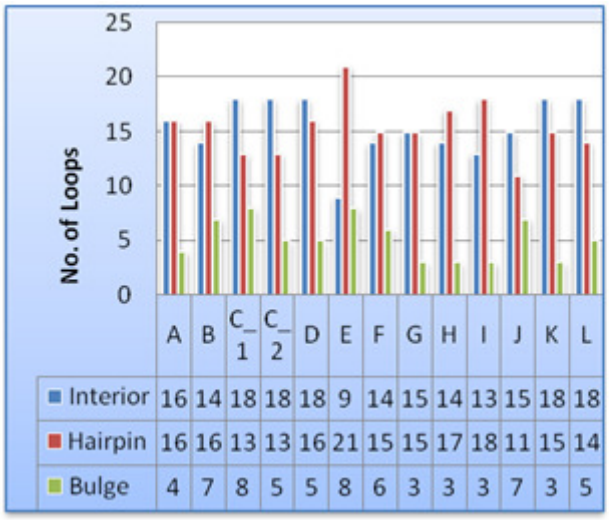

Fig. 3 : Number of loops form Representative RNA secondary structure for each cluster.

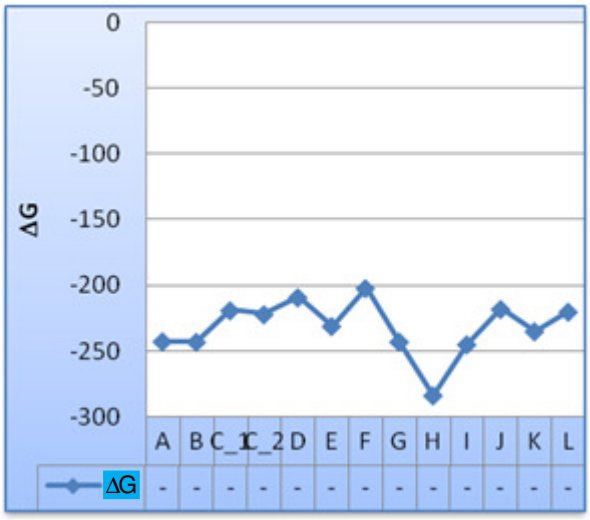

Fig. 4 : Negative free energy of the representative RNA structure for each cluster.

relatedness. Cluster D and F showed considerable difference of -6.0 kcal /mol, moreover they have been given the least negative free energies. As mentioned before, higher the ΔG more stable the molecule. It also comprehends that more stable ribosomal RNA in the species would represent lesser mutation and hence lesser speciation events over the period of time. These two clusters belong to three *Haliotrema, Metahaliotrema lithrinitrema*. Species from the genus exhibit faster rate of divergence. On the contrary, Species of the cluster H

Geomapping

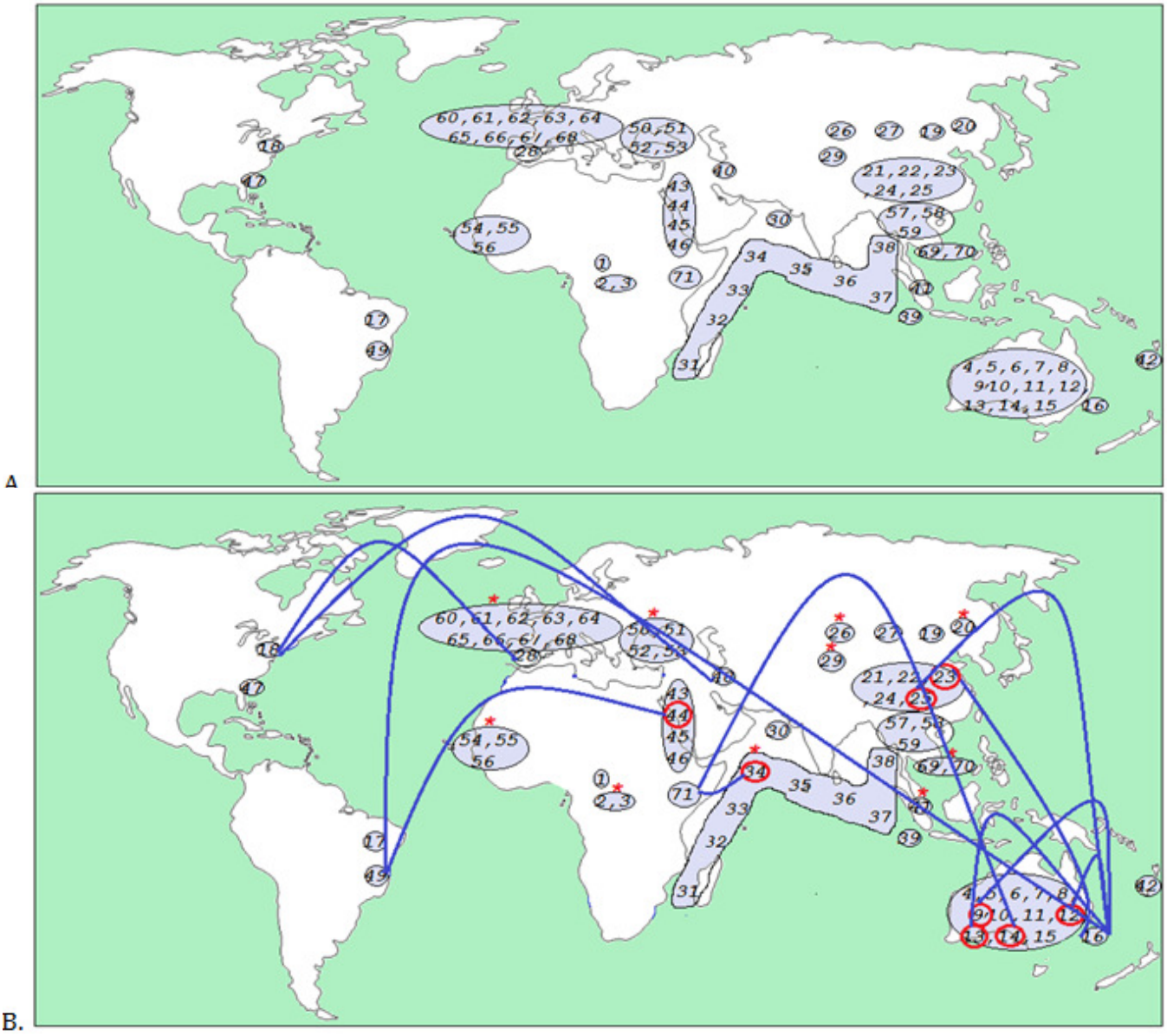

Fig. 5 (A & B) : Geo-mapping of 71 species, A- Manual plotting; B-Clade connections [* represents group of species from same genus that did not relate with others and circle represents that species belong to the same genus].

with the genus *Ligophorus* (ΔG = -284.40 kcal/ mol) will exhibit gradual rate of divergence. Clusters C_1, J and L represented almost equal amount of ΔG, showing similar pattern of evolutionary conservation. Those of moderate ΔG for the clusters would follow a general trend of speciation. The higher distinctions in negative free energy of some cluster also indicate that over the period of time they will soon tend to follow a different route of evolution. Since current study has considered family Ancyrocephalidae, genus with such significant features will represent a new family over the period of time. Three types of loops are formed in the secondary structure of RNA molecule (cluster/representative species) with unique pattern of occurrence (Fig. 2). The formation of loops, as mentioned earlier, is almost conferred by negative free energy, resolves stability and constancy of the entire molecule.

The present secondary structure is characterized by number and pattern of secondary structure in RNA molecule. Number of loops varied for individual cluster species (Fig. 3). Number of hairpin loops (11-21) was highest whereas bulge loops (3-8) were found to be least in number. In this section of result, the most important analysis to be inferred was to find out the coincidences between number of loops and negative free energies. Sum of number of loops had fallen 33 to 39 did not show greater distinctions among clusters. Cluster C_1 and D

Table 1 : Genus with respective species (family Ancyrocephalidae) investigated in the study.

Genus	Species	Host	Locality	Env.	Acc. ID
Actinocleidus	*A. recurvatus* Mizelle & Donahue, 1944	*Lepomis gibbosus*	Canada	M	AJ969951
	A. mogurndae Gussev, 1955	*Siniperca chuatsi*	China	F	DQ157667
Ancyrocephalus	*A. paradoxus* Creplin, 1839	*Sander lucioperca*	Kurish Gulf	M	AJ969952
	A. percae Ergens, 1966	*Perca fluviatilis*	Germany	M	KF499080
	B. tecta Venkatanarasaiah, 1984	*Pampus argenteus*	Hainan	M	KJ571012
	B. gussevi Lim, 1995	*Pomadasys hasta*	Sungai Buloh	M	KJ571007
	B. sp. Malaysia Priesner, 1933		Malaysia	M	AF387509
Bravohollisia	*B. maculates* Venkatanarasaiah, 1984		China	M	KJ571008
	B. rosetta Venkatanarasaiah, 1984	*Pomadasys hasta*	Sungai Buloh	M	DQ537364
	B. sp. 1 XW-2006 Priesner, 1933		Malaysia	M	DQ537365
	E. coronatus Pariselle *et al*, 1991	*Tilapia dageti*	Senegal	M	HQ010030
Enterogyrus	*E. sp. 1 AS-2010*	*Sarotherodon galilaeus*	Senegal	M	HQ010032
	E. sp. 2 AS-2010	*Sarotherodon galilaeus*	Senegal	M	HQ010031
	E. annulocirrus Yamaguti, 1968	*Pachyurus junki*	I-W P. Ocean	M	EU836195
	E. mehen Solar *et al*, 2012	*Pachyurus junki*	I-W P. Ocean	M	HQ615997
	E. aspistis Plaisance & Kritsky, 2004	*Pachyurus junki*	I-W P. Ocean	M	AY820614
	E. berenguelae Plaisance & Kritsky, 2004	*Pachyurus junki*	I-W P. Ocean	M	AY820615
Euryhaliotrema	*E. johni* Tripathi, 1959	*Pachyurus junki*	I-W P. Ocean	M	EU836193
	E. microphallus Yamaguti, 1968	*Pachyurus junki*	I-W P. Ocean	M	AY820617
	E. pirulum Plaisance & Kritsky, 2004	*Pachyurus junki*	I-W P. Ocean	M	AY820618
	E. triangulovagina Yamaguti, 1968	*Pachyurus junki*	I-W P. Ocean	M	AY820619
	E. sp LSJ-2011		I-W P. Ocean	M	HQ615997
	H. angelopterum Johnston & Tiegs, 1922	*Chaetodontidae*	I-W Islands	M	AY820620
	H. aurigae Yamaguti, 1968	*Chaetodontidae*	S W Parite	M	EU836198
	H. bihamulatum Zhang, 2001		China	M	DQ537378
	H. chrysotaeniae Young, 1968		Brazil	M	AF026115
	H. cromileptis Young, 1968	*Epinephelus coioides*	Australia	M	EU523146
	H. ctenochaeti Young, 1968		China	M	EU836199
	H. digyroides Zhang, 2001	*Epinephelus coioides*	China	M	DQ537377
	H. epinepheli Young, 1968	*Pinephelus fasciatus*	Australia	M	EU836201
	H. fleti Young, 1968	*Pinephelus fasciatus*	Australia	M	DQ157661
	H. geminatohamula Bychowsky & Nagibina, 1970	*Pinephelus fasciatus*	Australia	M	DQ157649
	H. grossecurvitubus Li & Chen, 2005		China	M	EU836204
Haliotrema	*H. johnstoni* Bychowsky & Nagibina, 1970	*Pinephelus fasciatus*	Australia	M	DQ157664
	H. kurodai Ogawa & Egusa, 1978	*Pinephelus fasciatus*	Australia	M	DQ537376
	H. leporinus Johnston & Tiegs, 1922	*Acanthurus nigrofuscus*	South China	M	EU836206
	H. macasarensis Yamaguti, 1963	*Platycephalus indicus*	China	M	EU836207
	H. macracantha Yamaguti, 1968		N. Caledonia	M	EU836208
	H. nanaoensis Pan & Zhang, 2000	*Epinephelus coioides*	Australia	M	DQ537373
	H. platycephali Yin & Sproston, 1948	*Epinephelus coioides*	Australia	M	FJ767866
	H. pratasensis Sun et al, 2007	*Acanthurus nigrofuscus*	South China	M	EU836209
	H. scyphovagina Yamaguti, 1968		I-W P. Ocean	M	AY820622
	H. shenzhenensis Wang *et al*, 2003	*Sciaenops ocellatus*	South China	M	DQ537372
	H. spirotubiforum Zhang, 2001	*Lutjanus vita*	Red Sea	M	DQ157656

Table 1 continued....

Table 1 continued....

	H. subancistroides Zhang, 2001	*Gerres lucidus*	Red Sea	M	EU836210
	H. sp. 1 TY-2005		Red Sea	M	DQ058213
	H. sp. 2 TY-2005		Red Sea	M	DQ058214
	H. sp. WXY-2005	*pinephelus fasciatus*	Australia	M	DQ157663
	H. sp. WXY-2007	*pinephelus fasciatus*	Australia	M	EF437158
	H. sp. ZHDDa	*pinephelus fasciatus*	Australia	M	DQ157658
Lethrinitrema	*L. zhanjiangense* Sun *et al*, 2014	*Lethrinus nebulosus*	S. China Sea	M	KJ571017
Ligophorus	*L. acuminatus* Euzet & Suriano, 1977	*Liza saliens*	Spain	M	JN996816
	L. angustus Euzet & Suriano, 1977	*Chelon labrosus*	Spain	M	JN996816
	L. cephalic Euzet & Suriano, 1977	*Liza cephalus*	Spain	M	JN996830
	L. confuses Gil Corrado, 1936	*Liza ramada*	Spain	M	JN996807
	L. heteronchus Euzet & Suriano, 1977	*Liza saliens*	Spain	M	JN996812
	*L. imitans*n Euzet & Suriano, 1977	*Liza ramada*	Spain	M	JN996815
	L. leporinus Zhang & Ji, 1981	*Liza cephalus*	China	M	DQ537380
	L. llewellyni Dmitrieva *et al*, 2007	*Liza haematocheila*	Sea of Azov	M	JN996823
	L. macrocolpos Euzet & Suriano, 1977	*Liza saliens*	Spain	M	JN996819
	L. mediterraneus Hargis, 1955	*Liza cephalus*	Spain	M	JN996827
	L. minimus Euzet & Suriano, 1977	*Liza saliens*	Spain	M	JN996817
	L. pilengas Sarabeev & Balbuena, 2004	*Mugil soiuy*	Sea of Azov	M	JN996824
	L. szidati Euzet & Suriano, 1977	*Mugil soiuy*	Sea of Azov	M	JN996806
	L. vanbenedenii Parona & Perugia, 1890	*Mugil soiuy*	Sea of Azov	M	DQ157655
Metahaliotrema	*M. geminatohamula* Bychowsky & Nagibina, 1970	*Sphyrna argus*	S. Brazil	M	DQ157646
	M. Mizellei Yamaguti, 1953	*Sphyrna argus*	China	M	DQ157647
Onchobdella	*O. atramae* Peters, 1857	*Hemichrom is fasciatus*	Africa	M	HQ010034
Pseudohaliot-rema	*P. Sphincteroporus* Yamaguti, 1953	*Siganus doliatus*	Australia	F	AF382058
Scutogyrus	*S. longicornis* Paperna & Thurston, 1969	*African Cichlids*	Africa	M	HQ010035
	S. minus Pariselle & Euzet, 1995	*Sarotherodon*	Africa	M	HE792779

Note : IWP = Indo West Pacific, N = North, S = South, W = West, M = Marine, F = Freshwater
All the sequences of present study was taken from NCBI database. Acknowledgement is due to all the contributors.

with highest number of loops (39) (Fig. 4) were contended with lower negative free energies, showing an anomalous behavior of the molecules.

Second highest number of loops were contented by E, B, A, C_2, K and L (38, 37, 36 & 36, 36 & 37 respectively) as a group. Their negative free energies did not coincide for number of loops. Clusters F, G, H, I and J had 34, 33, 34, 34 and 33 loops respectively with highly varied negative free energies. The anomalies between loops and ΔG are occurred due size of hairpin, bulge and interior loops and unique pattern of nitrogenous bases in RNA. It also was confirmed that all cluster were evolutionary distinct. The great anomalous behavior of species representing cluster confirmed that individuals tend to evolve at faster rate than those of lesser coincidences with loops and ΔG. This finding confirmed that species belonging to different genus had a specific pattern of evolution and later on distributed in different regions. Apart from ΔG, the equal number of loops of D and C_1 showed that member belonging to these cluster had been following the same pattern of evolutions. The same hypothesis have been imposed for A & C_2, B & E, and F, G, H, I & J with almost equal number of loops, representing strong ancestral relatedness among species. Though ΔG was not very well peered for the clusters, it had been due to size and number of nitrogenous bases of RNA molecules. Only ΔG does not account for relatedness of species in a clade, loops also insight into evolutionary relationship. Comparatively, three types of loops represented uniqueness, stability, conservation pattern, evolutionary relatedness and range of ancestral lineage.

DISCUSSION

The molecular distinction among species from different genus provided an understanding over evolutionary process and expressed ancestral lineage to the origin of a new species. The phylogenetic tree with 13 clusters demonstrated the inter-genus relationship as few of them like *Haliotrema* was distributed into eight clades, showing the genus richness and high evolution in the family Ancyrocephalidae. Some of them remains highly conserved like *Ligophorus, Scutogyrus* & *Enterogyrus* and were not distributed in any clades. Distribution was further supported by RNA secondary structure of representative species in the folded form showed paired and unpaired (loops) bases. Number of different loops and negative free energies coincide the relatedness between genus. *Haliotrema, Ancyrocephalus, Ligophorus* & *Euryhaliotrema* were all found to relate with other clades. It was strengthened further by structural analyses of RNA molecule. Qualitatively, bases which are bonded tend to stabilize molecule due to higher negative free energy whereas unpaired bases tend to destabilize the molecule due to lesser negative free energy (Svoboda and Di Cara, 2006). Quantitatively, loop that are more in number destabilize the secondary structure because they require more positive free energy(Ding, 2006). Therefore, cluster A, B, and H are the most stable and cluster C_1, C_2, D, E, F, G, I, J, K and L are lesser stable structure, signifying that organisms belonging to the particular cluster will be of equal stability in terms of negative free energy of their RNA molecules, and hence, will follow the same pattern of origin and evolution (Shabalina, 2006 and Schuster, 2006). Although, negative free energy and number of loops showed noticeable variations within all clusters but a correlation between the two parameters have been established. Except cluster D, F, E, K and H, remaining eight clusters (cluster A, B, G & I), (cluster C_1, C_2, J and L) represented equal stability, conservation pattern and sympatric speciation events (Fig. 4). This was further strengthened by equal number of loops developed in the representative molecule. Loops more in number but smaller in size are formed with less negative free energy whereas loops less in number but larger in size require more negative free energies (Aalberts and Nandagopal, 2010). Evidently, both, size and number of loops are accounted for estimating out the stability of a molecule (Zhang *et al*, 2008). The pattern of evolution and relatedness among species is reflected by the development of loops and their sizes which in turn account for the overall stability of RNA (Petrillo *et al*, 2006). Evolution, most of the time, rises the level of complexity that is strictly coincided with the necessities of situations (Stewart, 2014). RNA having more complex secondary structure presents with more loops and small sizes whereas molecule with lesser loops and large sizes shows lower level of complexity (Adami *et al*, 2000). Cladistic analysis corroborated that even after great speciation events, molecular information were maintained by species as two different species from two different genus, represented the cladistic relationship and had fallen in the same cluster (Fig. 1). Although, genus were distributed in different geographical zones (Fig. 6) but represented a particular group in the same family from different genus, indicating a common ancestral lineage, so their evolutionary history can be traced back to thousands of year (Fozail *et al*, 2015).

CONCLUSION

The family Ancyrocephalidae shows species richness due to having dual evolutionary features in the family. Phylogenetic study confirmed the monophyletic and paraphyletic feature which was further supported by secondary structure analyses of representative species. Cladistic analysis giving strong clues about ancient lineage, origin and range of similarity was comprehended by secondary structure of 28S rRNA. Species distribution strengthened intra genus relationship, divergence, and migration over period of times. In the phylogenetic tree, clustering and cladistic hypothesis was supported by zoogeographical (geo-mapping) distribution in different zones of the world.

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A Combination Study in Some Members of Monocotylidae (Monogenea) in Molecular Phylogeny Employing 28SrRNA along with Geographical Distribution

Fozail Ahmad[1], D. Singh[2], P. V. Arya[3]

Department of Zoology, Dyal Singh College (University of Delhi), Lodhi Road, New Delhi, 110003

Abstract: ***Evolution and interrelatedness among the same group of organism have been explored and debated in many ways. Traditional tools of phylogenetic investigation presented preferred scene for a considerable time. The stage was taken by the incorporation of molecular tools in later stages. Considering a combination of host specific parasite may be more informative in terms of investigating evolutionary lineage of an organism. Recently attempts have been made to incorporate secondary rRNA structure for consideration of phylogenetic studies. Present investigation is an attempt to investigate a family i.e., Monocotylidae (Class: Monogenea) for the same. Cues from geographical distribution and molecular tools have been employed in the study. The finding supports that host play substantial role in the formation of new species. Species distribution strengthened intra genus relationship, divergence and migration over period of times.***

Keywords: Zoogeographical distribution, Monocotylidae, Speciation, 28S rRNA

1. Introduction

Monocotylidae a family of monogena with more than 100 species, parasitic on chondrichthyan fishes (skin, gills, nasal cavities), the phylogeny of the family attempted on morphological and molecular basis (Chisholm et al. 1995; Chisholm et al. 2001 and Glennon et al. 2006). Finding the phylogenetic relationship (clade and cluster) among species of different genus and/or species from a particular family with their zoogeographic distribution may present evolutionary clue for diversity and speciation (Vaillant et al. 2013). Evaluation of zoogeographical distribution (Arya & Singh 2015) together with molecular clue may present evolutionary history including probable origin of the organisms (Rogers 2007; Brumfield & Edwards 2007 and Fozail et al., 2015a & b). Monogenean parasites have utilized for indirectly study of their host, zoogeographical diversity, distribution, migration and settlement over period of time(Arya & Singh 2015)(Mendlová et al. 2012)(Šimková & Morand 2008). Monocotylidae offers a broader range for evolution and zoogeographical distribution on account of multiple sites onto host (Leslie et al., 2001 and Fehlauer-Ale & Littlewood 2011). Their exposure to various sites on the same host, may be accounted for them to have special genetic compositions in order to face the different protective sites developed by chondrichthyan fishes, which is also necessary for their survival in the varying environment (Fels & Kaltz 2006). Measurement of structural parameter of 28S rRNA parameters (bond energy, geometrical features, base composition etc.) and its comparison is proved as the best methods to study molecular phylogeny and correlation with zoogeographical distribution (Tuplin et al. 2002). Phylogenetic characters of rRNA basically include bulges, loops, helices and separation of single strands as they have been conserved throughout the evolution (Lescoute 2005). Secondary structure of ribosomal RNA provides substantial information regarding evolutionary relationship that cannot be simply inferred from cladistic analyses using simple RNA sequences(Keller et al. 2010; Chaudhary & Singh 2013 and Fozail et al., 2015a & b). RNA also provides necessary information regarding the development of biomarker of individual species(Gilad et al. 2008).

Present work is an attempt of utilizing 28S rRNA, secondary structure and zoogeographical distribution reports of the parasite to investigate phylogenetic relationship along with probable pattern of speciation.

2. Materials and Methods

Genus & Species Selection-
Total 39 species from 12 genus of this family were selected based upon the availability of their 28S rRNA in NCBI, their host, distribution and environment were confirmed from literature (Table-1).

Multiple Sequence Alignment (MSA) by ClustalW- 28S rRNA sequences were aligned by ClustalW multiple alignment (Thompson *et al*. 1994) with default settings. Sequence alignment in MEGA 6 and phylogenetic tree prepared using NJ method (Figure -5)

Molecular Phylogenetic Analysis- The Kimura-2 parameter model to estimate distances for correcting the transition bias. Most parsimonious tree was secured using the close-neighbor-interchange algorithm. Bootstrap 1000 replications for every species. Subsequently, MSA were exported as part of the result (Fig: 4a-e). This was mainly exercised for analyzing the genus divergence, speciation and average similarity among species.

Inferring Secondary Structure of 28S rRNAs- Based upon the best alignment score of the sequences in each cluster, aligned using ClustalW. The inference of the secondary structure using Mfold (http://mfold.rna.albany.edu), at a fixed temperature of 37^0 C, structure was analyzed for bulges, stems, loops and negative free energy (ΔG). Every

cluster had been associated with its common rRNA averaging, evolutionary phenomenon.

Geo mapping- All the selected species (Table-1) were marked on simple world map manually (Figure -5) for the global scenario of the species relatedness and diversity. Later on joined with reference to their respective cluster for inferring molecular relatedness globally.

3. Result

Construction of Phylogenetic Tree- Phylogenetic tree forming seven clusters, consisting of two or more than two species showing evolutionary cross relationship. In the tree, Cluster A - G had 9, 2, 8, 3, 4, 2 and 9 species respectively. Cluster A with three clusters exhibited very poor bootstrap value having drastic difference among the three clusters, indicating a huge fluctuation in the event of speciation. In all, two clusters belong to the same genus and represent significant relationship and overall variations among four different genus.

Table 1: Genus with respective species (family Monocotylidae) investigated in the study

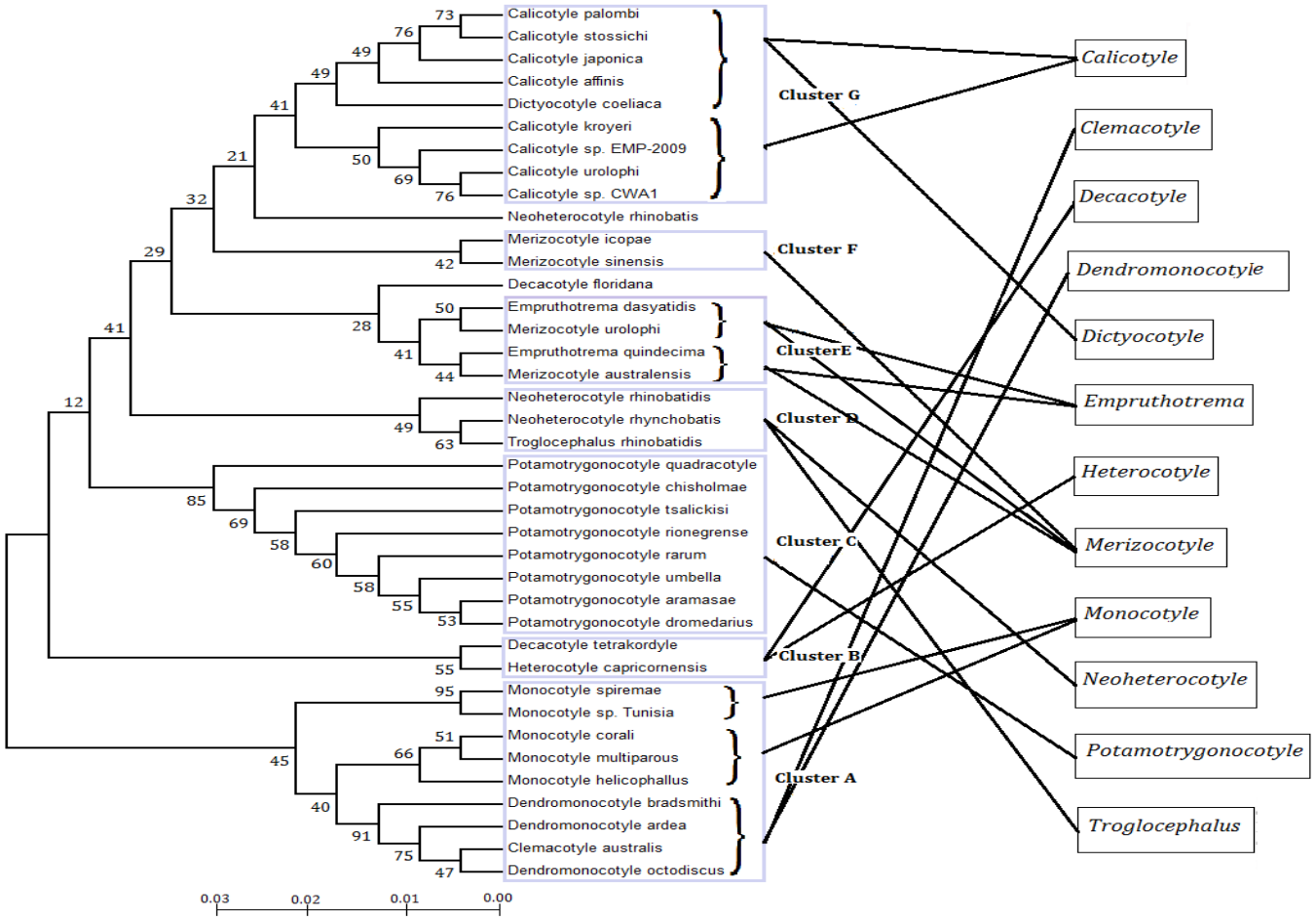
Sl.	*Genus*	*Species*	*Host*	*Envir-onment*	*Country/Area*	*Accession ID*
1	*Calicotyle*	*C. affinis* Scott, 1911	*Chimaera monstrosa*	M	N. A. Ocean	AF382061
		C. japonica Diesing, 1850	*Squalus mitsukurii*	M	Japan	AB485996
		C. kroyeri Diesing, 1850	*Anacanthobatis folirostris*	M	Mexico	AF279748
		C. palombi Euzet & William, 1960	*Mustelus mustelus*	M	N. A. Ocean	AF131709
		C. stossichi Braun, 1899	*Mustelus norrisi*	M	Mexico	AF279751
		C. urolophi Chisholm et al, 1991	*Urolophus spp.*	M	Australia	AF279752
		C. sp. CWA1 Chisholm et al, 2000	-	-	-	AF279750
		C. sp. EMP Perkins et al, 2009	-	-	-	FJ971978
2	*Clemacotyle*	*C. australis* Young, 1967	*Aetobatus narinar*	M	Australia	AF348350
3	*Decacotyle*	*D. floridana* Chisholm et al, 1998	*Aetobatus narinari*	M	Mexico	AF348357
		D. tetrakordyle Chisholm & Whittington, 1998	*Taeniura lymma*	M	Australia	AF348358
4	*Dendrocotyle*	*D. ardea* Chisholm & Whittington, 1998	*Pastinachus sephen*	M	Australia	AF348351
		D. bradsmithi Macleay, 1881	*Myliobatis australis*	F	Australia	FJ971986
		D. octodiscus Hargis, 1955	*Dasyatis americana*	M	N. A. Ocean	AF348352
5	*Dictyocotyle*	*D. coeliaca* Nybelin, 1941	*Raja naevus*	M	N. A Ocean	AY157171
6	*Empruthotrema*	*E. dasyatidis* Whittington & Kearn, 1992	*Dasyatis fluviorum*	F	Australia	AF348345
		E. quindecima Chisholm & Whittington, 1999	*Taeniura lymma*	M	Australia	AF348346
7	*Heterocotyle*	*H. capricornensis* Chisholm & Whittington, 1996	*Himantura fai*	M	Australia	AF348360
8	*Merizocotyle*	*M. australensis* Beverley-Burton and Williams, 1989	*Himantura fai*	M	Australia	AF348348
		M. icopae Beverley-Burton & Williams, 1989	*Rhinobatos typus*	M	Australia	AF348349
		M. sinensis Timofeeva, 1984		M	Taiwan	FJ514075
		M. urolophi Chisholm & Whittington, 1999	*Urolophus paucimaculatus*	M	Tasmania	AF348347
9	*Monocotyle*	*M. corali* Chisholm, 1998	*Pastinachus sephen*	M	Australia	AF348353
		M. helicophallus Beverley-Burton & Williams, 1990	-	M	Australia	AF348355
		M. multiparous Beverley-Burton & Williams, 1990	*Himantura uarnak*	M	Australia	AF348356
		M. spiremae Beverley-Burton & Williams, 1990	*Himantura uarnak*	M	Australia	AF348354
		M. sp. Tunisia Beverley-Burton & Williams, 1990	-	M	Tunisia	AF387511
10	*Neoheterocotyle*	*N. rhinobatidis* Young, 1967	*Rhinobatos typus*	M	Australia	AF026107
		N. rhinobatis Pillai & Pillai, 1976	*Rhinobatos typus*	M	Australia	AF348362
		N. rhynchobatis Tripathi, 1959	*Rhinobatos typus*	M	Australia	AF348363
11	*Potamotrygonocotyle*	*P. aramasae* Tripathi, 1959	*Paratrygon aiereba*	F	Brazil	JN379514
		P. chisholmae Mayes et al, 1981	*Potamotrygon motoro*	F	River basin (USA)	JN379519
		P. dromedarius Mayes et al, 1981	*Potamotrygon hystrix*	F	Brazil	JN379518
		P. quadracotyle Mayes et al, 1981	-	F	Brazil	FJ755807
		P. rarum Mayes et al, 1981	*Potamotrygon schroederi*	F	Brazil	FJ755809
		P. rionegrense Mayes et al, 1981	*Potamotrygon cf. motoro*	F	Brazil	FJ755810
		P. tsalickisi Mayes et al, 1981	*potamotrygonid*	F	River basin (USA)	JN379513
		P. umbella Mayes et al, 1981	*Potamotrygon*	F	Brazil	FJ755808
12	*Troglocephalus*	*T. rhinobatidis* Young, 1967	*Rhinobatos typus*	F	Australia	AF348364

Note : M= Marine; F : freshwater. All the sequences for the present study was taken from NCBI database. Acknowledgement is due to all the contributors.

Second cluster (B) having species from two different genus and represented 55% of considerable bootstrap value. Cluster C reflected a constant bootstrap value (53-59 %) for all species except *Potamotrygonocotyle quadracotyle* in the cluster, all species belong same genus. The bootstrap value of the cluster could be average to 55%. Cluster D with species from two different genus and considerable bootstrap values. Cluster E had two clusters from two different genus. Cluster F all species from the same genus not connected by considerable bootstrap values and Cluster G forming two clusters, one with species from same genus whereas, other with one species from the genus *Dictocotyle*. Both clusters with significant bootstrap (70%). The phylogenetic tree of selected species from different genus shown the level of similarities, point of deviation and time of speciation between genus that is in the same cluster. Two species from two different genus might have evolved from same ancestor regardless of any high bootstrap value. Cluster in the tree represented that a group of species might have evolved from a common ancestor, thousand years ago. This result is further supported in the subsequent sections of the result.

Secondary Structure Analysis- The inferred secondary structure of 28S rRNA by Mfold of representative species from seven cluster exhibited the evolutionary distinction between species and clusters as well (Figure -2) also provided the stability of molecules in terms of negative free energy (ΔG). Representative species were selected by multiple sequence alignment of species from each cluster, and the most conserved sequence of the species was considered based on alignment score given by ClustalW. Formation of secondary structure is characterized by the bulge loops, interior loops and hairpin loops conferred by negative free energy of molecule. Higher the negative free energy (ΔG), more stable the molecule. Negative free energy of cluster A - G (rRNA from species) were predicted to be -261 kcal/mol, -229.3 kcal/mol, -260.2 kcal/mol, -264.3 kcal/mol, -247.1 kcal/mol, -244.2 kcal/mol and 218.1 kcal/mol respectively (Figure -3). The negative free energies of cluster A, C and D fall in the range of -260 kcal/mol and discrete by -2.5 kcal/mol approximately, representing that species from both groups had followed similar pattern of evolution. Anomaly to this observation can be accounted as the varying number of different loops directly affects stability of molecule. Cluster E and cluster F had an average negative free energy of -245.5 kcal/mol (discrete by approximately ΔG =-3.9 kcal/mol), shown to be correlating each other and representing evolutionary relatedness. In case of cluster B and G, ΔG was highly discrete by -11kcal/mol, signifying a distant re

Three types of loops are formed in the secondary structure of RNA molecule (cluster/representative species) with unique pattern of occurrence (Figure -2). The formation of loops, as mentioned earlier, is almost conferred by negative free energy, resolves stability and constancy of the entire molecule.



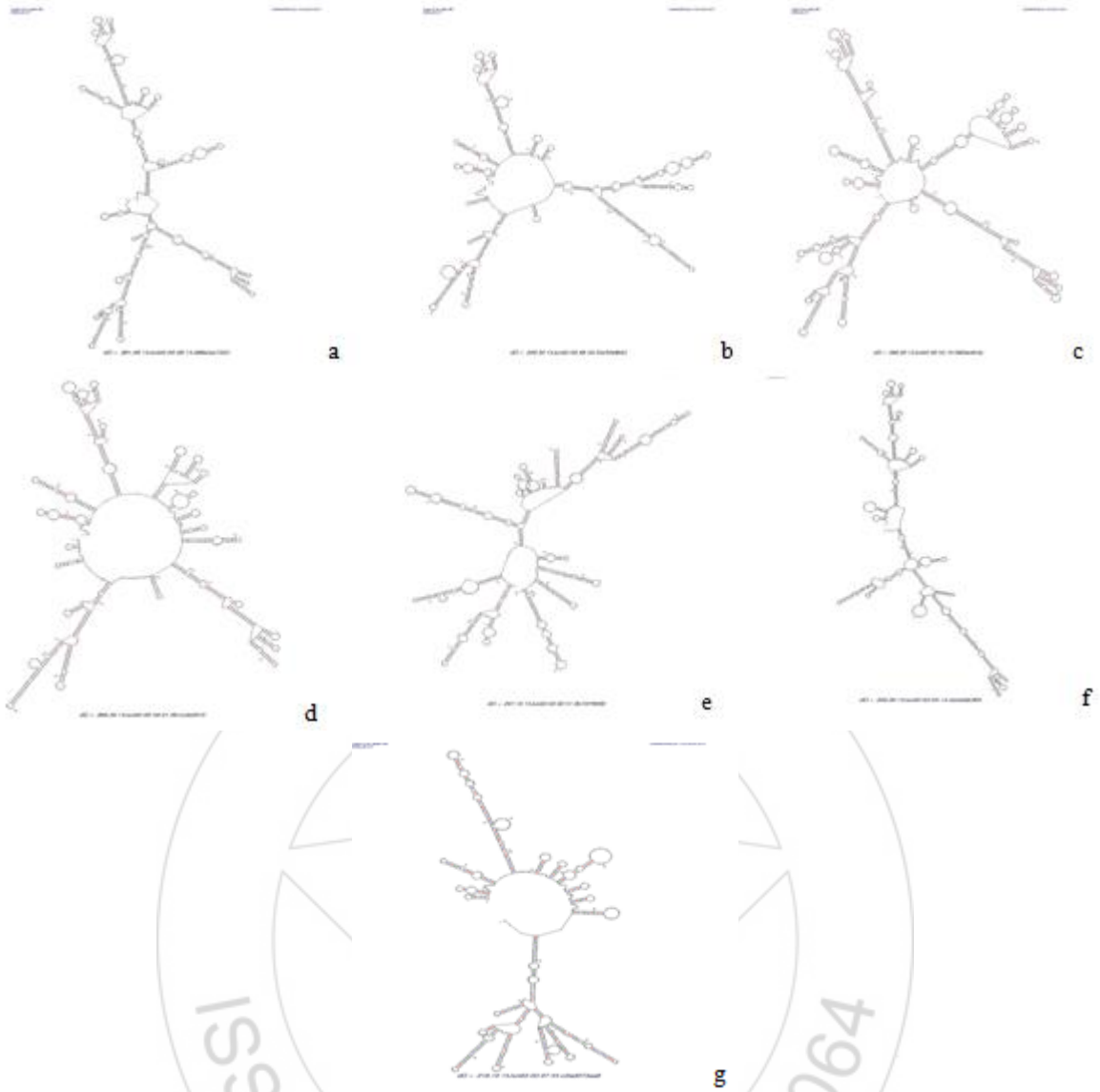


Figure 2: Secondary structure of 28S rRNA of 39 species represented by 12 genus clustered in 07 clusters family Monocotylidae

In present analysis, the sum of interior loops and hairpin loops is equal, although, their number varied for individual clusters (Figure-3). Number of bulge loops (3-6) was found to be least for all clusters. Cluster D with highest negative free energy (Figure-4) represented 40 loops, second most in number. Cluster A with the second highest **ΔG** developed a total of 35 loops that did not seem to coincide with its negative free energy (-261 kcal/mol) which should have been, thermodynamically, second most of all, mainly due to specific pattern and number of nitrogenous bases participated in forming the loops.

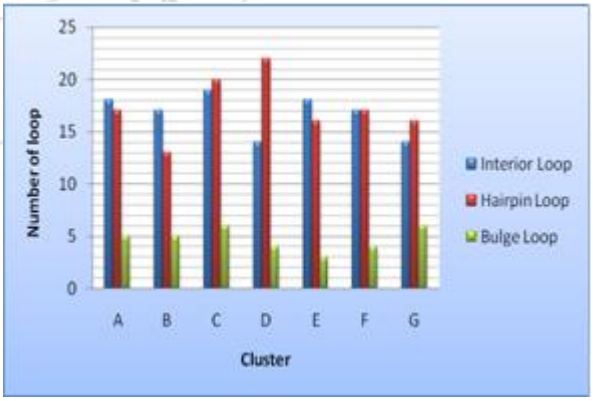


Figure 3: Number of loops from of respective RNA secondary structure for each cluster

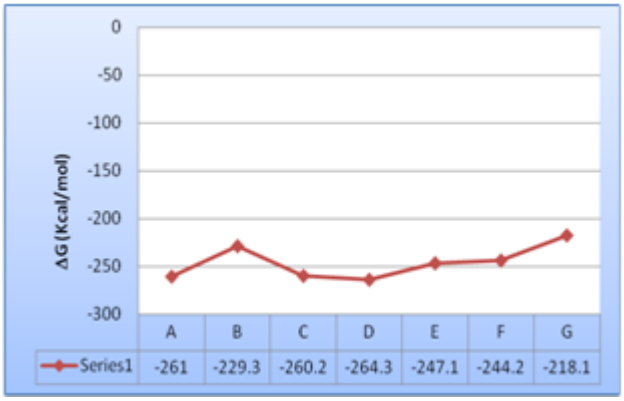


Figure 4: Negative free energy of the representative RNA structure for each cluster

Cluster C surprisingly had the greatest number (45) of loops in total. Only cluster A tend to deviate in terms of number of loop, otherwise negative free energy was peered for the three related clusters relating that species belonging to different genus had a specific pattern of evolution and later on distributed in different regions. Cluster E and F with almost equal amount of ΔG developed equal number of loops, representing strong ancestral relatedness among species. Cluster B and G were discrete by a great energy difference of -11 kcal/mol but number of loops were discrete by one loop, that could not be accounted for concern. Hence, it can be concluded that these two groups were descended from distinct ancestral lineage. Comparatively, three types of loops represented uniqueness, stability, conservation pattern, evolutionary relatedness and range of ancestral lineage.

Multiple Sequence Alignment Analysis

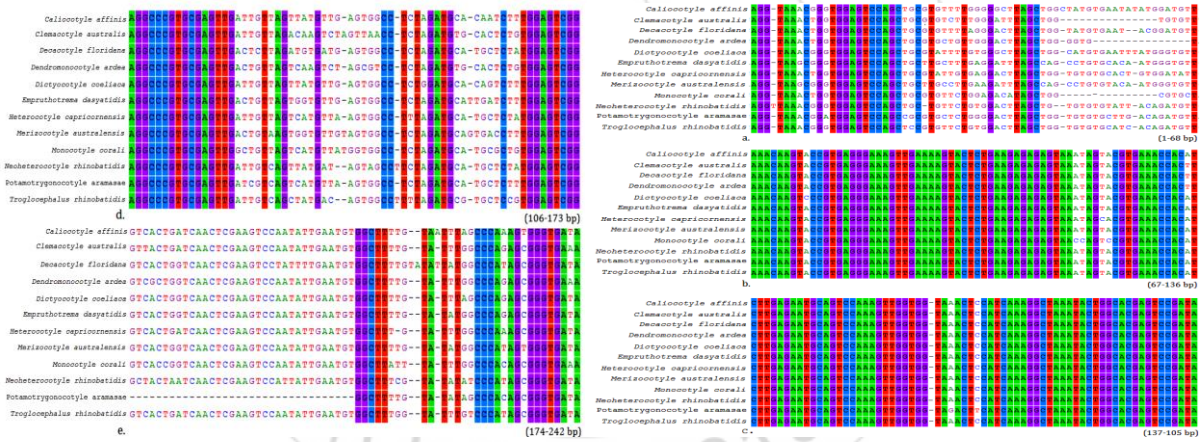
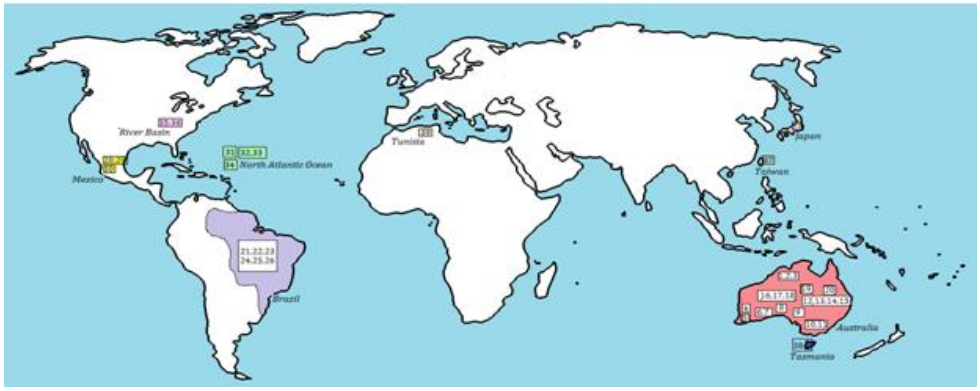


Figure 5: ClustalW alignment of 12 sequences of 28S ribosomal RNAs.

Conserved bases were shown to be highlighted with four different colors (Figure-5a- e). Out of 242 base pairs, 125 base pairs were found mismatching along with gaps (insertion & deletion). Figure-5b with bp 67-136 showed better alignment wherein six mismatches were maintained & Figure-5c with bp 137-105 had one indel and two mismatches. Figure-a, d & e represented high level of mutation over alignment. These simple alignment results provided an overview on genus variability and divergence for speciation.

Geo Mapping



4. Discussion

The molecular distinction among species from different genus provides an understanding over evolutionary process and expressing ancestral lineage to the origin of a new species. RNA in the folded form show paired and unpaired (loops) bases (Chen et al. 2005). Qualitatively, bases which are bonded tend to stabilize molecule due to higher negative free energy whereas unpaired bases tend to destabilize the molecule due to lesser negative free energy (Greatorex et al. 2002 and Geisberg et al. 2014). Quantitatively, loop that are more in number destabilize the secondary structure because they require more positive free energy (Trotta 2014). Thus, cluster A, C and D are the most stable and Cluster B, E, F and G are less stable structure, signifying that organisms belonging to the particular cluster would be of equal stability in terms of negative free energy of their RNA molecules, and therefore, could follow the same pattern of origin (Sun & Caetano-Anollés 2008). From first to seventh cluster, each organism representing its own cluster, exhibiting distinctions in term of number of neighbor/sister clade and 28S rRNA secondary structure. Although negative free energy and number of loops showed noticeable variations within all clusters with an established correlation between the two parameters. Except cluster B and G, remaining five clusters (cluster A, C & D) and (cluster E and F) represent equal stability, conservation pattern and sympatric speciation events (Figure-4). This was further supported by equal number of loops developed in the representative molecule. Cluster B and G with their respective higher and lower number of loops and negative free energies were not coinciding with other clusters in number of loops and ΔG. Because each group of organisms possess the particular pattern of evolution for ribosomal RNA. The differences among clusters about ΔG were mainly accounted due to the size of loops. Loops more in number but smaller in size are formed with less negative free energy whereas loops less in number but larger in size require more negative free energies (Katz 2003). Evidently, both, size and number of loops are accounted for estimating out the stability of a molecule (Zhang et al. 2008). The pattern of evolution and relatedness among species is reflected by the development of loops and their sizes which in turn account for the overall stability of RNA (Wongsurawat et al. 2012). Evolution, most of the time, rises the level of complexity that is strictly coincided with the necessities of situations. RNA having more complex secondary structure presenting with more loops and small size whereas molecule with lesser loops and large sizes show lower level of complexity (Gevertz 2005). Cladistic analysis corroborated that even after great speciation events, molecular information were maintained by species as two different species from two different genus represented the cladistic relationship and had fallen in the same cluster (Figure -1). Although, they were distributed in different geographical zones (Figure -6) but represented a particular group in the same family from different genus, indicating a common ancestral lineage. So their evolutionary history can be traced back to common points. A major reason is observed about species richness in the family Monocotylidae and why cladistic relationship represents inter genus similarity. The one reason behind speciation in Monocotylidae is that whenever infecting sites are changed or switched (*e.g*. from gill to inner wall of the body cavity), genetic composition automatically gets changed, determined by the extent of parasitism and resistance of host (Fels & Kaltz 2006 and Millanes et al. 2014). This creates a major molecular change followed by physiological variations, waved into conserved domain of nucleic acids (Thompson et al. 2001). Over the period of time, the developing variation is stacked and then a time reaches when the molecularly distinct species appear with novel feature and said to follow a new route for a different lineage (Nancy and Moran 1998).

The finding paves way to a hypothesis that host plays substantial role in the formation of new species especially for monogenetic parasites. Cladistic analysis giving strong clues about ancient lineage, origin and range of similarity was comprehended by secondary structure of 28S rRNA. Species distribution strengthened intra genus relationship, divergence, and migration over period of times. In the phylogenetic tree, clustering and cladistic hypothesis was supported by zoogeographical distribution of Monocotalidae in different regions of the world.

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RESEARCH ARTICLE

COX-1 STUDIES IN EVALUATION AND ASSESSMENT OF MOLECULAR DIVERSITY AMONG GYRODACTYLIDAE, DIPLECTENIDAE, DIPLOZOIDAE AND DICTILOPHORIDAE FAMILIES (CLASS: MONOGENEA)

[1]Fozail Ahmad, [1,*]Priya Vrat Arya and [2]Singh, H.S.

[1]Department of Zoology, Dyal Singh College, University of Delhi, New Delhi-110003, India
[2]Department of Zoology, Ch. C. S. University, Meerut-250004, India

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ABSTRACT

Proteins being mirror to molecular signature of an organism, their potential in assessment of molecular diversity may be useful. Comparison among the organisms or on a larger scale of family may further be taken to give insight on the molecular journey of the organisms. Present paper deals with the study of COX-1 in four families *viz*., Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae (Class : Monogenea) using structural and other significant parameters. In all 16 species have been extensively studied across four families. Results reflecting peculiar diversity on molecular level suggesting divergence based evolution in the form of molecular molding.

Key Words: Cytochrome C Oxidase, Monogenea, Secondary Structure, Evolution.

INTRODUCTION

Proteins are more conserved than nucleic acids during evolution, providing strong platform to study conserved aspects of their structure as well as function (Butland *et al*., 2005; Socolich *et al*., 2005; Sicheritz-Ponten, 2001). Among them Cytochrome C Oxidase is one of most conserved protein and oldest one on the earth (Sicheritz-Ponté *et al*., 1998; Castresana *et al*., 1994). Cytochrome oxidase reduces oxygen to water making it essential enzyme for aerobic metabolism (Collman *et al*., 2007; Ekici *et al*., 2014). It creates a proton gradient as an intermediate step in the conversion of redox energy to ATP (Rottenberg, 1998). The enzyme complex of the electron transport chain with 13 subunits is of mixed genetic origin (Li *et al*., 2006). The three largest subunits (I-III) are encoded by mitochondrial genomes (Breek *et al*., 1997) and carry out known catalytic functions of the enzyme and show homology between eukaryotes and prokaryotes (Steffens *et al*., 1987; Smits *et al*., 2007). Other 10 subunits encoded by nuclear genome (Lenka *et al*., 1998; Wolz *et al*., 1997). The mixed origins of COX give challenge of study the evolutionary relatedness of two distinct genetic systems (Wu *et al*., 2000). COX-I, the largest subunit of the holoenzyme is important in enzyme function and only subunit conserved in all heme–copper oxidases from prokaryotes to eukaryotes (Soto *et al*., 2012). It is incorporated into the mitochondrial inner membrane, containing 12 transmembrane helices and three redox centers, heme-a, heme-a3, and CuB (Clemente *et al*., 2013). Evolution in terms of classification and placing monogeneans help integrate the large group to identify proper position in taxonomic class. A study was initiated to study the evolution of COX-I in Monogeneans and examined its protein sequences from 16 species for four families, Gyrodactylidae, Diplectenidae, Diplozoidae and Dictilophoridae. The finding may furnish a space to enumerate ancestral lineage and evolutionary pattern among selected families.

Corresponding author: Dr. P. V. Arya,
Department of Zoology, Dyal Singh College, University of Delhi, New Delhi-110003, India.

MATERIALS AND METHODS

Selection of Protein Sequences: Cytochrome C oxidase-1 was selected for 16 species from four different families based upon the availability of particular type of protein sequences for sufficient number of species in a particular family, in order to carry out analytical studies. All sequences had varying length, differ by one or two amino acids with no phylogenetic issue at all. The Gyrodactylidae, Diplozoidae, Diplectanidae and Dictylophoridae had 5, 2, 6 and 3 selected species respectively.

Phylogenetic Analysis: Sequences were subjected to alignment using ClustalW (inbuilt in MEGA 6) for multiple sequence alignment (Thompson *et al.,* 1994) with the default gap and extension penalties. The phylogenetic tree generated using neighbor joining (NJ) method in MEGA 6. The average pathway method to calculate the branch length all over the sequences. Most parsimonious tree was chosen by the close-neighbor-interchange algorithm.

Pair-wise Sequence Alignment: Pair-wise alignment was done for 3-D structure. One protein sequence from each family was taken and executed into NCBI-PBLAST (Protein-Basic Local Alignment Search Tool). The sequence with the highest score was chosen for structural modeling. The number of mutation over amino acids and comparative evaluation among 04 sequences from families was also done.

Protein Structure Prediction: Homolog protein sequences were processed in SWISS-MODEL for structure prediction and identifying its quality predicted from features of the target-template alignment for model building based on the target-template alignment using Promod-2. Insertions and deletions were remodeled using a fragment library. Side chains were then remodeled followed by regularization by force field (Guex, *et al*., 1997).

The global and per-residue model quality were assessed using the Q-Mean scoring function (Benkert, *et al*., 2011).

RESULTS

Multiple Sequence Alignment: In multiple sequence alignment every sequence was approximately contented with 145 amino acids except sequences from *Gyrodactylus* species. Initially, sequence alignment was performed with full amino acid length and later it was trimmed for being highly dissimilar and mutative (fig. 1). After removing non-matching sequences a total of 77 amino acids with conserved sites were obtained. First block of MSA reveled that rate of mutation was slow among species of the genus *Gyrodactylus*. Out of five sequences, over MSA, one block is observed for mutation or mismatching, imparting the protein Cytochrome C Oxidase-1 with high conserved occurrence in the genus.

Overall Cytochrome C Oxidase-1 has been carrying random mutation events in some genus while others with less or no mutation throughout speciation and diversification. Globally, with 77 residues, only 20 sites found conserved, indicating cytochrome c oxidase-1, a significant conservative protein for phylogenetic analysis. Residues in larger red block could be omitted without any considerations (fig. 1), though, as per the individual genus sequences are concerned, they have negligible mismatches and significant conservation sites. Overall divergence among sequences covered a broad range of mutation (fig. 2). Each group/taxon with a particular range of divergence as in case of the family Gyrodactylidae, 50-55% of mean divergence was observed. Those of Diplectenidae, Diplozoidae and Dictilophoridae had 36-70%, 22-28% and 7-9% of mean divergence respectively. Range of mean divergence characterizes to the rate of change, larger the range, faster the rate of change in amino acid composition. Diplectenidae exhibited great variability in terms of protein conservation.

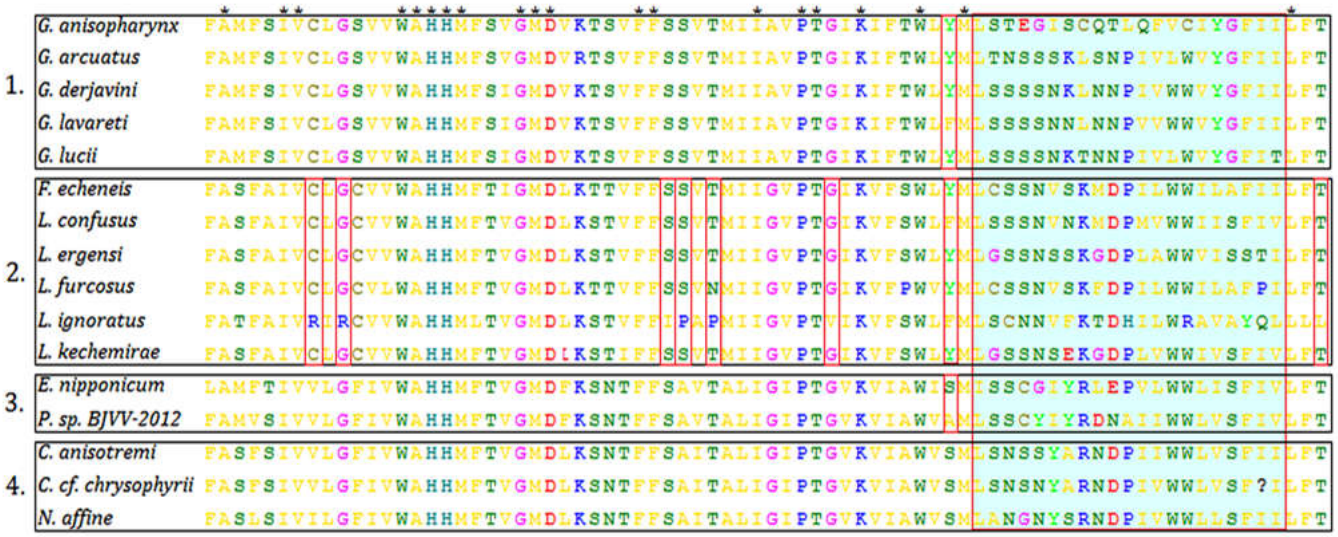

Figure 1.Multiple sequence alignment of 16 protein sequences. Number as 1-4; families Gyrodactylidae, Diplectenidae, Diplozoidae & Dictilophoridae respectively. *conserved amino acid residues; red box nonconserved regions in the sequences. Similar amino acids are given the same color; red block dissimilarities of residue in the particular genus

Contrast observed by the species of the genus *Lamellodiscus*. Six sequences of the genus reflected great diversity within the species. Other two genus *Diplozoidae* and *Dictilophoridae* with 2 and 3 species sequences had one and no mismatches respectively.

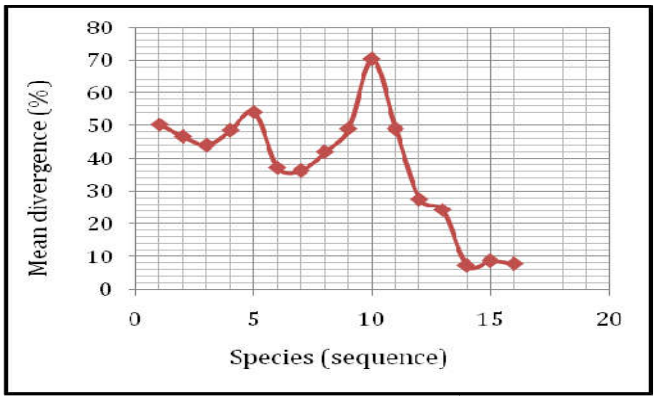

Figure 2. Plot of mean divergence between 16 sequences. Mean calculated in the form of a matrix using MEGA6 and then plotted

Pair-wise Sequence Alignment: Using NCB-PBLAST selected proteins from each genus were run and homolog were retrieved from the result with higher similarity percentage in order to assume 3-D structure of the Cytochrome C oxidase-1 (fig. 3). All of the sequences had similarity score above 85-95% that made them easy for protein homology modeling.

The query sequences were selected on the basis of their conservatory behavior in multiple sequence alignment. The phylogenetic trees from all methods produced the similar taxon group except UPGMA, projected out the out-group which was the only difference among all (fig. 4). In the process of sequence manipulation and tree construction, no sequence was given as out-group.

The first group, Gyrodactylidae, in the MSA showing just one mutation coincided with its clade about large branch of *Gyrodactylus anisopharynx* in all phylogenetic trees. So, this species may or may not be regarded as out-group or root of the tree, depending upon the kind of analysis being performed. Clade also shown monophyletic mode of species divergence, confirming small mutation among protein sequence.

Figure 3. Pair-wise sequence alignment of 4 sequences selected from each family. b *Gyrodactylus anisopharynx;* b. *Lamellodiscus furcosus;* c. *Eudiplozoon nipponicum* & d. *Neoheterobothrium affine.* Blocks- conserved/matching amino acid residues

Other group Diplectenidae in MSA depicted observable variation at various sites as it had 7 mismatches in residues and have been the group to have highest variability in the sequences. The variations in the sequences led to the greater rate of speciation than others under investigation. Other two groups Diplozoidae and Dictilophoridae were amazingly found to share a common origin (Fig. 4), though not as monophyletic but paraphyletic evolutionary pattern. As individual clade, Diplozoidae and Dictilophoridae both separately showing monophyletic pattern of evolution, suggesting origin from two different ancestors.

Protein Structure Prediction

For structural variations protein were modeled using Swiss-Model server and structure of four sequences was predicted (Fig. 5). Despite of sequence variability in cytochrome c oxidase-1 significant similarity was observed for every protein with reference to structure and function as well. In order to compare the four proteins we intended to set parameters those of Swiss-Model generated itself that include local quality estimation, development of α-helix and β-sheet structure etc. The local quality estimation (LQE) in (Fig. 6). LQE can be manipulated for justifying the variable amino acid numbers. On one axis graph showing number of residues and predicted local similarity to target on the other axis. *Gyrodactylus anisopharynx* had 250 residues and drawn a good similarity score of 0.5-0.8, a considerable range to target sequence. Other 03 sequence of *Lamellodiscus furcosus, Neoheterobothrium affine and Eudiplozoon nipponicum* are having number of amino acids just half of *Gyrodactylus anisopharynx* and hence showing lesser similarity score, 0.3-0.8 to their target sequences.

More precisely only *Lamellodiscus furcosus* had poor score to its target. This may be related with the above result of MSA in which high mutation had occurred throughout the alignment. Over local similarity, being homologous to each other, sequence coincided in structure and functions.

Fig. 6 representing a less dissimilarity in all monomeric structure as the complete protein is made up of 13 polypeptides. So, it is confirmed that structurally Cytochrome C Oxidase-1 remain conserved even if one compares individual polypeptides from different family. There might have mutation by environmental or ecological factors and great speciation event would have led the conserved protein to keep unique amino acids composition conserved, tending no change in structure. Feasibility of differences in Cytochrome comes from residues participating in core formation of protein whose removal or deletion would not affect the structural topology and so the function. Structurally all proteins are monomeric as a key enzyme in aerobic metabolism by functions. Proton pumping heme-copper oxidases represent the terminal, energy-transfer enzymes of respiratory chains in prokaryotes and eukaryotes.

Evaluation & comparison of Secondary Structure

Cytochrome C Oxidase-1 protein secondary structure was further elaborated and then compared so as to establish a clear distinction among them to identify the probable function of a protein from 3-D structure using a series of method (Fig. 8).

In secondary structure (Fig. 8) α-helix and β-sheet are very common to occur, depending upon the intrinsic propensity of amino acid sequence in a protein.

Phylogenetic Analysis

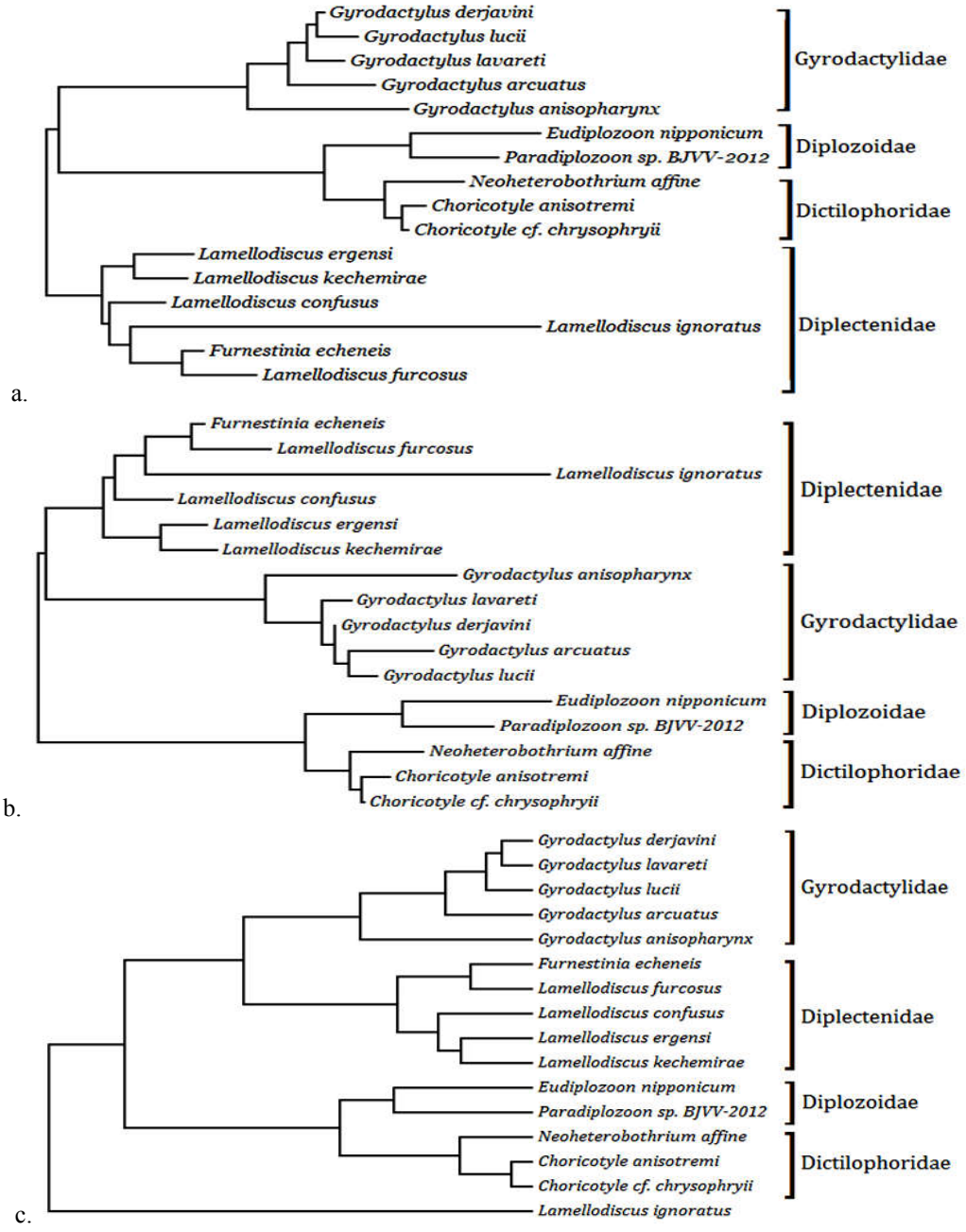

Figure 4. Phylogenetic trees a. Neighbor Joining; b. Maximum Parsimony & c. UPGMA.

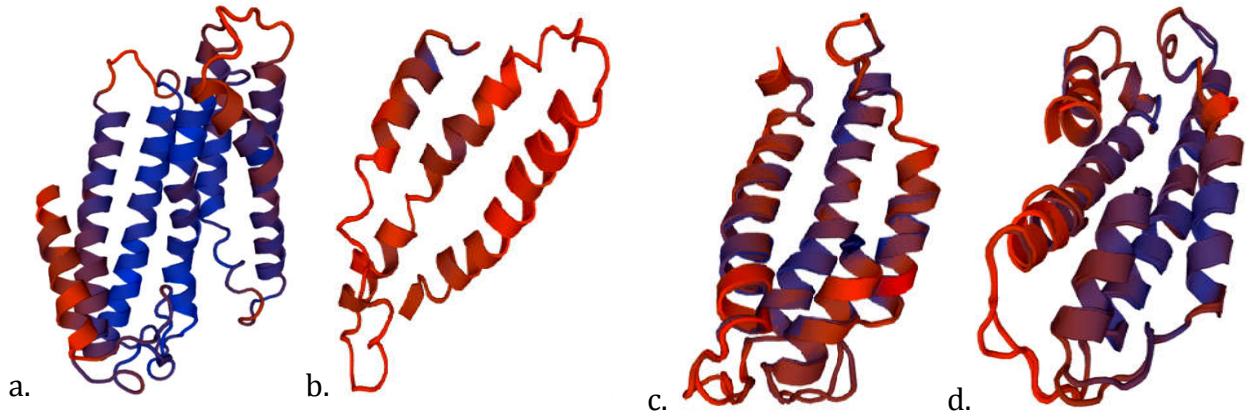

Figure 5. Predicted protein structure of a. *Gyrodactylus anisopharynx;* b. *Lamellodiscus furcosus;* c. *Neoheterobothrium affine* & d. *Eudiplozoon nipponicum*

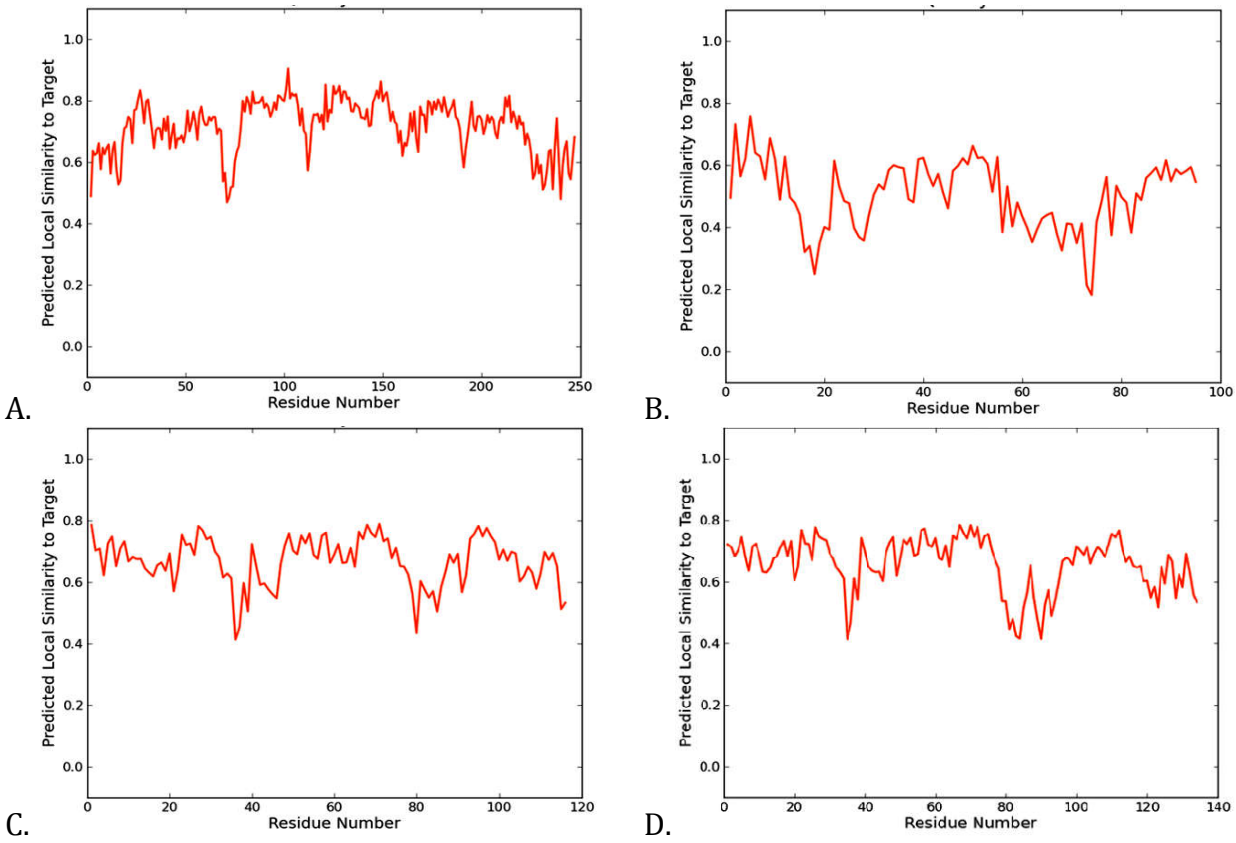

Figure 6. Local estimation of side chains to target sequences. A. *Gyrodactylus anisopharynx;* B. *Lamellodiscus furcosus;* C. *Neoheterobothrium affine,* & D. *Eudiplozoon nipponicum*

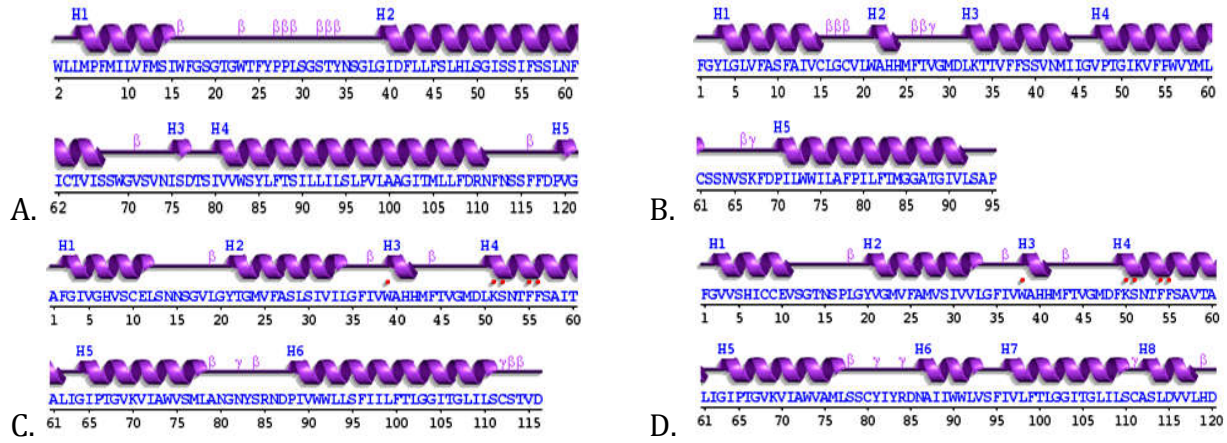

Figure 7. Open form secondary structure of Cytochrome C Oxidae-1. A. *anisopharynx;* B. *furcosus;* C. *affine* & D. *nipponicum*

Fig. 7A which belongs to *anisopharynx,* although, had 240 amino acid residues but only 120 residues were shown in the structure in order to coincide with other groups where *furcosus* had the least number of residues. Helix and sheet were indicated as H and β respectively.

For *anisopharynx* a total of 6 helices, out of which only 3 larger and rest of the sequence tend to develop β sheets. In comparison to *furcosus*, it contain 4 larger α helices and a smaller one. Likewise *affine* 5 larger α helices and 2 smaller ones. Highly significant number of α helices was developed into *nipponicum* as 5 larger and 3 smaller ones with just 120 amino acid residues. From the reference of stability, β sheet are more stable than α-helix and tend to show lesser mutation in the course of evolution.

As per results more α-helix greater the mutation or lesser the β sheet lesser the mutation. Resultantly least number of α-helix in *anisopharynx* has made it more stable than remaining three. And therefore, the evolution in that particular protein will be more than others. This result is consistent with the evolution of proteins under adverse conditions. In an order of stability to fix the relative evolution of four groups of Cytochrome C Oxidase-1, it can be represented as; *anisopharynx* > *furcosus* > *affine* > *nipponicum.* Accordingly their rate of evolution was understood with the order of relative stability and so the pattern of evolution. *G. anisopharynx* was evolved at slowest rate with highest stability and in contrast *Eudiplozoon nipponicum* evolved at fastest rate with least stability for protein.

DISCUSSION AND CONCLUSION

Measurement of sequence parameters including MSA, PSA and local quality estimation for inferring out the 3-D structure, reveals a number of facts over evolution of COX-I among monogeneans. Problems faced in carrying the study was the lack of availability of complete sequence of COX-I for monogeneans, therefore, analysis over the gene duplication and gene divergence could not be performed that would have certainly strengthened our finding for evolutionary aspect in different monogeneans and would have provided strong clues for their relatedness from across the globe. MSA provide an initial and comparative understanding of protein variability (Blackburne and Whelan, 2013). Separately, all 04 groups support intra-genus relationship by having specific mutation sites. The first group, Gyrodactylidae, with five species had highly conserved pattern in protein sequences (Fig. 1).

Only one site is found mutated with a mean divergence of 45-50% (fig. 2) with other sequences. The unique feature about the family is the monophyletic evolution of the species as shown in phylogenetic tree (Fig. 3) showing a linear evolutionary pattern from a common ancestor, withdrawing our attention towards possible relationship among species by fast but sensitive mutation. Knowingly, Gyrodactylidae represent the most diverse species for maximum number of geographical distribution. Family is rich in both number of species and adaptation to various ecological conditions. Most importantly, Gyrodactylidae show most stable form by having developed least α-helix structure (Fig. 8). Evolution in terms of gene duplication events have not been considered for COX-I in monogeneans because it fails to provide enough cue on mutation events that would be sufficient enough to create a new path of genus/group. Evolution has taken place in the protein of the family but it does not necessarily mean to have a new species or group in return.

Family Diplectenidae exhibited eight point mutations even after MSA and sequence editing had done, indicating a higher rate of mutation in the family with higher level of speciation and divergence. The tendency was supported by the molecular phylogeny of the group in the phylogenetic tree that they follow dual route (monophyletic and paraphyletic) of evolution. Among them is *Lamellodiscus ignoratus* exhibited longest branch (Fig. 4), an indication of maximum mutation in gene besides other family member. A significant variation in the mean divergence (Fig. 2) for Diplectenidae further strengthens higher species variability among the members of the group. The observation can be further rationalized with ecological attributes and geographical distribution for a clear scenario over the entire family.

In earlier studies of zoogeographical distribution and molecular phylogeny on *Lamellodiscus*, the family had found not confined in to a particular geographical zone rather it had been dispersed across the globe with significant phylogenetic anomaly (Fozail Ahmad *et al*., 2015). Members of the group were found in almost each geographical region, providing a strong support to our current study. The third group, Diplozoidae shows a single point mutation in MSA with 22-27% of mean divergence that may have either increased or decreased if more sequence had incorporated. Surprisingly, this group represents phylogenetic relationship (Fig. 4) with Dictilophoridae and forms a separate taxon.

The feasibility of monophyletic evolution or more precisely, co-evolution of COX-I in both of the group may have taken place and close relatedness among members can be inferred. As an individual group of Monogeneans, mean divergence (7-10%) of Dictilophoridae is least from others with no point mutation in MSA. Structurally, both of them are very similar in terms of having number of α-helix is 6 and 7 for Diplozoidae and Dictilophoridae respectively (fig. 8). These finding are supported by local quality estimation of protein sequences while modeling their three dimensional structures. Both show almost equal range of similarity for their target sequence.

Overall, four groups in the study provides a generalized evolutionary distinction of COX-I protein of Monogenean families in terms of sequence and structure. The four groups are highly diverging members of parasitic class, representing variability in conserved protein. Monogeneans can be evaluated on the basis of such analysis for their origin and evolution. Further studies can be performed with more families/group in order to justify the ancestral lineage. This finding just gives an idea of evolutionary relatedness in all families/genus in term of COX-I protein changing over the period or may provide the beginning of evolution of class Monogenea.

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IN-SILICO PHYLOGENETIC TOOLS EMPLOYED ON SOME MEMBERS OF FIVE MAJOR FAMILIES OF MONOGENEA *VIZ.*, MONOCOTYLIDAE, ANCYLODISCOIDIDAE, ANCYROCEPHALIDAE, CICHLIDOGYRIDAE AND POLYSTOMATIDAE FOR INVESTIGATING THEIR RELATEDNESS AND GLOBAL DIVERSITY DISTRIBUTION

Fozail Ahmad, Dharmendra Singh, P. V. Arya* and H. S. Singh[1]

Department of Zoology, Dyal Singh College (University of Delhi), New Delhi - 110 003, India.
[1]Department of Zoology, Ch.C. S. University, Meerut - 250 004, India.
*e-mail: zoology.dsc@gmail.com

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ABSTRACT : The global diversity and the relatedness among the members of the same group remain a key attraction for evolutionary diversity. Members of parasitic Class Monogenea is extensively investigated during recent past. Present paper is an attempt to explore the concept of relatedness and global diversity evolution in five major families of this class using various *in-silico* tools. Study involve investigations on 227 species using 28S rRNA data and its geomapping co relations.

Key words : Geomapping, phylogeny, evolution, Monogenea.

INTRODUCTION

Enumerating the present time diversity of lower organisms and comprehending how they diversified in ancient time, are the points of milestones in evolutionary biology, ecology and conservation biology (Pariselle *et al,* 2011). The estimation of past parasitic biodiversity and present diversification is remained in its initial stage (Dobson e*t al,* 2008). Efforts with multiple approaches have been carried out to present a convincing answer to these questions. Being an ideal taxon for investigation of past diversifications and present diversity, monogeneans have been extensively studied for number of important reasons (Poulin, 2002). Monogenea form a diverse group with thousands of species (Cribb, 2002). They don't show diversifications in numbers only but are the group among flatworms to have undergone an adaptive radiation, ecological adaptation, parasitism, multiple host relationship, adaptation from being external to internal parasite on the same host and morphological versatility (de León *et al,* 2010; Karvonen *et al,* 2012 and Vanhove *et al,* 2013). Apart from these features, host switching is a common phenomena in monogeneans at all the branches of its phylogeny making analysis easier to explore for a link between ecological characteristics of host and diversity of parasites, and to control for the phylogenetic history of their associations (Bakke *et al*, 2002; Badrane *et al,* 2001 and Reeves *et al,* 2015). As a whole it is quite difficult to estimate species and parasitic diversity, still there is a chance with good range of possibility of analyzing into families and subfamilies (Gerasev, 2004). For all (approximately 4000) species, a total of 35 families have been classified followed by 250 genus designated in the literature and at various databases (Türkay Öztürk1 *et al,* 2014). Out of these families, Gyrodactylidae, Monocotylidae, Ancyrocephalidae, Capsilidae, Cichlidogyridae, Polystomatidae and Diplectanidae are among constantly studied and providing a novel hypothesis of evolutionary relatedness of their member species (Williams, 1991). Each of them possesses distinct features in terms of morphology, physiology, host specification, co-evolution and ecological patterns (Mladineo *et al,* 2013). Families like Ancylodiscoididae and Polystomatidae and members of Dactylogyrids are afforded with the members of fresh water bodies, making a geographic linking among those of other fresh water species across the globe (Vanhove *et al*, 2014). Incorporation of information into family analysis have been paid attention due to encompassing a range of diversity richness in monogeneans with a vital understanding over all aspects of parasitism, making evolutionary study more interesting and easier at the secondary stage of analyses (Cribb *et al,* 2002 and Fozail *et al,* 2015a-c).

Geographical study on monogeneans does not exactly show their origin and hence it needs to be strengthened further, since their distribution merely demonstrates a clue to the root of diversification (Badets *et al,* 2009 and Fozail *et al,* 2015a-c). Together with molecular phylogeny and zoogeographical tracking as a combinatorial approach to the ancient history may provide an insight to common origin and diversification of this taxon (Poisot *et al,* 2011).

Phylogeny itself is not capable of resolving this problem, however a molecular pattern among members of the group can be established in order to understand parasitic diversity with all due consideration of features mentioned above (Telford, 2006).

In present study, we intend to present the prevalence of major families in different geographical zones and their evolutionary relatedness using molecular data in order to understand their possible pattern of occurrence/ diversification/relatedness.

MATERIALS AND METHODS

Selection of families

Selection of families (Table 1) is based upon diversity of family and the previous phylogenetic analyses being performed by us and genomic data of species exists in NCBI (National Center for Biotechnology Information).

Table 1 : Summary about families selected for the study.

Sl.	Family	Total genus	Total species	rRNA type
1.	Ancylodiscoididae	6	27	28S
2.	Ancyrocephalidae	12	72	28S
3.	Cichlidogyridae	1	23	28S
4.	Monocotylidae	12	39	28S
5.	Polystomatidae	15	44	28S

Molecular Phylogenetic Analysis

Initially nucleotide sequences of all species for all families were retrieved from NCBI. The sequences for separate family were aligned using Multiple Sequence Alignment (MSA) program with clustalW. Subsequently, each MSA was subjected to MEGA6 for inferring phylogenetic tree. The average pathway method was used to calculate the branch length depicted in the number of variations all over the sequences. Resultantly, the most parsimonious tree was chosen by the close-neighbor-interchange algorithm by keeping bootstrap value of 1000 replication.

Geomapping and Cladistic Comparison of families

Geomapping of each family was done on physical world map. Later on occupied positions by species on the map were connected to infer their geographical pattern and parasitic diversity. Phylogenetic tree for each species were represented with clades/cluster so as to determine intra genus relationship and to strengthen geographical occurrence.

RESULTS

Construction of Phylogenetic Tree

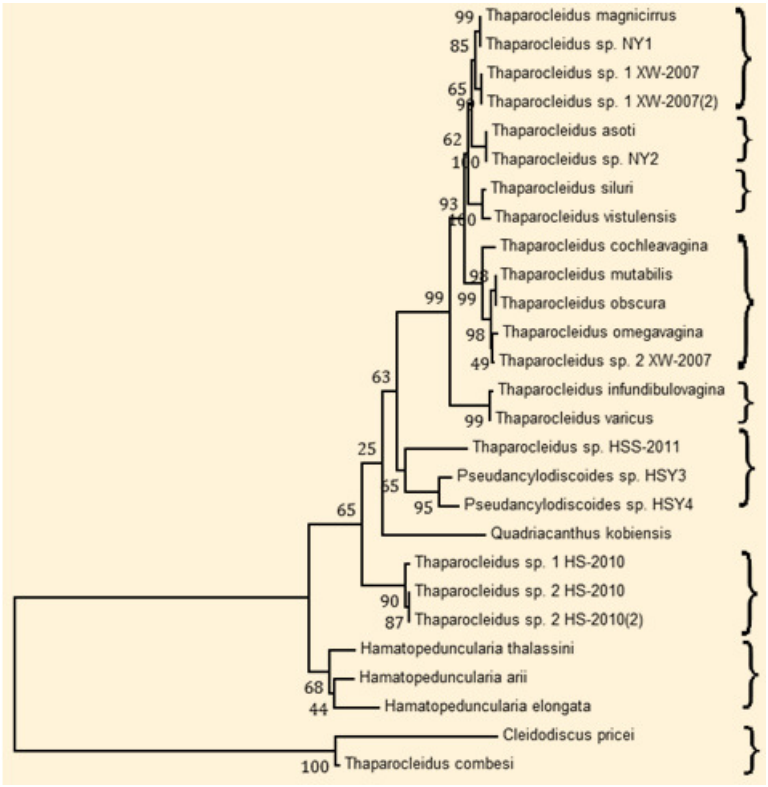
After MSA sequences were processed for tree construction, five trees were constructed using MEGA6 for each family (fig. 1). Number of species for each family in the phylogenetic tree varied due to unavailability of molecular (rDNA) data in NCBI. Later on trees were grouped into clades/cluster. Number of clades in each tree differed because number of species was not equal for all families. Possible error was minimised by focusing onto the geographical distribution of members into families and not clades (later section). Bootstrap values exhibited significant variations over branches and rendered to be 70% as standard value to significance.

The family Ancylodiscoididae (fig. 1 A) gave a total of nine cluster wherein, many sister clades were present. Evolutionarily, species followed distinctive root of diversity as shown by branch length of its phylogenetic tree. Although, members of this family are less in number, approximately 27, but formation of nine clades signifies that parasitic diversity has deep root so far as evolution is concerned. They have been evolving at a much faster rate than the members of other families in the study.

The family Ancyrocephalidae (fig. 1 B) with highest number of species formed highest number of clades that has been coincided with its length of phylogenetic tree. Family Cichlidogyridae (fig. 1 C) with 23 species had five clades that followed a conserved root of evolution. Family Polystomatidae and Monocotylidae (fig. 1 D & E) with 44 and 39 species respectively had showed equal number of clades, following almost adequate pattern of evolution.

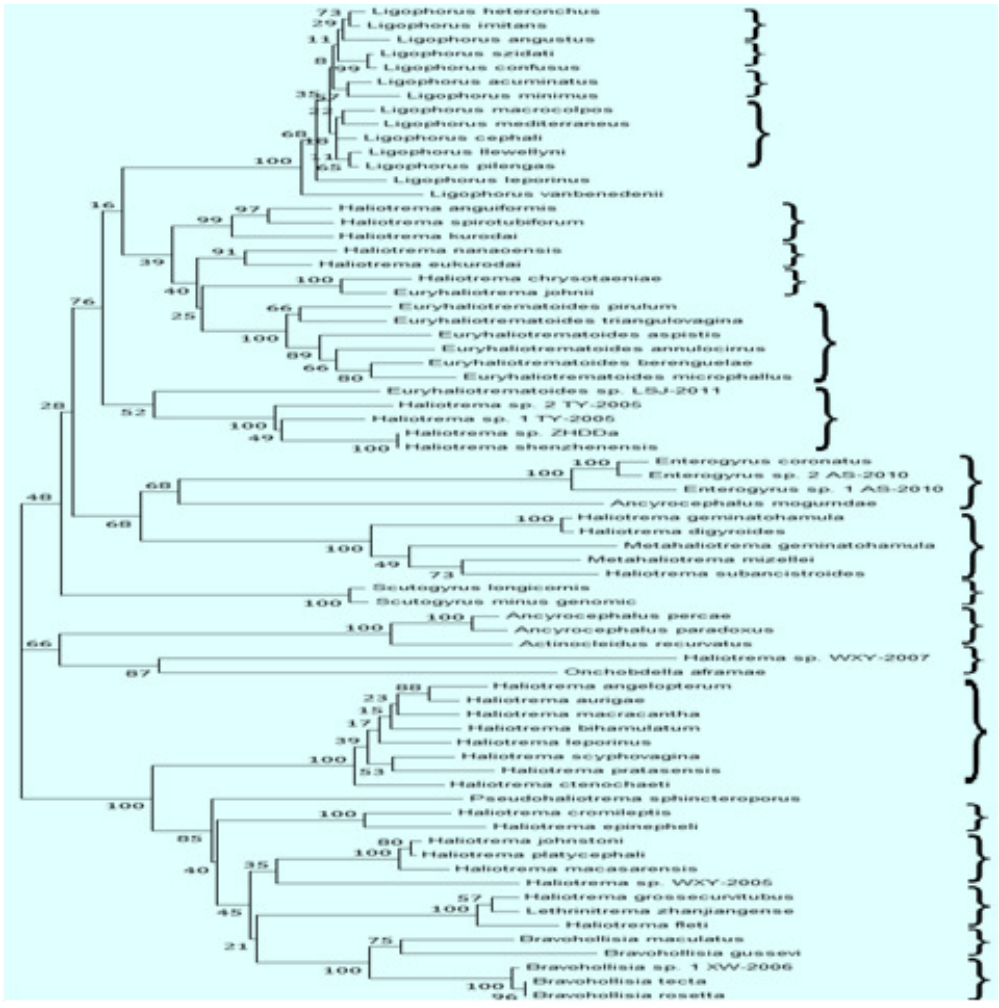
Clade versus Geomapping

Family distribution was not bound to a specific location except certain families. Ancyrocephalidae with highest number of species and clades found to be distributed in all sub-continents. This family was more related with Australian zones and less propagated in other zones. Phylogenetic patterns, although, did not reveal that which group of species was more prevalent still smaller number of clades reflected rapid pace of variability among members of this family. China in parallel to Australia displayed a thorough distribution along with Indo-west Pacific Ocean (fig. 2). Members of Ancyrocephalidae were distributed over all geographic zones including Africa, Europe, and North & South America (fig. 2). This was pretty agreeable to the pattern of formation of cluster in the phylogenetic tree but it had deviated from the number and geographical distribution that most of the species should not have been found in confined in the specific locations rather it should have been equally dispersed. Therefore, it has been confirmed that reason behind high number of cluster in the phylogenetic tree is



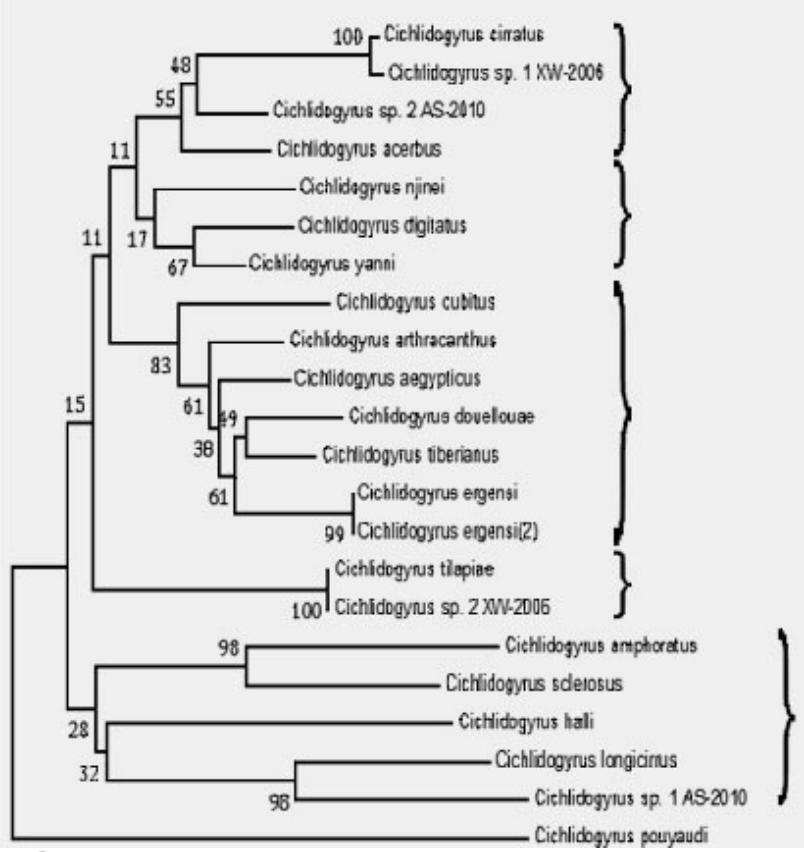
vast geographical distribution as environmental and ecological factors have caused over all changes in members including molecular and physiological variations. Clades presented in this study have shown that if more species fall in the same cluster then rate of diversification is less and if clades are formed with less species then rate of diversification is higher in that particular taxon. On the other hand, highly diversified taxon greatly distributed across the globe, expected to be found in all regions of the world. Majority of members of the family Ancylodiscoididae were falling into Indian water bodies and few of them were distributed into other sub-continent. Importantly, other aspect of the information could be comprehended as they have been widely found in Indian zones; they would have followed a route back to fresh water lineage. Their origin would have aroused through river systems and then turned into brackish and marine organism at lateral stage of evolution, resolving a clue

A

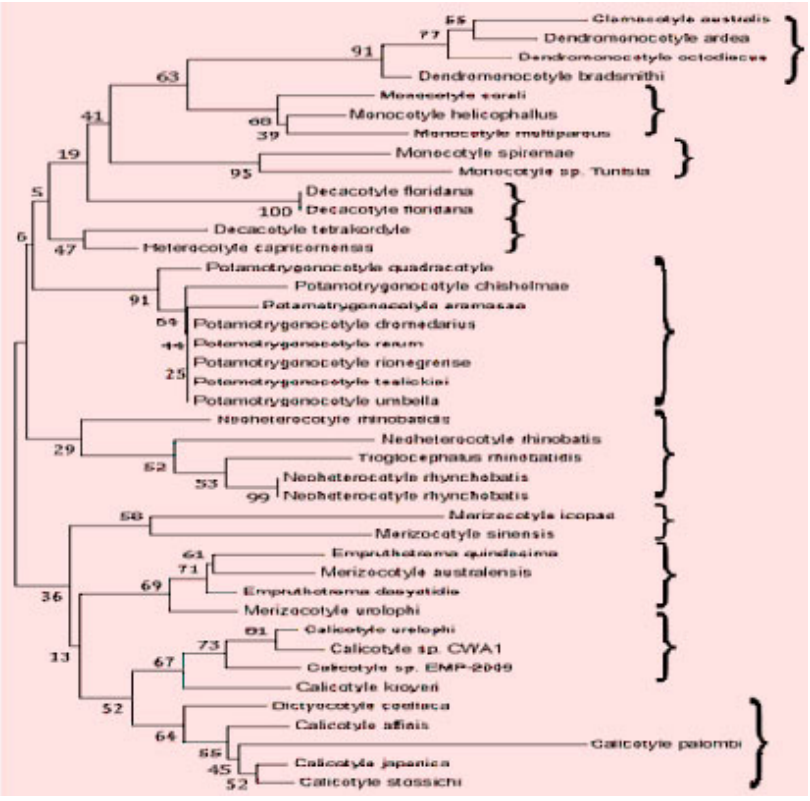


B

Fig. 1 continued...



C



D

Fig. 1 continued...

towards tracing of ancestral lineage and ancient history as well. Moreover, confinement in a particular location would decrease down the variability factor due to environmental and ecological constancy. Here number of clades did not matter efficaciously but prevalence did for Ancylodiscoididae. Most African and South African countries afforded the family Cichlidogyridae with least number of species in the study. According to the number of clades in its phylogenetic tree, distribution was shown to be normal. Out of 23 members only three from non African regions, showing a lesser variability among genus and good compatibility in molecular pattern of species. Reason behind lesser number of species in the family could be hypothesized by ecological and environmental features of a particular place. Besides this limited dispersion and geographical separation could have been one of the reasons leading to minor variability among members. Family Monocotylidae had a better coincidence between number of clades and geographical spots, it contained 11 clusters and distributed in all regions except China and Europe. Australian and American zones afforded more species than any other part. Out of these geographies, maximum members were confined to Australian regions representing a higher frequency of conservation as a group among all others. Although, clusters had varied a bit from dispersion but it totally depends upon number of species in a clade. Apart from Australian zones, North & South America regions also kept significant number of Monocotylidae along with North Atlantic Ocean. Even after confining in a specific location, species represent wide molecular pattern, signifying that all of the individuals in that particular region would show great variability in their nucleic acid composition. It has been supported by the distribution of families Monocotylidae and Ancyrocephalidae itself as both of them have been found in specific zones but molecularly represent higher diversity as far as evolutionary relatedness is

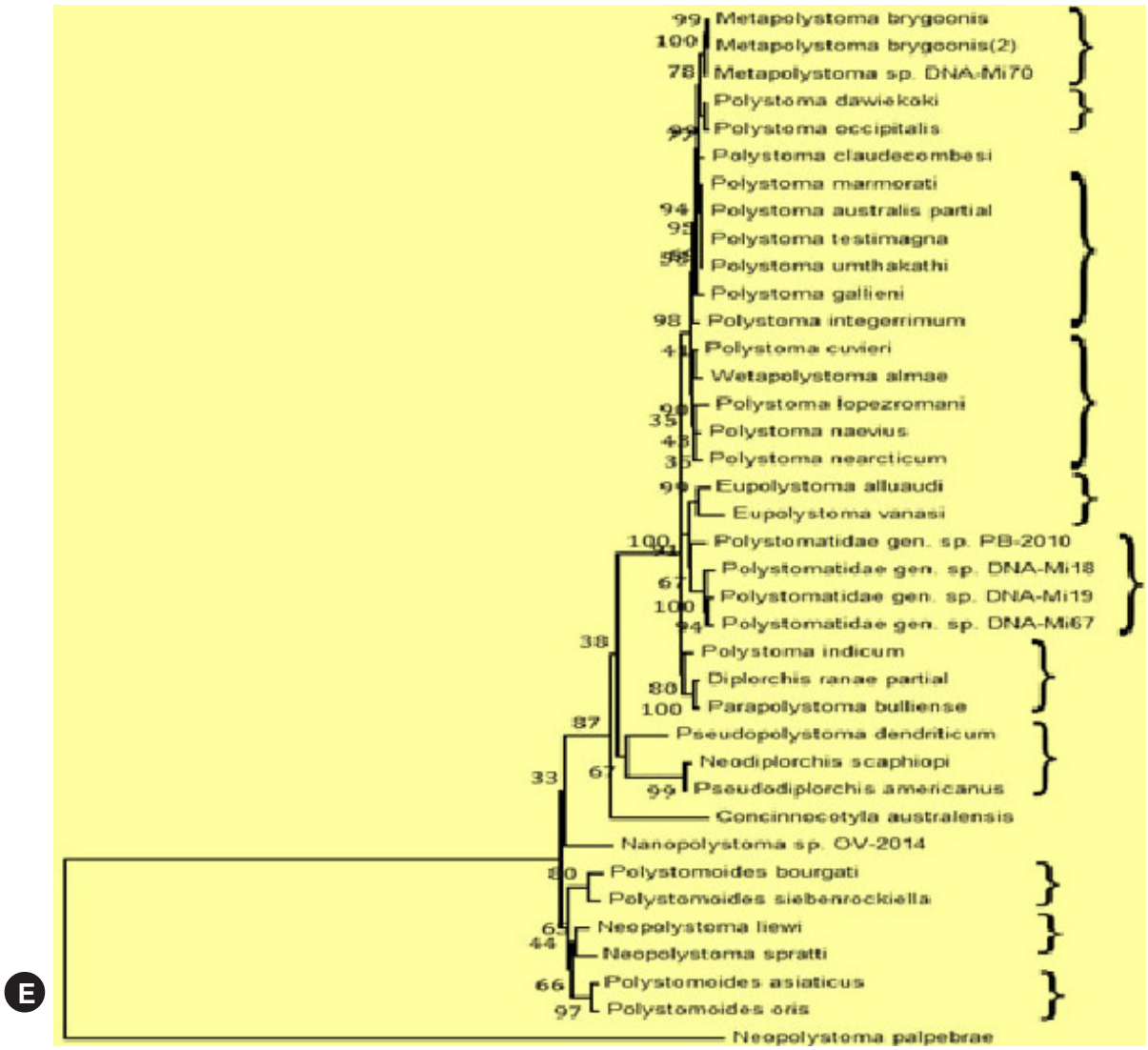


Fig. 1 : Phylogenetic tree representing of five different families-
A. Ancylodiscoididae : In all 27 species from 6 different genus studied; **B.** Ancyrocephalidae : In all 72 species from 12 different genus studied; **C.** Cichlidogyridae : In all 23 species from 1 genus studied; **D.** Polystomatidae : In all 44 species from 15 different genus studied & **E.** Monocotylidae : In all 39 species from 12 different genus studied.

concerned. Ecological and environmental elements would have definitely caused such anomalies in individuals. Therefore, it is not limited to Monogeneans only but other member from different class would face the same environmental attributes. Family Polystomatidae represented the best coincidence between number of clades and geographical patterns. Eleven clusters with 44 species were given to this family and their distribution came out to be equal in all regions of the world. No sub-continent was left unoccupied from Polystomatidae. Resultantly, such expression strengthen the fact about molecular conservation and parallel evolution and show that even after being exposed to various environmental and ecological conditions, individual were not much affected to the extent of totally different route of diversity and evolution. On the other hand they possess the magnificent tendency to conserve their molecular composition for a longer period of time.

DISCUSSION AND CONCLUSION

Among all family Ancyrocephalidae showed the greater prevalence followed by family Polystomatidae (fig. 2), confirming that these two families are the most diversified among others in the study. It was supported by cladistic analyses wherein species were clustered with two or three members. This finding coincides well with evolutionary relatedness among species of the same families that more the clades more the distribution/ diversification. Other families did not represent similar pattern of diversification as they showed conserved or confined origin to a specific location. Ancylodiscoididae

Table 2 : Summary of 227 species studied.

Family : Ancylodiscoididae		
Sl.	**Species**	**Location**
1.	*Hamatopeduncularia arii*	India
2.	*Hamatopeduncularia thalassini*	India
3.	*Hamatopeduncularia elongata*	India
4.	*Cleidodiscus pricei*	Lake Norman
5.	*Notopterodiscoides notopterus*	India
6.	*Pseudancylodiscoides* sp. HSY3	India
7.	*Pseudancylodiscoides* sp. HSY4	India
8.	*Quadriacanthus kobiensis*	India
9.	*Thaparocleidus asoti*	India
10.	*Thaparocleidus caecus*	Southeast Asia
11.	*Thaparocleidus cochleavagina*	India
12.	*Thaparocleidus combesi*	India
13.	*Thaparocleidus infundibulovagina*	India
14.	*Thaparocleidus magnicirrus*	India
15.	*Thaparocleidus mutabilis*	India
16.	*Thaparocleidus obscura*	India
17.	*Thaparocleidus omegavagina*	India
18.	*Thaparocleidus siluri*	India
19.	*Thaparocleidus varicus*	India
20.	*Thaparocleidus vistulensis*	India
21.	*Thaparocleidus sp. 1 HS-2010*	India
22.	*Thaparocleidus sp. 1 XW-2007*	India
23.	*Thaparocleidus sp. 2 HS-2010*	India
24.	*Thaparocleidus sp. 2 XW-2007*	India
25.	*Thaparocleidus sp. HSS-2011*	India
26.	*Thaparocleidus sp. NY1*	India
27.	*Thaparocleidus sp. NY2*	India
28.	*Hamatopeduncu laria arii*	India
29.	*Hamatopeduncularia thalassini*	India
Family: Ancyrocephalidae		
30.	*Actinocleidus recurvatus*	Canada
31.	*Ancyrocephalus mogurndae*	China
32.	*Ancyrocephalus paradoxus*	Kurish Gulf
33.	*Ancyrocephalus percae*	Germany
34.	*Bravohollisia tecta*	Hainan
35.	*Bravohollisia gussevi*	Sungai Buloh
36.	*Bravohollisia* sp. Malaysia	Malaysia
37.	*Bravohollisia maculates*	China
38.	*Bravohollisia rosetta*	Sungai Buloh
39.	*Bravohollisia* sp. 1 XW-2006	Malaysia
40.	*Enterogyrus coronatus*	Senegal
41.	*Enterogyrus* sp. 1 AS-2010	Senegal
42.	*Enterogyrus* sp. 2 AS-2010	Senegal
43.	*Euryhaliotrema annulocirrus*	I-W P. Ocean
44	*Euryhaliotrema mehen*	I-W P. Ocean
45.	*Euryhaliotrema aspistis*	I-W P. Ocean
46.	*Euryhaliotrema berenguelae*	I-W P. Ocean
47.	*Euryhaliotrema johni*	I-W P. Ocean
48.	*Euryhaliotrema microphallus*	I-W P. Ocean
49.	*Euryhaliotrema pirulum*	I-W P. Ocean
50.	*Euryhaliotrema triangulovagina*	I-W P. Ocean
51.	*Euryhaliotrema* sp. LSJ-2011	I-W P. Ocean

Table 2 continued...

Table 2 continued...

52.	*Haliotrema angelopterum*	I-W Islands
53.	*Haliotrema aurigae*	S W Parite
54.	*Haliotrema bihamulatum*	China
55.	*Haliotrema chrysotaeniae*	Brazil
56.	*Haliotrema cromileptis*	Australia
57.	*Haliotrema ctenochaeti*	China
58.	*Haliotrema digyroides*	China
59.	*Haliotrema epinepheli*	Australia
60.	*Haliotrema fleti*	Australia
61.	*Haliotrema geminatohamula*	Australia
62.	*Haliotrema grossecurvitubus*	China
63.	*Haliotrema johnstoni*	Australia
64.	*Haliotrema kurodai*	Australia
65.	*Haliotrema leporinus*	South China
67.	*Haliotrema macasarensis*	China
68.	*Haliotrema macracantha*	N. Caledonia
69.	*Haliotrema nanaoensis*	Australia
70.	*Haliotrema platycephali*	Australia
71.	*Haliotrema pratasensis*	South China
72.	*Haliotrema scyphovagina*	I-W P. Ocean
73.	*Haliotrema shenzhenensis*	South China
74.	*Haliotrema spirotubiforum*	Red Sea
75.	*Haliotrema subancistroides*	Red Sea
76.	*Haliotrema sp. 1 TY-2005*	Red Sea
77.	*Haliotrema sp. 2 TY-2005*	Red Sea
78.	*Haliotrema sp. WXY-2005*	Australia
79.	*Haliotrema sp. WXY-2007*	Australia
80.	*Haliotrema sp. ZHDDa*	Australia
81.	*Lethrinitrema zhanjiangense*	S. China Sea
82.	*Ligophorus acuminatus*	Spain
83.	*Ligophorus angustus*	Spain
84.	*Ligophorus cephalic*	Spain
85.	*Ligophorus confuses*	Spain
86.	*Ligophorus heteronchus*	Spain
87.	*Ligophorus imitans*n	Spain
88.	*Ligophorus leporinus*	China
89.	*Ligophorus llewellyni*	Sea of Azov
90.	*Ligophorus macrocolpos*	Spain
91.	*Ligophorus mediterraneus*	Spain
92.	*Ligophorus minimus*	Spain
93.	*Ligophorus pilengas*	Sea of Azov
94.	*Ligophorus szidati*	Sea of Azov
95.	*Ligophorus vanbenedenii*	Sea of Azov
96.	*Metahaliotrema geminatohamula*	S. Brazil
97.	*Metahaliotrema Mizellei*	China
98.	*Onchobdella atramae*	Africa
99.	*Pseudohaliotrema Sphincteroporus*	Australia
100.	*Scutogyrus longicornis*	Africa
101.	*Scutogyrus minus*	Africa
Family: Cichlidogyridae		
102.	*Cichlidogyrus amphoratus*	Africa
103.	*Cichlidogyrus falcifer*	Africa
104.	*Cichlidogyrus sclerosus*	Uganda
105.	*Cichlidogyrus sp. 1 AS-2010*	

Table 2 continued...

Table 2 continued...

106.	*Cichlidogyrus* sp. 1 XW-2006	
107.	*Cichlidogyrus* sp. 2 AS-2010	
108.	*Cichlidogyrus* sp. 2 XW-2006	
109.	*Cichlidogyrus amphoratus*	Africa
110.	*Cichlidogyrus acerbus*	Africa
111.	*Cichlidogyrus aegypticus*	Africa
112.	*Cichlidogyrus digitatus*	Africa
113.	*Cichlidogyrus acerbus*	Africa
114.	*Cichlidogyrus aegypticus*	Africa
115.	*Cichlidogyrus arthracanthus*	Africa
116.	*Cichlidogyrus arthracanthus*	Africa
117.	*Cichlidogyrus cubitus*	Benin
118.	*Cichlidogyrus ergensi*	Benin
119.	*Cichlidogyrus cubitus*	Benin
120.	*Cichlidogyrus njinei*	Cameroon
121.	*Cichlidogyrus cirratus*	Israel
122.	*Cichlidogyrus cirratus*	Israel
123.	*Cichlidogyrus tiberianus*	Israel
124.	*Cichlidogyrus pouyaudi*	Kogon River
125.	*Cichlidogyrus yanni*	Kogon
126.	*Cichlidogyrus douellouae*	Mékrou Rive
127.	*Cichlidogyrus halli*	Phongolo
128.	*Cichlidogyrus tilapiae*	South Africa
129.	*Cichlidogyrus longicirrus*	Ghana
	Family: Monocotylidae	
130.	*Caliocotyle affinis*	N. A. Ocean
131.	*Caliocotyle japonica*	Japan
132.	*Caliocotyle kroyeri*	Mexico
133.	*Caliocotyle palombi*	N. A. Ocean
134.	*Caliocotyle stossichi*	Mexico
135.	*Caliocotyle urolophi*	Australia
136.	*Caliocotyle sp. CWA1*	
137.	*Caliocotyle sp. EMP*	
138.	*Clemacotyle australis*	Australia
139.	*Decacotyle floridana*	Mexico
140.	*Decacotyle tetrakordyle*	Australia
141.	*Dendrcocotyle ardea*	Australia
142.	*Dendrcocotyle bradsmithi*	Australia
143.	*Dendrcocotyle octodiscus*	N. A. Ocean
144.	*Dictyocotyle coeliaca*	N. A Ocean
145.	*Empruthotrema dasyatidis*	Queensland
146.	*Empruthotrema quindecima*	Australia
147.	*Heterocotyle capricornensis*	Australia
148.	*Merizocotyle australensis*	Australia
149.	*Merizocotyle icopae*	Australia
150.	*Merizocotyle sinensis*	Taiwan
151.	*Merizocotyle urolophi*	Tasmania
152.	*Monocotyle corali*	Australia
153.	*Monocotyle helicophallus*	Australia
154.	*Monocotyle multiparous*	Australia
155.	*Monocotyle spiremae*	Australia
156.	*Monocotyle sp. Tunisia*	Tunisia
157.	*Neoheterocotyle hinobatidis*	Australia
158.	*Neoheterocotyle rhinobatis*	Australia

Table 2 continued...

Table 2 continued...

159.	*Neoheterocotyle rhynchobatis*	Australia
160.	*Potamotrygonocotyle aramasae*	Brazil
161.	*Potamotrygonocotyle chisholmae*	River basin (USA)
162.	*Potamotrygonocotyle dromedarius*	Brazil
163.	*Potamotrygonocotyle quadracotyle*	Brazil
164.	*Potamotrygonocotyle rarum*	Brazil
165.	*Potamotrygonocotyle rionegrense*	Brazil
166.	*Potamotrygonocotyle tsalickisi*	River basin (USA)
167.	*Potamotrygonocotyle umbella*	Brazil
168.	*Trogocephalus rhinobatidis*	Australia
	Family: Polystomatidae	
169.	*Diplorchis ranae*	
170.	*Madapolystoma sp. DNA-Mi18*	
171.	*Madapolystoma sp. DNA-Mi19*	
172.	*Madapolystoma sp. DNA-Mi67*	
173.	*Metapolystoma sp. DNA-Mi70*	
174.	*Nanopolystoma sp. OV-2014*	
175.	*Neodiplorchis scaphiopi*	
176.	*Polystomoides oris*	
177.	*Polystomatidae gen. sp. PB-2010*	
178.	*Diplorchis ranae*	
179.	*Polystomoides asiaticus*	Africa
180.	*Polystoma claudecombesi*	Africa
181.	*Polystoma dawiekoki*	Africa
182.	*Concinnocotyla australensis*	Australia
183.	*Neopolystoma palpebrae*	Australia
184.	*Concinnocotyla australensis*	Australia
185.	*Polystoma integerrimum*	Europe
186.	*Polystoma indicum*	India
187.	*Polystoma occipitalis*	Ivory Cost
188.	*Pseudopolystoma dendriticum*	Japan
189.	*Metapolystoma cachani*	Madagascar
190.	*Metapolystoma brygoonis*	Malagasy
191.	*Diplorchis ranae*	Africa
192.	*Madapolystoma sp. DNA-Mi18*	Africa
193.	*Madapolystoma sp. DNA-Mi19*	Africa
194.	*Madapolystoma sp. DNA-Mi67*	Australia
195.	*Metapolystoma sp. DNA-Mi70*	Australia
196.	*Nanopolystoma sp. OV-2014*	Australia
197.	*Neodiplorchis scaphiopi*	Europe
198.	*Polystomoides oris*	India
199.	*Neopolystoma spratti*	Malaysia
200.	*Neopolystoma liewi*	Malaysia
201.	*Polystomoides siebenrockiella*	Malaysia
202.	*Polystoma naevius*	Mexico
203.	*Polystoma gallieni*	Morocco
204.	*Polystomoides bourgati*	Nigeria
205.	*Parapolystoma bulliense*	Northern Queenland
206.	*Neopolystoma orbiculare*	Palaearctic region
207.	*Polystoma cuvieri*	Paraguay
208.	*Polystoma lopezromani*	Paraguay
209.	*Eupolystoma vanasi*	South Africa
210.	*Polystoma australis*	South Africa
211.	*Polystoma marmorati*	South Africa

Table 2 continued...

Table 2 continued...

212.	*Polystoma testimagna*	South Africa
213.	*Polystoma umthakathi*	South Africa
214.	*Eupolystoma alluaudi*	Togo
215.	*Wetapolystoma almae*	Tropical Peru
216.	*Pseudodiplorchis americanus*	USA
217.	*Polystoma nearcticum*	USA
218.	*Neopolystoma spratti*	Malaysia
219.	*Neopolystoma liewi*	Malaysia

Table 2 continued...

220.	*Polystomoides siebenrockiella*	Malaysia
221.	*Polystoma naevius*	Mexico
222.	*Polystoma gallieni*	Morocco
223.	*Polystomoides bourgati*	Nigeria
224.	*Parapolystoma bulliense*	NorthernQueenland
225.	*Neopolystoma orbiculare*	Palaearctic region
226.	*Polystoma cuvieri*	Paraguay
227.	*Polystoma lopezromani*	Paraguay

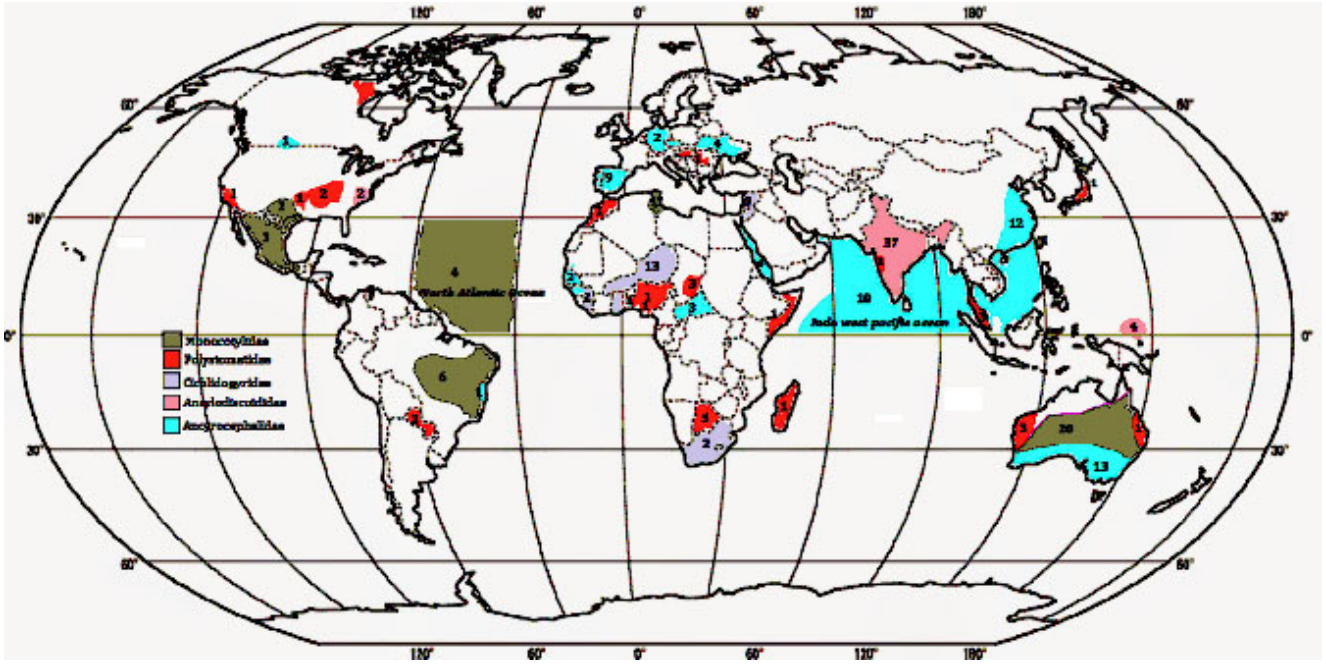


Fig. 2 : Geomapping of species from five major families (numbers representing number of species in the respective region).

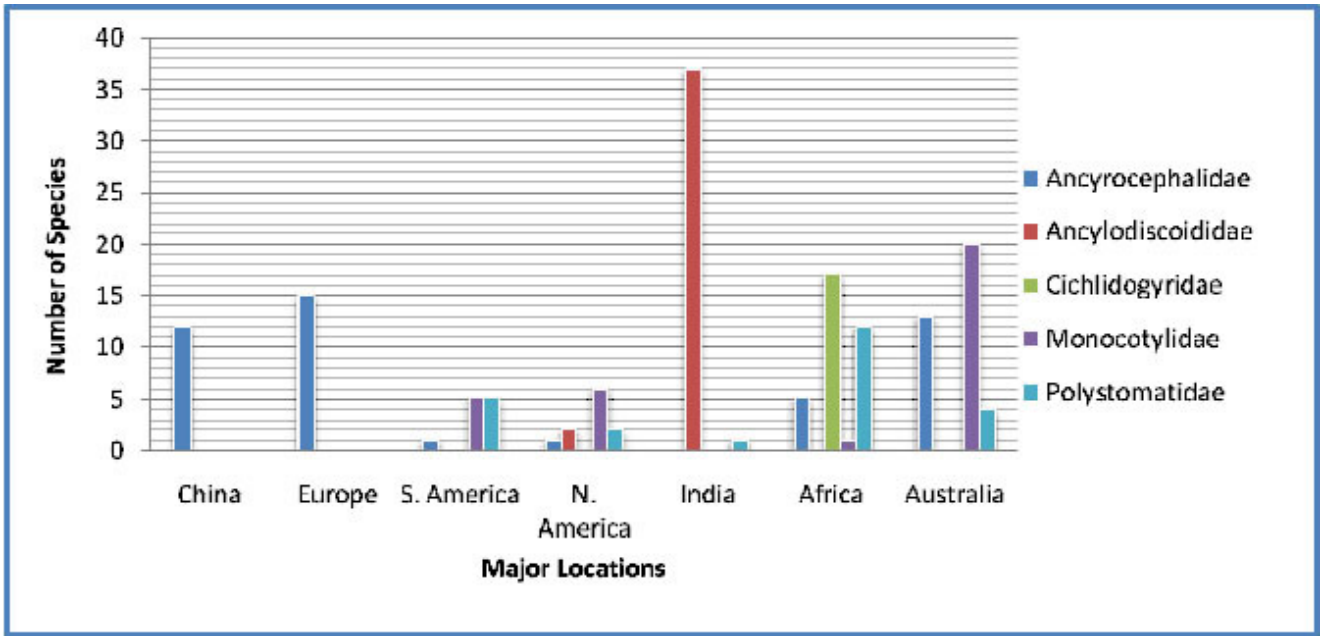


Fig. 3 : Families showing zoogeographical distribution of selected five families in major zones of the world.

and Cichlidogyridae represented significant level of conservation being confirmed by both geomapping and clustering as well. Another aspect of this conservatory point could be accounted as the robustness of the species, genus or families as they possessed the potential to confront the changing environmental and ecological conditions. This finding provides a range of enumerations that how species went prevalent into specific geographical zones of the world and what was the amount of change that caused their migration to other corner of the globe. Monogeneans have versatile nature to switch from one place to another and rapidly change morphology and become adapted, suggesting that families are specific to their member species and allow evolving when exposed to suitable environmental conditions.

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REVIEW ARTICLE

REVISITING DIVERSITY AND GEOGRAPHICAL DISTRIBUTION OF EIGHT MINOR FAMILIES *VIZ.,* ANOPLODISCIDAE, AXINIDAE, CAPSALIDAE, CICHLIDOGYRIDAE, HETERAXINIDAE, HEXABOTHRIIDAE, BOTHITREMATIDAE AND TETRAONCHOIDAE OF CLASS MONOGENEA

[1]Fozail Ahmad, [2]Sharma, C., [1]Aggarwal, V.P. and [1*]Arya, P.V.

[1]Department of Zoology, Dyal Singh College (University of Delhi), New Delhi-110003, India
[2]Department of Zoology, I.P. College, Bulandshar-203001 (UP), India

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ABSTRACT

Members of class monogenea are widely distributed all over the world in diverse ecosystems. Based on their relatedness they are assigned to respective families. Based on global diversity majority of representation comes from some main or major families. These major families accounted for most of the diversity of the class around the globe. Most of the contemporary study revolves around major families dealing various aspects including taxonomic explorations or molecular explorations. Present investigations is an attempt to revisit diversity and geographical distribution of few minor families of this class which are somewhat ignored in major contemporary studies. Based on global representation and species diversity eight minor families *viz.,* Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae were selected for the further investigation. A systematic effort was made towards understanding diversity, distribution and milestone chronology of the family

KEY WORDS: Monogenea, Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae.

INTRODUCTION

Monogeneans are widely distributed all over the world and are represented as the most dominating forms of helminth group parasitizing the external surface of fish. Monogeneans represent a diverse group with several thousand species recorded in many database, books and various literatures (Rohde, 1976; Pandey and Aggarwal, 2008). The class is diverse, not only in terms of number of species but in morphology, ecology, adaptation and host switching. Monogenea are the only class among the parasitic flatworms to have undergone an adaptive radiation (Brooks and McLennan, 1993). Due to radial diversification they seem to have developed a large number of species. Moreover, this diversification has caused them to expand and colonize the internal as well as external organ of amphibians and fishes. In their life cycle, Monogeneans also represent alternation of generation and are hermaphrodite that makes them to have a direct life cycle. Due to such an alternating life strategies and adaptations to parasitic life, they have been regarded as very successful parasites. Monogeneans comprise two very distinct groups, the Monopisthocotylea and Polyopisthocotylea. The two groups differ considerably, with important implications for morphology, mode of infection, pathogenicity, treatment and host response. Three major Monogenean families were recently studied in details mainly for their prevalence, rich diversity, versatile ecological behavior and multiple forms of evolution (Fozail *et al.,* 2015a). In order to elaborate the evolutionary aspect, in addition to origin and ecological situations, species need to be accounted for totality and existence in various geographical zones.

Corresponding author:* ***Arya, P.V.*
Department of Zoology, Dyal Singh College (University of Delhi), New Delhi-110003, Idnai.

Since, monogeneans are widespread across globe, each geographical zone have been occupied by their occurrence that provide easy platform to explore diversity of parasites (Fozail *et al.*, 2015b). A particular environment definitely impacts over the survivability of individual and prompt to adapt the present condition. Each geographical zones possess a characteristic features wherein species get to adapt a specific and particular mode of survival. Adaptation can be regarded as the change in morphology, genetic composition and extent of parasitism.

Almost all monogeneans comprise such versatile nature. In many cases, species get to extinct due to unfavorable ecological conditions and many a times it vanishes from a particular region. In contrast to these situations, monogeneans manage to survive even if they are forced to change their specific host. The widespread prevalence of monogenean species indicates that most of parasite families are resilient to the changing environment and may exist in varying ecosystem. In the present work we have summarized the minor monogenean families with a focus on description of geographical distribution, their discoveries, identification and diversity.

MATERIALS AND METHODS

Minor families of the class were selected for the study based on quantification of diversity of genus in the family. Anoplodiscidae, Axinidae, Capsalidae, Cichlidogyridae, Heteraxinidae, Hexabothriidae, Bothitrematidae and Tetraonchoidae were identified as less studied members of the class monogenea having lesser number of corresponding genera and species. These families are accounted in terms of validity, host specificity and diversity.

Family Anoplodiscidae

One of the monotypic families, Anoplodiscidae (Tagliani, 1912) of monogenean parasite occurs on sparid fishes from Australian, Mediterranean and Japanese waters. The only genus of this family *Anoplodiscus* (Sonsino, 1890) accommodates the only species *Anoplodiscus cirrusspirali* collected from Mediterranean Sea. Four more species were described over the period of time, identified as *A. australis* (Johnston, 1930), *A. spari* (Yamaguti, 1958), *A. cirruspiralis* (Roubal, Armitage and Rohde, 1983) and *A. tai* (Ogawa, 1994). During a survey of marine fishes in Brazil, Ogawa and Egusa (1981) exclusively studied the systematic position of the genus and provided validation of *A. australis* and *A. spari* from the host *P. pargus*.

Family Axinidae

First described by Monticelli (1903). The family is represented by four genera and seven species; *Allopseudaxine katsuwonis* (Ishii, 1936) Yamaguti, 1943 on *Katsuwonus pelamis* (Linnaeus) (gills) from Arecibo (Williams and Bunkley-Williams 1996), *Axine yamagutii* (Meserve, 1938) form open sea off coast (Meserve 1938), Mexico, *Axinoides jimenezi* (Caballero & Bravo-Hollis, 1969) on *Tylosurus crocodilus* (Caballero and Bravo-Hollis 1969), Mexico, *Axinoides oceanicum* (Caballero, Bravo-Hollis & Grocott, 1953) on *Tylosurus crocodilus* from Oceano Pacifico del Norte (Caballero *et al.,* 1953), Panama, *Axinoides raphidoma* (Hargis, 1956) on *Tylosurus crocodilus* (Caballero and Bravo-Hollis 1969), Mexico, *Chlamydaxine resplendens* (Caballero, Bravo-Hollis & Grocott, 1954) on *Tylosurus crocodilus* from Oceano Pacifico del Norte (Caballero *et al.,* 1954), Panama and *Oligapta kruidenieri* (Crane, Kritsky & Kayton, 1979) on *Thyrinops pachylepis* (Crane *et al.,* 1979), El Salvador.

Family Capsalidae

The first described Capsalid by Muller (1776) was *Entobdella hippoglossi* from the skin of *Hippoglossus hippoglossus.* Presently Capsalidae comprises approximately 200 described species in 9 subfamilies and 45 genera. Elasmobranchs, teleosts and primitive sturgeons are identified as the host of identified species. Some of them can affect host fishes due to their direct life cycle. Few of them are found to be adversely affecting their host in aquaculture and are even causing epizootic events, whereas some are among the largest monogeneans, concealing onto the host. Paradoxically one of the species is the most studied and known of all parasites. Graham Kearn (1998) represented a very meticulous report on the life of *Entobdella soleae* from the skin of *Solea solea* in Europe. In fact, more can be known about *E. Soleae* than any other monogenean (life cycle, migration, geographical distribution, host specificity etc.) as the species is represented as a typical parasitic flatworm. In contrast to *E. Soleae*, *Neobenedenia melleni* is very infamous in infecting number of teleost species in aquaculture. As it known that most of the monogenean species show legendary feature for their strict-host specificity, but *Neobenedenia melleni* is famous for the broadest host-specificity of any monogenean parasite; recorded from more than 95 species in more than 32 families from 5 order of wild and captive teleost. One of the legendary species of capsalids is known to be the *Benedenia seriolae*, a long standing parasite *Seriola* species in Japan.

This species may occur anywhere in the world. The family Capsalid comprises several members that claim to fame within the monogenean diversity; the first of it, camouflage to conceal, longest host range etc. This family also possesses the longest generic names courtesy of Yamaguti (1966) *Lagenivagino pseudobenedenia.*

Family Cichlidogyridae

Cichlidogyridae occur in West Africa, Madagascar, Asia and Neotropics. African species of Cichlids harbor monogenean parasites representing only those of *Cichlidogyrus* Paperna (1960), *Scutogyrus* Pariselli and Euzet (1995), *Onchobdella* Paperna (1968), and *Gyrodactylus* Nordmann (1832) are found on the gills of these fishes. Among these the genus *Cichlidogyrus* represents the most diverse group with 85 nominal species recorded from 75 host species. This genus also displays species richness ranging from 1 to 22 species per host species. The host-specificity of this family is also very different in terms of infecting single host that accounts for 50 members of them to be *oioxenous* and 35 members are accounted for being *stenoxenous* (infesting two or more host species). These features of members in the family had provided that, after performing phylogenetic analyses, their specificity was greater than was initially supposed and thus present diversity of monogenean species parasitizing explained just because of existence of cryptic species.

Family Heteraxinidae

Identified by Price (1962) this family has the smallest number of member as one species, *Cemocotyle trachuri* from a single genus *Cemocotyle*. During a study of monogenean parasite from the Swan River Estuary, a large collection of parasite of related family Microcotylidae was made. There found to be a close resemblance between Heteraxinidae and Microcotylidae and thus collected parasites were placed in later one. Most of the work has underestimated this family.

Family Hexabothriidae

The first hexabothrid was discovered by Kuhn (1829), over 70 species have been identified from almost as many host species. The Hexabothriidae Price (1942) comprises of polyopisthocotylean members exclusively parasitic on the gills of chondrichthyan fishes. At present, taxonomy of the family is in a state of convulsion; Kristky and Boeger (1989) have gone through only comprehensive revision and recognized 13 genera with few suspected species for recognition. It has been difficult to determine species relationship on the basis of selection of appropriate characters for the family, it further adds on to make proper classification much more tedious.

Family Bothitrematidae and Tetraonchoidae

Identified by Bychowsky (1957), Bothitrematidae comprises of only one species, *Bothitrema bothi* MacCallum, 1913. Previously, Bothitrematidae was considered a super family of Dactylogyridae Yamaguti (1963). Later concurrence with Bychowsky and his associates placed the family into Tetraonchoidae as both families share close similarities. This super family Tetraonchoidae includes genera *Paratetraonchoides* and *Pseudotetraonchoides,* Bychowsky (1965), *Tetraonchoididae*.

DISCUSSION

The eight families are revisited in the paper provided a scenario of all families of monogenean parasite wherein observation over minor families may be elaborated in context of geographical distribution. All families equally, by means of geographical distribution showing a lower degree of occurrence in a particular area. As per the high density of species in a specific area is concerned, it is the family Cichlidogyridae that strictly occur in South Africa, and with small number in Madagascar. We have mentioned in the previous work that richness of a particular member from a particular area (geographical area/location) is an indication of its origin. And definitely, taxonomic and phylogenetic status, from across the globe fall into the same geographical zone, confirming their classification into the updated record. More detail molecular investigation is required to establish relative evolutionary linkage/lineage of these families. This study may give a motivation to take up detailed molecular investigation for establishing relative evolutionary tree for all the members in the class.

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