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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

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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

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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*.

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 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).

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).

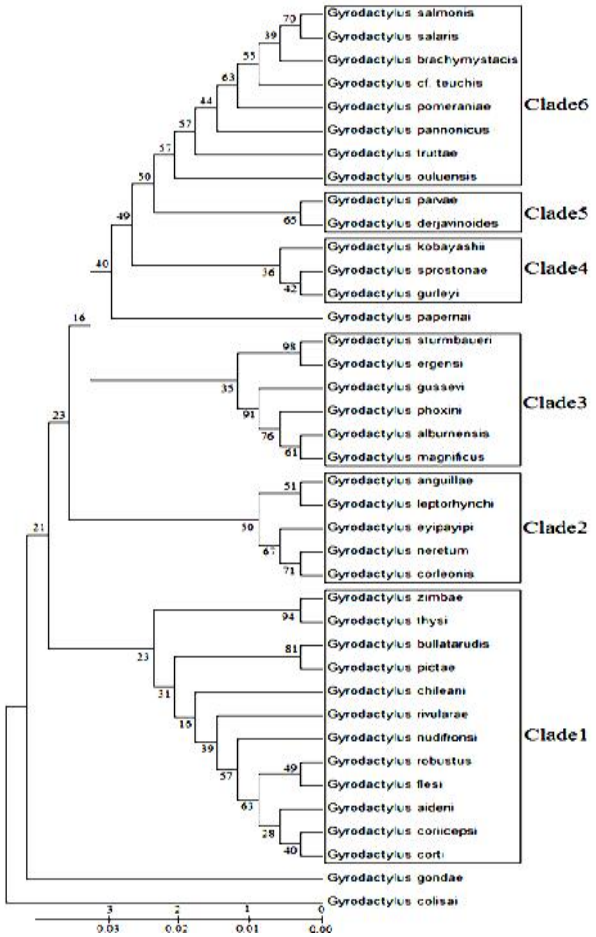

Figure 1 Phylogenetic tree (Neighbor joining) using 28S rRNA sequences for the 39 species of genus *Gyrodactylus.*

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.

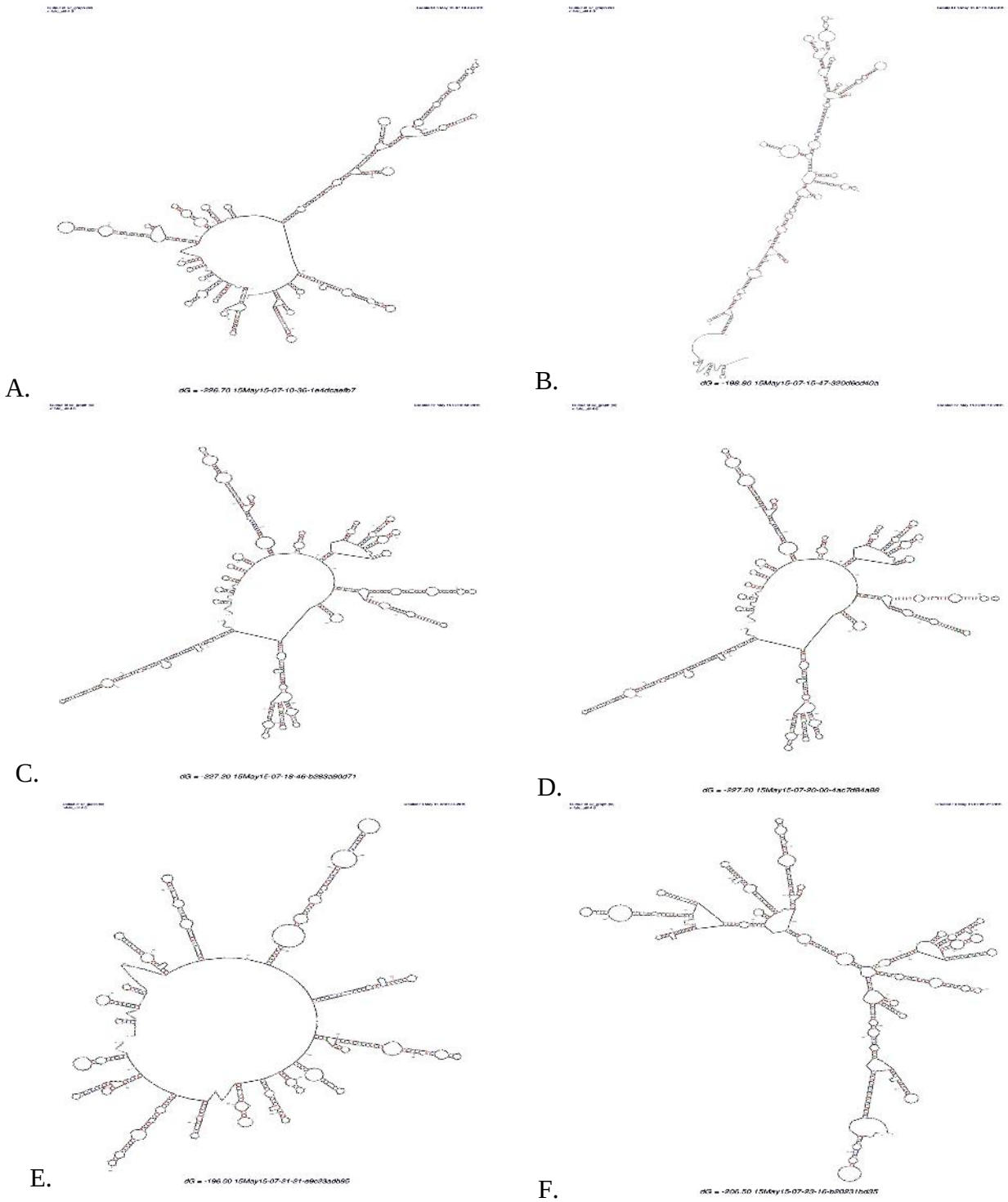

Figure 2 28S rRNA Secondary structure of **A.** *G. alburnensis*, **B.** *G. pictae*, **C.** *G. corti*, **D.** *G. stumbaeuri*, **E.** *G. corleonis*, **F.** *G. truttae*

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.

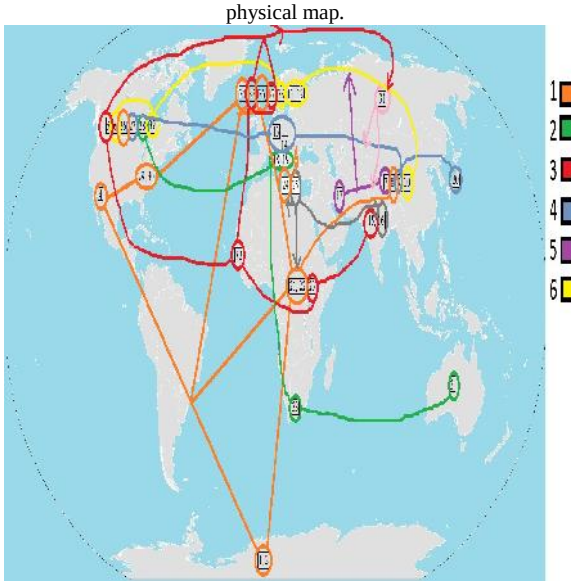

Fig.4 Geo mapping of selected species of genus *Gyrodactylus* and clade connectivity. Each number representing respective clade.

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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