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RESEARCH ARTICLE

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

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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

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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]

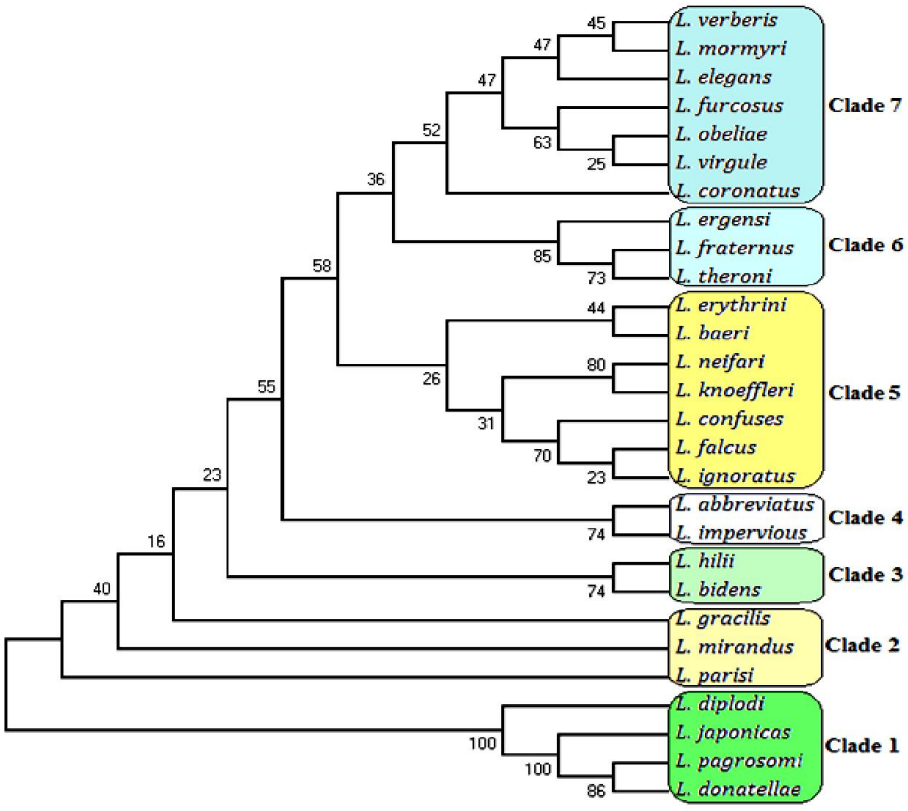

Fig. 1. Phylogenetic tree of 28 species of the genus *Lamellodiscus*, constructed using NJ method

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.

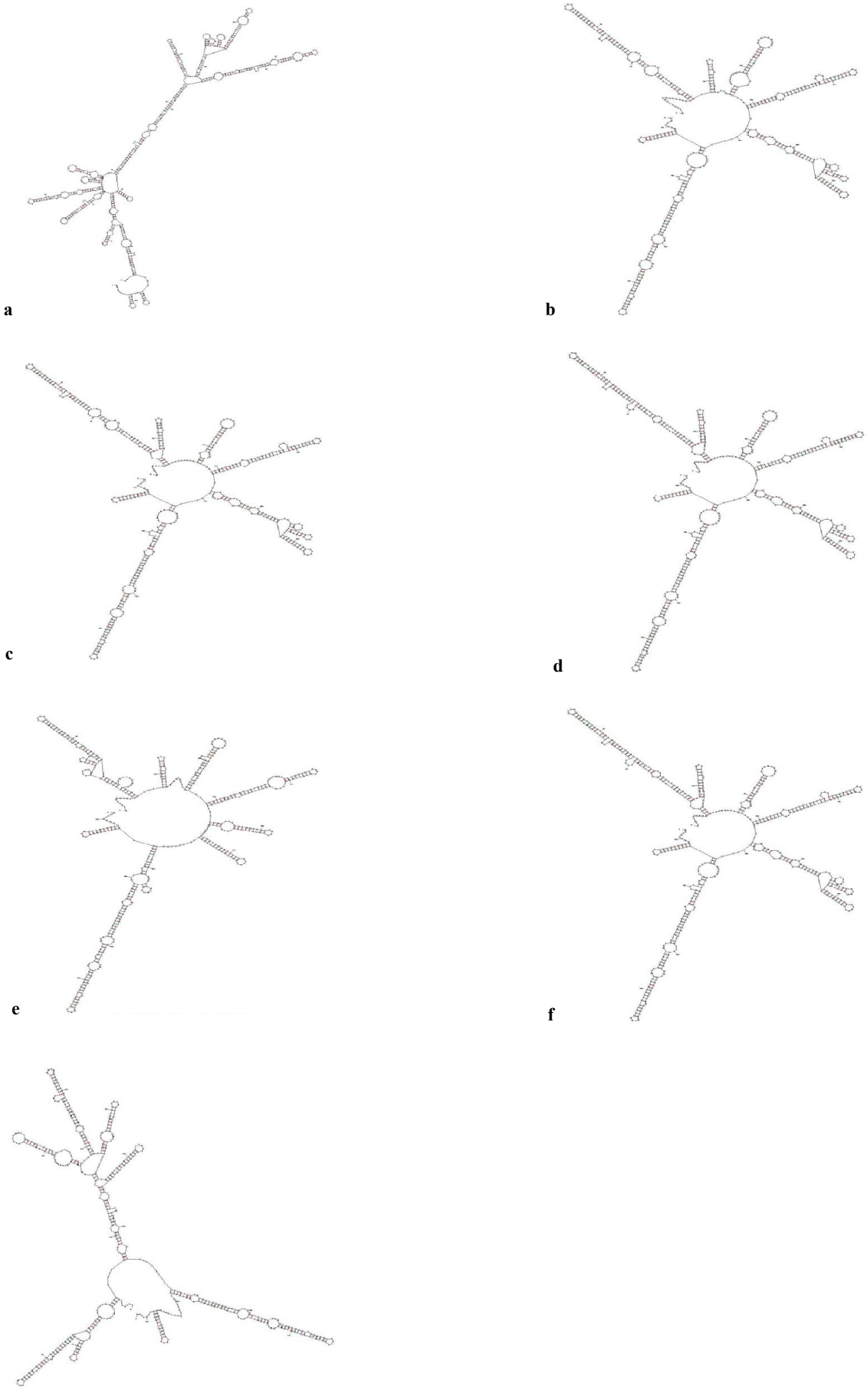

Fig. 2. Secondary structure of 7 representative 18S rRNAs from seven clades, a. *L. japonicas, b. L. mirandus, c. L. bidens, d. L. impervious, e. L. baeri, f. L. fraternus, L. mormyri*

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.

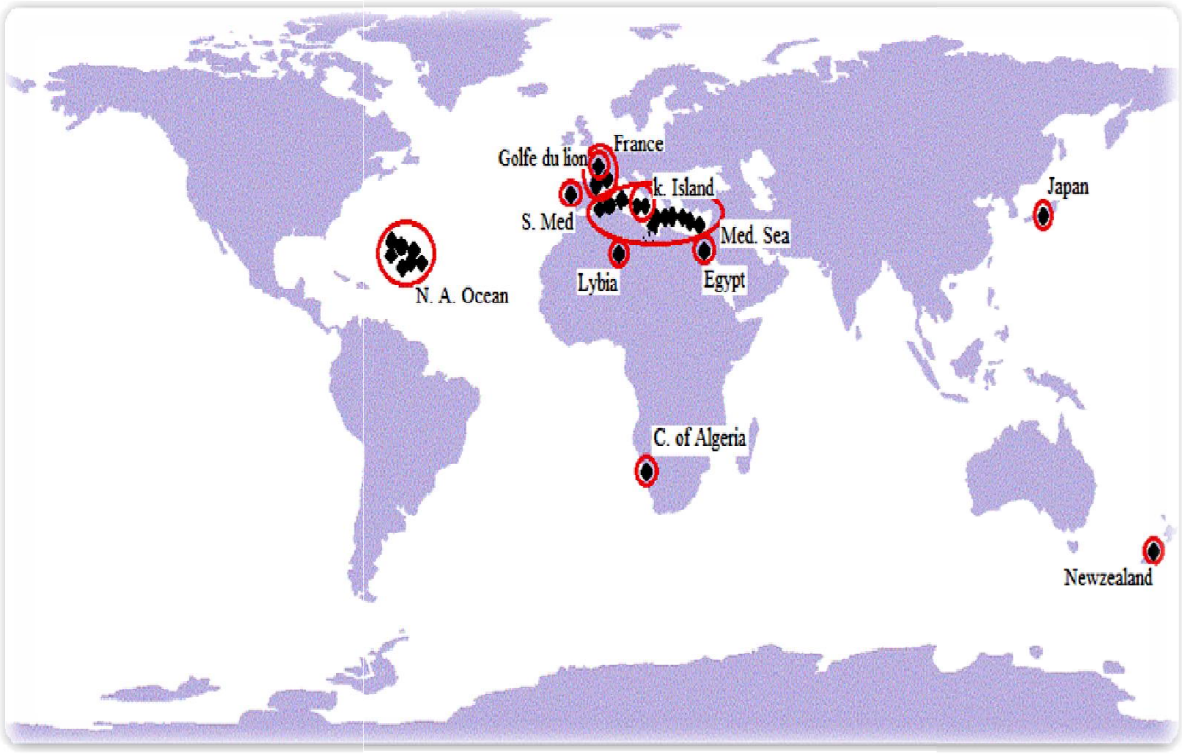

Fig.3. Geo mapping of selected species of *Lamellodiscus* distributed at 11 different geographical regions (A= Atlantic, C=Coast, K= Kerkennah, Med=Mediterranean, N=North, S= Spanish)

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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