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Comparative studies of Indian (Assam), Chinese, UK's and Iranian *Dactylogyrus lamellatus* Achmerow, 1952 for structural conservedness using Phylogenetic and Molecular analysis inferred from 28rDNA

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Abstract: **The present communication deals with the molecular comparison of *Dactylogyrus lamellatus* Achmerow, 1952 (collected from Assam, India) and same species available from other different continents using molecular marker, 28S rDNA. Since, they are morphologically more or less similar, comparative study for sequence conservedness has been made for Indian species and species from different countries using ExpaRNA. Besides this, the study is also supported by motif prediction using meme suite software which can be considered as a promising tool for monogenean species identification. Phylogenetic relationships have also been inferred using neighbour-joining (NJ) and maximum parsimony (MP) methods.**

***Keywords: Dactylogyrus lamellatus*, Assam, 28S rDNA, phylogenetic analysis.**

I. INTRODUCTION

Monogenea is a class of parasitic flatworms that are mostly found on fishes and a few lower aquatic invertebrates. Most monogeneans are browsers that move about freely on the piscine body surface feeding on mucus and epithelial cells of the skin and gills. However, a few adult monogeneans remain permanently attached to a single site on the host. Dactylogyrus is the largest monogenean genus, parasitizing many fish species. During survey of fresh water fishes, species of *Dactylogyrus lamellatus* was found harboring an exotic fish, *Ctenopharyngodon idella*. This host and parasite both is non native to India. In the North-East region of India (Assam), *D. lamellatus* Achmerow, 1952 was found from *C. idella* introduced in India from Japan in 1957 for the purpose of experimental culture and weed control. During the course of study, we have compared the Indian parasite with the species reported from Iran, China, South China and UK using 28S ribosomal DNA marker.

Conserved genes like 28S ribosomal RNA (28S rRNA) are very useful tool for molecular taxonomic studies. rDNA has several characteristics, including tandemly repeated genes, different rates of divergence between spacers and coding genes and concerted evolution which provide a wide range of phylogenetic resolution ranging from kingdoms to populations (Gerbi, 1985, Hillis and Dixon, 1991).

Since they are same species, they ought to have conserved regions (motifs) in the sequence. Number of motifs, their frequency and position must be similar. Since the morphological differences could not be seen, an attempt has been made to evaluate validation of species, *Dactylogyrus lamellatus.* Therefore, secondary structure for each sequence has also been generated separately by ExpaRNA software (online software) to compare the sequence conservedness. The conserved sequences are found in all species but at distinct positions. In the present study, the probable reasons for this genetical variation in sequence of *D. lamellatus* from India and abroad are discussed in detail and it is also believed that these changes might be responsible for the gene shifting.

II. MATERIALS AND METHODS

1. Collection of host fishes

Live fishes for the present investigation were collected from Brahmaputra river at the site of Guwahati, Assam (26°11'N and 91°44'E) and were also purchased from the local fish markets of Guwahati *viz*., VIP fish market, Pandu fish market, Uzaan fish market and Gudhuli fish market. The identification of piscine hosts were made with the help of classical works of Mclnerny and Gerard (1958), Misra (1959), Srivastava (1968), Nelson (1984) and Day (1989) and also with the help of Ichthyologists. Sometimes identification of piscine hosts was confirmed at "Fish Base". Immediately after capture, fish were sacrificed by a sharp blow on the top of the head and dissected. Specimens recovered were kept individually in 100% ethanol for molecular biological studies.

2. Parasite collection, identification, and extraction, DNA Isolation and amplification and sequencing of monogeneans

Methods of collection, extraction, amplification and sequencing of monogeneans were followed from Chaudhary and Singh (2010). For this study, we used 28S region of rRNA gene and available the primers used for this region. An aliquot (3 µl) of amplicon was checked on 1.5-2% agarose-TBE gels, stained with ethidium bromide and visualized under ultraviolet light as per protocol suggested by M/S Amarsham Pharmacia Biotech (U.K.). For sequencing, the amplification product was purified by Chromous PCR clean up Kit (#PCR 10) according to manufacturer's instructions. Both DNA strands were sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer using same primers which were used for amplification reaction.

3. Method of Phylogenetic analysis

DNA sequences were aligned using Clustal W (Thompson *et al*., 1994). DNA sequences of this species available in the gene bank were also downloaded and used in the phylogenetic analysis. Sequences were uploaded on the Basic Local Alignment Search Tool (BLAST) to search for the most similar reference sequences and positions of the 28S gene were determined (available at www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using the neighbour-joining (NJ) and maximum parsimony (MP) methods with the help of MEGA ver. 5.0 (Tamura *et al*., 2011). Bootstrap resampling (1,000 pseudoreplicates) was done and a bootstrap consensus tree produced. The motifs and their regular expressions were predicted with the help of online available MEME software (Timothy *et al*., 1994). Conservedness in loops of RNA secondary structures was predicted with help of ExpaRNA (Simth *et al*., 2010). Percentage of Guanine (G) and Cytosine (C) was calculated using GC calculator (http://www.genomicsplace.com/gc_calc.html).

III. RESULTS AND DISCUSSION

D. lamellatus Achmerow, 1952 collected from India was compared molecularly with same species reported from South China, China, United Kingdom and Iran, using 28S rDNA marker. 28S rDNA sequence of *D. lamellatus* from India (JQ926200) shows 99% similarity with *D. lamellatus* (JX524549) from Iran, *D. lamellatus* (AY307019) from South China and *D. lamellatus* (EF100533) from China. But *D. lamellatus* (AJ969948) from United Kingdom exhibited 98% similarity with Indian species. Both NJ and MP analyses inferred from 28S rDNA sequences gave similar topology with minor change (**Fig. 1 A and B**). Both trees exhibited phylogenetic relatedness and evolutionary pattern of *D. lamellatus* from India and with same species *D. lamellatus* from Iran, China and United Kingdom and other species of the genus *Dactylogyrus* Diesing,1850 (AY548929, EF100531, AY548928, EF100538 and AY548427). They originated from same ancestor, forming two clusters (Cluster 'A' and 'B'). Cluster 'A' shows two lineages for the genus *Dactylogyrus*. Lineage one includes two sister clades: one has *D. lamellatus* (reported from India, China and South China) showing close relatedness and second sister clade has *D. lamellatus* (reported from Iran and United Kingdom), which are more close

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than first sister clade. Lineage two includes *D. petruschewskyi* (AY548427) *and Dactylogyrus sp.* (EF100538). Cluster 'B' includes *D. inversus*, *D. kikuchii* and *D. gotoi* which are distant. The results suggest that the species, *D. lamellatus* introduced in India with their host. This is supported by the validation of species, *D. lamellatus* reported from India. The Indian (Accession Number JQ926200), Chinese, Iran, United Kingdom and Czech Republic isolates (in sister clades) are almost identical with very low variation which implicates that they are same species of parasite from different geographical regions.

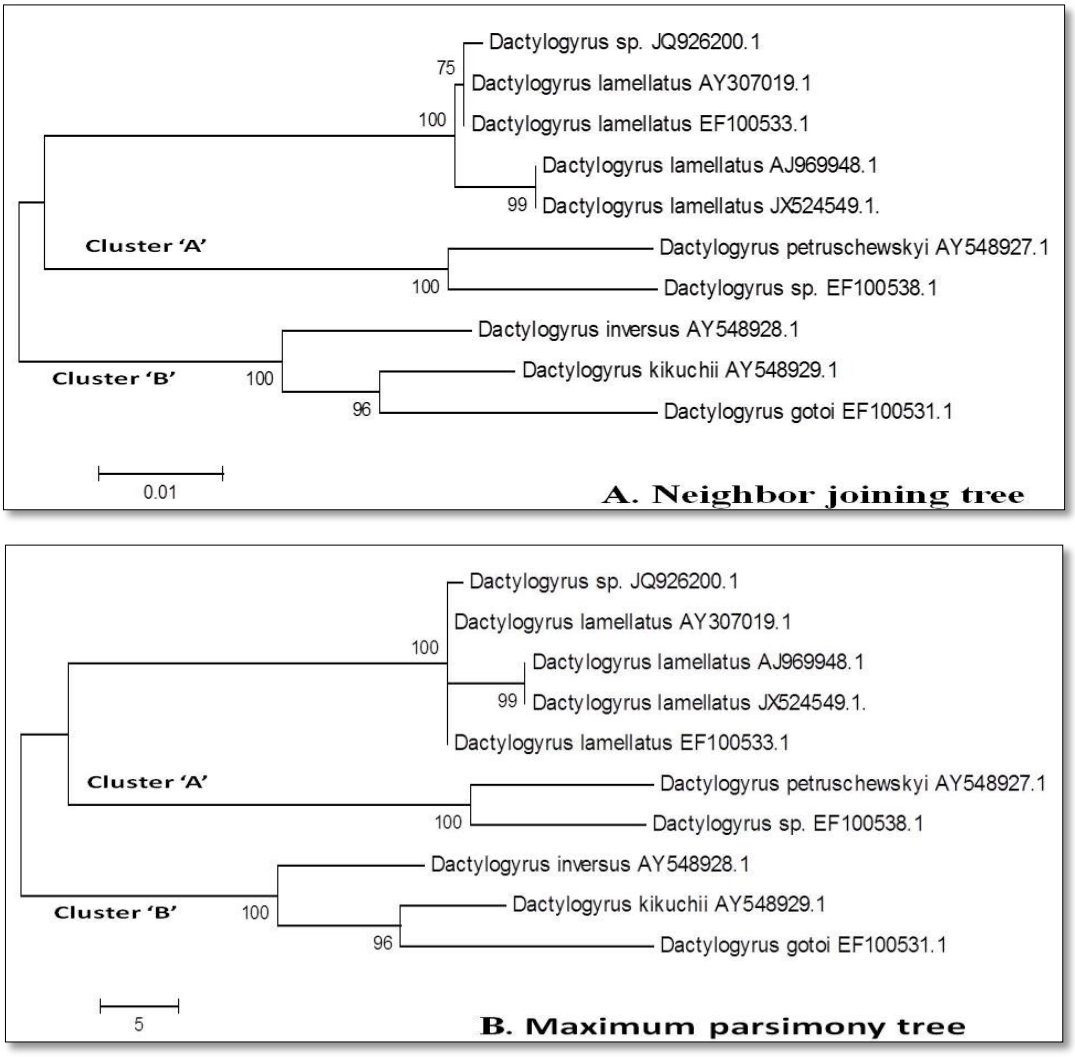

Fig. 1: Phylogenetic trees showing relationships between the *D. lamellatus* and related species

Secondary structure predictions

Percentage of Guanine (G) and Cytosine (C) was calculated using GC calculator (http://www.genomicsplace.com/gc_calc.html). It was found 49.3% in species, *D. lamellatus* (accession no. JQ926200) from India, 49.7% in same species (AY307019) from South China, 49.6% (in EF100533) from China, 50.3% (in AJ969948) from United Kingdom and 50.5% (in JX524549) from Iran. Thus, a structured RNA with higher GC content is likely to have more stable secondary structure.

Study of the RNA secondary structure further confirmed the conserved regions throughout sequence along with various types of loops (hairpin, interior, multi, bulge and exterior loop) constructed by non-matched bases of sequence. Secondary structures (**Fig. 2**) for *D. lamellatus* reported from various continents and subcontinents generated using ExpaRNA software. It shows species specific, topological differences. Besides the topology pattern, these structures infer the consensus sequences using seven colour code patterns (violet, blue, light green, yellow, red, pink and sky blue). These colour coded bases are exactly similar in a particular loop but at different position in sequences. In all RNA secondary

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structures generated, Colour coded conserved regions in secondary structure further confirms that all have similar conserved regions in their genetic material but at different positions. It is noted that same genetic material of all the same species have some variations, incorporated in its position, to adapt to different environmental conditions.

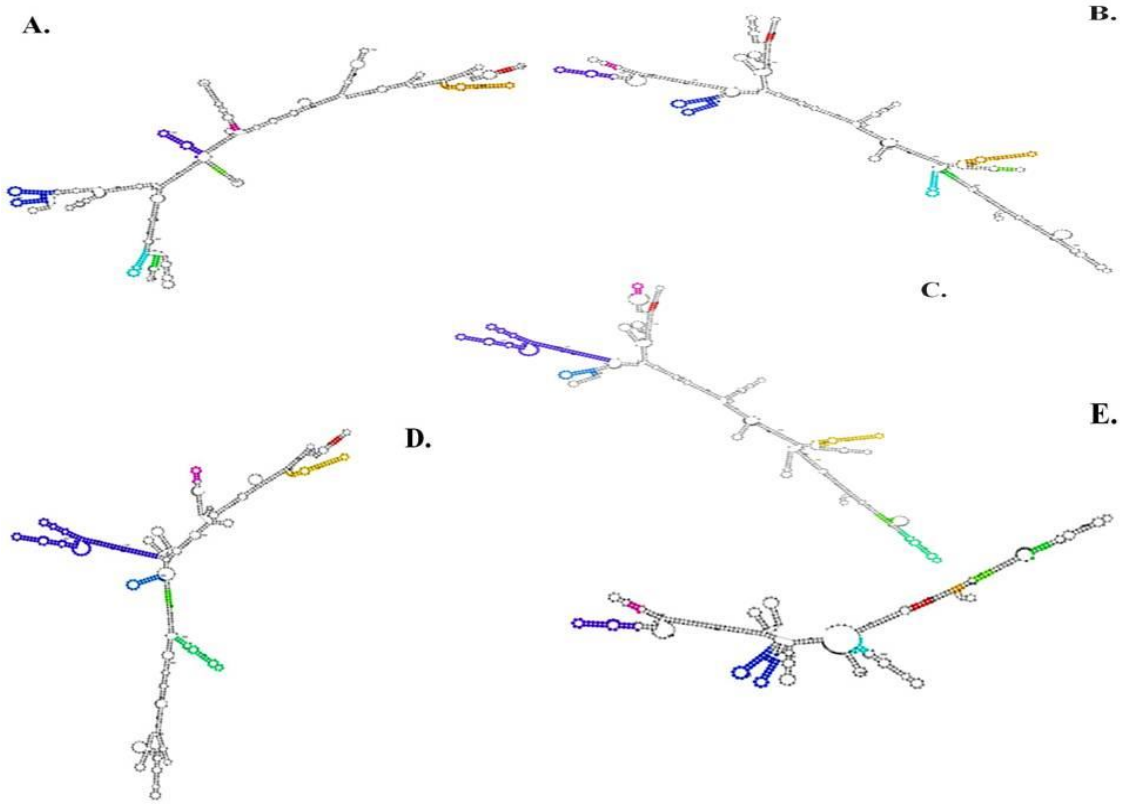

Fig. 2: RNA Secondary structures of species, *Dactylogyrus lamellatus* and related same species showing conserved regions in different loops.

A. *D. lamellatus* (India) B. *D. lamellatus* (South China) C. *D. lamellatus* (China)

D. *D. lamellatus* (United Kingdom) E. *D. lamellatus* (Iran)

Motif predictions

The motif prediction further confirms and verifies conserved regions (motifs) in each sequences of species, *D. lamellatus* and for this, nucleotide sequences of 28S r DNA region of *D. lamellatus* (from India, China, South China, Iran and United Kingdom) have been used for the motif identification with the help of online available software, MEME. The results of MEME showed three different kinds of motifs in 28S rDNA sequences of *D. lamellatus*. Minimum motif width is of 41 bases and maximum motif width of 50 bases. Base length of motif one and motif three obtained is 50 bases. However, motif two includes 41 bases. Motif one is observed as sky blue, two as deep blue and three as red colour. Motif one (**Fig. 3**), motif two (**Fig. 4**) and motif three (**Fig. 5**) are shown. Motif one 4 times, motif two again 4 times and motif three 2 times are repeated in Indian sequence of *D. lamellatus*. While Motif one 4 times, motif two 3 times and motif three 1 times are repeated in sequence of *D. lamellatus* of Iran and Motif one is repeated four times and motif two 5 times & motif three 2 times repeat in other sequences of same *D. lamellatus* (**Fig. 6**). The position of motifs in Indian sequence is clearly distinct from others. The order of motif was found different in sequences of India and Iran but same in all (in AY307019, EF100533 and AJ969948) showing definite shifting in motif localization (**Table 1**). The shifting pattern is clearly seen in combined block diagram of motifs (**Fig. 6**). This change may be because of changes in climatic conditions. The p-value of the motifs has been also found to be different for each sequence. The combined P-value of all the three motifs of *D. lamellatus* is 5.44e-44 (in JQ926200), 6.85e-44 (in AY307019), 6.93e-44 (in EF100533), 6.02e-44 (in AJ969948) and 1.85e-44 (in JX524549) for all sequences respectively.

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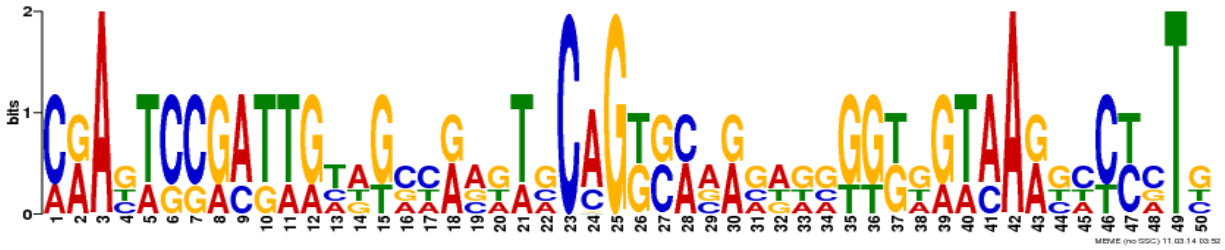

Fig. 3: Motif one, base length is 50 bases

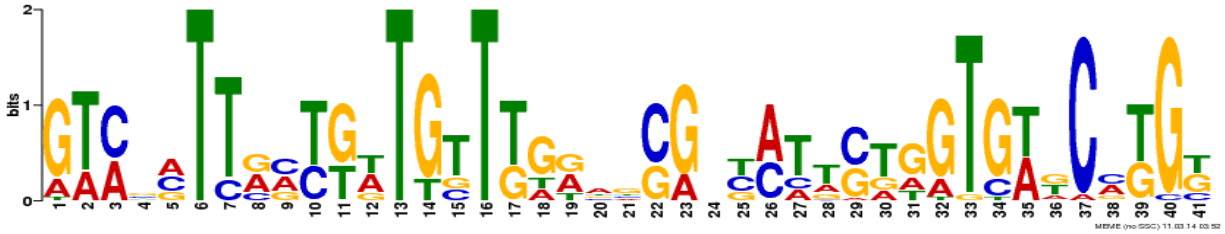

Fig. 4: Motif two, base length is 41 bases

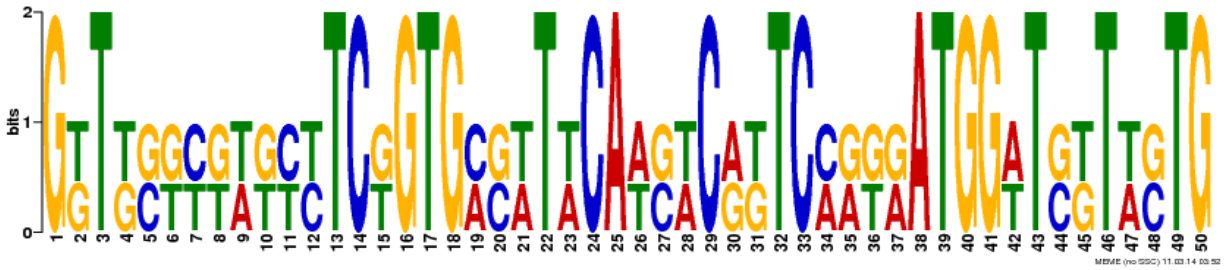

Fig. 5: Motif three, base length is 50 bases

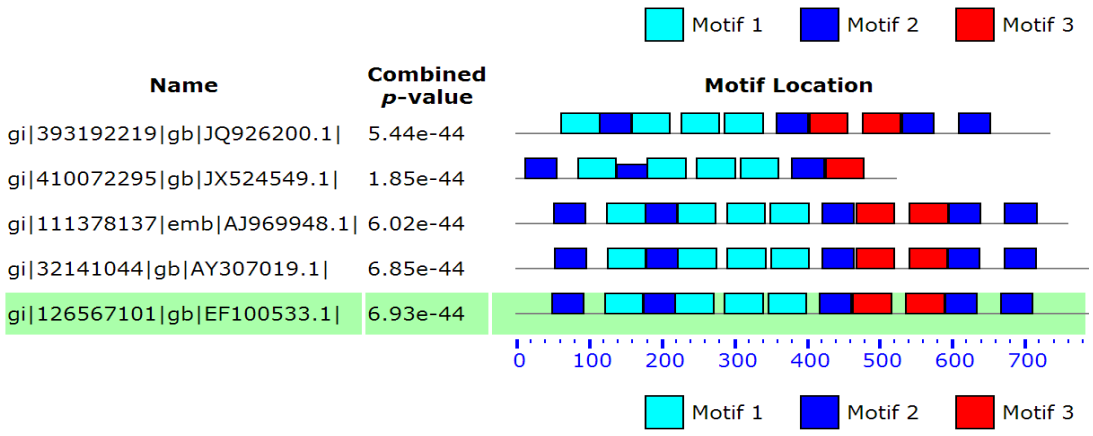

Fig. 6: Combined block diagram of all three motifs

Table 1: Position of three types of Motif in each sequence showing disposition and shifting in motifs

Accession no. with country	Motif 1	Motif 2	Motif 3
JQ926200 (India)	63-112 160-209 228-277 288-337	116-156 359-399 332-572 610-650	405-454 478-527

JX524549 (Iran)	86-135 182-231 250-299 310-359	13-53 139-179 381-421	427-476
AJ969948 (United Kingdom)	126-175 223-272 291-340 351-400	53-93 179-219 423-463 597-637 674-714	469-518 543-592
AY307019 (South China)	127-176 224-273 292-341 352-401	54-94 180-220 423-463 496-636 673-713	469-518 542-591
EF100533 (China)	123-172 220-269 288-337 348-397	50-90 176-216 419-459 592-632 669-709	465-514 538-587

From above Table 1, it is evident that all three motifs in its number and disposition remained more or less identical in *D. lamillatus* reported from U.K., China and S. China. However, in *D. lamillatus* reported from India, presence of similar motifs was found but they were found at different dispositions (Fig.6 and Table 1). Besides this, *D. lamillatus* reported from Iran exhibited deletion in number of nucleotide sequence in motif 2 and 3 (Fig.6 and Table 1). Not only this, motif 2 located at 139-179 got reduced (Fig.6 and Table 1).

IV. CONCLUSION

This molecular analysis reveals the genetic similarity between same species of *D. lamellatus* from different regions with minor variations. These variations are probably because of gene shifting. Reasons for genetic variations are due to their geographical distribution or adaptation with their host in different regions. If these variations are not occurring simultaneously, it may be altering host-parasite interactions.

At molecular level, this genetic comparison between Indian species and same species from other parts of the world may provide further clues to the understanding of the evolution of the species. RNA secondary structure analysis along with motif prediction could be a valuable tool for species discrimination. These findings should help to guide the global researcher in their goal to develop therapeutic models targeting parasites at their genetic levels.

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