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DNA Barcoding and Molecular phylogeny of some heteropterans in Shola Forest, Southern Western Ghats, Kerala

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Abstract: *Phylogenetic relationships among aquatic insects in Shola Forests of Southern Western Ghats using molecular data were examined. In the present study, Mitochondrial Cytochrome Oxidase subunit 1 (COI) gene of seven aquatic bugs are sequenced and analysed the genetic diversity of bugs. For seven species viz., Anisops paranigrolineata Brooks, 1951(family Notonectidae), Enithares hungerfordi Brooks, 1948 (family Notonectidae), Helocoris rotundatus Montandon, 1908 (family Naucoridae), Perittopus horvathi Lundblad, 1933 (family Veliidae), Metrocoris indicus Chen & Nieser, 1993 (family Gerridae) , Sigara (Tropocorixa) graveleyi Hutchinson, 1940 (Corixidae) and Limnometra anadyomene Kirkaldy, 1901 (family Gerridae) this is the first ever molecular study which has been done in heteropterans in Shola forest. The COI sequences of these seven species have been added to the existing database at GenBank NCBI which can be used for their identification. The result shows hierarchical increase in K2P mean divergence at different taxonomic levels (0. 05% at intraspecific, 10.27% at interspecific and 18.7 % at intergeneric levels). Maximum Likelyhood tree study reveals the phylogenetic status of Shola insects.*

Keywords: *Aquatic bugs, CO1, Genetic Diversity, K2P Divergence*

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I. Introduction

Aquatic and semi-aquatic insects (Hemiptera) are remarkably diverse with 4,656 species (326 genera and 20 families) which are found worldwide except in Antarctica [1]. DNA based species identification and phylogenetic analysis of animals have being used for decades. The first molecular systematic study with focus on Heteroptera was by Wheeler and his collegues [2], who combined 31 morphological characters with partial18S rDNA sequence data (670 bp). It has been used for species delimitation and identification for several families of Hemiptera ([3] and [4]). According to Menon *et al*. [5] and Damgaard [6], DNA based identification of heteropteran species has limited utility. However, the recent studies underscored the importance of DNA barcoding for identification of true bug species ([7] and [5]). Sequence variation generally shows large interspecific but small intraspecific divergences, and species frequently form clearly distinguishable clusters a distance-based phylogenetic tree. The homogenization of mitochondrial DNA sequences within a species, regardless of population size, is an intriguing phenomenon that has prompted study and speculation as to its evolutionary origin and significance [8]. The resulting 'barcoding gap' appears to represent a 'genetic signature' for species [9]. Jung *et al*. [7] presented COI variations in species of Heteroptera from Korean Peninsula and added 1,689 specimens from Canadian National Collection of Insects. Park *et al*. [4] expanded this database for analysis of phylogenetic relationship amongst species are also finding out with DNA barcoding. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution. Some notable works incorporating molecular data into phylogeny studies of Heteroptera are carried out elsewhere [10] and [11]. Sholas are tropical montane forests situated in the higher mountain tracts, (above 1500 m) of the Western Ghats which is a well-known biodiversity hotspot with thousands of plant and animal species, many of them being still unexplored. This study is a pioneer work in regard to aquatic insects solely in Shola Forests.

II. Methodology

2.1 Specimen Collection

Aquatic insects of the suborder heteroptera were collected by 'all out search' method from three shola forest area (Mathikettan Shola, Pampadum Shola and Anamudi Shola) located (N 09º 58' 07.9'' - N 10º 11' 34.2'' ; E 077º 11' 39.9''- E077º17' 01.50'') in the Southern Western Ghats, Kerala (Figure 1) and preserved in ethanol for further analysis. Seven heteropteran species were selected for the present study (Table 1). The collected specimens were identified using relevant literature and confirmed by Dr. Ping-ping Chen and Dr. Nico Neiser, The Netherlands.

2.2 DNA Isolation and Polymerase Chain Reaction

Genomic DNA was extracted from legs of aquatic insects by using DNeasy® Blood and Tissue Kit (Qiagen) as per the manufacturer's protocol. The quality of DNA isolated was checked using agarose gel electrophoresis. Polymerase chain reaction (PCR) amplification of COX1 was conducted in 20 µl reaction volume and which were in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). COX1 primers such as LCO (5' -GGTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') [12] were used for the amplification of CO1gene with the following thermo-profile: initial denaturation at 95°C for 5 min, 10 cycles at 95°C for 30 sec, annealing at 45 °C for 40 sec and extension at 72°C for 1 min, with a final extension at 72 °C for 7 min, followed by indefinite hold at 4 °C.

2.3 Sequencing and Phylogenetic Analysis

Sequencing was conducted with the PCR primers by Dideoxy Sanger standard method. Sequences were firstly edited manually for excluding the ambiguous and skipped bases and files were converted into FASTA format and submitted to the NCBI GenBank through sequin. The similar sequences were obtained from NCBI database by BLAST [13]. Around 14 sequences were retrieved from GenBank (NCBI) and aligned with target sequences for phylogenetic analysis. Multiple sequence alignments were prepared using Clustal omega and divergences were analysed by Kimura-2-Parameter (K2P) model of base substitution in MEGA software version 7 (Molecular Evolutionary Genetic Analysis) [14]. Nucleotide composition was calculated by the BioEdit software. The statistical confidences were evaluated by 1000 non-parametric bootstrap replicates for ML analysis. The Kimura two-parameter model [15] the most commonly used and standard model in DNA barcoding studies, was used to create Maximum Likelihood (ML) Tree to compute genetic distances.

III. Results

3.1 General features of mitochondrial DNA of specimens sequenced

Seven heteropteran insects were sequenced and their accession numbers, sequence size and nucleotide composition are given in Table 1. The size of the sequences ranged from 455 to 658 bp. Nucleotide compositions of these mt-genomes are biased toward adenines and thymines. The A+T content varied from 63.22% to 68.39% (Table 1). The maximum AT content was found in Metrocoris indicus (68.39%) and the minimum was observed in Heleocoris rotundatus (63.22%). They belong to the families Gerridae and Naucoridae respectively.

3.2 Genetic Distance

Pair-wise genetic distance of CO1 sequences was calculated using Maximum Composite Likelihood model. All positions containing gaps and missing data were eliminated. The analysis involved 22 nucleotide sequences. There were a total of 390 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Metrocoris indicus had genetically lesser distance with Enithares woodwardi (0.160) followed by Gerris argenticollis (0.166) and maximum distance of 0.242 observed with Heleocoris rotundatus. Enithares hungerfordii had lesser genetic distance with Paraplea frontalis (0.111) while maximum distance with Anisops paranigrolineatus (0.165). Heleocoris rotundatus had genetically lesser distance Enithares hungerfordi (0.174) and maximum distance with Anisops paranigrolineatus (0.204). Limnometra anadyomene had maximum genetic distance with Heleocoris rotundatus (0.217) and minimum genetic distance with Parapleahalei (0.153). Genetic distance of Perittopus horvathi was less with Gerris incognitus (0.118) and maximum with Helocoris rotundatus (0.203). Sigara graveleyi showed lesser genetic distance with Paraplea haveleyi (0.163) and maximum with Helocoris rotundatus (0.226). Anisops paranigrolineatus had maximum genetic distance with Sigara bicoloripennis (0.210) while minimum with Paraplea frontalis (0.134). The intra and interspecific KP divergence of sequences were also calculated with the similar sequences retrieved from GenBank. The intraspecific K2P divergence were done in Anisops paranigrolineatus. They were morphologically similar but showed 0.05 % intraspecific divergence. The interspecific divergence was calculated in the species Sigara, Paraplea, Enithares, Gerris, Metrocoris and Perittopus (Table 2). Sigara graveleyi had lowest genetic distance (0.098) with Sigara truncatipala. Enithares hungerfordii showed lesser genetic distance (0.086) with Enithares woodwardi. The genetic distance between Metrocoris indicus and Metrocoris deceptor was 0.105. The average interspecific divergence was found to be 10.27%. The inter-generic genetic distance was found to be the maximum in genus Limnometra with other species under observation ranging from 0.207 to 0. 240 (Table 3) and overall average is 18.7 %.

3.3 Phylogenetic Analysis

The phylogenic relationships of aquatic insects in Shola forest was studied using CO1 molecular marker with Maximum Likelihood Method (Figure 2). The ML tree was prepared and clade stability was estimated using one thousand non-parametric bootstrap replications. Phylogenetic tree revealed the formation of two major clusters. As the morphology shows the congeneric inter- specific species are found to be in neighbouring clade along with their identical species of GenBank. CO1 genes with related species name

clustered cohesively in our present data. Metrocoris indicus was clustered with similar one (M. deceptor) with highest bootstrap value (99%). Species under single family share one clade at a time. Species under the family Notonectidae were Anisops paranigrolineatus and Enithares hungerfordii were shown in a single cluster. The sister group to E. hungerfordii is E. tibialis and they share a recent common ancestor. The family notonectidae (Anisops paranigrolineatus and Enithares hungerfordi) is also a sister group to the family naucoridae (Heleocoris rotundatus). The congeneric species of genus Sigara (corixidae) formed a separate clade showing close relationship amongst them.

IV. Discussion

Aquatic and semi aquatic heteroptera play a remarkable role in aquatic ecosystems of Shola forests. The molecular level species identification and phylogenetic analysis proved a greater representation of heteropteran bugs by adding more species from Western Ghats to the existing database. The sequences showed higher AT content than GC as is inspected in Insect mitochondrial DNA. [16]. A+T content is a general feature of the COI mitochondrial DNA region in arthropods [17], [18], and [19]. Studies of Damgaard et al [20] revealed nucleotide composition of Halobates is AT biased and it was ranging from 67.1% to 74.4%. Similar frequencies were reported by Simon et al [18] in other hemimetabolous insects. Same pattern of nucleotide composition in heteropterans was reported by Sperling et al., [21], Habeeb and Sanjayan [22], Zhang et al., [23] , Raupach et al., [24] and Kaur and Sharma [25]. Two specimens of Anisops paranigrolineatus from the present study showed only 0.2% of sequence divergence, while 0.8% was the intraspecific divergence of some heteropterans by Jung et al [7]. Tembe et al. [26] found the average intraspecific divergence as 0.4%. Raupach et al. [24] analysed DNA barcodes of 457 species of Heteroptera and foundintraspecific pairwise distances to be less than 2.2%. It is because of the strong nearness of The interspecific divergence study confirmed the results of the earlier works done by Hebert et al. [27] and Li et al [28]. For the insects, the interspecific divergence has been suggested to be 3% by Hebert et al [27] and 5% by Li et al. [28]. In the present study, Gerris incognitus and Gerris argenticolllis pair showed maximum of 12.4 % inter specific divergence and minimum of 6.7% which is between Gerris incognitus and Gerris pingreensis. Slightly greater value given from the present study suggesting that all of the species are well separated species. The same result has been reported by the Harbhajan Kaur and Kanu Sharma [25]. The intra and interspecific and intergeneric divergence study show a hierarchical increase. In short, CO1 is a useful molecular marker for identifying and analyzing the heteropterans. Jung et al. [24] studied Korean species of Apolygus and found three of them formed a single complex Neighbour joining cluster. In this study the genus Sigara, Metrocoris and Enithares showed monophyly as reported by Tembe et al [25] in the family Pentatomidae. Studies about the termites by Singla et al [29] revealed the CO1 DNA barcoding importance to interpret the mono, Para and polyphylogeny.

V. Tables and Figures

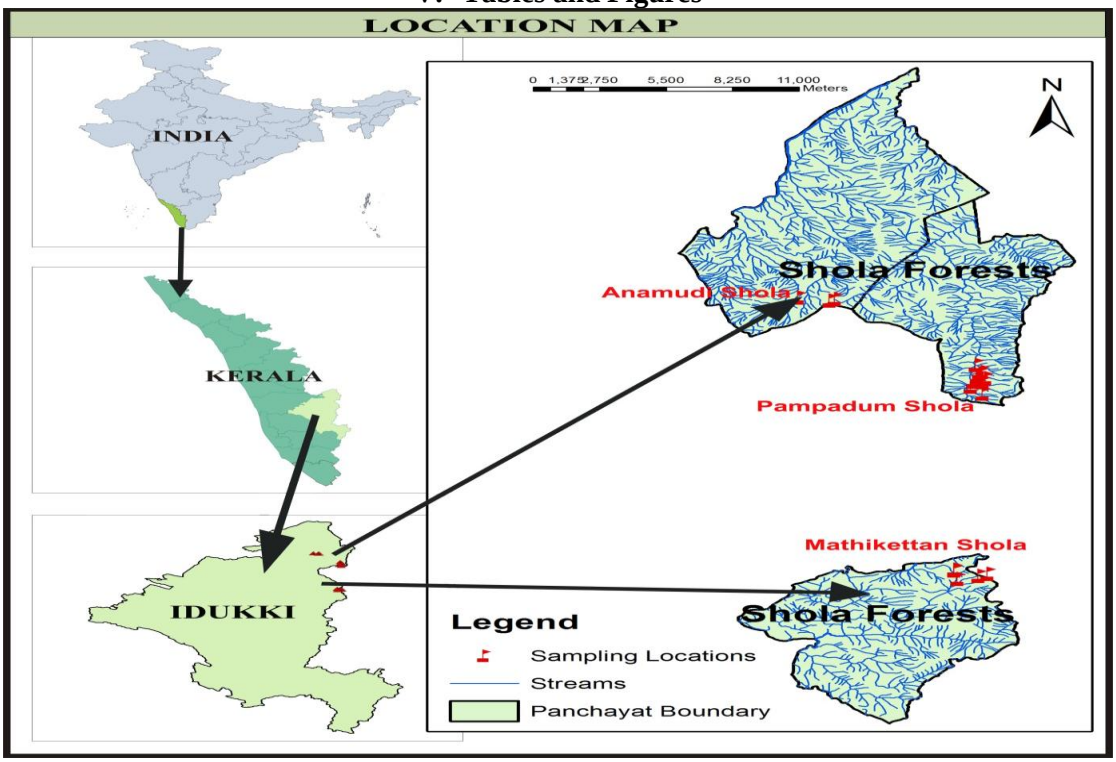

Figure 1: Location map of Sample collection

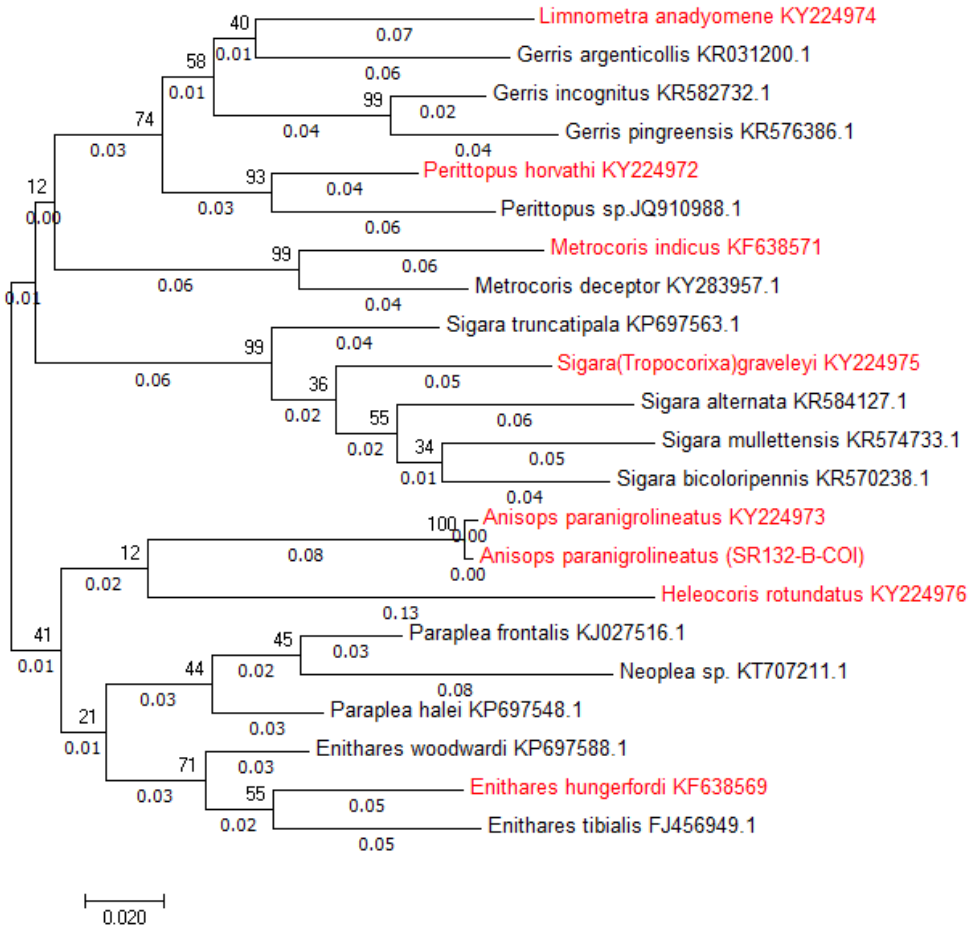

Figure 2: Maximum Likelihood (ML) tree of cytochrome oxidase subunit 1 (CO1) sequences of aquatic bugs from the Indian waters along with the similar sequences. Red colour indicates the sequences of the present study.

Table 1: Accession number, sequence size and nucleotide composition of aquatic insects of Shola forest

S. No.	Taxa (Family)	Sample code	Accession No.	Length (bp)	Nucleotide Composition AT	GC
1	*Anisops paranigrolineatus* (Notonectidae)	SR132-A	KY224973	658	67.02%	32.98%
2	*Enithares hungerfordi* (Notonectidae)	SR46-A	KF638569	658	66.57%	33.43%
3	*Heleocoris rotundatus* (Naucoridae)	SR46-G	KY224976	658	63.22%	36.78%
4	*Perittopus horvathi* (Veliidae)	SR600-7	KY224972	652	68.25%	31.75%
5	*Metrocoris indicus* (Gerridae)	SR46-E	KF638571	658	68.39%	31.61%
6	*Limnometra anadyomene* (Gerridae)	SR132-D	KY224974	658	68.09%	31.91%
7	*Sigara(Tropocorixa) gravaleyi* (Corixidae)	SR600-2	KY224975	455	66.37%	33.63%

Table 2: Table 3: Percentage of genetic distances (Kimura 2- Parameter) of CO1 sequences of Heteropterans in Shola Forests

Anisops_paranigrolineatus_KY224973																					
Anisops_paranigrolineatus_KY953213	0.005																				
Enithares_hungerfordi_KF638569	0.162	0.159																			
Enithares_tibialis_FJ456949.1	0.162	0.156	0.099																		
Enithares_woodwardi_KP697588.1	0.137	0.134	0.084	0.087																	
Gerris_argenticollis_KR031200.1	0.199	0.196	0.200	0.180	0.171																
Gerris_incognitus_KR582732.1	0.181	0.178	0.197	0.174	0.151	0.122															
Gerris_pingreensis_KR576386.1	0.180	0.177	0.193	0.177	0.162	0.131	0.064														
Heleocoris_rotundatus_KY224976	0.201	0.201	0.175	0.178	0.184	0.225	0.204	0.214													
Limnometra_anadyomene_KY224974	0.204	0.201	0.197	0.174	0.152	0.128	0.128	0.146	0.218												
Metrocoris_deceptor_KY283957.1	0.177	0.173	0.186	0.160	0.151	0.164	0.186	0.189	0.222	0.175											
Metrocoris_indicus_KF638571	0.173	0.170	0.202	0.183	0.158	0.164	0.188	0.205	0.244	0.190	0.106										
Neoplea_sp._KT707211.1	0.144	0.147	0.152	0.162	0.131	0.227	0.195	0.188	0.198	0.195	0.202	0.204									
Paraplea_frontalis_KJ027516.1	0.132	0.135	0.112	0.143	0.123	0.177	0.154	0.169	0.182	0.187	0.170	0.179	0.105								
Paraplea_halei_KP697548.1	0.137	0.140	0.126	0.130	0.105	0.151	0.125	0.153	0.200	0.154	0.161	0.168	0.133	0.073							
Perittopus_horvathi_KY224972	0.171	0.168	0.173	0.176	0.149	0.144	0.115	0.118	0.201	0.146	0.172	0.183	0.169	0.142	0.118						
Perittopus_sp.JQ910988.1	0.179	0.175	0.175	0.165	0.163	0.140	0.134	0.151	0.214	0.158	0.175	0.190	0.203	0.152	0.131	0.088					
Sigara(Tropocorixa)graveleyi_KY224975	0.204	0.207	0.192	0.179	0.173	0.189	0.180	0.190	0.224	0.187	0.208	0.201	0.204	0.183	0.160	0.202	0.196				
Sigara_alternata_KR584127.1	0.194	0.191	0.205	0.191	0.181	0.203	0.217	0.209	0.201	0.187	0.201	0.222	0.211	0.184	0.192	0.213	0.202	0.120			
Sigara_bicoloripennis_KR570238.1	0.205	0.208	0.185	0.191	0.178	0.186	0.194	0.184	0.238	0.190	0.200	0.207	0.198	0.173	0.167	0.189	0.202	0.117	0.102		
Sigara_mullettensis_KR574733.1	0.192	0.195	0.186	0.177	0.155	0.180	0.178	0.180	0.210	0.191	0.174	0.184	0.188	0.138	0.145	0.183	0.194	0.121	0.114	0.093	
Sigara_truncatipala_KP697563.1	0.190	0.187	0.193	0.174	0.159	0.164	0.172	0.171	0.217	0.168	0.178	0.194	0.192	0.183	0.145	0.170	0.180	0.098	0.117	0.099	0.114

VI. Conclusion

From this study, it is concluded CO1 DNA markers were useful in detecting genetic similarities, genetic diversities and genetic distances within species and genera and between species and among genera, which is sufficient to throw light on their genetic constitution. The molecular studies have to be extended to more species in Western Ghats especially in Shola region as they are still unexplored. Unfortunately hardly any heteropteran sequence data is available in GenBank from this area. Therefore expansion of the database with CO1 and other genes of heteropteran sequences of Western Ghats is being suggested.

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