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Genetic diversity and conservation of South Indian Mayfly, *Petersula courtallensis* Sivaramakrishnan, 1984 (Ephemeroptera: Leptophlebiidae)

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Abstract

The aim of this study was to measure variation in the genetic structure for south Indian mayfly species, *Petersula courtallensis* with wide geographic ranges. The mtCOI sequences of 492–522 bp were obtained for eight individuals collected at eight different study sites during July 2014 to February 2015. Two clusters were resolved based on the distribution of individuals in the Neighbor-Joining tree. This south Indian mayfly species, *P. courtallensis* had high intraspecific differences between sites across wide spatial distribution. The results presented here support the conclusion that *P. courtallensis* is a species complex. Results also indicated that a general pattern of high genetic diversity between the western and eastern streams. The levels of COI diversity observed suggest the presence of two genetic distinct populations and their genetic diversity should be conserved in the long term.

Keywords: Mayfly, *Petersula courtallensis*, genetic variation, DNA barcode, India

1. Introduction

Studies of spatial patterns of genetic diversity in stream insect populations highlight the interplay of both recent ecological and historical evolutionary factors in the composition of insect communities at a particular point of time in a catchment^[1, 2]. Many stream mayfly species show typical patterns of hierarchical genetic structure, with homogeneity among sampling sites within streams and the accumulation of divergence among catchments^[1]. However, the scale and magnitude of genetic divergence among populations varies within and among taxa^[2-4]. In addition to current connections among populations especially of adjacent streams, long term historic processes reflected in range expansions and contractions, population subdivision or secondary contact are also highlighted in the study of spatial factors of genetic diversity^[4].

Studies of several insect species in subtropical streams in South-East Queensland have found interesting patterns of genetic variation, with the greatest levels of genetic variation at the smallest spatial scale, between pools within a single stream^[6-8]. Similar studies in North America have also reported this effect for some species, for example for the trichopteran, *Gumaga griseola* in Arizona^[9] and mayfly *Baetis bicaudatus* (Ephemeroptera: Baetidae), from the Rocky Mountains, Colorado^[10]. These findings are contrary to expectations of isolation by distance. Though these patterns that larvae in a single stream pool represent the offspring of a small number of matings and that movement of larvae between pools within a stream is limited. Further evidence to support this idea comes from the fact that many species show significant deviations from Hardy–Weinberg proportions (randomly across sites and loci), found in a caddisfly^[6], a water strider^[5], a baetid mayfly species in southeast Queensland^[6-8], a baetid^[11] and a heptageniid mayfly in Europe^[12]. Furthermore, at least for one species, a tasimiid caddisfly, there was as much genetic variation between two sampling years in the same pool as there was between pools at each time^[6].

The rationale behind choosing *Petersula courtallensis*, for the present study is that it is an endemic, gondwanan relict with fairly wide distribution in streams of the Western Ghats, a biodiversity hotspot of global importance. The genus *Petersula* established by Sivaramakrishnan^[13] for the species *P. courtallensis* from southern Western Ghats. *Petersula* appears to belong to the *Meridialaris* lineage as delineated by Pescador and Peters^[14]. *Petersula* and *Kimminsula* belong to a complex of several related genera in Sri Lanka and southern India.

Apparently, the *Kimminsula* - complex is a derivative of an ancient Gondwanian fauna, and drifting India played a significant role in the dispersal of the ancestor of the *Kimminsula* - complex to the hill streams of Sri Lanka and southern India [15, 16]. Two species were recorded belongs to *Petersula* viz. *P. courtallensis* and *P. nathani* from main falls stream of Courtrallam and Annamalai hills, respectively. *P. courtallensis* is described based on nymph and imago. The species *P. courtallensis* is distributed throughout the Western Ghats. It is endemic to the Western Ghats. The aim of this study is to measure variation in the genetic structure for south Indian mayfly species, *Petersula courtallensis* with wide geographic ranges.

2. Material and Methods

2.1 Study area

The present study, samples were collected from streams and river basins of the southern Western Ghats during July 2014 to February 2015 (Fig. 1).

2.2 General features of sampling stations

All stations have shared some common features as follow: 1. streams drain undisturbed forested areas or village level landscapes, 2. stream orders ranges from I to IV, 3. anthropogenic impacts on streams are either very much limited or totally absent, 4. their selection across three states is neither systematic nor stratified, 5. most of the streams consist of highly heterogeneous substratum with extreme degrees of habitat diversity and 6. most of them are perennial streams and very few are intermittent in flow.

2.3 Specimen collection, preservation and identification

The larvae of *P. courtallensis* were collected in river basin with an aquatic D-net; in streams by using kick net method (mesh size; 0.5 to 1.0 mm) the substratum viz., bed rocks, boulders and cobbles were vigorously disturbed strictly restricted to one m² area; allowing the current to carry organic debris, including insects, into the net. Collected specimens were preserved in 100% ethyl alcohol for morpho and molecular studies. Collected samples were brought to laboratory and were examined under stereo-zoom microscope (Magnus, MSZ-TR) and identified using relevant taxonomic literature provided by Sivaramakrishnan [13].

2.4 DNA extraction, PCR and sequencing

DNA was extracted using DNeasy Blood and Tissue kit (Qiagen). Whole individuals were first soaked overnight in the extraction buffer with proteinase K at 56 °C, leaving the gut and chitinous body parts intact. A partial region of mitochondrial COI gene was amplified using universal primers C1-J-1718 (5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3') and C1-N-2191 (5'-CCC GGT AAA ATT AAA ATA TAAACT TC-3') designed by Simon [17]. PCR was carried out in a 30 µl volume with 100 ng template DNA, 10 pmol of each specific primer, 200 mM of each dNTPs, 1.0 unit of *Taq* DNA polymerase and 1x *Taq* buffer containing 1.5 mM MgCl₂. For the primer pairs, the protocol used was: initial denaturation at 94 °C for 2 min, followed by 35 cycles with each cycle consisted of denaturation at 94 °C for 30 s,

primer annealing at 46 °C for 30 s and extension at 72 °C for 1 min and by a final extension step of 72 °C for 10 min. The PCR products were visualized on 1.2% agarose gels and the most intense products were selected and purified by gel extraction kit (Qiagen) following the manufacturer's instructions. The purified products were sequenced commercially by Amnion Biosciences Pvt. Ltd, Bangalore, India. In all cases, DNA was sequenced from complementary strands, with sufficient overlap for the larger genes to ensure accuracy of the results.

2.5 ClustalX analysis

The chromatogram of sequences were visualized and edited in Bio Edit and aligned by eye. Edited sequences were confirmed in BLAST analysis for identity of closely related species sequences. The sequences were aligned using the program ClustalX (CLUSTAL 2.1). The mtCOI sequences were deposited in GenBank (Table 1). Neighbour-Joining (NJ) tree and intraspecific genetic divergence values were performed based on the Kimura 2-parameter (K₂P) model using MEGA 5 [18].

3. Results

The mtCOI sequences consisted of 492–522 bp were obtained from eight individuals collected at the eight different study sites. The sequences were aligned and edited using the program ClustalX. Similarities and differences among sequences within species were examined using K₂P pairwise comparisons and Neighbor-Joining (NJ) tree. K₂P distance comparing pairs of individuals varied from 0.000–0.258 with a mean of 0.153 and a median of 0.238 (Table 2). The NJ tree was created to determine (i) how many individuals were in each cluster, (ii) relatedness of each cluster and (iii) to analyze the inference that a cryptic species complex had been discovered. Two clusters were resolved based on the distribution of individuals in the NJ tree (Fig. 2). One cluster was formed based on eastern side populations due to low genetic diversity within five eastern side streams (PC1, PC2, PC3, PC4 and PC5) and another cluster was formed based three western side streams (PC6, PC7 and PC8) (Fig. 2).

The studied South Indian mayfly species, *P. courtallensis* had high intraspecific differences among sites across wide spatial distribution sampled. Pairwise distance comparisons for *P. courtallensis* had a maximum distance of 0.258. All the individuals shared conserved sequence differences that allowed for accurate appointment in NJ tree. Sequence divergence within each cluster was >2%, which did contain two population that produced genetic differences. The results indicated that a general pattern of high genetic diversity between the western and eastern streams, whereas low genetic diversity within western and eastern streams (Table 2; Fig. 2). Despite high levels of genetic diversity overall, the geographic distribution of the mtDNA for the short COI fragment revealed differentiation among the eight streams. Eastern side streams, especially those on upper flow unit bedrock, tend to drain smaller watersheds, and there was a strong and positive correlation between diversity and watershed size.

Table 1: Details of *Petersula courtallensis* collected and identified from the streams and rivers of the Western Ghats.

Code	Locality	Latitude & longitude	Date of collection	GenBank Accession Number
PC1	Kanyakumari (Tamil Nadu)	08.25.03 N, 77.23.485 E	11.xi.2014	LC217984
PC2	Karumandiammankovil (Tamil Nadu)	08.35.4 N, 77.13.285 E	21.iii.2015	LC217985
PC3	Gadananathi (Tamil Nadu)	08.48.045 N, 77.18.053 E	20.vii.2014	LC217986
PC4	Kannupullimettu (Tamil Nadu)	08.56.2035 N, 77.12.2574 E	17.vii.2014	LC217987
PC5	Kodaikanal (Tamil Nadu)	10.16.15.39 N,77.33.1583 E	01.ii.2015	LC217988
PC6	Athirapalli falls (Kerala)	10.17.0808" N, 76.34.0852 E	16.x.2014	LC217989
PC7	Silent Valley (Kerala)	11.06.495 N, 76.25.524 E	18.x.2014	LC217990
PC8	Nandini hole (Karnataka)	13.23.2352 N, 75.10.4702 E	03.ii.2015	LC217991

Table 2: Pairwise differentiation between the 8 samples of *Petersula courtallensis* using mitochondrial COI.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
PC1								
PC2	0.002							
PC3	0.002	0.004						
PC4	0.006	0.008	0.004					
PC5	0.045	0.047	0.047	0.051				
PC6	0.238	0.241	0.241	0.244	0.232			
PC7	0.252	0.255	0.255	0.258	0.253	0.062		
PC8	0.246	0.249	0.249	0.252	0.240	0.141	0.129	

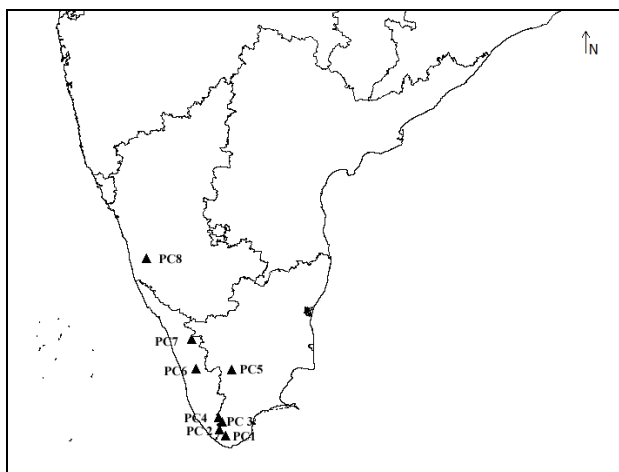


Fig 1: Map showing sampling sites for *Petersula courtallensis* for the study.

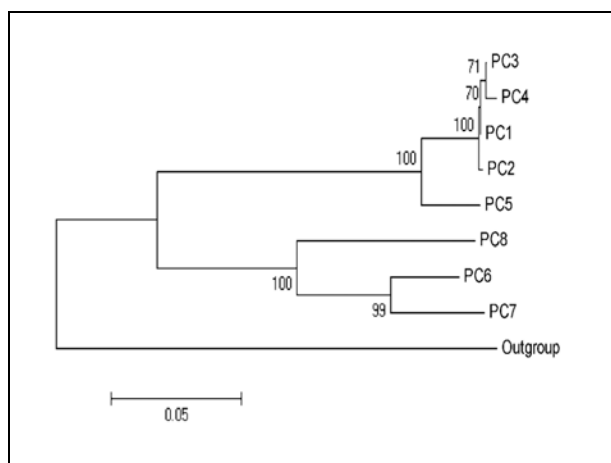


Fig 2: Neighbor-joining tree constructed using DNA barcodes for 8 samples of *Petersula courtallensis* from the Western Ghats, India.

4. Discussion

Many insect species remain undescribed worldwide. One challenge in the effort to quantify species diversity is the presence of cryptic species complexes that represent a group of closely related species which cannot be easily resolved

using traditional morphological characters. The introduction of barcoding has given researchers a new set of characters to detect and identify these organisms. In the last decade, many cryptic species complexes have been discovered or resolved for insect populations considered a single, polymorphic species or possibly a problematic species [19, 20]. Recent analysis of diversity for aquatic insect communities demonstrates more diversity than previously thought [21]. The results presented here support the conclusion that *P. courtallensis* is a species complex. The levels of COI diversity observations suggest the presence of two genetic distinct populations for *P. courtallensis*. The majority of sequences aggregated into cluster 1 (five individuals from Tamil Nadu), with the remaining sequences were classified cluster 2 (two individuals from Kerala and one from Karnataka).

Present study on the widely distributed endemic mayfly, *P. courtallensis* reveals a striking pattern of genetic subdivision at a special scale of 257 km on the eastern side of the Western Ghats and 510 km on the western side from north to south. A significant pattern of hierarchical genetic structures that correlates with local variation in surface and bedrock geology and with differing eco-climatological regions over long temporal span was detected in the context of conservative dispersal ability across long distances separated by barriers. The results are in agreement with previous findings in suggesting that population connectivity in aquatic insect communities can be quite low over short distances, despite the potential for overland dispersal during adult life stages [22,23,24]. A better understanding of dispersal and ovipositional behavior and their relationships with gene flow and finer scale landscape features are required to determine the general importance of such barriers [31].

The potential influence that these environmental differences could have on local demography of stream insects could thus drive the differences in genetic diversity between eastern and western streams. History is an alternative explanation for the correlation between geology and genetic structure and diversity. The result indicated that the lower diversity of the Eastern streams may be the presence of thick vegetations, geographical isolations and population bottlenecks. However, patterns of genetic diversity within and among natural populations may also have been influenced by post-pleistocene colonisation, historic or recent population

bottlenecks, landscape features, contemporary ecological selection processes, anthropogenic impacts and climate change. Furthermore, distinguishing between the influences of these processes on the genetic structure of *P. courtallensis* requires a detailed genetic analysis of both nuclear and mitochondrial DNA variation from populations throughout a greater portion of its South Indian range.

Future work can be focused on application of highly variable nuclear markers which may enable determination of whether populations are ecologically interdependent (that is, if population vital rates are affected by dispersal). Whether the apparently low dispersal rates are specific to the landscape within the study area or whether this is a species-level trait remains to be determined. The significant population genetic structure and isolation by distance are mostly likely the result of limited gene flow, and may have been augmented by regional differences in the temporal variability of population sizes related to local variation in bedrock and surficial geology as pointed out by Addison ^[32]. Protection of this genetic diversity may be vital in retaining a species' adaptive capacity and evolutionary potential. The present study reveals high genetic diversity between the eastern and western side populations. Of these, we are of the opinion that both populations deserve the highest level of conservation effort.

5. Conclusion

Genetic diversity is essential for the maintenance of the evolutionary potential of species and accurate projections of the future distribution of intraspecific genetic variability. The mtCOI sequences of 492–522 bp were obtained for eight individuals collected at eight different study sites. Two clusters were resolved based on the distribution of individuals in the Neighbor-Joining tree. This South Indian mayfly species, *P. courtallensis* had high intraspecific differences between sites across wide spatial distribution. The results presented here support the conclusion that *P. courtallensis* is a species complex. The levels of COI diversity observed suggest the presence of two genetic distinct populations and their genetic diversity should be conserved in the long term.

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