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Molecular characterization of some drosophilid species from biodiversity rich Uttarakhand state of India

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Abstract
Uttarakhand state in Central Himalayan region of India, bestowed with varied ecological habitats ranging from near tropics to the mid-elevation forests dominated by cool-temperate taxa; is truly a drosophilid biodiversity hotspot. With more than 90 species reported so far from the state and several new to science, it could definitely be a potential arena for systematics, cytogenetics and evolutionary biology research. Though, in past some researchers have worked towards exploration of drosophilid diversity, they have mainly concentrated their efforts on taxonomic data-basing and other research implications have largely been neglected. This work is the first earnest attempt towards mitochondrial DNA based molecular characterization of some drosophilid species inhabiting Garhwal region of Uttarakhand. Molecular data and sequence analysis of mtCOII region is provided here, which would help in resolving taxonomic ambiguities between closely related sibling species and strengthen the drosophilid taxonomic database of the region.

Keywords: COII gene, *Drosophila*, Garhwal, mitochondrial DNA.

1. Introduction
Drosophila is among the best-studied model organisms in modern biology, with now twelve fully sequenced genomes available in public domain [1]. In spite availability of such large genetic and genomic resources, meagre is known so far concerning the phylogenetics, ecology and evolutionary history of species inhabiting biodiversity rich Uttarakhand region of India. Drosophilidae is a large family of acalyptrate Diptera of worldwide distribution. The German Dipterist, Hermann Lowe first used the family name Drosophilidae in his several papers published in 1862. The first complete catalogue of the family listed more than 2,500 species in 55 genera [2]. Drosophilidae is the most diversified family with two subfamilies, Steganinae and Drosophilinae. Further, Yassin *et al.* [3] documented that this family encompasses 4,000 described species distributed in 76 genera. At present, the database on Taxonomy of Drosophilidae compiled by Gerhard Bächli, (dated 2014/07) reports 6,568 species of which, *Drosophila* Fallén 1823 is the largest with more than 1,150 species recorded so far [4].
The Indian subcontinent, bestowed with highly varied geographic conditions, also has its significant share in distribution of world *Drosophila* fauna. Considerable data regarding drosophilid fauna was collected through extensive field explorations in different ecological habitat [5]. The drosophilid flies thus obtained since long have enriched our knowledge in various field *viz.*, systematics, ecology, evolutionary biology, genetics and behavioural biology as well. Around more than 300 drosophilid species have been recorded so far from varied eco-geographical zones in India [6, 7]. Central Himalayan region of India, especially Uttarakhand, encompasses highly varied tropical to temperate regimes in span of just few hundred kilometres due to its variable altitudinal terrains all over, thus offering an abode for the rich and luxuriant flora and fauna. Earlier researchers in this region have mainly concentrated their efforts on taxonomic data-basing of the drosophilid fauna with around more than 90 species being reported so far [8], significant number of them are new to the world of science but their other research implications like in molecular systematics and evolutionary biology have been largely neglected.

Drosophilidae, being a large acalyptrate Dipteran family shows a wide distribution, in this region as well. The previous studies, however, have focused on the taxonomic classification based on morphological characters; phylogenetic evaluations especially in this region have never been conducted. Many times taxonomic ambiguities have been observed due to morphological and meristic similarities. Modern taxonomic approaches include analysis of a host of other traits, including anatomy, physiology, behavior, geography and genetic constituent. Hitherto, in recent past a wide variety of protein- and DNA-based methods have been used for the genetic characterization of drosophilid species. The analysis of short, standardized genomic regions (DNA barcodes) can discriminate morphologically similar species. In particular, the mitochondrial regions like Cytochrome oxidase subunits (COI, COII) and 16S rRNA, conserve as a uniform target for a molecular-identification system.

Thus, with advent of plethora of molecular techniques and recent sequencing of the genomes of many *Drosophila* species and their expanding implication in comparative biology all over the world, it is important to present before the *Drosophila* research community, a better taxonomic framework and relationships of the common and novel species reported by us from Uttarakhand region. This work is the first earnest attempt towards mitochondrial DNA based molecular characterization of some drosophilid species inhabiting Garhwal region of Uttarakhand. Molecular data and sequence analysis of mtCOII region is provided here, which would not only help in resolving taxonomic ambiguities between closely related sibling species and strengthening the drosophilid taxonomic database of the region but may also help us to understand the process of ecological adaptations that occurred over the history of this group.

2. Materials and Methods

2.1 Study specimens

Ten drosophilid flies analyzed in this study were collected in different sampling surveys during April-2013 and March-2014 at Srinagar-Garhwal (550 m asl, Lat. 30° 22´ N and Long. 78° 78´ E) and Kandoliya forest (1680m asl, Lat. 30° 80' N and Long. 78° 46' E) in Pauri District, Garhwal region of Uttarakhand (Table 1).

Table 1: Species studied, Collection locality and GenBank Accession Number (COII gene)

S. No.	Species	Sampling Station	GenBank Accession Number
1.	*Drosophila busckii*	Srinagar, Garhwal	KF601930
2.	*Drosophila immigrans*	Srinagar, Garhwal	KF601931
3.	*Drosophila melanogaster*	Srinagar, Garhwal	KF601932
4.	*Drosophila repleta*	Srinagar, Garhwal	KF601933
5.	*Zaprionus indianus*	Srinagar, Garhwal	KF601934
6.	*Drosophila bifasciata*	Kandoliya, Pauri	In Process
7.	*Drosophila nepalensis*	Kandoliya, Pauri	In Process
8.	*Scaptomyza himalayana*	Kandoliya, Pauri	In Process
9.	*Scaptomyza tistai*	Kandoliya, Pauri	In Process
10	*Zaprionus grandis*	Kandoliya, Pauri	In Process

2.2 Sample collection

The cosmopolitan species of fruit fly were easily collected from the rotten fruits, fungi, flowers and other vegetables materials rich in carbohydrate, but the collection of drosophilid flies from the natural habitats required the application of a large range of techniques. The following methods were used during the present survey. The specimens studied were preserved in 70% ethanol.

(i) Trap bait method: Collection was made largely through the use of small containers baited with yeasted banana or some others fermenting fruits such as oranges, tomato, guava, apples etc, suspended by strings from the branches of bushes and the trees. This method was found very effective for collecting species particularly belonging to the two sub-genera, *Sophophora* and *Drosophila* of the genus *Drosophila*. The banana trap design of Prof. Toda, was also used [9].

(ii) By net sweeping: Unlike *Drosophila*, members of the others genera of the family Drosophilidae are rarely or only occasionally attracted towards fermenting fruits. Thus, collection by a modified insect net over natural feeding sites such as decaying fruits and leaves, wild grasses and cultivated vegetation was therefore done to capture these flies.

(iii) Direct collection with aspirator: This method was frequently employed to trap those flies showing occasional appearance and also under the situations where net sweeping was rather not possible. Such flies were caught directly with the help of an aspirator while they were either courting or resting over the leaves, petals, fungi etc.

2.3 Identification and Morphological Study

Collected flies were etherized, categorized and species were identified under margined stereo zoom microscope with the help of study of head, thorax, wing, body length and genital structures [10].

External morphology of adult flies was examined under a stereomicroscope and metric characters were measured with an ocular micrometer. To observe the detailed structures of male and female terminalia and other respective organs were detached from the adult body and whole body was cleared by warming in 10% KOH solution around 100 °C for 15-20 minutes, and thus observed in a droplet of glycerol under a light microscope. The established morphological terminology and the definitions of measurements and indices were mostly followed [11-13]. The examined specimens as well as the holotype and paratypes of all species have been deposited in the Cytogenetics Laboratory, Department of Zoology, H.N.B Garhwal University, Chauras Campus, Srinagar-Garhwal, Uttarakhand, India.

2.4 Establishment of Stock culture

The individual females, which could not be identified, were

isolated and allowed to breed in separate vials containing standard laboratory food medium. The progeny thus obtained from such single gravid females was used for species identification study.

2.5 DNA Extraction from Single fly

The isolation procedure was standardized to isolate DNA for PCR from single fly-Each fly was mashed in 50 µl of fly lysis buffer (20 mM Tris-Cl pH 8.5, 1.5 mM EDTA, 30 mM NaCl and 250 µg/ml of freshly prepared Proteinase K). The mixture was incubated at 25-37 °C for 45 minutes. Finally, the Proteinase K was inactivated at 95 °C incubation for 5 minutes. Typically, 3 µl of this DNA preparation was used for 25 µl PCR reaction volume.

2.6 PCR Amplification of COII Region

PCR was performed in reaction mixture of 25 µl and 39 cycles of PCR were performed in a Gradient DNA thermo-cycler (Techne). Primer details (Table 2), PCR mixture details (Table 3) and the thermo cycling parameters (Table 4) are given in the following tables:

Table 2: Primer details

Gene	Primer Sequence	Product Length	Reference
CO II	F1- 5' ATGGCAGATTAGTGCAATGG 3'	688 bp	O'Grady 1999 [14]
	R1- 5' GTTTAAGAGACCAGTACTTG 3'		

Table 3: PCR Mixture Preparation

Components	Final Concentration in single PCR Tube	Volume (µl)
Autoclaved distilled water		17.8 µl
Taq Assay Buffer (10X) with $MgCl_2$	1X	2.5 µl
dNTPs Mix (10 mM each)	2.5 mM	0.5 µl
Primer (10 pmole/µl)	0.1 pmole/µl	0.5 µl F 0.5 µl R
Taq polymerase (3U/µl)	1U/ µl	0.2 µl
DNA Template	10-30 ng	3.0 µl
Total		**25.0 µl**

Table 4: Thermal Cycle Conditions

Cycle	Denaturation		Annealing		Extension	
	Temperature	Time	Temperature	Time	Temperature	Time
First Cycle	Initial denaturation 94 ˚C for 3 min					
39 Cycles	94 ˚C	30 sec	55 ˚C	45 sec	72 ˚C	1 min
Last cycle	Final extension 72 ˚C for 7 min					
Hold	4 ˚C					

2.7 DNA Sequencing

The PCR products were visualized on 1.5% agarose gels and the most intense products were selected for sequencing. Products were labeled with the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc., Foster City, California, USA) and sequenced bidirectionally using an ABI 3730 capillary sequencer following the manufacturer's instructions.

2.8 Sequence Analysis and Submission

Sequences were aligned using Clustal W. Length differences were resolved by inserting alignment gaps and positions that could not be aligned unambiguously were excluded. Base composition, number of transition and transversion between species were estimated from aligned sequences using MEGA 6.0 (Molecular Evolutionary Genetic Analysis). Genetic relationships among drosophilid species were estimated based on the pairwise matrix and sequence divergence was calculated. Phylogenetic history was inferred using the Maximum Parsimony (MP) method.

2.9 Sequence Submission

Partial mitochondrial COII sequences were submitted in NCBI GenBank for accession numbers. Five species viz., *Drosophila busckii*, *Drosophila immigrans*, *Drosophila melanogaster*, *Drosophila repleta* and *Zaprionus indianus* were provided with accession numbers KF601930 to KF601934, respectively while for other five species accession numbers are awaited.

3. Results and Discussion

Stop codons were absent from all amplified sequences of COII and the amplified sequences were on average 633 bp in length (range 618-640). This also indicates NUMTs (Nuclear DNA Sequences originating from mtDNA sequences) were not sequenced. In addition, because of the higher copy number of mitochondrial DNA, some studies have shown that NUMTs are detected though in a very small percentage. Moreover when detected, NUMTs regularly show indels or diagnostic mutations (e.g. stop codons) so as to reveal their presence. So in the present study, we detected no signs of pseudogenes. Further, the COII region, analysis revealed overall average nucleotide frequencies as A = 32.90%, T/U = 39.60%, C = 13.80%, and G = 13.70%. For estimating ML values, a tree topology was automatically computed. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Species-wise base composition is depicted in Table 5.

Table 5: Base composition (%) at first, second, and third codon positions of COII gene

	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
Drosophila bifasciata	40.9	13.4	33.2	12.5	641.0	41	17.8	26.6	15.0	214.0	50	5.6	43.0	.9	214.0	31	16.9	30.0	21.6	213.0
Drosophila nepalensis	41.1	11.7	33.5	13.8	618.0	41	18.0	26.8	13.7	205.0	50	2.4	45.1	2.4	206.0	32	14.5	28.5	25.1	207.0
Drosophila busckii	38.6	15.1	31.7	14.6	690.0	39	18.3	27.4	15.2	230.0	47	10.0	38.1	5.2	231.0	30	17.0	29.7	23.6	229.0
Drosophila immigrans	39.5	13.7	33.3	13.4	693.0	41	16.5	27.7	14.7	231.0	46	9.1	41.8	3.0	232.0	31	15.7	30.4	22.6	230.0
Drosophila melanogaster	39.3	13.1	33.7	13.9	624.0	41	16.7	28.2	13.9	209.0	47	6.7	43.3	3.4	208.0	30	15.9	29.5	24.6	207.0
Drosophila repleta	38.4	15.5	33.3	12.8	709.0	39	17.7	29.1	13.9	237.0	46	10.5	39.2	4.6	237.0	30	18.3	31.5	20.0	235.0
Scaptomyza himalayana	38.0	15.1	32.1	14.8	635.0	41	17.9	26.4	15.1	212.0	44	9.0	41.0	5.7	212.0	29	18.5	28.9	23.7	211.0
Scaptomyza tistai	39.4	14.0	32.6	14.0	629.0	40	18.1	25.7	15.7	210.0	47	6.7	43.5	2.9	209.0	31	17.1	28.6	23.3	210.0
Zaprionus indianus	39.0	14.4	33.0	13.5	687.0	40	19.2	27.5	13.1	229.0	43	8.7	43.2	4.8	229.0	34	15.3	28.4	22.7	229.0
Zaprionus grandis	41.7	11.9	33.0	13.4	640.0	41	18.2	26.2	15.0	214.0	52	2.3	43.5	1.9	214.0	32	15.1	29.2	23.6	212.0
Avg.	39.6	13.8	32.9	13.7	656.6	40	17.8	27.2	14.5	219.1	47	7.2	42.1	3.5	219.2	31	16.4	29.5	23.0	218.3

As expected, average transitional pairs were more frequent than transversional pairs. In the COII region the estimated Transition/Transversion bias (*R*) is 1.42. Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+G+I) [15]. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 715 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 [16]. In Table 6 each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row are to be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. Codon positions included were 1st+2nd+3rd+Noncoding.

The transitional bias suggests that this is a recently evolved group or slowly evolving genes. A transition bias in these genes means that there are few multiple substitutions and that the data therefore have phylogenetic signal [17]. Overall the lower rate of transversions should lead to better resolution of deep divergence events because of low saturation effects.

Table 6: Transition Vs Transversion Bias in COII gene

	A	T/U	C	G
A	-	*7.20*	*2.52*	**4.33**
T/U	*6.00*	-	**12.66**	*2.49*
C	*6.00*	**36.20**	-	*2.49*
G	**10.42**	*7.20*	*2.52*	-

Sequence divergence among species was also calculated. In the COII region, the mean genetic distance computed for all the species was found to be **0.139**. The highest sequence diversity (**0.178**) was between *Drosophila melanogaster* (of subgenus *Sophophora*) and *Drosophila busckii* (of subgenus *Dorsilopha*) and the lowest value (**0.083**) was between species *Drosophila melanogaster* and *Drosophila nepalensis* both belonging to subgenus *Sophophora*. Pair-wise genetic distance values between sequences based on COII using MEGA 6 are given in Table 7.

Table 7: Estimates of Evolutionary Divergence between all Species based on COII Sequences

		1	2	3	4	5	6	7	8	9	10
1	*Drosophila bifasciata*										
2	*Drosophila nepalensis*	0.105									
3	*Drosophila busckii*	0.145	0.151								
4	*Drosophila immigrans*	0.135	0.105	0.157							
5	*Drosophila melanogaster*	0.143	0.083	0.178	0.151						
6	*Drosophila repleta*	0.151	0.136	0.159	0.149	0.170					
7	*Scaptomyza himalayana*	0.142	0.149	0.154	0.140	0.161	0.174				
8	*Scaptomyza tistai*	0.107	0.119	0.156	0.152	0.143	0.157	0.118			
9	*Zaprionus indianus*	0.150	0.135	0.164	0.137	0.175	0.169	0.148	0.131		
10	*Zaprionus grandis*	0.113	0.092	0.119	0.119	0.123	0.140	0.124	0.111	0.111	

The evolutionary history was inferred using the Maximum Parsimony method (Figure 1). Tree #1 out of 2 most parsimonious trees (length = 471) was considered. The consistency index is 0.653928 (0.500000), the retention index is 0.329218 (0.329218), and the composite index is 0.215285 (0.164609) for all sites and parsimony-informative sites (inparentheses).

The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [18] in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 715 positions in the final dataset.

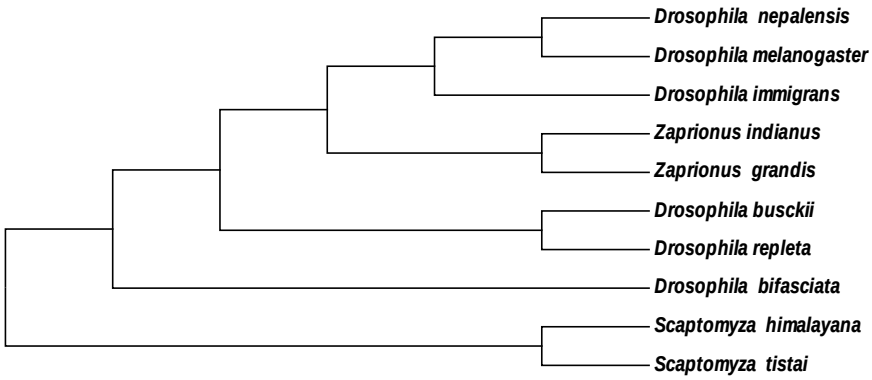

Fig 1: Maximum Parsimony tree analysis

4. Conclusion

Considering the high rate of discovery of novel drosophilid species from this region, molecular characterization tools can be useful to speed up the initial recognition of new units that may represent undescribed species. Towards the cause, this study was the first earnest attempt towards mitochondrial DNA based molecular characterization of ten drosophilid species inhabiting Garhwal region of Uttarakhand. It provides a framework for the further development of simple, low expense, PCR-based assays that can clearly differentiate virtually all drosophilid species from the region. This work would not only help in resolving taxonomic ambiguities between closely related sibling species and strengthening the drosophilid taxonomic database of the region but also to understand the process of ecological adaptations that occurred over the history of this group.

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