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Molecular Characterization of Ephemeroptera (Mayfly) Inhabiting River Alaknanda, Uttarakhand Based on Mitochondrial Control Region

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Abstract The present work focused to investigate molecular pattern in the Mitochondrial COl gene region so as to acquire a few initial data on genetic diversity and molecular structure of the Order Ephemeroptera in River Alaknanda, Uttarakhand. Overall 34 samples were collected from 8 sites located at different altitudes. We distinguished 17 polymorphic sites in an average 543 bp fragments acquired for the mitochondrial control region. The average nucleotide frequencies were A=24.87% T/U=35.84%, C = 20.20% G = 19.09% and Transition/Transversion bias (R) of 1.641 indicating that this is a recently evolved group or slowly evolving genes. These acquisitions will provide a base line data for future studies on macroinvertebrates from the region. This approach will remarkably widen application of DNA barcoding in biodiversity studies.

Keywords Ephemeroptera, Macroinvertebrates, Mitochondrial COl, DNA barcoding, Alaknanda.

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Introduction

Ephemeroptera (Mayfly) is a monophyletic order of semi pterogyte insects, consisting of 3000 species 400 genera and 42 reported families (Brittain and Sartori 2003). Mayflies present in freshwater and brackish water habitats and are major part of food chain. Mayflies (Order Ephemeroptera) are fundamental part of mountain streams being subsidiary producers and major fish food (Cranston 1995). They are also worked as an indicator of biological pollution. In Uttarakhand though ephemeropteran have been reported from most of the rivers, identification afar generic level remains to be done. Due to this region they are ideal objects for phylogenetic, systematic and taxonomic studies (Kumar et al. 2016). To our knowledge no molecular work of this kind was undertaken on mayflies in Uttarakhand. Despite an expanding demand of taxonomic proficiency in the aquatic expertise in the aquatic sciences, the number

of taxonomists continues to dwindle (Wheeler et al. 2004). Moreover morphology based indentification of mayflies species is incredibly demanding. Taxonomic keys ordinarily exist only for a certain life stage. Hebert et al. (2003a) prescribed that integrating DNA barcoding into conventional taxonomic tools would be a powerful approach for revealing hidden biodiversity. Furthermore, rapid and authentic than conventional methods alone. DNA barcoding can conquer the questions related to morphological identifiction and can provide a possibility for making regular species level identifications.

DNA sequences can be simply acquired, examined and elucidated. These tools are highly precise for macroinvertebrate species identification (Hebert et al. 2003b). Molecular techniques have demonstrated convenient to identify ambivalent life stage of macroinvertebrates and provide an authentic and quick approach to form regular identification of recognized species. The COl region of Mt DNA is especially useful for governing inter and intra-specific phylogenetic relationships at the genus and species level and within families (Caterino and Sperling 1999, Logan 1999, Trewick 2000). DNA sequencing of COl gene region has proven useful for understanding species diversity (Blaxter et al. 2004, 2005) and is often included as part of integrated taxonomic studies (Gurvey et al. 2001, Sharley et al. 2004 Sivaramakrishnan et al. 2014, Stribling et al. 2008).

Materials and Methods

Study area

The study area falls in the State of Uttarakhand, in the Northern part of India between the latitudes 28°43'—31°27'N and longitudes 77°34'—81°02'E. River Alaknanda arises from the satopanth and the Bhagirathi Kharak glacier at an imprecise altitude of 3641m asl in the Garhwal region of the State of Uttarakhand. For collection of seasonal ephemeropteran samples, 08 sites constituting dissimilar altitudinal zones were selected depending on their easy approachability. These selected sampling sites were Mana, Vishnuprayag, Birahi, Nandprayag, Karanprayag, Rudraprayag, Srinagar and Devprayag (Table 1).

Table 1. Location of sampling sites on River Alaknanda.

Sl. No.	Sampling sites	Latitude	Longitude	Elevation (m asl)
1	Mana	30°46′14′′	79°29′40′′	3150
2	Vishnuprayag	30°33′44′′	79°34′35′′	1445
3	Birahi	30°24′29′′	79°23′16′′	1040
4	Nandprayag	30°19′54′′	79°18′57′′	855
5	Karanprayag	30°15′46′′	79°12′58′′	760
6	Rudraprayag	30°17′14′′	79°17′43′′	620
7	Srinagar	30°13′46′′	79°47′15′′	535
8	Devprayag	30°08′43′′	78°35′52′′	465

Collection and identification of samples

Benthic macroinvertebrates of Order Ephemeroptera were collected from all the selected sampling sites on Alaknanda River catchment. Samples were collected seasonally following stratified random sampling (Cummins 1962) using kick-method and modified Surbers square foot sampler (Welch 1952).

Identification was carried out to the lowest recognizable level usually genera, in the laboratory with the help of keys by Usinger (1950), Ward and Whipple (1959), Needham and Needham (1962), New (1996), Macan (1979), Tonapi (1980) and Edington and Hildrew (1995). Ephemeropteran samples were used for mitochondrial DNA sequence analysis.

Sample preservation

The collected ephemeropteran samples were brought to the laboratory and preserved in 90% v/v ethanol in 2 ml cryopreservatory vials. These samples were then kept at 4°C for further use. For the DNA extraction, single specimen was used. The ephemeropteran individuals were surface cleaned with sterile deionised distilled water. The tissue from the thorax region were taken and immediately used for DNA isolation.

DNA extraction

The chemicals and reagents used for DNA extraction were as per specified standard protocol (Sambrook et al. 1989). Following protocols were used for isolation of DNA.

Each sample was meshed for 5—10 seconds with

a pipette tip containing 50 µl of squishing buffer without depressing the pipette plunger (so that sufficient liquid escapes from the tip). The remaining squishing buffer was expelled into sample mixture. The sample was incubated at 25 —37°C (or room temperature) for 20—30 minutes, Proteinase K was inactivated by heating to 95°C for 1—2 minutes.

Primer selection and PCR amplification

For the amplification of the COl region of approximately 548 bp, forward primer LCO1490 (5'–GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Vrijenhoek 1994) were used. The primer set used in the PCR reaction resulted in the amplification of the homologous fragments from all the tested ephemeropteran species.

The PCR reaction mixture was prepared in a total volume of 50 µl with 100 ng of genomic DNA, a 2.5 nm concentration each of the dATP, dTTP, dCTP and dGTP, 1oo ng each of the forward primer and reverse primer, 3U of taq DNA polymerase enzyme and 1X Taq DNA polymerase assay buffer (10X) and the remaining volume of with glass distilled water (Banglore Genei, India). The PCR reactions were conducted on Mj Mini-BIO RAD Thermal cycler.

The PCR reactions cycles consisted of initial denaturation for 5 minutes at 94°C, 35 cycle of 94°C for 30 seconds (denaturation), 60°C for 40 seconds (annealing) and 72°C for 40 seconds (extension) and followed by the final extension of 72°C for 10 minutes. After amplification 2.5 µl sample buffer was added to the amplified products. The products were then analyzed by electrophoresis in 1.8% (W/v) agarose gel at 70 v for about 3 h using 0.5×TAE buffer (Tris-acetate 40 mM, (pH 8.0). EDTA 1mM pH 8.0 (Sambrook et al. 1989).

Sequence analysis

Sequence of all ephemeropteran individuals were single aligned utilizing the program Clustal X 2.0 (Larkin et al. 2007). Length dissimilarity was determined by inserting alignment gap and position that could not be alingned unambiguously were excluded. The degree of sequence disparity was calculated by equating pair-wise contrasting of sequence dissimilarity over all ephemeropteran individuals. All inclusive base composition number of transition and transversion from aligned sequence and pair-wise evolutionary distance between haplotypes was resolved using molecular evolutionary genetic study (MEGA) version 6.0 (Tamura et al. 2013, 2004).

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	A	T/U	C	G
A	–	6.66	3.75	**12.47**
T/U	4.62	–	**12.3**	3.55
C	4.62	**21.82**	–	3.55
G	**16.25**	6.66	3.75	–

Results and Discussion

The amplified sequences of mayflies were on approximate 543 in length. NUMTs (Nuclear DNA sequences originating from mtDNA sequences) were not sequenced. Moreover, because of the higher copy number of mtDNA, few studies have allowed that NUMTs find out though in a very low percentage. Furthermore, when find out, NUMTs regularly exhibit mutations so as to unveil their presence, which was not remarked in current analysis. In the present study we recognize no indication of pseudogenes. Additionally, this mitochondrial region analysis unveiled overall average nucleotide frequencies A=24.87% T/U = 35.84%, C = 20.20% G=19.09% for evaluating ML values, a tree topology was automatically enumerated. The maximum log likelihood for this computation was - 6034.0588. The analysis involved 17 nucleotide sequences. There were an overall 536 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0 (Tamura et al. 2013).

Table 2 shows maximum likelihood determine of substitution matrix for all sequences. Every entry is the possibility of substitution (r) from one base (row) to another base (Column). Substitution pattern and rates were evaluated under the Tamura-Nei model (Tamura and Nei 1993). Rates of distinct transitional substitutions are exhibited in bold and those of transversional substitutions are shown in italics. Relative

Table 3. Base composition % for mitochondrial region of Order Ephemeroptera.

Samples	T (U)	C	A	G	Total
Heptagenia sulphurea Hs1	36.1	19.9	25.7	18.2	653.0
Heptagenia sulphurea Hs2	35.5	20.2	25.9	18.4	640.0
Heptagenia sulphurea Hs3	35.5	20.5	25.5	18.5	643.0
Heptagenia sulphurea Hs4	36.1	19.5	25.5	18.9	640.0
Heptagenia sp. Hsp 1	36.9	18.4	26.7	18.0	651.0
Heptagenia sp. Hsp2	36.5	18.5	26.7	18.3	644.0
Heptagenia sp. Hsp3	36.6	18.3	26.2	18.8	644.0
Baetis rhodani Br1	38.2	18.4	22.5	20.9	652.0
Baetis rhodani Br2	39.6	17. 9	23.2	19.3	647.0
Baetis rhodani Br3	38.2	18.4	22.6	20.7	651.0
Baetis rhodani Br4	37.6	18.5	22.6	21.3	628.0
Heptagenia sp. Hsp4	36.6	18.6	26.4	18.3	644.0
Heptagenia sp. Hsp5	37.6	17.6	26.9	17.8	635.0
Heptagenia sulphurea Hs5	36.0	19.7	25.8	18.5	644.0
Baetis muticus Bml	33.9	20.6	23.7	21.8	625.0
Baetis muticus Bm2	34.0	19.3	22.1	24.6	606.0
Baetis muticus Bm3	34.3	19.0	21.3	25.4	606.0
Average	35.8	20.1	24.5	19.6	631.1

values of r were examined when assessing them. For simplicity, sum of r value made equal in 100 (Table 2).

Maximum likelihood estimate of transition/transversion

The evaluated transition/ transversion bias (R) calculated was 1.641. Substitution pattern and rates were evaluated under the Tamura-Nei model (1993). The nucleotide frequencies are A=24.87% T/U =35.84%, C=20.20% G=19.09%. For evaluating ML values a tree topology was automatically calculated. The maximum log likelihood for this computation was -6034.0588. The analysis involved 17 nucleotide sequences. There were an overall 536 positions in the final dataset (Table 3).

Evolutionary analyses were handled in MEGA

Table 4. Overall estimates of evolutionary divergence between sequences.

	H. sulphurea Hs1	*H. sulphurea* Hs2	*H. sulphurea* Hs3	*H. sulphurea* Hs4	*H.*sp. Hsp1	*H.*sp. Hsp2	*H.*sp. Hsp3	*B. rhodani* Br1
H. sulphurea Hs1								
H. sulphurea Hs2	0.007							
H. sulphurea Hs3	0.007	0.013						
H. sulphurea Hs4	0.017	0.009	0.022					
H. sp. Hsp1	0.098	0.102	0.104	0.109				
H. sp. Hsp2	0.098	0.102	0.104	0.109	0.004			
H. sp. Hsp3	0.102	0.106	0.108	0.113	0.013	0.009		
B. rhodani Br1	0.308	0.311	0.315	0.319	0.295	0.289	0.295	
B. rhodani Br2	0.305	0.314	0.316	0.322	0.303	0.298	0.303	0.013
B. rhodani Br3	0.316	0.319	0.324	0.327	0.303	0.297	0.303	0.006
B. rhodani Br4	0.319	0.321	0.326	0.330	0.306	0.300	0.306	0.015
H. sp. Hsp4	0.109	0.115	0.113	0.121	0.030	0.032	0.041	0.301
H. sp. Hsp5	0.113	0.117	0.119	0.123	0.019	0.022	0.028	0.311
H. sulphurea Hs5	0.002	0.009	0.009	0.019	0.100	0.100	0.104	0.305
B. muticus Bm1	0.296	0.302	0.304	0.307	0.309	0.314	0.322	0.302
B. muticus Bm2	0.333	0.335	0.340	0.341	0.333	0.333	0.339	0.250
B. muticus Bm3	0.347	0.349	0.354	0.355	0.344	0.344	0.350	0.258

Table 4. Continued.

Samples	*B. rhodani* Br2	*B. rhodani* Br3	*B. rhodani* Br4	*H.* sp. Hsp4	*H.* sp. Hsp5	*H. sulphurea* Hs5	*B. muticus* Bm1	*B. muticus* Bm2	*B. muticus* Bm3
H. sulphurea Hs1									
H. sulphurea Hs2									
H. sulphurea Hs3									
H. sulphurea Hs4									
H. sp. Hsp1									
H. sp. Hsp2									
H. sp. Hsp3									
B. rhodani Br1									
B. rhodani Br2									
B. rhodani Br3	0.019								
B. rhodani Br4	0.024	0.020							
H. sp. Hsp4	0.306	0.309	0.311						
*H.*sp. Hsp5	0.320	0.319	0.322	0.038					
H. sulphurea Hs5	0.302	0.313	0.316	0.111	0.115				
B. muticus Bm1	0.302	0.311	0.316	0.325	0.328	0.293			
B. muticus Bm2	0.253	0.258	0.258	0.345	0.350	0.330	0.208		
B. muticus Bm3	0.261	0.265	0.265	0.356	0.361	0.344	0.213	0.011	

6.0. The transition bias demonstrates that this is a recently evolved group or slowly evolved genes. A transition bias in these gene means that there are few several substitutions and that the data therefore have phylogenetic signal. Mostly the lower rate of transversions should used to effective resolution of profound separation.

Mostly evaluates of evolutionary divergence between sequences was also computed. The numbers of base substitution per site from between sequences are shown in Table 4. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 17 nucleotide sequences. All positions carrying gaps and missing data were removed. There were a total of 536 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0 (Table 4).

The no. of base substitutions per site from averaging overall sequence pairs was shown. Analyses were conducted using the Kimura2-parameter model. The analysis involved 17 nucleotide sequences. All positions containing gap and missing data were removed. There were total 543 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0.

Taijma's test of neutrality the no. of segregating site was 202, the nucleotide diversity per site was 0.22140 and the Taijma's test statics D was -0.02728 and the theta (θ) were 0.22302 (Table 5). This test differentiates the number of segregating sites per site with the nucleotide assortment. Site is observed segregating if in a comparison of number of sequences, there were 2 or more nucleotide at the sites : Nucleotide assortment is explained as the average number of nucleotide dissimilarities per site between 2 sequences. It was earlier shown that if the population passes through a bottleneck, D can be remarkably positive or negative depending on the population history.

Thus, findings of the present study reveal molecular characterization of selected ephemeropteran Taxa inhabiting River Alaknanda in Uttarakhand. This will be instrumental in solving many problems related to taxonomy of this important order of aquatic insects. This work renders a base line data on mitochondrial control region of Order Ephemeroptera (Mayflies)

Table 5. Results from Tajima's neutrality test. N=Number of sequences, S=Number of segregating sites, Pi = Nucleotide diversity, and D is the Tajima test statics.

N	S	Pi	θ	D
39	202	0.22140	0.22302	-0.02728

which would be the premise for a better understanding of an important indicator group of macroinvertebrates for future study on them associated to population, taxonomic, systematic, and genetic studies. In future similar studies should be sustained out so as to detect variation in genetic structure based on molecular markers. Further the present study on mitochondrial control region from one of the ephemeropteran species could be utilized for relative studies in future with other macroinvertebrates.

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