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Intraspecific Genetic Diversity of Two Black Fly Species (Diptera: Simuliidae) from South India using DNA Barcode Based RFLP Analysis

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ABSTRACT

The population genetic diversity of two black fly species (*S.* (*S.*) *gurneyae* and *S.* (*S.*) *nilgiricum*) was investigated along an elevational gradient in two hills of South India by mitochondrial gene sequence of cytochrome coxidase subunit I (COI) based Restriction Fragment Length Polymorphism (RFLP). By comparing the similarity of the bands produced by three restriction endonuclease enzymes, the genetic distance was calculated among sampling sites. The mean Kimura two parameter divergences of two black fly species between sampling sites was observed less than 13% and the higher genetic differentiation occurred at higher elevational sites (>1000 m). These result suggest that hypothesis of speciation is related with higher elevational site in black flies.

Key words: COI gene, *S.* (*S.*) *gurneyae*, *S.* (*S.*) *nilgiricum*, elevation

INTRODUCTION

Black flies (Simuliidae) are a worldwide family of nematocerous Diptera that is well known for the hematophagous behavior of females and are transmission of the causative agents of parasitic diseases to birds and mammals. Some black fly species are vectors of filarial worms that cause onchocerciasis (river blindness). Black fly larvae constitute important component of running water habitats and used as bioindicators for water quality assessment (Harwood and James, 1979). According to the review of Adler and Crosskey (2015), 2,177 living and 12 fossil species of black flies have been recorded throughout the world. Of these, 61 named and 13 unnamed species in seven subgenera of the genus *Simulium* have been described in India. In general, Indian simuliids are nectar feeding habit but some species in northern India (*S.* (*E.*) *aureohirtum*, *S.* (*S.*) *christophersi*, *S.* (*S.*) *himalayense*, *S.* (*S.*) *indicum*, *S.* (*S.*) *novolineatum*, *S.* (*S.*) *rufibasis*, *S.* (*S.*) *striatum* and *S.* (*G.*) *tenuistylum*) are serious pests causing bloodsucking nuisance and local allergic reactions in human (Dhiman *et al*., 2014; Singh and Tripathi, 2003).

The vacational tourist spots (Nilgiri and Palani hills) of South India harbored higher number of *Simulium* species and conductivity, total dissolved solids and substrates in submerged water determined the distribution of black flies in Palani and Nilgiri hills (Dinakaran *et al*., 2009; Anbalagan *et al*., 2011, 2014a). The *S.* (*S.*) *gurneyae* (Senior-White, 1922) and *S.* (*S.*) *nilgiricum*

(Puri, 1932) are commonly found in Palani and Nilgiri hills, respectively (Anbalagan *et al*., 2011, 2014a). Therefore, these two black fly species were selected for the present study due to their ample distribution and abundance. The *S*. (*S*.) *gurneyae* is characterized by having tarsal claw with small basal tooth and yellowish mid femur in the female adults, mid tibia with yellowish gray and gradually darkened apically in the male adults and ventral pair of two filaments in respiratory gill much thinner than those of middle pair in the pupae (Anbalagan *et al*., 2014b). The morphological characters of *S*. (*S*.) *gurneyae* are dark brown mid femur in the females, brownish black mid tibia in the males and respiratory gill ventral pair of two filaments as thick as those of middle pair in the pupae (Anbalagan *et al*., 2014b).

Simulium members are structurally homogenous, difficult to identify or even impossible in many instances. Cytological identification is potential to identify larval stages only by using the giant polytene chromosomes (Conflitti *et al*., 2013). In recent years, molecular approaches in the taxonomy achieved rapid advancement for accurate identification of species by DNA barcodes. The DNA analysis has the advantage that differentiation can be undertaken at any developmental stage (Shaikevich, 2007). The DNA barcoding analysis received enough pledge for identification of black fly (Conflitti *et al*., 2013; Pramual *et al*., 2011). Although, genetic diversity studies using Restriction Fragment Length Polymorphism (RFLP) provided high-quality of outcomes in various species of insects, DNA barcode based RFLP is an effective tool to analyze polymorphism in insect species and consent to the identification of samples at any developmental stages (Shaikevich, 2007; Walsh *et al*., 2011).

Genetic diversity studies in black flies have been conducted on *Simulium gravelyi* by Random Amplified Polymorphic DNA (RAPD) (Anbalagan *et al*., 2012), *Psilopelmia* by internal transcribed spacer 1 of the nuclear ribosomal RNA gene (Tang *et al*., 1998), *Cnephia* by DNA barcode gene (Conflitti *et al*., 2013) and *Simulium tani* by DNA barcoding genes (COI and COII) (Low *et al*., 2014). Less attention has received on DNA barcode based RFLP analysis in black fly. Therefore, the current study aimed to determine the intraspecific genetic diversity of two black species (*S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum*) and to estimate the degree of genetic differentiation along elevational gradient, by using DNA barcoding gene of COI based RFLP analysis.

MATERIALS AND METHODS

Study area: Sampling was carried out in five streams in each Palani hills wildlife sanctuary and National Park and Nilgiri biosphere reserve (Fig. 1). Both locations are familiar tourist spots (Kodaikanal in Palani hills and Ooty in Nilgiri hills) in South India and these locate on the Southern part of Western Ghats. These two hills are heavily influenced by two monsoons (South-West and North-East), however, the maximum rainfall is received during South-West monsoon (June-August). The five streams were selected from lower elevation to higher elevation in each hill as following, Palani hills: Kumbakkarai (site 1), Moolayar (site 2), Kurusedi (site 3), Silver cascade (site 4) and Fairy falls (site 5) and Nilgiri hills: Kallar (site 1), Barliar (site 2), Coonoor (site 3), Manavalla (site 4) and Segur (site 5) (Fig. 1).

Sampling methods: Samplings of five streams were carried out between September, 2014 and January, 2015. In each sampling site, the population of *S*. (*S*.) *gurneyae* in Palani hills and *S*. (*S*.) *nilgiricum* in Nilgiri hills were identified and their larvae and pupae collected separately. The larval specimens were stored in 90-100% ethanol in the field and pupae were reared in a plastic container with wet condition until emergence. The emerged adults were identified and

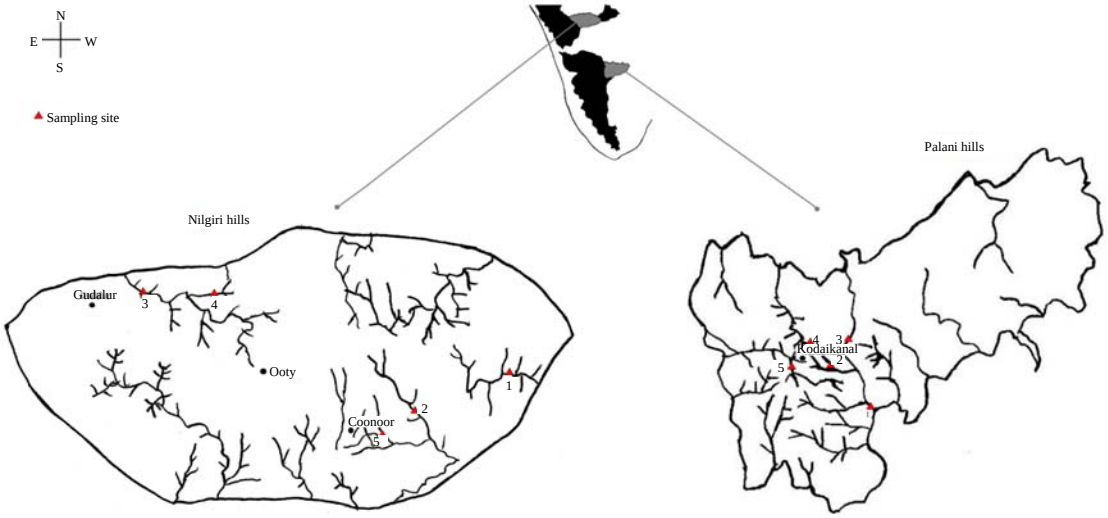

Fig. 1: Sampling locations in two hills of in Southern Western Ghats

Table 1: Physical and chemical parameters (Mean±SD) of sampling sites in two hills of Southern Western Ghats

	Palani hills					Nilgiri hills				
Sampling site	1	2	3	4	5	1	2	3	4	5
Latitude (N)	10º13'	10º11'	10º13'	10º14'	10º13'	11º20'	11º20'	11º19'	11º32'	11º30'
Longitude (E)	77º37'	77º31'	77º36'	77º31'	77º28'	76º52'	76º49'	76º48'	76º40'	76º41'
Elevation (m)	280	1080	1300	1600	2100	350	400	1350	870	970
Water temperature (ºC)	24 (1)	22 (2)	19 (1)	19 (1)	18 (2)	22 (1)	20 (1)	17 (1)	19 (1)	18 (1)
Dissolved oxygen (mg L^{-1})	11 (1)	12 (2)	9 (1)	9 (1)	10 (1)	9 (1)	10 (1)	11 (2)	12 (2)	8 (1)
pH	7.1 (0.2)	7.1 (0.1)	6.9 (0.1)	6.9 (0)	6.8 (0)	6.9 (0.1)	6.6 (0.2)	6.9 (0.2)	6.9 (0.2)	6.6 (0.1)
Total dissolved solids (mg L^{-1})	450 (30)	316 (10)	121 (8)	80 (10)	90 (15)	315 (24)	60 (8)	40 (5)	120 (15)	20 (3)
Conductivity (µ mhos)	0.1	0.09	0.04	0.01	0.01	0.12	0.07	0.01	0.01	0.01
Current velocity (cm sec^{-1})	0.05	0.04	0.06	0.05	0.06	0.03	0.05	0.04	0.06	0.04
Stream width (m)	9	8	5	6	1	2	3	1	2	4
Stream depth (cm)	25	10	10	8	6	10	5	5	5	10
Bedrock (%)	50	70	40	0	50	0	70	50	0	10
Boulders (%)	30	20	30	40	20	80	20	30	40	40
Pebbles (%)	20	10	20	40	30	20	10	20	40	40
Sand (%)	0	0	10	20	0	0	0	0	20	10

confirmed to the respective species. The physical parameters of water temperature, stream width and depth, substrates density were noted in each study site. The chemical variables in water of dissolved oxygen, pH, total dissolved solids and conductivity were measured using portable water analysis tester (PCS Testr 35, Eutech instruments, India). Latitude, longitude and altitude of sampling sites were taken with the help of Global Positioning System 12 (Garmin, India). The physical and chemical parameters of streams in two hills are given in Table 1.

DNA barcode based RFLP analysis: Genomic DNA from larva was isolated with the QIAamp genomic DNA isolation kit (Qiagen GmbH, Germany). The mitochondrial protein coding gene of cytochrome c oxidase subunit I (COI) was amplified by polymerase chain reaction using the primers LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994). The PCR reaction (20 µL) was conducted using the Exprime taq premix, Genet Bio Inc. (Korea) and the following volumes of

reagents: 2 μL of DNA extract, 10 μL of Mastermix, 1 μL of each primer (10 mM) and 6 μL of sterile water. The thermal cycling profile was: 5 min at 94ºC and 35 cycles of 30 sec at 94ºC, 60 sec at 58ºC and 1 min at 72ºC, with a final extension at 72ºC for 7 min. The amplified PCR products were identified by electrophoresis, using a 1% agarose gel and then purified using the Hiyield Gel/PCR-DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan). Finally, purified PCR products were sequenced by the automated DNA sequencer Model: 3500 (Applied Biosystems, Foster City, CA, USA). The DNA sequences were aligned using ClustalW and analyzed using MEGA version 6.0 (Tamura *et al*., 2012). The COI sequences are available in GenBank under accession numbers.

The amplified products were digested separately with KpnI (Promega), VspI (Asel) and MspI (Hpall) restriction endonucleases. Restriction enzyme digestions were performed in a 30 μL volume. The master mix digest consisted of 6 μL of the COI PCR product, 2 μL (1U) of enzyme, 2 μL 10X Buffer (Tango) and 20 μL Milli-Q. All mixtures were incubated for 1 h at 37ºC. The digested products were visualized on a 1% agarose gel.

Data analysis: Contigs of COI of newly described species and eight outgroup taxa were possibly obtained from GenBank were assembled and edited in SEQUENCER 5.0 (Genecodes, 2011). Protein-coding sequence of COI was imported into MEGA version 6 (Tamura *et al*., 2013) for alignment by amino acid. After the determining of the open reading frame, sequences were translated to amino acid and then aligned with ClustalW. The aligned amino acid was then back-translated to nucleotides and retaining the codon positions. Then the aligned sequences were taken for analysis. The genetic diversity indices were calculated based on the presence/absence of (1/0) of restriction fragments in gel. The unequal variance of test (Welch F-test) was calculated for identifying the heterogeneity of the restriction fragments among sampling sites. Further, the genetic distance of two black fly populations between sampling sites was measured using Jaccard similarity index and a phylogram was drawn by Neigbour Joining (NJ) clustering method with bootstrap support of 100 (PAST version 3.05).

RESULTS

The larvae of *S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum* from five streams in each hill were collected and genomic DNA extraction was performed. In these species, 650-700 bp of the mtDNA COI gene was successfully amplified (Fig. 2). The alignments of contigs consisted of 704 positions, of these *S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum* made up to 689 and 699 bases, respectively. The aligned contigs were checked in NCBI-BLAST to confirm the species similarity, which were matched with *S*. (*S*.) *bezzi* with 90% similarity. The mean base frequencies of *S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum* were A, 0.38; C, 0.17; G, 0.16 and T, 0.29, the estimated transition/transversion rate ratios were K_1 = 3.542 (purines) and K_2 = 1.875 (pyrimidines) and the overall transition/transversion ratio was 1.259. The mean Kimura two parameter divergence of *S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum* in the inter-site pairwise comparisons varying from 9.23-13.22%. The five sites of each hill showed inter-site pairwise distances was less than 13.22%.

Further, to study the intra-specific genetic diversity of COI gene in *S*. (*S*.) *gurneyae* and *S*. (*S*.) *nilgiricum* between sampling sites, PCR-RFLP was analyzed. In this analysis to digest the COI gene, we used three standard restriction endonucleases of *Kpn*I, *Vsp*I and *Msp*I. The restriction sites in aligned COI gene sequences of two species are obviously shown in Fig. 3. After digestion of COI gene in two black fly species by three restriction endonucleases, we obtained the outcomes of three obvious fragments (497, 267 and 127 bp) in *Msp*I, two fragments (468, 241 bp) in *Vsp*I and remains uncut in *Kpn*I (Fig. 4). Based on the restriction fragments obtained by three

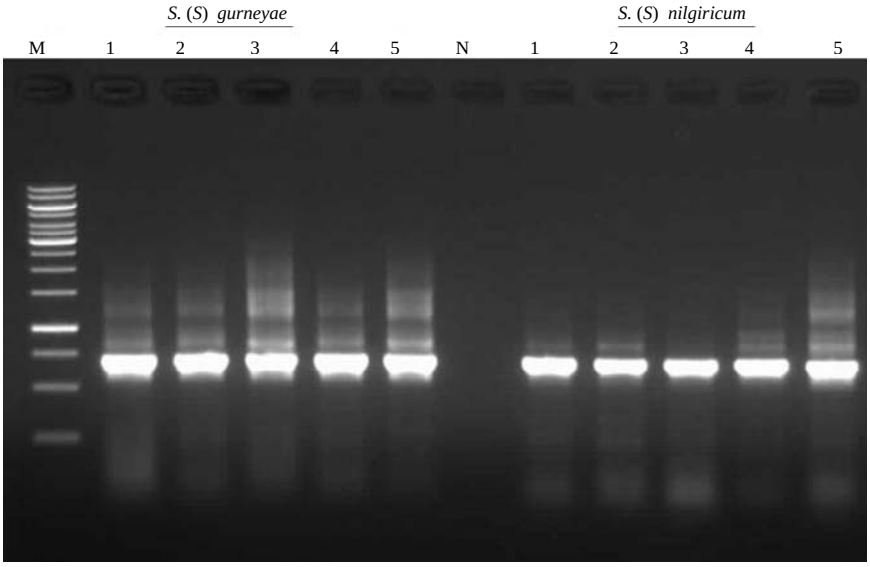

Fig. 2: Cytochrome c oxidase submit I gene amplification of two black fly species in sampling locations of Southern Western Ghats, M: 1 kb marker, N: Negative control, 1-5: Sampling sites

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                                    /KpnI                                   /MspI
S. gurneyae      ---CAAGGGTTTGC--TATGAT---TGTGTTATCCCCCACCGAGCAGGATCAAAAAATGA
S. nilgiricum    GGGAAAACGTTTGCATTATAATATATGTAGTACGTCCGACCACACCGGGTCAAAGAAAGA
                     **  ******  *** **   ***  **   ** ***   * ** ***** ** **

S. gurneyae      TGTATTTAAATTTCGATCTGTTAATAATATTGTAATAGCTCCGGCTAATACTGGCAATGA
S. nilgiricum    AGTATTTAAGTTTCGGTCTGTTAAAAGTATTGTAATAGCCCCGGCTAGTACAGGTAAAGA
                 ******** ***** ******** * ************ ******* *** ** ** **

S. gurneyae      TAAAAGTAACAACACTGCTGTAATAACAACAGATCAAACAAATAAAGGTATTCGATCAAA
S. nilgiricum    TAAAAGTAATAATACTGCTGTAATAACAACAGATCAAACAAATAAAGGTATTCGATCAAA
                 ********* ** **************************************************
                           /MspI                                   /VspI
S. gurneyae      TGTAATTCCATTTGATCGTATATTAATAATAGTTGTAATAAAATTTACAGCACCTAAAAT
S. nilgiricum    TGTAATTCCATTAGATCGTATATTAATAATAGTTGTAATAAAATTTACAGCCCCTAAAAT
                 ************ ****************************************** ********

S. gurneyae      AGATGAAATTCCTGCTAAATGTAAAGAAAAAATAGCTAAATCTACTGAAGCTCCAGCATG
S. nilgiricum    TGATGAAATTCCTGCTAAATGTAAAGAAAAAATAGCTAAATCTACAGAAGCTCCAGCATG
                 ************************************************ **************

S. gurneyae      AGCAATTCCAGAAGATAAAGGAGGGTATACTGTTCATCCTGTTCCAGCTCCTGCTTCTAC
S. nilgiricum    GGCAATTCCAGAAGATAAAGGAGGGTAAACTGTTCAACCTGTTCCAGCTCCTGCTTCTAC
                 *************************** ******** ************************

S. gurneyae      TATACTACTTGCTAATAACAAGGTAAGTGAAGGGGGAAGTATTCAAAAACTTATATTATT
S. nilgiricum    TATACTACTAGCTAATAGAAGAGTTAAAGAGGGGGGTAATATTCAAAAACTTATATTATT
                 ********* *******  *  ** *  ** ***** * **********************
                          /MspI                  /VspI
S. gurneyae      TATTCGAGGGAATGCTATATCAGGAGCTCCTAATATTAGAGGAACTAATCAATTTCCAAA
S. nilgiricum    TATTCGAGGGAAAGCTATATCAGGAGCTCCTAATATTAAAGGAACTAATCAATTTCCAAA
                 ************ ************************ **********************

S. gurneyae      TCCCCCAATTATAATTGGCATTACTATAAAAAAAATTATTACAAAAGCATGTGCTGTTAC
S. nilgiricum    TCCCCCAATTATAATAGGTATAACCATGAAAAAAATTATTACAAAAGCATGAGCTGTGAC
                 **************** ** ** ** ** ************************ ***** **
                                                    /MspI
S. gurneyae      AATAACATTATAAATTTGATCATCTCCAATTAGAGATCCAGGATGGCCTAACTCAGCTCG
S. nilgiricum    AATCACATTATAAATTTGATCATCTCCAATTAAGGATCCTGGGTGACCTAATTCAGCTCG
                 *** ***************************  ***** ** ** ***** ********

S. gurneyae      AATTAGTATTCTAAGAGAAGTTCCTACTATTCCTGCTCAAGCTCCAAAAATAAAATATAA
S. nilgiricum    AATTAGTATTCTTAGAGAGGTTCCTACTATTCCGGCTCAAGCCCCAAAAATAAAGTATAG
                 ************ ***** *************** ******** ************ ****

S. gurneyae      AGTTCCAATATCTTTAT-TATTTGTGTAAACACAAAAA-
S. nilgiricum    GGTTCCAATATCTTTATGTATTTATGGACACAAGCAAAC
                 ***************** ***** ** * ***   ***
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Fig. 3: Nucleotide sequence variants of the cytochrome oxidase I gene in *S.* (*S.*) *gurneyae* and *S.* (*S.*) *nilgiricum*. Asterisks indicate homology between two black fly species sequence. *Kpn*I, *Vsp*I and *Msp*I restriction endonuclease sites are in slash type

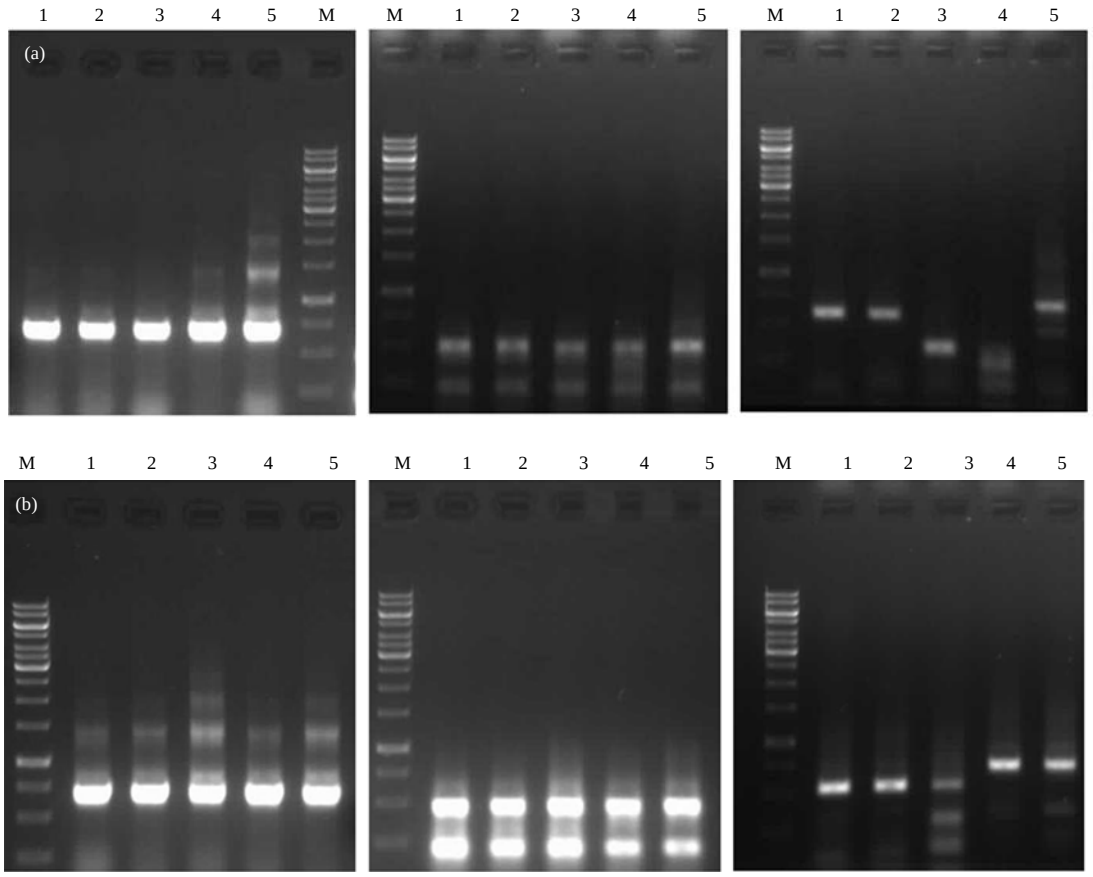

Fig. 4(a-b): PCR-RFLP of (a) *S.* (*S.*) *gurneyae* and (b) *S.* (*S.*) *nilgiricum* partial COI with restriction endonucleases of *Kpn*I, *Vsp*I and *Msp*I. M: 1 kb marker, 1-5: Sampling sites

Table 2: Genetic diversity indices for two black fly population in sampling sites

	S. (*S.*) *gurneyae*					*S.* (*S.*) *nilgiricum*				
Sampling site	1	2	3	4	5	1	2	3	4	5
α-diversity indices										
Polymorphism (%)	16.00	18.00	24.00	24.00	18.00	20.00	21.00	25.00	17.00	17.00
Dominance (D)	0.33	0.33	0.25	0.25	0.33	0.16	0.17	0.17	0.20	0.25
Simpson (1-D)	0.66	0.66	0.75	0.75	0.66	0.83	0.83	0.83	0.80	0.75
Shannon (H)	1.09	1.09	1.38	1.38	1.09	1.79	1.79	1.79	1.60	1.39
Evenness (H/S)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Brillouin	0.59	0.59	0.79	0.79	0.59	1.10	1.09	1.10	0.96	0.79
Menhinick	1.73	1.73	2.00	2.00	1.73	2.44	2.45	2.45	2.23	2.00
Margalef	1.82	1.82	2.16	2.16	1.82	2.79	2.79	2.79	2.49	2.16
β-diversity indices										
Whittaker	0.667					1.647				
Harrison	0.083					0.206				
Routledge	0.178					0.371				
Wilson-Shmida	3.333					4.765				
Mourelle	0.416					0.596				

enzymes, the genetic diversity indices were calculated for two species (Table 2). The percentage of polymorphism ranged from 16-24 in *S.* (*S.*) *gurneyae* and 17-25 in *S.* (*S.*) *nilgiricum*. The site 3 (1300 m) and 4 (1600 m) had the highest percentage of polymorphism in *S.* (*S.*) *gurneyae* and

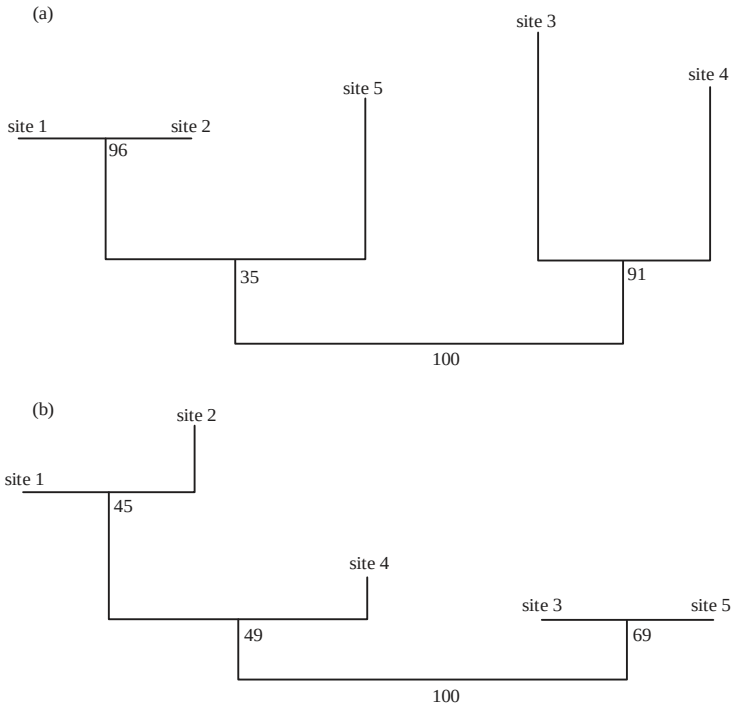

Fig. 5(a-b): Kimura two parameters divergence of (a) *S.* (*S.*) *gurneyae* and (b) *S.* (*S.*) *nilgiricum* between sampling sites by Neighbour Joining cluster analysis, bootstrap value is given below the node

S. (*S.*) *nilgiricum* polymorphism was high in the site 3 (1350 m). The alpha diversity indices indicate that site 4 and 5 from Palani hills and the site 3 from Nilgiri hills had the highest value of genetic diversity. The beta diversity indices resulted in the lower genetic similarity was observed among the population of *S.* (*S.*) *gurneyae* rather *S.* (*S.*) *nilgiricum* population.

The unequal variance of test (Welch F test) was performed to identify the heterogeneity of the restriction fragments among sampling sites. This result showed that lower heterogeneity was occurred between sampling sites for 2 species (*S.* (*S.*) *gurneyae*: $F = 0.1136$, $p = 0.976$; *S.* (*S.*) *nilgiricum*: $F = 0.3011$, $p = 0.874$). Inter-site pairwise distances based on Kimura two parameters were calculated for 2 black fly population in sampling sites by Neighbour Joining cluster analysis. In this analysis, two major clades formed in both species: first clade with 3 sites and second clade with 2 sites (Fig. 5). The higher divergence values were obtained between site 3 (1300 m) and 4 (1600 m) in *S.* (*S.*) *gurneyae* and site 3 (1350 m) and site 5 (970 m) in *S.* (*S.*) *nilgiricum* populations, indicating large elevational sites had the higher genetic variability.

DISCUSSION

The application of DNA marker techniques (expressed sequence tags, microsatellites, mitochondrial DNA, random amplified polymorphic DNA and restriction fragment length polymorphism) are useful for understanding of genetic diversity of insects and for mapping agriculturally and medically significant genes in insect pests (Behura, 2006). The genetic variation of *S.* (*S.*) *gurneyae* and *S.* (*S.*) *nilgiricum* populations in the present study area was performed using DNA barcode based RFLP. This result indicated that the high levels of genetic diversity found at

higher elevational site but lower genetic differentiation was observed in two black fly population between sampling sites. This may be associated with water temperature and other physical and chemical variables. The genetic diversity of chironomid communities in streams is reliable to altitude revealed by COI gene sequencing analysis (Loayza-Muro *et al*., 2013). The greater genetic diversity of *S.* (*S.*) *gravelyi* was observed at high elevational site (1590 m) using RAPD (Anbalagan *et al*., 2012). Further, dissolved oxygen in stream had a strong relationship with nucleotide diversity of *S.* (*S.*) *tani* in Malaysia (Low *et al*., 2014). The distributions of black flies, from cytotypes and molecular forms to full species, typically are related with stream profiles of pH, stream size and water temperature (Grunewald, 1981; Maraun *et al*., 2013).

Conversely, lesser genetic diversity was observed at lower elevational sites (<700 m) in the present study. It reflects that lower elevational populations have less chance of genetic exchange between other subpopulations (Anbalagan *et al*., 2012) and lower species or sex available at lower altitudes rather higher altitudes (Maraun *et al*., 2013). Genetic differentiation occurred along single steep altitudinal gradients and can usually be linked to the dispersal abilities of the species (Hodkinson, 2005). Compared to other studies of stream insect population suggests an increasing genetic diversity with maximum water temperature and decreased with elevational gradients (Jacobsen *et al*., 1997). Additionally, anthropogenic impacts is a major factor for predicting insect population in streams (Dinakaran and Anbalagan, 2007) and this factor increasing the diversity and abundance of filter feeding organisms (e.g., black flies and chironomids) in streams (Anbalagan *et al*., 2014b).

We conclude that DNA barcode based RFLP analysis is an effective tool for studying genetic diversity of black flies. The result of this study suggests that the higher genetic diversity of *S.* (*S.*) *gurneyae* and *S.* (*S.*) *nilgiricum* occurred at high elevational sites (>1000 m). This finding is pointed out to the need for further research on methods to understand dispersal pattern between high elevational sites.

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