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Materials and Methods

The methodology adopted in the present investigation is represented under the following headings:

Phase - I

- Diversity of Orthopterans

Phase - II

- Distribution of Orthopterans among Different Host Plants

Phase - III

- Population Dynamics of Acridids

Phase - IV

- Geometric Morphometric Analysis

Phase - V

- Molecular Phylogenetic Analysis of Acridids

Phase - I

3.1 Diversity of Orthopterans

Study area

Diversity of Orthopterans were carried out from different geographical regions of Coimbatore District, Tamil Nadu, India during August, 2018 to March, 2020 (Table -3 & Fig - 3). Coimbatore lies at 11°1′6″N 76°58′21″E in south India at 411 m above sea level on the banks of the Noyyal river, in northwestern part of Tamil Nadu. It is surrounded by the Western Ghats Mountain range to the west and north, with reserve forests of the Nilgiri biosphere reserve as well as the Anaimalai and Munnar ranges. The district is highly endowed with rivers, hills, flora and fauna due to its close proximity to the Western Ghats. This place is one of the biodiversity hotspots of the Western Ghats and the second largest city in Tamil Nadu after Chennai. It covers an area of 642.12km^2 (247.92 sq mi).

Table - 3

Latitude and Longitude of different location of the study area

Sites	locality	Latitude	Longitude	Altitude
I	Marudhamalai	11°02'33.1"N	76°52'10.1"E	513m
II	Saibaba Colony	11°01'15.2"N	76°56'55.4"E	433m
III	Thenkarai	10°56'51"N	76°50'33"E	456m
IV	Thudiyalur	11°04'32.3"N	76°56'00.8"E	458m
V	Singanallur	11°00'02.5"N	77°01'46.6"E	390m

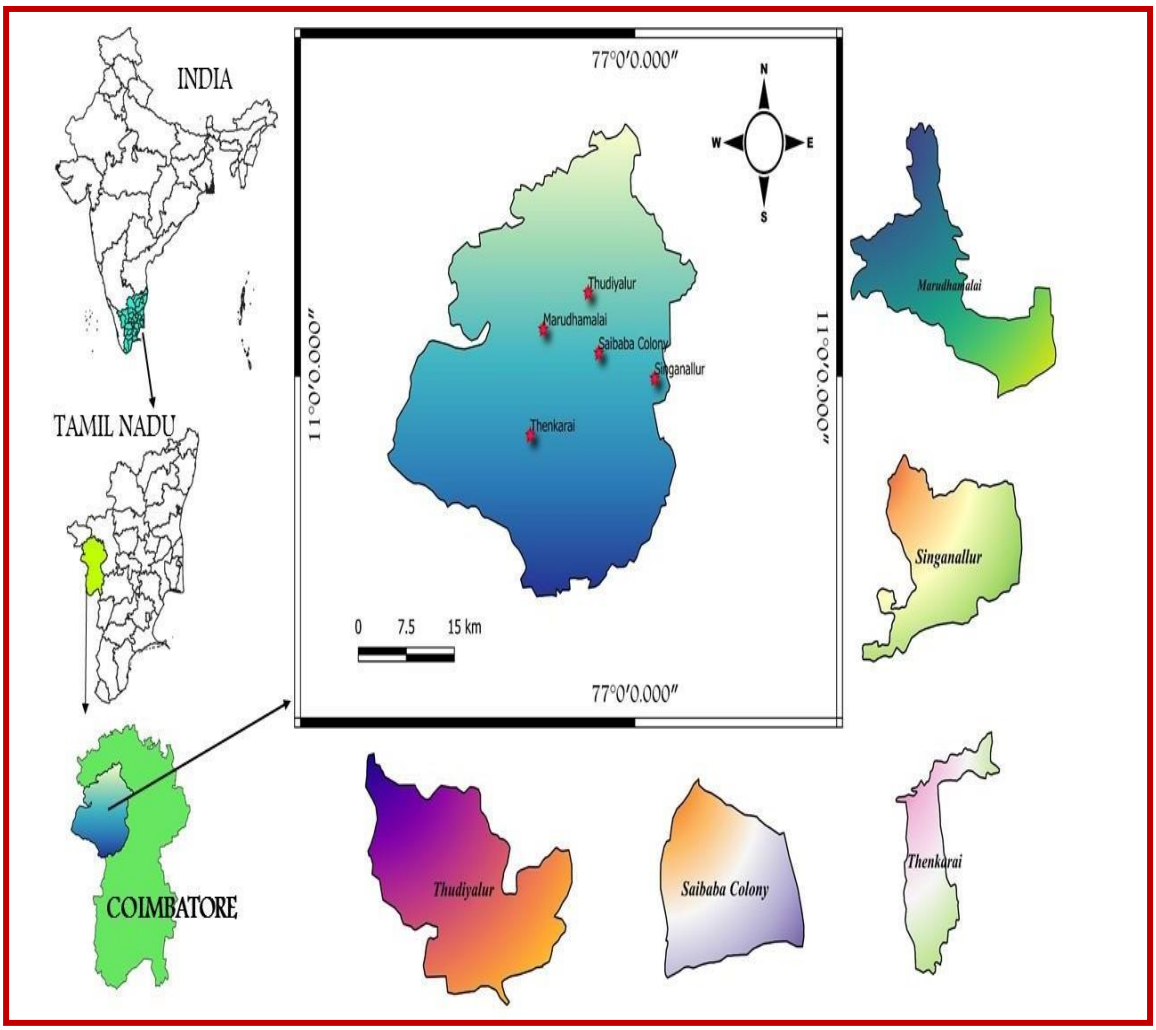

Figure - 3

Study area map of Coimbatore, Tamil Nadu, India

Characteristics of different sites

Each site has a distinct ecological feature and they were selected according to the characteristics of vegetation structure and degrees of human disturbances.

Site I: Marudhamalai (rural area) is located in the foothills about 12 km west from the city of Coimbatore. This place is home to many species of birds, plants and animals. Hills provide feeding and breeding ground for various species of insects. It also provides essential habitat for rare or endangered species. This area is covered along with dry deciduous forest and semi- evergreen mixed scrub.

Site II: Saibaba colony (urban area) is situated in the middle of the city, just 4 km north of the city of Coimbatore. It is one of the posh localities in Coimbatore. It is also home to the Bharathi Park, which provides a habitat for varieties of fauna and flora.

Site III: Thenkarai (sub-urban) is a scenic village located in the foothills of western ghats about 17 km away from the city of Coimbatore and on the way to Siruvani dam. The region has a high level of humility and is home to a diverse range of plants and animals.

Site IV: Thudiyalur (sub-urban) is located around 10 km north of Coimbatore's city centre. It is one of the fast-developing areas as it is located close to many industries. This area contains the moderate region of vegetation structure and human interference.

Site V: Singanallur (urban) is a major residential locality of Coimbatore city. It is situated on the banks of the Noyyal River, which flows to the southern boundary of the locality. It is one of the major wetland areas in Coimbatore.

Different seasons of the study area

Coimbatore has a semi-arid climate with moderate to higher temperatures throughout the year. The mean maximum and minimum temperatures in summer and winter vary between 35°C to 18°C. The average annual rainfall is around 700 mm (27.6 in) with the influence of both northeast and the southwest monsoons contributing to 47% and 28% respectively to the total rainfall. The northeast monsoon chiefly contributes to the rainfall in the district and summer rains are negligible. The southwest monsoon also brought certain rainfall but not at the level of the northeast monsoon.

Climatically study area was classified into four distinct seasons: **Post-monsoon** (January to March) comprised lower temperature with low pluviosity. **Summer** (April to

June) is generally a dry season with higher temperatures. **Pre-monsoon** (July to September) comprised scantly rainfall and moderate temperature. **Monsoon** (October to December) is characterized by heavy rainfall and lower temperature based on the northeast monsoon, which is prevalent in the study area.

Collection of Orthopterans

Orthoptera faunal composition was sampled twice a month from the study area in the morning (7 am to 9 am) and evening time (4 pm to 6 pm) through direct searching and entomological sweep net method (8.89 cm in diameter and 50.8 cm in length) (Fig - 4). Early in the morning and late in the evening, Orthopterans were less active and capturing way was easy. Efforts were made to standardize the sampling technique by sweeping each site for half an hour on every occasion. The sweep net method generally provides an accurate estimation of grasshopper diversity on grasslands (Evans *et al.*, 1983; Larson *et al.*, 1999). The sweep net sampling is the most commonly used method to evaluate grasshopper species composition (Joshi *et al.*, 1999). Hand collection was also adopted in cases where using an insect net was unsuitable.

(a) Sweep net method

(b) Search out method

Figure - 4

Methods of species collection (a & b)

Preservation of Orthopterans

Collected Orthopterans were brought to the Laboratory, Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore. The samples were placed into a wooden cage with a transparent plastic net. The number of Orthopterans caught were counted and transferred into the insect-killing bottles which containing cotton wool soaked with chloroform. The specimens were later separated into different taxonomic groups and specimens were followed the dry preservation method. They were pinned using an ordinary bell pin that is placed vertically through the thorax, slightly to the right of the center. The examined specimens were then properly marked with the place of collection, date of collection and its scientific name. Finally, fully dried specimens were stored in a standard insect box. Naphthalene balls were kept inside these boxes to prevent the decomposition of dry specimens and from the attack of ants or other insects.

Photography

Collected and identified Orthopterans from the fields were photographed using Canon: Model (EOS 600D), Lens EFS 18 -55 mm and Samsung note 10+. Some species were directly photographed from the study area. Other species were photographed in the laboratory when insects position is not suitable in the field.

Identification

Orthopterans identification up to species level was done with the help of binocular stereo zoom dissecting microscope using the keys of Kirby (1914); Dey and Hazra (2003); Shishodia *et al*. (2010); Gupta and Chandra (2017); Rajapandian and Natchiappan (2020) and description available on the ‘‘Websites http://bugguide.net and (http://www.orthoptera.org) Orthoptera Species File Online’’ based upon certain mentioned characteristics like size, colour, texture, body length, pronotum, tegmina, wing venation, hind femur and ovipositor site. Identified Orthopterans were confirmed with help of an expert from the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India. After identification and counts of species were made in all the sites, Orthopterans caught were immediately released in the sampled site in order to avoid impoverishing the environment. A small number of the dominant and rare species were collected for further examination.

Data analysis

Species richness was calculated as the Number of species/Total number of species ×100. The statistical analysis of the species data was done using the data analysis tool pack available in MS Excel 2010. The most common diversity indices such as Shannon-Wiener index, Simpson index and Margalef richness were calculated to describe and compare the diversity of species among different families, different sites as well as different seasons using the software PAST (Paleontological Statistical Software) version 2.02 (Hammer *et al.*, 2001). Moreover, the Bray-Curtis similarity index was performed in the same software to study the degree of similarity and dissimilarity of species composition among different families, sites and seasons. The similarity dendrograms obtained from the results of cluster analysis were plotted.

Phase – II

3.2 Distribution of Orthopterans among Different Host Plants

Vegetation types in Coimbatore

The forests of the Coimbatore district spread over an area of 693.48km2 against a district area of 7433.72 km2. The forests area is responsible for the cool weather, the green landscape and the clean air of the district. Coimbatore forest division is broadly divided into the following segments based on geographical variance: The Nilgiris slopes reserved forests, the plain forest around Mettupalayam, Vekkiangadu Valley, Naichenpalayam Valley, Thadagam Valley, Bolampatty Valleyand Walayar Valley. Five types of vegetation were found in Coimbatore namely Southern wet temperature, Southern tropical wet evergreen, Southern tropical moist deciduous, Southern tropical dry deciduous and Southern tropical thorn forests.

Soil types in Coimbatore

The soils of the Coimbatore district can be broadly classified into six major soils types viz., red calcareous soil, black soil, red non-calcareous, alluvial and colluvial soil, brown soil and forest soil. Deep red soil and black soil are the major soil types of the district. The highlands are predominantly occupied by black soil, which is dark grey to greyish brown. The rich black soil of the region has contributed to Coimbatore's flourishing agriculture and successful growth of cotton that served as a foundation for the establishment of the textile industry.

Collection of host plants

In order to assess the host preference and correlation of plant species with Orthopterans, plants were collected from same habitats as the Orthopterans in the study area. Both monocot and dicot plants as host for Orthopterans were recorded in the study area. The following variables were recorded in each site: name of plant species, family, location, number of plants and habitat.

Identification of host plants

Collected plants were identified based on morphological characters including colour, length, leaves, flowers and margins using taxonomic literatures (Gamble and Fischer, 1915-1936; Matthew, 1995 & 1999; Nair and Henry, 1983; Henry *et al.,* 1987 &1989; Chandrabose and Nair, 1987 & 1988; Singh *et al.,* 2000). Finally, these species were authenticated by Horticulture Department, Government Botanical Garden, Udhagamandalam, The Nilgiris (Appendix - I). The botanical name and vernacular name were verified using reputed websites such as The Plant List (2013), FRLHT etc.

Habitats of Orthopterans

The study area was classified into three different habitats such as grasslands (natural grasslands and grasslands with shrubs), agricultural lands (paddy, maize, corn and groundnut) and ground surface (soil and human altered sites) were illustrated in figure - 5.

Grasslands: It was covered with a variety of green grass, shrubs, climbers, creepers and weeds which grew to a maximum height and densities towards the monsoon season. During the post-monsoon season, the plants remained short, dry, pale and brown.

Agriculture fields: Orthopterans were collected from cultivated and harvested fields. Paddy, maize, corn and groundnut are some of the major products from agriculture and these fields support the survival and development of various species of insects. The grass and other weeds also sprouted in the field during the rainy season, attaining a maximum height.

Ground surface: Observation of Orthopterans on the ground (soil) were classified into the ground category. There was an undergrowth of grass and other plants which was uprooted for building constructions.

(a) Grasslands

(b) Agricultural lands

(c) Ground surface

Figure - 5

Orthopterans collected from different habitats (a, b & c)

Data analysis

The diversity of plant composition and common host plant of grasshopper among different regions of the study area was calculated. Species richness was determined as the total number of species present in the studied site. Software PAST version 2.02 (Hammer *et al.*, 2001) was used to perform the following statistical analyses: Principal Component Analysis (PCA) was performed in order to study the pattern of host plants variation among different species and families of Orthopterans. Moreover, to explore the similarity and dissimilarity of host plat preference among Orthopterans were also approached to cluster analysis. Different diversity indices such as Shannon-Wiener index, Simpson index and Margalef richness were executed to calculate the diversity of Orthopterans among different host plants. The total number of plant species and Orthopterans species in each site were included in the statistical analyses to determine the correlation coefficient between the composition of plant species and Orthopterans species using MS Excel 2010.

Phase - III

3.3 Population Dynamics of Acridids

Population dynamics of acridids were conducted from different ecological sites of Coimbatore (Fig -1 & table - 1) from January to December, 2019.

Collection procedure

Acridids populations were collected from the different ecological zones of the study area using sweep nets and handpicking method (Fig - 4). Acridids were surveyed inside a 10 m^2 area, thus, with the five plots, sampling a total area of 50 m^2 for each zone. Sampling was conducted twice a month during January to December, 2019 from 7 am to 9 am and 4 pm to 6 pm.

Weather report

During the study period, the meteorological parameters such as maximum and minimum temperature, relative humidity and rainfall (Table - 4) of the study area were recorded to assess the influence of these factors on the population dynamics of acridids in the study area. Monthly mean weather variables data were collected from the meteorological department, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Monthly maximum temperature was ranged from 36.7^{o}C to 27.8^{o}C (April and December); the minimum temperature was varied from 24.9^{o}C to 17.9^{o}C (May and January); humidity was

altered from 85% to 41% (March and May); rainfall fluctuated from 0 to 246.9 mm (February and October).

Table - 4

Weather report during the study period of January to December, 2019

Months	Maximum Temperature (°C)	Minimum Temperature (°C)	Humidity (%)	Rain fall (mm)
January	29.9	17.9	42	2.2
February	33.1	21.4	44	0
March	35.7	23.5	41	18.2
April	36.7	24.5	43	23.6
May	35.8	24.9	85	77.8
June	33.5	24.8	55	21
July	31.8	23.7	58	8.5
August	29.9	23	66	221.3
September	30.9	23.4	65	57.3
October	30.7	22.7	62	246.9
November	29.6	22.2	60	167.1
December	27.8	21.2	62	36

Data analysis

Dominance (D) of different species in acridid communities was determined according to the method by Buschini and Woiski (2008). D = (Abundance of a species/Total abundances recorded) × 100. Different species of acridids samples collected at the five different places were merged into combined samples. The correlation coefficient between acridids and monthly mean meteorological parameters were carried out using MS Excel 2010.

Following statistical analyses were performed using the software PAST (Paleontological Statistical Software) version 2.02 (Hammer *et al.,* 2001). Site-specific and season-specific acridids species were calculated using Principal Component Analysis (PCA). Similarity and dissimilarity of acridids population among sites and season were analysed using the Bray-Curtis cluster.

Phase – IV

3.4 Geometric Morphometric Analysis

Morphometric measurements

Studies on inter and intraspecific variations of acridids, six morphometric parameters studied were Body Length (BL) from head to the end of the abdomen, Antenna Length (AL) from base to the end of the last segment, Head Length (HL) from the base of antennae and pronotum, Pronotum Length (PL) distance between the base of head and tip of the first two pairs of wings, Fore Wing Length (FWL) and Hind Femur Length (HFL) were illustrated in figure - 6. The measurements of various parts in collected acridids were obtained in millimeters (mm) using vernier calliper. The body parts length measurement (mm) in all population were evaluated twice in order to reduce the quantity error.

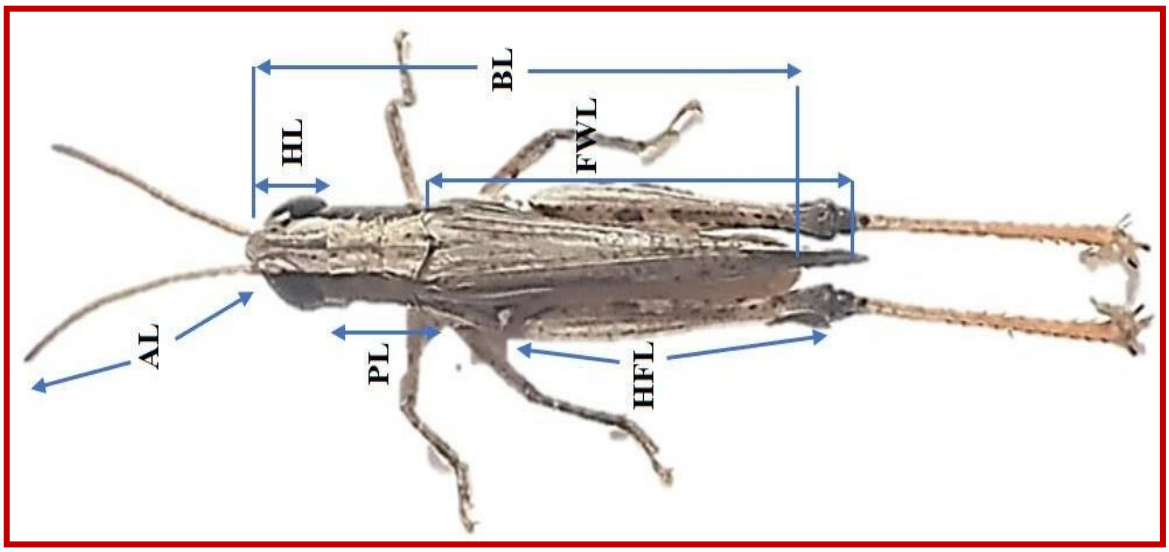

Figure - 6

Morphometric measurements of acridid

Note: BL-Body Length, AL- Antenna Length, HL- Head Length, PL- Pronotum Length FWL- Forewing Length and HFL- Hind Femur Length

Wing preparation

In this study, only the right forewings of acridids were used to compare the wing shape and co-variation between the populations. The right forewings were carefully removed from the body of adult acridids using dissecting needles, scalpels and forceps. The

wings thus removed were placed on a glass slide and examined under a microscope to describe the venation pattern of acridids.

Image processing

In order to capture the wing shape of acridids, each wing image was photographed with the same scale using a digital camera attached to a Phase-contrast stereomicroscope (MZ2000 Micros Austria with consistent magnification 10X/22) connected with the scope image version 9.0 image processing software with consistent resolution (1024×768 10 f/s).

Wing veins nomenclature

Wing veins nomenclature were identified using the published taxonomic keys (Grauvogel- Stamm *et al.,* 2000; Bethoux and Nel, 2001 and 2002; Petit *et al.,* 2006; Bai *et al.,* 2016; Rouibah *et al.,* 2019). Proximal, distal, anterior and posterior fields were used to describe the detailed position of landmarks in the wing of acridids populations. The six major longitudinal veins in acridids are the costa, subcostal, radius, media, cubitus and anal veins.

Data acquisition

Morphometrics parameters

The statistics parameters, mean ± standard deviation and variance were employed using PAST software. One-way ANOVA was used to compare the values obtained for the different parameters using SPSS. The P values less than 0.05 were considered statistically (95%) significant. Duncan's multiple range tests (DMRT) of the results for each parameter were used to determine whether the mean values differed significantly. Principal component analysis (PCA) and box plot were performed using PAST software v 2.02 (Hammer *et al.,* 2001) to compare the correlation matrix among selected species. Additionally, cluster analysis and survivorship curve were performed using the same program to study the similarity among parameters.

Geometric morphometrics

tps-Util: The wing photograph files were first converted in the tps-Util program version 1.76 to minimize bias in digitizing landmark locations of the specimen in the tps file.

tps-Dig: The x, y coordinates of landmark points on each species wing were subsequently digitized and measured by tps-Dig program version 1.40 in order to

characterize the shape variation for this trait. The digitization and linear measurement (Pixel) in all wings were evaluated twice in order to reduce the quantity error. The scale bar was used to standardize landmark distances to the same absolute scale across all images.

tps-Relw: Based on the landmark points, morphometric variation in the forewing shape was assessed separately for each sex by relative warp ordinations using the program of tps-Relw version 1.69, to explore the covariance between a set of variables and variance between two shapes. The relative warps were computed with the default weighting factor $\alpha = 0$, in order to weigh all landmarks equally.

tps-Small: Additionally, tps-Small program version 1.34 was performed to estimate the correlation between Procrustes and tangent space distance of the male and female wings to ensure that the amount of shape variation in the data sets was adequately represented after projection in the tangent space.

CVA: Canonical variate analysis was adopted to assess the intergroup variation and identify the individual species. CVA is a linear combination of the original variables that separate groups. In geometric morphometrics, CVA combines with visualization tools to give an insight into the covariation between the shape variables.

Phase – V

3.5 Molecular Phylogenetic Analysis of Acridids

Genomic DNA isolation

Total genomic DNA was isolated from the tissue samples of acridids (leg) were done using the EXpure Tissue DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd.,

Lysis/homogenization: Grind approximately 50mg of the sample with 500 µl of lysis buffer in a 2 ml microcentrifuge tube. Add 4 µl of RNAse and 500 µl of neutralization buffer into it. Vortex the content and incubate the tubes for up to 1 hour at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion and then centrifuge the tubes for 10 minutes at 10,000 rpm. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml microcentrifuge tube without disturbing the pellet. Add 600 µl of chloroform isoamyl alcohol and do hand mixing vigorously and centrifuge the tubes for 10 minutes at 10,000 rpm. Carefully transfer 600 µl of aqueous phase into a fresh 2ml microcentrifuge tube.

Binding: Add 600 µl of binding buffer to the content and mix thoroughly by pipetting and incubating the content at room temperature for 5 minutes and transfer 600 µl of the contents to a spin column placed in 2 ml collection tube. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through. Reassemble the spin column and the collection tube then transfer the remaining 600µl of the lysate. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.

Washing: Add 500 µl washing buffer I to the spin column and centrifuge at 10,000 rpm for 2 minutes and discard flow-through. Reassemble the spin column and add 500µl washing buffer II and centrifuge at 10,000 rpm for 2 minutes and discard the flow through. Dry spin the tube for 5 minutes at 10,000 rpm and transfer the spin column to a sterile 1.5-ml microcentrifuge tube.

Elution: Add 100 µl of elution buffer at the middle of the spin column. Care should be taken to avoid touch with the filtrate. Incubate the tubes for 2 minutes at room temperature and centrifuge at 10,000 rpm for 2 minutes. The buffer in the microcentrifuge tube contains the DNA. DNA concentrations were measured by Qubit fluorometer or 1% Agarose Gel electrophoresis.

PCR protocol

The polymerase chain reaction is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3`end of a custom-designed oligonucleotide when annealing to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA. The PCR product was sequenced using the primers (Folmer *et al.*, 1994) were shown in table - 5.

Composition of the Taq Master Mix: Taq DNA polymerase is supplied in 2X Taq buffer, 0.4mM dNTPs, 3.2mM MgCl2 and 0.02% bromophenol blue.

Table - 5

Details of primer used in this study

Primer Name	Sequence Details	Number of Base	Reference
LCO1490	5'GGTCAACAAATCATAAAGATATTGG3'	25	(Folmer *et al.*, 1994)
HCO2198	5'TAAACTTCAGGGTGACCAAAAAATCA3'	26	

Add 5 µl of isolated DNA in 25 µl of PCR reaction solution (1.5 µl of forward primer and reverse primer, 5 µl of deionized water and 12 µl of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

i) Denaturation: The DNA template is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA. **ii) Annealing:** The mixture is cooled to anywhere from 55℃. This allows the primers to bind (anneal) to their complementary sequence in the template DNA. **iii) Extension:** The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers and adding nucleotides onto the primer in a sequential manner, using the target DNA as a template (Table - 6).

Table - 6

Different stages of PCR Condition

Stages	Temperature	Time	Cycle
Initial denaturation	95 ^{o}C	2 min	25 cycles
Denaturation	95 ^{o}C	30 sec	
Annealing	55 ^{o}C	30 sec	
Extension	72 ^{o}C	2 min	
Final extension	72 ^{o}C	10 min	
Hold	4 ^{o}C	∞	

Purification of PCR production

Removed unincorporated PCR primers and dNTPs from PCR products by using a montage PCR clean-up kit (Millipore). Sequencing reactions were performed using a ABI PRISM® Big DyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme).

Sequencing protocol

The samples were resuspended in distilled water and sequencing was performed using ABI 3730 XL Genetic Analyser. The sequences were trimmed and edited using ClustalW and Bio Edit v 7.2.5 software. Obtained sequences were submitted to National Centre for Biotechnology Information (NCBI) GenBank and also authenticated.

Data analysis

The nucleotide sequence from each specimen was compared with barcode sequences on NCBI using Basic Local Alignment Search Tool (BLASTn). The haplotypes sequences were retrieved from NCBI GenBank and used for comparisons. Multiple sequence alignment of the closely related sequence was done with MAS (Multiple align show).

The nucleotide composition and AT bias were calculated using MEGAv.6.2 (Tamura *et al.,* 2013). Substitution rates, saturation test and test of selection for the evolutionary divergence: other parameters of the sequences including the rate of transitions (Ts) and transversions (Tv) at the first, second and third codon positions, were calculated and subsequently plotted against the uncorrected p distance for all the three codons to assess the saturation using MEGA v.6.2 (Tamura *et al.,* 2013).

Moreover, the average genetic divergence and inter-nucleotide divergence were calculated using the Kimura-2-parameter model to explore the extent of overlap between interspecific divergence. Estimation of substitution matrix and Transition/Transversion bias was performed in the same model using MEGAv.6.2 (Tamura *et al.,* 2013).

Investigations of the sequence saturations were done using DAMBE 5.3.10 (Xia, 2013) for estimating the rate of transition (Ts) /transversion (Tv) versus the genetic distance (F84). A further test for substantial saturation of the sequences was checked by using the method of Xia *et al.* (2003) and Xia and Lemey (2009) (DAMBE). The rate of synonymous (Ks) and non- synonymous substitutions (Ka) was calculated using DAMBE 5.3.10 (Xia, 2013).

Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-2336.9339) is shown. Initial tree(s) for the heuristic search were obtained

automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).

The evolutionary history was inferred to provide a graphic representation of the patterning of divergence between species using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.55495708 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. The phylogram of the haplotypes and the sequence of the present study was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA v.6.2 (Tamura *et al.*, 2013).