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Evolutionary Relationship between Heteropternis Respondents and Pternoscirta Sauteri on the 12S rRNA Sequence

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Abstract: ***Evolutionary relationship of the heteropternis respondents was studied based on the 12SrRNA sequence to compare and establish relationship among the selected species with their Chinese counterpart .acridid species of heteropternis respondents selected analysis of 12SrRNA accession number was JQ247658 .Chinese species of Pteroscirta sauteri 12SrRNA accession number was AY247195nucleotide base pair length of heteropternis respondens 338 base pairs G+C content 30.18%,A+T Content 69.82 % and 331 base pairs Pteroscirta sauteri G+C Content 27.49% A+T Base pairs 72.51%.***

Keywords: Heteropternis respondens, Pteroscirta sauteri, mtDNA, RAPD, AFLP, Microsatellite

1. Introduction

Insects represent a major life form on earth. Nearly 900,000 insect species have been discovered by now, comprising 75% of all the recorded animal species. Insects are found in almost all types of ecosystems; in the deserts as well as in the Antarctic. Insects comprise the largest species composition in the entire animal kingdom and possess a vast undiscovered genetic diversity and gene pool that can be better explored using molecular marker techniques. Existing trends of application of DNA marker techniques in diverse domains of insect ecological studies show that mitochondrial DNA (mtDNA), microsatellites, random amplified polymorphic DNA (RAPD), expressed sequence tags (EST) and amplified fragment length polymorphism (AFLP) markers have contributed significantly for progresses towards understanding genetic basis of insect diversity and for mapping medically and agriculturally significant genes and quantitative trait loci in insects. Apart from conventional marker systems, novel approaches including transposon display, sequence-specific amplification polymorphism (S-SAP), polymerase chain reaction (PCR) markers have been identified as alternate marker systems. Besides, whole genome microarray and single nucleotide polymorphism (SNP) assays are becoming more popular to screen genome-wide polymorphisms

2. Materials and Methods

Experimental animal
Acridids commonly known as short-horned grasshoppers are being much used by researchers, as they were good models for many scientific inquiries, besides their pest status. In Tamilnadu, Acridids live in almost all types of terrestrial ecosystems, which are mainly inhabited by diverse plant species ranging from grasses to trees. This insect group occurs as a dominant one both in agricultural and natural ecosystems by having significant distribution and impact on the ecosystem dynamics.

Acridid species were chosen to study their host-plant association, temporal distribution and abundance from various localities having natural and agroecosystems in nine districts within southern parts of the Tamilnadu state. These parameters were assessed and consolidated with the field study carried out during one year period (Jan - Dec 2009) as per Muralirangan *et al*. (1993).

Collection
species of Acridids of the family Acrididae were collected from cropland, grassland and forest ecosystems using sweep net technique and search out methods from various localities in nine districts within southern parts in the state of Tamilnadu, India [Latitude: 11º 00’ N and Longitude: 78º 00’ E]. Species level identification of all the collected Acridids were performed with the tallying of identification keys (Kirby, 1914; Dirsh, 1975; COPR, 1982Bhowmik, 1985; Muralirangan *et al.*, 1992; Srinivasan and Muralirangan, 1992; Mandal *et al.*, 2007).

Genomic DNA was extracted from leg muscle tissue of individual specimens using a simple proteinase K/SDS method. Leg muscle tissue was ground and incubated in 0.02 mol/L Tris-HCl (pH 8.0), 0.01 mol/L EDTA, 0.5% SDS, and 50 mg/mL of proteinase K overnight at 50°C. This mixture was extracted with phenol/chloroform, and DNA was precipitated with ethanol as described by Sambrook et al (1998).

Isolation of nuclear DNA Preparation of Chemicals and Reagents for DNA extraction

Stock solutions
10% SDS: 10g SDS (Sodium Dodecyl Sulphate) was dissolved in 100ml of distilled water (autoclaved and stored at RT).
1M Tris-HCl
(pH 8.0): 6.057g Tris was dissolved in 50ml of distilled water (autoclaved and stored at RT).
5M NaCl: 14.96g NaCl was dissolved in 50ml of distilled water (autoclaved and stored at RT).
0.5M EDTA
(pH 8.0): 0.7306g EDTA (Ethylene diamine tetra acetic acid) was dissolved in 10ml of distilled water (autoclaved and stored at RT).

Working solutions

DNA extraction buffer: 100mM Tris-HCl (pH 8.0) : 0.5 ml from 1M stock

10mM EDTA (pH 8.0) : 0.1 ml from 0.5M stock 1.4M NaCl: 1.4 ml from 5M stock 1% SDS : 0.5 ml from 10% stock 0.2% Mercaptoethanol : 0.01 ml The volume was made up to 5 ml with sterile distilled water. The mercaptoethanol was added just before the extraction.

RNAase stock: 10mg RNAase was dissolved in 1 ml of 10mM Tris-HCl (pH 8.0) and 15 mM NaCl.

Proteinase-k: 100 mg proteinase-k was dissolved in 1 ml of sterile distilled water.

1X TE buffer: 10mM Tris-HCl (pH 8.0) – 10 µl from 1M stock and 1mM EDTA (pH 8.0) – 2 µl from 0.5 M stock. The volume was made up to 1 ml with sterile distilled water. Fifty milligram of muscle tissue taken was ground with liquid nitrogen in a miniature pestle and the homogenate was incubated at 67ºC in an extraction buffer containing 100mM Tris-HCl, pH 8.0, 10mM EDTA pH 8.0, 1.4M NaCl, 1% SDS and 0.2% mercaptoethanol. The homogenate was treated with 150µg/ml proteinase-k at 50ºC for 4-12 hrs. It was then extracted with phenol-chloroform (1:1 mixture). The homogenate was centrifuged for 5 min at 10,000 rpm under room temperature. The upper aqueous phase was collected and treated with equal volume of chloroform-isoamylalcohol (24:1 mixture) by gentle mixing. It was centrifuged for 5 min at 10,000 rpm under room temperature. The collected upper aqueous phase was precipitated for DNA with cold absolute ethanol. It was again centrifuged for 45 seconds at 10,000 rpm under room temperature. The pellet was collected and dissolved in 400 µl of 1M NaCl. It was treated with 2 µl of RNAase and incubated at 37ºC for 30 min. Later 1 ml 51 of cold absolute ethanol was added to precipitate the DNA at -70ºC for 30 min. The sample was centrifuged for few minutes at 10,000 rpm at 4ºC. The supernatant was discarded, the DNA pellet was washed with 70% ethanol and the centrifugation was once again repeated. The DNA pellet was collected in microfuge tubes; the ethanol content was evaporated and then dissolved in 1X TE buffer. The quantity of the isolated DNA was spectrophotometrically tested at 260 nm and confirmed with the agarose gel electrophoresis.

PCR amplification of 12S rDNA

The primers for amplification of 12S rDNA- D2 region of approximately 550bp were Forward primer: 5'TACCGTGAGGGAAAGT TGAAA3' and Reverse primer: 5'AGACTCCTTGGTCCGTGTTT3'. PCR reaction mix was prepared in a total volume of 25µl with 100ng of genomic DNA, a 2.5mM concentration each of dATP, dTTP, dCTP and dGTP, 100ng 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 with glass distilled water (Bangalore Genie, India). The PCR reactions were conducted in PTC-1148 MJ MiniGradient Thermal Cycler (BIO RAD, USA). PCR reaction cycles consisted of initial denaturation for 5min at 94ºC, 40 cycles of 94ºC for 30 s (denaturation), 55ºC for 45sec (annealing) and 72ºC for 30sec (extension) and followed by the final extension of 72ºC for 10min. The amplicons were run through 2% agarose gel electrophoresis along with 100bp DNA ladder (Genie, Bangalore, India) and purified for sequencing process using the DNA elution kit as per manufacturer's protocol.

The amplified specific region of 12S rDNA was sequenced by dideoxy chain termination method (Sanger *et al.,* 1977), using Big Dye Terminator Version 3.1" Cycle Sequencing Kit in ABI 3130 Genetic Analyzer in accordance with manufacturer's instructions. The sequences of 12S rDNA among the populations have been deposited through Sequin in NCBI-GenBank. ClustalW is a widely used system for aligning any number of homologous nucleotide or protein sequences. All the sequences were aligned using ClustalW (Thompson *et al.,* 1995). The aligned 12S rDNA sequences of all the populations were subjected to genetic diversity and phylogenetic studies (provided as *Annexure*). Nucleotide diversity among the samples was estimated and sequences were compared by standard search algorithm (BLASTP) of NCBI (Altschul *et al.,* 1997). Sequence length, nucleotide composition and percentage and phylogenetic tree was constructed. All the analyses were conducted using MEGA version 5 (Tamura *et al.,* 2007). The reliability of trees was tested by bootstrapping.

Data and phylogenetic analysis

The 12S rDNA fragments of 17 species from the Acrididae were sequenced in this study and the 12S rDNA fragments of 17 grasshoppers were downloaded from GenBank (Tab. 2). All species of the in-group belonged to Acrididae and their samples were collected from Tamilnadu, and their sequences of 12S rDNA were submitted to NCBI. The out-group, Pyrgomorpha conica, from Swissland, belongs to the Pyrgomorphidae of the Pyrgomorphoidea, whose sequences of 12S rDNA were sequenced by Flook et al (1999). In this study, the classification of the Acridoidea is based on the Orthoptera Species File (OSF)

All sequences were aligned using Clustal X1.83 (Thompson et al, 1997) with parameters set to default. Alignments were improved by comparison to the secondary structures and regions of uncertain alignment were omitted from subsequent analyses. Bases composition and sequence variability were examined using the software package MEGA5.0（ Tamura et al, 2011). MEGA5 (Tamura et al. 2011) is an integrated program that carries out all four steps in a single environment, with a single user interface eliminating the need for interconverting file formats. At the same time, MEGA5 is sufficiently flexible to permit using other programs for particular steps if that is desired. MEGA5 is, thus, particularly well suited for those who are less familiar with estimating phylogenetic trees. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.

Five different types of phylogenetic analyses were performed based on Maximum Likelihood, Neighbor Joining, Minimum Evolution and Maximum Parsimony and UPGMA methods. Trees saved below the burn-in generations were discarded, and a majority-rule consensus tree of the remains was calculated, providing posterior probabilities for each clade. For the ME analysis, the TamuraNei's nucleotide substitution model was selected with pair-wise deletion of gaps, meanwhile, the interior

branch test with 500 replicates was used to assess the confidence that could be attached to the individual nodes.

Molecular Phylogenetic analysis is based on measuring the evolutionary distance between a pair of sequences having the number of nucleotide/amino acid substitutions. To compute the variations among sequences, there are many 54 biologist-friendly software tools available on line, those tools not only make it possible to use computational and statistical methods but also allow to select methods and algorithms best suited to understand the function, evolution and adaptation of genes and species. The goal of phylogenetic analysis of DNA sequences is to analyse evolutionary relationships among a group of related sequences by producing a tree representation of the relationships.

Table 1 Acridid species selected for the molecular phylogenetic analysis (12S rDNA)

S.No.	Species	Subfamily	Acc. No
1	*Heteropternis respondens*	Oedipodinae	JQ247658
2	*Pternoscirta sauteri*	Oedipodinae	AY247195

3. Results

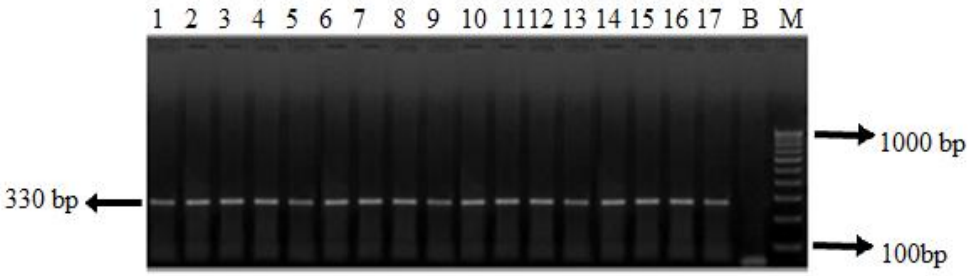
Acridids, plague locusts and grasshoppers continue to be responsible for food shortages in many parts of the world by inflicting damage to many crops and are good model insect system for scientific investigations, therefore Acridids are considered as a key insect group. There has been tremendous information available on their host selection, eco-geographical distribution and abundance, ecology, behaviour and acoustic communication, population dynamics. However, there is a vast lacuna in the taxonomy and systematics aspects of the grasshoppers including molecular information. To fulfil this gap the present study has been taken up with and objective to venture into the molecular aspects of genetic diversity and the genomic level among the local population of the selected insect species from different parts of the state of Tamilnadu,

12S rDNA of the selected Acridids

The genomic DNA isolated from the field collected populations Acridids were subjected to PCR amplification of the highly conserved region of 12S rDNA. The amplicons were sequenced and the gene sequences were submitted to Genbank of NCBI under the accession numbers JQ247658, AY247195 the 12S rDNA gene sequence data of the field collected 56 populations were analyzed for length, GC content, nucleotide polymorphism, sequence divergence, phylogenetic relationships and similarity/ identity of the sequence.

12SrDNA sequence variation in field populations of Acridids

A single specific PCR product was amplified from genomic DNAs samples for each of selected field populations of grasshoppers with the primer pair
FP: 5'TACCGTGAGGGAAAGTTGAAA3'
RP: 5'AGACTCCTTGGTCCGTGTTT3'



The PCR products (amplicons) were sequenced. The sequences were aligned to generate consensus for each of the sample excluding the forward and the reverse primer sequences.

Evolutionary relationship of the heteropternis respondens was studied based on the 12SrRNA sequence to compare and establish relationship among the selected species with their Chinese counterpart .acridid species of heteropternis respondens selected analysis of 12SrRNA accession number was JQ247658 .Chinese species of spathosternum prasiniferum of 12SrRNA accession number was AY247195nucleotide base pair length of heteropternis respondens 338 base pairs G+C content 30.18%,A+T Content 69.82 % and 331 base pairs Pteroscirta sauteri G+C Content 27.49% A+T Base pairs 72.51% .

Phylogenetic analysis Nucleotide Frequencies at conserved

NCBI	T(U)	C	A	G	T
AY247195	42.4	11.2	28.0	18.4	125.0
JQ247658	42.9	11.1	27.0	19.0	126.0

Phylogenetic analysis –Nucleotide Frequencies at variable

NCBI	T(U)	C	A	G	T
AY247195	42.2	8.7	31.6	17.5	206.0
JQ247658	40.1	12.7	29.7	17.5	212.0

Phylogenetic analysis –Nucleotide Frequencies –parsim-info:

NCBI	T(U)	C	A	G	T
AY247195	39.6	9.0	32.1	19.4	134.0
JQ247658	37.4	12.4	31.3	18.4	147.0

Phylogenetic analysis –Nucleotide Frequencies for singleton

NCBI	T(U)	C	A	G	T
AY247195	42.4	11.2	28.0	18.4	125.0
JQ247658	42.9	11.1	27.0	19.0	126.0

Today evolutionary relationship are built from molecular data , DNA marker such as mtDNA ,RAPD , AFLP, microsatellites and ESTs have been used as popular marker system in insect geneticsresearch.

Pternoscirta sauteri
- **Kingdom :** Animalia
- **Phylum :** Arthropoda
- **Class :** Insect
- **Order :** Orthoptera
- **Family :** Acrididae
- **Sub family :** Oedipodinae
- **Tribe :** Locustini
- **Genus :** *Pternoscirta*
- **Species :** *sauteri*

AY247195
>gi|30059844|gb|AY247195.1| Pternoscirta sauteri 12S ribosomal RNA gene,
partial sequence; mitochondrial gene for mitochondrial product
ATGAGTTAAGGTCTTTAAACCTAAAGAATTGGGCG
GTATTTCATTCCATTTAGAGGAACCTATCTCATAA
TTGATAATACACGATTTATTTTACTTGATTTATTTGT
TTGTATATCTCCGTTATCAGAAAATCTTTATGG
AGTTGAAATTTTCTTGTTTCTCATTAGGAATTATTTC
AGGTCAAGGTGCAGCTTATAATCAAGGGTATGA
TGGGTTACAATAATTTAATATTATTATGGATTTGAT
TTTGTAATATTTTAATGAAGGTGGATTTAATAGT
AATTTGAATTATTTAATTTGATTGATATTGGCTCTG
AGATGTGTACACTAT
Length = 331 base pairs
Molecular Weight = 98952.00 Daltons, single stranded
Molecular Weight = 199984.00 Daltons, double stranded
G+C content = 27.49%
A+T content = 72.51%
Nucleotide Number Mol%
A 100 30.21
C 32 9.67
G 59 17.82
T 140 42.30

Heteropternis respondens **(Walker, F., 1859)**
- **Kingdom :** Animalia
- **Phylum :** Arthropoda
- **Class :** Insect
- **Order :** Orthoptera
- **Family :** Acrididae
- **Sub family :** Oedipodinae
- **Tribe :** Epacromiini
- **Genus :** *Heteropternis*
- **Species :** *respondens*

>JQ247658
>gi|380750547|gb|JQ247658.1| Heteropternis respondens isolate PS-
95 12S ribosomal RNA gene, partial sequence; mitochondrial
TTAACTTGCTGTCTGGGTATTATTAGTTAGGATCTT
TAAACCCAAAGAATTTGGCGGCATTTCATTCCAT
TTAGAGGAACCTGTCCCGTAATTGATAATACACGA
TTGGTTATACTTAATTTATGCTTGTATATCTCCGT
TATCAGAATGTCTTTTTAGAGTAATAAAATTTTCTT
AATTCTTAATTTAGAATTATTTCAGGTCAAGATG
CAGTTTTAGTTAAGTGGAGATGGGTTACAATGATTT
TGTATTTGTCGGTTTTTCTTATGAGAATTTTTGA
TTAAGGTGGATTTAATAGTAATTTATTAACTTTATT
ATGAAAGAGCGCGCACACATTC
Length = 338 base pairs
Molecular Weight = 101259.00 Daltons, single stranded
Molecular Weight = 204368.00 Daltons, double stranded
G+C content = 30.18%
A+T content = 69.82%
Nucleotide Number Mol%
A 97 28.70
C 41 12.13
G 61 18.05
T 139 41.12

4. Consulsion

The eukaryote genomes contain a significant proportion of repetitive DNA of various types. Two of the tandem repetitive DNA classes, that is, ribosomal (rDNA) and satellite DNA (satDNA), are very different in genetic role.

Entomologists are getting more accustomed with the refinement of marker systems and are applying the new techniques to study insect genomes more efficiently particularly in relation to their phylogeny.
- The phylogenetic relationships among all taxa within the Acrididae (Orthoptera: Acridoidea)

- The character based and lineage based species concept has both taxonomic and evolutionary information.
- Evolutionary relationship among species groups could be brought out having as much of their comparative bionomical traits including molecular information.
- Molecular data have powerful advantages over morphological information, especially in terms of large number of characters, wide spread distribution and a much better approximation of their evolution to that expected from simple models. In this study nucleotide gene sequence of the 12S rDNA of selected 2 species of Acrididae, 1from India and 1 China were used as sample taxa
- The sequence constitutions and variations were analyzed and the molecular phylogenetic trees were reconstructed based on the combined sequence data of 12S rDNA.
- The data were subjected to analysis using MEGA 5. The sequence data of all the Acridid species studied were analysed using pair wise sequence divergence, phylogenetic tree construction based on Maximum Likelihood, Neighbour Joining, Minimum Evolution and Maximum Parsimony and UPGMA methods including bootstrap support revealed consistently established monophyletic origin of the family Acrididae.
- Hence, it is proposed that the global genomic profiling of the genetic material including 12S, 18S, 28S, mtDNA, and numtDNA put together analyzed for the global evolutionary pattern can throw light on the phylogenetic relationship and other information regarding the global distribution and functional behavior of the Acridids.

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