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RESEARCH ARTICLE

MOLECULAR TAXONOMY AND GENETIC DIVERSITY ANALYSIS OF *THEREAPETIVERIANA*L.

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ABSTRACT

The introduction of DNA barcoding has highlighted the expanding use of the mtDNA gene, cytochrome c oxidase I (COI), as a genetic marker for species identification (Dawnay *et al*., 2007). In the current study, COI gene based barcoding was employed to identify the seven spotted cockroach, *Thereapetiveriana.* The genetic diversity amongst this cockroach collected from various locations in Tamil nadu, India was analysed by the banding patterns obtained in RAPD PCR. This was further validated using PCR RFLP studies on the mtDNA gene, COI. The results highlighted the impact of varying environmental factors on the genetic makeup of these species.

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INTRODUCTION

Hebert *et al*. (2003) proposed that a DNA barcoding system for animal life could be based upon sequence diversity in cytochrome c oxidase subunit 1 (COI). They established that diversity in the amino acid sequences coded by the 5' section of this mitochondrial gene was sufficient to reliably place species into higher taxonomic categories (from phyla to orders). DNA barcoding refers to the technique of sequencing a short fragment of the genomic DNA, the "DNA barcode," from a taxonomically unknown specimen and performing comparisons with a reference library of barcodes from known species in order to establish a species-level identification. Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals. Genetic diversity can be analysed by various techniques like RAPD, RFLP, Protein fingerprinting etc. Species identification by DNA barcoding is a sequencing-based technology. Once the sequence information of the target specimen is obtained, it is possible to compare this information to a sequence library from known species (Hajibabaei *et al*., 2007). Some, among the many available repositories are comprehensive and include sequences from several segments of DNA (e.g. GenBank), but others are restricted to a specific marker (e.g. BOLD). *Thereapetiveriana* (Blattodea, Polyphagidae) belongs to a basal family of cockroaches (Grandcolas, 1993, 1997) and was reported from peninsular India (Princis, 1964). Also called the "desert cockroach", "seven-spotted cockroach" or "Indian domino cockroach", T.petiveriana is a fossorial species of crepuscular cockroach, inhabiting rubbish heaps around human habitations as well as in humus accumulations in certain isolated pockets of the scrub jungles and semiarid zones of south India (Grandcolas 1993). The current study is an attempt at using the recent molecular techniques to generate Bar-codes for identification of this species and analysing the effect of environmental factors on their genetic diversity.

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MATERIALS AND METHODS

Sample collection and identification

Insect samples (*Thereapetiveriana*) were collected from various parts of Southern India namely – Tiruvallur, Tambaram, Nungambakkam and Ambattur. They were identified using their systematic criteria. Live samples were transported to laboratory and processed for molecular attributes.

Isolation of genomic DNA

Genomic DNA was isolated from the cockroaches by Phenol-Chloroform method (Muhammad Zeidan and Henryk1991). Isolated nucleic pellet was washed with 70% ethanol and resuspended in TE buffer (10mM TrisHCl, 1mM EDTA, pH

7.4). Quality and quantity of the DNA was assessed were analysed by agarose gel electrophoresis and UV Spectrophotometry respectively.

PCR and DNA Sequencing

Internal regions of 900bp of COI gene was amplified using gene specific primers. Amplification was carried out in a 20µl reaction set up containing primers, deoxyribonucleotidetriphosphates (dATP, dCTP, dGTP and dTTP), template DNA sample and Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected to thermal cycling reactions consisting of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (46 s at 47°C), and extension (1 min at 72°C), with a final extension (10 min at 72°C). The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and DNA sequencing was performed by Sanger's method (ABI 3730).

Sequence Analysis

The nucleotide sequences for COI gene obtained was compared with its related species using BLASTn tool. Portable software Molecular Evolutionary Genetics Analysis (MEGA) version 5 was used to construct maximum likelihood (ML) tree for the obtained sequences to identity it's inter and intra species relationships. The Distance Matrix Explorer, an action menu of MEGA5 was used to compute the pairwise difference between the obtained target sequences to its maximum aligned sequences.

RAPD analysis

RAPD was performed using the 3 primers - OPA 7, OPA10 and OPA11.Amplification was carried out in a 25µl reaction set up containing primers, deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), template DNA sample and Prime TaqDNA polymerase. The reaction tubes were subjected for Thermal cycling reactions consisted of an initial denaturation (3 min at 94°C) followed by 40 cycles of denaturation (45 sec at 94°C), annealing (1 min at 37°C),and extension (1 min at 72°C), with a final extension(7 min at 72°C). The results were analyzed using 1.5% agarose gel stained with ethidium bromide. From the DNA banding pattern, scoring table was generated for the obtained scoring data. Dendrogram was constructed from Similarity matrix values using UPGMA algorithm. A Statistical software package SPSS v 16 was used to develop dendrogram.

PCR RFLP analysis

Restriction digestion of the COI gene amplicons obtained from four different locations of *Thereapetiveriana L* was performed. Amplification was carried out in a 20µl volume containing COI primer (Eurofins, India), deoxyribo nucleotide triphosphates (dATP, dCTP, dGTP and dTTP) (Biotools, Spain), template DNA sample and Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisted of an initial denaturation (3 min at 94°C) followed by 32 cycles of denaturation (30 sec at 94°C), annealing (1 min at 47°C) and extension (1 min 20 sec at 72°C), with a final extension(7 min at 72°C). PCR products were visualized using 1.5% agarose gel stained with EtBr (20mg/ml). The molecular weight of the bands was estimated using 1Kb DNA Ladder as reference. The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and subjected for restriction digestion using DpnI enzyme. Restriction digestion was performed for 20µl containing 1000ng of PCR product, 10u of DPNI enzyme and 1X of buffer. The tubes were subjected for restriction digestion at 37°C for 2hrs. Restricted digested samples were subjected to Polyacrylamide gel electrophoresis for visualization and to record the obtained data. The cast plates were assembled. Followed by the addition of 12% Separating gel solution (4ml Acrylamide, 2.5ml of Tris HCL (pH 8.8), 0.1ml of 10% SDS, 0.1ml of 10% APS, 0.004ml of TEMED and 3.3ml of distilled water) between the glass plate. Immediately the gel was overlaid with ethanol to exclude air and to obtain an even interface over the gel. After the polymerization of separating gel, 5% stacking gel solution was poured immediately on top of the separating solution and wells were formed by placing combs over the stacking gel solution (1.19ml Acrylamide, 0.875ml of Tris HCL (pH 8.8), 0.07ml of 10% SDS, 0.07ml of 10% APS, 0.007ml of TEMED and 4.76ml of distilled water). The gel was allowed to polymerize. Upper and lower reservoir of electrophoresis unit was filled with electrophoresis running buffer. Restriction digested sample and the DNA ladder were loaded in the respective lanes. The tank was then connected to power supply and the gel was run at 100V until the dye reaches bottom. Detection of DNA in the gel is performed by silver staining method since this method can detect DNA even at ng level. Scoring table was generated with the banding pattern and subjected to phylogenetic analysis (Sambrook *et al*., 2000).

RESULTS AND DISCUSSION

DNA barcodes have been proposed as a detour to provide new species identification and discovery, as the taxonomists would have spent decades to discover 10-15 million species using current description and publications (Daniel *et al*., 2006). The mitochondrial enzyme cytochrome oxidase c is a highly conserved electron transport protein coded by multiple genes containing regions that evolved at different rates (Lunt *et al*. 1996). These markers were proposed as DNA barcodes because of their potential for identification of putative regulatory elements, as they harbor sufficient sequence diversity, individually or in combination, to distinguish among species level phylogenetics (Sass *et al*., 2007). The identification of insects based on their DNA can be performed even with immature insects or fragments of puparium and adult insects, is a faster approach andalso facilitates the successful validation of classical taxonomical methods (Harvey *et al*., 2003; Mazzanti *et al*., 2010). According to Amendt *et al*., (2004), Polymerase chain reaction (PCR) amplification of suitable regions of the genome, sequence analysis of the amplicons obtained, and alignment of the data with reference sequences is the usual and recommended method. The DNA isolated from the collected insect samples were analysed and quality and quantity were found to be good. Amplicons obtained after PCR were visualised at 900bp for COI gene (Fig.1). Obtained sequences from the purified PCR product was compared with nucleotide database (BLASTn) and was found to be congeneric to *Thereapetiveriana*, the seven spotted cockroach.

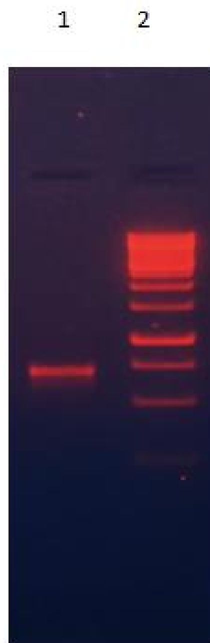

Fig. 1. COI gene amplification. Lane 1: COI from Cockroach, Lane 2: 1Kb DNA Ladder

Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Hence, genetic diversity plays an important role in the survival and adaptability of a species (Frankham and Richard 2005). Among the common molecular approaches used for analyzing genetic diversity, two prominent ones are RAPD PCR and PCR RFLP. In RAPD (Randomly Amplifed Polymorphic DNA) analysis, arbitrary primers are used resulting in the amplification of several discrete DNA fragments, which are then separated on agarose gels to visualise the DNA banding pattern (Williams *et al*., 1990). The presence or absence of bands can be scored and the data converted into similarity matrices for calculation of genetic distances (Ellsworth, Rittenhouse and Honeycut, 1993). In the current study, primary screening of the 4 samples using 3 primers namely OPA 7, OPA10 and OPA11 resulted in a number of amplicons and were selected to develop a fingerprint. Using OPA 10 eleven amplicons were obtained, while OPA11 generated thirteen amplicons. The gel was visualized to generate a scoring table and used for constructing a dendrogram (Fig. 2). The Dendrogram showed that the samples isolated from Tambaram and Nungambakkam formed the primary cluster, which in turn was related to the one collected from Tiruvallur, while the sample from Ambattur was the distinct sample forming the distant cluster.

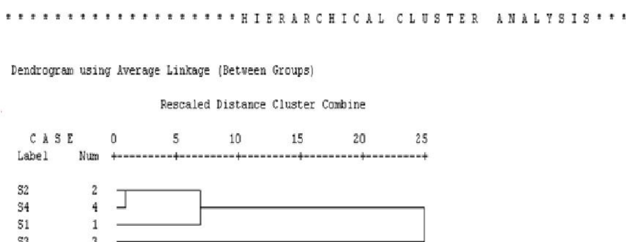

Fig. 2. Dendrogram analysis of *Thereapetiveriana* S1 – Tiruvallur, S2 – Tambaram, S4 – Nungambakkam, S3 – Ambattur

Table 1 illustrates the total number of bands generated for *Thereapetiveriana* L using RAPD decamers namely OPA9, OPA10 and OPA11. These showed high level of polymorphism within the four samples of *T. petiveriana* L. A maximum of 66.6% polymorphism was observed in OPA9 and OPA11 RAPD primers (Table 1) exemplifying the fact that genetic variation is high within the insect samples isolated from four different locations. RFLPs give highly reproducible patterns but variations in fragment lengths between individuals or species can arise either when mutations alter restriction sites, or result in insertions/deletions between them (Burr *et al*., 1983). PCR-RFLP analysis of the COI gene, using the isolated genomic DNA from 4 locations was performed using DpnI restriction enzyme. This was carried out to understand the genetic variation of the conserved coding region (COI) in the mitochondrial genome of the same sample from various environmental conditions. The results, observed in the agarose gel (Fig.3) and dendrogram analysis was concurrent to the RAPD results. Three major bands were observed in all samples, while the sample from Ambattur showed two additional faint bands.

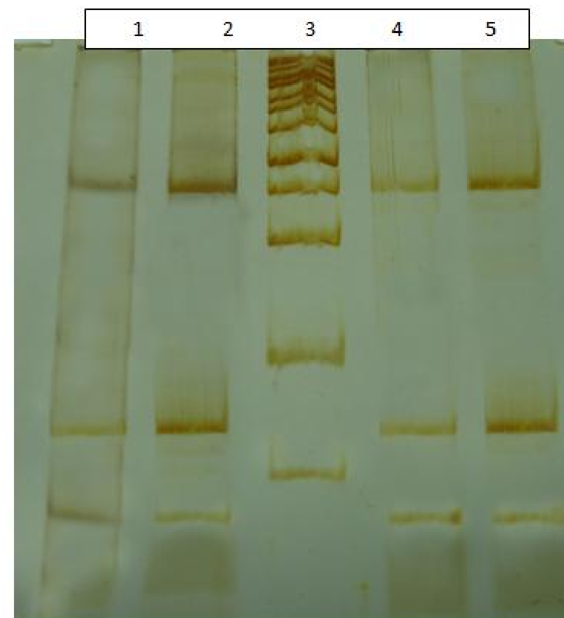

Fig. 3. RFLP analysis of *T.petiveriana*. Lane 1-Tiruvallur, Lane 2 – Ambattur, Lane 3 – 1KB DNA Ladder, Lane 4 – Nungambakkam, Lane 5 –Tambaram

Table 1. Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers for *Thereapetiveriana* L.

Primer	Monomorphic bands	Polymorphic bands	Total no of bands	% of polymorphism
OPA 9	1	2	3	66.66
OPA 10	3	4	7	57.14
OPA 11	2	4	6	66.66

RAPD and RFLP techniques were used to document the genetic variation among the species collected from the four localities. Their phylogenetic relationships were established and checked for accumulation of variations which may influence the diversity of the species. The RAPD-PCR and

PCR-RFLP showed genetic diversity amongst the samples collected from different geographical regions. Dendrogram showed the extent of similarity and dissimilarity between the samples of various geographical locations. Results showed that each species was not genetically identical. The samples from Tambaram and Nungambakkam showed low genetic variation whereas the samples from Tambaram and Ambattur displayed significant genetic variation, probably hinting at the variations incurred in the genotypes of the species owing to the environmental conditions in the particular region.

Conclusion

Various insects have shown the ability to adapt quickly to the surrounding climatic changes, which can be attributed to the sudden mutations occurring in the genome of the organism. This fact has been exploited to understand the changes in the climate of various geographical regions. The current study is a contribution to the ongoing environmental study pertaining to the observation of the climatic changes occurring on the earth. Minute changes in the environment are reflected on the genotype of these insects, which is gaining attention in the scientific community. The current study also validates the use of molecular techniques like RAPD-PCR and PCR-RFLP to understand and elucidate the genetic diversity in the living world.

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