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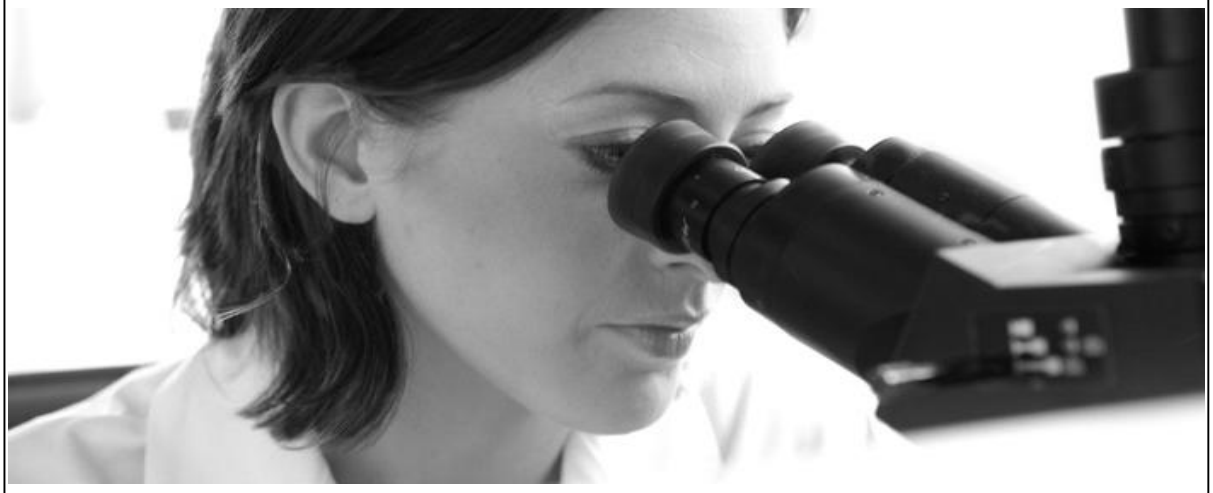

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RESEARCH ARTICLE

MORPHOMETRIC ANALYSIS AND GENETIC VARIABILITY OF THEREA PETIVERIANA (POLYPHAGIDAE:BLATTODEA) AS REVEALED BY RANDOM AMPLIFIED POLYMORPHIC DNA

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ABSTRACT

Cockroaches are known to carry human pathogens and can potentially transmit diseases to people. Additionally Cockroaches are a source of allergens and can cause allergic reactions and asthma in people especially children. *Therea petiveriana* is commonly known as seven spotted cockroach belongs to a basal family Polyphagidae of cockroaches and was reported from peninsular India. The morpho based phenotype is the product of the interaction between genes and environment. Genetic diversity can be analyzed by various techniques like RAPD, RFLP, Protein fingerprinting etc. For molecular analysis the cockroach species *Therea petiveriana* were collected from twelve different localities of southern parts of Tamil Nadu, India of leaf litters by adapting hand picking methods. About twelve populations of *T.petiveriana* were collected from different localities of Tamilnadu from the rice field and were subjected to RAPD analysis using twenty random decameric primers. Out of the twenty RAPD primers tested, three primers have produced clear, consistent and reproducible RAPD bands for all the populations. UPGMA based dendrogram analysis grouped all the populations of *T.petiveriana* into many clusters based on the genetic similarity coefficient.

INTRODUCTION

Cockroaches are one of humanity's most familiar and least loved insects. They are easily recognized by their flattened, oval-shaped body, head always covered by the pronotum, chewing mouthparts, leathery appearance of their forewings, membranous hind wings, a very well developed anal area and long spiny legs with large flattened coxae covering all thoracic sternites. They have a pair of cerci in the last abdominal tergite. Only in males the last abdominal sternites has a pair of accessory structures for copulation known as styles (Cornwell, 1968). Cockroaches are an important public health problem. They can eat and contaminate food and can give off a disagreeable odor. Cockroaches are known to carry human pathogens and can potentially transmit diseases to people. Additionally Cockroaches are a source of allergens and can cause allergic reactions and asthma in people especially children (Jeffrey Hahn, 2002). The most common roach are the American cockroach (P*eriplaneta Americana*, the German cockroach (*Blattella germanica*) and Seven spotted cockroach (*Therea petiveriana*) (Robinson, 2005; Uneke, 2007).

Therea petiveriana is commonly known as seven spotted cockroach belongs to a basal family (Polyphagidae) of cockroaches (Grandcolas, 1997) and was reported from peninsular India (Princis, 1964). It is a fossorial species, 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. The adults exhibit a strong tendency towards gregarious activities. They are essentially crepuscular, and during nonactive periods they remain subterranean. Adult males and females are macropterous, but flightless. When disturbed, they raise their wings and evert on either side, on the second and third abdominal segments, a pair of brown pouches (Livingstone and Ramani, 1978; Brossut and Sreng, 1985). These organs, described in various species of Polyphagidae (Grandcolas, 1997), are absent in the nymphal instars. Normally, the pouches are never visible and are withdrawn into the body cavity. The exposed surface of the pouch reveals numerous chitinous pleats of varying heights, which bear whorls of fine setae that are generally enveloped with secretion (Livingstone and Ramani, 1978).

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Morphological characteristics are an important source of information for many areas of biological investigation, including systematics and taxonomy. Most studies in these areas are done using meristic and morphometric characteristics. Meristic characteristics are generally countable and informative for species, genera and higher taxonomic levels. However, in interspecific and populational studies, these measurements are not informative and it is therefore necessary to obtain information on morphometric characteristics. These characteristics are generally quantitative phenotypic values obtained from continuous measurements and ratios in which classes are often defined based on means and standard deviation (Bookstein *et al*., 1985).The morpho based phenotype is the product of the interaction between genes and environment. Phenotypic variation is then an expected outcome of more than one factor. It can be scored by measurable changes in anatomy, morphology, physiology, life history, behavior, etc. (West-Eberhard, 1989; Gadagkar and Chandrashekara, 1990).

Genetic diversity can be analyzed 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, 2007). The RAPD technique is a polymerase chain reaction (PCR) based assay that was developed to detect polymorphisms in genomic DNA (Welsh and McClelland, 1990; Williams *et al*., 1990). Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identification (Laguerre *et al*., 1996; Selenska-Pobell *et al*., 1996). Hence, the present investigation was carried out to establish morphometric study and genetic variability analysis of different isolates of *Therea petiveriana* (Blattodea, Polyphagidae) collected from various locations in southern parts Tamil Nadu using the technique of RAPD-PCR.

MATERIALS AND METHODS

Therea petiveriana were collected from leaf litters adopting hand picking methods from Raja Doraisingam Government Arts College Campus, Sivagangai district, Tamilnadu, India. All the collected species were brought in to the laboratory in a clean and air filled plastic boxes in alive condition. Each species were subjected to morphometric analysis. The morphometric characters such as body weight (fresh & dry), body length, body width, fore wing length & width, hind wing length & width, antennae length and length of fore, middle & hind legs were analyzed. For each species 10 different sized insects were chosen and their characters were individually analyzed. The analyzed data were tabulated and subjected to various statistical analyses. For molecular analysis the cockroach species *Therea petiveriana* were collected from twelve different localities of southern parts of Tamil Nadu, India (Table 1) of leaf litters by adapting hand picking methods. All the collected species were brought in to the laboratory in a clean and air filled plastic boxes in alive condition and each species were used for genomic DNA analysis.

DNA Extraction and RAPD-PCR Analysis

The collected insects were transformed into a new sterilized specimen bottle for DNA isolation. DNA was extracted using phenol-chloroform-isoamyl alcohol method (Maniatis *et al*., 1982) with some modifications. 20ng of DNA was dissolved in 20µl PCR reaction buffer containing 10mM Tris-HCl (pH 9.0), 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 0.2mM dNTPs, 20 pM of primer and 0.5 U of DNA polymerase. Twenty primers (RAPD Kit A1 to RAPD Kit A20) obtained from Integrated DNA Technologies (IDT), USA were used for RAPD-PCR studies. PCR technique was conducted according to the methods of Williams *et al*. (1990) with initial heat step (94ºC for 5 minutes), 40 cycles of denaturation (94ºC for 1 minute), annealing (36ºC for 1 minute) and extension (72ºC for 2 minutes) and a finial extension step (72ºC for 7minutes). Amplification was performed using a programmable thermal Cycler PTC-150 (MJ Research, USA). The products of PCR and DNA size markers [λ DNA digested with EcoRI and HindIII (Bangalore GeNei, India)] were loaded onto a 1.6% Tris-Borate-EDTA agarose gel (Sambrook *et al*., 1989) and run for 4hours at 50V. The gels were stained with ethidium bromide (0.5 µg) and photographed using DP-001FDC Photo Documentation System (Vilber Lourmat, France). The RAPD products were analysed using the Bioprofile 1D software (Vilber Lourmat, France). Cluster analysis was performed and dendrogram was plotted based on pair wise genetic distance estimated using the unweighted pair group method with arithmetic mean (UPGMA) based on Nei (1978).

RESULTS

For morphobased analysis the measurement of the body characters such as body weight (fresh and dry), length and width of the body, antenna length, length and width of fore and hind wings, length of fore, middle and hind legs for both sexes are examined and presented in table 2. In this analysis the maximum body weight (fresh (4.48±0.31) and dry (0.34±0.07)), body length (2.13±0.08) and width (1.26±0.06) are observed in female but the antennae length (1.94±0.65) is maximum in male species (**Table 2**).

Table 1 Locations, collection method, collection date and codes for the different isolates of *Therea petiveriana* collected from Tamilnadu.

Sl. No.	Location	Code	Collection Date	Collection method
1.	Alagar Hills, Madurai	I	18.10.14	Hand Picking
2.	Viraganoor Dam, Madurai	II	26.10.14	Hand Picking
3.	Nagamalai, Madurai	III	26.10.14	Hand Picking
4.	Thiruparangundram, Madurai	IV	25.10.14	Hand Picking
5.	RDGA College campus, Sivagangai	V	15.10.14	Hand Picking
6.	Thiruppuvanam, Sivagangai	VI	28.10.14	Hand Picking
7.	Manamadurai, Sivagangai	VII	22.10.14	Hand Picking
8.	Kalaiyarkovil, Sivagangai	VIII	02.11.14	Hand Picking
9.	Puliyangudi, Ramanathapuram	IX	12.10.14	Hand Picking
10.	Paramagudi, Ramanathapuram	X	12.10.14	Hand Picking
11.	Parthipanoor, Ramanathapuram	XI	17.10.14	Hand Picking
12.	Sathiragudi, Ramanathapuram	XII	17.10.14	Hand Picking

The fore and hind wings length and width of *T.petiveriana* species are individually analysed and the results are given in **table 2**. In this analysis the highest length of the forewing

(1.76±0.08) and hindwing (0.88±0.10) are recorded in the female species but the width of both fore and hind wings are higher in male species. Length wise analyses of legs (fore, middle & hind) of the *T.petiveriana* species are studied and the results are given in the **table 2**. Maximum length of the fore, middle and hind legs are recorded in the female species than male. In this analysis the data of body fresh weight and body length is subjected to correlation analysis and positive correlation was observed in both the sexes of the species studied and negative correlation was observed for of body dry weight and body length in the both sexes of this species.

Table 2 Morphometric characters of both sexes of the selected species *Theera petiverina* in RDGA College campus, Sivagangai District, Tamil Nadu

Sl.No.	Morphometric characters	Values	
		Male	Female
1	Fresh Body weight (mg)	2.17±0.19	4.48±0.31
2	Dry Body weight (mg)	0.27±0.07	0.34±0.07
3	Body Length (cm)	1.87±0.04	2.13±0.08
4	Body width (cm)	0.86±0.02	1.26±0.06
5	Antenna length (cm)	1.94±0.65	1.16±0.27
6	Forewing height (cm)	1.42±0.19	1.76±0.08
7	Forewing width (cm)	1.22±0.58	0.9±0.12
8	Hindwing length (cm)	0.72±0.14	0.88±0.10
9	Hindwing width (cm)	0.38±0.05	0.36±0.11
10	Foreleg length (cm)	1.16±0.15	1.36±0.24
11	Middle leg length (cm)	1.68±0.16	1.9±0.23
12	Hind leg length (cm)	2.06±0.23	2.32±0.23

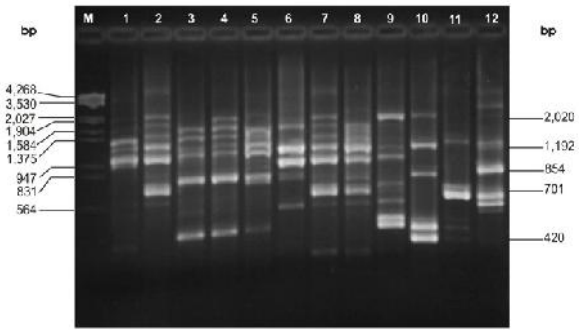

Fig.1a Random Amplified Polymorphs DNA of Different isolates of *Therea petiveriana* generated by the primer RAPD Kit A6

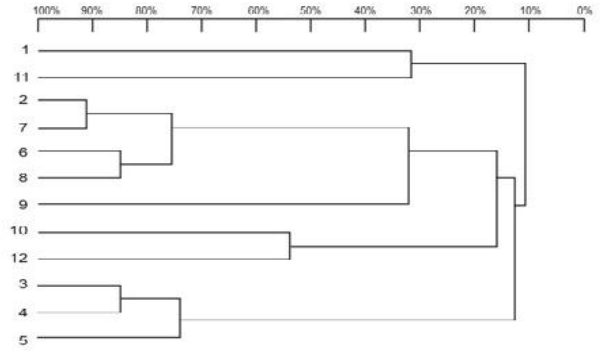

Fig. 1b Dendrogram with homology coefficient % (UPGMA) of *Therea petiveriana* generated by the primer RAPD Kit A6

In the present study the molecular genetic diversity of the *T.petiveriana* is analyzed using the RAPD PCR technology. Twenty RAPD (RAPD Kit A1 to Kit A20) decameric primers were tested for their ability to differentiate the twelve different isolates of *T.petiveriana*. Of all the primers tested, RAPD Kit-A6 ($^{5'}$GGTCCCTGAC$^{3'}$), Kit-A12 ($^{5'}$TCGGCGATAG$^{3'}$) and Kit-A16 ($^{5'}$AGCCAGCGAA$^{3'}$) yielded clear amplification patterns for isolates of *T.petiveriana*. These three primers amplified a total of 165 scorable bands in the molecular weight range of approximately 265bp to 3100bp. Dendrogram and similarity index were constructed based on the RAPD profiles generated by Kit A6, A12 and A16 primer using UPGMA (Bioprofile 1D software).

The primer Kit-A6 amplified a total of 65 scorable bands and the size of the amplified products varied from 410bp to 2030bp (**Fig. 1a**). The amplification patterns showed a maximum of seven bands in the isolates IV and IX (**Fig. 1a: lane 4 & lane 9**), and a minimum of three bands in isolate I (**Fig. 1a: lane 1**) for the primer Kit-A6. The dendrogram exhibited four major clusters, comprised of *T.petiveriana* isolates I and XI in first cluster, isolates II, VI, VII, VIII and IX in second cluster, isolates X and XII in third cluster and isolates III, IV and V in fourth cluster (**Fig. 1b**). The similarity index revealed maximum of 92% relatedness of *T.petiveriana* isolates II and VII, and minimum of 13% of genetic similarity of isolate I with all the isolates (**Fig. 1c**).

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.00											
2	0.20	1.00										
3	0.13	0.18	1.00									
4	0.13	0.33	0.69	1.00								
5	0.13	0.12	0.75	0.67	1.00							
6	0.29	0.73	0.15	0.15	0.15	1.00						
7	0.22	0.92	0.18	0.18	0.18	0.80	1.00					
8	0.25	0.83	0.18	0.18	0.18	0.89	0.91	1.00				
9	0.13	0.33	0.22	0.40	0.44	0.22	0.36	0.20	1.00			
10	0.13	0.20	0.18	0.25	0.29	0.13	0.22	0.13	0.25	1.00		
11	0.33	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	1.00	
12	0.13	0.18	0.18	0.18	0.25	0.25	0.20	0.22	0.22	0.57	0.25	1.00

Fig. 1c Similarity index based on RAPD profiles of *Therea petiveriana* generated by the primer RAPD Kit A6

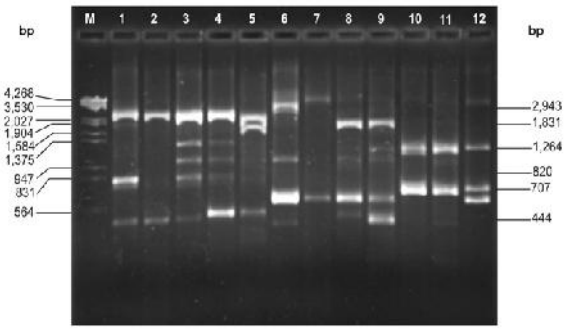

Fig.2a Random Amplified Polymorphs DNA of Different isolates of *Therea petiveriana* generated by the primer RAPD Kit A 12

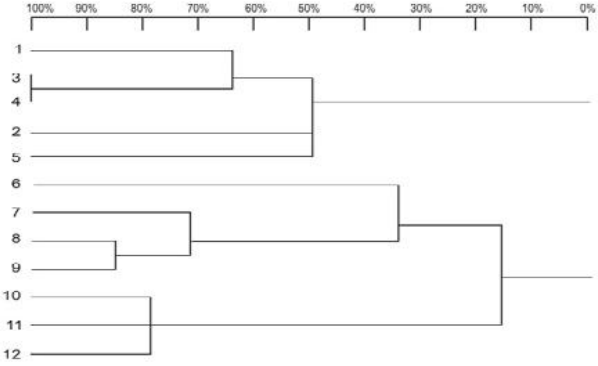

Fig. 2b Dendrogram with homology coefficient % (UPGMA) of *Therea petiveriana* generated by the primer RAPD Kit A12

The primer Kit-A12 produced 47 scorable fragments with the molecular weight ranges from 425bp to 2,973bp (**Fig.2a**). A maximum of five bands in isolates III and IV, and a minimum of three bands in isolates II, VII and X were generated by Kit-A12 primer. The dendrogram constructed from the RAPD profile of Kit-A12 revealed two distinct and separate clusters (**Fig. 2b**). One of the cluster grouped isolate I to V and the other grouped isolate VI to XII. The genetic similarity of 100% was recorded among the isolates of III and IV (**Fig. 2c**). The primer Kit-A16 produced a total of 53 scorable fragments in the molecular weight range of 265bp to 1990bp (**Fig. 3a**). The RAPD profile showed maximum of seven fragments in isolate IV and a minimum of two fragments in isolate I for this primer. The UPGMA dendrogram analysis generated for the RAPD profile of Kit-A16 showed two major clusters, one comprised isolate I to IV and the other included isolate V to XII (**Fig. 3b**). The similarity index (**Fig. 3c**) revealed 100% relatedness of *T.petiveriana* isolates V and IX and the least of 18% was recorded among isolates IV and VI.

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.00											
2	0.50	1.00										
3	0.67	0.33	1.00									
4	0.67	0.33	1.00	1.00								
5	0.50	0.50	0.33	0.33	1.00							
6	0.00	0.00	0.29	0.29	0.00	1.00						
7	0.00	0.00	0.33	0.33	0.00	0.40	1.00					
8	0.00	0.00	0.29	0.29	0.40	0.33	0.80	1.00				
9	0.00	0.00	0.25	0.25	0.33	0.29	0.67	0.88	1.00			
10	0.00	0.00	0.00	0.00	0.00	0.19	0.19	0.19	0.33	1.00		
11	0.00	0.00	0.00	0.00	0.00	0.19	0.19	0.19	0.29	0.80	1.00	
12	0.00	0.00	0.00	0.00	0.00	0.19	0.19	0.19	0.29	0.80	0.67	1.00

Fig. 2c Similarity index based on RAPD profiles of *Therea petiveriana* generated by the primer RAPD Kit A12

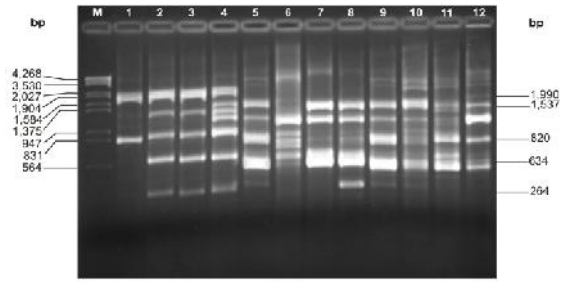

Fig.3a Random Amplified Polymorphs DNA of Different isolates of *Therea petiveriana* generated by the primer RAPD Kit A 16

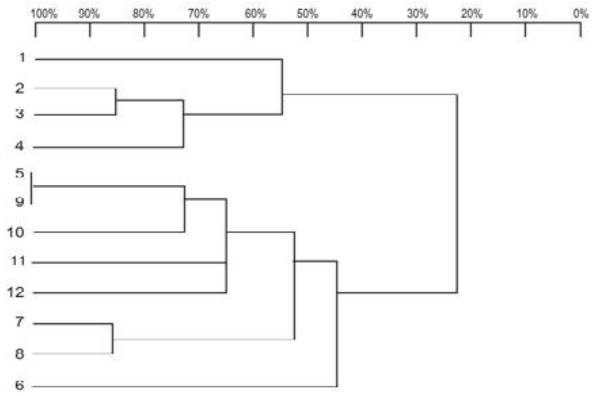

Fig. 3b Dendrogram with homology coefficient % (UPGMA) of *Therea petiveriana* generated by the primer RAPD Kit A16

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.00											
2	0.57	1.00										
3	0.67	0.89	1.00									
4	0.50	0.73	0.80	1.00								
5	0.33	0.22	0.25	0.40	1.00							
6	0.29	0.20	0.22	0.18	0.44	1.00						
7	0.22	0.22	0.22	0.22	0.57	0.25	1.00					
8	0.22	0.22	0.22	0.20	0.50	0.22	0.86	1.00				
9	0.33	0.22	0.25	0.40	1.00	0.44	0.57	0.50	1.00			
10	0.29	0.20	0.22	0.36	0.89	0.40	0.50	0.44	0.88	1.00		
11	0.33	0.22	0.25	0.40	0.75	0.44	0.29	0.25	0.75	0.67	1.00	
12	0.33	0.22	0.25	0.80	0.75	0.22	0.29	0.25	0.75	0.57	0.75	1.00

Fig. 3c Similarity index based on RAPD profiles of *Therea petiveriana* generated by the primer RAPD Kit A16

Among these three primers (Kit-A6, A12 and A16), Kit-A6 produced the most polymorphic amplification patterns that could distinguish almost all the isolates from each other. The RAPD profile of different isolates of *T.petiveriana* is generated by Kit-A6 primer revealed great degree of genetic polymorphism among the isolates.

DISCUSSION

The application of morphometric methods was proposed in the early 1960s by taxonomists who argued that taxonomy and systematics should be based on the use of multivariate statistical analysis of morphological characters as opposed to the use of underlying evolutionary or biological information. Morphometrics has the advantage over other methods such as electron microscopy or biochemical techniques (electrophoresis, protein and DNA analysis), that the organisms need not be destroyed, and can therefore be used for future studies. Insects are eminently suitable subjects for morphometric studies because their hard exoskeleton is easily measured and is largely free from the physical distortions suffered by many soft-bodied animals. Morphometric techniques can be used to identify and distinguish between morphologically similar groups of organisms when no single diagnostic character is available. Morphological variation within and between populations has been and still is the most widespread and practical method of classification of living and preserved organisms. Studies on variation among populations can be carried out using the morphometric technique. It can also be used to assess affinities and in many cases justify synonymy or recognize new taxa at the species level (Sokal & Crovello, 1970).

In this analysis the morphological characters such as body weight (fresh & dry), body length, antennal length, no. of antennal segments, fore and hind wings length & width and the length of fore, middle and hind legs of both male and female species of *Therea petiveriana* have been done. Mostly the maximum of results was observed in the female species when compared to male species. This similar trends based analysis was also studied in *Blatteila germanica, Supeila longipalpa and Diploptera punctata* by Chiang *et al.* (1989); in *Zonocerus variegates* (Barrientos, 1996); in *Aphis craccivora* (Mehrparvar *et al.*, 2012).

Various studies have reported that the morphometric analysis is a most precious tool in the field of taxonomic identification studies, comparative analysis, evolutionary studies and anatomical studies. Initially the identification of insect species only based on the morphological characters such as body size, colour, appearance, length and width of organs but a few years back the structure of genitalia was also included in the identification of species. At the same time this type of identification is not a conformed one and it is tentative. Followed by the developmental of molecular biology and biotechnological tools such as DNA isolation, PCR amplification technology, DNA sequencing analysis, phylogenetic analysis, DNA barcoding and DNA fingerprinting were applied in the field of taxonomic studies and make the identification is conformed one. At the moment a day these technologies were mostly used in the field of molecular systematic biology.

Genetic variation within a species has three components: genetic diversity (the amount of genetic variation); genetic differentiation (the distribution of genetic variation among populations); and genetic distance (the amount of genetic variation between pairs of populations). Molecular markers are used to describe and estimate genetic variation (Felsenstein, 1997). To be of evolutionary or taxonomic importance, geographic variation has to be genetically determined, at least to a large extent (Atchley, 1983). Variation in the quality of habitats can generate environmental variation in morphometric traits (Smith and Patton, 1988) which may be confounded with genetic variation. Laboratory rearing under standardized conditions can be used to control for environmental effects in studies of the genetic determination of morphometric differentiation (Bryante, 1977; Bryante and Turner, 1978; Sorensen and Sawyer, 1989).

In the present study the molecular genetic variability of the *T.petiveriana* is analyzed using the RAPD PCR technology. Twenty RAPD (RAPD Kit-A1 to Kit-A20) decameric primers were tested for their ability to differentiate the twelve different isolates of *T.petiveriana*. Of all the primers tested, RAPD Kit-A6 ($^{5'}$GGTCCCTGAC$^{3'}$), Kit-A12 ($^{5'}$TCGGCGATAG$^{3'}$) and Kit-A16 ($^{5'}$AGCCAGCGAA$^{3'}$) yielded clear amplification patterns for isolates of *T.petiveriana*. Among these three primers (Kit-A6, A12 and A16), Kit-A6 produced the most polymorphic amplification patterns that could distinguish almost all the isolates from each other. The RAPD profile of different isolates of *T.petiveriana* is generated by Kit-A6 primer revealed great degree of genetic polymorphism among the isolates. Similar mode of studies was also recorded in different primer of the same species by Sheeba *et al.* (2014). Neekhra *et al.* (2012) have also reporte the RAPD-PCR genetic variability analysis in the two different species *P.americana* and *B.germanica* and revealed that RAPD-PCR technique is useful for molecular taxonomy. The same RAPD-PCR study was also done in other insect species *Earias vitella* by Deivendran *et al.* (2015).

RAPD markers are playing an important role in the analysis of genetic diversity of a large number of insect species. The simplicity and reproducibility of the PCR-based assays, added to their higher multiplex ratio and capacity to detect higher levels of polymorphisms, makes them a lucid method to obtain intraspecific genetic variation in the insect species, where no prior sequence information is available (Kumar *et al.*, 2001). In the present study, RAPD markers generated from two random primers revealed sufficient polymorphism to characterize genetic variation within the *T.petiveriana* populations. The genetic variability among the populations of *T.petiveriana* could be attributed to varied ecology and geography of the collection sites. And also, the insect species undergoes and experiences different pattern of stresses and strains caused by our farming practices. These RAPD-PCR analyses can find a wide use in the field of identification and differentiation of closely related species and population within species. Genetic variability revealed is of great importance in species diagnostics as the pattern of bands revealed by RAPD-PCR are often species specific. It is finally concluded that some of the fragments are unique in this species which might be used for diagnostic purposes and comparative analysis.

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