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Molecular identification of mosquito vectors using genomic DNA isolated from eggshells, larval and pupal exuvium

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Abstract. Correct and precise identification of mosquito vectors is important in many respects including development of vector control strategies. Conventional identification methods have limitations for sibling and closely related species of mosquitoes, stage and quality of the specimen used and this could be overcome by DNA-based identification methods using molecular markers such as nuclear ribosomal internal transcribed spacer (ITS) which do not demand intact or undamaged specimen. Genomic DNA is usually isolated from whole mosquito, legs, wings etc. Alternate sources for genomic DNA isolation such as eggshells, larval and pupal exuviae were explored in this study by amplifying the ITS markers. Standardization of genomic DNA extraction and ITS amplification were carried out with laboratory specimens. The same was applied to specimens collected from the field. The results show that PCR amenable genomic DNA could be isolated from fresh exuviae collected in the laboratory and not from older and/or field specimens. But exuviae of larvae and/or pupae collected in the field reared to adulthood in the laboratory yielded PCR amenable genomic DNA. The results also revealed that the ITS2 marker could very well differentiate *Aedes aegypti* and *Aedes albopictus* by producing amplicons of ~330 bp and ~520 bp, respectively. The genomic DNA from these alternate sources also supported the species-specific PCR to distinguish the *Culex vishnui* subgroup mosquitoes.

INTRODUCTION

Correct identification of the insect vector is one of the important factors in the study of the arboviral diseases (Cook *et al.*, 2005). In addition, the precise identification of the target species has direct medical and practical implications, particularly in developing vector control strategies. In the past, mosquito taxonomy has been achieved mostly by using morphological characteristics, cyto-genetics and isoenzyme markers. Recently, the molecular approach has greatly improved the accuracy of species identification (Kumar *et al.*, 2007). Genomic DNA based molecular methods of species identification are advantageous as they can be applied to specimens and situations unsuitable for morphological taxonomy, for DNA might be obtained and analyzed from specimens in all developmental stages, of both sexes, fresh, preserved in alcohol, dried or frozen (Marrelli *et al.*, 2006). These methods could be applied not only to sibling species but also to members of closely related groups with overlapping morphological characters (Garros *et al.*, 2005). The methods have been applied to important groups of mosquito species complexes containing vector mosquitoes (Goswami *et al.*, 2005). Vectorial and behavioural variations found among these species groups or complexes constitute the major reason that needs accurate and precise identification (Garros *et al.*, 2004).

Isolation of PCR amenable genomic DNA is a prerequisite for the molecular approach of species identification. This could be

achieved using whole mosquito (Kumar *et al.*, 2007), legs (Ruiz *et al.*, 2005), wings (Garros *et al.*, 2004) etc. In the yesteryears, exuviae of insects such as butterflies (Feinstein, 2004), honey bees (Gregory & Rinderer, 2004) and dragonflies (Watts *et al.*, 2005) have been used to isolate genomic DNA and had been used for identification, trait selection etc.

Molecular markers such as Internal Transcribed Spacers (ITSs) of ribosomal DNA genes, third domain (D3) of 28S rDNA gene, mitochondrial Cytochrome oxidase C subunit I and II (COI & COII), Cytochrome oxidase B, 16S rRNA gene are helpful in species identification, phylogenetic analyses and other related studies. Among these molecular markers, commonly used for mosquito taxonomy, the internal transcribed spacers 1 and 2 (ITS1 & ITS2) of the ribosomal DNA are useful for distinguishing among closely related species of various genera such as *Anopheles* (Marrelli *et al.*, 2006), *Culex* (Toma *et al.*, 2000) and *Aedes* (Beebe *et al.*, 2007).

In this study, the ITS markers are used for the identification of the vectors of dengue and Japanese encephalitis viruses viz., *Aedes aegypti*, *Aedes albopictus* and *Culex tritaeniorhynchus*, *Culex vishnui* and *Culex pseudovishnui*, respectively. Along which, the usefulness of the left out parts in various instances of the life cycle of the mosquito, has been explored as source for genomic DNA.

MATERIALS AND METHODS

Laboratory specimens

The vector mosquito samples were received from the cyclic mosquito colony maintained at CRME, laboratory. Initially the eggs of *Aedes* spp. and rafts of *Culex* spp. were allowed to hatch separately in enamel coated iron trays filled 3/4th of its volume with tap water. When the eggs and rafts hatched, the left-out shells were collected, air-dried on blotting paper and transferred into a fresh, sterile micro-centrifuge tube and stored at –20°C until used. The hatched out larvae were fed with dog biscuit and yeast powder, finely ground and blended in the ratio of 3:2. Ten 3rd instar larvae were reared individually for each experiment. The exuviae were collected when the larvae molted into 4th instar larvae, pupae and adults. The experiment was carried out over time period for up to 9 days post-eclosion i.e. the pupae were allowed to emerge into adults and the exuviae were allowed to be in the same water in which the pupae were bred. Samples were collected after 0 to 9 days post-eclosion. These exuviae were air dried on blotting paper then transferred into fresh, sterile micro-centrifuge tubes and stored at –20°C until use. The corresponding adults were also preserved at –20°C.

Field specimens (exuviae)

The larval and pupal exuviae of the vector mosquitoes were collected from appropriate breeding habitats viz., cement tanks and grinding stones for *Ae. aegypti*, tree holes and leaf axils for *Ae. albopictus*, and paddy field for the *Cx. vishnui* subgroup mosquitoes. The collected specimens were immediately air dried on blotting paper and transferred into a clean and sterile micro-centrifuge tube. Then the samples were brought to the laboratory and stored at –20°C until use.

Field specimens (immatures)

Field mosquito samples were collected in and around Madurai city (Lat.: 9°58' N; Lon.: 78°10' E), one of the urban locality in the state of Tamil Nadu, southern India. The eggs of *Ae. aegypti* were collected within the city and those of *Ae. albopictus* from a sylvatic area, the Alagar koil hills, some 20 kms North of Madurai, using ovitraps; egg-rafts of *Culex* species in paddy fields at Kodimangalam, a nearby village within a radius of 20 kms.

The ovipads and a raft collected in the fields were allowed to hatch and the larvae reared as described above. The exuviae were collected air-dried and stored –20°C until processed. The other larvae collected were also reared to adulthood. The adults emerged were identified with morphological keys and then stored at –20°C for further processing.

DNA extraction and PCR

Except for the eggshells, all the exuviae were processed singularly for genomic DNA isolation. DNA extraction was carried out using 'DNA Extraction Solution' kit (Genei, Bangalore, India), following manufacturer's instruction with minor variations which is as: each sample was homogenized in 100 µl of DNA Extraction Solution and incubated for 20 minutes at room temperature. The homogenate was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh micro-centrifuge tube and equal volume of 100% ethanol was added. DNA was then pelletted by centrifuging at 10,000 rpm for 5 minutes. The DNA pellet was washed twice with same volume of 95% ethanol with spin at 5000 rpm for 5 minutes. A final wash was given with same volume of 70% ethanol. The DNA pellet was air-dried and dissolved in 10 ml of DNAse free deionized water.

PCR reaction was carried out in a volume of 25 µl containing 2.5 µl of 10X Taq polymerase buffer with 1.5 mM $MgCl_2$, 1.5 µl of dNTP mix (100mM each), 1.5 units of Taq polymerase enzyme, 2.4 nM of each primer, and 3 µl of the template DNA. The ITS2 primers (Porter & Collins, 1991) viz., 5.8S 5'-ATC ACT CGG CTC ATG GAT CG-3' and 28 S 5'- ATG CTT AAA TTT AGG GGG TAG TC-3' were used to identify the *Aedes* spp. whereas, the species diagnostic primers of ITS1 region described by Toma *et al.* (2000) were used to identify the *Culex* spp.

The thermal profile used to amplify ITS2 region from *Aedes* species was initial denaturation at 94ºC for 10 minutes followed by 25 cycles of denaturation at 94ºC for 60 seconds, annealing at 50ºC for 60 seconds and elongation at 72ºC for 60 seconds and a final elongation at 72ºC for 10 minutes. For the amplification of diagnostic ITS1 fragments from *Cx. vishnui* subgroup initial denaturation was carried out at 96ºC for 12 minutes followed by 35 cycles of denaturation at 96ºC for 30 seconds, annealing at 52ºC for 30 seconds and elongation at 72ºC for 90 seconds followed by a final elongation at 72ºC for 4 minutes. The reactions were carried out in Mastercycler (Eppendorf, Germany) thermal cycler. The PCR amplified DNA fragments were visualized in a 1.2% agarose gel stained with Ethidium bromide on a UV transilluminator (Vilbert Lormet, France).

RESULTS & DISCUSSION

It is understandable that whole mosquito or its body parts contain cells that ultimately yield genomic DNA. But the exuviae biochemically does not contain any nucleic acid and still yields genomic DNA useful in molecular taxonomic studies. This was explained as "When molting occurs, the lining of the foregut, hindgut, and all the tracheae pull out and become deposited with the molted skin" (Bertholf, 1925).

In the field collection, eggs of both the *Aedes* species could be obtained on the ovipad, whereas only a single raft of *Cx. tritaeniorhynchus* could be collected in the field.

The PCR amplification of ITS1 region distinctly differentiated the three species of the *Cx. vishnui* subgroup viz., *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui* by producing 536bp, 344bp and 246bp fragments respectively as reported by Toma *et al.* (2000) (Fig. 1). The PCR amplification of ITS2 region produced 2 distinct fragments of ~330bp and ~520bp for *Ae. aegypti* and *Ae. albopictus* respectively. This is suggestive of the usefulness of exuviae as a reliable source of genomic DNA in molecular taxonomy of mosquitoes.

When the above PCR reactions were carried out with genomic DNA extracted from exuviae collected on 1st day to 9th day post-eclosion no PCR amplification was seen *i.e.* PCR-amenable DNA could not be isolated from 24 hours or still older samples. Similarly exuviae collected directly from the field could not yield PCR amenable genomic DNA. This could be attributed to the decaying action of bacteria and other microbes on the small number of DNA yielding dead epithelial cells attached to the exuviae and the degradation of DNA along with cells. Although, the larval and pupal skins of larvae collected in the field and reared to adulthood in the laboratory yielded genomic DNA that

was found useful in the PCR reactions (Figs. 2 & 3).

However, in this study, the time span study was not carried out with eggshells since in our field survey for collection of exuviae, rafts/eggs, larvae etc., we could not find any eggshell. Therefore, it was presumed that any time span study with eggshells be of academic interest and of no practical utility.

The results are promising that the left-out parts of mosquitoes are as informative as the adult mosquitoes, when molecular studies are concerned. These left-out materials are very much useful in molecular identification of 3 important vector mosquitoes belonging to the *Cx. vishnui* subgroup, which are very difficult to be separated in the adult stage, as they have similar morphological characters which some times vary within species and overlap between species in a proportion of specimens (Reuben *et al.*, 1994). Although, for the *Aedes* vectors, morphological characters are quite reliable for the identification of the species, this technique will enable the early identification of the species before the adult emergence. In this study, however, the ITS2 primers have distinguished the two *Aedes* species distinctly; bur further it needs to be studied whether these primers could distinguish other species of *Aedes* mosquitoes those commonly breed in sympatry with the sylvan / rural tree-hole breeder *Ae. albopictus*.

Use of the left out body parts such as skins, eggshells etc., for genomic DNA isolation, has advantages over using part of

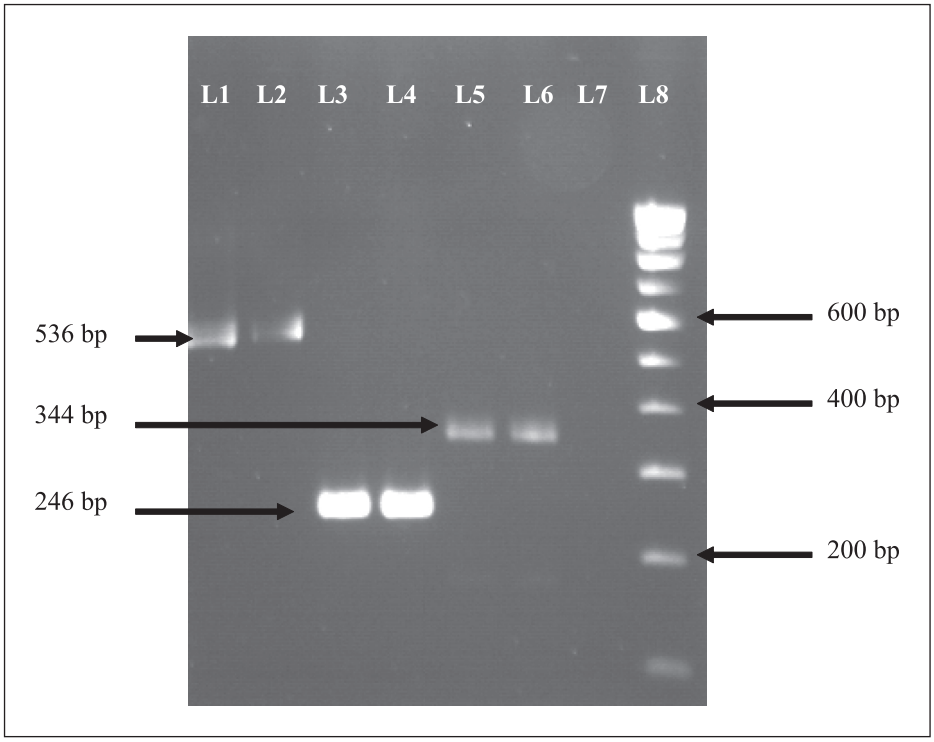

Figure 1. Gel picture showing species-specific PCR amplification of ITS-1 region of *Culex vishnui* subgroup mosquitoes collected from laboratory.

The amplicons of three different size viz., 536 bp, 344 bp and 246 bp are specific for *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui*, respectively. Lanes 1 & 2 show amplification from pupal exuvium and adult, respectively of *Cx. tritaeniorhynchus*; lanes 3 & 4 identify *Cx. vishnui* from pupal exuvium and adult, respectively, while lanes 5 & 6 identify *Cx. pseudovishnui* from pupal exuvium and adult, respectively. Lane 7 is negative control and lane 8 shows 100 bp molecular weight marker (Genei, Bangalore, India).

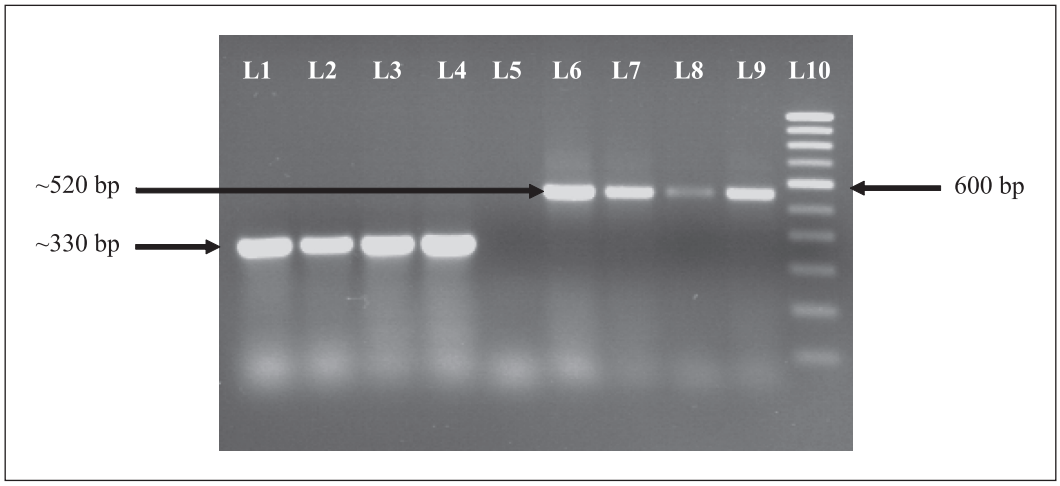

Figure 2. Gel picture showing amplification of ITS-2 region of *Aedes* species. The ~330 bp fragments in lanes 1-4 represent different genomic DNA sources of *Ae. aegypti*. Similarly the ~520 bp fragments in lanes 6-9 represent different genomic DNA sources of *Ae. albopictus*.

Lanes 1-4 show ~330 bp product specific for *Ae. aegypti* amplified from eggshells, larval & pupal exuvium and adult mosquito, respectively; similarly lanes 6-9 show ~520 bp product apecific for *Ae. albopictus* amplified from eggshells, larval & pupal exuvium and adult mosquito, respectively. Lanes 5 & 10 represent negative control and molecular weight marker (Genei, Bangalore, India).

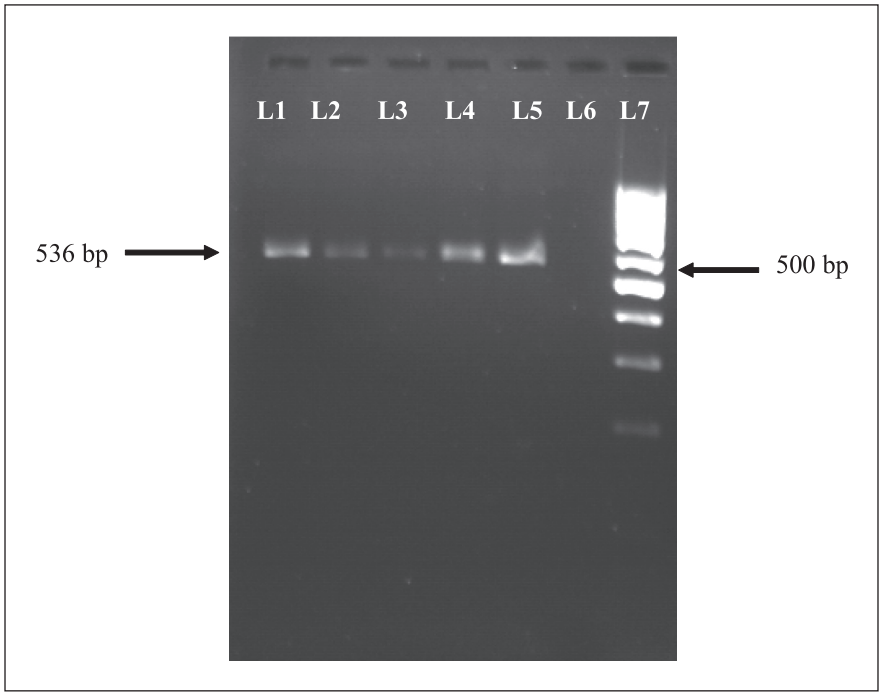

Figure 3. Gel picture showing amplification of ITS-1 region of the mosquito vector *Culex tritaeniorhynchus* collected from paddy field. Each lane represents isolation of genomic DNA from different sources.

L1 – DNA from eggshells; L2 – DNA from larval exuvium; L3 – DNA from pupal exuvium; L4 – DNA from adult wings; L5 – DNA from adult mosquito; L6 – Negative control; L7 – 100 bp molecular weight marker (Genei, Bangalore, India).

mosquito body such as wings, legs or abdominal segments etc. for molecular identification of cryptic and/or sibling species, as the use of later is prone to lose key morphological characters; for example most of the anophelines have key characters in wings and legs, few species of *Culex* have diagnostic characters in their legs and the genitalia, the most important distinguishing character, present in the final segment of the abdomen might be lost when body parts are used. In such cases it is advisable to retain and preserve the intact adult mosquito with all its morphological characters undamaged; Also, use of these materials spares the whole mosquito for further morphological identification, virus isolation, vouchering etc (Porter & Collins, 1991; Crabtree *et al.*, 1995; Cornel *et al.*, 1996).

The use of left out parts of mosquitoes especially the eggshells, overcome the difficulty, as faced by Beebe *et al.* (2007), of PCR amenable genomic DNA extraction from eggs older than 8 weeks, as dried eggs of *Aedes* spp. are known to hatch and produce viable larvae even after eight months.

Overall the ITS sequences distinguish vector mosquito species, however, information on intra-specific and geographic variation is scarce. Marrelli *et al.* (2006) suggest few precautions to be considered in future studies viz., (i) voucher specimens, assigned to the DNA sequences, need to be deposited in collections; (ii) intra-specific variations should be thoroughly evaluated; (iii) ITS2 and other molecular markers, considered as a group, will provide more reliable information; (iv) data about biology of vector populations that are missing should be prioritized; (v) the molecular markers are most powerful when coupled with traditional taxonomic tools. Use of exuvium would help vouchering the intact adult specimen as such, whose DNA sequence is being studied and thereby overcoming the problems notified by Marrelli *et al.* (2006).

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