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RAPD-PCR characterization of insecticide resistant and susceptible *Anopheles* species

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Abstract

Anopheles barbirostris is the major vector of Timor filaria, and *A. jamesii* is of Bancroftian filaria in Srilanka, and is a suspected vector of malaria as it can support the sporogenic cycle of *Plasmodium vivax.* Deltamethrin screening revealed that *A. jamesii* was susceptible to deltamethrin ($LC_{50} =$ 0.0025 ppm; $LT_{50} =$ 11.38 min), while *A. barbirostris* was resistant ($LC_{50} =$ 3.802 ppm; $LT_{50} =$ 20.28 min). Genomic DNA isolated from the two species were used for characterisation of the insecticide resistance and susceptibility using Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR). 15 random primers produced 59 bands in *A. barbirostris* and 31 bands in *A. jamesii.* Out of these, 58 bands were polymorphic between the two species. The genetic distance calculated using FREE TREE software indicated 0.91549 (Nei and Li method) and 0.83824 (Jaccard method). The matrices for dissimilarity (1.358) and similarity (0.150) between the two species calculated using NTSYSpc 2.2 showed polymorphism of 88.9 %. Genetic variance between *A. barbirostris* and *A. jamesi* is probably the reason for the former to be resistant, while the latter is susceptible to deltamethrin.

Key words: Polymorphism; deltamethrin; insecticide resistant; RAPD-PCR; dissimilarity matrix.

Introduction

The genus *Anopheles* (Diptera: Culicidae: Anophilinae) consists of approximately 484 species, out of which 100 species serve as vector of malaria and they alone are responsible for 500 million cases of malaria each year.[1] In addition they are obligate intermediary hosts for numerous other diseases such as filaria, dengue fever, elephantiasis, etc. Since many mosquitoes are members of morphologically indistinguishable or similar species complexes, identification or characterization at the molecular level is necessary, which can be achieved by the use of RAPD-PCR .

Large scale use of organochlorine (DDT/ BHC), carbamates, organophosphates and synthetic pyrethroids, has led to the widespread development of resistance due to selection for certain genes, with adverse impact on the human health.[2] Deltamethrin, a synthetic

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pyrethroid ester, is the most widely used insecticide, and resistance is now extremely widespread and threatens the success of world-wide vector control programmes.[3]

Two primary protein mechanisms of insecticide resistance are mutation causing alterations in the target sites and increase in the rate of insecticide metabolism by the enzymes responsible for detoxification. Methods of resistance include thickening of the cuticle of the vector to facilitate less permeation of the insecticide, metabolic resistance via over-expression of metabolizing P450 mono-oxygenases and glutathione-S-transferases, and the *kdr* sodium channel mutations which render the action of insecticides ineffectual.[3] Genetic make-up of the different species vary as well as the impact of insecticide among the different species also vary. Random Amplified Polymorphic DNA (RAPD)[4] permits scores of markers to be assayed on DNA extracted from a single mosquito. The analysis requires fairly a small amount of template DNA which need not be double stranded or purified.[4]

RAPD bands may display a high degree of polymorphism, and screening multiple primers against taxa of interest has proven to be a means of quickly identifying species-specific markers.[5] In the present study, two species of *Anopheles* from Mizoram (*A. barbirostris* Wulp and *A. jamesii* Theobald) were subjected to insecticide bioassay to assess the status of resistance or susceptibility to deltamethrin and to characterize the resistant and susceptible species by RAPD-PCR.

Materials and Methods

Collection and characterization of mosquitoes

A. barbirostris and *A. jamesii* (Fig. 1) were collected from two county areas, Lengpui and Sihhmui, Mizoram, by larval-dip method and identified based on key features.[6-8]

Screening of deltamethrin susceptibility

A baseline susceptibility test in terms of LC_{50} and LT_{50} was performed by exposing the 3rd instar larvae to 1% deltamethrin (K-orthrine - 2.5% deltamethrin active ingredient) according to WHO protocol.[2] Mortality was counted after 2 h of exposure. The laboratory assays were conducted in 18 x 28 cm

Figure 1. Adults of *Anopheles barbirostris* and *A. jamesii*.

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plastic cups containing 100 ml of distilled water. A known concentration of deltamethrin was prepared. Fifty early 3rd instar larvae were placed in each cup. The untreated control was maintained for each day's assay. The cups were held at 27-29°C and 65-75% relative humidity. The dead larvae were counted, removed and discarded. The data were adjusted by Abbott's formula. The LC_{50} and LT_{50} were calculated by log probit.[9]

Isolation of genomic DNA

Isolation of *Anopheles* genomic DNA was standardized based on earlier procedures,[10] in terms of quantity, RNA contamination and DNA shearing. Adult *Anopheles* was homogenized and further homogenized using lysis buffer (20 and 80 µl - 50 mM Tris-Hcl, pH 8.0; 5 mM EDTA, pH 8.0; 100 mM NaCl, 1% SDS). RNAse (4 µl, 10 mg/ml) was added with a working solution of 2 µg/µl and incubated at 37°C for 1 h. Again 400 µl of lysis Buffer was added and incubated for 10 minutes. Further, proteinase K (50 µl - 2 µg/µl) was added and incubated at 55°C for 2 h. In addition, PCI (500 µl - 25:24:1; saturated with 10 mM Tris buffer, pH 8.0 and 1 mM EDTA) was added, mixed well and spun at 10,000 rpm for 10 minutes at 4°C. The supernatant was mixed with 3 M sodium acetate (1/10th volume of the supernatant) and ice cold isopropanol (0.7th volume of the supernatant) and precipitated overnight at -20°C. It was centrifuged at 12,000 rpm for 10 minutes at 4° C. The DNA pellet thus formed was air-dried and re-suspended in TE Buffer (15 µl- pH 7.5 - 10 mM Tris; 1 mM EDTA). After DNA dissolved in the TE Buffer, it was subjected to gel electrophoresis.

RAPD-PCR of genomic DNA

The reaction was carried out for 15 µl/ reaction in which the reagents were added in the PCR tubes. The 15 primers tested were shown in Table 1. The cocktail mixture was made for 11 reactions in an 1.5 ml Eppendorf tube. Since the stock concentration of the different primers were different, different µl of primer ranging from 0.24-0.40 µl were added to different PCR tubes. 13.8 µl each of the cocktail mixture was added to the different PCR tubes containing different primers. Then, water was added needed to make the volume to 15 µl in each tubes. Gradient PCR was carried out at different annealing temperatures as required by the different primers. After completion of 30 PCR cycles, the products were subjected to gel electrophoresis with 1.5% agarose gel and 2.5 kb DNA marker was run.[11,12]

Table 1. List of random primers tested.

Primer name	Primer Sequence **(5'--->3')**	Annealing Temp. (°C)
MA 05	TGCGCCCTTC	40
MA 06	CTGCTGGGAC	36.1
MA 09	GACGGATCAG	30
MA 11	GTTGCCAGCC	36.9
MA 12	ACCGCGAAGG	30
MA 14	TGGGCGTCAA	36.1
MA 15	GGCGGTTGTC	36.9
MA 23	AGGCGATAAG	36.1
MA 24	TGACCCGCCT	39.2
MA 26	GACGTGGTGA	30
MA 27	CCGACAAACC	36.1

Agarose gel electrophoresis

0.8% of agarose gel was used to run the genomic DNA sample in which 0.8 g of agarose is dissolved in 100 ml of 1X TBE buffer (pH 8.3; Stock TBE solution 500 ml of 10X - Trisbase 53.89 g, boric acid 4.205 g, EDTA 27.5 g; 10 ml of 10X TBE and 90 ml of sterile Milli-Q water). The required amount of agarose was dissolved in 1X TBE by boiling in microwave oven for less than 1 min. The agarose was then cooled till vapors were not visible on it. Ethidium bromide (10 mg/ml) was

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added (2.5 µl to 20 ml of the gel solution) and then poured to plate. The solidified gel was then placed in electrophoretic tank filled with 1X TBE Buffer. The DNA sample was loaded in the gel with a loading buffer [5 µl of DNA + 1 µl of loading buffer (10 mg/ml) (- 2.5 mg of bromophenol blue/xylene cyanol, 4 g of sucrose, store at 4°C)] and were usually subjected to 100 volt until the dye was run till the end of the gel. For PCR products, the gel concentration used was 1.5%.[12]

Data analysis

Genetic distance was calculated using FREE TREE software.[13] The program NTSYSpc 2.2 was used for the analysis of the results of RAPD.[14] A matrix was generated using SIMQUAL option for similarity analysis and SIMGEND option for dissimilarity analysis.

Result and Discussion

Table 2 shows lethal activity of delatamethirn on the mosquitoes. The mortality ranged from 40-80% and was rarely 100%. *A. jamesii* was found to be susceptible to deltamethrin (LC_{50} = 0.0025 ppm; LT_{50} = 11.38 min); whereas, *A. babirostris* was resistant (LC_{50} = 3.802 ppm; LT_{50} = 20.28 min) (Table 3). The resistance of *A. babirostris* to deltamethrin could be due to mutation causing alterations in the target sites or increase in the rate of insecticide metabolism by the enzyme responsible for detoxification.

Although morphologically very similar, one species was susceptible while the other was resistant to deltamethrin which necessitate the use of RAPD-PCR to establish the genetic dissimilarity (Fig. 2). Screening of 15 random primers was performed in which 90 bands were produced, 58 of which differed between the two species.

Genetic distance using FREE TREE gave the value 0.91549 using Nei and Li method and 0.83824 using Jaccard method (nucleotide divergence = 1.00000; repetition count = 500; boot strap value =100%) (Table 3). The presence or absence of bands were represented by 1 and 0, respectively, and entered in NTSYSpc 2.2 analysis software. The dissimilarity matrix value generated using SIMGEND option was 1.358 and the similarity matrix value using SIMQUAL option was 0.150. These values represent the level of dissimilarity and similarity between the two *Anopheles* species. The % polymorphism (88.9), resolving power (70), polymorphic information content (0.44), effective multiplex ratio (0.89), marker index (0.39) were also calculated.

Table 2. LC_{50}, LT_{50} and RAPD band attributes and similarity and dissimilarity matrices between *A. barbirostris* and *A. jamesii*.

Parameters		Value
LC_{50} - ppm	*A. barbirostris*	3.8020
	A. jamesii	0.0025
LT_{50} - min	*A. barbirostris*	20.28
	A. jamesii	11.38
Total bands		90
Polymorphic bands		80
Resolving power (RP)		70
Polymorphic information content (PIC)		0.44
Effective multiplex ratio (EMR)		0.89
Polymorphism (%)		88.9
Marker index (MI)		0.39
SIMQUAL similarity matrix - NTSYS		0.150
SIMGEND dissimilarity matrix- NTSYS		1.358
Genetic distance (Nei and Li) – FREE TREE		0.91549
Genetic distance (Jaccard) – FREE TREE		0.83824

The RAPD method has proven useful for species identification and it was chosen because of its simplicity and applicability to a wide range of species without need for prior molecular work (e.g. cDNA or genomic libraries).[4,15] Using this trial and error approach, species-specific markers can be identified. This technique has the advantage of reflecting the genome of individuals represented by several genetic loci providing information

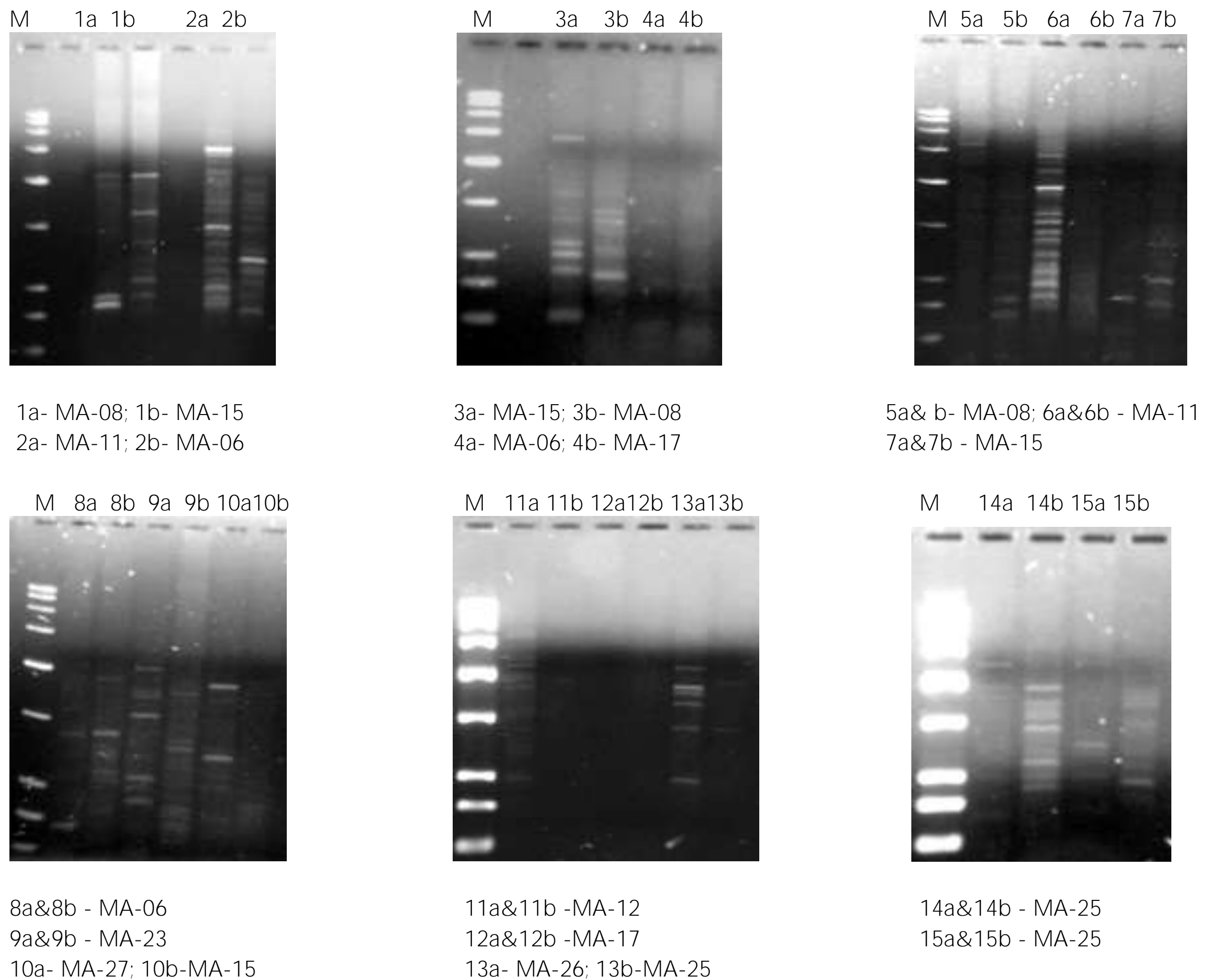

Figure 2. RAPD-PCR results using 15 random primers on the DNA extracted from *A. barbirostris* and *A. jamesii* (M - 100-3000 bp DNA ladder used as size marker; 1-15 represents lanes; a - *A. barbirostris* and b - *A. jamesii*).

on genome structure. Caution should be taken when interpreting RAPD results as the presence of a band may confirm loci, but absence of a band does not confirm the absence of loci due to the variability in the products generated. A number of controls must be run to overcome this problem. RAPD-PCR has its uses in mosquito genetics as it generates a fingerprint of the genome and is regarded as a dominant marker. Thus, RAPD-PCR and analysis of its results showed that the two morphologically similar *Anopheles* species show genetic variance which is probably the reason for *A. barbirostris* to be resistant, while *A. jamesi* is susceptible to deltamethrin.

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REFERENCES

1. Sturchler D (1989). How much malaria is there worldwide? *Parasitol Today*, 5, 39-40.
2. World Health Organization (2006). Guidelines for laboratory and field testing of mosquito larvicidaes. WHO/ CDS/ WHOPES/G-CDPP/2006.1.
3. Bouwman B, Sereda HB & Meinhardt HM (2006). Simultaneous presence of DDT and pyrethroid residues in human breast milk from a malaria endemic area in South Africa. *Environ Pollut*, 3, 902-917.
4. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*, 18, 6531-6535.
5. Arnold ML, Buckner CM & Robinson JJ (1991). Pollen-mediated introgression and hybrid separation in Louisiana Irises. *Proc Natl Acad Sci USA*, 88, 1398-1402.
6. Das BP, Rajagopal R & Akiyama J (1990). Pictorial key to the species of Indian Anopheline mosquitoes. *J Pure Appl Zool*, 3, 131-162.
7. Nagpal BN & Sharma VP (1994). *Indian Anophelines*. Scientific Publishers, New Delhi, pp. 416.
8. Nagpal BN & Sharma VP (1987). Survey of mosquito fauna of northeastern region of India. *Indian J Mariol*, 24, 143-49.
9. Finney DJ (1971). *Probit Analysis*. Cambridge, Cambridge University Press.
10. Rivero J, Ludmel U, Norming S, Martha P, Yasmin R, Palis & Flor H (2004). Opptimization of extraction of mosquitoes DNA suitable for PCR-based techniques. *Int J Trop Insect*, 3, 266-269.
11. Williams JGK, Hanafey MK, Rafalski JA & Tingey SV (1993). Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol*, 218, 704-740.
12. Sambrook J, Fritsch EF & Maniatis T (1989). *Molecular Cloning. A Laboratory Manual, 2nd edn*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Pavlicek A, Hrda S & Flegr J (1999). FreeTree - Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. *Folia Biol*, 45, 97-99.
14. Rohlf F J (1998). NTSYSpc 2.2. Numerical taxonomy and multivariate analysis system. *Exeter Software: Applied Biostatistics*.
15. Wilkerson RC. Parsons TJ, Albright DG, Klein TA & Braun MJ (1993). Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: *Anopheles*). *Insect Mol Biol*, 1, 205-211.

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