Iranian J. Publ. Health, Vol. 31, Nos. 3-4, pp.133-137, 2002

Use of Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) and ITS2 PCR assays for differentiation of populations and putative sibling species of *Anopheles fluviatilis* (Diptera: Culicidae) in Iran

*[1] *SR Naddaf Dezfouli, [1] MA Oshaghi, [1] H Vatandoost, [1] E Djavadian, [1] Z Telmadarei, [2] & M Assmar*

[1] Dept. Medical Entomology and Vector Control, School of Public Health and Institute of Health Research, Tehran University of Medical Science, Tehran, Iran.
[2] Dept. Medical Parasitology, Pasteur Institute of Iran, Tehran, Iran.

Abstract
Anopheles fluviatilis complex is known to be a vector of malaria in Iran. Since mosquitoes of this species cover a wide geographical range in Iran, they might have evolved into different separated populations. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) assay was used to differentiate geographic populations of this species. DNA was extracted from individual mosquitoes from 8 localities in 4 south and southeast provinces and amplified in PCR reactions using 18 single primers of arbitrary nucleotide sequence. Results of RAPD-PCR showed that Kazeroun populations could simply be differentiated from other populations using a diagnostic fragment amplified with primer UBC-306. But other populations could not be differentiated either visually or by means of statistical analysis. Moreover ITS2 fragments of some selected specimens were amplified using a pair of universal primer and sequenced as a key standard for detection of putative sibling species. Sequence analysis of the ITS2 fragments revealed a very high (100%) homology among the populations. These findings are crucial in epidemiological studies concerning relatedness of geographic populations and vector movement in the region. Results of RAPD-PCR and ITS2 analysis suggest that this taxon in Iran comprises of only one species with a low genetic variation among geographic populations.
Key words: Anopheles fluviatilis; ITS2, RAPD-PCR, Iran.

Introduction

Anopheles (*cellia*) *fluviatilis* James is known as a vector of malaria in Iran. It is distributed on the foothills of Zagros Mountains from southwest to south with some patchy distribution in the southeast at altitudes ranging from 50 to 1100 meters. This species has been found to play an important role in transmission of malaria in some areas of Iran while in others, despite endemic malaria, it has never been found infected (4). Since mosquitoes of this species cover a wide geographical range in Iran (fig. 1), they might have evolved into different separated populations or even morphologically undistinguishable species. Studies based on examination of banding patterns of polyten chromosomes showed that *An. fluviatilis* comprises of three reproductively isolated species in India designated as S, T and U (13). Recently, Manonmani *et al*., (8) using a PCR-based method have studied the ribosomal DNA (rDNA) of the specimens from two areas of Orissa state in India and identified two groups of internal transcribed spacer 2 (ITS2) sequences which were named X and Y by the authors. Later on, the species X and Y were shown to be identical to species S and T respectively (6). The cytological and rDNA-ITS2 PCR assays could be applied at species level for diagnosis of putative sibling species but are not efficient at population level. Random amplified polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR)-based technique which provides a quick and effective means for initial detection of cryptic species (14) but, perhaps the most appropriate use of this method is to identify intraspecific variations at the population level that represent subsets of the whole species gene pool (as reviewed in reference 2). This method has also been used to monitor dynamics of non-indigenous species following their introduction into a new geographical area (5).
The objective of this study was to use RAPDs to determine the rate of genetic variation of geographically distributed populations of *An. fluviatilis* in Iran . We also sought to differentiate the putative sibling species by sequencing the ITS2 fragments of some selected specimens as a key standard.

*Corresponding author. Tel: +98 21-6953311-20. Fax: +98 21 6465132, E-mail: snaddaf_2001@yahoo.com

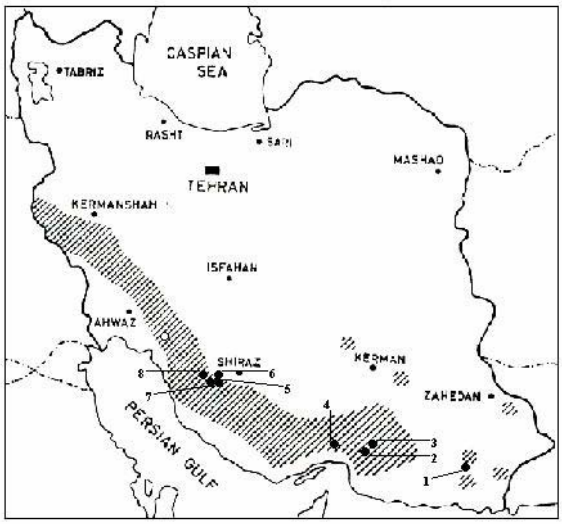

Fig 1. Distribution *of Anopheles fluviatilis* in Iran denoted by hatched areas (Reproduced from Eshghi et al. 1976). Black dots represent collection sites, 1, Daman; 2, Khosroabad; 3,Datehshoor; 4,Siahoo; 5, Djadas; 6, Islamabad; 7, Dadin; 8, Pirsabz.

Materials and Methods:

Mosquito collections

Adults and larvae of *An. fluviatilis* were collected from 8 different localities in four south and southeastern provinces of Iran including Hormozgan, Sistan & Baluchistan, Kerman and Fars. Table 1 lists the locations, sample sizes and collection methods and fig. 1 shows the geographical locations of all sampling sites. The collected larva specimens were reared to adult stage in insectery. All the specimens were identified using Shagodian's morphological key (12) and then stored at –20 ºC for further investigation.

Table 1: Details of *Anopheles fluviatilis* specimens used in this study.

Province	Area/village	Habitat/sample size	Type of collection	Date of collection
Hormozgan	Siahoo/Siahoo**	Animal and human baits (45)	Hand catch	Aug.- Sep.2001
Sist. & Balu	Daman/Abchekan*	Shelter pit (48)	Hand catch	Mar. 2001
Kerman	Kahnouj/Khosroabad*	Shelter pit (30)	Hand catch	Feb. 2001
Kerman	Kahnouj/Darehshoor*	Shelter pit (38)	Hand catch	May. 2001
Fars	Kazeroun/Djadas**	Stream (larva) (25)	Hand catch	Jul 2000
Fars	Kazeroun/Islamabad*	Human dwelling (19)	Total catch	Nov. 2000
Fars	Kazeroun/Dadin*	Stream (larva)(12)	Hand catch	Nov. 2000
Fars	Kazeroun/Pirsabz**	Stream (larva) (24)	Hand catch	Jul. – Sep 2002

The number of asterisks (*) indicates the number of specimens subjected to sequencing

Molecular method

DNA extraction: DNA was extracted from individual dried mosquitoes by homogenization of samples using a pestle grinder followed by the DNA extraction protocol of Ballinger-Crabtree *et al*. (1). The concentration of DNA samples were quantified by both running them on an agarose gel along with a known concentration of DNA molecular weight marker (MWM III, Roche) and UV spectroscopy. Working solutions were adjusted to 5ng/µl in double distilled water. The DNA samples were stored at 4 ºC, and to avoid degradation due to repeated freezing and thawing, were not frozen at any time.

RAPD-PCR Amplification: The procedures described here are those of Ballinger-Crabtree *et al*. (1) with minor modifications. Simply each 25µl reaction contained 10mM Tris-HCl, pH 8.3, 50mM KCl, 2 mM Mg Cl_2, 200µM of each dNTP (Pharmacia, Biotech), 30ng of each primer, 0.8U of taq DNA Polymerase (Amersham Pharmacia Biotech Inc.) and 10 ng of DNA. Reactions were overlaid with 30µl of mineral oil and amplified in a thermal cycler (Techne USA) programmed for one cycle at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 2min and a final extension step at 72°C for 7min, all with a minimum ramp time. In all the amplification, a negative control, which consisted of all reaction components expect the template DNA was included. Eight µl of amplification products were run along with a 100 bp ladder marker on a 1.2% agarose gel containing ethidium bromide for 3 hours at 60 V.

The gels were observed on a UV transluminator and digital photographs were provided using a gel documentation equipment (Vilber Lourmat, France). The approximate molecular weight of amplification products was calculated using PhotoCaptMW software, version 99.03. We used 17 decamer primers (UBC-304, UBC 306, UBC 308, UBC 310, A-07, AB-01, AB-03, AB-04, AB-07, AB11, AB-19, B1, B-07, B-08, M-04, S-20 and R-11) and one 17 base primer (M13) in this study, all obtained from Roche Molecular Biochemicals (Table 2).

Moreover the ITS2 fragments of some 11 specimens representing different populations were amplified using 5.8S and 28S primers and PCR reaction conditions of Manonmani *et al*. (8). Five µl of PCR products were electrophoresed on a 1.2% agarose gel to verify the size of the products and the remainders were purified using a gel band purification Kit (Amersham Pharmacia Biotech Inc). The concentration of DNAs recovered from the gels was quantified by UV spectroscopy and subjected to sequencing in an automatic sequencer. Sequencing was performed for both strands and the consensus data was deposited in Genbank database with the following accession Nos. AF509342, AF509344-47, AF509349-50, AF509352-53, AY172564 and AY172567.

Table 2. Names and sequences of primers used in this study

Primer	Sequence (5'-3')
A-08	GTGACGTAGG
B-01	GTTTCGCTCC
B-07	GGTGACGCAG
B-08	GTCCACACGG
AB-01	CCGTCGGTAG
AB-03	TGGCGCACAC
AB-04	GGCACGCGTT
AB-07	GTAAACCGCC
AB-11	GTGCGCAATG
AB-19	ACACCGATGG
M-04	GGCGGTTGTC
R-11	GTAGCCGTCT
S-20	TCTGGACGGA
UBC-304	AGTCCTCGCC
UBC-306	GTCCTCGTAG
UBC-308	AGCGGCTAGG
UBC-310	GAGCCAGAAG
M13	GTAAAACGACGGCCAGT

Results

To screen for diagnostic RAPD markers all the primers were tested on 6 individuals from 4 localities and 8 primers that produced bright and consistent bands were selected for larger samples. The data was first examined for fragments that were unique and conserved among all individuals of a given population**.** Moreover, the similarity of fragment profiles produced by the 3 best primers, UBC-306, UBC-304 and UBC-310, for each individual was compared with that of every other individual in a pair wise fashion and the similarity index (SI) between each pair was calculated according to the formula $SI = 2N_{AB}/(N_A + N_B)$ developed by Nei and Li (9), where N_{AB} is the number of shared amplified fragments for specimen A and B, and N_A and N_B are the total number of amplified fragments for specimen A and B respectively. Then the mean similarity index within and between populations were assessed**.** We could differentiate clearly the *An. fluviatilis* population of Kazeroun area from Fars province either visually using a single fragment produced by UBC-306 (fig.2) or by similarity index (SI). An approximately 1.5 Kbp marker produced by UBC-306 primer was absent in all 80 individuals (100%) of Kazeroun populations but present in 161 out of 162 (~99%) individuals of 4 other populations. This pattern was consistent for all the specimens collected from 4 villages of Kazeroun area including Islamabad, Djadas, Dadin and Pirsabz. We tried to favor the PCR conditions for amplification of UBC-306 1.5 Kbp marker in reactions containing DNAs from Kazeroun specimen by using serial dilutions of template DNA ranging from 2 to100 ng/µl and changing the amount and the type of *Taq* polymerase, but in none of them the 1.5 Kbp marker was amplified. Such unique diagnostic band was present in all populations of Sistan & Baluchistan, Kerman, and Hormozgan provinces. Although there were some differences in patterns of RAPD-PCR products among these populations, however they were not consistant and could not be differentiated either visually or by calculation of SI.

The sequences of ITS2 region of some 6 selected specimens from Kazeroun area, which displayed diagnostic fragment profiles, and 5 others including 2 from Hormozgan, 1 from Sistan & Baluchistan, and 2 from Kerman were generated and aligned with each other. All the ITS2 fragments were found to be 374 nucleotides in length with GC content of the 55%, which was concordant with those of other *Anopheline* species (3, 10, 11). The ITS2 sequences were free of ambiguities and no intraspecific variation was observed either in length or nucleotide composition. This suggests that the mosquitoes of *An. fluviatilis* in the studied areas are composed of one consensus type.

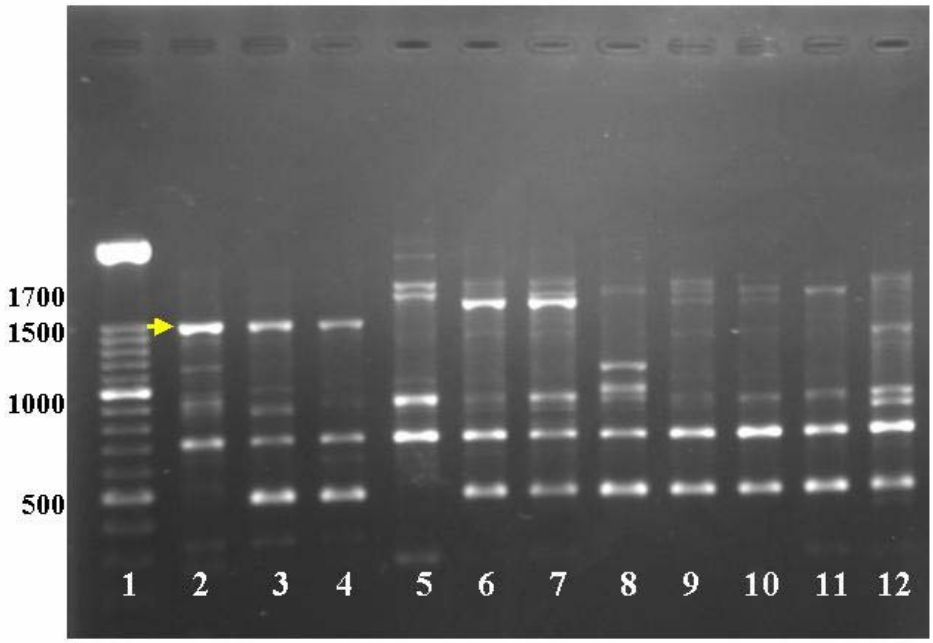

Fig. 2. Fragments amplified using UBC-306 primer on DNA extracted from individuals of 2 populations of *Anopheles fluviatilis* originated from Kazeroun and Iranshahr. Lane 1, 100 bp ladder; lanes 2-4, Iranshahr population; lanes 5-12, Kazeroun population. The arrowhead indicates the 1.5 kbp fragment which lack of it is diagnostic for Kazeroun populations.

Discussion

Amplification of DNA with a single 10 base-long primer of arbitrary DNA sequence is a Polymerase Chain Reaction (PCR) based technique, which yields a series of discrete fragments. This method reveals large number of polymorphisms, which can be used as genetic markers in research involving species diagnostics, population differentiation, and genetic fingerprinting. This method has shown to function well with insects (7), as these organisms have a relatively large genome size, which increases the probability of finding polymorphism. Differentiation between species or populations by RAPD-PCR might be accomplished either visually using some unique diagnostic bands or by statistical analysis of frequency of data for a group of fragments. In the latter one the genetic relatedness of groups, i.e. species, subspecies or populations is estimated by analyzing the variations within and among those groups and then the relatedness of unknown groups with those in the database is determined. However, statistical analysis has been shown to cause 10- 30% misclassifications for unknown population (1) and if more fragments in the discriminant analysis were included the complexity of the test would increase.

There are instances in which 95% individuals of a population or 99% individuals of a subspecies have been classified correctly using a single fragment amplified by a single 10-

mer primer of arbitrary sequence (1,7). In this study we were able to differentiate 99% of individuals of Kazeroun populations of *An. fluviatilis* with a single marker produced by a10-mer primer. Screening for such markers makes RAPD-PCR a friendly, faster, and cheaper procedure for identification of sibling species or discriminating among populations. Besides, they may be of great value in epidemiological studies in which an estimate of genetic relatedness of different geographic populations of a species will be an important tool in the study of vector movement.

It is known that the ITS2 region is highly conserved at the intra-species level but very variable at inter-species level (2) and has shown to be a useful tool for diagnosis of closely related species such as *An. gambiae* complex (10) and *An. maculipennis* complex (11). Studies of Manonmani *et al.* (8) on the Indian specimens of the *An. fluviatilis* complex revealed two sequence groups within the complex. However sequence analysis of ITS2 region of Iranian specimens showed they were all identical and indicating the presence of only one species of the complex in the region. Cytotaxonomical assay by Indian collaborative (Nanda et al, personnel communications) on some Iranian specimens collected from Iranshahr area revealed that they belong to species T**.** Further cytological and molecular analysis is needed to clarify the status of thess taxa and their relationship with transmission of malaria in Iran.

Acknowledgments

This work has been supported by a short grant from WHO EMRO to the authors.

We gratefully acknowledge the assistance of Dr Rassi and the staff of Public Health Research Centers (affiliated to School of Public Health and Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran) particularly Mr. Pakari, Ghasemi, Djalali and Akbarzadeh in Bandar abbas, Kazeroun and Iranshahr for supplying us with mosquito specimens. We also thank Dr. Z. Zamani for reviewing the manuscript and the staff of parasitology department of Pasteur Institute of Iran for supporting this study.

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