rDNA-ITS2 BASED SPECIES-DIAGNOSTIC POLYMERASE CHAIN REACTION ASSAY FOR IDENTIFICATION OF SIBLING SPECIES OF *ANOPHELES FLUVIATILIS* IN IRAN

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Abstract. A species-specific polymerase chain reaction (PCR) assay using primers already designed, based on differences in the nucleotides of the second internal transcribed spacer (ITS2), was used to identify the species composition of the *Anopheles fluviatilis* complex in Iran. All the amplified DNA samples obtained from specimens collected from different areas using different collection methods yielded to a fragment of 450 bp size, a PCR product corresponding to the species denoted as Y. Some 21 ITS2 region of Iranian specimens were sequenced and compared with the already published sequence data of species Y from India. The sequence data of the Iranian specimens were 100% identical to that of the Indian specimens, and hence confirmed the PCR assay results. Species Y is presumably species T in India, which has no role in the transmission of malaria, whereas mosquitos of *An. fluviatilis* are known as a secondary vector in Iran. This conflict will remain to be solved by further biological and molecular studies.

INTRODUCTION

Anopheles fluviatilis has been reported from Oman, Bahrain, eastern Saudi Arabia, Iran, Iraq, Pakistan, Afghanistan, India, Sri Lanka, Bangladesh, the Union of Myanmar, Thailand, Indonesia and Indochina. It is considered a main vector of malaria in Pakistan, India and Bangladesh (Gilles and Warren, 1993). In Iran, mosquitos of this species are distributed on the foothills of the Zagros Mountains, from south-west to south, with some patchy distribution in the south-east at altitudes ranging from 50 to 1,100 m.

An. fluviatilis is known as a secondary vector of malaria and to be responsible for transmission of the disease with semi-stability in Fars, Hormozgan and Khozestan provinces (Eshghi *et al*, 1976). Different biological studies have shown distinct differences among populations of this species in feeding preference, resting behavior and infection rates (Eshghi *et al*, 1976; Gunasekaran *et al*, 1994; Edalat, 1998). Subbarao *et al* (1994) identified three reproductively isolated species in India designated as S, T and U, based on the banding patterns of polytene chromosomes. Species S was found to be highly anthropophilic (~91%), while species T and U are almost totally zoophilic (Nanda *et al*, 1996). Regarding the limitations and complexity inherent in the examination of polytene chromosomes, many attempts have been made to develop alternative species diagnostic procedures, including allozymes, cuticular hydrocarbon profile and more recently, several DNA-based approaches. DNA-based methods have shown to be rapid and reliable with no limitation to a specific developmental stage or sex, and are increasingly replacing other diagnostic methods. One of these methods has been to analyze the structure and sequence of hypervariable regions of the genome shared by all species and then to devise diagnostic assays based on species-specific differences in these regions. Ribosomal DNA (rDNA) is a region of particular characteristics, which makes it a suitable target fragment for diagnosis of cryptic species (Collins and Paskewitz, 1996). Advantage was taken of differences in one of its regions *ie* internal transcribed spacer (ITS2), to develop species-specific primers that could differentiate successfully two species (known as X and Y) of *An. fluviatilis*, by producing discriminative bands of 350 and 450 bp length (Manonmani *et al*, 2001). In this study we report the use of these primers for the identification of species composition of the *An. fluviatilis* complex samples collected from different areas in Iran. Since there were chances for some interspecific variations to be in areas other than those from which species-specific primers for X and Y species were designed, we also made a comparison between the obtained DNA sequences of the ITS2

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region of some Iranian specimens and already published sequence data of the Indian counterparts.

MATERIALS AND METHODS

Adults and larvae of *An. fluviatilis* were collected from different areas in five south and southeastern provinces of Iran (Hormozgan, Fars, Sistan and Baluchistan, Kerman and Boshehr). To cover different biological forms, various collection methods, including aspirating mosquitos in human dwellings and cattle sheds and outdoors in pit shelters, total catches in human dwellings and cattle sheds and night collection on human and cattle baits were used. The larva specimens were collected from margins of grass-sheded slow running streams and some of them were reared to the adult stage in an insectary. Using available morphological keys, all the adult mosquitos were identified, and mosquitos of *An. fluviatilis* were marked and kept in separate tubes for further investigation.

Mosquito genomic DNA extraction

Mosquito genomic DNA was extracted using a modified method already described by Ballinger-Crabtree *et al* (1992). The dried individual mosquitos were ground to powder in a 1.5 ml microfuge with a pestle grinder and resuspended in 200µl of lysis buffer (100mM Tris-HCl, pH 8.0, 50mM EDTA, 1% sodium dodecyl sulfate, 0.15mM spermine, 0.5mM spermidine) and 10µl of a 20 mg/ml solution of proteinase K. Suspensions were incubated overnight at 50˚C, followed by gently extracting DNA twice with buffered 70% phenol/chloroform/water and once with chloroform (Applied Biosystem, USA). DNA was precipitated by the addition of 2 volumes of ice-cold ethanol followed by keeping the solutions at-20˚C overnight. They were then microfuged at 13,000 rpm for 10 minutes to pellet DNA. The pellets were air-dried and resuspended in 100µl of sterile redistilled water.

PCR and sequencing

To amplify the ITS2 region of DNA specimens, two sets of primer were used (Table 1). The first set, which are complementary to conserved 5.8S and 28S rDNA regions, produce a band of approximately 500bp, encompassing the areas from which the species-specific primers for the X and Y species were designed. Each 25µl reaction contained 10mM Tris-HCl, pH 8.3, 50mM KCl, 2.5 mM $MgCl_2$, 200µM of each dNTP (Pharmacia, Biotech), 50ng of each primer, 0.5U of taq DNA Polymerase (Amersham Pharmacia Biotech, Inc) and 0.05% of DNA from a whole mosquito as template. Reactions were overlaid with 30µl of mineral oil and amplified in a thermal cycler (Techne USA) programed for one cycle at 94˚C for 5 minutes followed by 25 cycles of denaturation at 94˚C for 1 minute, annealing at 50˚C for 2 minutes and extention at 72˚C for 2 minutes and a final extention step at 72˚C for 7 minutes. Five microliters of each amplification product along with a 100 bp ladder marker was run on a 1.2% agarose gel containing ethidium bromide. For the sequencing of the PCR products, 100µl of 21 selected amplified samples representing different areas and methods of collection was provided and purified using a gel band purification Kit (Amersham Pharmacia Biotech, Inc). The concentration of DNAs recovered from gel was quantified by UV spectroscopy and subjected to sequencing in an automatic sequencer. Sequencing was performed for both strands and

Table 1
Primers used for amplification of ITS2 region and for species diagnostic assay.

Primers used for amplifying the ITS2 region (5´ to 3´)
ATCACTCGGCTCATCGATCG (Complementary to 5.8S region)
ATGCTTAAATTTAGGGGGTAGTC (Complementary to 28S region)
Primer used for species diagnostic PCR (5´ to 3´)
CACCCCCAAATTGTACAGTGGA (Species X-specific)
ATTCGTAACCCTGGAACCTTA (Species Y-specific)

consensus data were deposited in the Genbank database with accession Nos. AF509342-AF509353 and AY172564-AY172567.

Species-diagnostic PCR

The species-diagnostic PCR was performed in reactions with the second set consisting of 5.8S primer and the two already designed species-specific primers. The conditions for PCR were the same except for the number of cycles and annealing temprature, which were further increased to 30˚ and 57.5˚C, respectively (Manonmani *et al*, 2001).

RESULTS

One hundred and ninety-nine out of 205 (~97%) samples successfully amplified with the species-specific primers turned out to be Y type, producing the approximately 450bp band (Fig 1). The other 4 samples were neither amplified with the conserved 5.8S and 28S primers, nor with the species-specific ones. The amplification could be carried out with as small an amount as 20ng of genomic DNA, and even with DNA samples kept at 4˚C for more than 15 months. The DNA extracted from 3 dried museum specimens collected about 17-39 years ago were also amplified successfully. Details regarding the collection of samples is given in Table 2. The sequencing results of the ITS2 region for all 21 specimens also revealed a pattern identical to that of the Y type.

DICUSSION

An. fluviatilis has been identified as a cryptic species in India. Three sibling species designated as S, T and U has already been described by Subbarao *et al* (1994), based on analysis of banding patterns in the polytene chromosomes. Mannonmani *et al* (2001) developed a set of species-specific primers by exploiting differences in the ITS2 region of specimens collected from some areas in India where S and T were the only prevalent sibling species of *An. fluviatilis*. The species-specific primers can amplify discriminative DNA fragments of 350 and 450 bp, representing X and Y types, respectively. Application of PCR assay using the species-specific primers for Iranian specimens indicated the presence of only one species in all the studied areas, which, according to the nomenclature of Manonmani *et al* (2001) is considered the Y species. This finding was well confirmed by the sequencing data, which, in alignment with Indian species Y, turned out to be 100% identical. It is suggested that species Y might be species T, which has already been identified cytotaxonomically by Manonmani *et al* (2001). If the biological features already described for species T in India are attributed

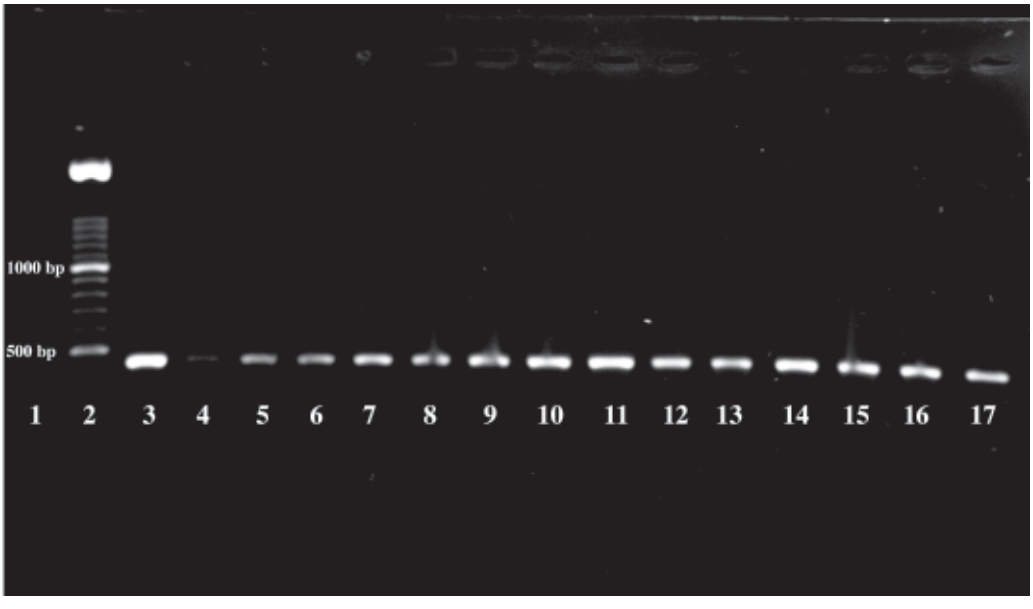

Fig 1- Species diagnostic PCR assay. Electrophoresis result of amplified DNA samples from individual mosquitos of *An. fluviatilis*. Lane 1, control with no DNA sample; lane 2 DNA molecular weight marker XIV (Boehringer); lanes 3-17, DNA sample collected from different areas in 5 provinces (lane 4, DNA sample extracred from a museum specimen collected about 39 years ago).

Table 2
Details of *Anopheles fluviatilis* samples used in this study.

Province	Region	Village	No. of specimens	Place of collection Habitat/Bait	Type of collection	Date of collection
Hormozgan	Minab	Chelou	3	Animal bait	Hand catch	May 1984
Hormozgan	Minab	Arengoon bala	3	Animal bait	Hand catch	Mar 2001
Hormozgan	Minab	Tom gohar	8***	Cattle shed	Total catch	Oct 2001
Hormozgan	Minab	Tom Basat	3***	Human dwelling	Total catch	Nov 2001
Hormozgan	Koveh	Siahoo	10	Animal bait	Hand catch	Jan 2001
Hormozgan	Koveh	Siahoo	4*	Animal bait	Hand catch	Aug- Sep 2001
Hormozgan	Koveh	Siahoo	9*	Animal bait	Hand catch	May 2000
Hormozgan	Koveh	Siahoo	8	Animal bait	Hand catch	Sep 2000
Hormozgan	Koveh	Siahoo	4*	Human bait	Hand catch	Sep-Nov 2000
Hormozgan	Koveh	Siahoo	10	Human bait	Hand catch	Dec 2000
Sistan & Baluchistan	Daman	Abchekan	9	Human dwelling	Total catch	Apr 2001
Sistan & Baluchistan	Daman	Abchekan	10*	Pit shelter	Hand catch	Mar 2000
Sistan & Baluchistan	Iranshahr	Iranshahr	5	Stream (larva)	Hand catch	Jul 2000
Sistan & Baluchistan	Ghasreghand	Ghasreghand	2*	Pit shelter	Hand catch	Jul 2000
Sistan & Baluchistan	Karevandor	Khash	1	Cattle shed	Hand catch	Apr 1963
Kerman	Kahnouj	Manoujan	8	Pit shelter	Hand catch	Jan 2001
Kerman	Kahnouj	Bargah	3	Pit shelter	Hand catch	Jan 2001
Kerman	Kahnouj	Khosro abad	5*	Pit shelter	Hand catch	Feb 2001
Kerman	Kahnouj	Khosro abad	11	Human dwelling	Total catch	Mar 2001
Kerman	Kahnouj	Khosro abad	2	Human dwelling	Total catch	Apr 2002
Kerman	Kahnouj	Dareh shoor	7	Cattle shed	Total catch	Apr 2002
Kerman	Kahnouj	Dareh shoor	5	Human dwelling	Total catch	Jun 2001
Kerman	Kahnouj	Dareh shoor	7*	Pit shelter	Hand catch	May 2001
Kerman	Kahnouj	Garmaei	5	Pit shelter	Hand catch	Mar- May 2001
Fars	Kazeroun	Djadas	6	Human dwelling	Hand catch	Nov 1999
Fars	Kazeroun	Djadas	3	Cattle shed	Hand catch	Jul 2000
Fars	Kazeroun	Djadas	2**	Stream (larva)	Hand catch	Jul 2000
Fars	Kazeroun	Islam abad	12	Human dwelling	Total catch	Nov 2000
Fars	Kazeroun	Dadin	5*	Stream (larva)	Hand catch	Nov 2000
Fars	Kazeroun	Islam abad	10*	Cattle shed	Total catch	Nov 2000
Fars	Kazeroun	Pirsabz	16***	Stream (larva)	Hand catch	Jul - Sep 2002
Fars	Khesht	Chiti	2*	Stream (larva)	Hand catch	Oct 2000
Boshehr	Dashtestan	Zir rah	1	Stream (larva)	Hand catch	Oct 2000

* Number of specimens subjected to sequencing.

to species Y, it is noticed that this presumed Y species in Iran is rather different from its Indian counterpart. Epidemiological surveys concerning the geographical distribution and range of this species in Iran have shown that this species is at least a secondary vector and could maintain malaria in some parts of the studied areas. Early studies in Kazeroun area showed a human blood index (HBI) as high as 50% for *An. fluviatilis* (Eshghi *et al*, 1976) and studies of Edrissian *et al* (1985), using the ELISA method, revealed a HBI of 5.1% for this species in Iran, whereas this value for species T in India has shown to be less than 1% (Nanda *et al*, 1996). In fact, the role of *An. fluviatilis* in transmitting malaria in the southern hilly areas of Iran has been well confined (Eshghi *et al*, 1976), but the T species in India has no role in transmitting malaria (Subbarao, 1997). For this conflict, one should first notice that the species-specific primers were designed based on a few nucleotide variations between different specimens in India. These primers have not been

examined yet for the 100% cytotaxonomical identification of species S and T. Therefore more evidence is needed to identify the species composition of this species complex in Iran clearly. Secondly, the *An. fluviatilis* complex is a newly divergent species and is not expected to observe high genetic variation within the complex. Studies of mtDNA variations in different Iranian and Indian populations of this species showed only 1% sequence variation among them (Oshaghi, 1998). To establish a convincing explanation for this problem, further biological, cytotaxonomical and molecular studies need to be done. Cytotaxonomically identified samples collected from Iran and India should be examined for variations in some other DNA target fragments to develop new species-specific primers. Some other DNA-based procedures, such as RAPD-PCR, capable of elucidating the probably existing intraspecific variations, might be employed as well.

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