Molecular characterization of *Anopheles fluviatilis* species complex in the Islamic Republic of Iran

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الخصائص الجزيئية للأنوفيلة النهرية بأنواعها المعقدة في جمهورية إيران الإسلامية

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الخلاصة: أجريت مقايسة بالتفاعل السلسلي للبوليمراز خاصة بالنَّوع للتعرُّف على تركيبة الأنواع المعقدة للأنوفيلة النهرية في جمهورية إيران الإسلامية. وقد نتج عن نماذج الدنا المضخمة التي جمعت من عينات مأخوذة من مناطق مختلفة قطع تتألف من 450 جزيئا قاعديا، وقد سُمِّيَ ناتج التفاعل السلسلي للبوليميراز الذي يناسب كل نوع من الأنواع باسم Y. ثم قورنت معطيات تسلسل المناطق ITS2 21 وهي مناطق الفاسحة الداخلية المُنْتَسَخة مع ما هو متوافر منها في بنك للجينات للتأكد من التطابق التام مع الأنواع Y في الهند. ويفترض أن النوع Y هو نفس النوع T الذي ليس له أي دور في نقل الملاريا في الهند، فيما يعرف أن الأنوفيلة النهرية من النواقل الثانوية للملاريا في جمهورية إيران الإسلامية.

ABSTRACT A species-specific polymerase chain reaction (PCR) assay was used to identify the species composition of the *Anopheles fluviatilis* complex in the Islamic Republic of Iran. All the amplified DNA samples from specimens collected from different areas yielded a fragment of 450 bp size, a PCR product corresponding to that of the species denoted as Y. The sequence data from 21 ITS2 [second internal transcribed spacer] regions were compared with those publicly available in the GenBank® database and confirmed that the specimens were 100% identical to species Y of India. Species Y is presumably the same as species T that has no role in transmission of malaria in India, whereas *An. fluviatilis* is known as a secondary vector of malaria in the Islamic Republic of Iran.

Caractérisation moléculaire du complexe d'espèces *Anopheles fluviatilis* dans la République islamique d'Iran

RESUME Une analyse PCR (amplification en chaîne par polymérase) spécifique à l'espèce a été effectuée pour identifier la composition des espèces pour le complexe *Anopheles fluviatilis* dans la République islamique d'Iran. Tous les échantillons d'ADN amplifié à partir des échantillons prélevés dans différentes régions ont donné un fragment de taille 450 bp, un produit de PCR correspondant à celui de l'espèce désignée Y. Les données de séquence de 21 régions ITS2 [second espaceur transcrit interne] ont été comparées à celles disponibles publiquement dans la GenBank® et ont confirmé que les prélèvements étaient 100 % identiques aux espèces Y d'Inde. L'espèce Y est vraisemblablement la même que l'espèce T qui n'a aucun rôle dans la transmission du paludisme en Inde, tandis que *An. fluviatilis* est connue comme vecteur secondaire du paludisme en République islamique d'Iran.

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Introduction

Anopheles (Cellia) fluviatilis James has been reported from Oman [*1*], Bahrain, Iraq, the Republic of Yemen [*2*], eastern Saudi Arabia [*3*], the United Arab Emirates [WHO/EMRO, unpublished document], the Islamic Republic of Iran [*4*], Pakistan [*5*], Afghanistan [*6*], India [*7*], Bangladesh [*8*] and China [*9*]. It is considered the main vector of malaria in Pakistan, India and Bangladesh [*10*]. In the Islamic Republic of Iran, mosquitoes of this species are found on the foothills of the Zagros mountains from the southwest to the south with some patchy distribution in the southeast at altitudes ranging from 50 to 1100 metres. It is known as a secondary vector of malaria and to be responsible for transmission of the disease on intermediate stability index in Fars, Hormozgan and Khuzestan provinces [*4*].

Different biological studies in the Islamic Republic of Iran have shown distinct differences among populations of this species in feeding preference, resting behaviour and infection rates. The human blood index for this species in the Jiroft and Kazeroun areas of Kerman and Fars provinces respectively was reported to vary from 3.4% to 28.6%. The sporozoite rate in these areas was 3.2% for Kazeroun, while in Jiroft, despite dissection of a large number of specimens, none were found to be infected. During the malaria epidemic of December 1957, a sporozoite rate as high as 11% was reported from the Behbahan area of Khuzestan province. Similar studies in Hormozgan province showed a sporozoite rate of 1.7% in the Chelou area, where this species was reported to have a marked tendency to rest indoors [*4*]. In other areas, including Rodan and Siahoo, this species has never been found to be infected [*4,11*].

Subbareo et al. [*12*] identified 3 reproductively isolated species in India based on the banding patterns of polytene chromosomes, designated as S, T and U. Species S was found to be highly anthropophagic (91%), while species T and U were almost totally zoophilic [*13*]. In view of the limitations and complexities inherent in examination of polytene chromosomes, many attempts have been made to develop alternative species diagnostic procedures using allozymes [*14*], cuticular hydrocarbon profiling [*15*] and, more recently, DNA-based approaches.

DNA-based methods have shown to be rapid and reliable with no limitation to a specific developmental stage or sex of mosquito and are increasingly replacing other diagnostic methods. One of these methods has been to analyse 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 common target of such methods because of the useful features of its sequence organization. It consists of tandemly repeated transcription units separated from each other by intergenic spacers (IGS). Transcription units contain coding regions alternating with spacer sequences: an external transcribed spacer (ETS), and 2 internal transcribed spacers (ITS1 and ITS2) [*16*]. The spacer sequences have been shown be highly variable in length and sequence, even between closely related species, making them all useful tools for species identification [*17*]. However, 2 important features in the ITS2 sequences of *Anopheles* species make them preferred targets. First, they are relatively short, less than 1 kilobase pairs (kbp), making the amplification of intervening ITS2 using primers from

highly conserved domains of flanking coding regions relatively simple. Second, the level of intraspecies variation in them is lower than the interspecific variation [*17*].

These characteristic features of ITS2 have been exploited by many researchers to develop PCR-based species diagnostic assays. Species A and D of *An. (Cellia) dirus* Peyton and Harrison were differentiated based on the ITS2 sequence [*18*]. Hackett et al. [*19*] distinguished *An. (Cellia) funestus* Giles from *An. (Cellia) rivulorum* Leeson and uncovered a cryptic taxon within the Funestus group based on sequence divergence in the ITS2 sequence. Identification of 6 sibling species of the *An. (Anopheles) maculipennis* Meigen complex [*20*] and 5 members of the *An. (Anopheles) quadrimaculatus* Say cryptic species [*21*] has been accomplished based on PCR assays using species-specific primers in the ITS2 region. Manonmani et al. [*22*] sequenced the ITS2 fragment of *An. fluviatilis* specimens collected from the Koraput and Malkangiri districts of Orissa state in India. These exhibited 2 different sequence patterns, designated by the authors as species X and Y [*22*]. They took advantage of the nucleotide differences in ITS2 fragments of species X and Y to develop species-specific primers, which together with the 5.8S primer could amplify discriminative 350 and 450 bp bands corresponding to species X and Y.

The main objective of this study was to determine the composition and distribution of members of the *An. fluviatilis* complex in the Islamic Republic of Iran using PCR assay and alignment of ITS2 fragments with those publicly available in the National Institutes of Health GenBank® database.

Methods

Mosquito collection

Adults and larvae of *An. fluviatilis* were collected from different areas in 5 south and south-eastern provinces of the Islamic Republic of Iran: Hormozgan, Fars, Sistan va Baluchistan, Kerman and Bushehr. Details about the collection of specimens are given in Table 1. The collection sites, together with the geographical distribution of *An. fluviatilis* mosquitoes in the country, are shown in Figure 1. Despite several attempts, we could not catch any specimen from the Behbahan area in Khuzestan province. To cover different biological forms, various collection methods were employed, including aspirating mosquitoes indoors from human dwellings and cattle sheds and outdoors from pit shelters, total catches in human dwellings and cattle sheds plus night collections on human and cattle baits. The larvae were collected from the margins of slow-running streams, most of them with floating vegetation, and reared to adult stage in an insectery. Using Shahgodian's morphological key [*23*], all the adult mosquitoes were identified and mosquitoes of *An. fluviatilis* were marked and kept in separate tubes for further investigation.

Molecular method

Mosquito genomic DNA was extracted from individual dried mosquitoes following homogenization of the sample using a pestle grinder and the DNA extraction protocol of Ballinger-Crabtree et al. [*24*]. A total of 199 specimens, representing different collection areas and methods were identified by PCR using the protocol developed by Mannonmani et al. [*22*]. More-

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

Province	Area/village	No. of samples collected (sequenced)	Habitat	Collection method	Collection date
Hormozgan	Minab/Chelou	3	Animal bait	Hand catch	May 1984
Hormozgan	Minab/Arengoonbala	3	Animal bait	Hand catch	Mar 2001
Hormozgan	Minab/Tom gohar	8 (3)	Cattle shed	Total catch	Oct 2001
Hormozgan	Minab/Tom basat	3 (3)	Human dwelling	Total catch	Nov 2001
Hormozgan	Siahoo/Siahoo	10	Animal bait	Hand catch	Jan 2001
Hormozgan	Siahoo/Siahoo	4 (1)	Animal bait	Hand catch	Aug–Sept 2001
Hormozgan	Siahoo/Siahoo	9 (1)	Animal bait	Hand catch	May 2000
Hormozgan	Siahoo/Siahoo	8	Animal bait	Hand catch	Sept 2000
Hormozgan	Siahoo/Siahoo	14 (1)	Human bait	Hand catch	Sept–Dec 2000
Sistan va Baluchistan	Daman/Abchekan	19 (1)	Shelter pit	Hand catch	Mar 2000 & 2001
Sistan va Baluchistan	Daman/Abchekan	5	Stream (larva)	Hand catch	Jul 2000
Sistan va Baluchistan	Ghasreghand/Zainedini	2 (1)	Shelter pit	Hand catch	Jul 2000
Sistan va Baluchistan	Khash/Karevandor	1	Cattle shed	Hand catch	Apr 1963
Kerman	Kahnouj/Manoujan	8	Shelter pit	Hand catch	Jan 2001
Kerman	Kahnouj/Bargah	3	Shelter pit	Hand catch	Jan. 2001
Kerman	Kahnouj/Khosroabad	5 (1)	Shelter pit	Hand catch	Feb 2001
Kerman	Kahnouj/Khosroabad	13	Shelter pit	Hand catch	Apr 2001
Kerman	Kahnouj/Darehshoor	7	Cattle shed	Total catch	Apr 2002
Kerman	Kahnouj/Darehshoor	5	Human dwelling	Total catch	Jun 2001
Kerman	Kahnouj/Darehshoor	7 (1)	Shelter pit	Hand catch	May 2001
Kerman	Kahnouj/Garmari	5	Shelter pit	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 (2)	Stream (larva)	Hand catch	Jul 2000
Fars	Kazeroun/Islamabad	12	Human dwelling	Total catch	Nov 2000
Fars	Kazeroun/Islamabad	10 (1)	Cattle shed	Total catch	Nov 2000
Fars	Kazeroun/Dadin	5 (1)	Stream (larva)	Hand catch	Nov 2000
Fars	Kazeroun/Pirsabz	16 (3)	Stream (larva)	Hand catch	Jul–Sept 2002
Fars	Khesht/Chiti	2 (1)	Stream (larva)	Hand catch	Oct 2000
Bushehr	Dashtestan/Zirrah	1	Stream (larva)	Hand catch	Oct 2000

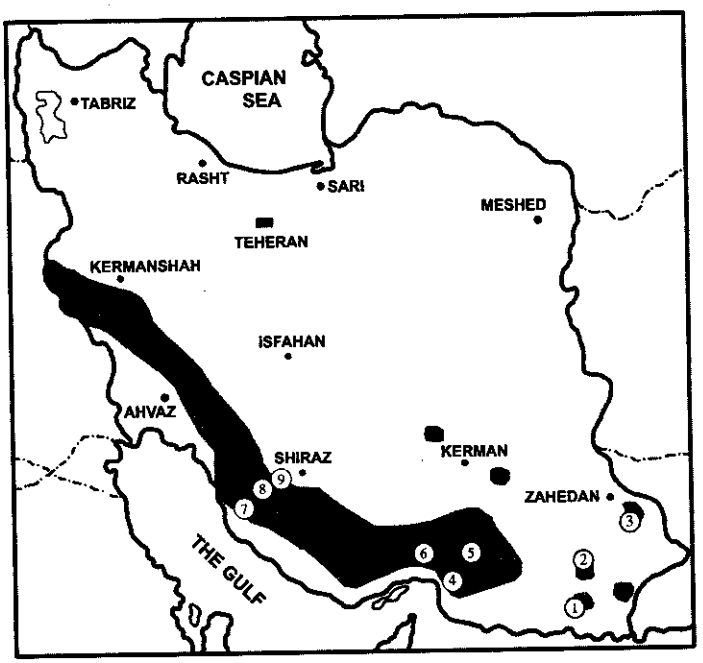

Figure 1 **Distribution of *Anopheles fluviatilis* in the Islamic Republic of Iran denoted by hatched areas (Reproduced from Eshghi et al. [4]). Encircled numbers represent collection areas: 1, Ghasreghand; 2, Daman; 3, Khash; 4, Minab; 5, Kahnouj; 6, Siahoo; 7, Dashtestan; 8, Khesht; 9, Kazeroun. Black circle shows Behbahan area where no specimens were obtained.**

over, the ITS2 fragments of some individuals were amplified using 5.8S and 28S primers and the PCR reaction conditions of Manonmani et al. [*22*], except for the amount of template which was reduced from 0.5% to 0.05% of a whole mosquito genomic DNA. The products were visualized on a UV transluminator following electrophoresis in a 1.2% agarose gel containing ethidium bromide. From each amplification, 100 µL was purified using a gel band purification kit (Amersham Pharmacia Biotech, New Jersey, USA). The concentration of DNA recovered from the gels was quantified by UV spectroscopy and subjected to sequencing in an automatic sequencer. Sequencing was performed for both strands and consensus data was deposited in the GenBank® database with accession numbers AF509342-AF509353 and AY172564-AY172567 using the *Blast* program (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Results

Analysis of the collected specimens showed that *An. fluviatilis* species is exophilic, exophagic and zoophilic. Except for Minab, the regions where this species was captured were all hilly or semi-mountainous. Most of the specimens were caught from outdoor habitats and on animal

baits. However, a few specimens (1%–5%) were caught on human baits.

All the specimens subjected to species-specific PCR assay yielded an approximately 450 bp band exclusively diagnostic for species Y. The amplifications could be carried out with an amount of genomic DNA as low as 20 ng and with DNA samples kept in 4 °C for more than 15 months. The DNAs extracted from 3 dried museum specimens collected about 17–39 years ago were also amplified successfully.

DNA sequences of the ITS2 region were generated for 21 specimens of the *An. fluviatilis*. The samples originated from different areas of the country (Table 1): 9 samples from Hormozgan, 2 from Sistan va Baluchistan, 2 from Kerman, and 8 from Fars province. All the ITS2 fragments were found to be 374 nucleotides in length. The GC content of the ITS2 fragments was 55%, which was concordant with those of other anopheline species [*20,21,25*]. All the ITS2 sequences were free of ambiguities and no intraspecific variation was observed either in length or nucleotide composition of the fragments. This suggests that *An. fluviatilis* ITS2 consensus sequences in the Islamic Republic of Iran are composed of one type.

Comparison of the sequence data with other *An. fluviatilis* ITS2 sequences submitted to GenBank® by Manonmani et al. [*22*] showed that they share 100% similarity with entry AF167299 and 97% with entry AF167299, both originating from India.

Discussion

Three cytotaxonomically diagnosed sibling species of *An. fluviatilis*, designated as S, T and U, have been described by Subbarao et al. in India [*12*]. Manonmani et al. [*22*] developed a set of species-specific primers by exploiting differences in the ITS2 region of some specimens collected from the Koraput and Malkingiri districts of Orissa state in India. Application of PCR assay using these primers for the same specimens could distinguish 2 species of *An. fluviatilis*, species X and Y as named by Manonmani et al. [*22*]. The identity of species X and Y may be the same as species S and T respectively, the only 2 sibling species of *An. fluviatilis* identified by cytogenic methods in these districts [*26*]. In a recent study led by the Indian Council of Medical Research (ICMR) in the above districts, out of 249 specimens cytotaxonomically identified as species S, 231 were identified as species X by PCR assay and, similarly, out of 153 specimens identified as species T only 128 were found to be species Y. The agreement of results between the 2 techniques was shown to be 93% for species X and 84% for species Y. It is suggested that the disagreements of 7% and 16% may be due to genetic subgroups within the sibling species [*27*].

No ITS2 fragment for species U has been published yet. Our molecular findings including PCR identification and ITS2 analysis confirmed the identity of *An. fluviatilis* in the Islamic Republic of Iran as species Y. Further cytotaxonomical assay by an Indian collaborative (Nanda et al., personal communication) on some the Iranian specimens collected from the Iranshahr area revealed that they belong to species T. Since the molecular findings for *An. fluviatilis* in all the studied areas, including Iranshahr, were identical, it can be concluded that the only prevalent sibling species in the country is T. It is known that the mosquitoes of species T in India are more zoophilic and are very poor vectors of malaria [*26*]. In the Islamic Republic of

Iran, however, they play an important role in transmission or maintenance of malaria in the region [*4*]. Such a different vectorial capacity between different allopatric populations of the same species has also been found in a number of other anopheline species.

Vector incrimination studies on the *An. (Cellia) culicifacies* Giles complex in India and Sri Lanka revealed that in India species B is a poor malaria vector, whereas in Sri Lanka, where on cytogenetic evidence only species B is found, *An. culicifacies* is known to be an important malaria vector [*28,29*]. Moreover, there are a number of cryptic species with different vectorial capacities that do not display species diagnostic differences in the polytene chromosomes. Such homosequential polytene chromosomes are found in some members of the *An. dirus* [*30*], *An. maculipennis* [*31*], *An. funestus* [*32*] and *An. culicifacies* complexes [*28*].

The *An. fluviatilis* complex is believed to be a newly diverged species and it is not expected to observe a high genetic variation within the complex. Studies of mitochondrial DNA variation in different Iranian and Indian population of this species showed only a 1% sequence variation among them [*33*]. The differences among the populations of this species in the Islamic Republic of Iran might be elucidated through application of some other DNA-based method such as random amplified polymorphic DNA (RAPD) PCR.

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