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rDNA-ITS2 Identification of *Anopheles pulcherrimus* (Diptera: Culicidae): Genetic Differences and Phylogenetic Relation with Other Iranian Vectors and Its Implications for Malaria Control

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ABSTRACT

***Anopheles pulcherrimus* Theobald has a wide distribution in western Asia and is a potential vector of malaria in Iran. We have examined the rDNA-ITS2 (internal transcribed spacer 2) region of *An. pulcherrimus* specimens collected during the two peaks of activity (May-June and October-November) from Sistan and Baluchistan province, southeastern Iran. There were no consistent differences between specimens originated from different ecological areas. Total amplified fragment is 490 bp, which is within the range of the records repeated from other anophelines. ITS2 was 350 bp long in all individuals examined with identical sequence in different populations. Sequence analysis revealed its differences with two other important malaria vectors in the region, *An. culicifacies* and *An. fluviatilis*. However, based on ITS2-derived phylogenetic tree, the nearest taxa to *An. pulcherrimus* is a new species related to *An. Culicifacies* and called species X in *An. culicifacies* species complex. These data may provide a better understanding on dynamics of malaria transmission in southeastern corner of Iran and neighboring countries. Moreover, the extent of the genetic variation in these mainly sympatric species could result in designing and application of species-specific diagnostic tools, which can facilitate the management of malaria control program in the region.** *Iran. Biomed. J. 7 (1): 1-6, 2003*

Keywords: *Anopheles pulcherrimus*, rDNA-ITS2, Molecular identification

INTRODUCTION

Anopheles pulcherrimus Theobald has a wide distribution in western Asia, extending from Lebanon, Syria and Iraq in the west, through Iran, Afghanistan, Pakistan and India in the east. It is also found in Turkey and Turkmenistan (central Asia), Caucasus in the north and Saudi Arabia and Bahrain in the south [1-3]. In Iran, it occurs mainly in Khozistan, Boshehr, Hormozgan and Sistan and Baluchistan provinces.

Slow moving streams, ditches, rice fields, pools, marshes and other types of waters with or without vegetation have been reported as the breeding sites for *An. pulcherrimus* larvae [4]. However, rice fields, weedy irrigation channels, marshes and any kind of clean stagnant water, overgrowth with vegetation, but not too heavily shaded, have been reported as the most favorable sites [5-8]. In Iraq, Al-Tikrity [4] reported that adults of this species were found in large numbers in human dwellings and animal shelters, while Abul Hab and Al-Kassal [9] described *An. pulcherrimus* as an indoor and outdoor resting species. Dispersal in great distances by wind has been cited for this species in Saudi Arabia [10].

The species has been reported to actively bite man and cattle, indoors as well as outdoors in northeast Afghanistan [11]. It has been incriminated as a vector of malaria in Iraq on epidemiological grounds [12] and in Central Asia [1] and the Kunduz area of northeastern Afghanistan through natural infection [2,13].

In Iran, malaria remains the most important parasitic disease, with an Annual Parasite Incidence (API) ranging from 17,000-55,000 cases. The

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country can be divided into three epidemiological zones, Zone I- area north of the Zagros Mountain, Zone II- western and southern Iran, and Zone III- southeastern Iran. About 80-85% of all malaria cases occurs in Zone III, in particular, in southeast of Kerman province, Sistan and Baluchistan province, and Hormozgan province. During the last 10 years, 3,000-8,000 imported cases of malaria have been detected yearly in different parts of Iran, mainly in Afghanistani and Pakistani immigrant populations. In fact, about 87% of malaria cases in Zone I, are imported. Apart from providing foci for malaria transmission, these imported cases are often chloroquine-resistant.

There are several suspected malaria vectors in Iran. *An. culicifacies* is the main vector in Sistan and Baluchistan province and is resistant to DDT, dieldrin and malathion [14]. *An. culicifacies* is a complex of at least four distinct species, but as yet only "species A" has been found in Iran [15; Djadid, unpublished data]. Secondary vectors in Sistan and Baluchistan province are considered to *be An. pulcherrimus*, *An. dthali*, *An. fluviatilis*, *An. stephensi* and *An. superpictus* [16-18].

To identify these mostly sympatric species, the main tool is the original pictorial key for nineteen Iranian anophelines, prepared by Mr. Shahgoudian [unpublished data], and revised later with minor changes by Dr. Saebi [unpublished data]. This key is very valuable in routine identification, but it is solely based on morphological characters of adults and fourth instar larvae. However, as Coluzzi [19] mentioned, the biological differences among sympatric sibling species are no less important than those characterized morphologically different species, and it seems that we need to review the species concept and the extent of genetic variation between and within suspected Iranian malaria vectors. On the other hand, during the last 8 years, we have conducted a series of molecular studies on Iranian vectors of malaria. By using PCR amplification of random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and ribosomal DNA cistron, mainly internal transcribes spacer-2 (ITS2) region, we have found new species within *An. maculipennis*, *An. culicifacies* and *An. fluviatilis* complexes from Iran [Djadid, unpublished data]. In an attempt to establish a molecular key to Iranian anophelines (19 species), the current study has been designed by using the sequence analysis of rDNA-ITS2 region. Here, we describe the ITS2 region of *An. pulcherrimus* and the second most common species in Sistan and Baluchistan of Iran. Based on the sequence data from other important anopheline vectors in the region, their phylogenic relation has been studied.

MATERIALS AND METHODS

Mosquitoes. Fifty-seven dried and alcohol preserved field specimens of *An. pulcherrimus* were collected by space spraying from four different geographical areas: Zabol, Iranshahr, Nikshahr and Chahbahar, districts in Sistan and Baluchistan province.

Genomic DNA extraction. DNA was extracted from individual mosquitoes using slight modifications of the techniques described by Ballinger-Crabtree *et al.* [20], Collins *et al.* [21] and Medina-Acosta and Cross [22]. Air-dried pellets have been re-suspended in 100 µl of double distilled water or TE buffer and stored in 4°C.

Oligonucleotide primers. The rDNA-ITS2 region was amplified using two specific primers for the conserved regions of the 5.8S and 28S genes (Fig. 1), based on the published nucleotide sequences of *Cx. tritaeniorhynchus* 5.8S rDNA [23] and *An. hermsi* 28S rDNA [24].

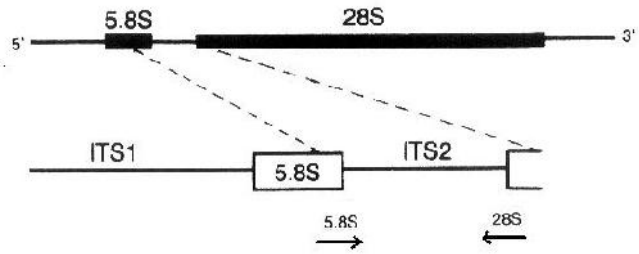


Fig. 1. Diagram of a single rDNA unit, the location and direction of primers used and the region of rDNA amplified and sequenced.

PCR amplification and gel electrophoresis. All PCR reactions were performed in a total volume of 25 µl. Each mixture contained 2.5 µl of 10X reaction buffer, 2 mM $MgCl_2$, 50 ng of primer, 0.001% gelatin, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 unit of Promega Taq polymerase, and sterile double-distilled water to 25 µl. Reactions were overlaid with 50 µl of mineral

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oil and amplified in a Hybaid Omnigene thermal cycler. For ITS2 PCR the protocol was: 94°C for 5 min; 25 cycles for 1 min at 94°C, 50°C for 2 min, 72°C for 2 min; then 72°C for 7 min. Following PCR, 5 µl of the amplification products was mixed with Ficol/Orange G loading buffer and subjected to electrophoresis in 6% polyacrylamide gel or 1% agarose (Appligen) in TBE buffer containing ethidium bromide, at 1.5 v/cm for 16 h (large gel) or 5 v/cm for 2 h (mini gel). The molecular weight marker for PCR product size determination was a ladder of 19 fragments (12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/506, 396, 344, 298, 220/201, 154/134 bp). Gels were viewed under UV light and photographed on black and white Polaroid 55 film or Ilford FP4 films.

Sequencing of PCR products. Aliquot of PCR product to be sequenced (1 µl) was checked for purity by agarose gel electrophoresis. The DNA concentration was estimated by comparison with size markers. The PCR product was purified from primer, buffer and other reagents using a PCR Clean-Up Kit (Roche Diagnostic, Germany). The purified PCR product was then ethanol precipitated and resuspended in 10 µl ddH_2O or TE buffer to a final concentration of 20-80 ng^{-1}. The sequence of ITS2 in three specimens of *An. pulcherrimus* was determined on an AB1 377 DNA sequencing system (Applied Biosystems) in National Research Center for Genetic Engineering and Biotechnology (NRCGEB), Iran.

Data analysis. Computer programs within the sites provided in GCG v.7.0 [25], PHYLIP [26] and ClustalW [27] were used to align DNA sequences and provide phylogenetic trees. Proposed secondary structure of ITS2 of *An. pulcherrimus* produced by using the RNAdraw (V1.1d2) program.

RESULTS AND DISCUSSION

In order to find the extent of inter- and intra-specific genetic variation in Iranian anophelines and its impact on epidemiology of malaria for designing the appropriate control measures, the current study has been designed using the sequence analysis of rDNA-ITS2 region. Ribosomal DNA has been widely used for phylogenetic studies at all taxonomic levels. In general, the greatest amount of variability occurs within the inter-genic spacer regions, whereas the most conserved sequences are found within the transcribed genes. Between these two extremes there are the internal transcribed spacer, which are processed out of the final rRNA product. The ITS is subject to secondary structural constraints related to processing, but exhibits substantial sequence variation in many groups of species. With regards to previous works by Djadid [unpublished data], *An. pulcherrimus* has a priority to studying inter- and intra-specific genetic variation, because it has been discriminated as a vector in Ghasreghand, Baluchistan [17, 18], and it seems to play a main role on maintenance of malaria in the region (i.e. Afghanistan and Pakistan).

The rDNA-ITS2 region of *An. pulcherrimus* specimens from Sistan and Baluchistan province of Iran has been amplified using 5.8 and 28 conserved primers. The total size of amplified fragment in all 57 specimens was 490 bp. This fragment has been sequenced in three individuals originated from Nikshahr, Iranshahr and Chahbahar. The sequence of *An. pulcherrimus* from Nikshahr has been submitted to GenBank (AF335105). However, no intra-specific variation has been found within specimens, comprising 91 bp of the 3' end of the 5.8s, ITS2 (350 bp) and 49 bp of the 5' end of 28s (Fig. 2, lower gel).

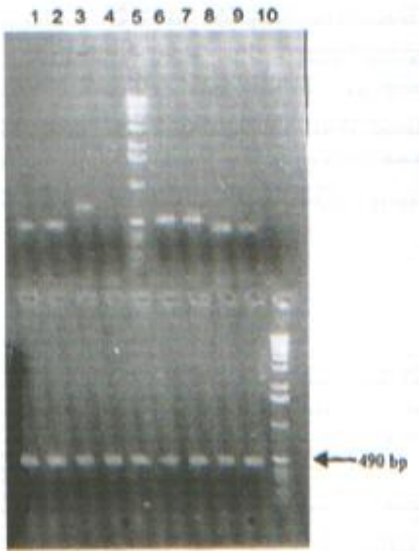


Fig. 2. PCR amplification of ITS2 region in main anopheline malaria vectors from different provinces in Iran. Upper gel: (1, 2), *An. pulcherrimu;* (3), *An. multicolor;* (4), *An. stephensi;* (5), Molecular Marker; (6 & 7), *An. fluviatilis;* (8 & 9), *An. maculipennis*, (10), negative control. Lower gel: *An. pulcherrimus* from different collection sites in Sistan and Baluchistan province, Iran.

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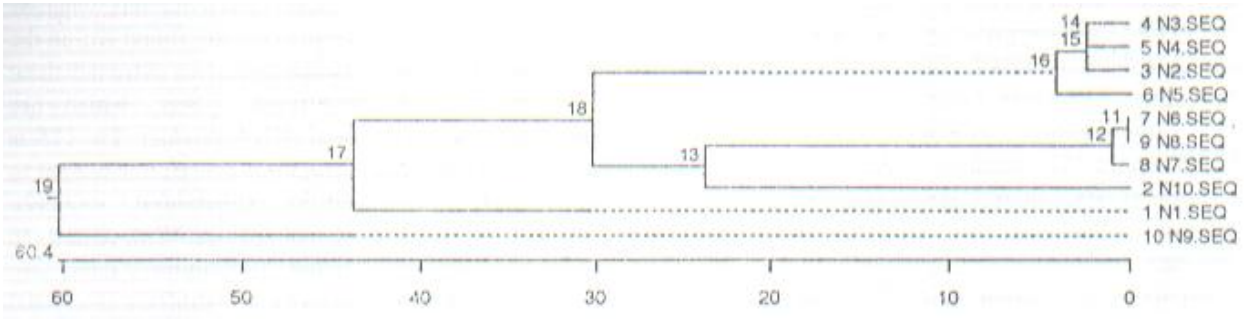


Fig. 3. Phylogenetic tree derived from ITS2 sequence in different malaria vectors in Iran. The nearest branch to *An. pulcherrinmus* (N9 SEQ) is a species related *to An. culicifacies* (N1 SEQ), which has been identified by Djadid (1997) and named after as species X in *An. culicifacies* species complex. Other sequences belong to *An. fluviatilis* species T (N2, N3, N4); *An. fluviatilis* from city of Minab (N5); *An. culicifacies* species A from Iran (N6, N7, N8); *An. culicifacies* species B from Pakistan (N10).

GC content of ITS2 region in this pecies is about 50.8% (A = 28.34%; T = 20.86%; G = 25.4%; C = 25.4%). Phylogenetic tree, using Clustal V method and based on sequence of ITS2 region in main anopheline species in Baluchistan, revealed that *An. pulcherrimus* (N9 SEQ) is more similar to a species related to *An. culicifacies* species complex (N1 SEQ), which has been found in the same area (Ghasreghand, Nikshahr, Baluchistan) (Fig. 3). Secondary structure of ITS2 in *An. pulcherrimus* was derived using the RNAdraw program (Fig. 4), which determines all optimal and sub-optimal secondary structures for an RNA molecule.

After defining the size, initiation and ending sequence of ITS2 in *An. pulcherrimus*, it was confirmed that the size of ITS2 is in expected range (299-564 bp) of other malaria vectors especially *An. culicifacies* (372 bp) and *An. fluviatilis* (375 bp), which present in the study area. The GC content (about 51%) is more similar to *An. maculipennis* species complex and *Aedes aegypti* (50%) than the other anopheline species (54-59%) [24, 28, 29]. Table 1 summarizes the sizes of amplified fragment, ITS2, GC content and the available accession numbers related to the major malaria vectors in Iran and Indian subcontinent. Although we could not find any size or sequence variation in the examined specimens within *An. pulcherrimus* populations, but these data were necessary for completion of the genome analysis in Iranian anophelines.

Comparison of ITS2 secondary structures in *An. pulcherimus* and other animals reveals that, most have a similar stem region. This region contains a putative ribosomal processing site in many organisms [30].



Fig. 4. Proposed secondary structure of ITS2 of *An. pulcherrimus* by using the RNAdraw (V1.1d2) program. Comparison of ITS2 secondary structures in *An. pulcherimus* and other animals reveals that most have a similar stem region, which contains a putative ribosomal processing site in many organisms.

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Table 1. The total sizes of amplified fragment by using 5.8s and 28s primers, ITS2 size, GC content and available accession numbers related to major malaria vectors in Iran and Indian subcontinent.

Anopheles Species/Complex	Total size of amplified band (bp)	Size of ITS2 (bp)	GC%	Accession Numbers*
An. pulcherrimus	490	350	50.8	AF335105
An. culicifacies	570	372	59.0	AF402296, AF402297
An. fluviatilis	562	375	53.3-54.7	AY049001, AY049003, AY049005, AF333384
An. subpictus	662-757	473-564	54.4-58.2	AF406613, AF406614, AF406615, AF406616, AY049004
An. maculipennis	453-462	313-367	49-51	AF436062, AY050639, AY050640

*All sequences submitted to GenBank by N.D.Djadid and co-workers during 2000-2001, and could be reached through NCBI sequence viewer.

The other part of this study concerns the phylogenetic relation of malaria vectors, which exist mainly as sympatric species; Sistan and Baluchistan province of Iran is neighboring Pakistan and Afghanistan, with almost the same anopheline species: *An. culicifacies*, *An. fluviatilis*, *An. stephensi*, *An. pulcherrimus*, and a strong gene flow within *An. culicifacies* and *An. stephensi* populations, specially in areas closer to border line.

Phylogenic analysis revealed that although *An. Fluviatilis* is the nearest texa to *An. Culicifacies*, a new member of *An. Culicifacies* (species X) is closer to *An. pulcherrimus*. This finding may support the presence of speciation process and the effect of different kinds of selection, which consider the balance achieved between gene flow and genetic drift. This is in concordance with the previous work of Djadid [unpublished data] on *An. maculipennis*, *An. culicifacies*, *An. stephensi*, *An. subpictus*, *An. gambiae* and *An. fluviatilis* by using RAPD, SSR, and ribosomal DNA cistron, mainly ITS2 region. Since then, we have followed this work by studying the molecular epidemiology and population genetics of other Iranian anophelines. This information could be used as a baseline data for further genome analysis in Iranian malaria vectors and may provide us a better understanding on the complexity of host-parasite interaction in malaria.

The potential value and importance of using tools that permit better identification of the vector species involved in transmission is obvious. These tools are now available and will enable both research scientists in the field and professionals concerned with malaria control to understand better the biology of malaria parasite transmission and to use this knowledge in devising more efficient vector-targeted control programs [31]. In conclusion, we hope that completion of current project on "molecular key to Iranian anophelines" provides (a) more data on the extent of inter- and intra-specific genetic variation in Iranian anophelines, (b) a species-specific diagnostic tool for identification of different species and sub-species and (c) better understanding on population dynamics of different malaria vectors in the region, which could be used in malaria control program in Iran and neighboring countries.

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