RIBOSOMAL DNA-ITS2 GENOTYPES OF THE MALARIA VECTOR *ANOPHELES SUPERPICTUS* (DIPTERA: CULICIDAE) FROM IRAN

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Abstract- Recent advances in DNA based technology have made available a wide range of molecular characteristics for taxonomic and systematic studies of malaria vectors. One region of the anopheline mosquito genome that has received particular attention is the ribosomal DNA. In this study, mosquitoes of the *Anopheles superpictus* collected from a wide geographical distribution of Iran were investigated for sequence variation within the ribosomal DNA ITS2. Two morphological forms of this species were identified based on adult female palpi and compared to genotypes identified by the rDNA ITS2 sequences. Sequence analysis of the ITS2 within and between populations identified three genotypes designated as X, Y, and Z. Genotype X occurred in north, west, south, and central regions whereas genotypes Y and Z were found sympatric in Baluchistan in southeast corner of the country. Totally 127 mutations occurred in the 518 bp region sequenced. The rate of genetic variation was 24.52% in which respectively 0.2%, 0.77% and 23.55% corresponded to 28S, 5.8S, and ITS2 regions. The length of ITS2 region was varied between populations; one group had 357 and other one 378 bp. These genotypes appeared to be evolving independently suggesting the possibility of cryptic species within taxon. This study is the first comparative study on morphological and genetic characteristics of *An. superpictus* and for the first time in the world reporting this taxon as a species complex.

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INTRODUCTION

Anopheles superpictus Grassi (Diptera: Culicidae) is known as a main malaria vector and the most widespread *Anopheles* species in Iran. It presents in almost all parts of the country including central plateau, plains of Alborz Mountains chain, south of Zagrous Mountains chain, and with low numbers in shore plains of the Persian Gulf and Caspian Sea (Fig. 1). This species has been collected in places with 2000 meters and in costal plains of Persian Gulf up to 50 meters height over the sea level (1, 2).

This species has a westward extension through Iraq till west of Europe, North of Africa, Middle-East and Arabian Peninsula and northward extension till south of Russia, and from east to Afghanistan and Pakistan (3-4). The range of geographical distribution of this species covers a number of different climate types, and in most parts it is incriminated as a main malaria vector (5-9). Thus there is the possibility that this species might be a mix of biological forms or group species. It is shown that more than half of the malaria vectors belong to sibling species which are morphologically indistinguishable but differ in genetics, ecology, biology, vectorial capacity, and host preference, blood

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Fig. 1. Geographical distribution of *Anopheles superpictus* in Iran.

feeding behavior, geographical distribution and resistance to insecticides (10, 11).

Presence or lack of a black spot/belt on the apical segment of adult female maxillary palps revealed two groups, A and B, within species. Also a number of diverse morphological characters at larval stage were found which were designated as typical and atypical form (12). Adults of this species have shown both exophagic and endophilic behavior and most of them rest in both human and animal dwellings (13, 14). Salivary gland dissection of this species in five provinces of the country showed 0.65-4.6% sporozoite infection (13). Polytene chromosome analysis of various *An. superpictus* populations of Southern Italy revealed one chromosomal polymorphism due to a paracentric inversion involving the central third of the 2L arm (15). It is known that presence of chromosomal polymorphism in *Anopheles* species is in relation to the vectorial capacity and power of malaria transmission (16).

Recent advances in DNA based technology have made available a wide range of molecular characteristics for taxonomic and systematic studies of mosquitoes. One region of the anopheline mosquito genome that has received particular attention is the ribosomal DNA (11). The rDNA gene family appears to occur mostly at a single haploid locus in mosquitoes (11, 17) and shows sequence similarity within copies of the rDNA unit among individuals in an interbreeding population or species (18). Internal transcribed spacer 2 (ITS2) region of rDNA unit has been useful for the identification of closely related anopheline mosquitoes. Species-specific sequence variation of ITS2 has been used to develop diagnostic PCR assays or to generate species-specific restriction fragment length polymorphism (RFLP) profiles for species identification, particularly for separating morphologically similar or identical species (19-24).

In this paper we use PCR-direct sequencing of the ITS2 region to follow the population structure of the current morphological species in the *An. superpictus* from Iran, and to determine the interspecies or intraspecies variation within the taxon.

MATERIALS AND METHODS

Mosquitoes were collected as larvae or as adults from different provinces in Iran. The larvae were reared through to adults where possible. Specimens were collected with standard methods including total and hand catch (Human and animal dwellings), pit–shelter, window trap (out let and in let) and animal and human biting in pick of mosquito activity (July to September). Material was identified as belonging to the *An. superpictus* using the morphological keys (25); presence or lack of a black spot/belt on the female palpi was scored for form A and B and presence of one or two pairs of palmate hairs on each abdominal segment of larvae was scored respectively for typical and atypical form of *An. superpictus*.

For genetic analysis genomic DNA was extracted from whole specimen using Collins method (26). All polymerase chain reactions were carried out in 0.5 ml Microfuge tubes in a 25 µl volume on an Eppendorf Personnel Thermo-cycler. The final PCR mixture contained 1–10 ng of template DNA, 20 pm of each primer, 1.25 mm $MgCl_2$, 2.5 mm of each dNTPs, 0.001% gelatin, 1 × *Taq* reaction buffer (Roche) and 1.0 unit of *Taq* DNA polymerase. The primers used for ITS2 amplification were those used in Beebe and Saul (21): 58S (forward) 5'-TGTGAACTGCAGGACACATG-3' and 28S (Reverse) 5'-TATGCTTAAATTCAGGGGGT-3'. Reaction cycles involved an initial denaturation at 94 °C for 4 min followed by twenty-five cycles of 94 °C for 30 sec, 53 °C for 1 min and 72 °C for 2 minute. PCR product was run on a 1.5% agarose gel. Restriction endonuclease digestion was carried out in a 0.5 ml

Microfuge tube containing 15 µl of PCR product, to which was added 2.5 µl of a stock solution containing 10 × Hinfl Buffer and 1 U of Hinfl per reaction (Roche) to give a final volume of 25.0 µl. The mixture was incubated in 37 °C for 1 h. The digested product was run on a 2.5% agarose gel at 60 V for 60 min for RFLP identification. The gel was stained with ethidium bromide (5 µg/ml) for 15 min and viewed on an ultraviolet transilluminator at 312 nm.

The ITS2 amplification products from a mosquito identified as form A or B (lack or having block spot on palpi) from different populations were subjected for sequencing. The ITS2 PCR was purified for direct sequencing using Qiagen PCR purification chromatography following the manufacture's directions. The PCR purified as above and all purified products were sequenced using a 373 ABI automated sequencer. Sequences were aligned using CLUSTAL X (27) software package. Similarity with other sequences in GenBank was assessed using FASTA search (http://www.ebi.ac.uk/fasta33/). Inter- and intra-population variability and phylogenetic analysis was carried out using MEGA2 (28).

RESULTS

Mosquitoes morphologically identified as group A and B or typical and atypical form of *An. superpictus* obtained from collection sites in Iran were subjected to genetic analysis using a PCR-RFLP technique to assess the presence or absence of restriction sites. The PCR products were then subjected to direct sequencing to assess the rate of DNA polymorphism and sequence insertion/deletions. The PCR products of the ribosomal region showed slightly amplified length polymorphism (PCR-ALP) between populations (Fig. 2). Subsequent sequence analysis confirmed the presence of two PCR products with 498 bp and 518 bp length in the mosquito genomes. This length variation was due to insertion/deletion events in the ITS2 region. The length of ITS2 sequences was 357 bp and 378 bp and could divide the populations into two main groups (Fig. 3).

The longer ITS2 sequences occurred in Baluchistan populations and the smaller one belongs to other populations. Out of 100 bp of sequences of 5.8S, four mutations and out of 40 bp of 28S only one substitution occurred. Initial screening of PCR products by various enzymes showed different RFLP profiles within populations. Hinfl showed three PCR-RFLP profiles, in which all populations, except Baluchistan, were identical and were designated as genotype X. Mosquitoes from Baluchistan displayed two different RFLP profiles occurred together which were designated as genotype Y and Z (Fig. 4).

There was no correlation between morphological patterns of neither adult female nor larvae and genotype status. The ITS2 region was sequenced for all the observed genotypes, including morphological forms and populations and the resulting twelve sequences were deposited in GenBank (AF941106-AF941117).

The rate of genetic variation between populations was 23.55% in which respectively 0.20%, 0.77% and 23.55% corresponded to 28S, 5.8S, and ITS2 regions. 40.94 percent (52 out of 127) of these variations was due to insertion/deletion events and 59.06% was due to substitutions. Totally 127 mutations occurred within the 518 bp length sequences, in which genotype Y and Z were varied in 124 and 122 sites from X. The two sympatric genotypes Y and Z differed by two nucleotides (0.4%). This sequence variation occurred almost equally throughout the ITS2 spacer. Phylogenetic analysis of sequence data showed that there are two main clads within populations in which one of them included two separate branches (Fig. 5). The phylogenetic tree revealed that three separate groups present within taxon, each putatively represents a separate species.

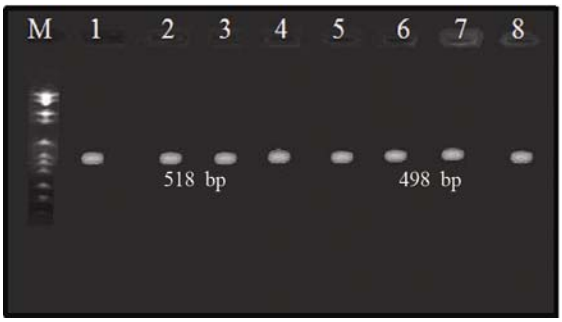

Fig 2. PCR amplification of rDNA-internal transcribed spacer II (ITS2) in different population of Anopheles superpictus from Iran. Note length polymorphism observed between 2-4 and 5-9 specimens which in order represent genotype X (498 bp) from Lorestan and Ardebil, and Y-Z (518 bp) from Baluchistan.

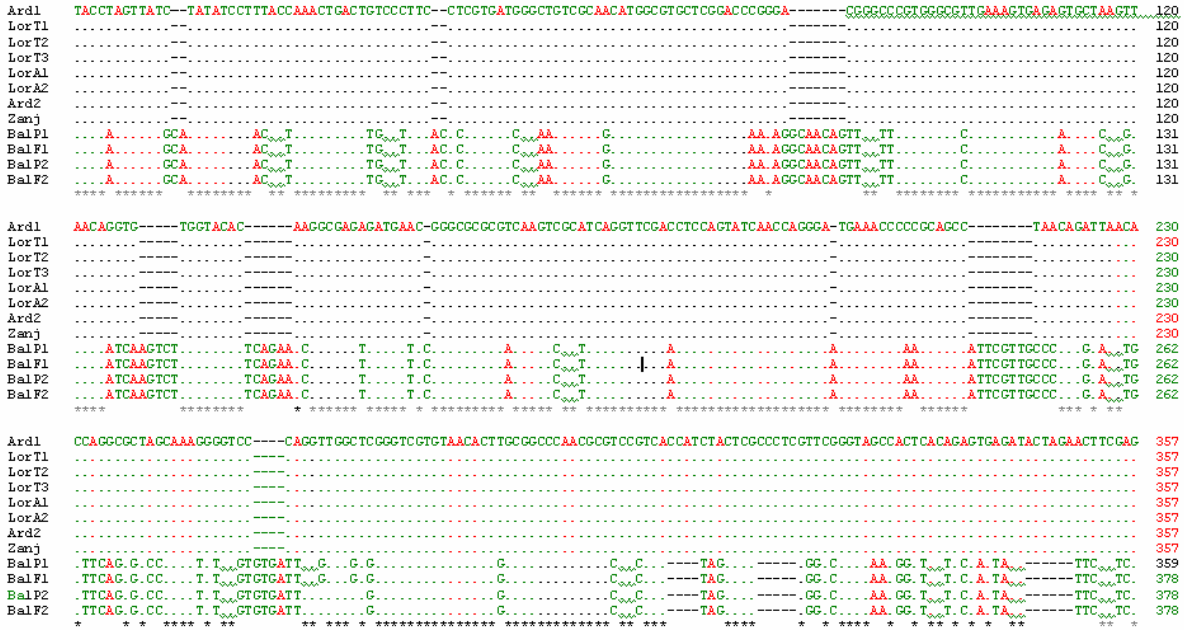

Fig. 3. Alignment of internal spacer number 2 (ITS2) DNA sequences of *Anopheles superpictus* population from Iran (Ard, Ardebil; LorT, Lorestan typic larval form; LorA, Lorestan atypic larval form; Zanj, Zanjan; Bal, Baluchistan province; *, variable; -, deletion; ., identical site.

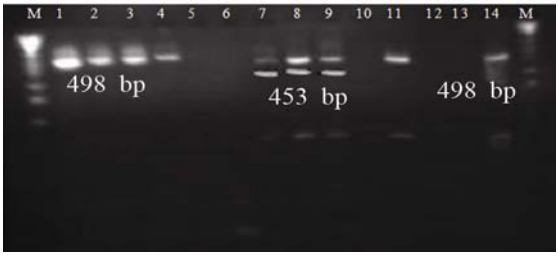

Fig 4. PCR-RFLP analysis of ITS2 region of *Anopheles superpictus* populations of Iran using Hinfl enzyme electrophoresed on a 2% agarose gel. Genotype X (498 bp) is undigested and genotypes Y/Z (518 bp) are digested into 453 and 65 bp. Numbers 1-4, 11, and 14: genotype X from different parts of Iran (except for Baluchistan), and numbers 7-9: genotypes Y/Z from Baluchistan.

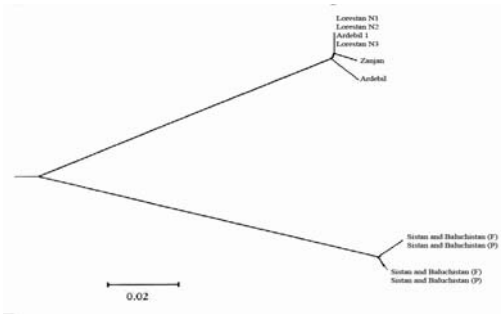

Fig. 5. Neighbor-Joining phylogenetic analysis of *Anopheles superpictus* population from Iran based on 498-518 bp of DNA sequences of rDNA-ITS2 region including 100 bp of 5.8S and 40 bp of 28S.

DISCUSSION

There appeared to be no correlation in the morphological characteristics of larvae or adults previously reported to identify *An. superpictus* populations with any of the genotypes identified in this study. These results would question the validity of using morphological characters to identify cryptic or closely related species.

The independent evolution of these rDNA gene families to detectable genotypes and the sympatric occurrence of two of these genotypes appear to suggest that a number of these genotypes exhibit natural mating barriers and thus possibly could represent separate species as previously described by (29).

Genotypes Y and Z occurred together in Baluchistan, while, genotypes X existed over a relatively large geographical range in the country including Ardebil in north west, Lorestan and Zanjan in west, and Kazeron in South.

Evidence for any natural hybridization between these genotypes may possibly manifest the status of the taxons if this is a group species or not. However, the observed genotypes have maintained two divergent paralogues (X and Y-Z) throughout this

range without any detectable sequence homogenization. The presence of a high genetic distance (25%) between populations highly supports the status of species as a species complex. This rate is far more than rate of intra-population or intra-species variation reported for other species. For instance, it was less than 0.1% and 2% for *An. gambiae* (30) and *An. sacharovi* (Sedaghat *et al*, 2003) respectively. It was about 4-5% between *An. culicifacies* species A and B (31) and 0-6% among the *An. gambiae* complex (31). It is probably premature at present to suggest species status for these genotypes, and further genetic studies with larger sample sizes over a wider geographical distribution are now required to determine the species status and distribution of this group.

Further support could also come from running parallel studies on a separate variable marker such as one from the mitochondria. It would appear, however, that the ITS2 sequence paralogues of genotype X and Y-Z are not undergoing any observable homogenization and have possibly become genetically fixed, because the sequence data of each genotype appear similar in all individuals examined from the populations examined. Also the role of malaria transmission of these genotypes should be checked by further entomological and epidemiological surveys. Previous studies in different areas of Iran have shown that the western or northern populations of this species (designated as species X) was playing important role in past.

North and east provinces, and in particular, northwest of Iran are mountainous, colder, and wetter than southeast corner, with a quite severe long cold seasons and short seasonal activity. The single main phylum comprising samples of most provinces of the country may represent form adapted to the colder, wetter, and temperate climates because it does not appear in the Sistan-Baluchistan province which is drier than other parts with a tropical climate. Conversely, the diverged sequences of Sistan-Baluchistan appear confined to the tropical climate, having failed to adapt to the colder and wetter northern and western regions. Presence of the big desert named Kavir-e Namak and Dasht-e Lut, a region of below 100 mm rainfall, may cause these two main groups of populations were separated and it may act as a barrier to gene flow, allowing the independent evolution of these groups into different genotypes. Sistan-Baluchistan is the contact region of three different zoological regions of Afrotropical, Palearctic, and Oriental. This may explain the presence of diverged sequences as well as a number of different *Anopheles* specie in the region (2).

Genotypes and sequences described here can be readily identified by DNA sequences, enabling studies into their distribution, biology and behavior to proceed in order to understand their role in the transmission of malaria. Further studies are now required to verify the species status of these sequences and study the other specimens from other countries in Europe, Asia, and Africa.

Conflict of interests

The authors declare that they have no competing interests.

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