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Molecular Characteristics of *Anopheles maculipennis* Meigen in Zanjan, North West of Iran, Inferred from ITS2 Sequence Analysis

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Abstract: The present study has been designed in order to verify the species composition within *Anopheles maculipennis* complex in North West of Iran. We determined ribosomal DNA sequences of the second internal transcribed spacer (ITS2) region from samples of *Anopheles maculipennis* Complex in Zanjan province. A total of 1536 specimens within the Complex were tested by Multiplex PCR, only *An. maculipennis* was found in this area. One clone out of four different individual mosquitoes of each field was generated with ITS2 PCR and half of them (192 samples) selected randomly for RFLPs. PCR-RFLP assay identified 2 haplotypes; haplotype I (99%) and haplotype II (1%). Twenty five sequences were generated comprising the 5.8S gene, the ITS2 and the 28S ribosomal gene. The alignment was 422 in length and percentage of GC content was 50.3% (26.07% A, 23.59% T, 26.78% C, 23.7% G). The ITS2 was 290 bp in length and two haplotypes were revealed varying by a single base (T↔C) at site 378. *An. maculipennis* is the dominant species anopheline of the province. ITS2 analysis revealed evidence of a slightly interaspecific variation among populations. However, further investigations on the genetic polymorphism among *An. maculipennis* populations and in particular within those belonging to the continental haplotype are required to support any hypothesis on differences in behavior across the distribution range for this potential malaria vector.

Key words: *Anopheles maculipennis*, internal transcribed spacer two, molecular characteristic, Zanjan, Iran

INTRODUCTION

Until the middle of twentieth century, malaria was endemic in many parts of Iran (Edrissian, 2006). Although it was finally eradicated from the Northern parts of the country, recent events such as re-emergence of malaria in some Trans-Caucasian countries of the former USSR (Romi *et al*., 2002), the occurrence of several indigenous cases of malaria in border provinces of Iran (Sadrizadeh, 1999) and littoral countries of Mediterranean and Black sea (Baldari *et al*., 1998; Alten *et al*., 2000; Sabatinelli and Joergensen, 2001; Kampen *et al*., 2003), in addition to the concern over global warming (Githeko *et al*., 2000) have lead to a renewed interest in the *Anopheles maculipennis* complex which includes both malaria vector and non vector species in Eurasia.

Vector control is an essential component of any malaria control program, the success of which relies on knowledge of vector species present in the area and their bionomics. Most of malaria vectors found in Eurasia are known to be complex of cryptic species. Members of these complexes may differ in biologic characteristic that have direct relevance to epidemiology and control of malaria such as vectorial efficiency, insecticidal resistance, feeding and resting preference. Therefore, mapping the distribution of cryptic species and understanding the bionomics and vectorial efficiency are essential for planning vector strategies.

Anopheles maculipennis complex the historical malaria vector in the present Eurasia and North America was exposed as the first sibling species complex of mosquitoes more than 80 years ago (Harbach, 2004). The complex comprises nine Palearctic members. Classically this Complex species are differentiated by eggshell morphology, larval chaetotaxy, isoenzyme analysis and cuticular hydrocarbon chromatography. Owing to lack of reliability and/or other disadvantages for techniques in practical routine application, alternative identification methodologies have been searched. A few years ago several techniques were used for molecular diagnosis of sibling species (Collins and Paskewitz, 1996; Collins *et al*., 2000; Krzywinski and Besansky, 2002). Among the most popular techniques are species specific primers and restriction fragment length polymorphism in PCR. Because the techniques are simple, inexpensive, very reliable and reproducible and provide discrete character states they

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can be useful for phylogenetic and population genetic analysis as well as diagnosis (Strickberger, 2000).

Correct vector identification is essential to assess the potential risk of malaria in the border provinces of Iran and devise appropriate control or monitoring strategies. Early records indicated the presence of *An. maculipennis* and *An. sacharovi* in North West of Iran (Manouchehri *et al*., 1992), but little is known about their present distribution, although *Anopheles maculipennis* and *An. sacharovi* are capable of malaria transmission. There are major discrepancies existing between Genbank entries and what are purportedly the same sequences aligned in the published papers (Linton *et al*., 2002). Also there are considerable variations in published alignments (Marinucci *et al*., 1999; Linton *et al*., 2003; Oshaghi *et al*., 2003; Sedaghat *et al*., 2003). Therefore, in the present study DNA sequences of nuclear Internal Transcribed Spacer (ITS2) from with mosquitoes were generated and the specimens were identified by comparison of ITS2 sequences in GenBank.

MATERIALS AND METHODS

Mosquito collection: Adult mosquitoes were collected from indoor and outdoor areas near larval habitats of Zanjan, Mahneshan, Taroom, Abhar and Ijrood districts of Zanjan province (North West of IRAN) by pyrethrum space spray and light traps during 2004-2005 (Table 1). Collected samples were transferred to the laboratory and identified according to standard keys. Females and males of *Anopheles maculipennis* complex were selected and stored at -20°C until examination.

DNA extraction: DNA was isolated according to the methods of Proft *et al*. (1999) and Linton *et al*. (2002) with minor modification. Mosquitoes were individually homogenized in 100 μL of grind buffer (0.5 M sucrose, 0.5 M Tris-HCl at pH 8.0, 0.5 M EDTA and 1% SDS). The tubes were incubated at 65°C for 30 min, 20 μL 5 M KOAc (pH 9.0) was added and placed in ice for another 30 min, then shaken before spinning at 1000 x g for 15 min. The supernatant was transferred to a clean tube with 200 μL of cold absolute ethanol and stored at -20°C for 2 h. The tubes were spun at 10000 x g for 10 min, then again washed twice with cold 70% ethanol by spinning at 5000 x g for 10 min. The pellet was air-dried and suspended in sterile water to give 50 μL of DNA solution. Four microliter of re-suspended DNA was used in each PCR reaction.

PCR: PCR was performed based on method described by Proft *et al*. (1999) and Kampern (2005). The sequence of the forward primer was complementary to a conserved region of 5.8S rDNA whereas the reverse primer annealed to a conserved 28S rDNA region. The PCR mixture had a total volume of 50 μL and contained 10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 250 mM dNTPs (Cinagen), 0.8 mM forward primer, 0.6 mM reverse primer and 1.25 Unites of *Taq* polymerase (Fermentas) using Gradient-Palm-Cycler (Corbett research, Australia). The thermo file consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 7 min.

The PCR products (7 μL amplicons) were mixed with 3 μL of loading buffer (bromophenol blue/xylene cyanole, Fermentas) and electrophoresed for approximately 1.5 h. at 84 V on standard 1.5% agarose gels with Tris-Borate-EDTA buffer and bands were stained with 1% Ethidium Bromide. DNA bands were visualized by illumination with 254 nm wavelength using Uvidoc (Uvitec, Cambridge, UK) and electrographs were saved on files.

RFLP: Total reaction volumes were 25 μL. Ten microliter of ITS2 PCR product were added to 200 μL PCR tubes containing 0.1 U of NCOI (Fermentas), 2.5 μL of Tongo buffer (33 mM Tris-acetate at pH 7.9, 10 mM Magnesium acetate, 66 mM Potassium acetate and 0.1 mg mL^{-1} BSA) and 12.4 μL of dd-H_2O. The tubes were incubated at the optimized enzyme activity temperature of 37°C for 16 h. according to the manufacture's instruction to ensure full cutting of fragments. For checking the digestive products, 10 μL of product in addition to 3 μL of loading buffer were electrophoresed.

Design of species specific primers and multiplex PCR: Differences in the ITS2 sequence of *Anopheles maculipennis* and *An. sacharovi* were

Table 1: Specimen trapping localities and GenBank accessing numbers for ITS2 sequences generated in this study

District	Locality	Co-ordinates	Altitude	GenBank accessing numbers
Abhar	Amid abad	36°11'N49°11'E	1575	DQ917918
Ijrood	Khir abad	36°17'N48°30'E	1750	DQ917922, DQ917924, DQ917925
Mahneshan	Leilan	36°45'N47°29'E	1970	DQ917912, DQ917913
	Saraghol	36°46'N47°30'E	1850	DQ917909, DQ917910, DQ917911
	Zamayem	36°45'N47°35'E	1800	DQ917919, DQ917920, DQ917921, DQ917923
Taroom	Zehtarabad	36°15'N48°30'E	350	DQ917926, DQ917927, DQ917928, DQ917914,
	Ghalat	36°14'N48°29'E	400	DQ917915, DQ917916, DQ917917
Zanjan	Gharaboteh	37°10'N47°40'E	1160	DQ860507,DQ917905, DQ917906, DQ917907 DQ917908

used to design species specific (reverse) primers MAC (5'..TGTGCCTCCCCGTTAGGTAA..3') and SAC (5' TCCCGTAGCTAGGAGCTGGT 3'). Combination of 5.8S universal forward primer with MAC and SAC reverse primers would generated PCR products of species specific lengths 310 bp for *An. maculipennis* and 400 bp for *An. sacharovi*. The primer sequence was selected on the criteria that they had similar length and melting temperatures and low properties to form primer-dimer with intra molecular secondary structures. The oligonucleotide primers were synthesized by Cinagen. The composition of the PCR mixtures were the same as for the amplication of the ITS2 region, except that the 28S primer was replaced with the species specific primers and annealing temperature was 58°C.

DNA sequencing: PCR products separated by electrophoresis on 1.5% agarose gels and bounds were excised with scalpel. DNA was recovered by using the DNA Gel extraction kit (Qiagen) according to the manufacturer's instructions and prepared for DNA sequencing. Sequencing was done by cycle sequencing on an ABI 3730 DNA analyzer (Applied Biosystems) using primer 5.8S and 28S as sequencing primers. All PCR products were sequenced in duplicate in both directions. Template DNA was retained at -70°C in Molecular Systematics laboratory of department of Parasitology in Zanjan University of Medical Sciences as voucher species.

Data analysis: Sequences were edited and aligned by using MUSCLE (Edgar, 2004) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) software packages. Similarity with other sequences in GenBank was assessed using BLAST (http://www.ncbi.nlm.nih.gov). Nucleotide sequences generated were submitted to GenBank and respective accessing numbers assigned (Table 1).

RESULTS

A total of 1536 specimens within the *Anopheles maculipennis* complex were tested by Multiplex PCR for species identification. Only one species, *An. maculipennis* was found in the study area. Twenty five sequences comprising the 3' end of the 5.8S gene, the ITS2 and the 5' end of 28S ribosomal gene were generated for 5 individuals from Zanjan, 7 from Taroom, 9 from Mahneshan, 3 from Ijrood and one from Abhar districts (Table 1). Sequences are available in GenBank under the following accession numbers: Garaboteh (DQ860507, DQ917905-DQ917908), Ghalat (DQ917915- DQ917917), Zehtarabad (DQ917914, DQ917926-DQ917928), Zamayem (DQ917919 - DQ917921, DQ917923), Saraghol (DQ917909-DQ917911), Leilan (DQ917912, DQ917913), Khirabad (DQ917922, DQ917924, DQ917925) and Amidabad (DQ917918). The alignment was 422 in length (including primers, 40 bp). Percentage of GC content for 422 bp was 50.3% (26.07% A, 23.59% T, 26.78% C, 23.7%G) in rich and unambiguous. The ITS2 was 290 bp in length and two haplotypes were revealed (Fig. 1) varying by a single base (T↔C) at site 378 (Fig. 2).

One clone from four different individual mosquitoes of each field collected samples listed in Table 1 were generated with ITS2 PCR and half of them (192 samples) selected randomly for RFLPs.

With using the ITS2 sequences generated in this study a PCR-RFLP assay was designed for the accurate and relatively quick identification of two haplotypes of *An. maculipennis*. The enzyme NCOI can recognize the nucleotide sequence.. C↓CATGG. Predicted fragments following digestion of the ITS2 with this enzyme were 219/203 for haplotype II and 219/159/44 for haplotype I. It should be noted that products below 50 bp are not easily detected in agarose gels and only fragments above this size are visible (Fig. 1). Haplotype I accounted for 190 (99%) of the specimens, complete digested with NCOI enzyme (DQ917906-DQ917928) and the two remaining both from Gharaboteh (DQ860507, DQ917905), shared haplotype II.

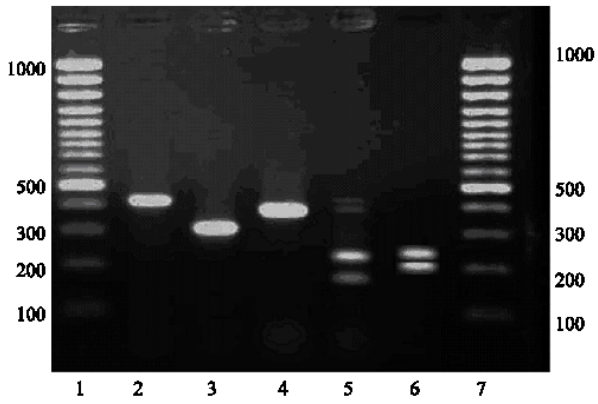

Fig. 1: Amplification of the entire internal transcribed spacer 2 and species-specific fragments for two *Anopheles* species. Lanes 1 and 7: DNA sizes ladder (bp); lane 2: ITS2 fragment of *Anopheles maculipennis*; lane 3: products of allele-specific PCR assay with primer MAC for *Anopheles maculipennis*; lane 4: products of allele-specific PCR assay with primer SAC for *Anopheles sacharovi*; lane 5: digested ITS2 fragment of *Anopheles maculipennis* with NCOI enzyme in haplotype I and lane 6: digested ITS2 fragment of *Anopheles maculipennis* with NCOI enzyme in haplotype II

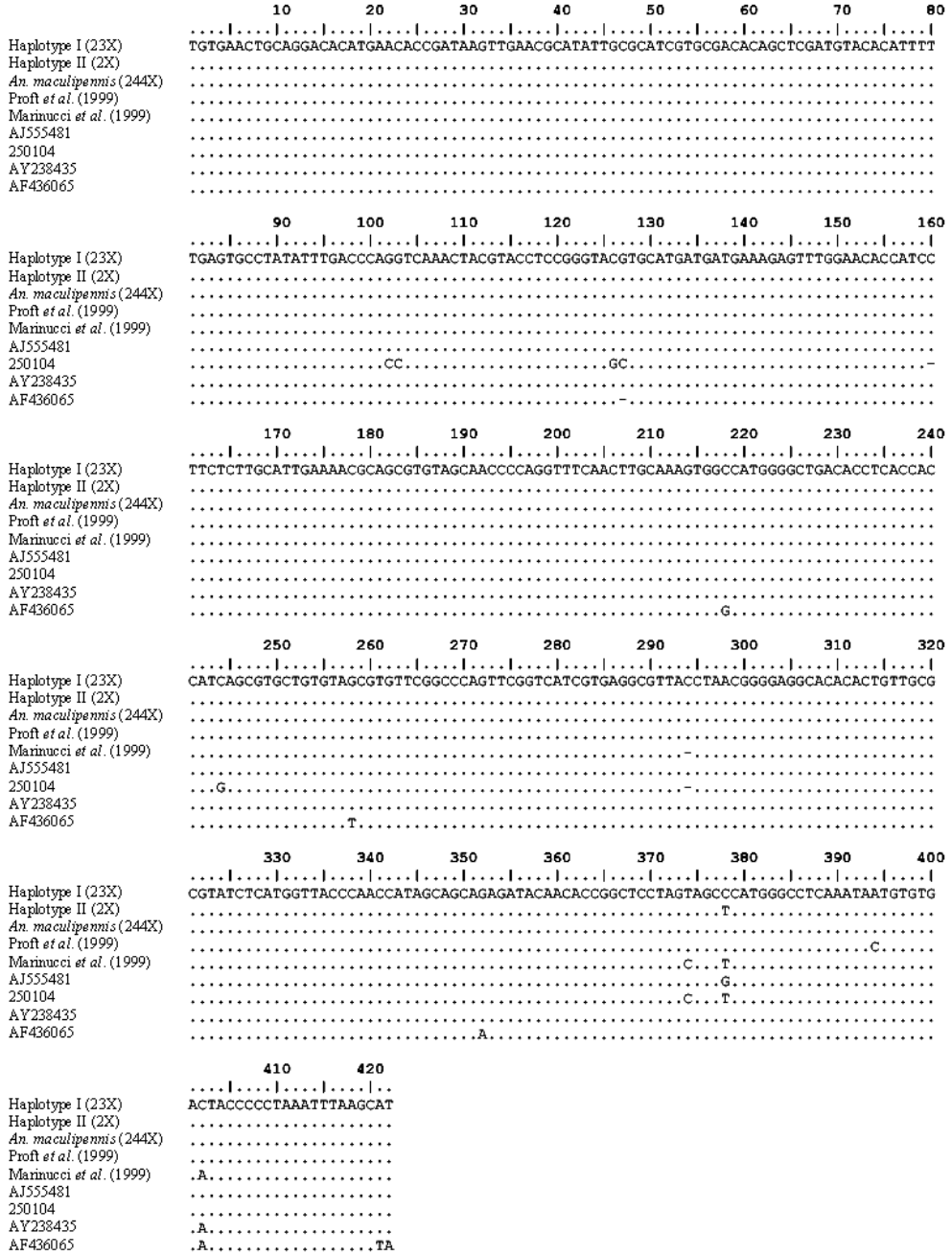

Fig. 2: Alignment of nucleotide sequences (5' to 3') of the ITS2 and flanking 5.8S (nucleotide 1-90) and 28S (381-422) coding regions of ribosomal DNA of the haplotype I and haplotype II of *Anopheles maculipennis* generated in this study and compared with the submitted and published sequences. (•) indicates identity with the sequence and (-) indicates indels

Previous studies that generated ITS2 sequence for *An. maculipennis* deposited 246 sequences in GenBank, which 148 from Greece (AF342713-AF342715, AF455818-AF455921, AF469847-AF469852, AF485807-AF485810, AF53552-AF53582), 52 from Iran (AF436065, AF53632-AF53637, DQ243829, AY137781-AY137816, AY238434, AY238435, AY533853, AY730264, AY730265, AY730267, AY730268, AY842514), 33 from Romania (AY579401,

AY634535-AY634566), 4 from Italy (AY238424-AY238427), 4 from Armenia (AY238430-AY238433), Yugoslavia (AY238428), Turkey (AY238429), Tajikistan (AJ555481), France (AY238429) and Germany (AY365010). All GenBank and published sequences were aligned with the haplotypes generated at current study (Fig. 2). Major discrepancies exist between the GenBank entries and what are purportedly the same sequences aligned in the published papers. Sixteen (3.8%) bases were variable among the sequences, twelve accounted for by three insertion, deletion and indel events in ITS2 region.

DISCUSSION

All wild captured mosquitoes tested in Zanjan province were identified as *An. maculipennis*. Analysis of the ITS2 sequences of this *Anopheles* showed that GC content was 50.3% and slightly interaspecific variations were in different populations and two haplotypes distinguished by one base change scattered in this province.

Although malaria has been eradicated from North-West and central regions of the country, but recently there have been some reports of an increase in malaria cases in the North-West. Combining these facts with major ecological and social changes such as the increased parasite pool resulting from travel to and from the southeast corner of the country and neighboring countries where malaria is endemic, the reintroduction of malaria in these regions becomes a realistic possibility and health authorities should use appropriate control or monitoring strategies.

Studies on the genetic structure of malaria vector populations can be used to infer the likely success of vector control strategies. They can highlight issues such as the impact of conventional methods (e.g., insecticide spraying) in reducing vector abundance, the reintroduction of vectors into formerly controlled areas, the spread of insecticide-resistance genes or the control of vectors by means of transgenic technology (Collins *et al*., 2000). The latter is a promising novel control strategy, although still subject to intense debate (Curtis *et al*., 1999). Should transgenic-based control be attempted, a natural first step would be to test its efficacy on islands, where confounding effects such as migration are expected to be lesser problems than on the continent.

The results of this study indicated that, *An. maculipennis* is the only species of the Maculipennis complex in the study area. Oshaghi *et al*. (2003) reported that *An. maculipennis* and *An. sacharovi* were present in this province and *An. maculipennis* contains 40% of them.

An. maculipennis is the dominant anophelines of the province with a high tendency toward resting indoors. This species has a high frequency in August and September at low altitudes of Zanjan province. Since this species is malaria vector in the region, we recommend that health officials pay careful attention to malaria control and monitoring programs and future ecological, molecular studies should be carried out on different populations of this species.

Percentage of GC content for the 422 fragment of ITS2 was 50.3%. These values are concordant with 50-60% GC values reported for other mosquitoes of subgenus *Anopheles* (Marrelli *et al*., 2005) and the Maculipennis Group (Marinucci *et al*., 1999; Proft *et al*., 1999; Linton *et al*., 2002; Nicolescu *et al*., 2004).

Our ITS2 sequences identified two haplotypes in the population of *An. maculipennis* in Zanjan province which were distinguished by one base change (transition T↔C). No interaspecific variation was noted in previous studies of Linton *et al*. (2002) from Greece and Sedaghat *et al*. (2003) and Djadid *et al*. (2007) from Iran. Comparing our sequences with those available in the literature, the high degree of homology was found for haplotype I. In particular this haplotype showed 100% nucleotide identity with sequences from Greece, Iran, Italy, Romania, Turkey, Armenia, Germany and France. Contrary to the consensus sequence of (Marinucci *et al*., 1999), Z50104 from Italy, AJ555481 from Tajikistan and AF436056 from Iran, did not completely match with two haplotypes.

Differentiation of the ITS2 between populations and species depends on many factors, including genetic drift, the relative number and size of repeats, rates of unequal crossover, gene conversion, immigration, number of loci and mating systems (Strickberger, 2000). The degree of differentiation observed within species, therefore is a balance struck between those processes that generate variability and those that lead to homogenization and fixation.

Concerted evolution of multi-gene families within species has resulted in rapidly evolving spacer regions, such as the ITS2 rDNA. Sequencing of these regions has been used to effectively determine the species composition of *Anopheles* mosquito complexes (Collins and Paskewitz, 1996). Djadid *et al*. (2007) surveyed different species of *An. maculipennis* complex in North part of Iran and identified three new species of *An. messaea*, *An. labranchia* and *An. atroparus* which have not reported before based on molecular analysis of rDNA-ITS2 sequences. Low levels of intra-specific variation in the ITS2 region has proven useful for the design of species-specific primers or PCR-restriction fragment-length polymorphism assays to differentiate

members of species complexes and for phylogeny reconstruction in mosquitoes. Previous studies of ITS2 sequences have shown little or no intra-specific variation in sibling species of *Anopheles*. Intra-specific variation in five colony strains of species of the *An. gambiae* complex was reported to range between 0 and 0.43% (Paskewitz *et al*., 1993). In contrast, Beebe *et al*. (2001) reported population-specific ITS2 sequences in the *An. bancroftii* group comparing populations from Queensland, Australia and the Western province of Papua New Guinea. However, further investigations of genetic polymorphism among *An. maculipennis* populations and in particular within those belonging to the continental haplotypes are required to support any hypothesis on differences in behavior across the distribution range for this potential malaria vector.

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