Molecular characterization of the *Anopheles maculipennis* complex in the Islamic Republic of Iran

M.A. Oshaghi,[1] M.M. Sedaghat[1] and H.Vatandoost[1]

تحديد الصفات الجزيئية لمعقَّدات الأنوفيلات المبقَّعة في منطقتَيْن من جمهورية إيران الإسلامية

محمد علي عشاقي، حسن وطن دوست، محمد مهدي صداقت

الخلاصة: استُخدمت المقايسة التشخيصية بالتفاعل السلسلي للبوليميراز PCR باستخدام بادئات مناوِعة للأنواع وبالتعيين المباشر للمتواليات للتعرُّف على معقدات الأنوفيلة المبقَّعة في المناطق المركزية والشمالية الغربية من جمهورية إيران الإسلامية. وقد جمعت العينات من تسع مقاطعات خلال أنشطة فصلية في العامين 2001 و2002، وتم التعرُّف المورفولوجي والمقايسة بالتفاعل السلسلي للبوليميراز مع التحديد المباشر للمتواليات. وقد أظهرت النتائج وجود نوعين فقط في منطقة الدراسة هما الأنوفيلة المبقَّعة (من نمط مايغن) والأنوفيلة الزخاروفية (من نمط فافر). وقد تأكدت هذه النتائج بالتشابه الشديد (99.2 — 100٪ من متواليا تهما) مع ما هو متوافر في بنك الجينات. وقد نوقشت المعطيات الجزيئية والتوزع النسبي لهذين النوعين في ما يتعلق بقدرتهما على نقل العدوى وبوبائيات الملاريا في المنطقة.

ABSTRACT A diagnostic polymerase chain reaction (PCR) assay using species-specific primers and direct sequencing was used to identify members of the *Anopheles maculipennis* complex in the north-west and central regions of the Islamic Republic of Iran. Specimens were collected from 9 provinces during 2 seasonal activities in 2001–2002, identified morphologically and subjected to PCR assay and direct sequencing. Results showed that only 2 species, *An. maculipennis* Meigen, and *An. sacharovi* Faver, were present in the area of study. This was confirmed by the high similarity (99.2%–100%) of their sequences with those available in GenBank. The molecular data and relative distribution of these species in relation to their vectorial capacity and the epidemiology of malaria in the region are discussed.

Caractérisation moléculaire du complexe *Anopheles maculipennis* dans deux régions de la République islamique d'Iran

RESUME Une PCR (amplification en chaîne par polymérase) diagnostique avec des amorces spécifiques d'espèces et un séquençage direct ont été utilisés pour la détermination des membres du complexe *Anopheles maculipennis* dans les régions du nord-ouest et du centre de la République islamique d'Iran. Des spécimens ont été capturés dans 9 provinces pendant deux activités saisonnières en 2001-2002, identifiés morphologiquement et soumis à une PCR et à un séquençage direct. Les résultats ont montré que seulement 2 espèces, *An. maculipennis Meigen* et *An. sacharovi Faver*, étaient présentes dans la zone d'étude. Cela a été confirmé par la forte similarité (99,2-100 %) de leurs séquences avec celles disponibles dans la GenBank. Les données moléculaires et la répartition relative de ces espèces par rapport à leur capacité vectorielle et l'épidémiologie du paludisme dans la région sont examinées.

[1]*Department of Medical Entomology and Vector Control, School of Public Health and Institute of Health Research, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran.*

Introduction

Mosquitoes of the *Anopheles maculipennis* complex have been incriminated as some of the main malaria vectors in Europe and the Middle East [*1*] and also in northern and central areas of the Islamic Republic of Iran [*2*]. The current situation of this taxon shows that there are 8 Palaearctic members within the complex: *An. atroparvus* Van Thiel, *An. beklemishevi* Stegnii & Kabanova, *An. labranchiae* Falleroni, *An. maculipennis* Meigen, *An. martinius* Shingarev, *An. melanoon* (as subalpinus) Hackett, *An. messeae* Falleroni, and *An. sacharovi* Favre [*1*]. These are closely related species and morphologically indistinguishable. Distinctive differences between them have been identified in mating patterns [*3*], inversion polymorphism in the polytene chromosomes of the larval salivary gland [*1*], and DNA sequences [*4–6*]. Generally, the 8 species occupy disjunctive geographic regions, although several do overlap in distribution. Of the 8, only 3, *An. labranchiae*, *An. atroparvus*, and *An. sacharovi* are important malaria vectors throughout their ranges.

Previous reports concerning members of the *An. maculipennis* complex in the Islamic Republic of Iran were based on morphological characteristics of eggs and recorded the presence of 5 members of the complex: *An. maculipennis*, *An sacharovi*, *An. melanoon*, *An. messeae*, and *An. subalpinus* [*7–9*]. Saebi has shown that *An. maculipennis sensu lato* is distributed in different north, west and central provinces of the country, including West and East Azarbijan, Kurdistan, Kermanshahan, Hamadan, Gilan, Mazanderan, Golestan, Khorasan, Semnan, Tehran, Markazi, Esfahan, Chahar-Mahal-Bakhtiary, Fars, Lorestan, and Kohkiluyeh Boyer-Ahmad provinces [*10*]. That study, however, did not determine which sibling species of the complex are present in those provinces, and since then no further studies have been carried out to determine current distribution.

Although malaria has been eradicated from northern and central regions of the country, because of major ecological and social changes such as increased travel to and from the south-east corner of the country and neighbouring countries, where malaria is endemic, there is a high risk of reintroduction in these regions. Since the 5 reported species in the Islamic Republic of Iran are capable of transmitting malaria [*2,11,12*], and because of the high risk of the resurgence of malaria transmission in the region, correct vector identification is essential to assess the potential risk of malaria, and to advise on appropriate control and monitoring strategies.

In our study we used the previously developed diagnostic polymerase chain reaction (PCR) system [*6*] that can differentiate between six members of the complex: *An. maculipennis sensu stricto*, *An sacharovi*, *An. messeae*, *An. melanoon*, *An. atroparvus* and *An. labranchiae*. The species-specific primers were designed based on differences in the nucleotide sequences in internal transcribed spacer regions (ITS2) of the ribosomal DNA that in combination with a universal primer could generate amplification products of different lengths, each unique for 1 species. In addition, DNA sequences of the ITS2 region were generated and the sequences compared with the ITS2 sequences in GenBank to confirm the results of species-specific PCR assays.

Methods

Mosquito collection was carried out in 9 provinces located in the semi-arid and

mountainous regions of north-west and central parts of the Islamic Republic of Iran: West Azarbijan, Kermanshahan, Zanjan, Tehran, Esfahan, Chahar-Mahal-Bakhtiary, Fars, Markazi, and Kohkiluyeh Boyer-Ahmad. Based on area and previous data on the distribution of species in each province, 2–5 villages were selected for sample collection. Sample collections were performed during two seasons, October–early December 2001 and June–early October 2002. To cover different ecological and biological factors, different techniques were used to collect adult mosquitoes from indoors and outdoors of stables and houses, and on human and animal baits. Individual, blood-fed, live females were transported into cups to lay eggs. Eggs were reared in insectaries to produce larvae. All the breeding places were also checked for larvae. Morphological investigation was performed to identify specimens using standard keys for larvae and adults of the *Anopheles* species of the Islamic Republic of Iran [*13*].

DNA was extracted from specimens following the phenol-chloroform extraction protocol [*14*]. The pellet was suspended in 100 mL TE buffer or double distilled water; 1–2 mL of suspended DNA was used in each PCR reaction. Amplification of ITS2 was carried out using the 5.8SF and 28SR primers of Collins and Paskewitz [*15*]. Diagnostic PCR assays were carried out using 5.8SF as the universal forward primer and 6 species-specific primers as reverse primers (Table 1). We carried out PCR using a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) and the reaction parameters described by Proft et al. [*6*]. Sequence data were obtained following PCR purification with the QIAgen PCR purification kit, and cycle sequencing reactions were read on an ABI 377 automated sequencer (PE Applied Biosystems). Following sequencing, the template DNA was dried and retained at –70 °C for future reference. Sequences were edited and aligned using *Sequencher* version 3.1.1 and *Clustal X* [*16*] software packages. Similarity with other sequences in GenBank was assessed using FASTA search (http://www.ebi.ac.uk/fasta33/).

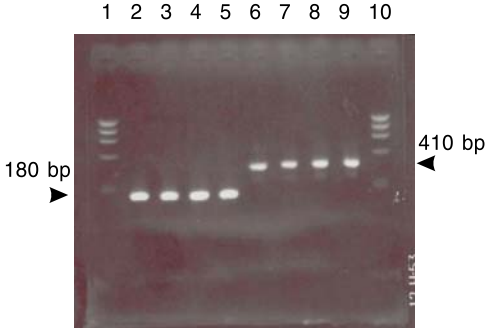
Results

The diagnostic PCR and sequencing results showed that only 2 species of the *An. maculipennis* complex, *An. maculipennis s.s.*

Table 1 **Characteristics of universal and species-specific primers used in this study**

Primer	Code	Nucleotide sequence (5´– 3´)	Length of PCR product (bp)
Universal forward	5.8S-UN	TGTGAACTGCAGGACACATG	494 (*sacharovi*)
Universal reverse	28S-UN	ATGCTTAAATTTAGGGGGTA	472 (*maculipennis*)
An. sacharovi	ASA	CAAGAGATGGATGTTTTACG	180
An. maculipennis	AMA	TATTTGAGGCCCATGGGCTA	410
An. atroparvus	AAT	CGTTTGGCTTGGGTTATGA	117
An. labranchiae	ALA	GTATCTCTGCTGCTATGGTC	374
An. melanoon	AML	TGCAAGTTGAAACCTGGGGC	224
An. messeae	AMS	GACGCCTCACGATGACCTT	305

and *An. sacharovi*, are present in the north-west and central parts of the Islamic Republic of Iran. The size of specific PCR products for *An. maculipennis s.s.* was 410 bp and for *An. sacharovi* 180 bp (Figure 1). Geographic distribution of these species in different provinces is as follows: in West Azarbijan and Zanjan provinces, which are more mountainous, both are present. The prevalence of *An. sacharovi* was 80% and 40% in these provinces respectively. In Tehran, Isfahan, Chahar-Mahal-Bakhtiary and Kohkiluyeh Boyer-Ahmad provinces we found only *An. maculipennis s.s.* and in Fars province only *An. sacharovi*. None of the *An. maculipennis* sibling species were found in Kermanshahan and Markazi provinces. In addition to *An. maculipennis s.l.*, other anopheline species which were found in the area of study were *An. superpictus*, *An. multicolour*, *An. pulcherimus*, *An. apoci*, *An. sergenti*, *An. turkudi*, *An. claviger* and *An. dthali*.



Line 1 and 10 molecular weight marker, lines 2–5: *An. sacharovi* and lines 6–9: *An maculipennis s.s.* The lengths of the polymerase chain reaction products are 410 bp for *An. maculipennis s.s.* and 180 bp for *An. sacharovi.*

Figure 1 **Specific polymerase chain reaction products for *An. sacharovi* and *An. maculipennis s.s.***

The rDNA-ITS2 region of both species was amplified successfully and was subjected to sequencing. We generated DNA sequences for 26 specimens from the north-west and central regions, *An. maculipennis s.s.* (20 specimens) and *An. sacharovi* (6 specimens). Inclusive of primers (43 bp), the ITS2 fragment lengths were 472 and 494 nucleotides for *An. maculipennis s.s.* and *An. sacharovi* respectively. The AT content was 49.36% in *An. maculipennis s.s.* and 52.23% in *An. sacharovi*. Total sequence divergence between the species, including indels, was 26.37%. The 24 bp length difference is accounted for by 10 indel events within the ITS2 region, at bases 149–152 (4 bp), 170–171 (2 bp), 189 (1 bp), 209–212 (4 bp), 224 (1 bp), 238(1 bp), 255(1 bp), 375 (1 bp), 403–405 (3 bp), 443–448 (6 bp) (Figure 2). In addition to the indels, 67 species-specific base substitutions were noted, of which 29 (43.3%) were transitions and 38 (56.7%) were transversions. No intraspecific variation was observed in either length or nucleotide composition of the fragments in all samples sequenced, and hence, each species shared the same haplotypes. Comparison of the sequence data with available ITS2 sequences in GenBank showed that *An. sacharovi* sequences share 100% similarity with entry Z83198 [*4*] and 99.35% similarity with entry of AF436062 of Djadid (unpublished data). The AF436062 is erroneously identified *An. maculipennis*, it is in fact *An. sacharovi* [*5*]. A similar situation was found for the *An. maculipennis s.s.* sequences in that they share 100% similarity with AF455820, AF342714, AF342713, AF342715, and 99.788% similarity with Z50104, 99.364% similarity with AF455819, and 99.153% similarity with AF455818 [*4,5*].

Sequences generated in this study have been submitted in GenBank under the following accession numbers: AY114205,

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            1                                                          60
sacharovi   ATCACTCGGCTCGTGGATCGATGAAGACCGCAGCTAAATGCGCGTCACAATGTGAACTGC
maculipe.   ............................................................

            61                                                         120
sacharovi   AGGACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATG
maculipe.   ............................................................

            121                                                        180
sacharovi   TACACATTTTTGAGTGCCTATTTTTGACCATCAGAAGTCAAACTACGTCGGCGGCGCCGT
maculipe.   ....................A......----CC.G...........A--.CT.CGG..

            181                                                        240
sacharovi   ACGTGCATAGATGATGAAAGATTTTGGGACGTAAAACATCCCATCTCTTGCATTGAATAC
maculipe.   ........-...........G.....A----.C.C.....TT-............-.A

            241                                                        300
sacharovi   CGTAGTGTGTAACA-CCCAGGGCTTCAACTTGCAAAGTGACCATGGGGCCAACACTTCAC
maculipe.   ..C..C.....G..A...CA..T..............G.........TG....C....

            301                                                        360
sacharovi   CGCCATCTTGTGCATGTGTAGTGTGTTCGGCCTAGCTTGGTTAACGTGAGGCGAACCCAA
maculipe.   .A.....AGCGTGC.......C..........C..T.C...C.T.........TTA..T.

            361                                                        420
sacharovi   CGGAGGAAGCACAATACAACTGCGCGTATCTCATGGTTCTAACCCAACCATAGCAACAGA
maculipe.   AC.G...G.....C-..TGT..................ACCC---..........G....

            421                                                        480
sacharovi   GATACAAAACCAGCTCCTAGCTACGGGAGTACATAGGCCTCAAATAATGTGAGACTACCC
maculipe.   .......C...G........TA------.CC...G................T........

            481         495
sacharovi   CCTAAATTTAAGCAT
maculipe.   ...............
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Total sequence variation is 91 (18.38%), which includes 24 indels (26.37%) and 67 substitutions (73.63%). Substitution comprises 29 (43.3%) transitions and 38 (56.7%) transversions.

Figure 2 **Alignment of internal transcribed spacer 2 nucleotide sequences of *An. maculipennis* s.s. and *An. sacharovi* generated in this study**

AY114206, AY114207, AY114209, AY114-210, and AY114211 for *An. sacharovi* and AF536337, AY137789, AY137792, AY13-7793, AY137794, AY137799, AY137800, AY137801, AY137805, AY137806, AY13-7807, AY137808, AY137809, AY137810, AY137811, AY137812, AY137813, AY13-7814, AY137815, AY137816 for *An. maculipennis s.s.*

Discussion

In this study, using the PCR assay and sequence data, we have found that only 2 members of the complex *An. sacharovi* and *An. maculipennis s.s.* exist in the northwest and central regions of the Islamic Republic of Iran. This is the first record based on both molecular and morphological tools

concerning the distribution of these species in the area of study, and is concordant with the results of Dow who reported 2 members of the complex, *An. maculipennis s.s.* and *An. sacharovi*, in the north-west and the central plateau [*7*].

The AT content for *An. maculipennis s.s.* and *An. sacharovi* (49.36% and 52.23% respectively) is in concordance with the 40%–50% AT values reported for other mosquitoes of the subgenus *Anopheles*, including members of the maculipennis and quadrimaculatus groups [*4–6,17*].

Identification of mosquito complex species based on PCR has proven to be an appropriate methodology. It is easy to apply in practice, works on all developmental stages of both sexes, and only small amounts of tissue are needed, which facilitates further extensive studies on the same specimens. In this study, since we had only *An. sacharovi* and *An. maculipennis* species, the species-specific primers were tested in the presence of just 2 DNA species. However, none of the other 4 species-specific primers in the PCR tube produced amplicons, therefore, we conclude that PCR assay is a reliable tool for identification of at least 2 sibling species of the *An. maculipennis* complex in the Islamic Republic of Iran.

Among the *An. maculipennis* sibling species, *An. sacharovi* is regarded as the most distinct member of the complex and morphologically distinguishable [*18*]. Based on morphological characteristics, early studies [*10,19–21*] demonstrated the presence of *An. sacharovi* in Tehran, Isfahan, Kohkiluyeh Boyer-Ahmad, and Chahar-Mahal-Bakhtiary provinces, and *An. maculipennis s.l.* in Kermanshahan and Markazi provinces. However, in our study we found no trace of them in these provinces. This could be due to sample size, insecticide application, improved mosquito control measures, or the huge climatic, socioeconomic and agricultural changes which have occurred over the past few decades in the regions of the study.

The presence of 2 important malaria vectors (*An. sacharovi* and *An. maculipennis*) of the *maculipennis* group, and the presence of other important malaria vectors such as *An. superpictus, An. pulcherimus*, and *An. dthali* in the area of study is particularly important for the epidemiology of malaria and vector control programmes in the region. Although malaria has been eradicated from north-west and central regions of the country, recently there have been some reports of an increase in malaria cases in the north-west [*22*]. Combining these facts with major ecological and social changes such as the increased parasite pool resulting from travel to and from the south-east corner of the country and neighbouring 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.

Acknowledgements

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Correction

Correlates of unintended pregnancy in Beheira governorate, Egypt. R.M. Youssef, I.I. Moubarak, Y.A. Gaffer and H.Y. Atta. *Eastern Mediterranean Health Journal*, 2002, Vol. 8 Nos 4/5, pages 521–36.

In the abstract: "…47.1% of all women used no contraception…" should read:

"…47.1% of women who reported unintended pregnancy were not using contraception…"

Table 1 title should read:

"Table 1 Pregnancy intention status in relation to social background of 880 women in Beheira governorate, Egypt"