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Original Article

Molecular Characterization of Mosquitoes (Diptera: Culicidae) in Northwestern Iran by Using rDNA-ITS2

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SUMMARY: Several mosquito species are vectors of disease; however, to understand their role in disease transmission, accurate species identification is of particular importance. Morphological identification is the main method used, but molecular techniques have emerged as a tool for the identification of closely related species. In this study, mosquitoes from the West Azerbaijan Province in northwestern Iran were characterized on the basis of their rDNA-ITS2 sequences. Nine populations of 6 species of mosquitoes belonging to the genera *Anopheles*, *Culex*, *Culiseta*, and *Ochlerotatus* were studied. To the best of our knowledge, ITS2 sequences of *Culiseta longiareolata* and *Culex hortensis* have been reported for the first time. In addition, ITS2 sequences of *Culex theileri* and *Ochlerotatus caspius* have been reported for the first time in Iran. Phylogenetic analysis based on ITS2 showed that subfamilies Anophelinae and Culicinae of the family Culicidae could be differentiated successfully and subgenera *Anopheles* and *Cellia* of the genus *Anopheles* were separated. The analysis showed that the genera *Culex*, *Culiseta*, and *Ochlerotatus* have diverged separately.

INTRODUCTION

Mosquitoes transmit several major diseases and can be considered as the most important insect vectors. Accurate identification of mosquito species could lead to understanding the role of each species in the disease transmission cycle and resulting in effective control programs designed on the basis of the bio-ecology of each species (1).

Morphological identification is the main method used for species identification; however, molecular techniques have emerged as a tool for the identification of closely related species. The molecular marker rDNA-ITS2 is used in molecular taxonomy for separating sibling species (2), in phylogeny for establishing evolutionary relationships among mosquito species (3), and in phylogeography and population genetics for assessing the geographic distribution of mosquitoes (4).

Molecular studies have been performed to characterize Culicidae in Iran and clarify taxonomy in some problematic situations, e.g., different species of the Iranian Oriental-Palearctic-Afrotropical Members of the genus *Anopheles* (5), *An. stephensi* (6), *An. hyrcanus* (7), *An. fluviatilis* (8), *An. superpictus* (9), and *An. maculipennis* (10).

On the basis of the above mentioned studies, a study of species from different taxa of the family Culicidae seems to be necessary. The West Azerbaijan Province in northwestern Iran is an important biogeographic region, being the corridor between Europe, Africa, and Asia. This province has a common border with 4 foreign countries: Armenia, Azerbaijan, Iraq, and Turkey. In this study, we characterized mosquitoes collected from different locations in the West Azerbaijan Province (11) on the basis of their rDNA-ITS2 sequences and investigated the use of this fragment in the phylogeny of 6 studied species and phylogeography of the medically important species *An. maculipennis*.

MATERIALS AND METHODS

Study area, sample collection, and morphological identification: All samples were collected from the West Azerbaijan Province, Iran (Table 1). The mosquitoes were collected monthly between May and November 2012. Larvae were collected using a standard dipping method. For collecting adult mosquitoes, several methods were used, such as hand catches, night landing catches on cows, pit shelter collection, and total catch. In the total catch method, a human or animal dwelling was covered by a white cotton cloth and all entrances and exits of the location were closed before spraying insecticides for less than a minute; and after 10–15 minutes, all windows and doors were opened and the white cotton cloth was carefully inspected for dead or knocked-out mosquitoes. All samples were identified to

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Table 1. The collection localities and ITS2 accession numbers of the investigated specimens

Species	Location	Altitude (m)	Latitude	Longitude	Collection site	Accession no.	No. of Sequenced samples
Anopheles maculipennis	WA-Sar3	994	36° 9′20.63″N	45°32′ 7.05″E	Beri-Soo	KF483834	3
Anopheles maculipennis	WA-Bav2	2,071	37°49′28.46″N	44°44′ 3.31″E	Bavan	KF483842	3
Anopheles maculipennis	WA-jal67	1,409	39°26′45.90″N	44°26′ 4.27″E	Yarim-Ghiye	KF483845	2
Anopheles superpictus	WA-Mah5	1,371	36°45′42.49″N	45°42′23.85″E	Mahabad	KF483835	3
Anopheles superpictus	WA-Kale	1,562	36° 9′40.37″N	45°24′41.30″E	Bewran-Sardasht	KF483844	2
Culex hortensis	WA-Mak2	1,400	39°24′31.87″N	44°26′11.77″E	Bazargan	KF483838	3
Culex theileri	Wa-Urm1	1,572	37°43′50.12″N	44°39′33.78″E	Koor-Abad	KF483841	4
Culiseta longiareolata	WA-Nagh34	1,313	36°57′28.22″N	45°21′51.71″E	Naghadeh	KF483837	3
Ochlerotatus caspius	WA-Baz4	1,411	39°17′21.80″N	44°25′29.81″E	Baghcheh-Joogh	KF483843	3

the species level by using the standard morphological key (12), and accuracy of the morphological identification was confirmed using PCR for 2–3 specimens of each species.

Genomic DNA extraction and ITS2 fragment amplification: Nine populations of 6 species were subjected to molecular analysis. Genomic DNA of the mosquitoes was extracted using the Bioneer AccuPrep® Genomic DNA Extraction Kit (Daejeon, South Korea), according to the manufacturer's instructions. The extracted DNA was diluted in TE buffer and maintained at 4°C. The desired ITS2 fragments were amplified using universal 5.8S (5′ ATC ACT CGG CTC GTG GAT CG 3′) as the forward primer and universal 28S (5′ ATG CTT AAA TTT AGG GGG TAG TC 3′) (13) as the reverse primer. The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 57°C for 50 s, and 72°C for 1 min and 72°C for 10 min. Accuracy and quality of the amplicons were examined using a 1.5% agarose gel and visualized by UV transillumination after staining with CinnaGen® safe stain (Tehran, Iran). High-quality amplicons of the desired size were sequenced.

Phylogenetic analysis: For phylogenetic analysis, ITS2 sequences from the same mosquito species were retrieved from GenBank (www.ncbi.nlm.nih.gov). The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model (14). Initial trees for heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 8.9461]). All positions containing gaps and missing data were eliminated. A total of 193 positions were found in the final dataset. Evolutionary analyses were conducted using MEGA6 (15).

RESULTS

In this study, 9 populations of 6 species of mosquitoes, including subfamilies Anophelinae and Culicinae, were investigated in the West Azerbaijan Province in northwestern Iran. The 9 populations are as follows: 3 populations of *An. maculipennis* (subfamily Anophelinae, genus *Anopheles*, subgenus *Anopheles*) from the north, center, and south of the West Azerbaijan Province, 2 populations of *An. superpictus* (subfamily Anophelinae, genus *Anopheles*, subgenus *Cellia*), *Culex hortensis* (subfamily Culicinae, genus *Culex*, subgenus *Maillotia*), *Culex theileri* (subfamily Culicinae, genus *Culex*, subgenus *Culex*), *Culiseta longiareolata* (subfamily Culicinae, genus *Culiseta*, subgenus *Allotheobaldia*), and *Ochlerotatus caspius* (subfamily Culicinae, genus *Ochlerotatus*, subgenus *Ochlerotatus*). The rDNA ITS2 region was successfully amplified and sequenced for a representative subset of the 6 species (Table 1).

The phylogenetic analysis based on the amplified fragment showed that the ITS2 sequences of the studied species could successfully differentiate between different taxonomic levels, including members of subfamilies *Anophelinae* and *Culicinae* and different genera (*Anopheles*, *Culex*, *Culiseta*, and *Ochlerotatus*) (Fig. 1).

With respect to the different populations of *An. maculipennis* (3 populations from different geographical regions in the study area) and *An. superpictus* (2 populations from wetland and mountainous areas), it seems that using the ITS2 fragment could differ the mentioned populations as well.

Regarding the presence of the *An. maculipennis* complex in this region, acquired sequences of 3 different populations of this species in the present study (KF483845, KF483842, and KF483834) and sequences of the ITS2 fragment of this species from other regions (FJ210887 from northwestern Iran (16), AY730267 from north of Iran [unpublished], DQ118166 from Greece (17), EF612526 from south of Iran [unpublished], HQ877951 from Turkey (18), and AY238423 from France (19)) were analyzed (Fig. 2).

DISCUSSION

To the best of our knowledge, this is the first report of characterization of the ITS2 fragment in the following 4 species of mosquitoes from Iran: *Culex theileri*, *Cx. hortensis*, *Culiseta longiareolata*, and *Ochlerotatus caspius*, where of 2 species are new to science (*Cx. hortensis* and *Cu. longiareolata*). Three of these species are known to transmit diseases such as dirofilariasis (*Cx. theileri*) (20), Usutu virus (*Cu. longiareolata*), and flaviviruses (*Oc. caspius*) (21–22). In addition, all 3 species transmit the West Nile virus (23). Presence of several important vectors in the region emphasizes the impor-

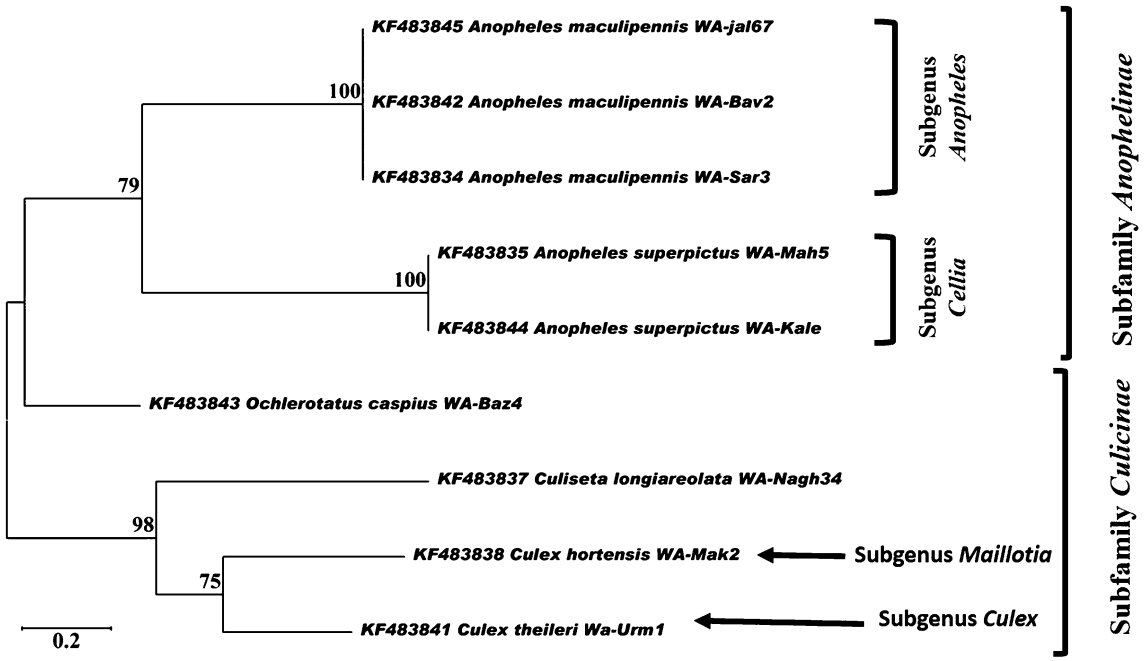

Fig. 1. Molecular phylogenetic analysis by the Maximum Likelihood method using 500 bootstrap replications. The tree with the highest log likelihood (-1506.1887) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

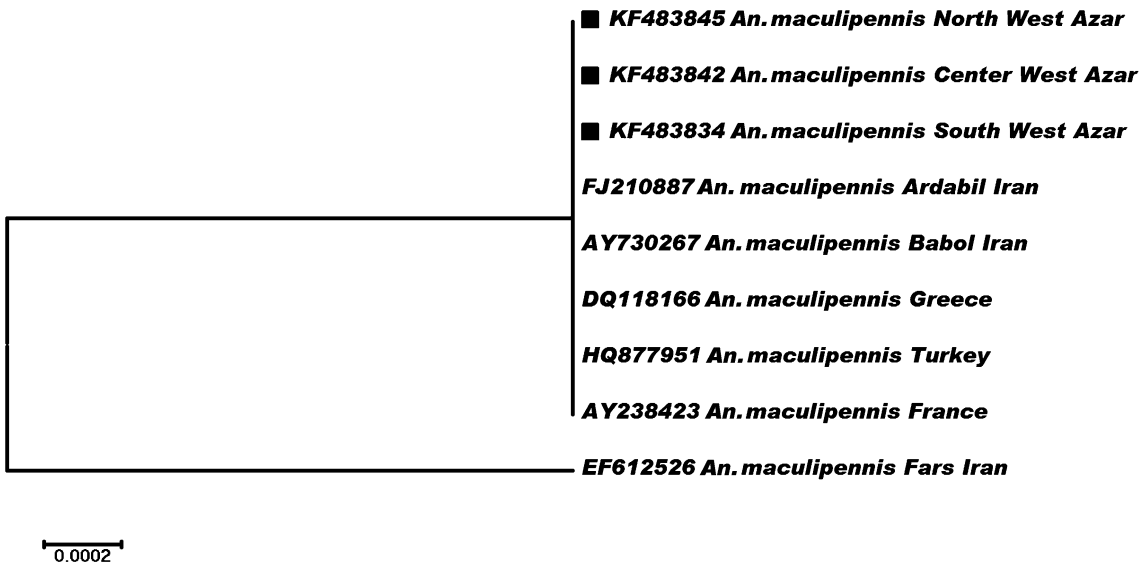

Fig. 2. Phylogenetic relationships based on DNA sequences of ITS2 rDNA fragment for 9 populations of *An. maculipennis*, (3 population from this study, indicated by ■ mark [KF483845, KF483842, and KF483834]), and (FJ210887 from northwest Iran (16), AY730267 from north of Iran [unpublished], DQ118166 from Greece (17), EF612526 from south of Iran[unpublished], HQ877951 from Turkey (18), and AY238423 from France (19)).

tance of accurate characterization at the molecular level, and the current report constitutes an important addition to the repertoire of tools available for accurate species determination.

The phylogeny analysis revealed that the species belonging to the subfamily Anophelinae were placed basal to the species of the subfamily Culicinae. This arrangement is consistent with the results of other studies. Miller et al. (1997) examined the phylogenetic relationships among 4 genera (*Aedes*, *Anopheles*, *Culex*, and *Toxorhynchites*) by using 18S and 5.8S rDNA sequences, which led to the placement of the genus *Anopheles* in a position basal to the other 3 examined genera (24). The same arrangement has been reported by another study based on 18S rDNA (25). Both subgenera of the *Anopheles* species (*An. maculipennis* belonging to subgenus *Anopheles*) and (*An. superpictus* belonging to subgenus *Cellia*) have been clearly differ-

entiated; however, a close relationship between *Anopheles* and *Cellia* could be suggested by considering the bootstrap values. This relationship is supported by the results of other molecular (26) and morphological (27) studies. In some cases, *Anopheles* and *Cellia* have been clustered together by using other molecular markers like *ND5* (28).

For differentiation of *An. maculipennis* populations, ITS2 has proven to be more efficient than the D2 region of 28 rDNA (29–30). However, the acquired sequences of 3 different populations of *An. maculipennis* in this study (KF483845, KF483842, and KF483834) showed no differences when compared with *An. maculipennis* from Greece (DQ118166), Turkey (HQ877951), or France (AY238423).

Further studies with larger sample sizes for re-evaluation of the phylogenetic relationship among the species by using other markers (i.e., mtDNA) are required.

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Conflict of interest None to declare.

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