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Molecular Analysis of ITS2 Fragment among Anopheles maculipennis Species Complex, West Azerbaijan Province, Iran

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Abstract

Background: *Anopheles maculipennis* complex species is considered as one of the most important species complexes with 12 species and significant role in the transmission of important diseases such as malaria, lymphatic filariasis and multiple Arboviral infections. The aim of the present study was to analyze Internal Transcribd Spacer (ITS) 2 fragment among *Anopheles maculipennis* species complex in West Azerbaijan Province, Iran and also to identify different species of *An. maculipennis* using ITS2 fragment.

Methods: Adult and larval specimens of different mosquitoes' species were collected from the northern, southern and central parts of West Azerbaijan Province. Adult mosquitoes were collected using standard methods of indoor and outdoor hand catch, human and animal bait and light traps. Also larvae were caught using dipping method during May- Sep 2016. After DNA extraction, ITS2 fragment was amplified and analyzed using Bioinformatics tools.

Results: Totally, 271 specimens belonged to Genus *Anopheles* [158 samples of *An. maculipennis* (adult: 50, Larvae: 57), 101 *An. claviger* (adult: 21, larvae: 80) and 12 specimen of *An. superpictus* (adult: 3, larvae: 9)] were collected from different parts of the province. Also, the presence of at least two species of *Anopheles maculipennis* species complex (*An .maculipennis* and *An. Persiensis)* was concluded.

Conclusion: Based on the reported differences between these species, accurate identification of these species in terms of their ecology, vectorial capacity and their insecticide resistance profile is recommended. Also, other molecular markers such as COI, should be examined for better resolution of species composition in *An. maculipennis*.

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Introduction

Anopheles maculipennis has been reported as the vector of some important diseases such as malaria (1), filarial nematodes (2), West Nile virus (3) and Sindbis virus (4). In addition to its role in the transmission of different diseases, the wide geographical distribution of *An. maculipennis* from northern Europe (5) through northern Africa (6) and the Middle East (7, 8), necessitates proper study of this species.

The complexity of the classification of this species, has led to considering this species as a species complex comprising of twelve species of which six ones (*An. atroparvus, An. labranchiae, An. maculipennis, An. messeae, An. persiensis*and *An. sacharovi)* have been reported from Iran (9). A new species (*An. persiensis*) has been reported for the first time from Iran (10). Although the identification of different species of this species complex based on morphological characteristics is almost impossible, because of the notable biological and behavioral differences between different species of this species complex, judgments about different aspects of the sibling species of *An. maculipennis* is reasonably difficult.

Despite the disappearing of malaria from some parts of Iran, due to the favorable weather conditions, the possibility of transmission still remains as a threat in different parts of country, especially during /after natural or political disasters like the re-emerging of malaria in some areas of West Azerbaijan Province after the collapse of the Soviet Union and border clashes between Armenia and the republic of Azerbaijan (11).

More than half of malaria vectors belong to species complexes and these species complexes are morphologically difficult or impossible to be recognized, but at the same time they are notably different in terms of genetics, biological and ecological aspects, including vectorial capacity, resistance to insecticides, host preference and geographical distribution (12).

During the time, several methods such as polytene chromosomes, cuticular hydrocarbons, hybridization and morphological characteristics of adult, larvae and eggs, have been used for identification of members of species complex such as *An. maculipennis.* Due to some limitations in the mentioned methods, new molecular methods such as PCR technique are being used widely in order to determine the species and population genetics of species complex (13-19). Among the used molecular markers, more attention has been paid to the Internal Transcribed Spacer (ITS) fragments. Because, in addition to variation in the order and arrangement of nucleotides, even the number of nucleotide sequences (length) are varied. Both ITS1 and ITS2 vary between and within species and therefore can be used in the identification of species complex (20-23).

DNA-based molecular methods using various markers have been employed for separation of different species of *An. maculipennis* species complex in many countries, including Italy, Romania, Great Britain, Greece and Russia. Given the wide distribution of this species in different parts of Iran (24-28) and on the other hand considering the transmission cycle of diseases transmitted by these species in the region, such as West Nile fever (29), the necessity of proving the genetic diversity of this species in Iran and the studied region (8) and the history of resistance to some insecticides (30) and identifying different species of *An. maculipennis* are felt more than ever.

Due to the efficiency of molecular markers to identify the close species of Anopheles (31, 32), this study aimed to analyze ITS2 fragment in *An. maculipennis* in West Azerbaijan Province and to identify its different species.

Material and Methods

Study area and sample collection

Adult and larval specimens of different mosquitoes' species were collected from the north, south and center of West Azerbaijan Province and geographical details have been presented in table 1. Adult mosquitoes were collected using standard methods of indoor and outdoor hand catch, human and animal bait and light traps. Also larvae were caught using dipping method during May- Sep 2016.

Caught specimens were transferred to the laboratory for species identification using morphological characters as described by widely used morphological keys (33). Isolated *An. maculipennis* samples were kept separately in freezer until the time of DNA extraction.

Table 1. Geographical details of sampling locations

District	Village/ Sample No.	Altitude	Longitude	Latitude
Urmia	Korabad /1	1354	44°60'5.51"E	35°29'49.0"N
	Korabad /2	1341	43°39'0.49"E	37°40'24.3"4N
	Korabad /3	1365	44°54'51.5"E	37°39'69.0"N
Bazargan	Yarimghieh /3	1389	45°46'4.17"E	36°16'45.65"N
	Yarimghieh /4	1405	45°31'11.72"E	35°17'31.84"N
Mahabad	KHorkhoreh /1	1371	45°42'13.85"E	36°45'22.49"N
	Mahabad /2	1304	45°44'12.8"E	36°48'24.18"N
	Mahabad /3	1438	45°40'32.42"E	36°23'45.33"N
	Mahabad /4	1315	45°42'23.45"E	36°35'42.41"N
Makoo	Sangar	1348	44°25'53.99"E	39°18'59.72"N
Miyandoab	Miyandoab /3	1538	45°40'32.42"E	36°23'45.32"N
	Heydarabad /1	1551	45°32'7.05"E	36°92'3.63"N
	Heydarabad /2	1562	45°24'40.30"E	37°40'9.37"N
	Abasabad /1	1138	45°43'13.39"E	37°19'23.59"N
	GHibjagh /2	1121	46°20'43.49"E	37°59'12.63"N

DNA extraction and amplification of ITS2 fragment

Genomic DNA was extracted using the previously described protocol (20) and also Takapouzist commercial kits (based on the manufacturer's protocol). Obtained DNAs were dissolved in sterile distilled water and kept in 5ºC for amplification of desired part (ITS2 and 5.8S rDNA fragment using universal primers).

The desired fragments were amplified using as forward primer universal 5.8S (5' ATC ACT CGG CTC GTG GAT CG 3') and as reverse primer universal 28S (5' ATG CTT AAA TTT AGG GGG TAG TC 3') (10). The PCR conditions

were 94°C for 5 min. followed by 30 cycles of 94°C for 45 s, 57°C for 50 s, 72°C for 1 min. and 72°C for 10 min. High quality amplicons of the desired size were sequenced.

Bioinformatics analysis

To ensure the accuracy of acquired sequences, they were analyzed. The similarity of the resultant sequences with other sequences in the Gene Bank (www.ncbi.nlm.nih.gov), were compared using BLAST software. Phylogenetic analysis were conducted using MEGA 6 software (34).

Results

Three species of Genus *Anopheles* were caught in current study; about 158 samples of *An. maculipennis* (adult: 50, Larvae: 57), 101 *An. claviger* (adult: 21, larvae: 80) and 12 specimen of *An. superpictus* (adult: 3, larvae: 9) were collected from different parts of the province. According to the aims of the current study, sixteen samples of *An. maculipennis*, from different parts, were subjected to amplification of ITS2 fragment and reactions were conducted successfully (Figure 1). Amplicons' length ranged from 224 bp to 410 bp.

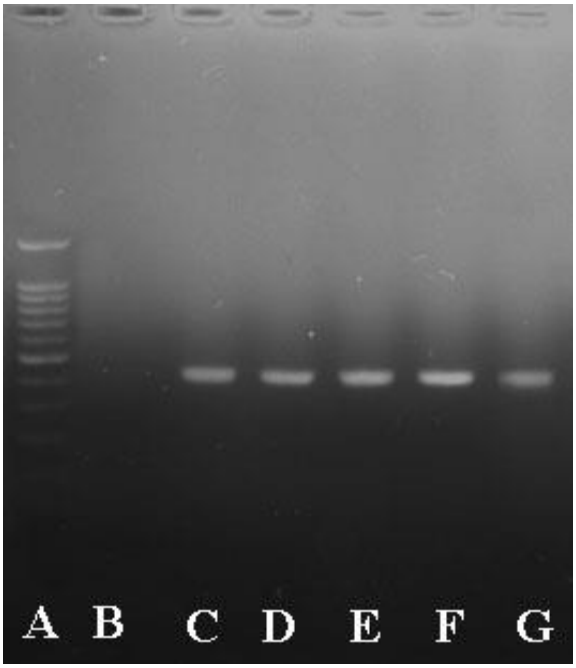

Figure 1. Amplification of ITS2 fragment among Anopheles maculipennis species complex from different parts of West Azerbaijan Province, Iran: A: molecular weight marker (100bp), B: negative control, C: Urmia, D: Bazargan, E: Makoo, F: Mahabad and G:Miyandoab

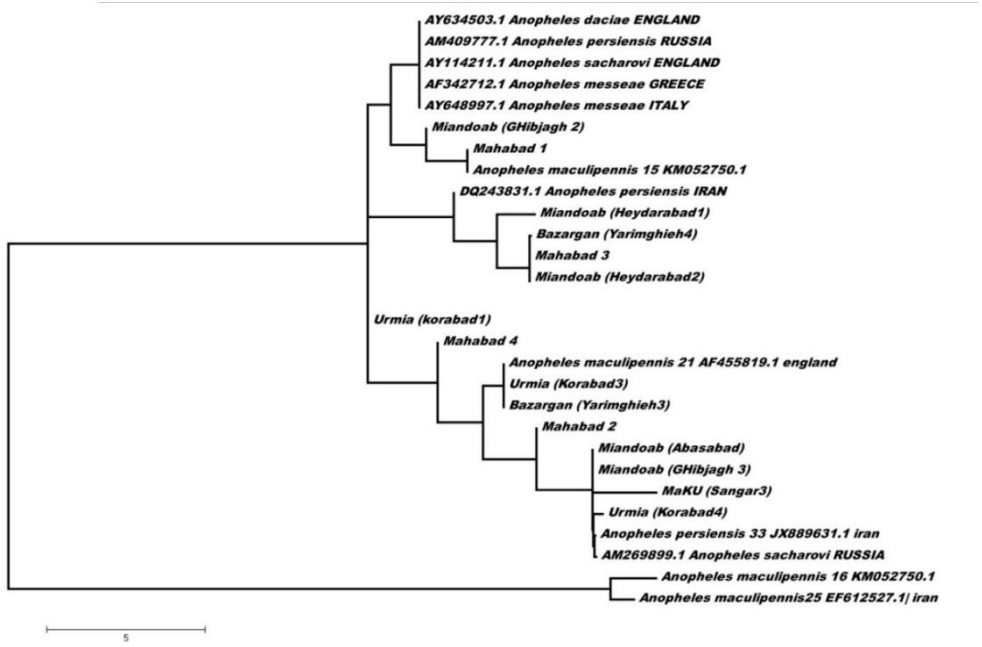

Figure 2. The resulting phylogenetic tree drawn of DNA ITS2 fragment in populations of An. maculipennis collected from different areas of West Azerbaijan Province

By comparing acquired sequences with the sequences of ITS2 registered in Gene Bank, high similarity (98%) was found with two species of *An. maculipennis* species complex including *An. maculipennis* (AF455819 England) and *An. persiensis* (JX88963.1 Iran).

A phylogenetic analysis based on current sequences indicates the high genetic diversity in studied samples across the West Azerbaijan Province. Due to the nature of ITS2, showing the genetic diversity, these results suggest the possibility of the presence of several species of *An. maculipennis* species complex in the study area.

The resultant phylogenetic tree, using acquired sequences and similar GeneBank sequences, suggests an accumulation of varied sequences in several branches and sub-branches. In addition to showing high genetic diversity, species complex suggest a separate allocation of *An. maculipennis* in the form of the branches (Figure 2).

Considering the proximity of the phylogenetic tree drawn in, it seems that at least two species of this complex (*An. maculipennis* and *An. persiensis*) are present in the studied areas. The presence of *An. Sacharovi* (another member of *An. maculipennis* species complex) could not be finalized using these results and further studies are needed.

Discussion

In the current study, the ITS2 fragment was amplified and analyzed in one of the main malaria (and other vector-borne diseases) vectors (*An. maculipennis* species complex) across the West Azerbaijan Province. Also, ITS2-based phylogenetic analysis, revealed the presence of at least two species of *An. maculipennis* species complex *(An. maculipennis and An.*

persiensis) in West Azerbaijan Province. Based on the findings of this study, it can be concluded that ITS2 fragment could be successfully used for differing several taxa levels and even geographical populations within the species of *An. maculipennis* species complex, which showed acceptable resolutions.

An important feature of ITS2 fragment is its proven role in identification of new species, in the case of first identification/description and reporting of *An. persiensis* by taking advantage of ITS2 fragment (10), while other markers such as Cytochrome Oxidase I (COI) in previous studies (8), could not identify this species in the study region. However, lack of identification of *An. persiensis* in the study with other markers can also be caused by failure to catching this species during the sampling process.

Two species have been identified based on molecular markers ITS2 in the present study, but in other study conducted by Djadid et al(2007) in five provinces (except for the West Azerbaijan Province), four other species (*An. sacharovi, An. labranchiae, An. atroparvus* and *An. messeae*) have been identified in addition to these two species (*An. maculipennis* and *An. persiensis*) (9). These results could be attributed to the wider study area in the mentioned study (five provinces: East Azarbaijan, Ardebil, Gilan, Khorasan and Mazandaran Provinces).

Research on other species with molecular methods showed higher confidence in the case of separation complex species and populations within species. This study as a comprehensive study on the species of *An. maculipennis* in West Azerbaijan Province could be followed by complementary studies using other molecular markers such as COI and bigger sample size which would be helpful in order to clarify the presence of different species of this species complex in the region. Sampling of all areas of the province and increasing the sample size in future studies can increase the chances of catching and identifying more species.

Conclusion

In order to determine the precise role of the species in the disease transmission chain and to develop control programs based on biological characteristics, accurate identification of important vectors of the disease is of particular importance. Accordingly, in the present study, ITS2 molecular markers in *An. Maculipennis* species complex in West Azerbaijan Province were investigated and the presence of two species (*An. Persiensis*, *An. maculipennis*) was concluded.

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