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 Transcribed 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.

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.

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.

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.
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.

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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

<table>
<thead>
<tr>
<th>District</th>
<th>Village/Sample No.</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Latitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urmia</td>
<td>Korabad/1</td>
<td>35°29'49.0&quot;N</td>
<td>44°60'5.51&quot;E</td>
<td>35°29'49.0&quot;N</td>
</tr>
<tr>
<td></td>
<td>Korabad/2</td>
<td>43°39'0.49&quot;E</td>
<td>37°40'24.3&quot;N</td>
<td>37°40'24.3&quot;N</td>
</tr>
<tr>
<td>Bazargan</td>
<td>Varamin/1</td>
<td>35°46.17&quot;E</td>
<td>36°16'45.65&quot;N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Varamin/2</td>
<td>45°11.72&quot;E</td>
<td>35°17'31.84&quot;N</td>
<td></td>
</tr>
<tr>
<td>Mahabad</td>
<td>Yarimghieh/1</td>
<td>36°23'45.32&quot;E</td>
<td>45°42'23.45&quot;E</td>
<td>36°23'45.32&quot;E</td>
</tr>
<tr>
<td></td>
<td>Yarimghieh/2</td>
<td>45°44'12.8&quot;E</td>
<td>36°48'24.18&quot;N</td>
<td></td>
</tr>
<tr>
<td>Makoo</td>
<td>Sangar</td>
<td>44°25'53.99&quot;E</td>
<td>39°18'59.72&quot;N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Miyandoab/1</td>
<td>45°40'32.42&quot;E</td>
<td>36°23'45.32&quot;N</td>
<td></td>
</tr>
<tr>
<td>Miyandoab</td>
<td>Heyranabad/1</td>
<td>45°24'40.30&quot;E</td>
<td>37°40'39.37&quot;N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abbasabad/1</td>
<td>45°43'13.39&quot;E</td>
<td>37°19'23.59&quot;N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GHibagh/2</td>
<td>46°20'43.49&quot;E</td>
<td>37°59'12.63&quot;E</td>
<td></td>
</tr>
</tbody>
</table>

#### DNA extraction and amplification of ITS2 fragment

Genomic DNA was extracted using the previously described protocol (20) and also Takapouziist commercial kits (based on the manufacturer’s protocol). Obtained DNAs were dissolved in sterile distilled water and kept in 5°C 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
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.
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 Azerbaijan, 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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