The molecular and morphological variations of *Culex pipiens* complex (Diptera: Culicidae) in Iran

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**ABSTRACT**

**Background & objectives:** Taxonomic status of *Culex pipiens* is well-known as many years with such a wide variety of morphological and biological characteristics. These changes have been the subject of extensive investigation by many researchers. There are a little information about the morphology and molecular data of *Cx. pipiens* complex in Iran. The taxonomic status of the complex is very important because of medical and veterinary importance and wide distribution in the country.

**Methods:** This study was carried out in 11 areas in Iran using dipping technique from April 2009 to October 2010. Molecular study was carried out using primers F1457 as forward and B1256 as reverse, which amplified Ace.2 gene and performed PCR-RFLP using *SacI* restriction enzyme.

**Results:** *Culex quinquefasciatus* found in south to central areas of Iran and reported as sympatric with *Cx. pipiens* in the central regions. *Culex pipiens* distributed in many areas of the country. Sequencing alignment of Ace.2 gene of *C. quinquefasciatus* and *Cx. pipiens* showed 6.5% variation in 46bp, especially in intron locus of gene. *Culex pipiens* complex from Iran are located in two separate clades with sister branches using phylogenetic sequencing tree.

**Interpretation & conclusion:** The male genitalia found as the most reliable diagnostic characters for identification of *Cx. pipiens* complex in Iran that confirmed by amplify the Ace.2 gene in the samples but we recommended the use of sequencing PCR products of microsatellite loci and *COI* gene in future study.

**Key words** Ace.2 gene; *Culex pipiens*; *Culex quinquefasciatus*; Iran; morphology; PCR- RFLP

**INTRODUCTION**

Taxonomic status of *Culex pipiens* Linnaeus complex is still controversial despite its medical and veterinary importance. Some researchers believed that *Cx. quinquefasciatus* Say (Giles 1906, as *Cx. fatigans*) species was placed in the subspecies of *Cx. pipiens*¹. Knight and Malek² listed the *Cx. pipiens* and *Cx. quinquefasciatus* as distinct species based on the studies of Sirivanakarn and White³ in Southeast Asia, Miles⁴ in Australia and Jupp⁵ in South Africa. Recent study indicated the occurrence of *Cx. quinquefasciatus* and the subspecies of *Cx. pipiens pallens* and *Cx. pipiens molestus*⁶-⁸.

*Culex pipiens* complex considered as the vector of arboviral pathogens such as West Nile, St Louis, Sindbis, and Equine encephalitis and other parasites such as *Wuchereria bancrofti*, *Dirofilaria immitis*, *D. repens* and *Plasmodium relictum*, *P. gallinaceum* causing bird malaria¹-⁹. By now, West Nile and Sindbis viruses have been reported in Iran¹⁰. Enzootic cycles of West Nile fever are involving host wild birds and *Cx. pipiens* complex¹¹.

*Culex pipiens*, *Cx. p. pallens* and *Cx. pipiens* form molestus and *Cx. quinquefasciatus* are important members of *Cx. pipiens* complex in the world. *Culex pipiens* distributed in most temperate and subtropical regions, while *Cx. quinquefasciatus* has spread in tropical climates in the world⁹,¹². Distribution of *Cx. pipiens* expressed in many parts of Iran whereas *Cx. quinquefasciatus* reported from the south of the country¹³-¹⁸ and *Culex pipiens* form molestus has been reported in Tehran Province located adjacent of the north of the country¹⁹.

Distribution patterns of *Cx. pipiens* and *Cx. quinquefasciatus* in Iran are very similar to their climatic distribution in North, South America and Africa. *Culex pipiens* restricted to temperate and subtropical regions in more northern areas of America whereas *Cx. quinquefasciatus* found in southern areas with tropical climate²,⁹,²⁰. The recent two species have overlapped and created hybrid forms in the central region of the North America and have not been studied in relation to hybrid species in Iran.

Morphological characters compared with other physiological and behavioral characters are important in taxo-
nomic studies. Although morphological characters such as the larval abdominal seta 1 of segments III–IV, siphon/ saddle index, shape of siphon, the number of the branches of seta 1a–S and 1b–S, DV/D ratio, the ratio of length cell R2/R2+3, the intersection of subcosta and costa with bifurcation of R2+3 in adults are important for diagnosis of Cx. pipientis complex species, but the recent characters cannot completely separate them. By now, the male genitalia considered as the most important morphological diagnostic character\(^6,9,21-23\). The variations of morphological and biological characters find in the local population, therefore, it necessary obtain more accurate data in relation to taxonomic terms of Cx. pipientis complex\(^2\). The final decision on taxonomic status of the species complex needs more complete information which are obtained from the study of different populations\(^1\).

Rapid and accurate identification of Cx. pipientis complex is important in the world. Morphological diagnostic methods are difficult, long-time and limited to males. The biochemical and molecular techniques introduced for identification of Cx. pipientis complex in 1995. Crabtree et al\(^{24}\) express the ITS gene for identification of Cx. pipientis complex, Cx. restuans and Cx. salinarius using PCR standard methods, but failed to identify the species complex. The other molecular techniques including; PCR and PCR-RFLP on microsatellite loci and, Ace.2, COI, ITS genes were studied for the separation of these complex species. Ace gene and microsatellite loci noted as the most important characters\(^9,25-28\). Malcolm et al\(^{25}\) mentioned to variation in the Ace gene. In an other study, Bourguet et al\(^{29}\) observed more variation, in the nucleotides of Ace.2 gene in Cx. pipientis and Cx. quinquefasciatus. Consequently, the Ace.2 cited as autosomal gene and its function is still unknown\(^9,25,29\).

Bourguet et al\(^{29}\) observed a little polymorphism in the same subspecies strains whereas found more difference as 37 of 710 sequences between Ace.2 gene in the Cx. pipientis and Cx. quinquefasciatus. Therefore, this gene can be quite useful as a tool for the separation of two species. Endonuclease enzymes identified and then cut the DNA strands in at specific locations using PCR-RFLP method. Bourguet et al\(^{29}\) could separate Cx. pipientis from Cx. quinquefasciatus using Ace.2 gene and Scal, as restriction enzyme. Site of nucleotides enzyme of Scal found in intron 2 on Ace.2 gene. Two sites of Scal enzyme recognized for Cx. quinquefasciatus whereas, one site found in Cx. pipientis species. Bourguet et al confirmed the accuracy of this method among the species collected in the world\(^{29}\).

There are scatter studies about the taxonomic status of Cx. pipientis complex in the country. In addition, the behavior and physiological differences of species could influence the epidemiology of the vector-borne diseases, therefore, it is necessary to obtain the information of the samples which are collected from the field.

MATERIAL & METHODS

Study area

World is divided to 5 strata-based on vegetation distribution and Köppen Climate Classification including cold and dry, cool and moist, hot and dry, warm and moist (subtropical), and warm and moist (tropical). Iran divided into 5 strata including: tropical warm and humid, subtropical warm and humid, hot and dry desert, cool and moist mediterranean, and cold and dry\(^{30}\). In this study, Chabahar (25°17’ N, 60°37’ E) and Nikshahr Cities (26° 04’ N, 60°37’ E) from Sistan and Baluchistan Province selected as tropical warm and humid, Jiroft City (28°5’ N, 57°8’ E) from Kerman Province, Borazjan City (29°15’ N, 51°12’ E) from Bushehr Province, Ahvaz City (31°19’ N 48°41’ E) From Khuzistan Province as subtropical warm and humid, Yazd City (54°04’ N, 31°59’ E) from Yazd Province and Kerman City (30°17’ N, 57°04’ E) from Kerman Province considered as hot and dry desert, Neka City (36°42’ N, 53°33’ E) from Mazandaran Province selected as cool and moist mediterranean, Mashhad City (36°18’ N, 59°36’ E) from Khorasan-e-Razavi Province and Hamadan City (34°48’ N, 53°33’ E) from Hamadan Province, Teheran City (35°45’ N 51°35’ E) from Teheran Province represented as cold and dry climate (Fig. 1).

![Map showing Culex pipientis and Cx. quinquefasciatus distribution in different study areas in Iran during 2009–10.](image)
Mosquito sampling and morphological studies

The study was conducted in 12 randomly selected areas using dipping technique in Iran from April 2009 to October 2010. *Culex pipiens* collected from different areas were transferred to the Entomology Laboratory, Department of Entomology and Parasitology, School of Medical Sciences, Tarbiat Modares University. The IV instar larvae were separated, and some parts of the adult body such as wings, were mounted using Canada balsam diluted with Xylene. Three caudal abdominal segments of male were removed, then was placed in KOH 10% at 20 to 30 min and washed with distilled water and placed in ethanol 96% for dehydration. The samples were mounted using slide, cover slide and Puri medium and identified using systematic keys.

Larval collection was conducted from different regions of the country using dipping method. The mosquito larvae collected from larval habitats were transferred to specific cage for rearing in Insectarium condition (22–25°C, 70–75% RH). The taxonomic figures were drawn using Zeiss microscope with a Nikon drawing tube accessory long arm (191/2 inches).

Molecular studies

In all, 137 samples comprised of 54 larvae, 46 males, and 37 females used to DNA extraction and amplification of *Ace.2* gene. DNA extracts from individual mosquitoes using a standard phenol-chloroform protocol. PCR reactions contained 1 ml template DNA, each forward and reverse primers at 0.20 mM were performed using BioNeer kit (AccuPower® PCR Premix Cat No: K-2012), this kit as lyophilized 0.2 cc tube has been prepared and its 50 μl volume are including 10 mM Tris-HCL (PH 9.0), 30 mM KCL, 1.5 mM MgCl2, 250 μM of each deoxynucleoside triphosphate (dNTPs) and 2.5 units of Taq DNA polymerase. Amplified products were visualized by 1.5% Agaros gel (Agarose, MP Sigma) electrophoresis in TBE buffer as mentioned earlier. PCR products were purified using AccuPrep® gel purification kit according to the manufacturer’s instructions. A portion of each purified PCR sample was subjected to DNA sequencing using a 373 ABI automated sequencer. Resultant sequences were aligned using CLUSTAL_X software by http://ebi.ac.uk/clustaw/.

The sequences comparison with the GenBank entries using Blast and the software for phylogenetic analysis online embedded in PubMed (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was performed using neighbor-joining method on combination of the data obtained from this study. Obtained sequences were submitted in GenBank under submission No. JF501651–JF501654 and JF430595.

RESULTS

Our findings indicated the presence of two species, *Cx. pipiens* and *Cx. quinquefasciatus*. The distribution of *Cx. quinquefasciatus* was limited to scattered areas of the southern Iran including: Ahvaz, Borazjan, Chabahar, Nikshahr, Jiroft, and Kerman cities extends to central Iran (Yazd City), where occurrence sympatric with *Cx. pipiens*. The distribution of *Cx. pipiens* was found in central and northern provinces of the country including: Yazd, Teheran, Hamadan, Neka and Mashhad cities (Tables 1 and 2).

Morphological study on 54 larvae samples showed the occurrence of *Cx. pipiens* in north and neighbors it.
Table 1. The variations of morphological characteristics of *Culex pipiens* complex larvae comparison with PCR-RFLP method, Iran 2009–10

<table>
<thead>
<tr>
<th>Area</th>
<th>Meteorological condition</th>
<th>No.</th>
<th>Mosquito species</th>
<th>Morphological characteristics in larvae</th>
<th>PCR-RFLP result</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Seta 1 on seg III–IV</td>
<td>Siphon/ saddle index</td>
</tr>
<tr>
<td>Mashhad (Northeast)</td>
<td>Cold and dry</td>
<td>3</td>
<td><em>Cx. pipiens</em></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Neka (North)</td>
<td>Cool and moist mediterranean</td>
<td>6</td>
<td><em>Cx. pipiens</em></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Teheran (neighbor North)</td>
<td>Cold and dry</td>
<td>5</td>
<td><em>Cx. pipiens</em></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
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<td>0</td>
</tr>
<tr>
<td>Yazd (Center)</td>
<td>Hot and dry desert</td>
<td>11</td>
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<td>11</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
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<td>7</td>
</tr>
<tr>
<td>Kerman (Near Center)</td>
<td>Hot and dry desert</td>
<td>6</td>
<td><em>Cx. pipiens</em></td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
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<td>2</td>
</tr>
<tr>
<td>Jiroft (neighbor South)</td>
<td>Subtropical warm and humid</td>
<td>6</td>
<td><em>Cx. pipiens</em></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Borazjan (neighbor South)</td>
<td>Subtropical warm and humid</td>
<td>7</td>
<td><em>Cx. pipiens</em></td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Nikshahr (South)</td>
<td>Tropical warm and humid</td>
<td>5</td>
<td><em>Cx. pipiens</em></td>
<td>1</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td><em>Cx. quin.</em></td>
<td>4</td>
<td>4</td>
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<tr>
<td>Chabahar (South)</td>
<td>Tropical warm and humid</td>
<td>5</td>
<td><em>Cx. pipiens</em></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>1</td>
<td>1</td>
</tr>
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</table>

Table 2. Variations of morphological characteristics in *Culex pipiens* complex adult comparison with PCR-RFLP method, Iran (2009–10)

<table>
<thead>
<tr>
<th>Area</th>
<th>Meteorological condition</th>
<th>No.</th>
<th>Mosquito species</th>
<th>Morphological characteristics in female</th>
<th>PCR-RFLP result</th>
<th>No.</th>
<th>Morphological characteristics in male</th>
<th>PCR-RFLP result</th>
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<td></td>
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<td></td>
<td></td>
<td>Costa &amp; subcosta intersect/ bifurcation of R2+3</td>
<td>RCell/ R2+3</td>
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<td>DV/D</td>
<td></td>
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<tr>
<td>Neka (North)</td>
<td>Cool and moist mediterranean</td>
<td>4</td>
<td><em>Cx. pipiens</em></td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hamadan (Northwest)</td>
<td>Cold and dry</td>
<td>4</td>
<td><em>Cx. pipiens</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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</tr>
<tr>
<td>Yazd (Center)</td>
<td>Hot and dry desert</td>
<td>9</td>
<td><em>Cx. pipiens</em></td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Teheran (neighbor North)</td>
<td>Cold and dry</td>
<td>–</td>
<td><em>Cx. pipiens</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4</td>
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</tr>
<tr>
<td>Kerman (Near Center)</td>
<td>Hot and dry desert</td>
<td>2</td>
<td><em>Cx. pipiens</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>–</td>
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</tr>
<tr>
<td>Jiroft (neighbor South)</td>
<td>Subtropical warm and humid</td>
<td>3</td>
<td><em>Cx. pipiens</em></td>
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<td>1</td>
<td>0</td>
<td>6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
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<td>2</td>
<td>3</td>
<td>6</td>
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<tr>
<td>Ahvaz (Southwest)</td>
<td>Subtropical warm and humid</td>
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<td><em>Cx. pipiens</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Cx. quin.</em></td>
<td>3</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Nikshahr (South)</td>
<td>Tropical warm and humid</td>
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<td><em>Cx. pipiens</em></td>
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<td>1</td>
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<td><em>Cx. quin.</em></td>
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<td>3</td>
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<tr>
<td>Chabahar (South)</td>
<td>Tropical warm and humid</td>
<td>6</td>
<td><em>Cx. quin.</em></td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
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</tr>
</tbody>
</table>
Results of morphological study of the samples were confirmed by PCR-RFLP method except the samples of Borazjan, Nikshahr and Jiroft cities.

Although seta 1 on abdominal segment III–IV reported as important diagnostic character between the recent species but may be unreliable among the samples of southern Iran (Table 1 and Fig. 2). The siphon/saddle index in larval stage calculated as the range of 2.29–3.3 for Cx. quinquefasciatus and 3.33–3.95 for the Cx. pipiens. This character in samples of north and neighbor it, indicated the presence of Cx. pipiens whereas found varied among the sample collected from the southern regions of the country.

Seta 1a–S and 1b–S, found as a range 2–9 branches, the range of 2–6 considered for Cx. pipiens and 6–9 calculated for Cx. quinquefasciatus. These larval characters were found more reliable in north than south areas. The shape of siphon in north indicated the presence of Cx. pipiens and confirmed by PCR–RFLP while, Teheran samples were not compatible completely. Although PCR–RFLP confirmed the presence of Cx. quinquefasciatus in southern Iran, but the recent morphological character was not reliable among the samples (Table 1 and Fig. 2).

In adults, DV/D ratio of male genitalia was found ranging between –0.2 and 2.37 for Cx. pipiens that was compatible completely by PCR–RFLP. RCell/R2+3 in our study were found in the range 1.65–6.99. This character found as 3.35–6.99 for Cx. pipiens and range of 1.65–3.3 for Cx. quinquefasciatus. Although the use of RCell/R2+3 was reliable for the samples collected from different parts of Iran; but was not compatible completely with PCR–RFLP method. Costa and subcosta intersections with bifurcation of R2+3 of the samples in some areas were not compatible completely with PCR–RFLP results (Table 2 and Figs. 3–4).

Molecular studies in most of the samples, especially in temperate area were compatible by morphological study. More morphological variations were observed in samples collected from central and southern Iran. Molecular study confirmed the occurrence of the species of Cx. quinquefasciatus in the central and southern of the country. In fact, the fragments of PCR–RFLP products
with 350, 230 and 120 bp, found associated with *Cx. quinquefasciatus*. Also, the fragments with 470 and 230 bp observed with *Cx. pipiens* in the whole samples of Teheran, Hamadan, Neka, Mashhad and some samples of Ahvaz and Yazd cities (Fig. 5). The sequence of nucleotides gene of *Cx. pipiens* in our study was similar to *Cx. pipiens* in California as Accession No. FJ948081. Ace.2 gene sequences of *Cx. quinquefasciatus* in our study was completely similar to sequence of these genes in the GenBank as Accession No. J948080. Alignment of our sequencing of two species *Cx. quinquefasciatus* and *Cx. pipiens* collected from Yazd area showed the variety about 6.5% in 46 bp especially in intron locus of gene (Fig. 6).

**Phylogenetic analysis**

The results of phylogenetic analysis of species *Cx. pipiens* and *Cx. quinquefasciatus* showed that the *Cx. pipiens* complexes from Iran are located in two separated clades with sister branches. Four specimens of *Cx. pipiens* from Iran as well as seven specimens from United States were located together in one lineage. One sample of *Cx. quinquefasciatus* from Iran as well as nine samples from Mexico, United States and Bangladesh were located together in one lineage. In this phylogenetic tree *Cx. restuans* was considered as an out group (Fig. 7).

**DISCUSSION**

**Morphometric studies**

Taxonomic status of *Cx. pipiens* complex has been considered as one of the important issues in taxonomic research. Harbach expresses the taxonomic status of *Cx. pipiens* complex as ambiguous and it is concerned as a part of morphological, physiological, and behavioral genetics. The main taxonomic characters for identification of *Culex* larvae, considered as siphon shape and its seta and siphon/saddle index. Length of siphon is related not only with the larval habitat contamination but also with geographic distribution. The siphon/saddle index of *Cx. pipiens* was cited in average 4.08 and the range of 3.48–4.63. This index for *Cx. quinquefasciatus* was reported with a range of 2.77–3.41 and average 3.11. In our investigation, the range of 2.29–3.3 for *Cx. pipiens* was cited in average 4.08 and the range of 3.48–4.63. This index for *Cx. quinquefasciatus* was reported with a range of 2.77–3.41 and average 3.11. In our investigation, the range of 2.29–3.3 for *Cx. quinquefasciatus* and 3.33–3.95 was allocated for *Cx. pipiens*. Azari-Hamidian and Harbach express this index >3.45 for *Cx. pipiens* and <3.45 for *Cx. quinquefasciatus*. In our study there were overlap of the values of siphon/saddle index of *Cx. quinquefasciatus* and *Cx. pipiens*. Considering, our morphological findings confirmed by PCR-RLFP method (Table 1), it seems that average of this index was influenced by larval habitats, climatic conditions, latitude and longitude.

Our results show that the abdominal seta 1 on segment III–IV is reliable for identification of *Cx. pipiens* complex. Although this character confirms the presence of *Cx. pipiens* in north and neighbor it of Iran, but not reliable for identification of *Cx. quinquefasciatus* in south and central areas of Iran (Table 1 and Fig. 2). In parallel, Harbach noted this character was unreliable in the center and northeast regions of the Arabian peninsula where hybrid populations of the species exist.

Our findings indicated that seta of 1a–S and 1b–S, were in the range of 2–9, the number of branches as 2–6
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Fig. 6: Alignments of Ace.2 gene sequences for *Cx. quinquefasciatus* and *Cx. pipiens* collected from Iran, compared with *Cx. quinquefasciatus* from Mexico in GenBank (FJ948080) and *Cx. pipiens* from California (FJ948081). "." Indicates similarity; "*" Indicates the absence of mutation; The highlighted sequences are two exons.

<table>
<thead>
<tr>
<th></th>
<th>Mexico, FJ948080 Cx.quin</th>
<th>Iran, JF501652 Cx.quin</th>
<th>California, FJ948081 Cx.pip</th>
<th>Iran, JF501654 Cx. pip</th>
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<td>GAGGAGATGTGAAATGCGAAGAACTGAGCAGTGAAGGACTGCTGTAACGAGCTTTGAG 60</td>
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<td></td>
<td>GTACACACGAAAGGACTTCTTGTGACGCGAAATTTGCGAAGACGAGCTTGGGA 120</td>
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|                  | AGTACTACTTCTTGTGAGTACAGAAGCAAGCGGAAATTTGCTAGTGACTTGG 179 | T                      | T.A                           | G.G.C                 | 180
|                  | TGGTGGAAGCTGATGACTAATAATTGAGAGACTGACACTGATATTAA 299 | G.A.C                 | A                            | A.G.C                 | 300
|                  | TTTTTAGTATAGTGAGCGGTATTTGCTATGGAAGCAACATTTATCAGATCTG 419 | T.T.T.T               | C                             | A                      | 420
|                  | TGCCTGTCTTCTCTGAGATGCTGCTTGAGCAGATGCTGCTCGATATCAGATCTG 477 | T.T.T.T               | C                             | C                      | 480
|                  | TCCAGGCAAGCGAGGACTTGATCCAGGGCGGACCGTACGTCAGACGCGCCTGCGATCG 537 | T                    | T                             | T                      | 540
|                  | GTGGATGCTTGGGATGCTGCTTGATGACGACGACGATGCTGCTCGATACGAGATC 597 | T                    | T                             | T                      | 600
|                  | GAAATCGGCGGATGCAAGGATAGCAGAAGATAGTGGCGAGGATAGTGGCGAGGATAGC 657 | T                    | T                             | T                      | 660
|                  | TTGGATGCTTGGGATGCTGCTTGATGACGACGACGATGCTGCTCGATACGAGATC 711 | T                    | T                             | T                      | 714

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Fig. 6: Alignments of Ace.2 gene sequences for *Cx. quinquefasciatus* and *Cx. pipiens* collected from Iran, compared with *Cx. quinquefasciatus* from Mexico in GenBank (FJ948080) and *Cx. pipiens* from California (FJ948081). "." Indicates similarity; "*" Indicates the absence of mutation; The highlighted sequences are two exons. 
found in Cx. pipiens and 6–9 as in Cx. quinquefasciatus. Similarly, Harbach\(^\text{12}\) noted the number of branches on Cx. quinquefasciatus was more than Cx. pipiens. Knight and Malek\(^\text{2}\) reported an average of 4 and a range of 2–9 branches in Cx. pipiens from Egypt\(^\text{12}\). The shape of siphon in most of the samples indicated the occurrence of the Cx. pipiens and confirmed by PCR–RFLP method. However, morphological and molecular study about the Cx. quinquefasciatus was not compatible completely (Table 1 and Fig. 2). Further support to this result also came from a previous study; Harbach\(^\text{12}\) noted that siphon of Cx pipiens is longer and narrower than Cx. quinquefasciatus. However, some population of Cx. quinquefasciatus is similar to Cx. pipiens in relation to shape of siphon.

Further support to this result also came from a previous study, seta 1 on larval abdominal segment III–IV found more valid than the other morphological characters for identification of the recent two species\(^\text{34}\). However, in our study the results of morphological study using seta 1 on abdominal segment III–IV were not compatible with molecular study.

In our study, dorsal arms of phalosoma in Cx. pipiens samples were described as divergent, broad and nearly truncate at the apex and divergent as the base toward the end and the ventral arm was narrow while dorsal arms in Cx. quinquefasciatus reported as narrow, sharp apex and parallel as the base toward the end. Also the ventral arm was flat and leaf shape. In addition, the DV/D ratio of the samples find as range of −0.2−2.37. The ratio calculated as −0.2−0.25 confirmed the occurrence of Cx. pipiens. Further support to these results also came from a previous study, Harbach\(^\text{12}\) reported that the ratio with range of −0.14 to zero means −0.09 for Cx. pipiens while, for Cx. quinquefasciatus range of 0.56–1.89 means 1.03. Knight and Malek\(^\text{2}\) cited as a range −0.02–0.14 for the population of Cx. pipiens in Egypt. Azari-Hamidian \textit{et al}\(^\text{17}\) reported the presence of Cx. quinquefasciatus in the Iranian islands of the Persian Gulf. Dehghan \textit{et al}\(^\text{35}\) expressed that the male genitalia is the main character to identify the species of Cx. pipiens complex.

In our research, RCell/R2+3 ratio for Cx. quinquefasciatus was in the range of 1.65–3.3 and for Cx. pipiens found as 3.35–6.99. Further support to these
results also came from a previous study, Harbach (1988) reported RCell/R2+3 ratio of Cx. pipiens female as 4.6–6 and average 5.3. Azari-Hamidian and Harbach reported the ratio of Cx. pipiens >4 while it has been measured <4 for Cx. torrentium. The ratio calculated range was between 2.8 and 3.3 for Cx. quinquefasciatus.

In our investigation the intersection of costa, subcosta with bifurcation of R2+3 was not compatible with PCR–RFLP result except the samples of Hamadan, Yazd and Chabahar areas (Table 2, Fig. 3). It seems that RCell/R2+3 were more reliable than the recent character for identification of the species of Cx. pipiens complex.

**Molecular studies**

Malcolm et al used the Ace.2 gene for discrimination of the members of Cx. pipiens complex. There are some reports about the Scal cutting enzyme to distinguishing of Cx. pipiens and Cx. quinquefasciatus. A Scal enzyme site that discriminates the Cx. quinquefasciatus and Cx. pipiens alleles located in intron 2. Ace.2 gene of Cx. pipiens digests to fragments for 470 and 230 bp by Scal cutting enzyme whereas three fragments with 350, 230 and 120 bp produced in Cx. quinquefasciatus. In fact, in hybrid species there are four fragments for 470, 350, 230, and 120 bp. In our study, none of the samples found with four fragments. Bourguet et al found two biological forms, Cx. pipiens form pipiens and form molestus with the similar fragments and resulted the occurrences of gene flow hypothesis among them. In our research, sequence aligning of Ace.2 gene for Cx. quinquefasciatus and Cx. pipiens showed 6.5% variation in 46 bp. In fact the variation in intraspecific was found more than the interspecific. Similarity, Bourguet et al noted nucleotide diversity occurred more in intron 2 (non-coding region) than other sites of the Ace.2 gene.

Cx. pipiens form molestus and Cx. pipiens are not genetically differentiated, with the former probably being and ecotype of the later. Culex pipiens and Cx. quinquefasciatus as shown both by their different ITS2 and Ace sequences; in the other hands, there are no way to discrimination of two biological forms of Cx. pipiens using ITS2 and Ace genes. Variation has not been found in two biological forms of pipiens and molestus based on literature of Ace gene sequences. Culex pipiens form molestus is unlikely to appear as a true species. Recent studies on microsatellite sequences indicate the occurrence of variation between these two biological forms, however, some reports indicated discrimination of two biological forms using PCR–RFLP on the COI gene. It should be mentioned, that two biological forms are considered as true species. However, this can be an interesting and significant topic in future research and the specification processes will be discussed.

In conclusion, the most important discriminative character of Cx. pipiens and Cx. quinquefasciatus found the male genitalia. The range distribution of Culex quinquefasciatus and Cx. pipiens in the country may be created as a hybrid species and need to more comprehensive research in the future.

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