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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 *Sca*I 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 *Cx. 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 Malek[2] listed the *Cx. pipiens* and *Cx. quinquefasciatus* as distinct species based on the studies of Sirivanakarn and White[3] in Southeast Asia, Miles[4] in Australia and Jupp[5] in South Africa. Recent study indicated the occurrence of *Cx. quinquefasciatus* and the subspecies of *Cx. pipiens pallens* and *Cx. pipiens pipiens* [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 malaria[1, 9]. By now, West Nile and Sindbis viruses have been reported in Iran[10]. Enzootic cycles of West Nile fever are involving host wild birds and *Cx. pipiens* complex[11].

Culex pipiens 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 world[9, 12]. Distribution of *Cx. pipiens* expressed in many parts of Iran whereas *Cx. quinquefasciatus* reported from the south of the country[13–18] and *Culex pipiens* form *molestus* has been reported in Tehran Province located adjacent of the north of the country[19].

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 climate[2, 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. pipiens* complex species, but the recent characters cannot completely separate them. By now, the male genitalia considered as the most important morphological diagnostic character[1, 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. pipiens* 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. pipiens* 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. pipiens* complex in 1995. Crabtree *et al*[24] express the *ITS* gene for identification of *Cx. pipiens* 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. pipiens* 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. pipiens* 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. pipiens* from *Cx. quinquefasciatus* using *Ace.2* gene and *Sca*I, as restriction enzyme. Site of nucleotides enzyme of *Sca*I found in intron 2 on *Ace.2* gene. Two sites of *Sca*I enzyme recognized for *Cx. quinquefasciatus* whereas, one site found in *Cx. pipiens* 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. pipiens* 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 48°31′ E) from Hamadan Province, Teheran City (35°45′ N 51°35′ E) from Teheran Province represented as cold and dry climate (Fig. 1).

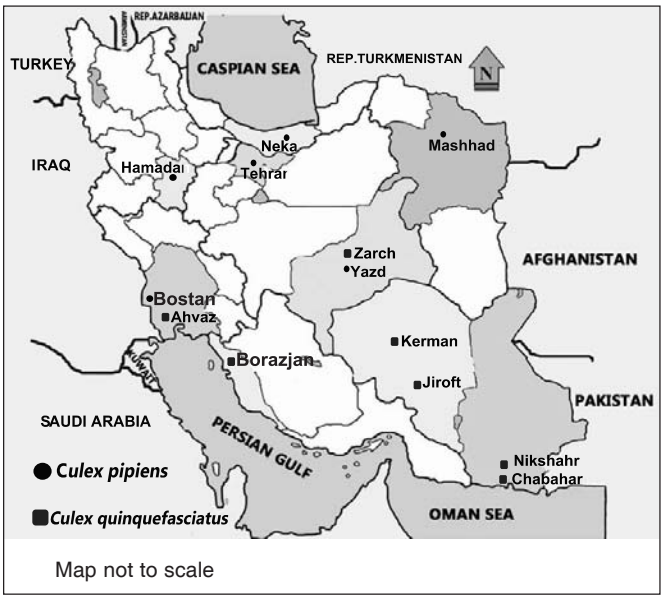

Fig. 1: Map showing *Culex pipiens* and *Cx. quinquefasciatus* distribution in different study areas in Iran during 2009–10.

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[13, 16, 20, 31].

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 (22–25ºC, 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[32]. 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 $MgCl_2$, 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 (0.089 M Tris, 0.089 M boric acid, and 0.5 MEDTA, Ph 8.0 stained ethidium bromide (Et-Br) at a concentration of 0.5 lag/ml, and run at 60–80 V for 60 min. Ultraviolet translumiator at 312 nm was viewed for PCR product.

Primers

In this study, we used the same primers based on Bourguet *et al*[29]. These primers amplify the Ace gene as length of approximately 710bp. The Sequences of primers were as Forward (F1457) 5'–GAGGAGATGTGGAATCCCAA–3' and Reverse (B1246)5'–TGGAGCCTCCTCTTCACGGC–3'. The PCR protocol included 5 initiation cycles (5 min at 95°C), followed by 30 cycles (30 sec 95°C, 30 sec at 61°C and 45 sec at 72°C) and 10 min final extension at 72°C.

PCR–RFLP

We used restriction enzyme to digest the standard PCR products based on Bourguet *et al*[29]. The PCR–RFLP reaction contained 3 µl of 10 × buffer, 7–10 µl PCR product, 1µl restriction enzyme 1–2 unit/µl and 10–20 µl distilled water in a final reaction valium of 30 µl. The contents of the tube mixed with brief shake and short spin. Because the maximum of the enzyme effect was 16 h the contents of the tubes covered by amount of mineral oil and placed inside a special rack and then put into water bath at 37°C for 16 h. PCR standard product digested with *Sca*I cutter restriction enzyme (recommended by the supplier Roche, Germany). This enzyme can be identifying sequence of 5′... AGT / ACT ... 3′ and 3′... TCA / TGA ... 5' DNA fragments. *Sca*I enzyme digested the PCR product and 5–10 µl of PCR–RLFP product added to 2–3 µl loading buffer for electrophoresis on 2% Agarose gel in TBE buffer as mentioned earlier.

Sequencing

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/* [33]. 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. Culex 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

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

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

Area	Meteorological condition	No.	Mosquito species	Morphological characteristics in female		PCR-RFLP result	No.	Morphological characteristics in male	PCR-RFLP result
				Costa & subcosta intersect/ bifurcation of R2+3	RCell/ R2+3			DV/D	
Neka (North)	Cool and moist mediterranean	4	*Cx. pipiens*	3	4	4	5	5	5
			Cx. quin.	1	0	0		0	0
Hamadan (Northwest)	Cold and dry	4	*Cx. pipiens*	4	4	4	4	4	4
Yazd (Center)	Hot and dry desert	9	*Cx. pipiens*	9	8	9	9	7	7
			Cx. quin.	0	1	0		2	2
Teheran (neighbor North)	Cold and dry	–	*Cx. pipiens*	–	–	–	4	4	4
Kerman (Near Center)	Hot and dry desert	2	*Cx. pipiens*	1	0	0	–	–	–
			Cx. quin.	1	2	2		–	–
Jiroft (neighbor South)	Subtropical warm and humid	3	*Cx. pipiens*	2	1	0	6	0	0
			Cx. quin.	1	2	3		6	6
Ahvaz (Southwest)	Subtropical warm and humid	5	*Cx. pipiens*	2	2	1	4	0	0
			Cx. quin.	3	3	4		4	4
Nikshahr (South)	Tropical warm and humid	4	*Cx. pipiens*	1	1	0	7	0	0
			Cx. quin.	3	3	4		7	7
Chabahar (South)	Tropical warm and humid	6	*Cx. quin.*	6	6	6	7	7	7

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–RLFP 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

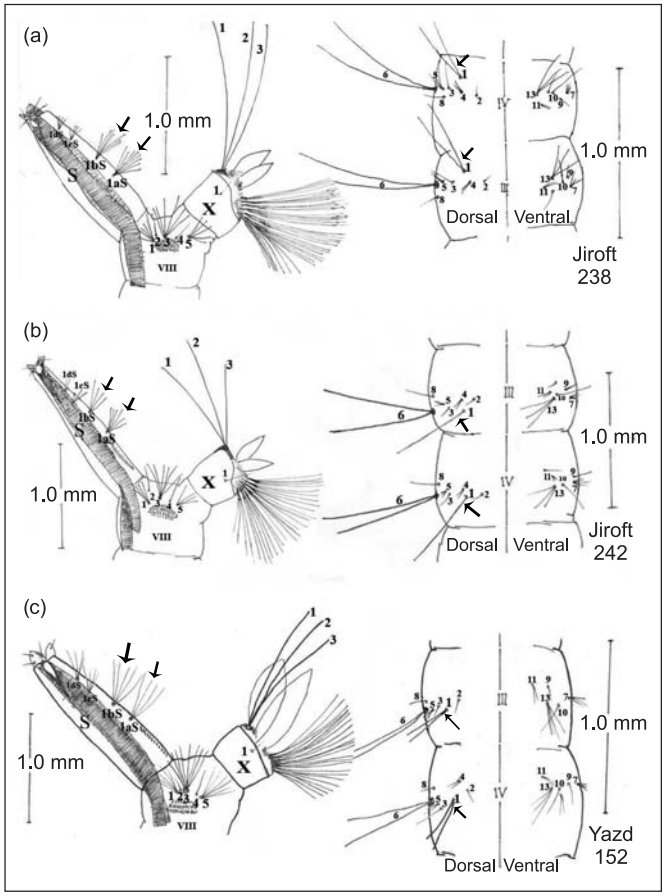

Fig. 2: Variation of morphological character of *Cx. pipiens* and *Cx. quinquefasciatus* larvae identified by PCR-RFLP method— (a) & (b): *Cx. quinquefasciatus*; and (c): *Cx. pipiens.*

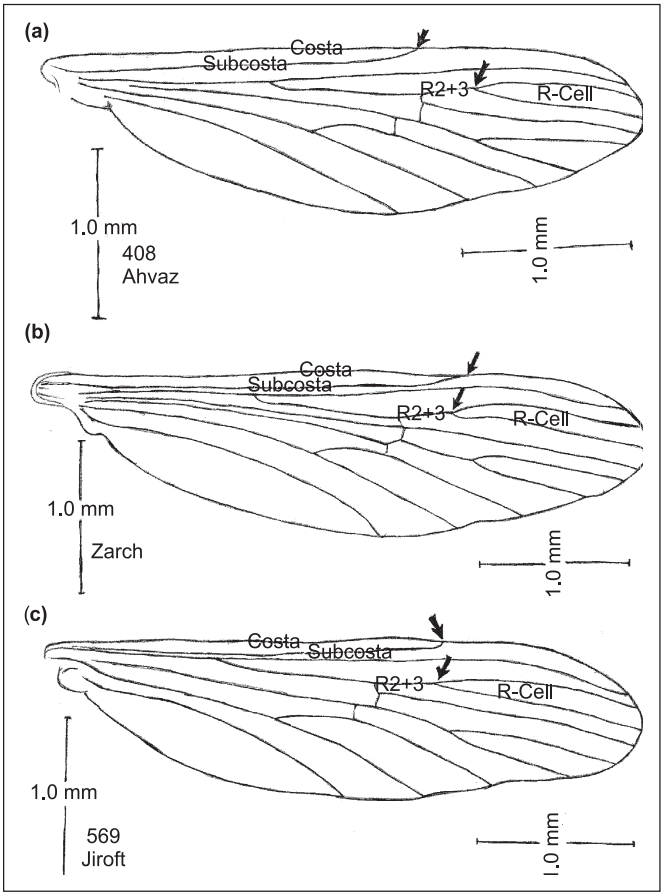

Fig. 3: Variation of vein venation in wings of *Cx. pipiens* and *Cx. quinquefasciatus*, identified by PCR-RFLP method— (a) & (c): *Cx. quinquefasciatus*; and (b): *Cx. pipiens*.

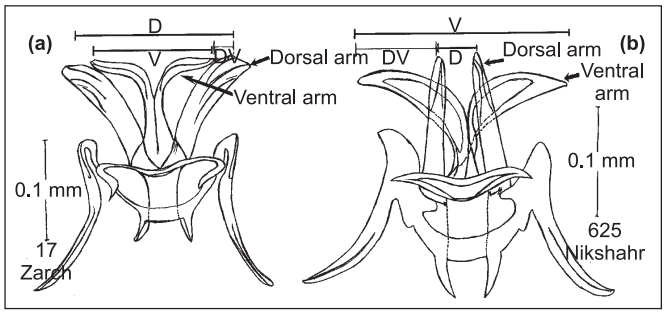

Fig. 4: The difference between dorsal and ventral arms of genitalia of *Cx. pipiens* and *Cx. quinquefasciatus* identified by PCR-RFLP method—(a): *Cx. pipiens;* (b): *Cx. quinquefasciatus;* (D): Distance between dorsal arms; V: Distance between ventral arms; and DV: Distance between dorsal and ventral arms.

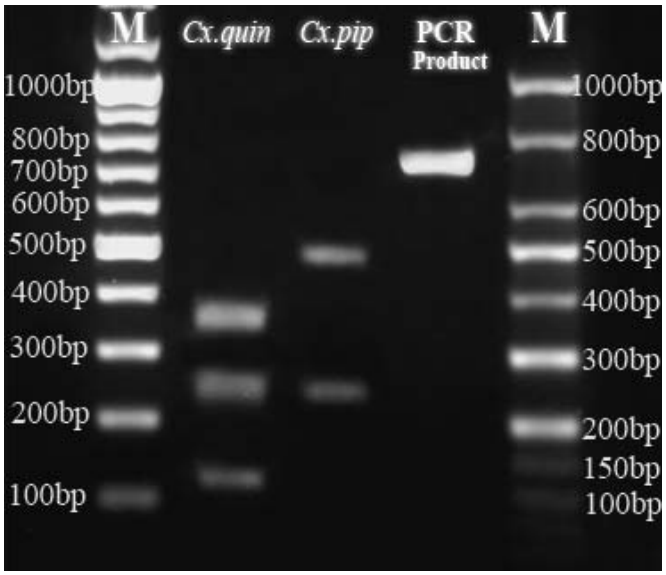

Fig. 5: Electrophoresis of PCR products and PCR-RFLP fragments of the *Ace.2* gene length 713 bp amplified by the primers F1457 (Forward) and B1246 (Reverse) in *Cx. pipiens* and *Cx. quinquefasciatus* collected from different parts of Iran. *Sca*I restriction enzyme created two fragments with 470 and 230 bp for *Cx. pipiens*, and 350, 230, and 120 bp for *Cx. quinquefasciatus*. PCR product from Yazd sample, M: Marker; *Cx. pip*: *Culex pipiens* Yazd sample; and *Cx. quin*: *Culex quinquefasciatus* Ahvaz sample.

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 linage. One sample of *Cx. quinquefasciatus* from Iran as well as nine samples from Mexico, United States and Bangladesh were located together in one linage. 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[1]. Harbach[22] 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[12]. 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[16] 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[22] 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

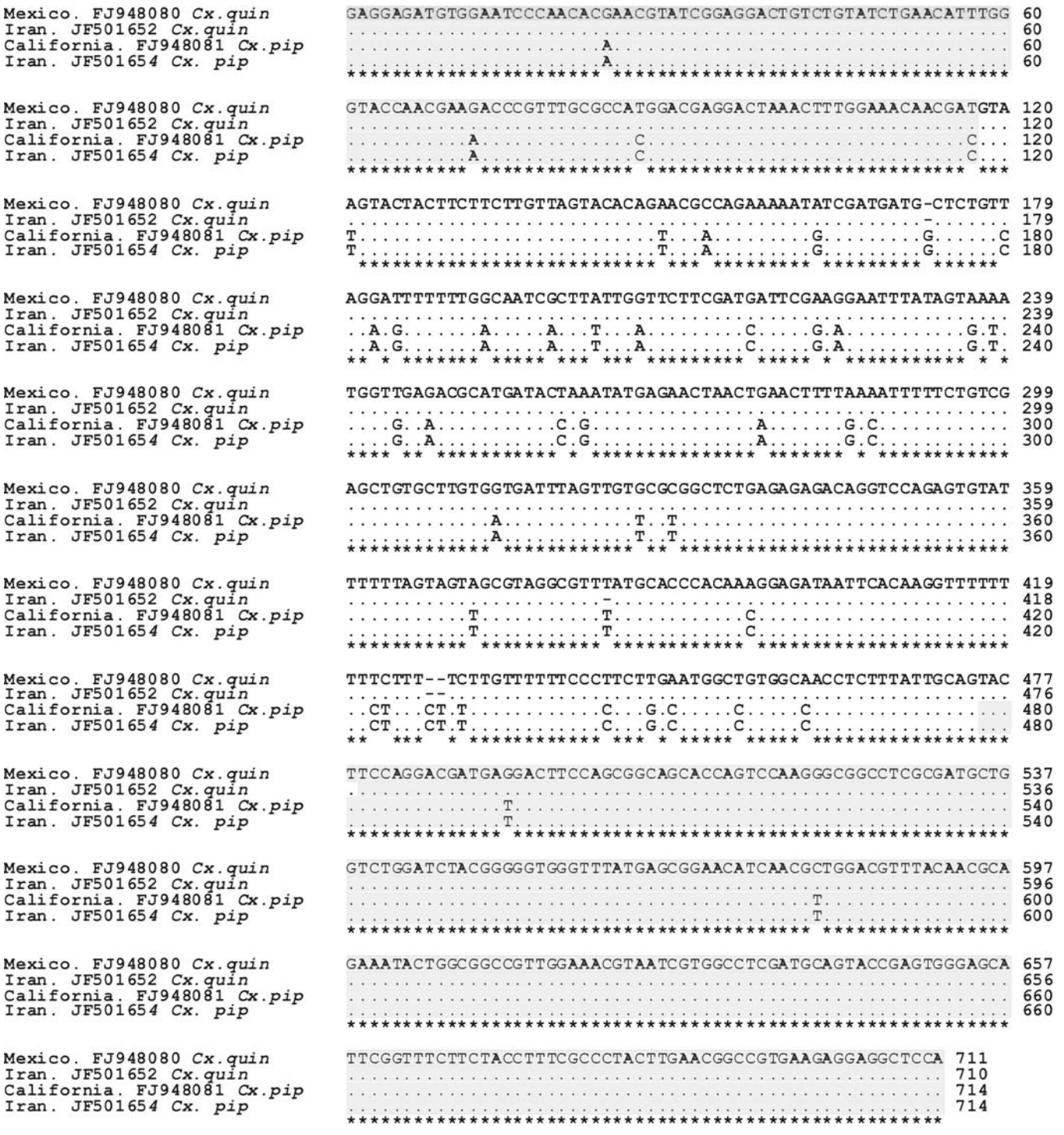

Fig. 6: Alignments of *Ace.2* gene sequences for *Cx. quinquefasciatus* and *Cx. pipiens* collected from Iran, compared with *Cx. quinquefasciatus* from Mexico in GenBank (FJ948080) and *Cx. pipiens* from California (FJ948081). "." Indicates similarity; "*" Indicates the absence of mutation; The highlighted sequences are two exons.

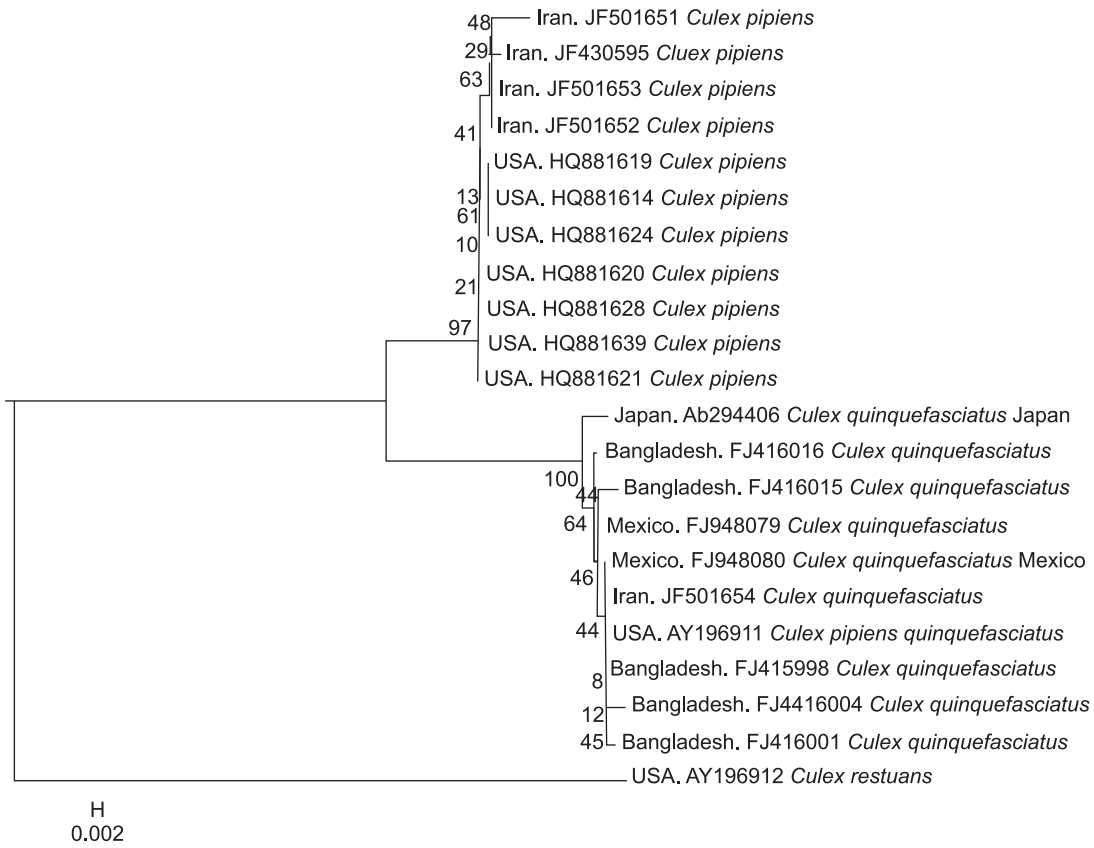

Fig. 7: The phylogenetic tree based on 714 bp of *Ace.2* gene sequences of *Cx. pipiens* and 710 bp of the same gene sequences of *Cx. quinquefasciatus*. The tree was constructed by the neighbor-Joining method. The bootstrap values are shown as numbers on the tree. The scale bar on the left indicate substitutions per site. The ace.2 sequences of *Cx. restuans* are used as out group.

found in *Cx. pipiens* and 6–9 as in *Cx. quinquefasciatus*. Similarly, Harbach[12] noted the number of branches on *Cx. quinquefasciatus* was more than *Cx. pipiens*. Knight and Malek[2] reported an average of 4 and a range of 2–9 branches in *Cx. pipiens* from Egypt[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[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[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[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[2] cited as a range –0.02–0.14 for the population of *Cx. pipiens* in Egypt. Azari-Hamidian *et al*[17] reported the presence of *Cx. quinquefasciatus* in the Iranian islands of the Persian Gulf. Dehghan *et al*[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[12]. Azari-Hamidian and Harbach[16] 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*[12, 16].

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*[25] used the *Ace.2* gene for discrimination of the members of *Cx. pipiens* complex. There are some reports about the *Sca*I cutting enzyme to distinguishing of *Cx. pipiens* and *Cx. quinquefasciatus*. A *Sca*I 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 *Sca*I 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[29]. In our study, none of the samples found with four fragments. Bourguet *et al*[29] 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*[29] noted nucleotide diversity occurred more in intron 2 (non-coding region) than other sites of the *Ace.2* gene.

Culex pipiens form *molestus* and *Cx. pipiens* are not genetically differentiated, with the former probably being and ecotype of the later. *Culex pipiens* and *Cx. quinquefaciatus* 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[36]. Variation has not been found in two biological forms of *pipiens* and *molestus* based on literature of *Ace* gene sequences[9, 29, 37]. *Culex pipiens* form *molestus* is unlikely to appear as a true species[38]. 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 [39]. 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 speciation 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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