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Cytochrome Oxidase-I Gene Sequencing Approaches to Identification of *Culex pipiens* Complex Collected in Baljurashi Province, Saudi Arabia

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Abstract: Some species of mosquitoes such as *Culex pipiens* complex are survive at different habitat conditions. They are considered of medically important vectors for some diseases, which causes a huge financial and medical problems to humans. From this point of view, species identification is the first step in the control of *Culex pipiens* complex. Previously identification of *Culex pipiens* complex is mainly done on the basis of a wide variety of morphological and biological characteristics. This can be difficult because diagnostic morphological features are often varied so little between species. So that in the present work, *Culex* species identification is performed by using DNA sequencing assay based on the mtDNA cytochrome oxidase-I (COX-I) gene. 56 mosquitoes specimens were collected from different infected areas in Baljurashi province, Al Baha, Saudi Arabia and grown for several generations in lab. COX-I gene amplification was carried out using the universal primers LCO1490 and HCO2198. Only 35 mosquito specimens had good amplified COXI target gene sequenced. These DNA sequences were compared with available sequences using basic alignment search tool in NCBI-Nucleotide database. Most of the examined specimens have high matching result in gene-bank with LC102132. However specimens T6, 8, 9, 15, 21, 45 & 51 have high similarity with JQ958371 *Culex pipiens* strain cf-3 and also specimen T35 has high similarity with KJ500032 *Culex pipiens* isolate 2AF. In conclusion, Sequence alignment and phylogenetic analysis of mitochondrial COX-I gene can be used as molecular tool for identification of *Culex* species.

Keywords: *Culex pipiens* Complex**,** Mitochondrial COX-I Gene, Sequence Alignment, Phylogenetic Analysis

1. Introduction

Mosquitoes are widely distributed insects all over the world because each of mosquitoes genera have many of species and they can survive in diverse habitats [1]. Genus *Culex* includes 200 species, many of them recognized as vectors of several diseases cause medical problems to human and animals [2].

The first step in the overcome and the control of mosquito-borne diseases is the identification of mosquito's genus and species. Traditionally, the identification of mosquito genus and species is mainly done on the basis of morphological characteristics. This method of mosquitoes identification can be difficult because some of the diagnostic morphological features may be damaged during collection or storage, and also these features are not present in all developmental stages [1, 3]. Genus *Culex* is not monophyletic [4]; [5] and the taxonomic categories are based on external morphological similarities that may not reflect natural relationships [6]. Moreover, Laurito and Almiron, (2013) [7] reported that the morphological identification of *Culex* species is difficult. Because of the recent anatomical studies indicated that, the

anatomical featured of *Culex* species larvae and adults are polymorphic and overlap among species.

Recently, DNA sequence analysis provides a more accurate way of *Culex* species complex identification and the use of molecular data, in combination to morphological methods, has resolved some long-standing taxonomic problems [8-10]. The increase in the number of new discovered DNA markers attributed to the accurate identification of mosquito species. Therefore, *Anopheles anthropophagus* and *Anopheles sinensis* can be identified more simply, rapidly, and accurately by using the ITS2 sequence as specific DNA marker than on the basis of the morphological characteristics ([11]; [12]. In addition Hebert *et al.* (2003) [13] suggested that DNA sequencing of the mitochondrial cytochrome oxidase I gene (COX-I) could allow DNA blasting and clustering that would facilitate the classification of *Anopheles* species [14]. Several studies have demonstrated that the COX-I gene is a valid molecular tool for identifying mosquito species [15]; [16] and revealing cryptic species [17].

Hebert *et al.* (2003a) [13] established that COX-I profiles, derived from the low-density sampling of higher taxonomic categories, ordinarily assign newly analyzed taxa to the appropriate phylum or order. The COX-I identification system will provide an accurate and accessible solution to the current problem of mosquito species identification [16]. Recently, Wang *et al.* (2012) [18] found that COX-I sequence differences among congeneric mosquito species were approximately 30 times higher than the average differences within species.

The aim of this study was to assess the role of COX-I gene as a valid molecular tool for identifying mosquito samples of *Culex pipiens* complex collected from infected sites in Baljurashi province (south of Saudi Arabia), El Baha.

2. Materials and Methods

2.1. Mosquito Samples Collection, Characterization and Incubation

The wild-type adults of mosquitos were collected from different infected spots of stagnant water in Baljurashi province (south of Saudi Arabia), El Baha, KSA. The collected samples were reared in the laboratory of Experimental Station of Dengue Fever Mosquitoes, King Abdulaziz University in special growing cages under specific conditions (the room temperature maintained 27°C to 29°C and 65% room humidity). The collected mosquitoes in different samples were identified morphologically for *Culex pipiens* complex (these data obtained by Alghamdi *et al.* 2017) [19]. All mosquito samples during laboratory incubation fed on the blood of adult pigeons to obtain the pure cultures. These pure cultures were reared again for some generations.

2.2. DNA Extraction

Genomic DNA of each mosquito samples was extracted following the procedures of Kress and Erickson (2012) [20]. Before DNA extraction adult females were prevented from feeding on animal blood and then 60 samples of adult females were used for total DNA extraction. Each adult sample was grounded in 50 µl of homogenate buffer (Insect Lysis Mix: 16.5g of GuSCN, 12 ml of 0.5 M EDTA pH 8.0, 6 ml of 1M Tris-HCL pH 8.0, 1 ml Triton X-100, 10 ml Tween-20 and made up to a final volume of 200 ml with ddH2O). The homogenate was incubated in the water bath for 45 minutes at 58°C with a quick addition of 5 µl of proteinase-K. Then incubate the tubes in ice for 30 minutes and centrifuged at 10000 rpm for 10 minutes; transfer the clear supernatant into fresh 1.5 ml Eppendorf tubes. To precipitate the DNA, 100 µl of 100% ethanol was added to the supernatant and incubated these tubes for 15 minutes at room temperature before transfer the supernatant to silica gel spin column. DNA was collected after rapid centrifugation of spin columns for two minutes at maximum speed. DNA was stuck on the silica gel of the column while the solution was passed down. Then DNA was washed twice by adding 500 µl of 70% ethanol to the spin column and centrifuge of spin columns for two minutes at 10000 rpm in each wash. Finally, transfer these columns to fresh 1.5 ml Eppendorf tubes and DNA solubilized by adding 50 µl ddH2O into the spin column. For collect DNA, centrifuge these tubes for one minute at 9000 rpm after incubation at 38°C for 10 minutes. The solubilized DNA samples were stored at -20°C till further experimental procedures.

2.3. COX-I Gene Amplification and Sequencing

The fifty-six isolated DNA samples were used for amplification of mitochondrial cytochrome-c oxidase-I (COX-I) gene by Polymerase Chain Reaction (PCR). The mitochondrial COX-I gene of approximately 710 bp fragment including primers was amplified by the forward primer LCOI1490: (5′-GGTCAACAAATCATAAAGATATTGG-3') and the reverse primer HCO2198: (5′-TAAACTTCAGGGTGACCAAA- AAATCA-3'), that are derived from Shaikevich (2007) [21].

The PCR reaction mixture used for COX-I gene amplification consisting of 10 – 50 ng of DNA template, 200 µM of dNTPs, 1 µ of Taq DNA polymerase, 1x Reaction buffers of PCR, 5 pmol concentration of each primer and complete the volume to 25 µl by ddH2O. While the PCR program used in this experiment started by initial denaturation at 94°C for 4 minutes, then followed by 32 cycles of denaturation at 94°C for 30 sec, primer annealing at 47°C for 40 sec and extension at 70°C for 60 sec. When these cycles completed followed by final extension at 70°C for 10 min. The amplification products were confirmed by 1% Agarose gel electrophoresis. The PCR products were given for commercial sequencing (Macrogen, Beotkkot-ro, Geumcheon-gu, Seoul, Korea).

2.4. DNA Sequences Alignment and Phylogenetic Analysis

The pattern of the evolution of *Culex* species was studied by

phylogenetic analysis. The sequences comparison with the GenBank entries using Blast and the software for phylogenetic analysis online embedded in PubMed from National Center for Biotechnology Information (blast.ncbi.nlm.nih.gov/Blast.cgi). All DNA sequences were performed to check for sequence homology with available sequences using basic local alignment search tool the LALIGN algorithm Smith-Waterman (SSE2, Michael Farrar 2006) in the online website (http://www.ch.embnet.org/cgi-bin/LALIGN_form_parser).
The aligned nucleotide sequences of the COX-I genes were concatenated in a single data matrix and create a phylogeny tree using Beast2 program (https://www.beast2.org.com) and Fig tree V1.4.3 program.

3. Results

3.1. COX-I Gene Amplification

The sixty extracted DNA samples were examined on 1.5% agarose gel for detecting the quality of these DNA samples (Figure 1). Only fifty-six isolated DNA samples were given clear and good bands. These DNA samples were used for amplification of mitochondrial cytochrome-c oxidase-I (COX-I) gene by Polymerase Chain Reaction (PCR).

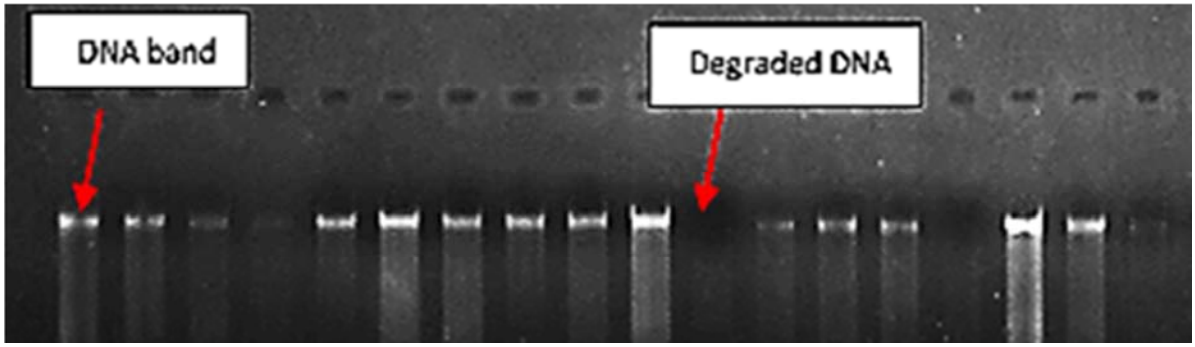

Figure 1. *Agarose gel picture of some extracted DNA samples for detect the samples with good isolated DNA.*

As shown in figure 2 an approximately 710 bp fragment including primers region of COX-I was amplified in 56 mosquito specimens using the universal primers LCO1490 and HCO2198. The amplification of the COX-I gene revealed that only 38 specimens amplified ~ 710 bp fragment specific for *Cx. pipiens* complex, while 18 specimens did not amplify this COX-I fragment.

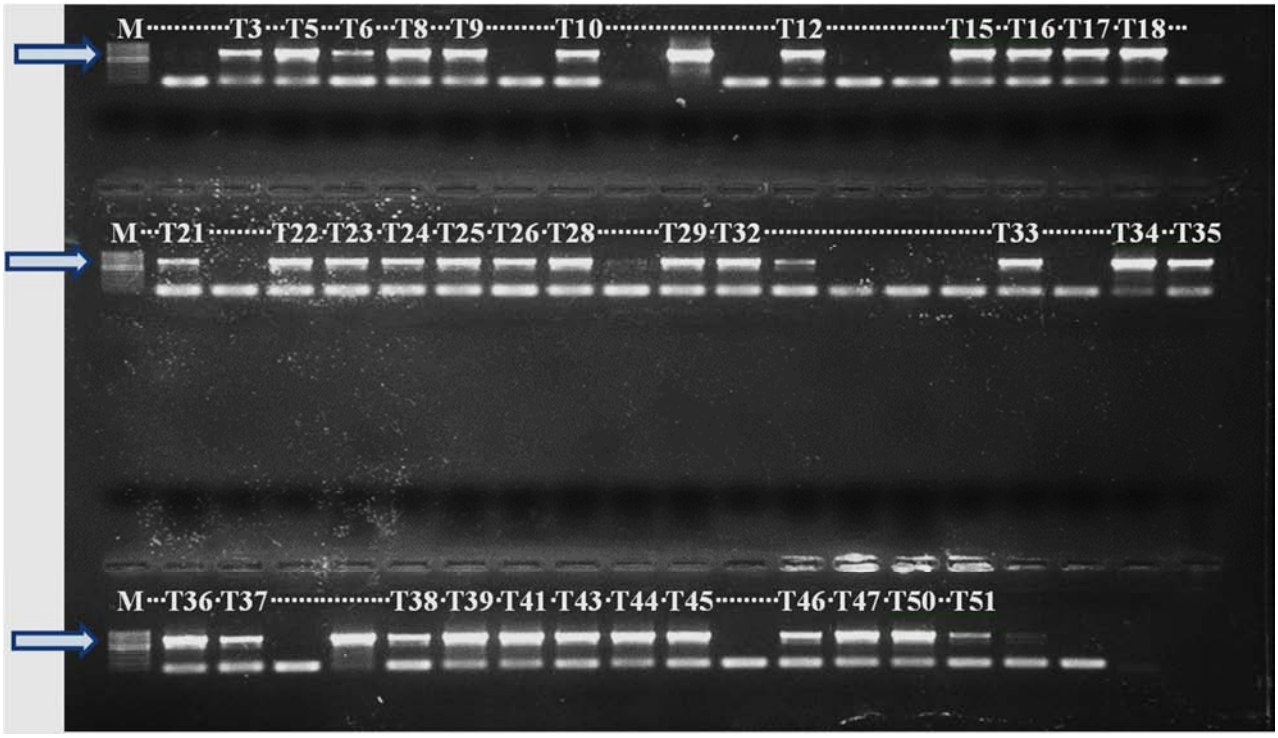

Figure 2. *Agarose gel picture expose the amplification of 710 bp COX-I gene in 56 mosquitoes specimens isolated from Baljurashi province, Baha, KSA. The three white arrows indicate 710 bp fragments and M is 100bp DNA ladder.*

3.2. COX-I Sequencing

Only 35 mosquito specimens had good amplified COX-I target gene sequenced and were used in the final analysis (Figure 2). These fragments were purified from gel and re-amplified before sequencing. The sequences were AT-rich, with an average of 67.8% AT content for all codes. Some sequencing samples had COX-I sequences containing ambiguous bases in the final 710 bases being called as heterozygous double bases.

3.3. DNA Sequences Blast, Alignment and Matching

All 35 DNA sequences were compared with available sequences using Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to check for sequence homology. Data illustrated in Table 1 exposed that, most of 35 specimens examined in this experiment have high matching result in gene-bank with LC102132 *Culex pipiens* mitochondrial COX-I gene for cytochrome c oxidase subunit 1 partial cds (Mixao *et al.* 2016). However specimens T6, T8, T9, T15, T21, T45 and T51 have high similarity with JQ958371 *Culex pipiens* strain cf-3 cytochrome oxidase subunit I (COX-I) gene, partial cds; mitochondrial (Oshaghi *et al.* 2012) and also specimen T35 has high similarity with KJ500032 *Culex pipiens* isolate 2AF cytochrome c oxidase subunit I (coxA) gene, partial cds; mitochondrial (Raharimalala *et al.* 2014). In addition specimens, T10, T32, T41 have no significant matching result in the gene bank.

Table 1. *Shown the blast results of each 35 specimens COXI gene sequences using NCBI blast-n database.*

Sample code	Gene-Bank			Reference
	Similarity (%)	Accession	Definition	
T17, T22, T23, T24, T25, T26, T28, T29	100	LC102132	*Culex pipiens* mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds, isolate: Cx_pip_Port-2168.	Mixao, V., Bravo Barriga, D., Parreira, R., Novo, M. T., Sousa, C. A,. Frontera, E., Venter, M., Braack, L. and Almeida, A. P (2016): Comparative morphological and molecular analysis confirms the presence of the West Nile virus mosquito vector, *Culex univittatus*, in the Iberian Peninsula. Parasit Vectors 9 (1), 601.
T03, T5, T16, T18 T35, T38, T43, T47	99			
T37	98			
T12, T23, T46, T50	97			
T33, T44	96			
T39	86			
T08	100	JQ958371	*Culex pipiens* strain cf-3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.	Oshaghi, M.A., Koosha, M. and Sedaghat, M. M. (2012): Sequence analysis of mtDNA COI barcode region revealed three haplotypes C/T/H within *Culex pipiens* complex.
T06, T09	99			
T15, T21	98			
T45, T51	97			
T34	96	KJ500032	*Culex pipiens* isolate 2AF cytochrome c oxidase subunit I (coxA) gene, partial cds; mitochondrial.	Raharimalala, F. N., Boukraa, S., Bawin, T., Zimmer, J.-Y. and Francis, F. (2014): Phylogenetic study of Wolbachia in Belgian *Culex pipiens*. (Unpublished)
T10, T32, T41	No significant results			

Aligned nucleotide sequences of the COX-I genes were concatenated in a single data matrix and create a phylogeny tree using Beast2 program (https://www.beast2.org.com) and Figure tree V1.4.3 program. The results of phylogenetic analysis of our 33 specimens and LC102132 *Cx. pipiens* showed that the *Cx. pipiens* complexes from Baljurashi province are located in two separated clusters with sister branches (Figure 3).

Evolutionary pattern of mitochondrial COX-I gene sequences of T17, T34, T39, T44, T50 and T51 were checked with COX-I gene sequence of LC102132 that was collected from NCBI-Nucleotide database. Phylogenetic analysis reveals that T17, T34, T50 and T44 specimens are more close to LC102132 respectively; all the sequences were branched as outgroup in the tree as shown in Figure 4. In addition T39 and T51 clustered in one group; indicates that they have high sequence diversity from the rest of the specimens and LC102132.

Further, mitochondrial COX-I sequences of T17, T34, T39, T44, T50 and T51 were aligned with the sequence of LC102132 by nucleotides using multiple sequence alignment on Clustal-O (1. 2. 4) in the online website (https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=clustalo). As shown in figure 5, alignment of our sequences with *Cx. pipiens* (LC102132) in gene-bank showed some similarities, variations and mutations which may give high variability with specimens T34, T39 and T51. However, sequences of T17, T44 and T50 specimens seems closer to LC102132.

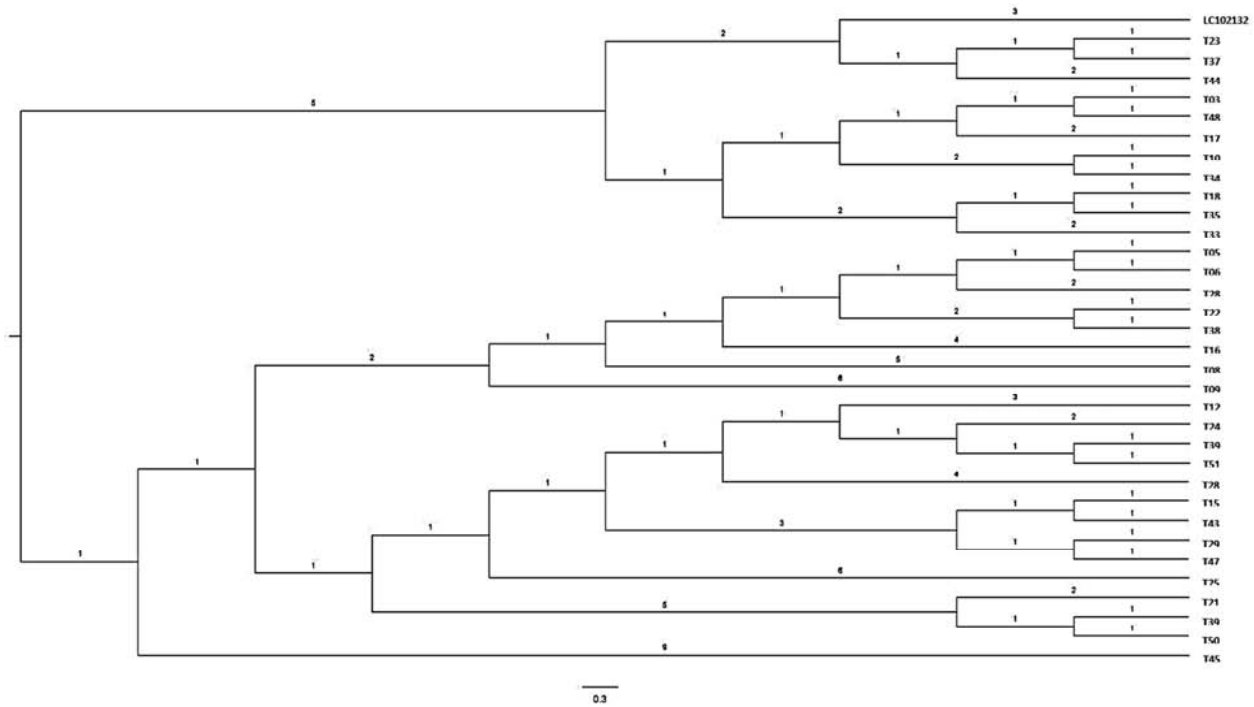

Figure 3. *Phylogenetic analysis of 32 mitochondrial COX-I gene sequences of Culex pipiens specimens from Baljurashi province, KSA. For phylogenetic analysis Beast2 program (https://www.beast2.org.com) and Fig tree V1.4.3 program were used.*

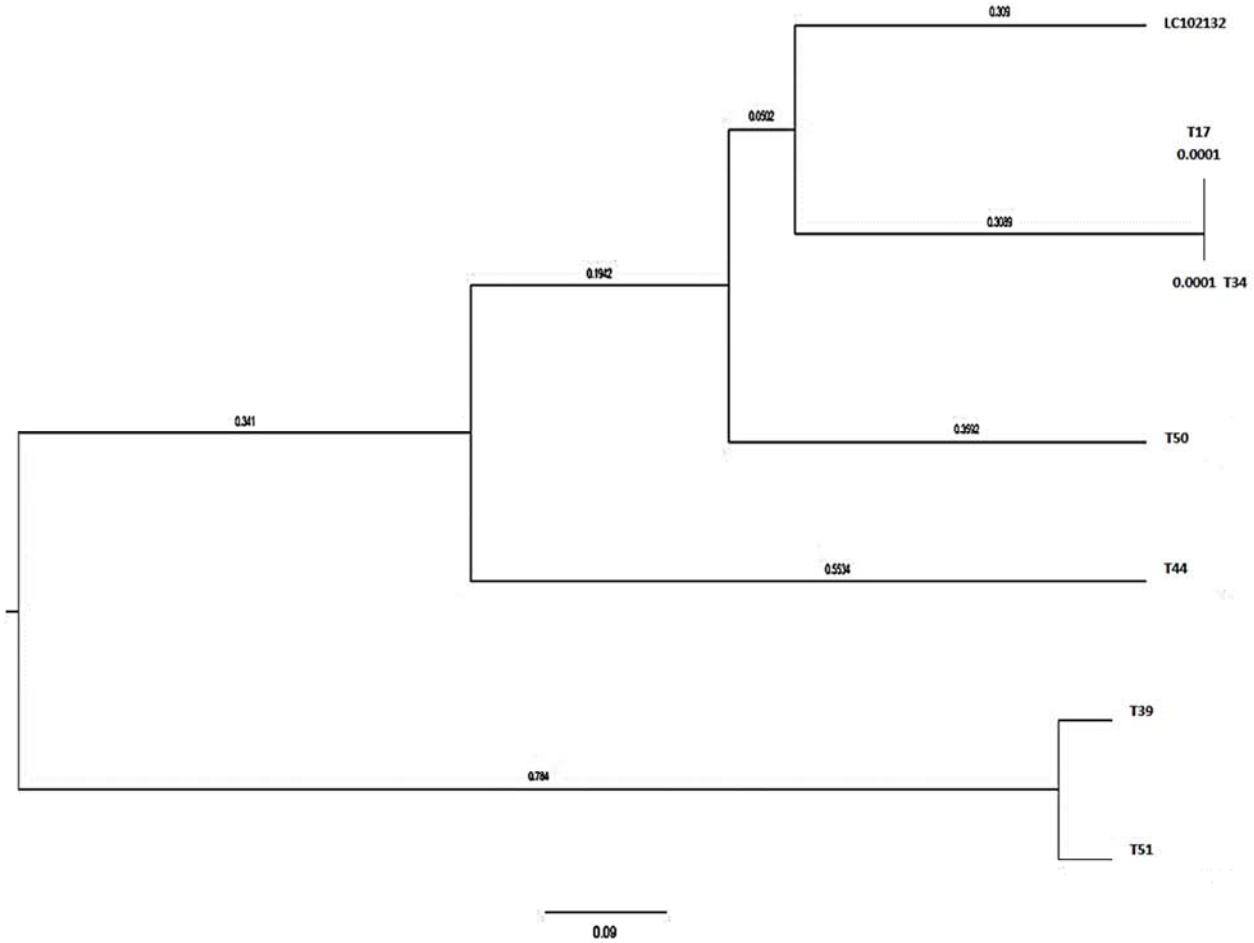

Figure 4. *Phylogenetic analysis of mitochondrial COX-I gene sequences of six Culex pipiens specimens from Baljurashi province, KSA. Noticeably, T17, T34, T50 and T44 are closely related to LC102132. While T39 and T51 both emerged as outgroup in the phylogenetic tree. For phylogenetic analysis Beast2 program (https://www.beast2.org.com) and Fig tree V1.4.3 program was used.*

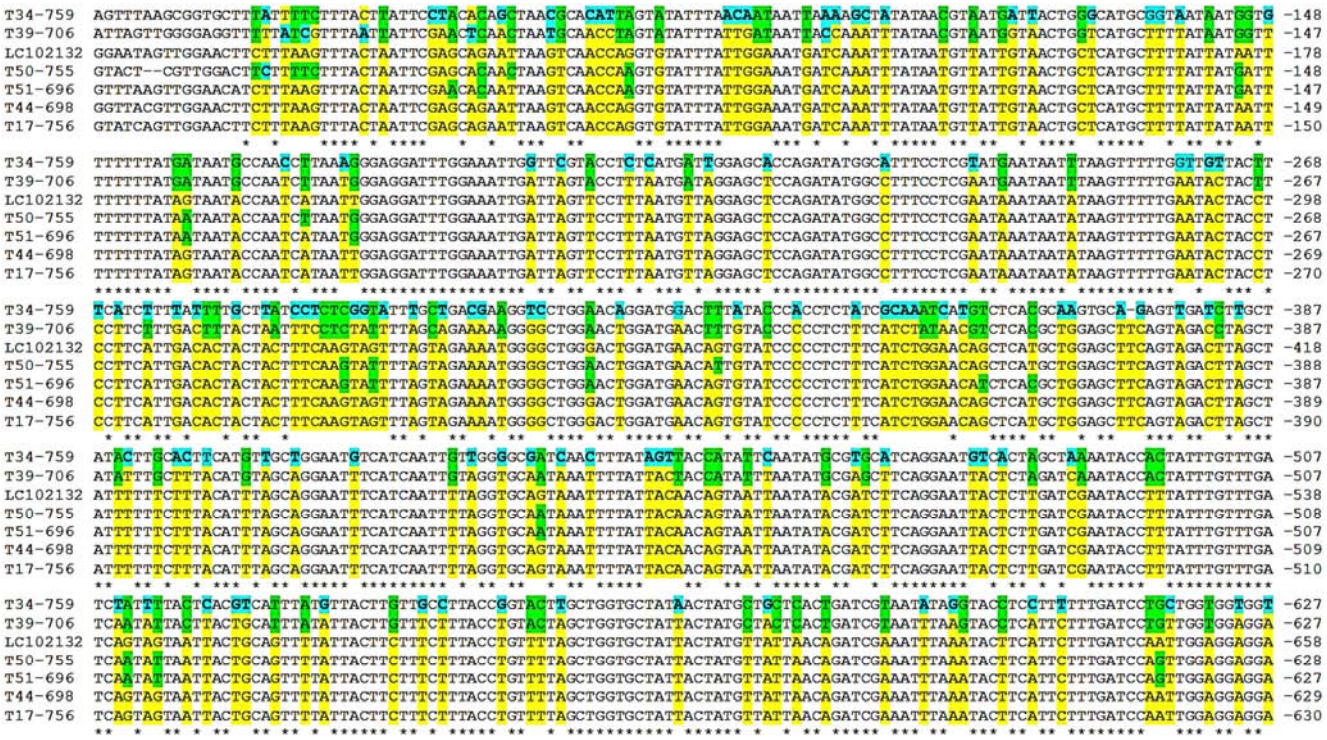

Figure 5. *Multiple sequence alignment of six mitochondrial COX-I sequences of Culex pipiens complex specimens collected from Beljourashi, KSA, compare with the sequence of LC102132 derived from Gene Bank. Sequence alignment was carried out in Clustal O (1.2.4). "*"Indicates the absence of mutation; bases highlighted with yellow color indicates similarity with LC102132; bases highlighted with light blue color indicates mutations occurred in one specimen only; bases highlighted with green color indicates mutations occurred in more than one specimen.*

4. Discussion

Taxonomic status and distribution patterns of *Culex pipiens* complex are the first step in the surveillance and control of these mosquitoes and their transmitted diseases. Morphological characteristics have been used as basic for mosquito genus and species identification. Furthermore, these morphological techniques are still not able to distinguish mosquitoes among species complexes [22]. So that the user of the molecular assays especially PCR-based techniques contributed much to taxonomic differentiation and declare the possible existence of hybridization between species [23]; [24].

Recently a diagnostic of specific molecular marker must demonstrate consistent differences between closely related mosquito species [25]. Ajamma *et al.* (2016) [22] investigated that, post-polymerase chain reaction (PCR) method used to identify variations in nucleic acid sequences, has been used to differentiate species within the *Anopheles gambiae* and *Culex pipiens* complexes. Data obtained from DNA sequences are largely used in molecular taxonomy for defining the genetic structure of vector species populations, for resolving phylogenetic relationships among or within groups of Culicidae [26]; [27], and also for the identification of species [28]. Several researchers concluded that mitochondrial genes are considered good markers for mosquito species complexes identification due to lack of introns, limited chance to recombination, and haploid nature of inheritance [24].

In most metazoans, the mitochondrial genome is a small circular DNA molecule with 17 kb containing about 37 genes with 13 protein-coding genes, 22 tRNA genes and two rRNA genes [15]. Some of these genes are used as taxonomic molecular markers such as small ribosomal subunit RNA (rrnS, 12S), large ribosomal subunit RNA (rrnL, 16S), cytochrome oxidase subunits (COX-1, 2 & 3) and cytochrome b apoenzyme (cob) [15]; [16].

The results of the present investigation exposed that the common distributed mosquito species in Beljorashi, Baha, KSA is *Culex pipiens* complex. In Saudi Arabia, *Culex pipiens* complex was found to be the dominant mosquito species compared to other medically important genera such as *Aedes* spp. and *Anopheles* spp. [19]; [29]; [30]; [31]. Also, the previous investigation occurred by Alghamdi *et al.* (2017) [19] indicated that depending on the morphological characters of adult mosquitoes in different samples collected from Beljorashi were *Culex pipiens* strain cf-3. However our results in the present investigation exposed that by using NCBI blast of mitochondrial cytochrome c oxidase subunit I (COX-I) sequence some of *Culex pipiens* samples (T06, T08, T09, T15, T21, T45 and T51) were *Culex pipiens* strain cf-3 and sample T34 was *Culex pipiens* strain 2AF. While the other 24 samples were highly similar to *Culex pipiens*, partial cds, isolate: Cx_pip_Port-2168 (Gene bank code: LC102132). Also the results of COX-I sequences alignment and phylogenetic analysis of six *Culex pipiens* specimens from Baljurashi province, KSA exposed that, T17, T34, T50 and T44 were closely related to LC102132. While T39 and

T51 both emerged as outgroup in the phylogenetic tree.

Recently, several investigations indicated that a fragment of the cytochrome-c oxidase subunit I (COX-I) has been employed for taxon barcoding [13], and to estimate genetic diversity among phylogenetically close species [32]. COX-I barcode amplifications and sequences have been used to successfully identify mosquitoes species [16]; [18]; [33] and also to recognize species complexes [34]; [35]. In addition, Cywinska *et al.* (2006) [33], worked on a short fragment of mtDNA from the cytochrome c oxidase 1 (COX-I) region was used to provide the first COX-I barcodes for 37 species of Canadian mosquitoes (Diptera: Culicidae) from the provinces Ontario and New Brunswick. Sequence variation was analyzed in a 617-bp fragment from the 5' end of the COX-1 region. Sequences of each mosquito species formed barcode clusters with the tight cohesion that were usually clearly distinct from those of allied species.

5. Conclusion

In conclusion, the mosquito *Culex pipiens*, predominantly exists all through the tropics area including Saudi Arabia acts as a vector causing several parasitic diseases. So that it is important to address their control with priority. In the present study, for molecular species identification, the cytochrome c oxidase subunit I (COX-I) mitochondrial gene has been used for assessing genetic diversity among different *Culex pipiens* complex isolates. This fragment was also used to compare phylogeographic patterns within closely related species [21]; [36]. One problem regarding the molecular analysis of COX-I gene is the ambiguous identification or the absence of clusters in trees of recently diverged species [37]. Therefore we need to develop new DNA markers, algorithms analysis for improvement of these subjects.

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