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Investigating the Genetic Diversity of *Culiseta longiareolata* Populations in Southern Iran: Insights from the *COI* Gene

Güney İran'daki Culiseta longiareolata Popülasyonlarının Genetik Çeşitliliğinin Araştırılması: COI Geninden Elde Edilen Bulgular

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ABSTRACT

Objective: *Culiseta longiareolata (Cs. longiareolata)*, a mosquito species found in Europe, Asia, and North Africa, is an important vector for various diseases like West Nile encephalitis, and malaria. This study aimed to assess the genetic diversity of the *Cs. longiareolata* mosquito population in Fars province, southern Iran due to the critical role of genetic diversity in shaping the adaptability and survival of mosquito populations and also adaptation of mosquitoes to environmental changes, insecticides, and pathogens.
Methods: In this study, samples of *Cs. longiareolata* mosquitoes were collected from 12 locations in Fars province, Iran. Larval stages were collected using the dipping method, while adults were captured with aspirators and mosquito nets, then stored at -20 °C for later DNA extraction. The *COI* gene was amplified via polymerase chain reaction (PCR) for genetic analysis. The sequencing was done by Bioneer Company in South Korea, and the results were analyzed using bioinformatics tools like Chromas, ClustalW2, and MEGA7. The final sequences were registered in GenBank for further analysis.
Results: The phylogenetic analysis using the maximum likelihood method revealed that *Cs. longiareolata* samples from Iran clustered with populations from various countries. The analysis involved 17 nucleotide sequences with 504 positions, using the Tamura-Nei model. The genetic diversity index was 0.0165, with 6 haplotypes and a haplotype diversity of 0.721. Demographic expansion indices suggested a potential expansion event in the population. A total of 274 mosquitoes were collected, with 18 examined by PCR and 6 sequenced for the study.
Conclusion: This study provides the first comprehensive genetic assessment of *Cs. longiareolata* populations in southern Iran using the *COI* gene which contributes valuable baseline data for future molecular and ecological studies. The findings reveal a moderate level of genetic diversity and clear genetic connections with Mediterranean and Middle Eastern populations, highlighting possible regional gene flow. The research highlights the significance of genetic diversity in comprehending mosquito dynamics and enhancing disease control efforts. Ongoing genetic monitoring is essential for managing vector-borne diseases.
Keywords: *Culiseta longiareolata,* genetic diversity, *COI* gene, phylogenetic analysis, vector-borne diseases

ÖZ

Amaç: Avrupa, Asya ve Kuzey Afrika'da bulunan bir sivrisinek türü olan *Culiseta longiareolata (Cs. longiareolata)*, bruselloz, kuş gribi, Batı Nil ensefaliti ve sıtma gibi çeşitli hastalıklar için önemli bir vektördür. Bu çalışma, Güney İran'ın Fars eyaletindeki *Cs. longiareolata* sivrisinek popülasyonunun genetik çeşitliliğini değerlendirmeyi amaçlamıştır.
Yöntemler: Bu çalışmada, İran'ın Fars eyaletindeki 12 lokasyondan daldırma yöntemi kullanılarak *Cs. longiareolata* sivrisinek örnekleri toplandı. Yetişkin sivrisinekler aspiratörler ve sivrisinek ağları kullanılarak toplandı ve daha sonra DNA ekstraksiyonu için -20 °C'de saklandı. Genetik analiz için *COI* geni polimeraz zincir reaksiyonu (PCR) yoluyla çoğaltıldı. Dizileme işlemi Güney Kore'deki Bioneer Şirketi tarafından yapıldı ve sonuçlar Chromas, ClustalW2 ve MEGA7 gibi biyoinformatik araçlar kullanılarak analiz edildi. Nihai diziler daha ileri analizler için GenBank'a kaydedildi.

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Bulgular: Maksimum olabilirlik yöntemi kullanılarak yapılan filogenetik analiz, İran'dan alınan *Cs. longiareolata* örneklerinin çeşitli ülkelerden gelen popülasyonlarla kümelendiğini ortaya koydu. Analiz, Temura-Nei modeli kullanılarak 504 pozisyonlu 17 nükleotid dizisini içeriyordu. Altı haplotip ve 0,721 haplotip çeşitliliği ile genetik çeşitlilik indeksi 0,0165 idi. Demografik genişleme indeksleri, popülasyonda potansiyel bir genişleme olayına işaret etti. Çalışma için toplam 274 sivrisinek toplandı, 18'i PCR ile incelendi ve 6'sı dizilendi.
Sonuç: Bu çalışma, İran'daki *Cs. longiareolata* popülasyonlarının küresel popülasyonlarla genetik olarak bağlantılı olduğunu ortaya koymuştur. Araştırma, sivrisinek dinamiklerini anlamada ve hastalık kontrol çabalarını geliştirmede genetik çeşitliliğin önemini vurgulamaktadır. Vektör kaynaklı hastalıkların yönetimi için devam eden genetik izleme şarttır.
Anahtar Kelimeler: *Culiseta longiareolata*, genetik çeşitlilik, *COI* geni, filogenetik analiz, vektör kaynaklı hastalıklar

INTRODUCTION

Mosquitoes are among the most important insects in the field of medicine and public health, known as vectors of numerous human and animal diseasefs (1-6). To date, more than 3,500 species of mosquitoes have been reported worldwide. Species belonging to the four genera *Culiseta*, *Anopheles, Aedes*, and *Culex* play a key role in the transmission of significant diseases such as malaria, filariasis, encephalitis, and arboviral diseases (7-16). Among these, *Culiseta longiareolata (Cs. longiareolata)* has attracted special attention as one of the important mosquito species. This species was first reported in Algeria (North Africa) (17) and is now found in diverse natural habitats such as ponds, valleys, streams, and even stagnant water in plastic containers, tires, and fountains (18-20). The geographic distribution of this mosquito includes the southern Palearctic, Mediterranean, European, and Asian regions (18-20).

Cs. longiareolata is easily distinguished from other species of the genus *Culiseta* by its distinctive white stripes on various parts of its body (21). This species is not only known as a nuisance pest, but also plays an important role in the transmission of various diseases to humans and animals. *Cs. longiareolata* is one of the mosquito species that directly and indirectly affects human and animal health and acts as an intermediate host for the transmission of several pathogens and is involved in the spread of diseases such as West Nile encephalitis, and malaria (22,23). Some aspects of the medical importance of this species are mentioned. First, West Nile Encephalitis: This viral disease caused by the West Nile virus is usually transmitted to humans and animals by mosquitoes. *Cs. longiareolata* is one of the mosquito species that can play a role in the transmission of this virus (24). This disease can cause severe neurological symptoms such as inflammation of the brain (encephalitis) and, in severe cases, death. Second, role in the transmission of parasitic diseases; avian malaria: *Cs. longiareolata* is known as a potential vector for the *Plasmodium* parasite in birds. This parasite causes avian malaria, which can have a significant impact on wild and domestic bird populations (24). While this disease does not directly affect humans, it can lead to a decrease in biodiversity and disruption of ecosystems. Third, role in the transmission of Malta fever; although Malta fever is mainly transmitted through the consumption of water or food contaminated with the bacterium *Brucella*, *Cs. longiareolata* can also play a role in the transmission of this disease (24). By transmitting the bacteria between different hosts, this mosquito can contribute to the spread of the disease in different areas. Fourth, human harassment; in addition to its role in the transmission of diseases, *Cs. longiareolata* also act as a nuisance pest for humans. The bite of this mosquito can cause itching, inflammation, and allergic reactions in people. This can lead to a decrease in quality of life and disruption of daily activities, especially in areas with high densities of this mosquito.

Considering the medical importance and wide geographic distribution of this species, studying the genetic diversity of *Cs. longiareolata* can provide valuable information about the population structure, genetic patterns, and phylogenetic relationships of this species. The *cytochrome c oxidase subunit I (COI)* gene is a common molecular marker in genetic and phylogenetic studies, widely used for species identification and evolutionary relationship analysis. Due to its relatively constant evolutionary rate and ability to distinguish species, this gene is a suitable tool for studying genetic diversity in different populations.

This study aims to investigate the genetic diversity of *Cs. longiareolata* in Fars province based on the *COI* gene due to the critical role and importance of genetic diversity in shaping the adaptability and survival of mosquito populations. This research seeks to analyze the population structure, identify haplotypes, and determine the phylogenetic relationships of this species in the region. The findings of this study can enhance our understanding of the distribution patterns, ecology, and role of this mosquito in disease transmission. Additionally, they provide essential information for control and management programs targeting this species.

METHODS

Study Area

The collection of Culiseta samples was carried out using the stratified sampling method. For this purpose, Fars province was initially divided into four regions: north, south, west, and east, and three sampling locations were selected from each region. In total, 12 locations were selected for sampling from Fars province for this project (Figure 1 and Table 1). This study was conducted with the approval of the Ethics Committee of Shiraz University of Medical Sciences (number: IR.SUMS.SCHEANUT.REC.1403.078, date: 10.11.2024).

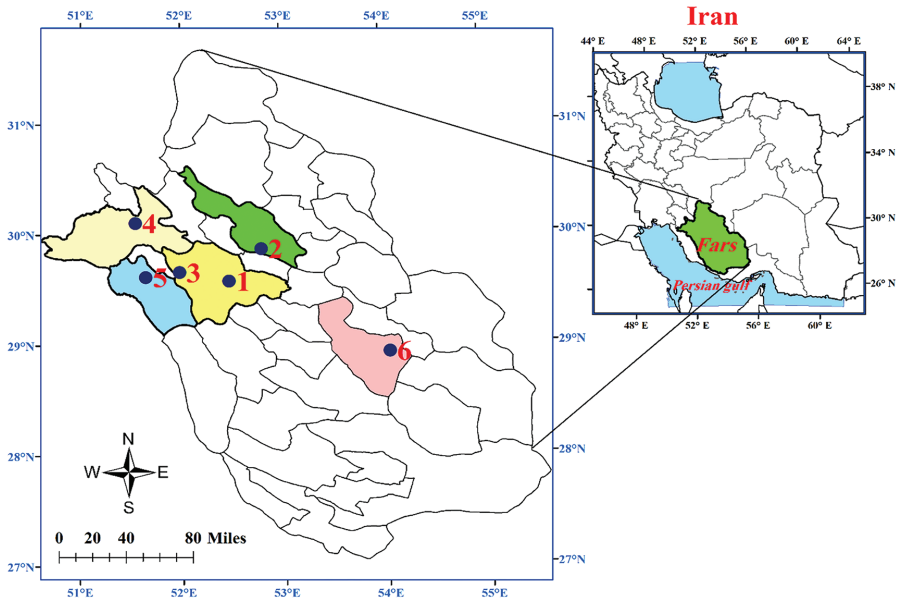

Figure 1. The geographical locations of the collection sites were mapped using ArcGIS Pro software, version 3.3.0 (Environmental Systems Research Institute). The collected samples were then identified based on valid keys and recorded in standard forms, and finally stored in a -20 °C freezer until DNA extraction (25-27). There are total of 6 location of study: Shiraz: 1, Marvdasht: 2, Dasht Arjan: 3, Nurabad: 4, Kazerun: 5, Vakilabad: 6 from Fars province, Iran

Table 1. Geographic characteristics of the collection sites for adult *Culiseta longiareolata*, detailing the number of adults collected at each location

Map codes for sampling sites	Sampling sites	Geographic coordination		Altitude (ma.s.l.)	Collected samples (n)	Adults analyzed by PCR (n)	Adults included in sequencing analysis (n)	Accession number in GenBank
		Latitude (N)	Longitude (E)					
1	Shiraz	29.579	52.475	1553	54	3	1	OR250657
2	Marvdasht	29.868	52.797	1599	42	3	1	OR250658
3	Dasht Arjan	29.660	51.984	2027	37	3	1	OR250659
4	Nurabad	30.107	51.547	978	44	3	1	OR250660
5	Kazerun	29.616	51.649	834	36	3	1	OR250661
6	Vakilabad	28.927	54.051	1383	61	3	1	OR250662
-	Total	-	-	-	274	18	6	-

PCR: Polymerase chain reaction, ma.s.l.: Meters above sea level

Mosquito Collection

Collecting adult samples at dusk to determine abundance, conduct genetic diversity studies, and analyze phylogenetic relationships in each of the study sites where no spraying was done, such as human, animal, and outdoor spaces, was carried out using an mouth aspirator and mosquito net.

DNA Extraction

DNA extraction from adult mosquitoes to determine genetic diversity was performed using the method described by Collins et al. (28). After DNA extraction, to determine the genetic diversity of the Culiseta DNA genome, we used a 721-bp region of the mitochondrial DNA-encoded *COI* gene amplified in a thermal cycler using forward primers (GGTCAACAAATCATAAAGATATTGG) and reverse primers (TAAACTTCAGGGTGACCAAAAAATCA) (29).

The polymerase chain reaction (PCR) reaction was carried out in a total reaction volume of 20 µL (4 mM of magnesium chloride, 1.5 µM of forward primer, 1.5 of reverse primer, 2 mM buffer, 150 mM of each deoxynucleoside triphosphate, 1 U Taq DNA polymerase, 40 ng of DNA and deionized water to correct volume). The thermal conditions for the PCR reaction consisted of an initial denaturation at 95 °C for 10 min; followed by 30 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and expansion at 70 °C for 1 min; with a final extension at 70 °C for 10 min (30).

Then, this amplified DNA was loaded onto a 1% agarose gel along with a 100 bp ladder marker. Staining with green viewer solution was performed to visualize the bands formed on the ultraviolet trans-illuminator. The PCR product was purified and sequenced (30).

Phylogenetic Analysis

For sequencing and analysis of the *COI* gene, we sent 30 microliters of the gene amplification product from those of suitable quality through the Takapouzist Institute to Bioneer Company in South Korea, and sequencing was performed using the primers used in the PCR reactions (29). The results obtained from the sequenced samples were reviewed and edited using the bioinformatics software Chromas (31). To examine the similarity of the obtained sequences with each other, ClustalW2 software was used, and to compare with the sequences registered in GenBank, the NCBI BLAST server available in the GenBank databasewas used. The MEGA7 software was used to examine phylogenetic relationships and draw the phylogenetic tree, and subsequently, the obtained sequences were submittedto GenBank (32).

Statistical Analysis

Genetic diversity indices, including nucleotide diversity (π), the number of segregating sites, haplotype diversity (Hd), and the average number of pairwise nucleotide differences (K_a, K_b), were calculated using DnaSP version 6.12.03. Population expansion was assessed through Fu and Li's D and F tests, Tajima's D, Fu's Fs, Strobeck's S, and Harpending's raggedness index (*r*) to infer potential demographic events. These tests were used to evaluate deviations from neutrality and detect signs of population expansion or selection. All analyses were based on *COI* gene sequences and performed using default parameters. Phylogenetic relationships were examined using the maximum likelihood (ML) method in MEGA X, applying the Tamura-Nei model with a gamma distribution (+G) to account for evolutionary rate heterogeneity across sites. A total of 504 aligned positions were used in the final dataset, excluding gaps and missing data from the analysis.

RESULTS

The evolutionary history was inferred using the ML method based on the Tamura-Nei model. The tree with the highest log-likelihood value (-943.91) is shown, with branch percentages indicating related taxa clustering. The initial tree was obtained using the Neighbor-Join and BioNJ algorithms on pairwise distances. The analysis included 17 nucleotide sequences, with 504 positions after removing gaps. The substitution model included a discrete gamma distribution for rate variation across sites, with estimated nucleotide frequencies of A=30.33%, T/U=39.29%, C=15.59%, and G=14.79%.

ML values were estimated based on an automatically calculated tree topology. Molecular phylogenetic analysis using the ML method showed that the *Cs. longiareolata* samples from Iran (codes OR250657 to OR250662) are clustered with other populations of this species from Türkiye, Malta, Portugal, Croatia, Spain, Greece, the Netherlands, Germany, and the United Arab Emirates. The genetic distance between these samples indicates a moderate genetic diversity among the different populations of this species. Based on the provided tables, 274 adult mosquitoes were collected from 6 regions in southern Iran, of which 18 samples were examined by PCR and 6 samples were sequenced. π was 0.0165, and 6 haplotypes with a Hd of 0.721 were observed. Demographic expansion indices (such as Fu and Li's D and F, Tajima's D, and R2) indicated that the population of this species may have experienced a demographic expansion event (Tables 2 and 3, Figure 1).

All genetic variability indices were computed using DnaSP v6.12 for a 504bp mitochondrial COI fragment from 17 *Cs. longiareolata* specimens.

π : Average number of pairwise nucleotide differences per site (unit: substitutions/site).

K_a and K_b: Average number of pairwise nucleotide differences per sequence (unit: substitutions/sequence); their identical values indicate they represent the same metric (likely duplicated in labeling).

Θs (theta, per site): Population mutation rate estimate based on the number of segregating sites, scaled per site (unit: substitutions/site).

Θg (theta, per sequence): Same as Θs, but scaled to the full sequence length (unit: substitutions/sequence).

Hap (number of haplotypes): Count of distinct mitochondrial haplotypes observed.

Hd: Probability that two randomly chosen haplotypes differ (dimensionless, range 0-1).

Variance of Hd: Sampling variance of haplotype diversity.

Population expansion indices were calculated using DnaSP v6.12 based on 17 mitochondrial COI haplotypes (504 bp).

Table 2. Genetic variability indices for *Culiseta longiareolata* samples from Southern Iran

Number of sequences	Number of sites	Number of segregating sites (polymorphic)	π	K_a	K_b	Θs	Θg	Number of haplotypes	Haploid diversity	Variance of haploid diversity
17	504	67	0.0165	8.353	8.353	0.0393	19.818	6	0.721	0.0075

π: Nucleotide diversity

Table 3. Population expansion indices for *Culiseta longiareolata* samples from Southern Iran

D^+	F^+	F_s	S	D	R_2
-3.571	-3.759	4.313	0.048	-2.437	0.2525

D^+: Fu and Li's D, F^+: Fu and Li's, F_s: Fu's Fs, S: Segregating sites, D: Tajima's D

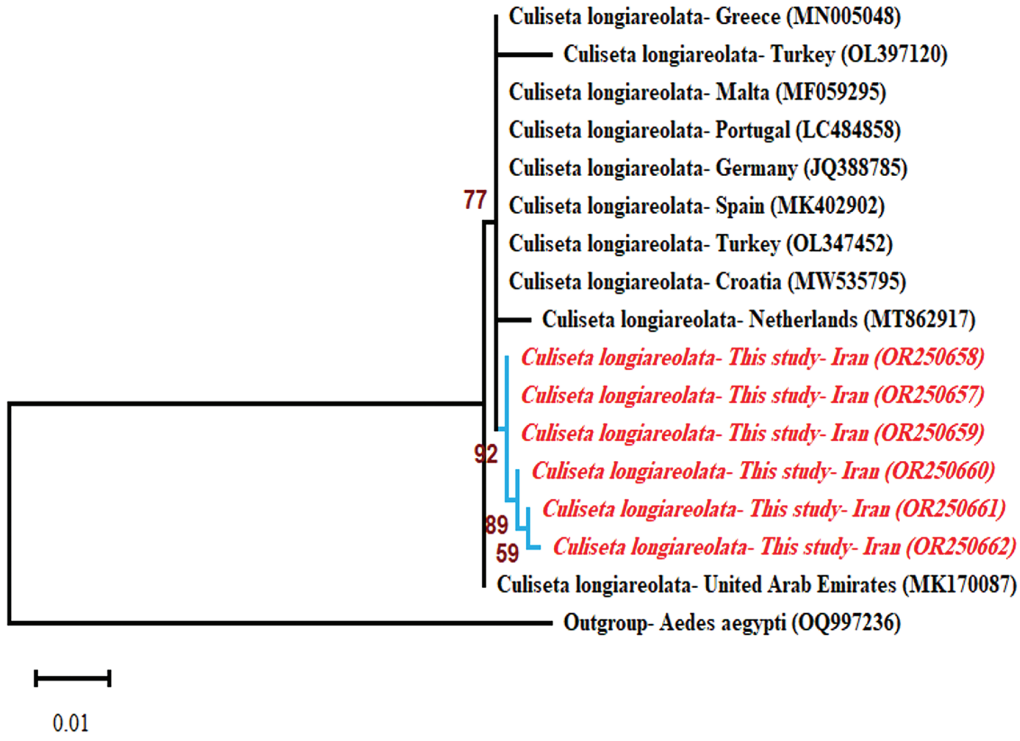

Figure 2. The evolutionary history was inferred by using the maximum likelihood method and Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-1244.36) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+non-coding. There were a total of 671 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

D^+ and F^+: Fu and Li's tests comparing singleton vs. internal mutations (negative values suggest recent expansion or positive selection).

Fs: Fu's statistic; strongly negative values indicate excess of haplotypes, consistent with population expansion.

S: Strobeck's statistic (ratio of segregating sites to pairwise differences); lower values support expansion.

D: Tajima's D; negative values reflect an excess of low-frequency variants, typical after demographic expansion.

R_2: Harpending's raggedness index; low values (e.g., <0.3) indicate a smooth mismatch distribution, supporting recent expansion.

DISCUSSION

Previous studies have shown that *Cs. longiareolata* has a wide geographical distribution in Asia, Europe and Africa (18-20). The findings of this research are also consistent with these data and indicate that the populations in Iran have a genetic connection with global populations. The medium to high level of genetic diversity indicates that the populations of this species are not completely isolated in different regions. Phylogenetic data indicate that the populations in Iran are likely interacting genetically with other regions, which could be due to natural migration or environmental influences. Population expansion indices (such as Fu and Li's D, Tajima's D) indicate a probable increase in population size in the past, and this growth could be due to climatic changes, the expansion of suitable habitats, and an increase in water resources in the environment. *Cs. longiareolata* is a vector of important viral and bacterial diseases such as West Nile encephalitis, brucellosis, and avian influenza (7-11,24). Considering these factors, studying the genetic diversity of this species can help predict population changes and provide more effective control measures to reduce diseases transmitted by this mosquito. This research is one of practical comprehensive studies on the genetic diversity of *Cs. longiareolata* in Iran and provides valuable information for environmental management and vector control programs.

Genetic studies on mosquito populations have shown that environmental factors, including temperature, humidity, and available breeding sites, significantly impact genetic variation (33). The phylogenetic relationships of *Cs. longiareolata* populations in Iran suggest possible genetic exchange with populations from neighboring countries, further supporting the role of migration in maintaining genetic diversity (34). Studies on mosquito-borne disease transmission emphasize that genetic diversity in vector populations can influence pathogen susceptibility and transmission efficiency (35). Genetic studies on mosquito populations in Iran have demonstrated that environmental heterogeneity—including regional variation in temperature, precipitation, and larval habitat availability—shapes population structure and genetic diversity. For instance, analyses of mosquito species across Iranian ecozones revealed significant genetic differentiation correlated with altitude and aridity gradients (34).

Notably, phylogeographic work on *Cs. longiareolata* in southern and western Iran identified shared haplotypes with populations from Iraq and Türkiye (35), providing direct molecular evidence of cross-border gene flow. Such migration likely counteracts genetic drift and sustains diversity in peripheral populations. Furthermore, studies on *Aedes caspius* and *Culex pipiens* in Iran have linked higher mitochondrial Hd with increased vector competence for West Nile virus and *Dirofilaria immitis* (1-3,11-12.36-38) underscoring the epidemiological relevance of genetic variation in local vector populations.

Additionally, research indicates that climatic shifts have historically played a role in mosquito population dynamics, leading to fluctuations in genetic diversity due to changes in habitat suitability (39). The presence of genetically distinct yet interconnected populations suggests that movement between regions may be more frequent than previously assumed, potentially facilitated by human activities and natural dispersal mechanisms (40). Such findings highlight the need for continuous surveillance and genetic monitoring of mosquito populations to mitigate the risks associated with emerging infectious diseases (41).

Incorporating molecular tools for vector surveillance can aid in identifying cryptic species and understanding their role in disease ecology, improving vector control strategies (42-44). Future research should focus on the impact of anthropogenic factors on genetic variation and how these changes may influence disease transmission dynamics. Strengthening collaborative research efforts across different geographical regions will enhance our ability to predict and manage vector-borne disease risks effectively. New studies suggest that genetic changes in mosquito populations are influenced by environmental factors, especially in areas with unstable climates (33). This adaptability may explain the persistence of *Cs. longiareolata* across diverse habitats, supporting their role as efficient disease vectors (37). The application of genomic sequencing in mosquito surveillance programs has provided novel insights into the evolutionary trajectories of different populations (38). Such approaches are essential for identifying emerging threats and assessing the impact of control measures (39). Furthermore, ecological studies have revealed that competition with other mosquito species, as well as interspecies hybridization, may also contribute to genetic differentiation in *Cs. longiareolata* (40). This underscores the need for integrated vector management programs that incorporate ecological, genetic, and epidemiological data (41).

Study Limitations

This study was limited by the small number of sequenced samples (n=6) due to budget and logistical constraints. Additionally, only one genetic marker (*COI*) was analyzed, which may not capture the full extent of genetic variation. Future research using multilocus approaches and larger datasets is recommended.

CONCLUSION

This study confirms that *Cs. longiareolata* populations in Iran share genetic connections with global populations, showing medium to high genetic diversity and this represents the first COI-based phylogenetic assessment of *Cs. longiareolata* in southern Iran that can show critical role of genetic diversity in shaping the adaptability of mosquito populations. The genetic exchange likely occurs through migration or environmental factors, with population expansion possibly driven by climatic changes and habitat availability. The research highlights the importance of genetic diversity in predicting mosquito population dynamics and improving disease control measures. Environmental factors and migration are key in maintaining genetic variation, which can affect disease transmission. Continued genetic monitoring is crucial for managing vector-borne diseases and improving surveillance strategies.

*Ethics

Ethics Committee Approval: This study was conducted with the approval of the Ethics Committee of Shiraz University of Medical Sciences (number: IR.SUMS.SCHEANUT.REC.1403.078, date: 10.11.2024).

Informed Consent: Consent was not required to participate.

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Footnotes

*Authorship Contributions

Concept: S.S., A.P., Design: A.H.R., Data Collection or Processing: R.S., Analysis or Interpretation: A.P., Literature Search: S.S., Writing: A.H.R.

Conflict of Interest: No conflict of interest was declared by the authors.

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