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Molecular Confirmation of *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae), *Culex torrentium* Martini, 1925 (Diptera: Culicidae), and *Culiseta longiareolata* Macquart, 1838 (Diptera: Culicidae) Using ITS2 DNA Barcoding

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Abstract

Purpose: This study aimed to identify *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae), *Culex torrentium* Martini, 1925 (Diptera: Culicidae), and *Culiseta longiareolata* Macquart, 1838 (Diptera: Culicidae) species using the internal transcribed spacer 2 (ITS2) region of ribosomal DNA and to provide molecular characterization of these species.

Method: Mosquito samples collected in Kocaeli between 2017 and 2020 were used in the study. Genomic DNA isolated from the samples was amplified by PCR using suitable primers for ITS2. Agarose gel electrophoresis results of the ITS2 region revealed different band lengths among the species, consistent with the structure of ITS2 exhibiting length polymorphism. The ITS2 sequences obtained from DNA sequencing were submitted to GenBank and compared with reference sequences in the NCBI database. Phylogenetic analyses were conducted using MEGA X software with 1000 bootstrap replicates.

Findings: Sequence analysis and phylogenetic tree construction confirmed that the analyzed samples belonged to *Culex pipiens*, *Culex torrentium*, and *Culiseta longiareolata*. The ITS2 region revealed length polymorphism across species on agarose gel and proved particularly effective in distinguishing between the morphologically similar species *Culex pipiens* and *Culex torrentium*.

Conclusion: The ITS2 region of ribosomal DNA is a reliable molecular marker for the identification and characterization of these three mosquito species, especially for discriminating between *Culex pipiens* and *Culex torrentium*, which are difficult to differentiate based on morphology alone.

Key words: *Mosquito, ribosomal RNA, DNA barcoding*

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ITS2 DNA Barkodlama Yöntemi ile *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae), *Culex torrentium* Martini, 1925 (Diptera: Culicidae) ve *Culiseta longiareolata* Martini, 1925 (Diptera: Culicidae) Türlerinin Moleküler Olarak Doğrulanması

Özet

Amaç: Bu çalışma, *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae), *Culex torrentium* Martini, 1925 (Diptera: Culicidae) ve *Culiseta longiareolata* Macquart, 1838 (Diptera: Culicidae) türlerini ribozomal DNA'nın internal transcribed spacer 2 (ITS2) bölgesini kullanarak tanımlamayı ve bu türlerin moleküler karakterizasyonunu sağlamayı amaçlamıştır.

Yöntem: Çalışmada 2017-2020 yılları arasında Kocaeli'de toplanan sivrisinek örnekleri kullanılmıştır. Örneklerden izole edilen genomik DNA, ITS2 için uygun primerler kullanılarak PCR ile çoğaltılmıştır. ITS2 bölgesinin agaroz jel elektroforezi sonuçları, türler arasında farklı bant uzunlukları ortaya koymuş olup bu durum, uzunluk polimorfizmi

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gösteren ITS2'nin yapısıyla tutarlıdır. Ardından DNA dizilemesinden elde edilen ITS2 dizileri GenBank'a gönderilmiş ve NCBI veritabanındaki referans dizilerle karşılaştırılmıştır. Filogenetik analizler, MEGA X yazılımı kullanılarak 1000 bootstrap tekrarı ile gerçekleştirilmiştir.
Bulgular: Dizi analizi ve filogenetik ağaç oluşturma, analiz edilen örneklerin *Culex pipiens*, *Culex torrentium* ve *Culiseta longiareolata* türlerine ait olduğunu doğrulamıştır. ITS2 bölgesi, agaroz jel üzerinde türler arasında uzunluk polimorfizmi göstermiş ve özellikle morfolojik olarak benzer olan *Culex pipiens* ile *Culex torrentium* türlerini ayırt etmede etkili olduğunu kanıtlamıştır.
Sonuç: Ribozomal DNA'nın ITS2 bölgesi, özellikle morfolojiye dayalı olarak ayırt edilmesi zor olan *Culex pipiens* ve *Culex torrentium* türlerinin ayrımında olmak üzere, bu üç sivrisinek türünün tanımlanması ve karakterizasyonu için güvenilir bir moleküler belirteçtir.

Anahtar kelimeler: *Sivrisinek*, *ribozomal RNA*, *DNA barkodlama*

1. Introduction

There are numerous phylogenetic studies on mosquitoes utilizing morphological and molecular markers. In particular, the mitochondrial cytochrome C oxidase I (COI) gene is among the genes frequently preferred in molecular barcoding studies [1,2]. The fact that the ITS2 barcode gene possesses conserved primer sequences belonging to many species has established this region as a prominent molecular marker suitable for comparative analyses across various taxa. However, the species-level discriminatory power of the ITS2 sequence in mosquitoes may remain limited in certain complex groups, similar to COI; this situation particularly manifests itself in the differentiation of distinct forms and species within the "*Culex pipiens (Culex pipiens* Linnaeus 1758 (Diptera: Culicidae)" complex [2].

Globally, the family Culicidae is represented by approximately 130 genera, 145 subgenera, and 3490 subspecies. In Turkey, 50 mosquito species have been identified to date, including genera such as *Aedes*, *Ochlerotatus*, *Culex*, *Culiseta*, *Uranotaenia*, *Coquillettidia*, *Orthopodomyia*, and *Anopheles*. While mosquitoes constitute a significant public health problem due to their characteristics as biological and mechanical vectors for human health, they are also economically impactful due to their extensive habitat diversity and flight capacity. Therefore, the reliability of species differentiation via morphological and molecular methods plays a critical role in population monitoring and vector control [3, 4].

While *Cx. pipiens* is widely observed on a global scale across America, Europe, Asia, Africa, and Australia, *Culex torrentium* Martini, 1925 (Diptera: Culicidae) (*Cx. torrentium*) has a more limited distribution, mostly in Central and Northern Europe. These species can show significant differences in terms of vector competence, specifically regarding pathogens such as West Nile virus (WNV), Sindbis virus (SINV), and Usutu virus (USUV). Studies report that *Cx. torrentium* may possess a higher vector potential than *Cx. pipiens pipiens* in certain regions [5].

Culiseta longiareolata Macquart, 1838 (Diptera: Culicidae) (*Cs. longiareolata*), a species of the genus *Culiseta*, is distributed over a wide geography covering Asia, Europe, the Mediterranean, and Africa. Its medical and veterinary importance is associated with avian diseases and its potential in the transmission of agents such as West Nile fever. Furthermore, the role of this species as a vector in certain regions is a noteworthy subject for research [6]. In the present study, the ITS2 sequences of *Cs. longiareolata* specimens collected from Kocaeli were analyzed, revealing a fragment length of 336 bp with a GC content of 45.53% and an intraspecific genetic distance ranging from 0 to 0.077. These molecular characteristics contribute to the genetic characterization of this species in Turkey and provide baseline data for future comparative studies.

The complexity of traditional morphological classification and the difficulties in distinguishing cryptic/sibling species have led to the adoption of molecular identification as a significant complementary approach. The ITS2 region of ribosomal DNA is a frequently used region for distinguishing closely related species due to its highly conserved structure and species-specific characteristics [2, 7, 8].

In Turkey, molecular studies on *Culex* mosquitoes have employed various genetic markers. Early studies conducted COI-based DNA barcoding of mosquitoes across different regions [9-11]. Kılıç et al. (2019) used RAPD markers to characterize *Cx. pipiens* populations in the Aegean region, reporting low genetic differentiation and high gene flow [12]. More recently, Akıner et al. (2022) investigated the distribution of the *Cx. pipiens* complex and *Cx. torrentium* in the Black Sea region using Ace-2 and CQ11 markers, confirming the presence of *Cx. torrentium* at seven locations. Although *Cx. torrentium* has been molecularly confirmed in the Black Sea region, ITS2-based verification of *Culex* and *Culiseta* species in the Marmara region has remained unexplored [13].

Therefore, this study aims to molecularly confirm the presence of *Cx. pipiens*, *Cx. torrentium*, and *Cs. longiareolata* in the Kocaeli region using ITS2 DNA barcoding, and to provide molecular characterization of these species in the Kocaeli region.

2. Materials and methods

2.1. Collection of samples

A total of 16 adult mosquitoes were used in this study. The mosquitoes were collected from four different localities during the summer period of 2017–2020. They were collected between the specified coordinates in Başiskele (40.640383, 29.938674 - 40.639618, 29.938382), Derince (40.771936, 29.816591 - 40.834515, 29.903672), İzmit (40.760071, 29.928734 - 40.822020, 29.924783), and Kartepe (40.682336, 30.136046 - 40.656733, 30.146763). Since the primary objective of this study was molecular verification, the specimens were initially sorted by morphological characters (e.g., wing venation, thoracic chaetotaxy, abdominal banding patterns) based on standard morphological descriptions and subsequently confirmed by ITS2 DNA barcoding [14]. Morphological identification of mosquito species can be challenging due to phenotypic plasticity and the presence of sibling species; therefore, molecular approaches provide a more reliable confirmation. These samples were preserved in 70% ethyl alcohol at -20°C until the DNA isolation process was performed.

2.2. DNA isolation and polymerase chain reaction

After removing the alcohol from the mosquitoes, DNA isolations were performed as recommended in the MACHEREY-NAGEL brand DNA isolation kit. Following DNA isolation, the ITS2 region was amplified using primers belonging to the 5.8S rDNA-ITS2-28S rDNA gene region shown in Table 1 [15]. A 5x Master mix was used to prepare the PCR reaction mixture. The reaction mixture recommended by the manufacturer was prepared for each sample. To detect DNA bands, a 1% agarose gel was prepared in TAE buffer using Safe T staining (as an alternative to ethidium bromide). Amplified PCR products were run on a horizontal gel electrophoresis device at 100 Volts for 30-45 minutes and visualized with a UV transilluminator. DNA sequence analysis was performed by BM Lab.

Table 1. Forward and reverse primers used in the PCR process for the ITS2 region

Primer Region	Primer Direction	Primer Sequence	Annealing temperature
ITS2 (5.8S rDNA-28S rDNA)	Forward	5′- TGTGAACTGCAGGACACATGAA-3′	57 ℃
	Reverse	5′- ATGCTTAAATTTAGGGGGTAGTC-3′	

2.3. Phylogenetic analysis

DNA sequences were viewed using the FinchTV program, and the sequences were individually saved in FASTA format via FinchTV. The forward and reverse complement reads of the sequences were aligned and compared using the ClustalW program. The ITS2 gene regions belonging to 16 mosquito samples were deposited in GenBank (S25:OP679871; S3: PV083809; S10: PV083810; S11: PV083811; S17: PV083813; S30: PV083815; S31: PV083816; S38: PV083817; S41: PV083818; S45: PV083819; S46: PV083820; S60: PV083821; S66: PV083822; S67: PV083823; S68: PV083824; S34: PV101465.1).

The similarities of the ITS2 sequences of the mosquito samples from this study were compared with other sequences registered in the NCBI database, and the species specified in Table 2 were used as outgroups in the phylogenetic tree. For the evolutionary analysis, the MEGA X software was used to determine the modeling approaches, genetic distance matrices, nucleotide compositions, nucleotide pair frequencies, and substitution matrices for the DNA barcode gene sequences of the samples. Phylogenetic analyses were conducted using MEGA X software (Kumar et al., 2018) with the Maximum Likelihood method and 1000 bootstrap replicates [16] (Figure 2).

Table 2. Accession numbers for mosquito species retrieved from NCBI were employed as outgroups in phylogenetic analyses based on the ITS2 DNA barcode gene

Species	**Accession Numbers**
Cx. pipiens	U22116.1
	MK972092.1
Cs. longiareolata	PQ309040.1
	KF483837.1
Cx. torrentium	U33037.1
	MK972014.1

3. Results

The ITS2 gene regions of 16 adult mosquitoes collected from Kocaeli province were amplified by PCR, analyzed, and registered in GenBank (see Materials and Methods for accession numbers). As a result, three species belonging to the *Culex* and *Culiseta* genera were identified.

When the G-C values for *Cx. pipiens* were examined, these values were found to be 55.5%, including outgroups. The nucleotide frequencies were 24.5% for Adenine, 20.1% for Thymine, 30.2% for Cytosine, and 25.3% for Guanine. Both the lowest and highest genetic distances were found to be 0. When the ITS2 gene region was examined in terms of nucleotide pair frequencies, no variation was observed within the 364 base pair region (data not shown in the table). The Tamura-Nei model [17] was used to determine substitution rates via the maximum likelihood method. Transitional substitution rates were calculated as 8.42 for A/G, 10.07 for T/C, 6.68 for C/T, and 8.14 for G/A.

Upon examining the G-C values for *Cx. torrentium*, the values were found to be 58.5%. When evaluated together with outgroups, the average G-C ratio was determined to be 58.3%. The nucleotide frequencies were 18.5% for Adenine, 23.2% for Thymine, 31.4% for Cytosine, and 26.9% for Guanine. To examine the ITS2 gene region, when one *Cx. torrentium* DNA sequence was compared with outgroups, and one variation was observed in the 234 base pair region. Values of 0 for parsim-info sites and 1 for singleton sites were obtained. Transitional substitution rates were calculated as 15.58 for A/C, 9.20 for T/A, 9.20 for C/A, and 11.74 for G/T. The lowest genetic distance for the *Cx. torrentium* species was found to be 0, and the highest genetic distance was 0.004 (data not shown in the table).

When the G-C values for *Cs. longiareolata* were examined, and the values were found to be 45.53%. When evaluated together with outgroups, the average G-C ratio was determined to be 45.72%. The nucleotide frequencies are 27.08% for Adenine, 27.38% for Thymine, 23.21% for Cytosine, and 22.32% for Guanine. When the ITS2 gene region of *Cs. longiareolata* was evaluated together with outgroups, a variation rate of 0.4% was detected in the 336 base pair region. The number of transitional pairs was 5, transversional pairs was 11, and the R value was determined to be 0.4. Transitional substitution rates were calculated as 8.4 for A/C, 9.77 for T/A, 9.77 for C/A, and 9.53 for G/T. The lowest genetic distance for the *Cs. longiareolata* species, including outgroups, was found to be 0, and the highest genetic distance was 0.077.

The agarose gel electrophoresis image of the ITS2 gene regions belonging to the *Cx. pipiens*, *Cx. torrentium*, and *Cs. longiareolata* species is shown in Figure 1.

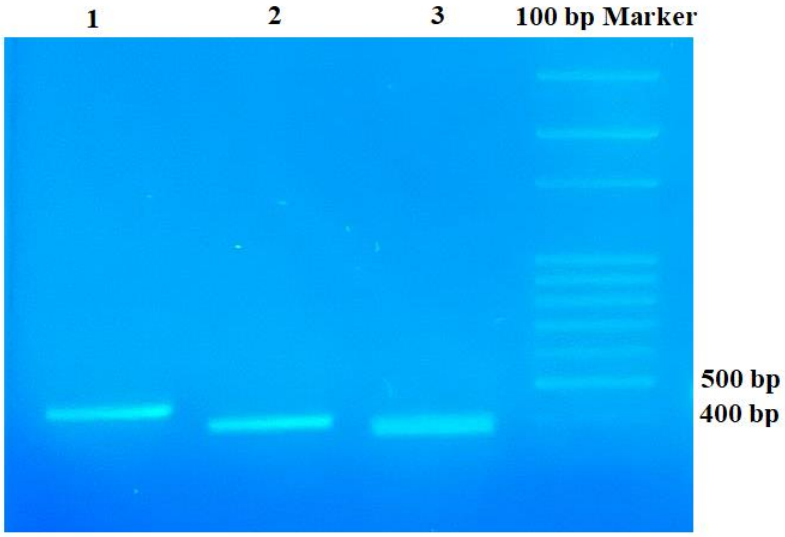


Figure 1. Agarose gel electrophoresis image of the ITS2 gene region. Lane 1: *Culex pipiens*, Lane 2: *Culex torrentium*, Lane 3: *Culiseta longiareolata*

The branches and clades belonging to *Cx. pipiens*, *Cx. torrentium*, and *Cs. longiareolota* species in the 1000-replicate bootstrap phylogenetic tree, constructed by including outgroups obtained from GenBank, are shown in Figure 2.

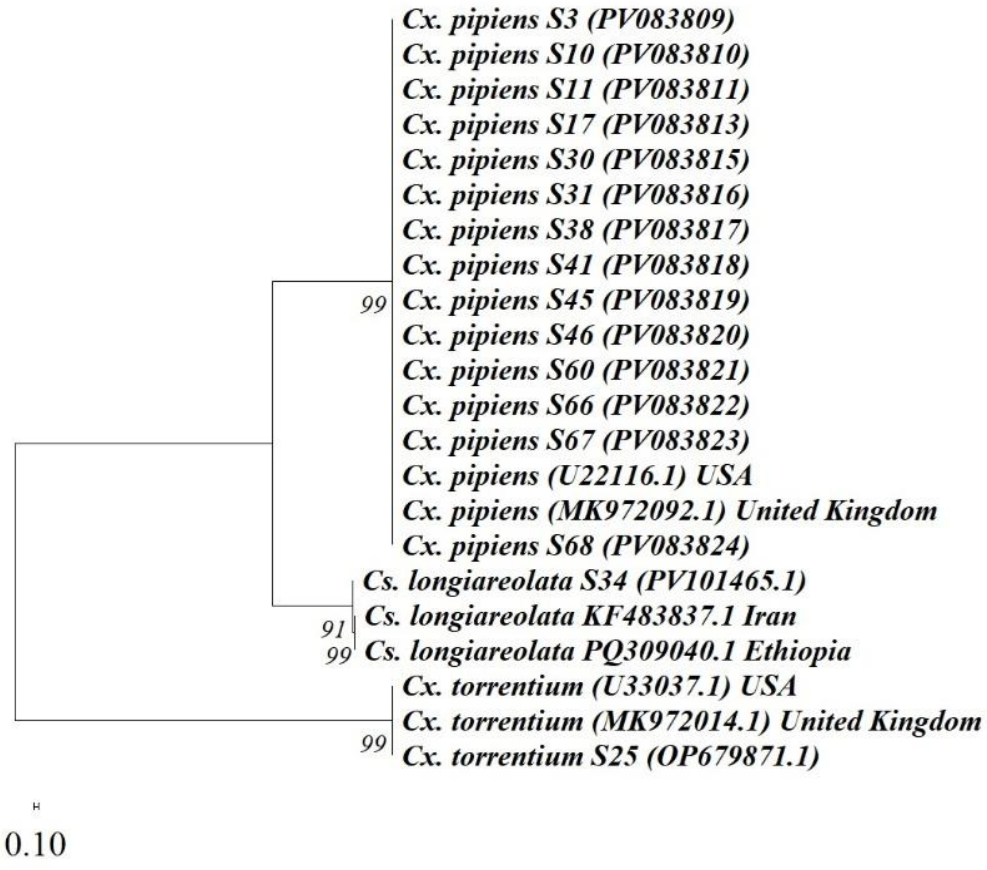


Figure 2. Phylogenetic tree constructed using the Maximum Likelihood method for the ITS2 gene region of *Culex pipiens*, *Culex torrentium*, and *Culiseta longiareolata*.

4. Conclusions and discussion

Molecular genetic studies are generally conducted on mosquitoes that carry human pathogens; these studies reveal that sibling species groups may be common in morphological taxonomy and that these species may differ biologically and ecologically. To date, the most extensive sequencing studies performed on mosquitoes have focused on the COI gene; however, different markers have also been proposed for mosquito barcoding in various studies [9, 11, 18]. One of these is ITS2, a non-coding region located between the 5.8S and 28S rRNA genes. Its high mutation rate allows this region to exhibit significant variability, highlighting it as a valuable marker that enables the detection of even small genetic variations [18, 19].

Ribosomal DNA regions, along with sequences of ITS2 and the mitochondrial COI gene, are among the most popular markers for understanding phylogenetic relationships and determining genetic variations in many organisms, including mosquitoes [15]. In our other conducted study, it was determined that the COI barcode gene was insufficient for distinguishing sibling species belonging to the *Cx. pipiens* complex [11]. Taking this situation into account, in the present study, the ITS2 gene—an intervening region obtained with primers belonging to the 5.8S rDNA and 28S rDNA regions-was sequenced, and Culex species were re-verified via NCBI-BLAST.

Upon examining Figure 1, different band sizes of ITS2 were observed for the three species identified in our study. This situation is a result of length variations in the ITS2 region. Indeed, Koosha et al. (2017) stated in their study that the base length of the ITS2 segment varies between 326–356 bp and that slight length polymorphism appears in ITS2 PCR products. It was reported that in the alignments performed, the 5.8S–ITS2–28S segment corresponds to a region with a total length of 478 bp containing 41 substitutions and 48 indels (insertion-deletion). There are suggestions that these significant intraspecific polymorphisms seen in samples registered in GenBank stem partially from 11 variations seen in microsatellite repeats within the ITS2 region [15].

In a previous study by Shaikevich and Zakharov (2010), the ITS2 region of *Cx. torrentium* was reported to be 52–54 bp shorter than that of *Cx. pipiens*, with an interspecific genetic distance of 12.5% between the two species and an intraspecific variability of 2% within *Cx. torrentium*. In our study, the length difference between the ITS2 regions of *Cx. pipiens* (364 bp) and *Cx. torrentium* (234 bp) was approximately 130 bp, which is larger than the difference reported in Russian populations. This length polymorphism was clearly visible in the agarose gel electrophoresis (Figure 1). The intraspecific genetic distance within our Cx. torrentium specimens was 0.004 (0.4%), which is lower than the 2% variability reported by Shaikevich and Zakharov (2010). This lower diversity is likely due to the geographically restricted sampling in our study (Kocaeli province only) compared to the broader Russian populations examined in their study [20].

The *Cx. pipiens* complex represents a group of mosquitoes that are very closely related in terms of taxonomic and genetic studies. According to the study by Miller et al. 1996, both ITS1 and ITS2 regions show a significant level of heterogeneity among mosquito species. This heterogeneity was observed as 41% nucleotide gaps and 21% gaps of 10 nucleotides or larger among *Culex* species. Furthermore, in the same research, it was reported that approximately 16% of the variations in ITS1 comprised base substitutions and insertions/deletions, whereas this rate was approximately 8% for ITS2. Approximately 50% of the variations in ITS1 and approximately 35% of the variations in ITS2 were associated with insertion/deletion events [21].

In a recent study conducted in the Black Sea region of Turkey, Akıner et al. (2022) employed Ace-2 and CQ11 markers to investigate the distribution of the *Cx. pipiens* complex and *Cx. torrentium*. Their study confirmed the presence of Cx. torrentium at seven locations and identified 22 haplotypes within the *Cx. pipiens* complex based on Ace-2 sequences. In the present study, we used the ITS2 region as an alternative nuclear marker to verify the species of *Culex* and *Culiseta* specimens collected from Kocaeli province. While Ace-2 and CQ11 markers enable the discrimination of *Cx. pipiens* biotypes (pipiens and molestus), ITS2 sequencing provides a complementary approach for species-level identification, particularly in distinguishing *Cx. pipiens* from *Cx. torrentium*. Together, these findings indicate that multi-marker approaches - combining nuclear genes (Ace-2, ITS2) and microsatellite loci (CQ11) - can offer a more comprehensive understanding of *Culex* species diversity in Turkey [13].

In a genetic characterization study from the Aegean region, Kılıç et al. (2019) used RAPD markers to analyze *Cx. pipiens* populations from 25 sublocations across 6 provinces. Their study revealed low genetic differentiation (GST = 0.07) and high gene flow (Nm = 6.61) among populations, indicating substantial genetic exchange across the region.

While RAPD markers provide population-level insights into genetic structure and gene flow, the ITS2 region employed in our study offers species-level resolution, particularly for distinguishing between morphologically similar species such as *Cx. pipiens* and *Cx. torrentium*. Together, these approaches contribute complementary information: population genetics data from RAPD markers and species confirmation data from ITS2 barcoding, both of which are essential for effective mosquito surveillance and vector control strategies [12].

Within the scope of this study, the ITS2 barcode gene was examined in three different species: *Cx. pipiens*, *Cx. torrentium*, and *Cs. longiareolata*. As seen in Figure 1, different band lengths were detected among the species, and these differences were confirmed; a good distinction was achieved via sequence analysis. However, similar to COI, the ITS2 sequence did not provide a distinction between *Cx. pipiens* form *pipiens*, *Cx. pipiens* form *molestus*, and *Cx. quinquefasciatus*, which are located within the Cx. pipiens complex; BLAST results also showed high similarity with these species. In contrast, despite the close relationship between *Cx. pipiens* and *Cx. torrentium*, ITS2 was able to offer an effective distinction between these two species. Consequently, it can be said that the ITS2 barcode is effective in species differentiation in mosquitoes and is a useful indicator specifically for the differentiation of closely related species such as *Cx. pipiens* and *Cx. torrentium*. These findings emphasize the necessity of combining ITS2 with other molecular markers and conducting additional studies in different regions to strengthen interspecific differentiation. However, this study is limited by its relatively small sample size (n=16) and single-region sampling (Kocaeli province). Future studies incorporating larger sample sizes and broader geographic coverage across Turkey will contribute to a more comprehensive understanding of the genetic diversity of these species.

Artificial Intelligence Statement
(X) This article did not use artificial intelligence.
( ) This article does use artificial intelligence.

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Data Availability Statement
( ) Option A (Public Repository): "The research data supporting this study are publicly available in [Name of Repository: e.g., Zenodo, Figshare, Dryad] with the permanent DOI link [URL/DOI]."
(**X**) Option B (Upon Request): "The raw data supporting the findings of this study are available from the corresponding author [Author Initials] upon reasonable request and within ethical boundaries."
( ) Option C (Third-Party Data): "The data used in this study were obtained from [Name of Institution/Source] and are subject to third-party restrictions regarding redistribution."
( ) Option D (No Data): "No new data were created or analysed in this study. Data sharing is not applicable to this article."

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