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Molecular Characterisation of Cytochrome Oxidase I and Internal Transcribed Spacer 2 Fragments of *Culiseta longiareolata*

Culiseta longiareolata'daki Sitokrom Oksidaz I ve Internal Transcribed Spacer 2'nin Moleküler Karakterizasyonu

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ABSTRACT

Objective: Among the mosquitoes, *Culiseta longiareolata* plays a notable role in the transmission of avian malaria, tularemia and arboviral diseases, including West Nile fever. We conducted this study to characterise the cytochrome oxidase I (COI) and internal transcribed spacer 2 (ITS2) fragments of *Cs. longiareolata* in northwestern Iran to determine the classification status of this species.
Methods: The COI and ITS2 fragments from six populations of *Cs. longiareolata* were amplified, sequenced and analysed. For phylogenetic analysis, the evolutionary history was estimated using the Tamura-Nei-based Maximum Likelihood approach.
Results: Thirteen sequences (six for ITS2 and seven for COI) from six populations of *Cs. longiareolata* were acquired and deposited into the GenBank. Phylogenetic analysis showed that the COI sequences from the current study cluster together with the same species from other parts of the world. Moreover, the ITS2 sequences of the current study and sequences retrieved from the GenBank, despite intraspecies variation, fall into a distinct clade with acceptable bootstrap values.
Conclusion: Notable genetic variations were observed between various *Cs. longiareolata* populations based on the evaluations of ITS2 and COI fragments. By conducting such studies, the exact classification status of this species can be determined.
Keywords: Mosquitoes, molecular systematics, phylogenetic analysis

ÖZ

Amaç: Sivrisinekler arasında *Culiseta longiareolata*, Batı Nil humması da dahil olmak üzere kuş sıtması, tularemi ve arbovirüs enfeksiyonlarının bulaşmasında önemli bir rol oynamaktadır. Bu çalışma, *Cs. longiareolata*'nın Sitokrom Oksidaz I (COI) ve Internal Transcribed Spacer 2 (ITS2) fragmanlarını karakterize etmek için İran'ın kuzeybatısında yapıldı.
Yöntemler: *Cs. longiareolata*'nın altı popülasyonundaki COI ve ITS2 fragmanları amplifiye edildi, sekans dizimi yapıldı ve analiz edildi. Filogenetik analiz için evrimsel tarih, Tamura-Nei tabanlı maksimum olabilirlik yaklaşımı kullanılarak tahmin edildi.
Bulgular: On üç sekans (ITS 2 için 6 ve COI için 7) elde edildi ve Genbank'ta saklandı. Filogenetik analiz, mevcut çalışmadaki *Cs. longiareolata*'nın COI dizilerinin, dünyanın diğer bölgelerinden gelen aynı türlerle birlikte kümelendiğini gösterdi. Buna ek olarak, çalışmadaki 6 *Cs. longiareolata* popülasyonunun ITS2 dizileri ve genbank formundan elde edilen diziler, tür içi varyasyonlara rağmen, kabul edilebilirön yükleme değerlerine sahip ayrı bir diziye yerleştirilmiştir.
Sonuç: *Cs. longiareolata* popülasyonları arasında ITS2 ve COI'ya bağlı olarak dikkate değer çeşitlilikte genetik varyasyon bulunmuştur. Bu sonuçlar, *Cs. longiareolata*'nın farklı popülasyonlarının daha büyük örneklem boyutlarıyla daha geniş ölçekte yapılacak daha ileri çalışmalar için bir başlangıç olabilir. Bu tür çalışmalar yapmak, bu türlerin kesin sınıflandırmasını belirleyebilir.
Anahtar Kelimeler: Sivrisinekler, moleküler sistematikler, filogenetik analiz

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Mosquitoes (Diptera: Culicidae), are important from a medical and veterinary point of view. Specific species of the this family play an important role in the transmission of diseases such as malaria, filariasis, and multiple arboviral diseases such as dengue fever, yellow fever, zika, chikungunya, sindbis, and West Nile fever (1). *Culiseta longiareolata* as a species of the genus Culiseta, has been distributed in the broad geographical range of Asia, Europe, the Mediterranean and Africa (2). The medical and veterinary importance of *Cs. longiareolata* is due to its role in the transmission of diseases such as avian malaria (3), tularemia (4). Also its probable role in the transmission of West Nile fever as experimental vector, highlights the importance of this species (5).

Cytochrome Oxidase I (COI), as the largest protein coding gene in the mitochondrial genome of eukaryotes, has been extensively used as a molecular marker in mosquitoes phylogenetic studies and in many cases it is also known as a mosquito species identification barcode (6).

Unlike coding regions that are almost constant between species, the internal transcribed spacer 2 (ITS2) region has a notable variation that can be used to distinguish populations of species. Sequencing of ITS2 regions of nucleotides is considered as an important modern systematic tool (7,8). This marker has been used in molecular studies of different mosquito species (9-12).

Considering the report of the presence of West Nile virus in the vectors of West Azerbaijan province (13) and since this virus possibly can be transmitted by *Cs. longiareolata*, as the secondary vector (5), the precisely characterization and identification of this vector is of notable importance. Given the increasing application of molecular markers in complementation and confirmation of the identification of important vectors, molecular characterization of this speciaes, as valuable basic information, can be useful.

The morphological characterization and identification of mosquito species of Iran, have been conducted in different parts of country and also West Azarbaijan Province where the *Cs. longiareolata* is one of the species reported from different regions of Iran and has a wide distribution across the country (14).

Due to the presence of *Cs. longiareolata* in a wide range of the world, the history of mosquito-borne diseases, which *Cs. longiareolata* plays a role in these diseases, and the need for a detailed study of this species (including characterization of its molecular markers), the current study was conducted to characterize the COI and ITS2 fragments of *Culiseta longiareolata* in West Azerbaijan Province, North West of Iran.

METHODS

Ethics Statement

Prior to the approval of all projects by the Urmia University of Medical Sciences, they are reviewed and endorsed by the ethical committee. The current project has been reviewed and approved by Urmia University of Medical Sciences' ethical committee under the number: IR.UMSU.REC.1397.265.

Study Area, Sample Collection and Species Identification

Mosquitoes' specimens were collected from six localities of three counties (Urmia, Khoy and Makoo), West Azerbaijan Province, North West of Iran (Figure 1).

Different habitats were examined during May-October 2019 for larvae collection using the standard dipping method (15). All samples were identified using standard morphological keys (16).

Genomic DNA Extraction and ITS2 Fragment Amplification

Specimens were amplified in triplicates from six populations of *Cs. longiareolata* for molecular analysis of both markers (totally 36 specimens for COI and ITS2). Bioneer AccuPrep® Genomic DNA Extraction Kit (South Korea) was used for genomic DNA extraction of mosquitoes. Extracted DNA was diluted in TE buffer and kept at 4 °C. The extracted genomic DNA was subjected to polymerase chain reaction (PCR) using super PCR MasterMix® (Yekta Tajhiz Azma, Tehran, Iran). The desired ITS2 fragments were amplified using the universal primers, forward-5.8S (5' ATC ACT CGG CTC GTG GAT CG 3') and reverse-28S (5' ATG CTT AAA TTT AGG GGG TAG TC 3') (17). The PCR conditions were 94 °C for 5 min followed by 30 cycles of (94 °C for 45 s, 57 °C for 50 s, 72 °C for 1 min) and 72 °C for 10 min.

For amplification of COI fragment, the universal primers forward: 5' GGAGGATTTGGAAATTGATTAGTTCC 3' and reverse: 5' CCCGGTAAAATTAAAATATAAACTTC 3', were used (18). The PCR program was set as follows: initial denaturation at 94 °C for 5 min; 30 cycles of (94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s] and a final extension at 72 °C for 7 min. The accuracy and quality of amplicons were tested on an agarose gel of 1.5% and visualized after staining with YektaGreen® safe stain (Iran) through ultraviyole transillumination. High quality amplicons were sequenced. After analyzing the sequences, entirely identical sequences were removed from each population, and only one sequence of each population was deposited into the Genbank.

Figure 1. Location of West Azerbaijan Province and sampling localities, 1- Makoo, Sangar: 39.316410, 44.432039, 2- Khoy, Marakan: 38.851780, 45.258208, 3- Urmia, Koor-Abad: 37.723190, 44.674631, 4- Ghahramanloo: 37.659869, 45.207550, 5- Nazloo: 37.651213, 44.983285, and 6- Moallem 37.546660, 45.033280 (Original basic map has been prepared from d-maps.com)

Phylogenetic Analysis

COI and ITS2 sequences of the same mosquito species were retrieved from GenBank for phylogenetic analysis (www.ncbi.nlm.nih.gov). The evolutionary history was estimated using the Tamura-Nei-based Maximum Likelihood approach (19). Initial tree(s) for heuristic analysis were automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pair distances calculated using the Maximum Composite Likelihood (MCL) method, then choosing topology with a higher log probability value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter =8.9461)]. For ITS2 sequences, all positions containing gaps and missing data were eliminated by analyzing the original chromatograms from acquired sequences as well as using bioinformatics tools. There were a total of 193 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (20).

Statistical Analysis

The methods and algorithms used in phylogenetic analyzes have statistical bases. The statistical bases of Tamura-Nei-based Maximum Likelihood approach, Neighbor-Join and BioNJ algorithms and MCL method, have been used as previously described (19,21,22).

RESULTS

In current study the COI and ITS2 fragments of 6 populations belonging to *Cs. longiareolata* were amplified in triplicates from which the sequence with the highest quality was analyzed and deposited in GenBank (Table 1).

The phylogenetic analysis of the COI fragments, including sequences from the 6 populations of the present study as well as sequences of this fragment in other genera and mosquito species, showed that the COI sequences of *Cs. longiareolata* from current study cluster together with the same species from other parts of the world (Figure 2). The intra-species variation of this gene sequence is significant among the 6 populations studied and other sequences retrieved from other countries. Sequences of other genera and species (retrieved from Genbank), were distinctly separated into separate branches and clades other with high bootstrap values (Figure 2). Genetic variation was also observed in COI, even from single site samples (MK863417 and MK863416, Ghahramanloo, Urmia).

The Phylogenetic analysis based on the amplified fragment showed that the ITS2 sequences of 6 populations of *Cs. longiareolata* of current study and sequences of this species retrieved form Genbank, in spite of intra-species variation, have been placed in a distinct clade with acceptable bootstrap values (Figure 3).

Sequences of other species and genera of mosquitoes were analyzed in addition to the sequences of this gene in 6 populations in order to test the ability of ITS2 to differentiate between other levels of taxa (species and genera). The results showed that ITS2 was able to successfully differentiate between different taxonomic levels, including members of different species and Genera (Figure 3).

DISCUSSION

Due to the rapid development of molecular phylogeny in diseases' vectors, in the present study, two widely used molecular markers (COI and ITS2), in the molecular systematic of *Cs. longiareolata* were amplified and analyzed. The transmission of some of important mosquito-borne diseases by this species reveals the necessity of studying this mosquito (23,24). Possible genetic variation among different populations of *Cs. longiareolata* was investigated by sampling of this species from several regions and amplification and analysis of both COI and ITS2 in different populations of this species. The possible efficacy of these two markers in separating different populations was also evaluated.

Although ITS2 is most commonly used to examine intra-species genetic diversity, its efficacy in identifying new species should not be overlooked. Even three new species in the *An. maculipennis* complex have been described partly based on nucleotide differences of ITS2 fragment (25). Notable genetic variation observed among different populations of *Cs. longiareolata* based on ITS2 needs further investigation to evaluate the potential for existence of different species with the same morphological structure in *Cs. longiareolata* (*Cs. longiareolata* or *Cs. longiareolata* complex). The results of a study analyzing the ITS2 that has identified *An. persiensis* that COI marker has not been able to separate it from other species of *An. maculipennis* complex (26), can be a proof of the effectiveness of ITS2.

Although the current methods incorporated the use of a 658-base-pair cytochrome *COI* gene region, as the DNA barcode (6), but in previous work efforts with mosquito species the resolution provided by COI barcodes is highly variable. The inefficiency of COI in separation of *Anopheles deaneorum* from *Anopheles marajoara* (27) or while studying *Culex* species, it has been stated that only

Table 1. The localities of sampling sites, geographical properties and NCBI-genbank accession no's of acquired sequences for COI and ITS2 fragments of *Cs. longiareolata,* North West of Iran

County	Localities	Geo. details		Altitude (m)	Accession no	
		Lon.	Lat.		COI	ITS2
Makoo	Sangar	44.432039	39.316410	1.348	MK863414	MK861163
Khoy	Marakan	45.258208	38.851780	948	MK863419	MK861162
Urmia	Nazloo	44.983285	37.651213	1.358	MK863420	MK861165
	Moallem	45.033280	37.546660	1.350	MK863415	MK861166
	Ghahramanloo	45.207550	37.659869	1.000	MK863417 MK863416	MK861161
	Koor-Abad	44.674631	37.723190	1.545	MK863418	MK861164

COI: Cytochrome oxidase I, ITS2: Internal transcribed spacer 2, Lon.: Longitude, Lat.: Latitude

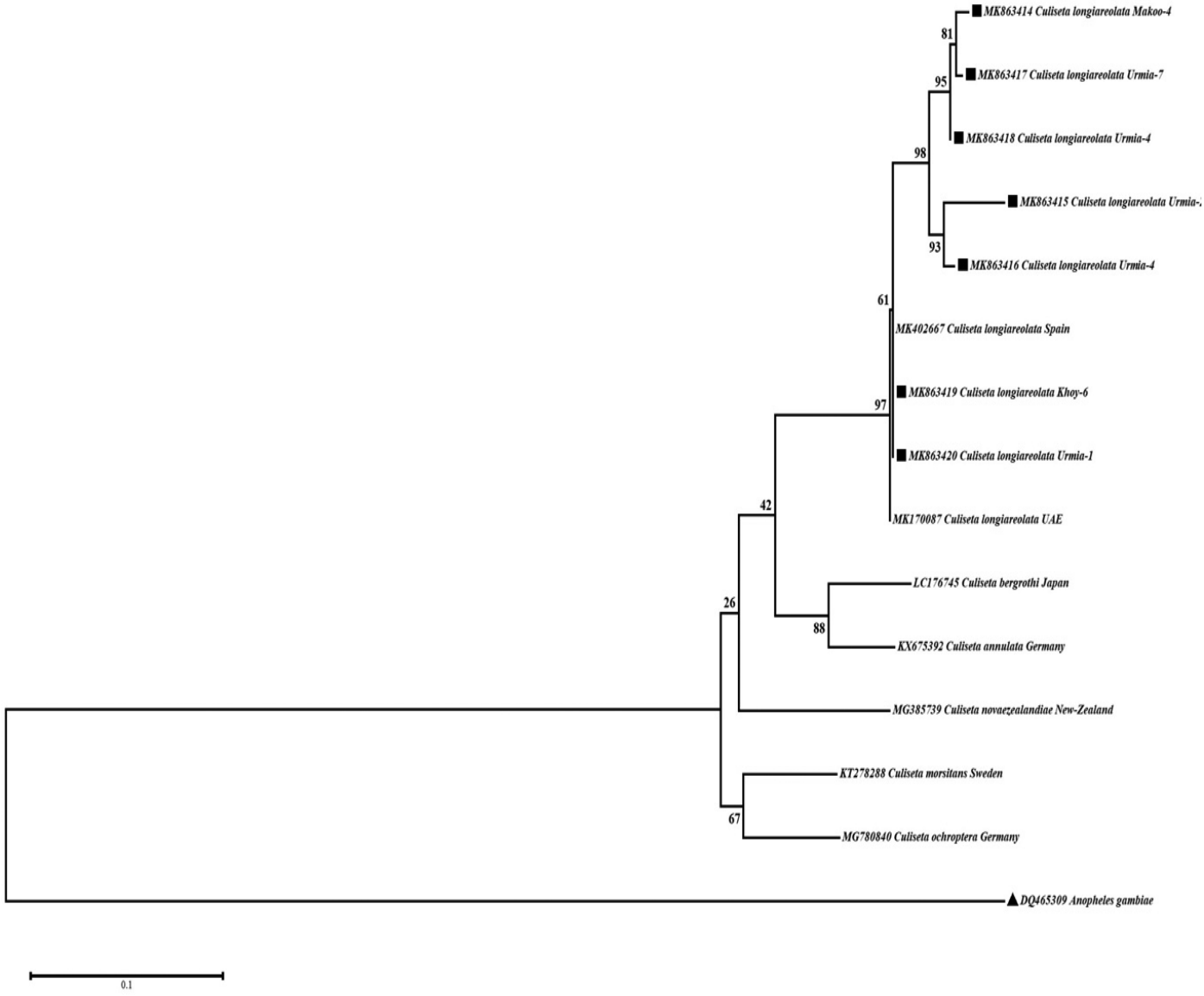

Figure 2. Molecular Phylogenetic analysis of COI fragment of *Cs. longiareolata*, by Maximum Likelihood method. The tree with the highest log likelihood (-3133.8388) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) 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. The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. ■ indicates the sequences of current study, ▲ *Anopheles gambiae* was used as outgroup

COI: Cytochrome oxidase I

42% of their samples clustered together with their conspecifics by utilizing the Neighbor Joining technique (28). Numerous other studies have reported ineffectiveness of COI (29,30). Comparatively, there are many reports of the effectiveness of COI as an effective complementary identification tool for mosquito species (31,32). Differences in reports of efficacy of COI, may be attributed to differences in the efficiency of this marker in different taxa and even species.

An important point in the present study is the remarkable consistency of the studied two markers in showing notable genetic diversity in different populations of *Cs. longiareolata*.

However, the results of the present study regarding *Cs. longiareolata* could be a weak confirmation of the possibility of finding a species complex, but conclusive conclusion about this species is not possible due to the small sample size in the present study. The results of the present study could be a prelude to further studies, at a wider scale, with larger sample sizes of different populations with a wider geographical distribution of *Cs. longiareolata*, using different markers as well as non-molecular studies. Also complementary studies could be useful for analyzing the intraspecific variation among different populations, as it was seen between Moallem and Ghahramanloo populations in current study. Conducting such studies can determine the exact classification status of this species.

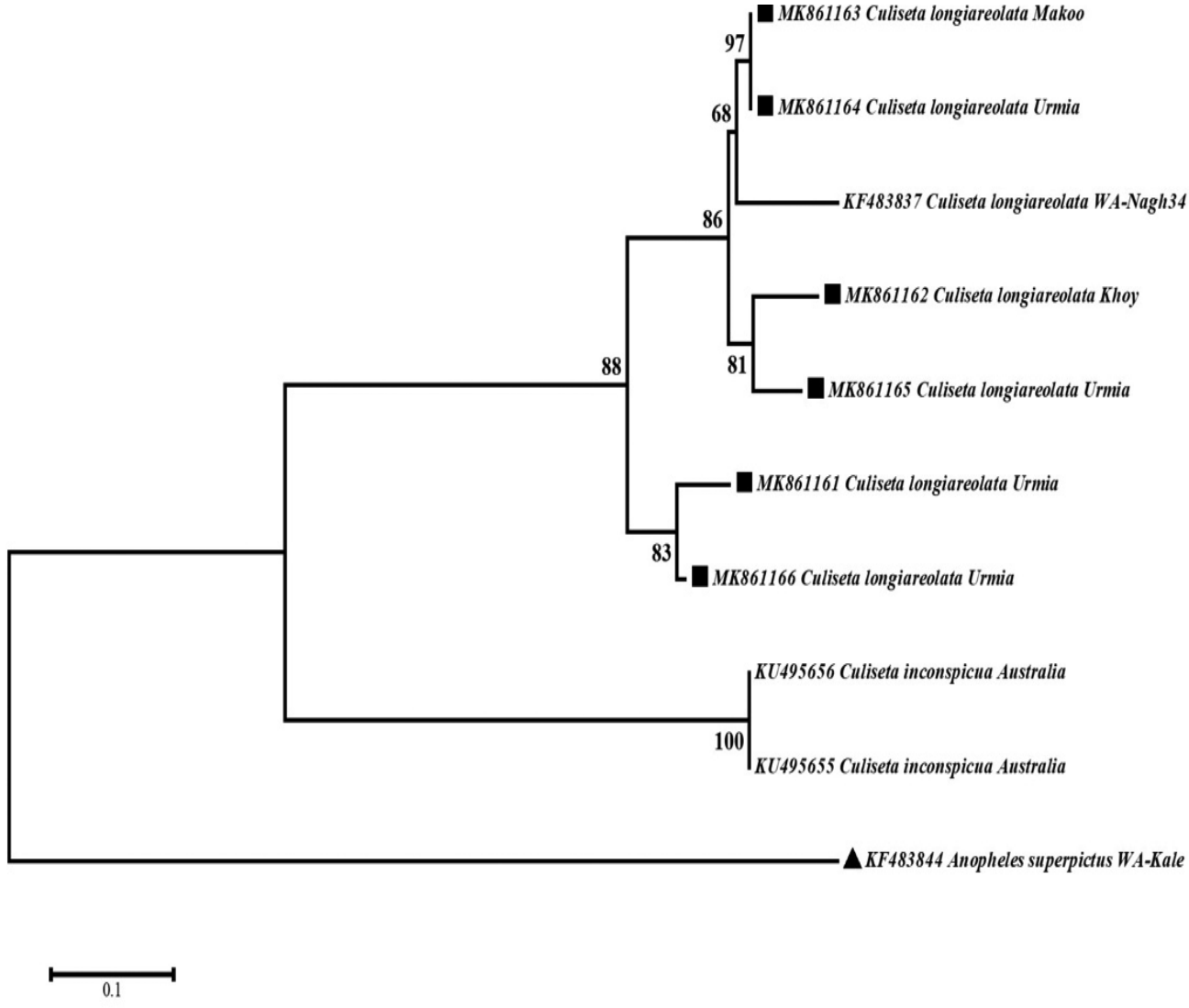

Figure 3. Molecular Phylogenetic analysis of ITS2 of *Cs. longiareolata*, by Maximum Likelihood method. The tree with the highest log likelihood (-579.0416) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) 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. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset. ■ indicates the sequences of current study, ▲ *Anopheles superpictus* was used as outgroup

ITS2: Internal transcribed spacer 2

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* Ethics

Ethics Committee Approval: The ethics committee of Urmia University of Medical Sciences approval (IR.UMSU.REC.1397.265) was obtained.

Informed Consent: Not applicable.

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* Authorship Contributions

Concept: A.R.C., Design: S.S., M.V., A.R.C., Data Collection or Processing: S.S., M.M.B., Analysis or Interpretation: M.V., M.M.B., A.R.C., Literature Search: S.S., M.M.B., Writing: S.S., M.V., M.M.B., A.R.C.

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