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Original Article

Phylogenetic Analysis of the Oriental-Palearctic-Afrotropical Members of *Anopheles* (Culicidae: Diptera) Based on Nuclear rDNA and Mitochondrial DNA Characteristics

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SUMMARY: The phylogenetic relationships of *Anopheles* spp. at the junction of Oriental, Palearctic, and Afrotropical regions in the Iranian plateau were investigated using molecular markers. A 711-bp mitochondrial DNA cytochrome oxidase C subunit I (COI) fragment and the entire second internal transcribed spacer (ITS2) region (286–576 bp) of the nuclear ribosomal DNA (rDNA-ITS2) were sequenced from 14 and 28 taxa, respectively. The analyses included 12 species within Anopheles and 4 within the Myzorhynchus Series of the subgenus *Anopheles*, 8 within Neocellia, 6 within Myzomyia, 3 within Paramyzomyia, and 1 within the Pyretophorus Series of the subgenus *Cellia*. The congruent tree topologies of both molecular markers strongly supported monophyly of subgenera *Anopheles* and *Cellia*. Phylogenetic trees constructed on the basis of ITS2 sequences could accurately categorize all of the series according to the classical taxonomy but could not distinguish Pyretophorus (*Anopheles subpictus*) from Paramyzomyia Series. Although sequence data of the COI region were available for only 14 species, the inferred trees revealed good classification among the series but could not show the monophyletic relationship of *Cellia* spp. Except for a few cases, the tree inferred from ITS2 sequences revealed the best classification for the species studied. The molecular data could significantly improve our understanding of the phylogenetic position of the taxa.

INTRODUCTION

Malaria is one of the most important infectious diseases in the world, with approximately 250 million clinical cases and over 600,000 deaths per year (1), caused by *Plasmodium* parasites (*P. falciparum, P. vivax, P. malariae*, and *P. ovale*) transmitted by approximately 70 species of *Anopheles* mosquitoes (2).

Anopheles mosquitoes are composed of 7 subgenera: *Anopheles* (189 species), which is distributed nearly worldwide; *Cellia* (239 species) Theobald, which is restricted to the Old World tropics; and the 4 Neotropical subgenera *Lophopodomyia* Antunes (6 species), *Kerteszia* Theobold (12 species), *Nyssorhynchus* Blanchard (33 species), and *Stethomyia* Theobald (5 species); and *Baimaia* (3–5). The subgenera *Anopheles, Cellia*, and *Nyssorhynchus* are further divided into hierarchical systems of informal taxonomic categories that include sections, series, groups, subgroups, and complexes (4).

The subgenus *Anopheles* includes 2 sections, each divided into 3 series with different species numbers (Table 1). The subgenus *Cellia* (mainly Oriental and also Afrotropical) includes 239 formally recognized species and a growing number of unnamed members of sibling species complexes, which are divided among 6 series (Table 1). Members of the Anopheles Series of the subgenus *Anopheles* and Myzomyia and Neocellia Series of the subgenus *Cellia* comprise species that are morphologically very similar (4). The taxa of *Cellia* and *Anopheles* subgenera occur in the oriental regions of Southern, Western, and Central Asia of the Palearctic region. Some taxa of these 2 subgenera such as *An. maculipennis s.l., An. sacharovi, An. culicifacies s.l., An. stephensi, An. fluviatilis s.l.*, and *An. superpictus s.l.* are the most important malaria vectors in Central Asia, including the Iranian plateau.

Iran, particularly the southeast part, is the junction for 3 different zoological regions, Afrotropical, Palearctic, and Oriental, where a number of different *Anopheles* spp. are found; the recent surveys of Iranian *Anopheles* identified at least 24–28 species (6,7).

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Table 1. Internal classification of genus *Anopheles*

Subgenus	Section	No. species	Series	No. species	Geographical distribution
Anopheles	Angusticorn	94	Anopheles	87	Old and New World
			Cyclolepteron	2	Neotropical
			Lophoscelomyia	5	Oriental
	Laticorn	95	Arribalzagia	24	Neotropical
			Christya	2	Afrotropical
			Myzorhynchus	69	Old World
Cellia	NA	239	Cellia	6	Afrotropical
			Myzomyia	69	Afrotropical, Mediterranean and Oriental
			Neocellia	33	Mainly Oriental, also Afrotropical
			Neomyzomyia	99	Afrotropical, Oriental, Australasian
			Paramyzomyia	6	Mainly Mediterranean, also east African and north Indian
			Pyretophorus	24	Oriental, Afrotropical

Adapted from Harbach 2004 (4).
NA, not applicable.

New species, or members of species complexes, have been added to the Iranian mosquito checklist; mostly members of species complexes such as *An. superpictus* X, Y, and Z (8,9) and *An. fluviatilis* T, U, and V (10–12) that are defined by DNA sequence data have been added to the checklist.

In total, 34 *Anopheles* spp. have been reported in the country, although some of them, including *An. martinius* of the Maculipennis Group, *An. cinereus, An. nigerrimus*, and *An. rhodesiensis rupicola*, have not been reported in recent years and not been included in the latest checklist. The classification of the 34 Iranian *Anopheles* spp. is shown in Table 2.

Among them, *An. (Cellia) stephensi, An. (Cellia) culicifacies s.l., An. (Cellia) fluviatilis s.l., An. (Cellia) superpictus s.l., An. (Anopheles) maculipennis s.l., An. (Anopheles) sacharovi*, and *An. (Cellia) dthali* are proven vectors of human malaria in the country (13–25). In addition, *An. (Cellia) pulcherrimus* is a suspected vector of malaria in southeastern Iran (26).

Recent developments in the field of DNA-based techniques, particularly PCR direct sequencing, have provided useful data for determining the phylogenetic relationships among numerous *Anopheles* spp. The sequences of the second internal transcribed spacer (ITS2) of ribosomal RNAs (rRNAs) and the cytochrome oxidase subunit I (COI) are the most widely used regions of the nuclear and mitochondrial genomes, respectively, to identify genetic variations and infer phylogenetic relationships. In addition to providing phylogenetic information, the ITS2 locus has become an increasingly popular tool for developing a diagnostic marker to differentiate between anopheline species, while mitochondrial DNA (mtDNA) markers have been widely used for many phylogenetic studies (19,27,28). Among the mitochondrion genes, the COI gene has been extensively used for phylogenetic analysis by itself or in combination with nuclear genes and has proven to be phylogenetically highly informative in many insect groups. DNA barcoding, a short standardized fragment of the mtDNA COI gene for accurate species identification, was first advocated in 2003, and since then, barcoding has proven useful for species delineation in a number of insect taxa (29,30), including mosquitoes (31–34).

In the present study, we aimed to develop phylogenetic topologies for the Iranian plateau Palearctic-Oriental-Afrotropical members of the *Anopheles* and *Cellia* subgenera of the genus *Anopheles* using the sequences of ITS2 and COI regions to infer evolutionary histories. Furthermore, the establishment of suitable molecular markers will be an important step toward our understanding of the molecular phylogeny within *Anopheles*.

MATERIALS AND METHODS

Sample collection and identification: The specimens used in this study, which were collected from 2005 to 2011, originated from various localities of different provinces of Iran. Various collection methods were employed, including aspirating mosquitoes indoors from human dwellings and cattle sheds and outdoors from pit shelters, pyrethrum spray space in human dwellings, and cattle sheds plus night collections on human and cattle baits. Larval collection from aquatic habitats was performed using the standard larval dipping technique. All specimens were identified using an Iranian *Anopheles* morphological key (35) and then stored at −20°C for further molecular investigation.

PCR, sequencing, and phylogenetic analysis: Genomic DNA was extracted from individual mosquitoes using the Qiagen DNA extraction kit, following the protocols recommended by the manufacturer (Qiagen, Hilden, Germany). The COI region of approximately 711 bp was amplified and sequenced using the primers (Table 3) and thermal cycling conditions explained previously (32,36). The variable expansion region of the ITS2 rDNA was amplified and sequenced using the thermal cycling program and primers reported by Beebe and Saul (37) (Table 3).

The PCR products were visualized on a UV transilluminator following electrophoresis on a 1.2% agarose gel containing ethidium bromide. The PCR products were purified from seized gels according to the supplier's guidelines (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and used directly for DNA sequencing in an automatic sequencer (Seqlab, Göttingen, Germany). The sequences were generated in both direc-

Table 2. Details of *Anopheles* spp. analyzed in this study and their sequence accession numbers

No.	Subgenus	Series	Species	ITS2		COI
				GenBank ID	Length (bp)	GenBank ID
1	*Anopheles*	Anopheles	*An. plumbeus*	JQ928897	335	JF966740
2	*Anopheles*	Anopheles	*An. sacharovi*	AY114208	314	KM389466
3	*Anopheles*	Anopheles	*An. maculipennis*	AY137814	292	JF966746
4	*Anopheles*	Anopheles	*An. messeae*	AY648996*	305	NA
5	*Anopheles*	Anopheles	*An. martinius*	NA	NA	NA
6	*Anopheles*	Anopheles	*An. labranchiae*	AY253849*	305	NA
7	*Anopheles*	Anopheles	*An. melanoon*	AM271001*	302	NA
8	*Anopheles*	Anopheles	*An. persiensis*	AY137844	286	NA
9	*Anopheles*	Anopheles	*An. atroparvus*	AY634532*	307	NA
10	*Anopheles*	Anopheles	*An. claviger*	AY129232*	346	JF966742
11	*Anopheles*	Anopheles	*An. algeriensis*	NA	NA	NA
12	*Anopheles*	Anopheles	*An. marteri*	NA	NA	NA
13	*Anopheles*	Myzorhynchus	*An. pseudopictus*	GU478907	430	KM389468
14	*Anopheles*	Myzorhynchus	*An. hyrcanus*	GU478906	430	JF966743
15	*Anopheles*	Myzorhynchus	*An. peditaeniatus*	AF543862*	451	NA
16	*Anopheles*	Myzorhynchus	*An. nigerrimus*	NA	NA	NA
17	*Cellia*	Myzomyia	*An. apoci*	AY445826*	404	JF966747
18	*Cellia*	Myzomyia	*An. dthali*	JF966738	380	KM389470
19	*Cellia*	Myzomyia	*An. rhodesiensis*	NA	NA	NA
20	*Cellia*	Myzomyia	*An. sergentii*	AY533851*	423	NA
21	*Cellia*	Myzomyia	*An. culicifacies A*	JF966735	370	JF966744
22	*Cellia*	Myzomyia	*An. fluviatilis T*	GQ926591	379	JF966741
23	*Cellia*	Myzomyia	*An. fluviatilis U*	GQ926589	379	NA
24	*Cellia*	Myzomyia	*An. fluviatilis V*	DQ344526*	379	NA
25	*Cellia*	Neocellia	*An. moghulensis*	JQ928806	378	KM389469
26	*Cellia*	Neocellia	*An. superpictus X*	AY941117	357	JF966745
27	*Cellia*	Neocellia	*An. superpictus Y*	AY941112	378	NA
28	*Cellia*	Neocellia	*An. superpictus Z*	AY941111	378	NA
29	*Cellia*	Neocellia	*An. pulcherrimus*	AY515172*	354	JF966748
30	*Cellia*	Neocellia	*An. stephensi*	EU346652*	471	JF966739
31	*Cellia*	Paramyzomyia	*An. multicolor*	AY564229*	547	NA
32	*Cellia*	Paramyzomyia	*An. turkhudi*	AY456391*	399	KM389467
33	*Cellia*	Paramyzomyia	*An. cinereus*	NA	NA	NA
34	*Cellia*	Pyretophorus	*An. subpictus*	GQ870337*	576	NA
35	NA	NA	*Culex pipiens*	JQ958369	336	JQ958372

Length (bp) of ITS2 sequences for each taxon is shown. The COI barcode sequences are all 670 bp. The sequences marked with an asterisk (*) were acquired from GenBank. NA indicates not successful in acquiring sequence. *Culex pipiens* was used as an outgroup.

Table 3. Details of the primers used in this study

Primer	Sequence 5′–3′	Mer (bp)	PCR size (bp)
5.8S (Forward)	TGTGAACTGCAGGACACATGAA	22	Variable 370–690
28S (Reverse)	ATGCTTAAATTAGGGGGTAGTC	22	
COI-F (Forward)	GGTCAACAAATCATAAAGATATTGG	25	711
COI-R (Reverse)	TAAACTTCAGGGTGACCAAAAAATCA	26	

tions, and the resultant chromatograms were edited and aligned using ClustalX (38). Homology of the sequences with GenBank sequences was assessed using FASTA search (http:/www.ebi.ac.uk/fasta33/). Consensus sequences for the ITS2 and COI regions were deposited in the GenBank database (Table 2). For species without samples in the present study, relevant sequences were acquired from public databases (http://www.ncbi.nlm). Owing to their different lengths, all sequences used for alignment were trimmed to obtain a consistent region. For the ITS2 region, the start and end points of the locus were determined using the Hidden Markov Models of the flanking 5.8 and 28S rRNA regions (39,40). Pairwise sequence divergence, using Kimura's 2-parameter distance algorithm, and neighbor joining (NJ) plus maximum parsimony (MP) trees presented herein were processed in MEGA 5 (41). The robustness of all phylogenetic trees was tested with a bootstrapping value in MP and NJ. The ITS2 and COI sequences of *Culex pipiens* were acquired and used as an outgroup (Table 2). For some species, there were no available data for the ITS2 and COI regions (Table 2).

RESULTS

ITS2 sequence analysis: The ITS2 region from samples of 15 taxa were successfully amplified, sequenced, and aligned together with ITS2 sequences of 13 other species obtained from GenBank (Table 2). Owing to the lack of successful collection or preserved specimens, the sequence data for *An. martinius, An. algeriensis, An. marteri, An. nigerrimus, An. rhodesiensis*, and *An. cinereus* could not been obtained. Moreover, there were no available data for these species in GenBank (Table 2). The PCR products and the region sequenced comprised a portion (18–139 bp) of the 5.8S rRNA gene, the entire region of ITS2 (286–576 bp), and a portion (17–59 bp) of the 28S rRNA gene. Owing to different lengths of the ITS2 sequences, the beginning and end of all sequences used for alignment were trimmed to obtain a consistent region. Therefore, the corresponding regions of the 5.8S rRNA gene (18 bp) and 28S rRNA gene (17 bp) were used, whereas the lengths of the ITS2 sequences were variable across the different taxa. The entire ITS2 region ranged from 286 bp for *An. persiensis* to 576 bp for *An. subpictus* (Table 2). The alignment of the ITS2 sequences showed a huge number of mutations (substitutions and indels) among the species. For species of the subgenus *Anopheles*, there were 422 (86%) polymorphic sites, with 384 (92%) were parsimony-informative sites. The alignment of the ITS2 sequences for *Cellia* spp. revealed 415 polymorphic sites (86%), with 381 (92%) parsimony-informative sites.

For the COI region, the sequences used for alignment and phylogenetic analysis were trimmed to 670 bp at the 5′ end of the COI gene. There were no indels in the COI alignment of the species studied, including both *Anopheles* and *Cellia* subgenera, but 207 (30%) sites were variable, of which 151 (73%) were parsimony-informative.

Molecular phylogenetic analysis: The phylogenetic trees constructed from the ITS2 sequences clearly separated the 2 subgenera *Anopheles* and *Cellia*. The tree could distinguish all series according to the classical taxonomy but could not differentiate *An. subpictus* of Pyretophorous Series from *An. turkhudi* and *An. multicolor* of Paramyzomyia Series (Fig. 1). *An. apoci* was clustered with *An. sergentii* and *An. dthali* of the Myzomyia Series of the subgenus *Cellia*. Four of 5 different series of *Cellia* were well classified, and all showed a monophyletic topology. Most of the nodes in the ITS2 tree were supported by bootstrap values with >50% of 1,000 replicates. In contrast to the morphological trees, all 9 species of Anopheles Series were clustered together according to the classical taxonomy. The species of Myzorhynchus Series formed a single major branch and were associated with those of Anopheles Series.

Although sequence data of the COI region were available only for a subset of the species (n = 14), the inferred trees did not support the monophyletic condition of the 2 main subgenera *Anopheles* and *Cellia* (Fig. 2). *An. claviger* and *An. plumbeus* of the Anopheles Series of the subgenus *Anopheles* showed a close relationship with *Cellia* spp. The inferred tree showed unexpected relationships between Neocellia (*An. pulcherrimus*) and Myzomyia (*An. dthali*) or Neocellia (*An. moghulensis*) and Paramyzomyia (*An. turkhudi*) Series (Fig. 2). In contrast to the ITS2 tree, *An. apoci* was clustered with the subgenus *Anopheles* spp. Except for 2 nodes, the bootstrap support values of all nodes in the tree were <50%. The combined analysis of COI and ITS2 sequence data revealed tree topologies that matched very well with those of the classical morphological taxonomy with 2 main clades for *Cellia* and *Anopheles* subgenera, but positioned *An. moghulensis* in-between the *Cellia* and *Anopheles* subgenera (Fig. 3). Different species

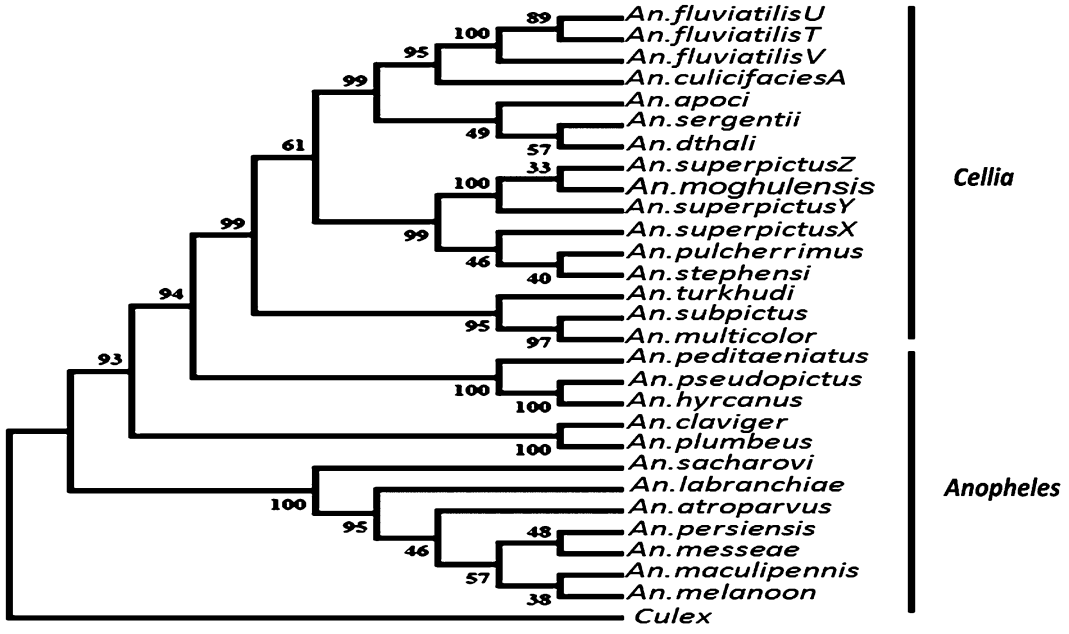

Fig. 1. Phylogenetic relationships from maximum parsimony analyses based on DNA sequences of ITS2 rDNA region for 28 Iranian *Anopheles* spp. *Culex pipiens* was used as an outgroup. Bootstrap values are indicated ant nodes.

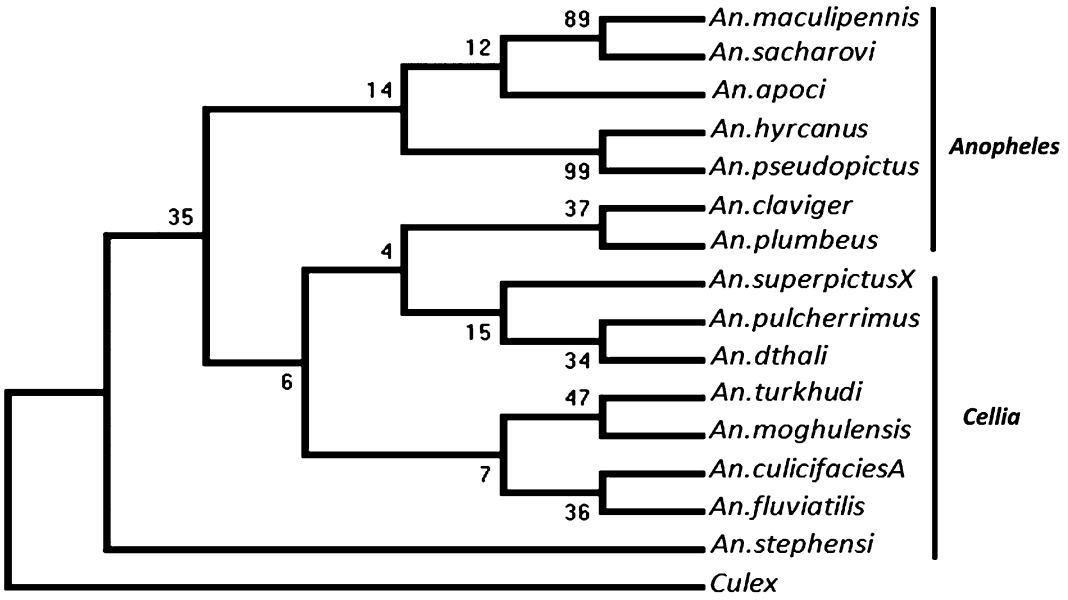

Fig. 2. Phylogenetic relationships from maximum parsimony analyses based on DNA sequences of mtDNA COI region for 15 Iranian *Anopheles* spp. *Culex pipiens* was used as an outgroup. Bootstrap values are indicated ant nodes.

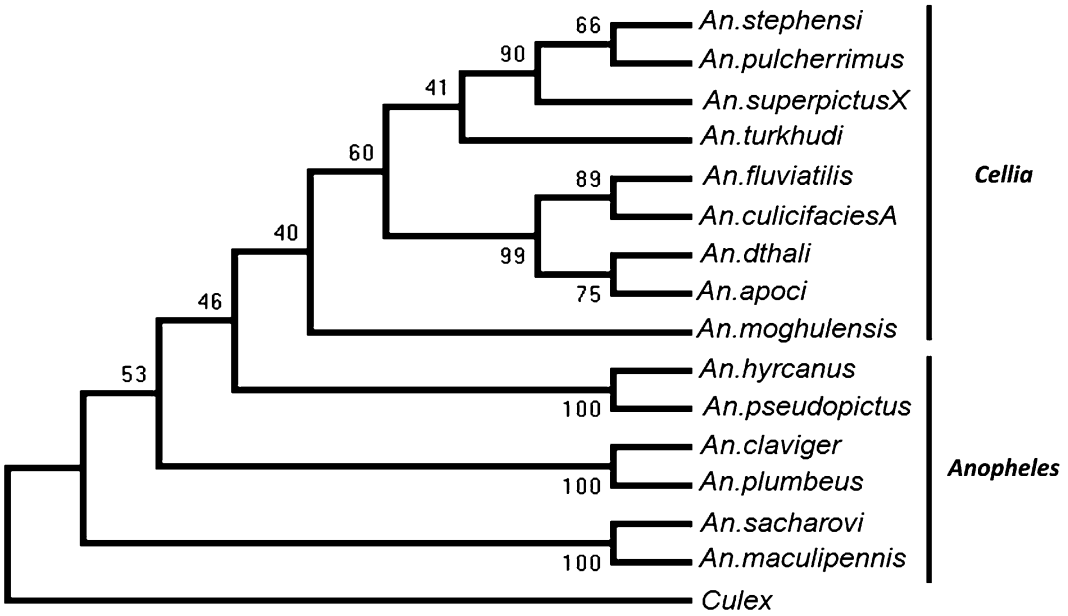

Fig. 3. Phylogenetic relationships from maximum parsimony analyses for combined DNA sequences of mtDNA COI and ITS2 rDNA regions for 15 Iranian *Anopheles* spp. *Culex pipiens* was used as an outgroup. Bootstrap values are indicated ant nodes. Only taxa with both sequences available were included.

within the series of *Cellia* or *Anopheles* were clustered together accurately, and except for a few cases, the bootstrap support values of most nodes in the tree were >60%.

DISCUSSION

The objective of the present study was to evaluate 2 molecular markers in order to infer evolutionary relationships among different species of the Anopheles and Myzorhynchus Series of the subgenus *Anopheles* and Myzomyia, Neocellia, Paramyzomyia, and Pyretophorus Series of the subgenus *Cellia* prevalent in Iran, the cross point of Oriental, Palearctic, and Afrotropical regions.

Both *Cellia* and *Anopheles* subgenera include a number of isomorphic and sibling species complexes such as the *An. maculipennis* complex, *An. hyrcanus* complex, *An. fluviatilis* complex, and *An. culicifacies* complex, each comprising several members.

Some species complexes such as the *An. fluviatilis* complex are recently diverged, and a few genetic polymorphisms have accumulated within the species (11). Therefore, selection of appropriate genes for analyzing phylogenetic relationships between these closely related taxa is important. The selected genes should have ade-

quate variable regions capable of classifying sibling or closely related species. In the present study, nuclear ITS2 locus and mitochondrial COI gene were selected, among which the former is the most variable region of the nuclear rDNA and the latter has been highly informative in many insect groups. Comparison of rDNA ITS2 and mtDNA COI utilities for the correct classification of the species revealed that ITS2 is favored on the COI gene. This finding is in agreement with the view of other researchers indicating that ribosomal gene, including ITS2, are more useful in molecular systematics of various taxa, including Anopheles (42–44). This locus has also been used in barcoding and DNA array technologies (45,46). Mohanty et al. inferred phylogenetic relationships among the species of the subgenus *Cellia* using the mitochondrial genes COI and COII and the rRNA gene (D3 and ITS2 regions). They showed that the D3 region of the 28S rRNA gene and the ITS2 region of rDNA were favored on the COI and COII regions of mtDNA in achieving the correct arrangement of the taxa and matching with that of the morphological classification, which may be owing to the variable degree of the rate of evolution of the different genes within the organism (47). In the present study, the phylogenetic tree inferred from ITS2 rDNA revealed the correct arrangement of 28 various anopheline taxa as per the traditional morphological classification of anophelines at the subgenera and series levels.

The confusing situation of *An. apoci* is discussable, which has been shown in the morphological key of Iranian *Anopheles* (35). The molecular trees based on DNA of ITS2 and the combined (ITS2 + COI) loci showed the association of *An. apoci* with the subgenus *Cellia* clade, whereas the tree inferred from COI alone revealed a significant association between this species and the Maculipennis Group of the subgenus *Anopheles*. Thus, ITS2 and COI trees do not conform to each other and support the results of phylogenetic analysis inferred from larval and adult morphological characteristics, respectively. To resolve the correct systematic of *An. apoci*, further molecular data from different genes are required.

An. subpictus is a member of the Pyretophorus Series of the subgenus *Cellia*, which was found to be associated with *An. multicolor* and *An. turkhudi*, members of the Paramyzomyia Series of the subgenus *Cellia*, in the phylogenetic tree inferred from the ITS2 sequences. Besides *An. multicolor* and *An. turkhudi*, *An. stephensi* and *An. superpictus*, members of the Neocellia Series of the subgenus *Cellia*, were the closest species to *An. subpictus*. This phenomenon was also noted in another phylogenetic analysis using 18S and 28S rDNA data sets, which showed close affinity of *An. subpictus* to *An. stephensi* (3).

The phylogenetic relationships between the members of Myzomyia and Neocellia Series are always problematic. In the present study, only ITS2 was phylogenetically informative and could distinguish the species of these 2 series from each other. This classification was also observed when D3 rDNA sequence data were used for phylogenetic analysis of the members of Myzomyia and Neocellia Series present in the Indian subcontinent (48). However, the number and diversity of species used in that study are less than those employed in the present study.

In conclusion, the present study investigated the molecular characteristics (rDNA ITS2 and mtDNA COI) of diverged species belonging to 6 series and 2 subgenera of the genus *Anopheles*. Such studies not only increase our understanding of anophelines taxonomy but also provide a platform for investigating different epidemiological phenomena such as vector prevalence and species divergence.

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Conflict of interest None to declare.

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