Review Article
Utility of Complete Mitochondrial Genomes in Phylogenetic Classification of the Species of Anopheles (Culicidae: Anophelinae)

Taghi Ghassemi-Khademi¹; Mohammad Ali Oshaghi²; Hassan Vatandoost²,³; *Seyed Massoud Madjdzadeh⁴; *Mohammad Amin Gorouhi⁵,⁶

¹Department of Biology, Faculty of Sciences, Shiraz University, Shiraz, Iran
²Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
³Department of Chemical Pollutants and Pesticides, Institute for Environmental Research, Tehran University of Medical Sciences, Tehran, Iran
⁴Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran
⁵Department of Vector Biology and Control, School of Public Health, Kerman University of Medical Sciences, Kerman, Iran
⁶Research Center of Tropical and Infectious Diseases Kerman University of Medical Sciences, Kerman, Iran

*Corresponding authors: Dr Seyed Massoud Madjdzadeh, E-mail: madjdzadeh@uk.ac.ir, Dr Mohammad Amin Gorouhi, E-mail: amin_gruhi@yahoo.com

(Received 30 Dec 2019; accepted 30 Mar 2021)

Abstract
Background: Among the blood-sucking insects, Anopheles mosquitoes have a very special position, because they transmit parasites of the genus Plasmodium, which cause malaria as one of the main vector-borne disease worldwide. The aim of this review study was to evaluate utility of complete mitochondrial genomes in phylogenetic classification of the species of Anopheles.

Methods: The complete mitochondrial genome sequences belonging to 28 species of the genus Anopheles (n=32) were downloaded from NCBI. The phylogenetic trees were constructed using the ML, NJ, ME, and Bayesian inference methods.

Results: In general, the results of the present survey revealed that the complete mitochondrial genomes act very accurately in recognition of the taxonomic and phylogenetic status of these species and provide a higher level of support than those based on individual or partial mitochondrial genes so that by using them, we can meticulously reconstruct and modify Anopheles classification.

Conclusion: Understanding the taxonomic position of Anopheles, can be a very effective step in better planning for controlling these malaria vectors in the world and will improve our knowledge of their evolutionary biology.

Keywords: Anopheles; Phylogeny; DNA, mitochondrial; Taxonomy; Malaria vectors

Introduction

Among a large number of insect species, only relatively few species feed on blood that attracts our attention (1). Blood-sucking insects cause very serious damages to humans and livestock. One way this happens is through the transmission of a large number of parasites. For example, it was estimated that trypanosomiasis in cattle cost the agriculture industry 5 billion United States dollars (USD) in one year (2). Some nuisance blood-sucking insects es-
blood-sucking insects, *Anopheles* mosquitoes have a very special position, because they transmit parasites of the genus *Plasmodium*, which cause malaria in humans in endemic areas. For example, *Anopheles gambiae* is one of the best known, because it is a vector of the most dangerous malaria parasite species to humans, *Plasmodium falciparum* (3).

Some species of *Anopheles*, are vectors for canine heartworm *Dirofilaria immitis*, also transmit *Wuchereria bancrofti* and *Brugia malayi* as filariasis -causing species and viruses such as o'nyong'nyong virus (ONNV) that causes O'nyong'nyong fever (4). The *Anopheles* genus contains 465 mosquito species belonging to seven subgenera. The most important taxa include *Anopheles* (cosmopolitan, 182 species), *Baimaia* (distributed in the Oriental, one species), *Cellia* (distributed in the Old World, 220 species), *Kerteszia* (12 species), *Lophodemomyia* (six species), *Nyssorhynchus* (39 species) and *Stethomyia* (five species), the last four taxa distributed in the Neotropical region (5, 6). The following species in this research were studied:

**Anopheles (Cellia) gambiae Giles, 1902**

*Anopheles gambiae* is a very important malaria vector throughout Africa south of the Sahara. This species, probably transmits some arboviral diseases, also it is a major filariasis vector and for this reason, it has received serious attention from entomologists (2). Also, *An. christyi* is not a malaria vector but is a closely related species to the *Anopheles gambiae* complex (7, 8).

**Anopheles (Cellia) arabiensis Patton, 1905**

This species is found widely distributed in Africa but shows a high preference for drier areas. Despite being a very important vector of malaria, it is not an important filariasis vector (8).

**Anopheles (Cellia) melas Theobald, 1903**

This species is found along the west African coast and it breeds in brackish waters. This species feeds more readily and regularly on man and it is a very important vector of both malaria and filariasis, especially in coastal areas (2, 8). This species act as a secondary vector of malaria in the same regions that *An. gambiae* or *An. arabiensis* occur. As mentioned, this species can play an important role in malaria transmission in coastal areas where it occurs in very high densities (9).

**Anopheles (Cellia) merus Dönitz, 1902**

This species is found in the east of Africa (8, 10) and it has an important role in the transmission of malaria along the Tanzanian coast (11) and more recently in Mozambique (12).

**Anopheles (Cellia) dirus species complex**

A document stated: “The danger from *An. dirus* is not only that it is very resistant to control within its habitat but that it is an extraordinarily efficient vector, so long-lived and anthropophilic that only a small population is necessary to maintain high malaria endemicity” (13). Generally, it is a very efficient vector for malaria (14).

**Anopheles (Cellia) farauti species complex and Anopheles hinesorum**

*Anopheles farauti* and *An. hinesorum* play an important role in malaria transmission. *Anopheles farauti* acts as an important vector of malaria in the Solomon Islands and the islands of Buka and Bougainville as well as Papua New Guinea (15). In comparison with *An. Farauti*, the species of *An. hinesorum* is restricted to locations with freshwater larval habitats (15, 16).

**Anopheles (Anopheles) atroparvus van Thiel, 1927**

*Anopheles atroparvus* previously has been found in Europe as common species with a preference for brackish water larval habitats.
But it has been found in freshwater habitats as well (9, 17).

Anopheles atroparvus is largely unable to transmit tropical strains of P. falciparum, but competent in supporting a European strain. This species is known to be involved in winter transmission of malaria at the start of the twentieth century in Britain, coastal areas in the Netherlands and Germany, (18) and other parts of Europe (19). In Portugal, An. atroparvus is the main malaria vector (20).

Anopheles (Nyssorhynchus) darlingi Root, 1926

This species is a lowland, riverine, forest-dwelling species and unable to survive in dry climates, for example, north-eastern Brazil (17). Anopheles darlingi is considered one of the most important malaria vectors in the Americas and the Neotropical region (21).

Anopheles (Cellia) minimus species complex

Anopheles minimus is a vector of malaria parasites throughout its respective distributions. This species is considered a primary and very important malaria vector in the hilly forested regions of mainland Southeast Asia (22).

Anopheles epiroticus Linton and Harbach

Anopheles epiroticus occurs most often along with the mainland coastal areas from eastern India to Thailand, southern Vietnam, and peninsular Malaysia (16). This species is a malaria vector species in southeast Asia (23).

Anopheles (Cellia) culicifacies species complex

Sibling species of Anopheles culicifacies include the species A, B, C, D, and E which are morphologically indistinguishable but there are many ecological, cytological, and behavioral differences between the members of this complex (15). The sibling species of An. culicifacies were reported from different parts of southeast Asia including Iran, Afghanistan, Pakistan, India, China (15, 24-27). Four species of this complex (A, C, D, E) have been considered as malaria vectors in India (15, 24-27).

Anopheles cracens

Anopheles cracens (=An. dirus B) is distributed in southern Thailand, Terengganu, Perlis, and Indonesia. This species is present in peninsular Malaysia (28). Anopheles cracens acts as a main vector of P. knowlesi in Kuala Lumpur. Also, this species can transmit P. falciparum and P. vivax in laboratory condition (28).

Anopheles (Cellia) punctulatus species complex

Anopheles punctulatus species complex is the main malaria vector but it is not common and only reported from the island of New Guinea (15, 16).

Anopheles (Nyssorhynchus) albitarsis species complex

The An. albitarsis complex includes six species widely distributed in South American countries including Argentina, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Venezuela, Paraguay, Peru, Panama, Guyana, and French Guiana and this complex is an important malaria vector in mentioned countries (17). This complex includes six species: An. albitarsis, An. oryzalimnetes, An. marajoara, An. deaneorum, An. jancouneae and An. albitarsis F. (29). Except for An. deaneorum, species of this complex are indistinguishable based on morphological characters (29).

Anopheles homunculus, Anopheles cruzii and Anopheles bellator

Adult females of An. homunculus which act as a secondary malaria vector are very similar to An. Cruzii morphologically (30). Anopheles homunculus has been found in Colombia, Venezuela, Brazil, Bolivia, Peru, Suriname, Guyana, and Trinidad (30). In the extra-Amazonian region, especially in the states within the range of the Atlantic forest, An. cruzii and An.
*Anopheles bellator* are vectors of autochthonous malaria, in a cycle that likely involves monkeys belonging to the genera *Cebus* and *Allouata* (30).

**Anopheles (Cellia) stephensi Liston, 1901**

*Anopheles stephensi* is the main malaria vector in the Eastern Mediterranean region and south of the Asia continent as well as in the Indian subcontinent (except Nepal and Sri Lanka; 15, 16, 25, 27, 31-35).

**Anopheles (Anopheles) sinensis species complex**

*Anopheles sinensis* is a member of the Hyrcanus Group in the Myzorhynchus Series (6, 15). This species is found in China and Korea and predominantly transmit malaria in these countries. *Anopheles sinensis* also found in Afghanistan, Taiwan, Japan, and the western part of Indonesia (Sumatra and West Kalimantan) (15).

**Anopheles laneanus Corrêa and Cerqueira, 1944**

*Anopheles laneanus* was suspected to be involved in human malaria transmission (36). It belongs to Kerteszia Subgenus. It is found in areas of Serra da Mantiqueira (in south-eastern Brazil) and other Latin American countries (36, 37).

**Anopheles (Cellia) maculatus Group**

The members of *Anopheles maculatus* group have a different role in malaria transmission. *Anopheles maculatus* is recognized as the main malaria vector in some parts of India, southern Thailand, and peninsular Malaysia (15).

**Anopheles (Anopheles) quadrimaculatus Say, 1824**

*Anopheles quadrimaculatus* is a common species in the United States of America, particularly in the eastern part of the country. This species also is found in Mexico and southern Canada including Ontario and Quebec (38).

In the meantime, mitochondrial DNA (mtDNA) has been the most commonly used genetic marker for the first generation of phylogeographic investigations. The animal mitochondrial genome is a small and closed circular molecule of 15000–20000bp and is highly variable in structure, content, organization, and quality of gene expression in different animals (39). The mitochondrial genome has several properties that make it particularly attractive as a genetic marker in evolutionary and phylogenetic studies because of the relative simplicity of extraction and simple sequence organization, maternal inheritance, free of recombination in most cases and relatively rapid rate of evolution, perhaps up to 10 times faster than nuclear DNA (40, 41). Recently, several mitochondrial (mtDNA) and DNA genomes have been used to estimating phylogenetic relationships among species belonging to the genus *Anopheles* (7, 37, 42-50). Altogether, using several genomes of mtDNA is better than using a single gene for phylogenetic analysis of animals, because multiple sequences (especially complete genome of mtDNA) have sufficient information about evolution and evolutionary process reconstruction (39). Therefore, a phylogenetic reconstruction based upon a single gene or a short DNA segment is highly likely to produce an incorrect tree topology (51). Several lines of evidence show that using the complete mitochondrial genome is a robust tool in order to gain complete phylogenetic relationships among taxa while using partial mitochondrial genes is not sufficient for this purpose (48-50, 52). Considering that there was no research on the efficacy of the complete mitochondrial genomes in phylogenetic classification of *Anopheles* mosquitoes and the fact that some species of *Anopheles* mosquitoes are dangerous vectors of various diseases, including malaria, the present study evaluated the efficacy of the complete mtDNA genomes in proper separation and detecting the taxonomic and phylogenetic status of some of the species belonging to *Anopheles* genus. Understanding the
taxonomic and phylogenetic status of these species is a very effective step in better identification and planning for controlling these dangerous species of mosquitoes in the world.

Materials and Methods

The complete mitochondrial genome sequences belonging to 28 species of Anophelinae subfamily and two species of culicinae subfamily (n=35) were downloaded from NCBI (Table 1). BioEdit 7.0.5.3 software (53) was used to create a DNA sequence alignment using Clustal W algorithm (54) in the obtained sequences. Also, the corresponding mtDNA sequences of Culex pipiens pallas, Culex pipiens pipiens, and Culex quinquefasciatus were used as outgroups in the analysis. Nucleotide composition of mtDNA of studied species from Anopheles genus (n=32) and their accession numbers (n=35) is shown in Table 1. These Anopheles species belong to four subgenera of Anopheles (n=3), Cellia (n=18), Kerteszia (n=6), and Nyssorhynchus (n=5) containing various series of Pyretophorus (n=7), Neocellia (n=2), Myzomyia (n=3), Neomyzomyia (n=6), Myzorhynchus (n=1), Anopheles (n=2), Argyritarsis (n=1), Albitarsis (n=4), and sub-genus Kerteszia (n=6). The evolutionary history was inferred using the Neighbor-Joining (55), Minimum Evolution (56) and Maximum Likelihood methods. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (57). Analyses involved 35 whole mtDNA nucleotide sequences, and all positions containing gaps and missing data were eliminated. Finally, there were a total of 14647 positions in the final dataset. All of the evolutionary analyses were computed using the Kimura 2-parameter method (58) and were conducted in MEGA6 (59). Analyses were visualized and edited by FigTree software v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

The number of base differences per sequence from averaging over all sequence pairs between groups (Table 2) and within groups (Table 3) was conducted in MEGA6 (59). Analyses were conducted using the Kimura 2-parameter model (58).

Results

Phylogenetic analysis of 28 species belonging to the genus Anopheles (n=32) was performed using complete sequences of the mtDNA. The average length of the mitochondria genome was calculated 15376.1bp. In 15376.1 bp, the average base composition of mtDNA sequences was: 37.9% T, 12.7% C, 40.1% A, and 9.3% G, showing a strong AT bias (78%). In this study, each subgenus was considered as a separate group, so in addition to the outgroup, 5 groups were determined and phylogenetic distances among these groups were calculated and results are shown in Table 2. As
the results indicated, the outgroup (n=3) was at a distance far from subgenera members and this implies the close phylogenetic distances between them. The shortest distances were obtained between subgenera Anopheles and Cellia and it means that these two subgenera are phylogenetically closest subgenera together. As it was mentioned, the highest distances were obtained between the outgroup (Culex sp.) and other groups. Molecular phylogenetic trees for complete mtDNA genomes were constructed using the ML, NJ, ME, Bayesian inference methods and they showed the same topology (Figs. 1, 2, 3, 4) and three sequences from the genus Culex sp. was used as the outgroups, and they were completely separated from other groups. Three phylogenetic trees revealed a great and main clade that all of the species belonging to Anopheles, Cellia, and Nyssorhynchus subgenera formed a monophyletic clade and the species belonging to subgenus Kerteszia were located close to this group (but not inside the group). In total, the species belonging to four subgenera were separated into four distinct groups. The species belonging to subgenus Cellia constructed a monophyletic clade with the highest supported monophyly value (≥93) in all of the three phylogenetic trees. Also, the clade of subgenus Anopheles with the highest supported values (≥99) was placed next to this group. The third clade belonging to the subgenus Nyssorhynchus was formed with the highest supported value (=100) in all of the four phylogenetic trees. Also, the fourth clade belonging to the subgenus Kerteszia was formed with the highest supported value (=100). In cluster of subgenus Cellia, two distinct groups were detected. The relationships of group 1 are as follows: \{((An. arabiensis + An. gambiae + An. coluzzii) + (An. melas + An. merus)) + An. cristi + An. epiroticus)\} + \{(An. stephensi + An. Maculatus) + (An. cu-licifacies + (An. minimus (2 seq.)))\} and the relationships of group 2 are as follows: \{(An. dirus + An. cracens) + (An. hinesorum + An. punctulatus) + An. farauti (2 seq.)\}. In group 2 we have a cluster with this model \{(An. dirus + An. cracens) + (An. hinesorum + An. punctulatus) + An. farauti (2seq.))\}. In the cluster of subgenus Anopheles, one distinct clade was detected. The relationships of the species belonging to this clade are as follows: \{An. sinensis + (An. quadrimaculatus + An. atroparvus)\}. Also, in the cluster of subgenus Nyssorhynchus, one distinct group was detected. The relationships of the species belonging to this clade is as follows: \{An. darlingi + [An. deane-orum + An. janconnae + (An. oryzalimnetes + An. albitarsis)]\}. In the cluster of subgenus Kerteszia, one distinct group was detected. The relationships of the species belonging to this clade are as follows: \{An. homunculus + An. bellator + ((A. cruzii (2 seq.)) + (An. laneanus + An. cruzii))\}. The highest phylogenetic differentiation within each group was seen within Anopheles and Cellia subgenera (respectively: 0.079 and 0.089) and the least phylogenetic differentiation was found within Kerteszia and Nyssorhynchus subgenera (respectively: 0.044 and 0.042). Also, the maximum phylogenetic distance was seen between outgroups and other groups and after them, the subgenus Kerteszia was in the most phylogenetic distance with three other groups of Nyssorhynchus, Anopheles, and Cellia (respectively: 1583.8, 1584.1, and 1590.5). Likewise, the least phylogenetic distance was found between Cellia and Anopheles subgenera (Equal to 1332.4).
Table 1. Taxonomic classification and details of mtDNA genomes of 28 Anopheles species and two Culex species as outgroups retrieved from GenBank (n=35; www.ncbi.nlm.nih.gov)

| Subgenus   | Series   | Species                  | T(U) | C    | A    | G    | Total | Accession Number |
|------------|----------|--------------------------|------|------|------|------|-------|------------------|
| Cellia     | Pyretophorus | Anopheles arabiensis     | 37.5 | 13.0 | 40.1 | 9.4  | 15369.0 | NC_028212       |
|            |          | Anopheles gambiae        | 37.5 | 12.9 | 40.0 | 9.5  | 15563.0 | L20934          |
|            |          | Anopheles coluzzii       | 37.6 | 12.9 | 40.1 | 9.4  | 15441.0 | NC_028215       |
|            |          | Anopheles melas          | 37.5 | 13.0 | 40.1 | 9.4  | 15366.0 | NC_028219       |
|            |          | Anopheles merus          | 37.5 | 13.0 | 40.1 | 9.4  | 15365.0 | NC_028220       |
| Neocellia  |          | Anopheles stephensi      | 37.9 | 12.5 | 40.4 | 9.2  | 15387.0 | NC_028223       |
|            |          | Anopheles maculatus      | 37.3 | 12.9 | 40.2 | 9.6  | 14850.0 | NC_028218       |
| Myzomyia   |          | Anopheles culicifacies   | 38.1 | 12.4 | 40.4 | 9.1  | 15330.0 | NC_027502       |
|            |          | Anopheles minimus        | 38.1 | 12.5 | 40.3 | 9.1  | 15411.0 | NC_028221       |
| Neomyzomyia|          | Anopheles dirus          | 38.0 | 12.7 | 40.2 | 9.2  | 15404.0 | L20934          |
|            |          | Anopheles cracens        | 37.9 | 12.8 | 40.0 | 9.3  | 15412.0 | NC_020768       |
|            |          | Anopheles hinesorum      | 37.6 | 12.7 | 40.4 | 9.4  | 15336.0 | NC_020769       |
|            |          | Anopheles punctulatus    | 38.0 | 12.1 | 40.7 | 9.2  | 15322.0 | NC_028222       |
|            |          | Anopheles farauti        | 37.8 | 12.8 | 40.1 | 9.3  | 15359.0 | KT895423        |
|            |          | Anopheles farauti        | 37.8 | 12.8 | 40.1 | 9.3  | 15358.0 | NC_020770       |
| Anopheles  | Myzorhynchus | Anopheles sinensis       | 38.0 | 12.5 | 40.3 | 9.2  | 14988.0 | NC_028016       |
|            |          | Anopheles atroparvus     | 37.4 | 13.0 | 40.0 | 9.6  | 15458.0 | NC_028213       |
|            |          | Anopheles quadrimaculatus| 37.1 | 13.4 | 40.3 | 9.3  | 15455.0 | L04272          |
| Nyssorhynchus| Argyritarsis | Anopheles darlingi      | 38.0 | 12.5 | 40.2 | 9.4  | 15385.0 | GQ918273        |
|            |          | Anopheles deaneorum      | 37.8 | 12.8 | 39.9 | 9.4  | 15424.0 | HQ335347        |
|            |          | Anopheles jancomae       | 37.7 | 13.0 | 39.9 | 9.4  | 15425.0 | NC_030717       |
|            |          | Anopheles oryzalimnetes  | 37.8 | 12.9 | 39.9 | 9.3  | 15422.0 | NC_030715       |
|            |          | Anopheles albitarsis     | 37.8 | 13.0 | 39.9 | 9.4  | 15413.0 | HQ335344        |
| Kerteszia  |          | An. cruzii               | 38.6 | 12.5 | 40.0 | 9.0  | 15472.0 | KU551289        |
|            |          | Anopheles laneanus       | 38.4 | 12.6 | 39.9 | 9.1  | 15446.0 | NC_030250       |
|            |          | Anopheles homunculus     | 38.7 | 12.5 | 39.9 | 8.9  | 15738.0 | NC_030248       |
|            |          | Anopheles bellator       | 38.3 | 13.0 | 39.9 | 8.8  | 15668.0 | NC_030249       |
|            |          | Anopheles cruzii         | 38.5 | 12.6 | 39.9 | 9.0  | 15449.0 | NC_027470       |
|            |          | Anopheles cruzii         | 38.6 | 12.4 | 39.9 | 9.1  | 15478.0 | KU551284        |
| Outgroups  |          | Culex pipiens pipiens   | 37.9 | 12.7 | 40.1 | 9.3  | 15376.1 |               |
|            |          | Culex quinquefasciatus   |       |      |      |      |       | http://jadh.tums.ac.ir |
|            |          | Culex pipiens pallens   |       |      |      |      |       | Published Online: March 31, 2021 |

Table 2. Genetic distances between subgenera of the genus Anopheles based on complete mitochondrial sequences

|          | Nyssorhynchus | Anopheles | Cellia | Kerteszia | Outgroup |
|----------|---------------|-----------|--------|-----------|----------|
| Nyssorhynchus| **** | 1378.9 | **** |
| Anopheles  | 1431.2 | 1332.4 | 1590.5 | **** |
| Cellia     | 1583.8 | 1584.1 | 1983.3 | 2121.4 | **** |
| Kerteszia  | 1999.1 | 1960.4 | 1960.4 | 1960.4 | **** |
| Outgroup   | 1999.1 | 1960.4 | 1960.4 | 1960.4 | **** |
Table 3. Estimates of average evolutionary divergence over sequence pairs within groups of *Anopheles* genus

| Group Name | Average divergence within Groups |
|------------|---------------------------------|
| *Nyssorhynchus* | 0.042                           |
| *Anopheles* | 0.079                           |
| *Cellia*    | 0.089                           |
| *Kerteszia* | 0.044                           |

**Fig. 1.** Neighbor-joining tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond
Fig. 2. Minimum Evolution tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond to the bootstrap value. The tree was rooted with three *Culex* spp. mtDNA sequences.
Fig. 3. Maximum Likelihood tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond to the bootstrap value. The tree was rooted with three *Culex* spp. mtDNA sequences.
Fig. 4. Bayesian phylogeny reconstructed based on using complete mitochondrial genome sequences of 28 Anopheles species. The values besides the branches are BI posterior probability values. The tree was rooted with three Culex spp. mtDNA sequences.
Fig. 5. Phylogeny tree of 26 Anopheles species based on the Maximum Likelihood (ML) analysis of nine protein-coding genes (PCGs) located on the heavy strand (7536bp; Peng et al. 2016)
**Discussion**

As mentioned, the species belonging to four subgenera were separated into four different and distinct groups. The species belonging to subgenus *Cellia* constructed a monophyletic clade in all of the four phylogenetic trees. Also, the clade of subgenus *Anopheles* was placed next to this group. The third and fourth clade belonging to the subgenera *Nyssorhynchus* and *Kerteszia*.
Kerteszia respectively was formed with the highest supported value in all of the three phylogenetic trees.

In the cluster of subgenus Cellia, two distinct groups were detected. The relationships of group.1 are as follows: [{((An. arabiensis + An. gambiae + An. coluzzii) + (An. melas + An. merus)) + An. christyi + An. epiroticus}) + {(An. stephensi + An. Maculatus) + (An. culicifacies + (An. minimus (2 seq.))})] and the relationships of group.2 are as follows: [(An. dirus + An. cracens) + (An. hinesorum + An. punctulatus) + An. farauti (2 seq.)]. In group.1 into the cluster of subgenus Cellia, we have a cluster with this model: {((An. arabiensis + An. gambiae + An. coluzzii) + (An. melas + An. merus)) + An. christyi + An. epiroticus}) + {(An. stephensi + An. Maculatus) + (An. culicifacies + (An. minimus (2 seq.))}). It should be mentioned that An. (Cellia) coluzzii is the molecular M form of An. gambiae (64) and as indicated, is located next to this species. Also, in the group.1 into the cluster of subgenus Cellia, we have another cluster with this model: {((An. stephensi + An. maculatus) + (An. culicifacies + (An. Minimus (2 seq.))})}. Both An. culicifacies and An. minimus are classified within a single series (Myzomyia) and a single group (Funestus) (6) and in this research, they were located within a single clade. Also, An. stephensi and An. maculatus are classified within the Neocellia series (6) and in this research, they were located within a single clade. In group.2 into the cluster of subgenus Cellia, both species An. dirus and An. cracens are classified within a single series (Neomyzomyia), group (Leucosphyrus) and subgroup (Leucosphyrus) (6), for this reason, they were located within a single clade. Also, three species of An. hinesorum, An. punctulatus and An. farauti are classified within a single series (Neomyzomyia) and group (Punctulatus) (6) and were located within a single clade. The species belonging to group.1 and group.2 are completely separate from each other, so eleven species: An. arabiensis, An. gambiae, An. coluzzii, An. Melas, An. merus, An. christyi, An. epiroticus, An. stephensi, An. maculatus, An. culicifacies, An. Minimus and five species: An. dirus, An. cracens, An. hinesorum, An. punctulatus, An. farauti, have distinct location from each other in phylogenetic trees (Figs: 1, 2, 3) and this subject should be considered in the control plans of these malaria vectors. In the cluster of subgenus Anopheles, both An. quadrimaculatus and An. atroparvus are classified within a single subgenus (Anopheles), section (Angusticorn), series (Anopheles), and group (Maculipennis) and so they were located in very phylogenetic distances together. Anopheles sinensis is classified within subgenus: Anopheles, section: Laticorn, series: Myzorhynchus and group: Hyrcanus (6), so this species was separated from the two other species. Besides, the least phylogenetic distance was found between Cellia and Anopheles subgenera (Equal to 1332.4) and this suggests that these two subgenera have very close phylogenetic relationships to each other. In addition, into the cluster of subgenus Nyssorhynchus, the species belonging to the clade of {An. deaneorum + An. janconnae + (An. oryzalinnetes + An. albitaris}), are classified under: subgenus: Nyssorhynchus, section: Argyritarsis, series: Albitarsis and group: Albitarsis (6). Also, An. darlingi is classified under: subgenus: Nyssorhynchus, section: Argyritarsis, series: Argyritarsis and group: Darlingi (6), so this species was separated from the other four species. In the cluster of subgenus Kerteszia, one distinct group was detected. As already mentioned, adult females of An. cruzii and An. homunculus which are the secondary malaria vectors are not morphologically recognizable because of high morphological similarities, so it is hard to differentiate these two species (30). In this research, three sequences belonging to the An. cruzii have been used, but two sequences with accession numbers:
KU551289.1 and NC_024740.1 were located within a single clade but the third sequence (Accession number: KU551284.1) (44), constructed a single clade with An. laneanus. Most likely, this sequence sample (with sample ID: PEC_2_7, from Sao Paulo (Brazil), is another form of An. cruzii, because An. cruzii has several sibling species (42, 43). So, this sequence has to be re-examined and based on the exact comparison of its sequence with other sequences of sibling species of An. cruzii, its correct name should be determined. Overall, in all of the four phylogenetic trees, subgenus Ker-teszia was separated from three other subgenera and after outgroups, this subgenus was in the most phylogenetic distances with them. Due to these results, it is suggested that this subgenus could be introduced as an independent genus from Anopheles, which makes it easy classifying Anopheles mosquitoes. Based on the four phylogenetic trees, subgenus Cellia sistered to subgenus Anopheles and it is consistent with previous studies (65). These two subgenera have minimum phylogenetic distance (=1332.4) and both Cellia and Anopheles subgenera (within a single cluster) sistered to subgenus Nyssorhynchus and among that, Ker-teszia subgenus has a more distinct location than the other three subgenera and based on Table 2, after the outgroup, it is placed at the maximum phylogenetic distances with other subgenera. In a study (7), nine protein-coding genes (PCGs) located on the heavy strand (7536bp) were used and their phylogenetic tree is shown in Fig. 5. Their results are very similar to the results of this study. However, the results of this study are certainly more accurate than their study. For example, in this study, Anopheles subgenus completely separated from Cellia subgenus, but in another study (7), An. atro-parvus and An. quadrimaculatus that belong to the subgenus Anopheles, were placed into the major clade which corresponds to the sub-genus Cellia. 

Also, in another study (66), phylogenetic relationships of anopheline mosquitoes were investigated using a cladistic analysis of morphological characters. The examined species were included: one Chagasia, three Bimellu, and 60 species representing all six subgenera of the genus Anopheles. The obtained phylogenetic tree is shown in Fig. 6. Six subgenera belonging to the genus Anopheles separated completely, but they used 163 morphological characters and biometry of these traits is time-consuming and involves human errors. Instead, in the current survey, using complete mtDNA genomes, four subgenera of Anopheles are separated with very high precision, so it is concluded that complete mtDNA genomes act better, faster, and more efficiently than that of morphological traits and using distinct genes in classifying the species of Anopheles. In total, each of the subgenera belonging to Anopheles, are demarcated with very high precision and each is completely considered as a monophyletic group (Figs. 3, 4). Finally, in the latest study, comparative evolutionary mitochondrialomics of 50 mosquito species (Anopheles, Culex, Armigeres, and Aedes) were evaluated (65). In the depicted trees, the phylogenetic relationships of four subspecies of Anopheles, exactly similar to the results of the current review but the phylogenetic relationships of the species are different. Besides, in the mentioned research, phylogenetic relationships of four species of mosquitoes were studied but in the present review, we focused on Anophelinae only, and the number of analyzed samples in this review is more than that of samples for Anophelinae in mentioned study. For this reason, it seems that the results of the current review are more accurate and reliable.

Conclusion

The results of the current review showed that the mitogenomes act very accurately in recognition of the phylogenetic and taxonomic status of Anopheles and provide a higher level of support than those based on individual or partial mitochondrial and nuclear genes and with using them, we can meticulously reconstruct
Anopheles classification and improve our knowledge about their evolutionary biology.

Acknowledgements

Our special thanks to anonymous reviewers who provided helpful comments on the first draft of the article. The authors declare that there is no conflict of interest.

References

1. Manguin S (2013) Anopheles mosquitoes—new insights into malaria vectors. Intech Open Press, Rijeka, Croatia.
2. Lehane MJ (2005) The Biology of Blood-Sucking in Insects. Cambridge University Press, UK.
3. Michel K, Suwanchaichinda C, Morlais I, Lambrechts L, Cohuet A, Awono-Amhene PH, Simard F, Fontenille D, Kanost MR, Kafatos FC (2006) Increased melanizing activity in Anopheles gambiae does not affect development of Plasmodium falciparum. Proc Natl Acad Sci. 103(45): 16858–16863.
4. Lehrer S (2010) Anopheles mosquito transmission of brain tumor. Med Hypotheses. 74: 167–168.
5. Freitas LA, Russo CA, Voloch CM, Mutauqua OC, Marques LP, Schrago CG (2015) Diversification of the genus Anopheles and a neotropical clade from the late Cretaceous. PLoS One. 10(8): e0134462.
6. Harbach RE (2013) The phylogeny and classification of Anopheles. In: Manguin S (Ed): Anopheles mosquitoes-new insights into malaria vectors, Intech Open Press, Rijeka, Croatia, pp. 3–55.
7. Peng XY, Zhou P, Duan XY, Qian ZQ (2016) The mitochondrial genomes of twelve Anopheles mosquitoes (Diptera: Culicidae) and their phylogenetic implications. Conserv Genet Resour. 8: 1–4.
8. Wiebe A, Longbottom J, Gleave K, Shearer FM, Sinka ME, Massey NC, Cameron E, Bhatt S, Gething PW, Hemingway J, Smith DL, Coleman M, Moyes CL (2017) Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. Malar J. 16: 85.
9. Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J, Patil AP, Temperley WH, Gething PW, Kabaria CW, Okara RM (2010) The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. Parasite Vector. 3 (1): 117.
10. White BJ, Kundert PN, Turissini DA, van Ekeris L, Linser PJ,Besansky NJ (2017) Dose and developmental responses of Anopheles merus larvae to salinity. J Exp Biol. 216: 3433–3441.
11. Temu EA, Minjas JN, Coetzee M, Hunt RH, Shift CJ (1998) The role of four anopheline species (Diptera: Culicidae) in malaria transmission in coastal Tanzania. Trans R Soc Trop Med Hyg. 92: 152–158.
12. Cuamba N, Mendis C (2009) The role of Anopheles merus in malaria transmission in an area of southern Mozambique. J Vector Dis. 46: 157–159.
13. Rosenberg R, Andre RG, Somchit L (1990) Highly efficient dry season transmission of malaria in Thailand. Trans R Soc Trop Med Hyg. 84: 22–28.
14. Obsomer V, Defourny P, Coosemans M (2007) The Anopheles dirus complex: spatial distribution and environmental drivers. Malar J. 6: 26.
15. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, Gething PW, Elyazar IR, Kabaria CW, Harbach RE, Hay SI (2011) The dominant Anopheles vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. Parasite Vector. 4: 89.

http://jad.tums.ac.ir
Published Online: March 31, 2021
16. Sinka ME, Rubio-Palis Y, Manguin S, Patil AP, Temperley WH, Gething PW, Van Boeckel T, Kabaria CW, Harbach RE, Hay SI (2011) Correction: The dominant Anopheles vectors of human malaria in the Americas: occurrence data, distribution maps and bionomic précis. Parasite Vector. 4: 210.

17. Sinka ME, Rubio-Palis Y, Manguin S, Patil AP, Temperley WH, Gething PW, Van Boeckel T, Kabaria CW, Harbach RE, Hay SI (2010) The dominant Anopheles vectors of human malaria in the Americas: occurrence data, distribution maps and bionomic précis. Parasite Vector. 3: 72.

18. Takken W, Geene R, Adam W, Jetten TH, van-der-Velden JA (2002) Distribution and dynamics of larval populations of Anopheles messeae and An. atroparvus in the delta of the rivers Rhine and Meuse, The Netherlands. Ambio. 31: 212–218.

19. Becker N, Petric D, Zgomba M, Boase C, Madon M, Dahl C (2010) Mosquitoes and their Control. Springer Press, Berlin.

20. Fernandes L, Briegel H (2005) Reproductive physiology of Anopheles gambiae and Anopheles atroparvus. J Vector Ecol. 30(1): 11–26.

21. Hiwat H, Bretas G (2011) Ecology of Anopheles darlingi Root with respect to vector importance: a review. Parasite Vector. 4: 177.

22. Garros C, van Bortel W, Trung HD, Coosemans M, Manguin S (2006) Review of the Minimus Complex of Anopheles, main malaria vector in Southeast Asia: from taxonomic issues to vector control strategies. Trop Med Int Health. 11: 102–114.

23. Linton YM, Dusfour I, Howard TM, Ruiz LF, Duc-Manh N, Ho-Dinh T, Sochanta T, Coosemans M, Harbach RE (2005) Anopheles (Cellia) epiroticus (Diptera: Culicidae), a new malaria vector species in the Southeast Asian Sundaicus Complex. Bull Entomol Res. 95: 329–339.

24. Vatandoost H, Emami SN, Oshaghi MA, Abai MR, Raeisi A, Piazzak N, Mahmoodi M, Akbarzadeh K, Sartipi M (2011) Ecology of malaria vector Anopheles culicifacies in a malarious area of Sistan va Baluchestan Province, southeast Islamic Republic of Iran. East Mediterr Health J. 17: 439–45.

25. Hanafi-Bojd AA, Vatandoost H, Oshaghi MA, Charrahy Z, Haghdoost AA, Sedaghat MM, Abedi F, Soltani M, Raeisi A (2012) Larval habitats and biodiversity of anopheles mosquitoes (Diptera: Culicidae) in a malarious area of southern Iran. J Vector Borne Dis. 49: 91–100.

26. Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A, Terenius O (2014) Isolation and identification of culturable bacteria from wild Anopheles culicifacies, a first step in a paratransgenesis approach. Parasites Vectors. 7: 419.

27. Hanafi-Bojd AA, Vatandoost H, Oshaghi MA, Haghdoost AA, Shahi M, Sedaraght MM, Abedi F, Yeryan M, Pakari A (2012b) Entomological and epidemiological attributes for malaria transmission and implementation of vector control in southern Iran. Acta Trop. 121: 85–92.

28. Amir A, Sum JS, Lau YL, Vythilingam I, Fong MY (2013) Colonization of Anophles cracens: a malaria vector of emerging importance. Parasites Vectors. 6: 81.

29. Motoki MT, Wilkerson RC, Sallum MAM (2009) The Anopheles albitaris complex with the recognition of Anopheles oryzalimnetes Wilkerson and Motoki, n. sp. and Anophele janconnae Wilkerson and Sallum, n. sp. (Diptera: Culicidae). Mem Inst Oswaldo Cruz. 104: 823–850.

30. Cardoso JDC, Bergo ES, Oliveira TMP, Sant’ana DC, Motoki MT, Sallum MAM (2012) New Records of Anopheles homunculus in Central and Serra Do Mar Biodiversity Corridors of the Atlantic Forest, Brazil. J Am Mosq Control Assoc. 2: 1–5.

31. Vatandoost H, Oshaghi MA, Abai MR,
Shahi M, Yaaghoobi F, Baghaii M, Hanafi-Bojd AA, Zamani G, Townson H (2006) Bionomics of Anopheles stephensi Liston in the malarious area of Hormozgan Province, southern Iran, 2002. Acta Trop. 97: 196–203.

32. Oshaghi MA, Yaaghoobi F, Abai MR (2006) Pattern of mitochondrial DNA variation between and within Anopheles stephensi (Diptera: Culicidae) biological forms suggests extensive gene flow. Acta Trop. 99: 226–233.

33. Abai MR, Mehravaran A, Vatandoost H, Oshaghi MA, Salim-Abadi Y, Rafi F (2008) Comparative performance of imagicides on Anopheles stephensi, main malaria vector in a malarious area, southern Iran. J Vector Borne Dis. 45: 307–312.

34. Gorouhi MA, Vatandoost H, Oshaghi MA, Raeisi A, Enayati AA, Mirhendi H, Hanafi-Bojd AA, Abai MR, Salim-Abadi Y, Rafi F (2016) Current Susceptibility Status of Anopheles stephensi (Diptera: Culicidae) to Different Imagicides in a Malarious area, Southeastern of Iran. J Arthropod Borne Dis. 10: 493–500.

35. Malhotra PR, Jatav CP, Chauhan RS (2000) Surface morphology of the egg of Anopheles stephensi stephensi sensu stricto (Diptera: Culicidae). Ital J Zool. 62: 147–151.

36. Sallum MAM, Forattini OP, Wilkerson RC (2000) Redescription of the adult and larva and first description of the pupa of Anopheles (Kerteszia) laneanus. J Am Mosq Control Assoc. 16: 86–92.

37. Oliveira TMP, Foster PG, Bergo ES, Nagaki SS, Sanabani SS, Marinotti O, Marinotti PN, Sallum MAM (2015) Mitochondrial Genomes of Anopheles (Ker-teszia) (Diptera: Culicidae) From the Atlantic Forest, Brazil. J Med Entomol. 53: 790–797.

38. Carpenter S, LaCasse W (1955) Mosquitoes of North America (North of Mexico). Berkeley: University of California Press, London.

39. Zhang WQ, Zhang MH (2013) Complete mitochondrial genomes reveal phylogeny relationship and evolutionary history of the family Felidae. Genet Mol Res. 12: 3256–3262.

40. Oshaghi MA (2005) mtDNA inheritance in the mosquitoes of Anopheles stephensi. Mitochondrion. 5: 266–271.

41. Ghassemi-Khademi T (2017) Evaluation of phylogenetic relationships of Antilopini and Oreotragini tribes (Bovidae: Artiodactyla) based on complete mitochondrial genomes. JWB. 1: 1–11.

42. Ramirez CC, Dessen EM (2000) Chromosomal evidence for sibling species of the malaria vector Anopheles cruzii. Genome. 43: 143–151.

43. Ramirez CC, Dessen EM (2000) Chromosome differentiated populations of Anopheles cruzii: evidence for a third sibling species. Genetica. 108: 73–80.

44. Oshaghi MA, Shemshad K, Yaghobi-Ershadi MR, Pedram M, Vatandoost H, Abai MR, Akbarzadeh K, Mohtarami F (2007) Genetic structure of the malaria vector Anopheles superpictus in Iran using mitochondrial cytochrome oxidase (COI and COII) and morphologic markers: a new species complex? Acta Trop. 101: 241–248.

45. Naddaf SR, Razavi MR, Bahramali G (2010) Molecular variation and distribution of Anopheles fluviatilis (Diptera: Culicidae) complex in Iran. Korean J Parasitol. 48: 231–236.

46. Swain S, Mohanty A, Tripathy HK, Mahapatra N, Kar SK Hazra RK (2010) Molecular identification and phylogeny of Myzomyia and Neocellia series of Anopheles subgenus Cellia (Diptera: Culicidae). Infect Genet Evol. 10: 931–939.

47. Paredes-Esquivel C, Harbach RE, Townson H (2011) Molecular taxonomy of
members of the Anopheles hyrcanus group from Thailand and Indonesia. Med Vet Entomol. 25: 348–352.

48. Mehravaran A, Oshaghi MA, Vatandoost H, Abai MR, Ebrahizadeh A, Roodi AM, Grouhi A (2011) First report on Anopheles fluviatilis U in southeastern Iran. Acta Trop. 117: 76–81.

49. Karimian F, Oshaghi MA, Sedaghat MM, Waterhouse RM, Vatandoost H, Hanafi-Bojd AA, Ravasan NM, Chavshin AR (2014) Phylogenetic analysis of the oriental-Palaearctic-Afrotropical members of Anopheles (Culicidae: Diptera) based on nuclear rDNA and mitochondrial DNA characteristics. Jpn J Infect Dis. 67: 361–367.

50. Neafsey DE, Waterhouse RM, Abai MR, Aganezov SS, Alekseyev MA, Allen JE, Amon J, Arcà B, Arensburger P, Artemov G, Assour LA (2015) Mosquito genomics. Highly evolvable malaria vectors: the genomes of 16 Anopheles mosquitoes. Science. 347(6217): 1258522.

51. Nikaido M, Rooney AP, Okada N (1999) Phylogenetic relationships among cetartiodactyls based on insertions of short and long interspersed elements: hippopotamuses are the closest extant relatives of whales. Proc Natl Acad Sci USA. 96: 10261–10266.

52. Krzywinski J, Grushko OG, Besansky NJ (2006) Analysis of the complete mitochondrial DNA from Anopheles funestus: an improved dipteran mitochondrial genome annotation and a temporal dimension of mosquito evolution. Mol Phylogenet Evol. 39: 417–423.

53. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acid S. 41: 95–98.

54. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple alignment through sequence weighting, positional-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

55. Saitou N, Nei M (1987) The neighbor-joining method: A new method for re-constructing phylogenetic trees. Mol Biol Evol. 4: 406–425.

56. Rzhetsky A, Nei M (1992) A simple method for estimating and testing minimum evolution trees. Mol Biol Evol. 9: 945–967.

57. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39: 783–791.

58. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16: 111–120.

59. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30: 2725–2729.

60. Win NZ, Choi EY, Park J, Park JK (2017) Molecular phylogenetic relationship of the subfamily Nymphalinae (Lepidoptera: Nymphalidae) in Myanmar, inferred from mitochondrial gene sequences. J Asia Pac Biodivers. 10: 86–90.

61. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19: 1572–1574.

62. Huelsenbeck JP, Ranala B (2004) Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Syst Biol. 53: 904–913.

63. Ghassemi-Khademi T (2018) New insight into the phylogeny of the orchid bees (Apidae: Euglossini). Journal of Wildlife and Biodiversity. 2(1): 19–35.

64. Coetzee M, Hunt RH, Wilkerson R, Della-Torre A, Coulibaly MB, Besansky NJ (2013) Anopheles coluzzii and Anopheles amharicus, new members of the
Anopheles gambiae complex. Zootaxa. 3619: 246–274.

65. Hao YJ, Zou YL, Ding YR, Xu WY, Yan ZT, Li XD, Fu WB, Li TJ, Chen B (2017) Complete mitochondrial genomes of Anopheles stephensi and An. dirus and comparative evolutionary mitochondrialomics of 50 mosquitoes. Sci Rep. 7 (7666): 1–13.

66. Sallum MAM, Schultz TR, Wilkerson RC (2000) Phylogeny of Anophelinae (Diptera: Culicidae), based on morphological characters. Ann Entomol Soc Am. 93: 745–775.