Review Article

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

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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 especially *Anopheles* genus act as a vector of diseases in different parts of the world that causes many problems for human activities (1, 2). Mosquitoes are found throughout the world except the south-pole (Antarctic). In many parts of their distribution, especially in tundra areas of the Northern hemisphere, mosquito populations reach pest and sometimes plague proportions. Mosquitos are the most familiar of all blood-sucking insects (1, 2). Among the

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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), *Lophopodomyia* (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 and *Anopheles christyi* (Newstead and Carter, 1911)

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. *Anophele 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.

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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 Lipis. 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 ma-laria vector in mentioned countries (17). This complex includes six species: *An. albitarsis*, *An. oryzalimnetes*, *An. marajoara*, *An. deaneorum*, *An. janconnae* 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 ar 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*.

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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

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taxonomic and phylogenetic status of these species is a very effective step in better identification and planning for controlling these dangerous spe-cies 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 pallens*, *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), *Kertezia* (n=6), and *Nyssorhynchs* (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). Also, the robustness of clades was calculated by the bootstrap method and thus, in this study, it was considered 50–60% as weak support (as bootstrap values), 64–75% as moderate support, 76–88% as good support and strong support as values >89% (60). In addition, Bayesian analyses of studied gene sequences were run with the parallel version of MrBayes 3.1.2 (61) on a Linux cluster with one processor assigned to each Markov chain under the most generalizing model (GTR+G+I) because overparametrization apparently does not negatively affect Bayesian analyses (62). Each Bayesian analysis comprised two simultaneous runs of four Metropolis-coupled Markov-chains at the default temperature (0.2). Analyses were terminated after the chains converged significantly, as indicated by the average standard deviation of split frequencies <0.01. Bayesian inference of phylogeny was conducted for 6,000,000 generations. Seven hundred bootstrap replicates were used as ML branch support values. The posterior probabilities equal/higher than 0.95 and bootstrap supports equal/higher than 70% were considered as strong support values (63). The obtained phylogenetic trees 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 mt DNA. 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

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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. christyi* + *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. punc-tulatus*) + *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 re-lationships 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 *Nyssorhychus* 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).

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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	15363.0	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
		Anopheles christyi	36.7	13.7	40.0	9.6	14967.0	NC_028214
		Anopheles epiroticus	37.6	12.8	40.1	9.5	15379.0	NC_028217
	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
		Anopheles minimus	38.6	12.0	40.5	8.9	15395.0	KT895423
	Neomyzomyia	*Anopheles dirus*	38.0	12.7	40.2	9.2	15404.0	JX219731
		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	JX219736
		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	*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
	Albitarsis	*Anopheles deaneorum*	37.8	12.8	39.9	9.4	15424.0	HQ335347
		Anopheles janconnae	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_024740
		Anopheles cruzii	38.6	12.4	39.9	9.1	15478.0	KU551284
		Avg.	37.9	12.7	40.1	9.3	15376.1	
Outgroups		*Culex pipiens pipiens*						HQ724616
		Culex quinquefasciatus						HQ724617
		Culex pipiens pallens						KT851543

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

	Nyssorhynchus	***Anopheles***	***Cellia***	***Kerteszia***	**Outgroup**
Nyssorhynchus	***				
Anopheles	1378.9	***			
Cellia	1431.2	1332.4	***		
Kerteszia	1583.8	1584.1	1590.5	***	
Outgroup	1999.1	1960.4	1983.3	2121.4	***

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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

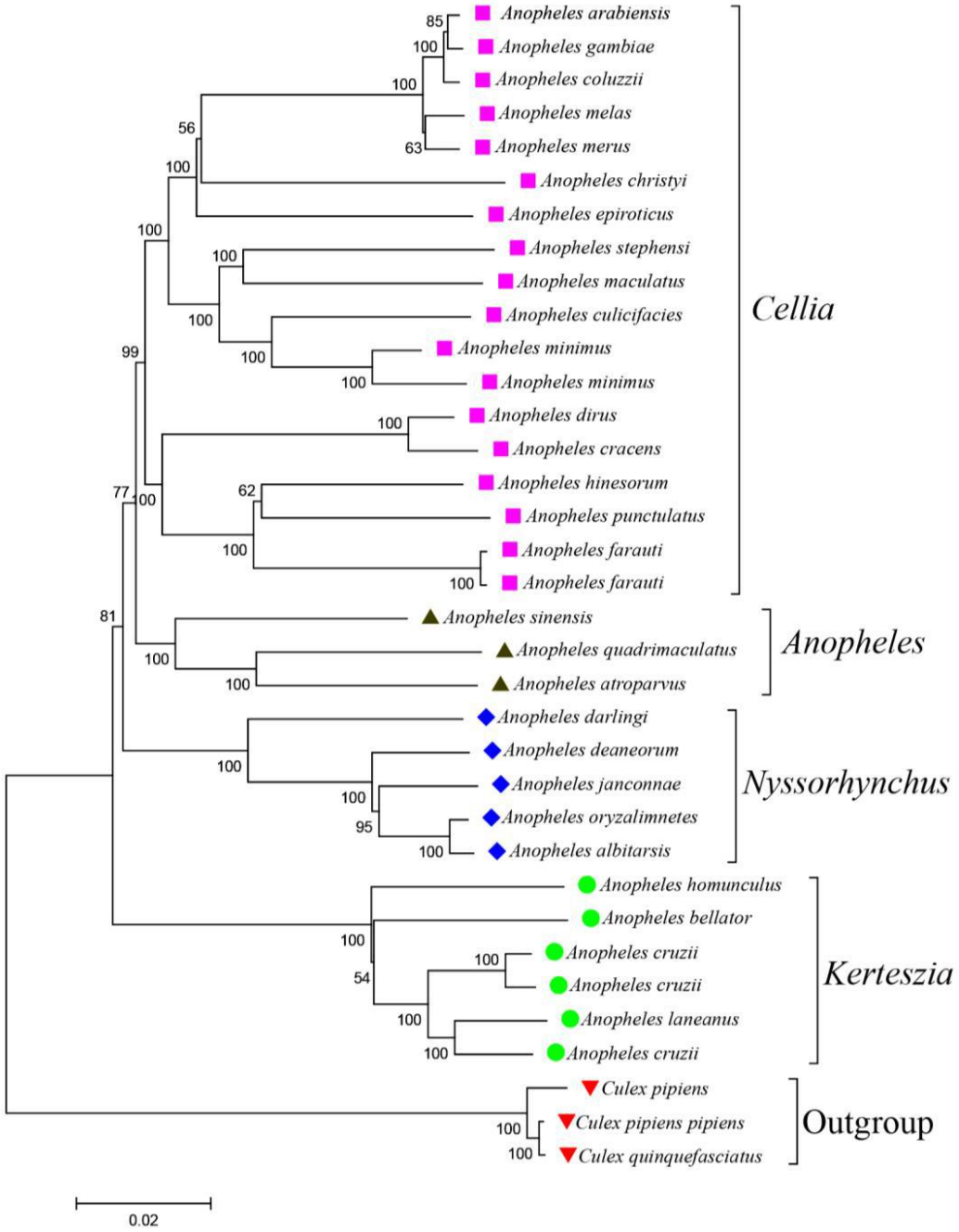

Fig. 1. Neighbor-joining tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond

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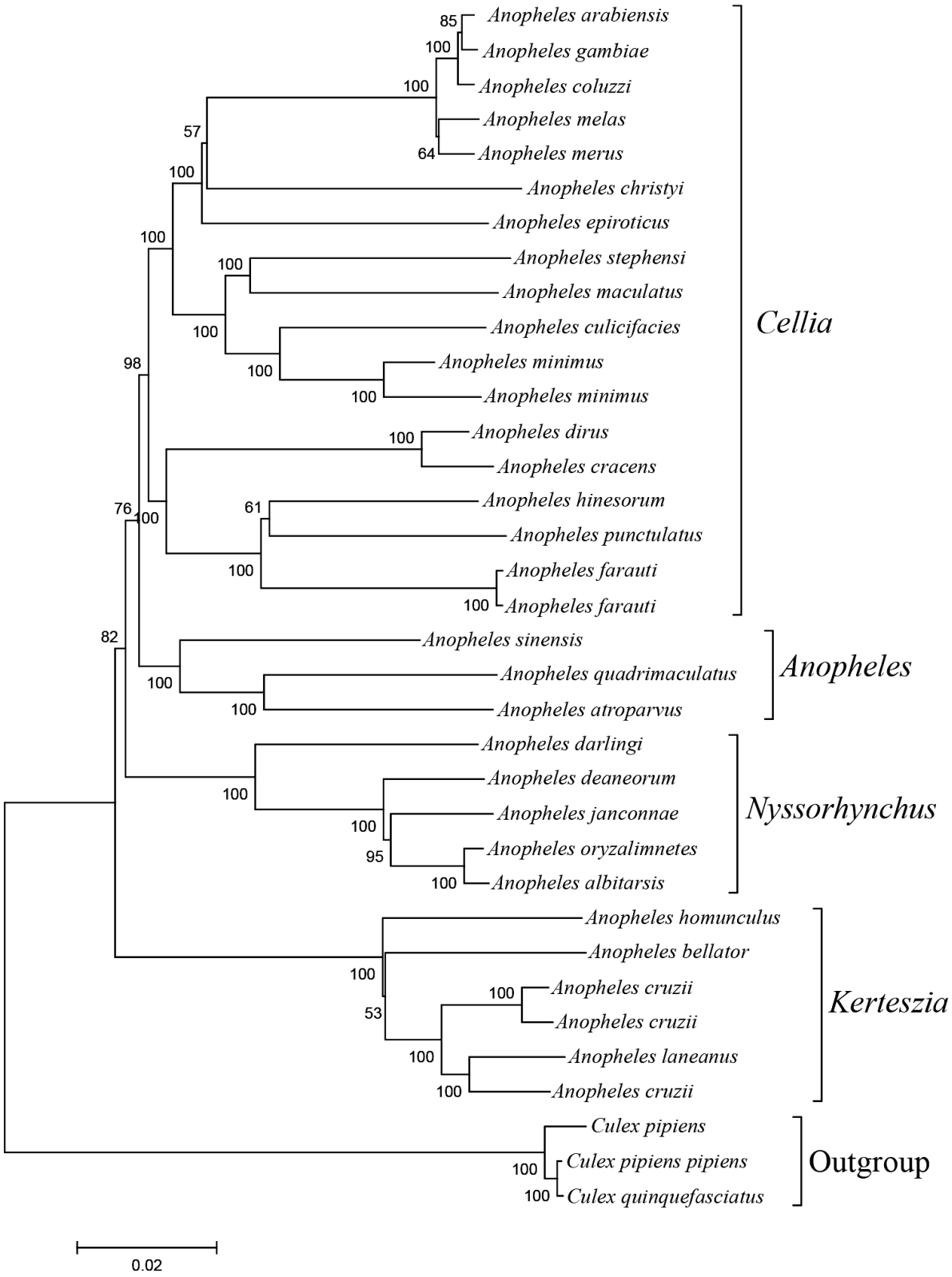

Fig. 2. Minimum Evolution tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond to the bootstrap value. The tree was rooted with three *Culex* spp. mtDNA sequences

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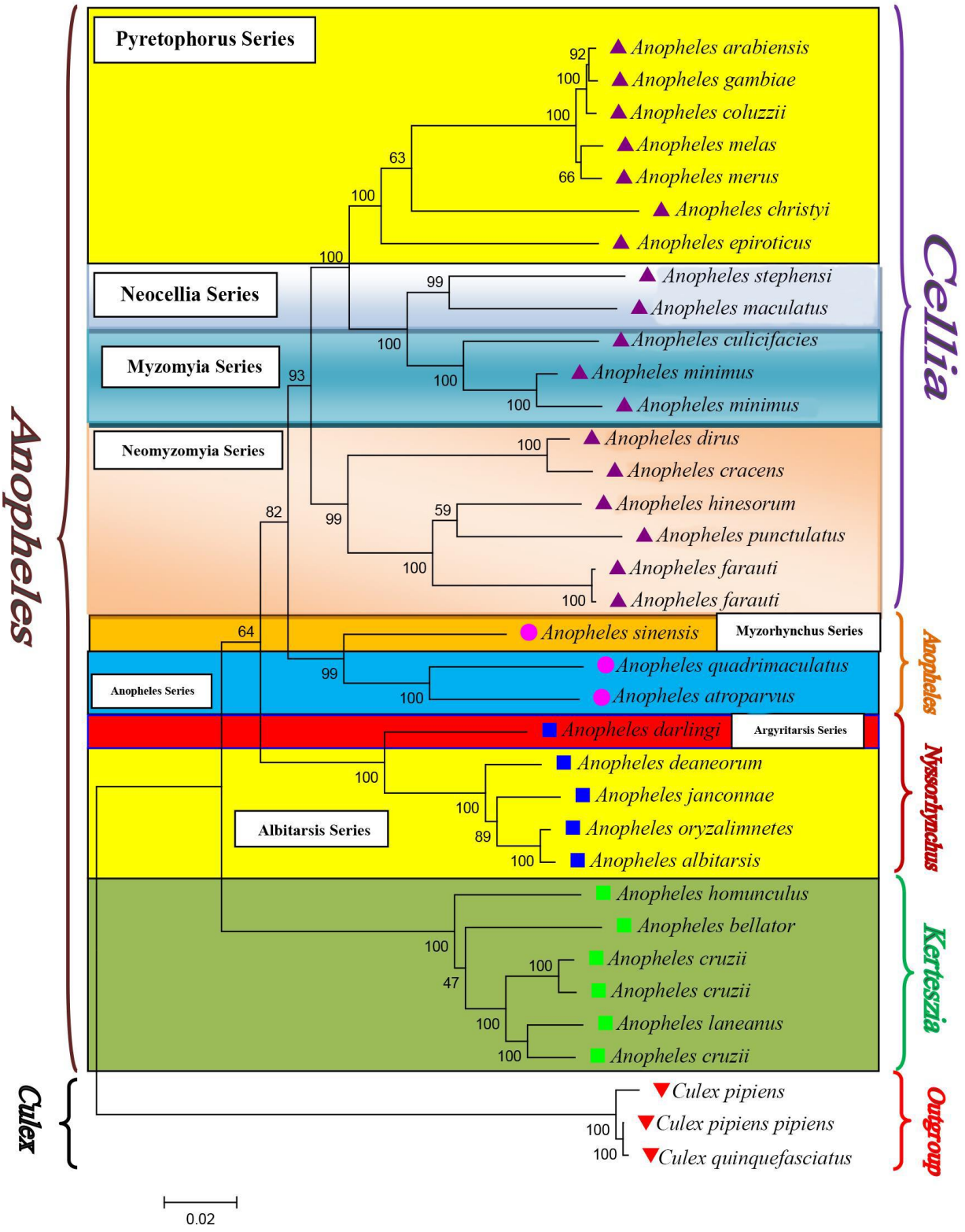

Fig. 3. Maximum Likelihood tree showing the phylogenetic relationships among 28 *Anopheles* species using complete mtDNA genomes based on Kimura 2-parameter. The numbers on each branch correspond to the bootstrap value. The tree was rooted with three *Culex* spp. mtDNA sequences

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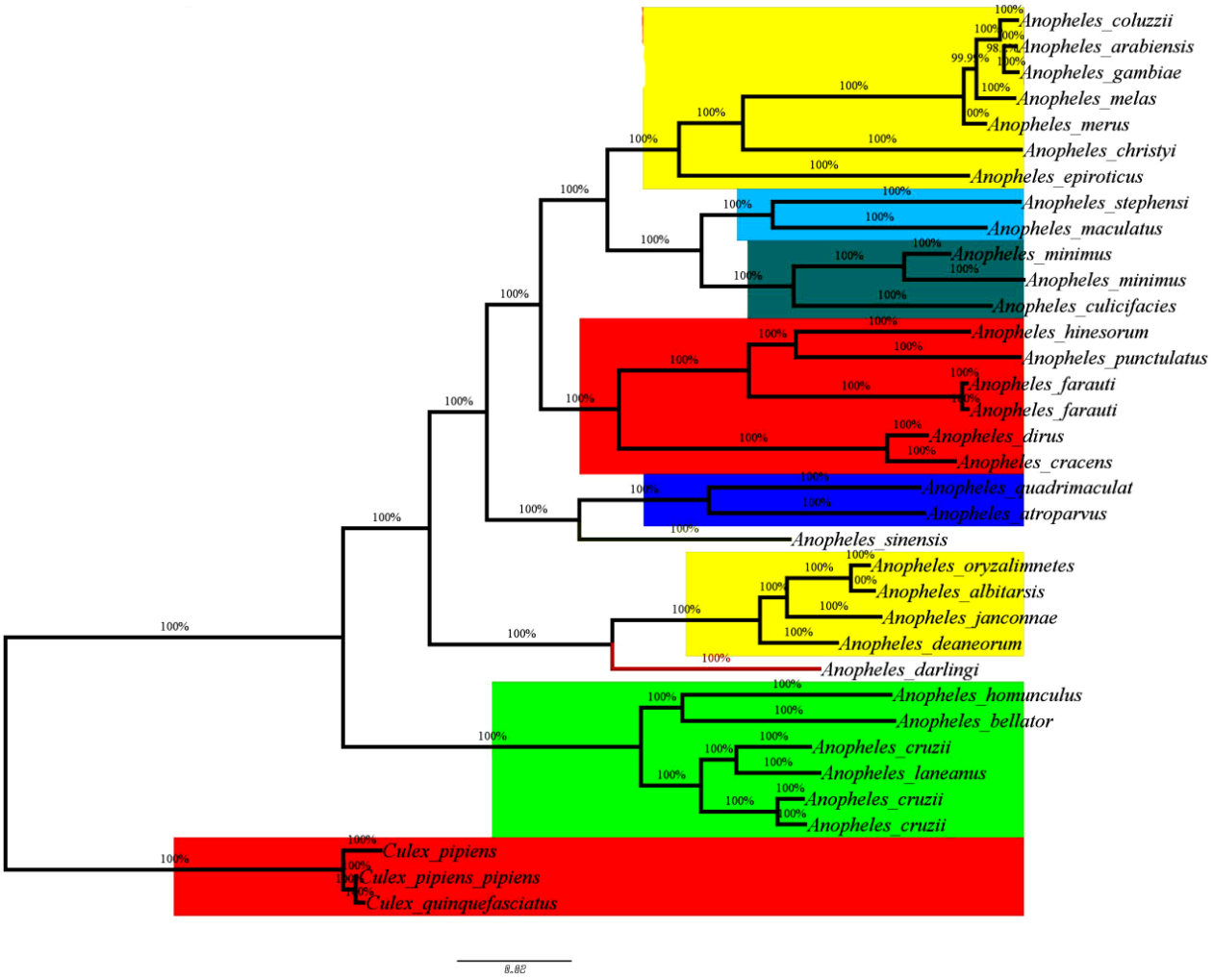

Fig. 4. Bayesian phylogeny reconstructed based on using complete mitochondrial genome sequences of 28 *Anopheles* species. The values besides the branches are BI posterior probability values. The tree was rooted with three *Culex* spp. mtDNA sequences

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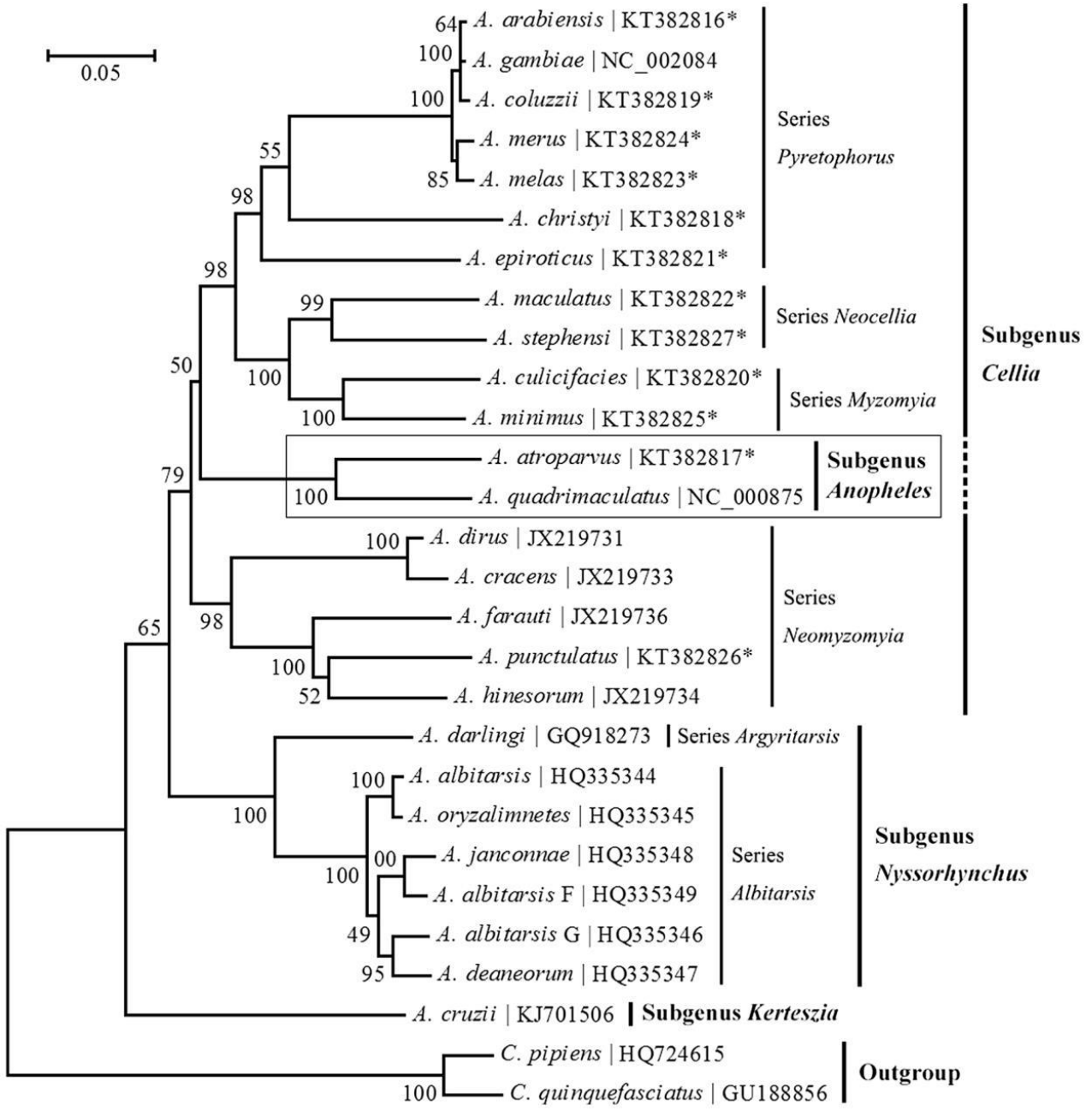

Fig. 5. Phylogeny tree of 26 *Anopheles* species based on the Maximum Likelihood (ML) analysis of nine protein-coding genes (PCGs) located on the heavy strand (7536bp; Peng et al. 2016)

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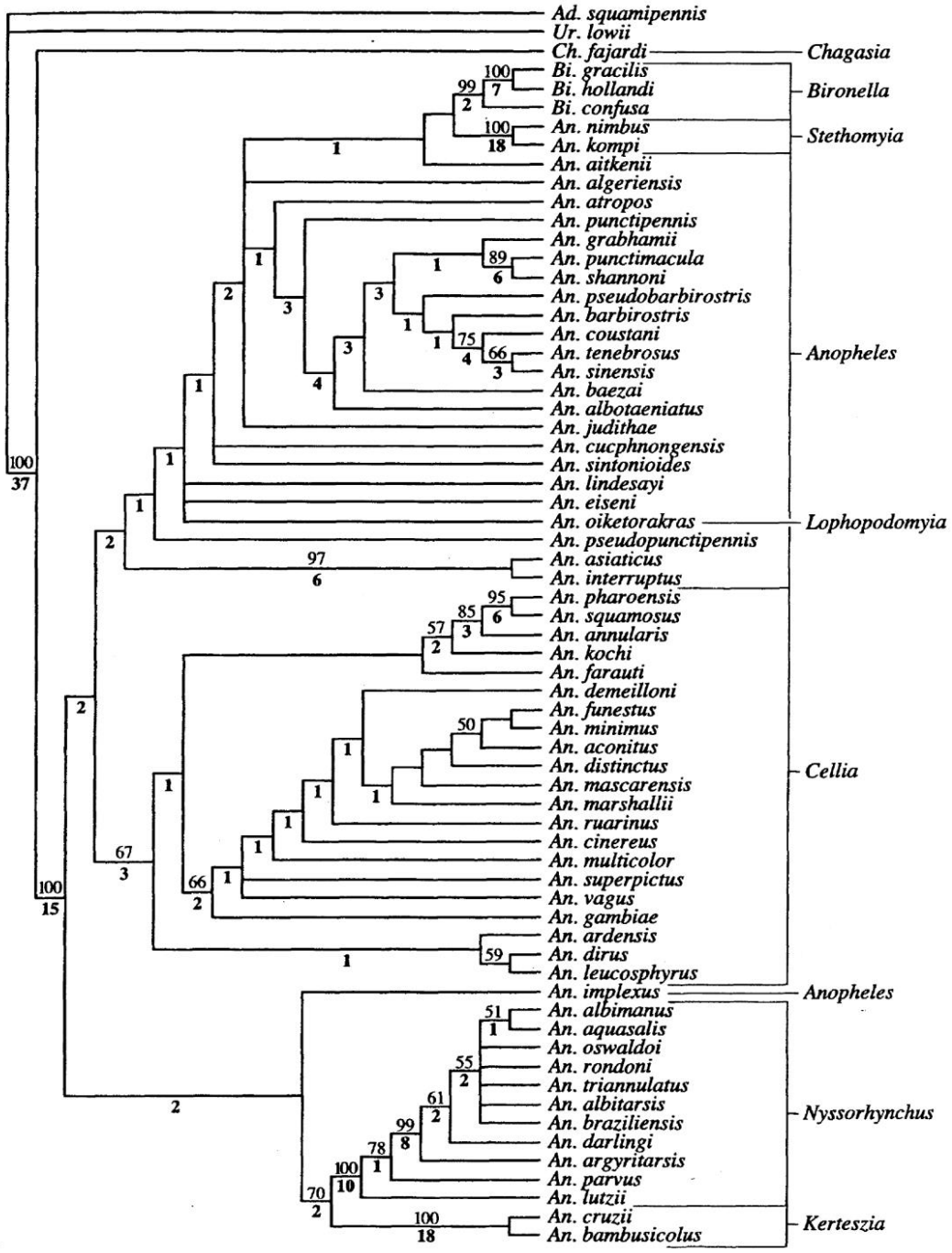

Fig. 6. Phylogenetic tree of Anophelinae (Diptera: Culicidae) based on morphological characters (163 morphological characters; Sallum et al. 2000)

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

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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*)}, and these seven species are located in very close phylogenetic distances together because these seven species are very similar morphologically and are classified within a single subgenus (*Cellia*) and a single series (*Pyretophorus*) (6). 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: Hyr-canus (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. oryzalimnetes* + *An. albitarsis*)}, 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:

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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, the subgenus *Kerteszia* 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, *Kerteszia* 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 *Bimnellu*, 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 mitochondriomics 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 series 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

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Anopheles classification and improve our knowledge about their evolutionary biology.

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