Complete mtgenome sequences of Anopheles peditaeniatus and An. nitidus and phylogenetic relationships of the genus Anopheles based on mtgenome sequences (Diptera: Culicidae: Anophelinae)

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Abstract

Background: Despite the medical importance of the genus *Anopheles* in the transmission of malaria and other human diseases, its phylogenetic relationships are not unsettled and the characteristics of mitochondrial genome (mtgenome) are not well understood.

Methods: The present study sequenced and analyzed the complete mtgenomes of *An. peditaeniatus* and *An. nitidus*, and investigated the characteristics and phylogenetic relationships of 76 complete mtgenome sequences in the genus *Anopheles* using Illumina sequencing and bioinformatics techniques.

Results: The complete mtgenomes of *An. peditaeniatus* and *An. nitidus* are 15416 and 15418 bp long, respectively, and include 13 PCGs, 22 tRNAs, two tRNAs and one control region (CR). These 76 mtgenomes are similar as earlier reports in insects in general characteristics, and however the *trnR* and *trnA* have a reversal arrangement to form "*trnR-trnA*" as reported in other mosquito genera. Their variations mainly occur in CR with a length of 493 - 886 bp, and six repeat unit types are identified for the first time and demonstrate some evolutionary signals. The subgenera *Lophopodomyia*, *Stethomyia*, *Kerteszia*, *Nyssorhynchus*, *Anopheles* and *Cellia*, are proposed to be monophyletic with the phylogenetic relationships of (*Lophopodomyia* + ((*Stethomyia* + *Kerteszia*) + (*Nyssorhynchus* + (*Anopheles* + *Cellia*)))). Four series Neomyzomyia, Pyretophorous, Neocellia and Myzomyia in *Cellia*, and two series Arribalzagia and Myzorhynchus in *Anopheles* are proposed to be monophyletic, and three sections Myzorhynchella, Argyritarsis and Albimanus and their subdivisions in *Nyssorhynchus* all appear polyphyletic or paraphyletic.

Conclusions: The study comprehensively uncovered the characteristics of mtgenome and the phylogenetics based on mtgenomes in the genus *Anopheles*, and provided an information frame for further study on the mtgenomes, phylogenetics and taxonomic revision of the genus.

Background

The genus *Anopheles* belongs to the subfamily Anophelinae in Culicidae. It is the most diverse genus in the Subfamily, including 475 formally named species and more than 50 unnamed members of species complexes worldwide [1]. It can transmit a variety of diseases, and is thought to be the most important group of insects in medicine. Mosquitoes of the genus *Anopheles* are the unique vectors of human malarial parasites, which causes 228 million cases and 405,000 deaths worldwide in 2018 [2]. In addition to malaria parasites, mosquitoes in *Anopheles* also transmit filarial parasites [3]. Some studies have shown that *Anopheles* mosquitoes also harbor viruses, collectively termed the virome, and some of these viruses are arboviruses, which multiply in the mosquito vectors before transmission to a vertebrate host, such as o'nyong-nyong [4]. Other viruses may infect insect hosts but not infect vertebrates, and are called insect-specific viruses (ISV) [5]. Due to the exceeding importance, mosquitoes of the genus are subject to more taxonomic studies than any other mosquito genus.

The classification of genus *Anopheles* started more than 100 years ago [6], in which it was treated as one of 18 genera in the Anophelinae, and *Cellia*, *Nyssorhynchus, Stethomyia* and *Kerteszia* were also treated as independent genera based on the morphological characteristics. Subsequently, the five genera were successively degraded as subgenera of the genus *Anopheles* based on the number and location of the specialized setae on male genital gonocoxites and other characteristics [7-9], and three additional subgenera, *Lophopodomyia*, *Baimaia* and *Christya* were established for the genus *Anopheles* [10-12]. Due to the diversity of species contained in subgenus *Anopheles*, *Cellia* and *Nyssorhynchus*, taxonomists divided some species into informal categories such as Sections, Series and Groups. Earliest phylogenetic studies for the genus *Anopheles* were mainly based on morphological characters and individual genes, different data sets and phylogenetic inference methods often lead inconsistent results, and therefore the phylogenetic relationship of *Anopheles* have not been well settled.

There have been a number of representative phylogenetic studies on the genus *Anopheles*. An analysis including 63 species in Anophelinae based on 163 morphological characters suggested the monophyly of the subgenera *Cellia*, *Nyssorhynchus*, *StethomyiaKerteszia* and *Lophopodomyia*. In *Nysorhynchus*, the three sections Albimanus, Argyritarsis and Myzorhynchella were suggested to be paraphyly; in *Cellia*, only series Cellia was considered to be monophyly; and in *Anopheles*, series Arribalzagia and Lophoscelomyia were considered to be monophyly, while series Cyclolepptеron+Arribalzagia was nested in series series Myzorhynchus [13]. Some further morphology-based studies also suggested the monophyly of subgenera *Nyssorhynchus*, *Cellia* and *Kerteszia*, and displayed the sister relationships between subgenera *Kerteszia* and *Nyssorhynchus* [12, 14, 15]. An analysis based on *COX1* + ITS2 dataset suggested the monophyly of the subgenus *Anopheles* (16 species included) and *Cellia* (18 species), and the analysis using ITS2 dataset alone resulted in the same conclusion but not for *COX1* dataset alone [16]. Two studies based on the nucleotides of 13 protein-coding genes of mtgenomes, including 50 and 33 species, both also supported the monophyly of subgenera *Anopheles*, *Nyssorhynchus*, *Cellia* and *Keterszia* [17, 18]. Generally, the monophyly of the subgenera *Anopheles*, *Nyssorhynchus*, *Cellia*, *Stethomyia*, *Keterszia* and *Lophopodomyia* have been suggested by most nowadays studies; however, the sections and series in the subgenera *Anopheles*, *Nyssorhynchus* and *Cellia* have not been well determined. There is a need to elucidate the phylogeny of the genus *Anopheles* using more species, more data and updated phylogenetic analysis approaches.

Mitochondria is a very important organelle in eukaryotic cells, which has a genome independent of the nuclear genome, namely “mitochondrial genome” (mtgenome) [19]. Mtgenome has the characteristics of small genome size, low level of recombination and maternal inheritance, and therefore it has been widely used as a molecular marker for identification of species, evolution, phylogenetic inference and population structure research [20, 21]. Since the publication of the first insect mtgenome (*Drosophila yakuba*) in 1985 [22], the number of insect mtgenomes have increased rapidly. Phylogenetic studies based on insect mtgenomes have shown good results in Diptera [23], Orthoptera [24], Coleoptera [25] and Hymenoptera [26]. So far, NCBI has housed the complete mtgenomes of 125 species in Culicidae, of which 74 species belong to the genus *Anopheles*. The Diptera mtgenome is mostly 14-20 kb long, including 37 genes: 13 protein-coding genes (PCGs), 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and a control

region (CR), and these genes are arranged in a compact circular genome [27]. The gene number and structure in all reported mosquito mtgenomes are similar to the typical mtgenomes of Diptera, and however, *trnA* and *trnR* of mosquitoes are rearranged to form "*trnR-trnA*" arrangement [17, 18, 22].

In the present study, we sequenced and annotated the complete mitogenomes of *An. peditaeniatus* and *An. nitidus* in the genus *Anopheles*, and comparatively analyzed the characteristics of 76 species of mtgenome sequences in the genus *Anopheles*. More importantly, we constructed and discussed the phylogenetic relationships of these 76 known mtgenome sequences. The study provided new insight of the mtgenomes characteristics and phylogenetic relationships in the genus *Anopheles*.

Methods

Sample collection and DNA extraction

Specimens of *An. peditaeniatus* and *An. nitidus* were collected from Yadong County (29°11'46"N, 95°12'11"E), Tibet, China in July 2014, and Tiebei County, Jilin Province, China (42°27'21"N, 128°06'18"E) in July 2013, respectively. All collected samples were preserved in individual vials in silica. After morphological identification in laboratory [28], these samples were stored in 100% alcohol, and housed at -20℃ until the DNA extraction. Total DNA was extracted from the individual adult mosquito using the Qiagen Genomic DNA Kit [29], and used for 350 bp library construction and Illumina high throughout sequencing of mitochondrial genome in Shenzhen Huitong Biotechnology Co. Ltd..

Mtgenomes assembly, annotation and characteristics analysis

The mtgenomes of *An. peditaeniatus* and *An. nitidus* assemblied and annotated using Mitos (http://mitos.bioinf.unileipzig.de/index.py) [30]. The annotation of 13 PCGs and two rRNA genes wase confirmed in reference of known mosquito mtgenomes, and corrected using Geneious v4.8.5 [31]. The secondary structures of tRNAs were predicted using tRNAscan-SE 2.0 [32], and the structure map of the mtgenomes were visualized using OGDRAW1.3.1 [33]. The base composition, codon usage, relative synonymous codon usage (RSCU), and amino acid content were computed with MEGA v.7.0.26 software [34]. The nucleotide composition bias was calculated using the formulas AT skew = [A − T] / [A + T] and GC skew = [G − C] / [G + C] [35], and the Three-dimensional scatterplot of the AT-Skew, GC-Skew and AT% was drawn using Origin Pro v.9.0 [36]. The selection pressure of 13 PCGs during the evolution process was analyzed by calculating Ka and Ks values using DnaSP v6.12.03. Sequence motifs in the CR were identified using the Tandem Repeats Finder program [37].

Phylogenetic analysis

Multiple sequence alignment of 13 PCGs was performed on the Translator-X Server (http://translatorx.co.uk/), in which MAFFT was used to align the amino acid sequences of 13 PCGs, and Gblocks was used to remove poorly aligned sites. Finally, the individual alignments were connected

together using SequenceMatrix [38] to obtain the amino acid tandem sequence of 13 PCGs. The best-fit substitution model for nucleotide datasets was selected by PartitionFinder 2 [39].

Phylogenetic analyses of the 76 *Anopheles* species of mtgenomes (two sequenced in the study and 74 known) were performed using the Maximum likelihood (ML) analysis in IQ-TREE 1.6.10 [40], and the Bayesian Inference (BI) analysis in MrBayes v.3.2.7a [41] using *Culex pipiens pallens* as the outgroup (Table 1). The bootstrap values were calculated with 1000 replicates for ML, and for BI, performed two independent runs, each with four chains, and these chains ran simultaneously for 1,000,000 generations, and the tree being sampling every 1000 steps with 25% burn-in rate. The phylogenetic tree was drawn using FigTreev.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Nucleotide composition and genome organization

The complete mtgenomes of *An. peditaeniatus* (MT822295) and *An. nitidus* (MW401801) are both circular, closed and double-stranded structures, with full lengths of 15,416 and 15,418 bp, respectively (Fig. 1). Both are composed of 37 genes (including 13 PCGs, 22 tRNA genes and two rRNA genes) and one control region (CR). There are 22 genes (nine PCGs and 13 tRNAs) located on the majority coding strand (J-strand), while the other 15 genes (four PCGs, nine tRNAs and two rRNAs) are on the minority strand (N-strand). Compared with the typical Diptera mtgenome (*Drosophila yakuba*), both *An. peditaeniatus* and *An. nitidus* have "*trnR-trnA*" rearrangements. The AT content of the mtgenomes of the two species are as high as 78.32% and 78.26%, respectively, which are significantly higher than their GC content (21.68%, 21.74%), showing obvious AT bias (Additional file 1: Table S1). The AT-skew of *An. peditaeniatus* (0.0322) is higher than the average AT-skew of all investigated mosquito mtgenomes (0.0283), whereas the AT-skew of *An. nitidus* mtgenome (0.0266) is lower than the average AT-skew value. The GC-skew in *An. peditaeniatus*(-0.1587) and *An. nitidus* (-0.1536) are higher than the average GC-skew value in mosquitoes investigated (-0.16048).

The three-dimensional scatter plot of the AT content, AT-skew and GC-skew of 76 mtgenomes in the genus *Anopheles* is shown in Fig. 2. The AT-skew with the range of variation from 0.005 for *An. gilesi* to 0.043 for *An. christyi*. However, all mtgenomes display negative GC-skews ranging from -0.207 for *An. parvus* to -0.136 for *An. punctulatus*. Most of the species of the subgenera *Nyssorhynchus* and *Cellia* have similar AT content and AT/GC-skew, which are closely distributed in the Three-dimensional scatter plot, whereas the species of the subgenera *Lophopodomyia, Stethomyia*, *Kerteszia* and *Anopheles* are widely distributed in the plot for AT content, AT-skew and GC-skew.

Protein-coding genes

The total nucleotide lengths of the 13 PCGs of *An. peditaeniatus* and *An. nitidus* is 11,223 and 11,168 bp, respectively. In the *An. peditaeniatus*, ATN is used as the start codon, except for *COX1* and *ND5* which use

TCG and GTG as the start codon, respectively, and in the *An. nitidus*, 13 PCGs initiate with ATN as the start codon, but *COX1* uses TCG as a start codon (Table 2).

The RSCU values of 76 species of mtgenomes in the genus *Anopheles* are presented in Additional file 2: Table S2. The mtgenomes of the *Anopheles* have relatively different usage frequencies of synonymous codons. In the 76 species, UUA is the most frequently used codon, followed by CGA, GGA, GCU. The amino acid Leu has the highest usage percentage for all 76 mtgenomes investigated with an average of 16.37%, followed by Phe (9.69%), Ile (9.31%) and Ser (8.48%), whereas Cys has the lowest percentage (0.99%). The usage percentages of amino acids seem no obvious difference among different subgenera (Fig. 3).

The non-synonymous (Ka) and synonymous (Ks) substitution ratio (Ka/Ks) of 13 PCGs are shown in the Fig. 4. The Ka/Ks ratios are all less than 1, and the *ND6* has the highest Ka/Ks ratio (0.203), followed by six genes (*ATP8*, *ND2*, *ND5*, *ND4L*, *ND4*, *ND3*) with Ka/Ks ratios of 0.098-0.152. Complex IV (*COX1*, *COX2* and *COX3*), Complex III (*CYTB*), *ND1* and *ATP6* have low Ka/Ks ratios with range from 0.022 (*COX1*) to 0.051 (*ND1*). These results imply all of these 13 PCPs experienced purifying selection, especially Complex IV, Complex III, *ND1* and *ATP6*.

Transfer RNAs, ribosomal RNAs and CR

The total length of 22 tRNAs of *An. peditaeniatus* and *An. nitidus* is 1475 bp and 1476 bp, respectively, and the length of these 22 tRNAs varies from 64 to 72 bp. All tRNAs can fold into the typical clover-leaf structure, containing four stems and loops except for *trnS2* which lost the dihydrouridine (DHU) arm. There are 22 mismatched base pairs(G-U) to be found in *An. peditaeniatus* tRNAs, and 21 mismatched base pairs(G-U) in *An. nitidus* (Additional file 3: Figure S1). In the two newly sequenced mtgenomes, *rrnL* is located between *trnL2* and *trnV*, and *rrnS* between *trnV* and CR. The length of the rRNAs is 2125 bp, with an AT content of 81.36% in *An. punctulatus*; 2122 bp, with an AT content of 81.39%% in *An. nitidus*.

The control regions (CRs) of the mtgenomes are both located between *rrnS* and *trnI* with their lengths of 575 and 580 bp, and their AT content of 94.43% and 93.62% (the highest among all mtgenome regions), respectively in *An. peditaeniatus* and *An. nitidus*. Six repeat unit types are identified in the CRs of the 74 species of mtgenomes in *Anopheles* (Additional fle 4: Fig S2). All species have the repeat unit type of 15-27 bp poly-T Stretch, which is located in front of other repeat unit types and just after 140-212 bp of conserved sequence. The poly-T Stretch is adjacently connected with the conserved motif 5'-CCCCTA-3' in the conserved sequence in 68 species, whereas the motif was substituted by 5'-ATTGTA-3' in *An. cracens* and *An. dirus*, and 5'-TTCCCC-3' in *An. kompi*, *An. nimbus*, *An. gilesi* and *An. pseudotibiamaculatus*. The second type is a 12-55 bp sequence with 2-6 repeats, which is just after the poly-T Stretch and exists in 54 species. The third type ([TA(A)]n Stretch) contains 22-91 repeats, which exists in 36 species. The fourth type is a 12-38 bp sequence with 2-5 repeats which are near *trnI* and exist in 40 species. The remaining two repeat unit types are found in only a few species, one of them is a 15-36 bp sequence which after the second type and exists in 5 species; and the last one is a 108-171 bp sequence, which is longest one among all six types and only exists in four species.

Phylogenetic relationships

Bayesian inference (BI) and Maximum-likelihood (ML) analyses produced two same topology of phylogenetic trees in the subgenus-level (Fig. 5-6). The six subgenera investigated, *Lophopodomyia*, *Stethomyia*, *Kerteszia*, *Nyssorhynchus*, *Anopheles* and *Cellia* all seem to be monophyly in both analyses, with posterior probability (pp) = 1 for every subgenus in BI (Fig 5) and bootstrap values (bv) ranging from 99% to 100% in ML analysis (Fig. 6). The subgenus *Lophopodomyia* is located at the base of these six subgenera, and the branch comprising the remaining five subgenera has the support of pp = 1 and bv = 71%. The two subgenera *Stethomyia* and *Kerteszia* form a monophyly with pp = 1 and bv = 89%, which was earliest derived but the *Lophopodomyia*. The branch containing the *Nyssorhynchus*, *Anopheles* and *Cellia* possess the support of pp = 1 and bv = 68%. The subgenus the *Nyssorhynchus* seems to be sister group with the monophyly *Anopheles* + *Cellia* that has pp = 1 and bv = 99%.

In the subgenus *Cellis*, four series investigated, Myzomyia, Neocellia, Pyretophorus and Neomyzomyia each seem monophyletic with pp = 1 and bv = 100% for all of these monophylies. The series Neomyzomyia would be earliest derived and sister with remaining three seriers, and the series Pyretophorus would be sister with series Myzomyia and Neocellia. In the subgenus *Anopheles*, two sections Angusticorn and Laticorn both seem polyphyletic, and in section Laticorn both series Arribalzagia (pp = 1 and bv = 96%) and Myzorhynchus (pp = 1 and bv = 100%) seem monophyletic. In the subgenus *Nyssorhynchus*, three sections investigated Myzorhynchella, Argyritarsis and Albimanus all seem polyphyletic, and in the section Argyritarsis, two series Argyritarsis and Albitarsis both seem polyphyletic as well.

On the other hand, internal relationships of the *Kerteszia* are different of BI tree and Ml tree: *An. homunculus* branched out earlier than *An. bellator* in BI-tree (Fig. 5), however, in ML-tree, *An. bellator* branched out earlier than *An. homunculus* (Fig. 6).

Discussion

Characteristics of the mtgenome sequences of the genus *Anopheles*

The length of 76 mtgenomes in the genus *Anopheles* ranges from 15,573 bp to 15,803 bp, and the length variation mainly occurred in the CRs, which is similar as earlier reported mtgenomes in insects [42, 43]. Each mtgenome sequence includes 37 genes, and the *trnR* and *trnA* have a reversal arrangement to form "*trnR-trnA*" in comparison of *Drosophila yakuba*, as those reported in other genera in Culicidae [22,45]. All tRNA genes can form a complete clover secondary structure, except for *trnS2* that lacks the DHU arm, which seem to be a common feature of metazoans [43]. The nucleotide composition for all species exhibits high AT bias with AT-skew values all positive and GC-skew all negative, similar as earlier reports in insects. The 13 PCGs mainly use ATN as the start codon and TAA as the stop codon, which is similar as other mtgenome sequences in insects [43]. The usage frequencies of synonymous codons and amino acids vary with the codon UAA having the highest usage frequency, followed by CGA and GGA, and the amino acid Leu to be most used, followed Phe and Ile. This is the detailed analysis for the usage

frequency for the first time, and may potentially contribute the biochemical and functional characteristics of mitochondrial genes.

The lengths of CRs are quite variable with range from 493 bp to 886 bp. The present study identified six repeat unit types for the first time. All mtgenome sequences investigated have the poly-T stretch, which may involve in the identification of the replication origin of mtDNA [44]. The remaining five repeat unit types vary in length and position among species, some of them seem different among subgenera to some extent. For example, the third type ([TA(A)]n Stretch) were not found in subgenera *Anopheles*, *Lophopodomyia* and *Stethomyia*, and the longest type only found in subgenera *Lophopodomyia* and *Kerteszia*. The CRs have been reported to be taxon-specific and of evolutionary information, and was used as an important evidence in the inference of phylogenetics in genus *Culex* and *Lutzia* and taxon [46]. However, the evolutionary information carried in the genus *Anopheles* does not seem stable and reliable.

Phylogenetics relationships

This present study suggests that these six subgenera investigated are all monophyletic, and the phylogenetic relationships among subgenera are *Lophopodomyia* + ((*Stethomyia* + *Kerteszia*) + (*Nyssorhynchus* + (*Anopheles* + *Cellia*)))).

A phylogeny study based on 163 morphological characters for 64 species in the subfamily Anophelinae with Approximations Weighting (AW) method in 2000 showed that the subgenera *Lophopodomyia*, *Stethomyia*, *Kerteszia*, *Nyssorhynchus* and *Cellia* were monophyletic, whereas the subgenus *Anopheles* polyphyletic. These two subgenera *Lophopodomyia* and *Stethomyia* were separately linked inside the subgenus *Anopheles* [13]. A further morphology-based phylogenetics analysis published in 2005 used 167 characters for 66 species in the Anophelinae with both Equal Weighting (EW) and Implied Weighting (IW) methods, which got the same results as described above [15]. All analyses from these three methods showed that the two subgenera *Nyssorhynchus* and *Kerteszia* were sister-group, and the AW and EW methods suggested a relationship (*Nyssorhynchus* + *Kerteszia*) + (*Cellia* + (*Lophopodomyia* + *Stethomyia* + *Anopheles*)), whereas the IW method suggested (*Anopheles* + *Lophopodomyia* + *Stethomyia*) + (*Cellia* + (*Kerteszia* + *Nyssorhynchus*)). For molecular-based phylogenetic analysis, a study using *COI*, *COII* and 5.8S rRNA for 47 species in the genus *Anopheles* with ML method in 2015 suggested the monophyly of the subgenus *Stethomyia*, *Kerteszia*, *Nyssorhynchus*, *Anopheles* and *Cellia* with the phylogenetic relationships *Anopheles* +(*Cellia* + (*Nyssorhynchus* + (*Stethomyia* + *Kerteszia*))) [48]. A study using a.a. sequences of 1,085 single-copy orthologous genes for 18 species of the subgenera *Nyssorhynchus*, *Anopheles* and *Cellia* with ML method in 2015 proposed that all of these three subgenera are monophyletic with the relationships (*Nyssorhynchus* + (*Anopheles* + *Cellia*)) relationship [49]. Our earlier study using all PCG nucleotide sequences of 50 mtgenomes in Culicidae with ML and BI method in 2017 showed that the subgenera *Nyssorhynchus*, *Anopheles* and *Cellia* are monophyletic with the relationships (*Nyssorhynchus* + (*Anopheles* + *Cellia*)) [17].

All these six subgenera included in these comprehensive phylogenetic analyses above were suggested to be monophyly except for the subgenus *Anopheles*, which was recognized as a polyphyly in two morphology-based inferences while as a monophyly in three molecular-based inferences. Importantly, the study based on 18 whole nuclear genomes showed that the subgenus *Anopheles* is monophyletic [49]. This present study supported the monophyly of these six subgenera, resulting from these molecular-based inferences. The studies based on 18 whole nuclear genomes [50] and 50 whole mtgenomes [17] suggested that the subgenus *Nyssorhynchus* be sister group with (*Anopheles* + *Cellia*), and the study supports the result. The study based on *COI*, *COII* and 5.8S rRNA suggested the sister relationship of the subgenera *Stethomyia* and *Kerteszia* [48], and the study supports the result. The subgenus *Lophopodomyia* were grouped with the subgenera *Anopheles* and *Stethomyia* in two morphology-based inferences [13, 15], whereas it was not included in the molecular-based inferences [17, 48, 49]. This study suggests that the subgenus *Lophopodomyia* be the sister with other five subgenera together. In general, the phylogenetic relationships constructed between morphology-based and molecular-based inference are quite different, and there is need of further studies with inclusion of more species and data to elucidate the among-subgenera relationships.

For the subgenus *Cellia*, four series Neomyzomyia, Pyretophorous, Neocellia and Myzomyia investigated all appear to be monophyletic (pp = 1 and bv = 100% for their clades), with the phylogenetic relationships of Neomyzomyia + (Pyretophorous + (Neocellia + Myzomyia)). The results are completely consistent with those of our earlier study that was also based on whole mtgenomes [17], and almost consistent with the phylogenetic study based on 18S, 28S, *COI* and *COII* data in monophyly and relationship [47]. However, the early morphology-based study in 2000 treated the four series as paraphyly [13]. These suggest that results stemmed from molecular and morphology are often conflicting as discussed above.

For the subgenus *Anopheles*, the two sections Angusticorn (only series Anopheles included) and Laticorn (two series Myzorhynchus and Arribalzagia included) both seem to be polyphyletic. The two series Myzorhynchus and Arribalzagia would be monopheletic (pp = 1 and bv ³ 96% for their clades), and if *An. lindesayi* were excluded, the series Anopheles would also be monopheletic (pp = 0.92 and bv = 85%), with the relations of (Anopheles + (Myzorhynchus + Arribalzagia)). The phylogenetic study based on *COI*, *COII* and 5.8S rRNA suggested the sections Laticorn and Angusticorn be polyphyletic, and inside the two series Anopheles and Myzorhynchus involved also be polyphyletic. In two morphology-based studies, one based on 163 morphological characters proposed the sections Laticorn and Angusticorn to be polyphyletic, the series Arribalzagia to be monophyletic, and the two series Myzorhynchus and Anopheles to be paraphyletic [13]. The another based on 167 morphological characters proposed the section Laticorn to be monophyletic, the section Angusticorn to be polyphyletic, the two series Arribalzagia and Myzorhynchus to be monphyletic, and the series Anopheles to be polyphyletic [15]. All of these four studies suggested that the section Angusticorn be polyphyletic, in which the series Anopheles be polyphyletic, and most of these studies proposed that the section Laticorn be polyphyletic, in which the series Arribalzagia be monophyletic and the series Myzorhynchus may be monophyletic.

For the subgenus *Nyssorhynchus*, three sections Myzorhynchella, Argyritarsis and Albimanus investigated, and their subdivisions in the three sections all appear polyphyletic or paraphyletic. The morphology-based study based on 163 morphological characters data suggested the three sections Albimanus, Argyritarsis and Myzorhynchella were paraphyletic [13]. In two molecular-based study, one based on *white* and *ND6* for 21 species in the *Nyssorhynchus* with BI method in 2010 [50] suggested the three sections be not monophyletic, and a another one based on *white*, *CAD* and *COI* for 32 species in *Nyssorhynchus* with BI method in 2013 showed the three sections to be polyphyletic, and the three series also to polyphyletic [51]. All of these four studies demonstrate that the taxonomy and phylogenetics of the subgenus are quite conflicted, and there is more necessarity to reconstruct the taxonomic system of the subgenus along the phylogenetic study.

Conclusions

This study sequenced and analyzed the complete mtgenomes of *An. peditaeniatus* and *An. nitidus*, and investigated the characteristics and phylogenetic relationships of 76 complete mtgenome sequences in the genus *Anopheles*. These mtgenomes are of general characteristics similar as earlier reports in insects, and however the *trnR* and *trnA* have a reversal arrangement to form "*trnR-trnA*" in comparison of *Drosophilayakuba* mtgenomes as those reported in other genera in Culicidae. Their variations mainly occur in CR regions with length from 493 bp - 886 bp, and six repeat unit types are identified for the first time, which demonstrate the evolutionary importance among subgenera to some extent. The subgenera *Lophopodomyia*, *Stethomyia*, *Kerteszia*, *Nyssorhynchus*, *Anopheles* and *Cellia* are all proposed to be monophyletic with the phylogenetic relationships of *Lophopodomyia* + ((*Stethomyia* + *Kerteszia*) + (*Nyssorhynchus* + (*Anopheles* + *Cellia*)))). Four series Neomyzomyia, Pyretophorous, Neocellia and Myzomyia in the subgenus *Cellia*, are proposed to be monophyletic, two series Arribalzagia and Myzorhynchus in the subgenus *Anopheles* are proposed to be monophyletic while the series *Anopheles* seems polyphyletic, and three sections Myzorhynchella, Argyritarsis and Albimanus and their subdivisions in the subgenus *Nyssorhynchus* all appear polyphyletic or paraphyletic. In general, there is need of further studies with inclusion of more species and data to elucidate the phylogenetic relationships in the genus.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data are available as tables and figures in the main document and its additional files. The GenBank accession numbers for the two mtgenomes produced in the present study are MW401801 and MT822295.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BC and JG conceived and designed the study. JG and BC performed the experiments and data analysis, and drafted the manuscript. ZTY, WBF, HY and XDL joined the specimens collecting and experiments. All authors read and approved the final version of the manuscript.

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Abbreviations

mtgenome: mitochondrial genome; PCGs: protein-coding genes; rRNAs: ribosomal RNA genes; tRNAs: transfer RNA genes; CR: control region; RSCU: relative synonymous codon usage; BI: Bayesian inference; ML: Maximum likelihood.

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Tables

Table 1 Detailed sequence information of mtgenomes used in the present phylogenetic analysis.

Sections/Series	Species	Total size (bp)	PCGs size (bp)	tRNA size (bp)	rRNA size (bp)	CR size (bp)	GenBank ID
Subgenus *Cellia*							
/Myzomyia	*An. aconitus*	15359	11224	1472	2114	519	NC039540
	An. culicifacies	15364	11194	1474	2121	535	NC028216
	An. culicifacies B	15330	11230	1474	2114	498	NC027502
	An. funestus	15356	11231	1477	2121	519	NC038158
	An. minimus	15411	11194	1476	2117	546	NC028221
/Neocellia	*An. maculatus*	14850	11188	1479	2108	N/A	NC028218
	An. splendidus	15362	11224	1477	2121	510	NC039397
	An. stephensi	15387	11190	1477	2117	551	NC028223
/Neomyzomyia	*An. cracens*	15412	11224	1482	2123	576	NC020768
	An. dirus	15406	11224	1478	2124	568	NC036263
	An. farauti 4	15412	11224	1482	2125	576	NC020770
	An. hinesorum	15336	11224	1479	2123	505	NC020769
	An. punctulatus	15322	11187	1477	2118	493	NC028222
/Pyretophorus	*An. arabiensis*	15369	11194	1477	2122	530	NC028212
	An. christyi	14967	11188	1477	2126	N/A	NC028214
	An. coluzzii	15441	11194	1478	2124	599	NC028215
	An. epiroticus	15379	11188	1479	2122	535	NC028217
	An. gambiae	15363	11230	1479	2125	519	NC002084
	An. melas	15366	11194	1477	2122	526	NC028219
	An. merus	15365	11188	1478	2121	525	NC028220
Subgenus *Anopheles*							
Angusticorn/Anopheles	*An. atroparvus*	15458	11175	1474	2161	614	NC028213
	An. eiseni geometricus	15696	11241	1474	2120	860	MF381678
	An. lindesayi	15366	11225	1475	2123	531	KX961140
	An. quadrimaculatus A	15455	11220	1473	2115	625	NC000875
Laticorn/Arribalzagia	*An. costai*	15433	11241	1473	2122	598	NC037794
	An. nr. costai	15434	11241	1473	2121	600	NC037821
	An. fluminensis	15429	11241	1474	2120	594	NC037818
	An. forattinii	15459	11241	1473	2125	615	NC037813
	An. medialis[*]	15409	11241	1475	2121	545	NC037789
	An. minor	15466	11238	1478	2123	594	NC037802
	An. peryassui	15417	11241	1474	2120	585	NC037790
Laticorn/Myzorhynchus	*An. coustani*	15408	11194	1475	2112	570	MT806097
An. nitidus	15418	11168	1476	2122	580	MW401801	
An. peditaeniatus	15416	11224	1477	2125	575	MT822295	
	An. sinensis	15418	11224	1473	2125	577	MF322628
Subgenus *Nyssorhynchus*							

Albimanus/Oswaldoi	*An. albertoi*	15385	11240	1475	2114	558	NC037804
	An. arthuri	15387	11240	1475	2114	560	NC037806
	An. benarrochi	15387	11240	1477	2116	556	NC037787
	An. evansae	15382	11240	1477	2115	553	NC037795
	An. galvaoi	15420	11240	1477	2150	555	NC037814
	An. goeldii	15391	11240	1477	2117	560	NC037810
	An. konderi	15395	11240	1478	2125	555	MF381685
	An. nuneztovari	15393	11240	1477	2117	562	MF381680
	An. oswaldoi	15380	11237	1477	2115	554	NC037793
	An. rangeli	15386	11240	1477	2114	558	NC037786
	An. rondoni	15385	11240	1477	2113	557	NC037815
	An. striatus	15385	11240	1476	2115	557	NC037801
	An. strodei	15388	11240	1475	2115	560	NC037808
	An. triannulatus	15401	11240	1477	2125	559	NC037800
Argyritarsis/Albitarsis	*An. albitarsis*	15413	11216	1477	2119	575	NC020662
	An. albitarsis F	15418	11216	1479	2121	578	NC030768
	An. albitarsis G	15474	11216	1480	2125	615	NC030766
	An. braziliensis	15397	11240	1480	2115	562	NC037791
	An. nr. braziliensis	15413	11240	1478	2116	578	MF381606
	An. deaneorum	15424	11216	1476	2121	581	NC020663
	An. janconnae	15425	11216	1480	2120	575	NC030767
	An. marajoara	15453	11240	1476	2132	584	NC037788
	An. oryzalimnetes	15422	11216	1479	2120	581	NC030765
Argyritarsis/Argyritarsis	*An. argyritarsis*	15403	11240	1481	2115	579	NC037807
	An. atacamensis	15412	11241	1476	2122	564	NC037792
	An. darlingi	15386	11240	1489	2122	554	NC014275
	An. lanei	15396	11240	1478	2116	567	NC037799
	An. sawyeri	15417	11240	1477	2116	599	NC037798
Myzorhynchella/	*An. antunesi*	15427	11242	1475	2118	595	NC037817
	An. guarani	15531	11241	1473	2119	700	NC037816
	An. lutzii	15341	11242	1475	2118	509	NC037820
	An. parvus	15444	11235	1470	2116	617	NC037805
	An. pristinus	15405	11241	1476	2117	581	NC037824
Subgenus *Kerteszia*							
	An. bellator	15668	11242	1477	2126	811	NC030249
	An. cruzii	15449	11230	1478	2116	600	NC024740
	An. homunculus	15739	11242	1475	2125	886	NC030248
	An. laneanus	15446	11242	1479	2124	591	NC030250
Subgenus *Stethomyia*							
	An. kompi	15505	11240	1476	2118	647	NC037827

	An. nimbus	15476	11240	1467	2121	628	NC037811
Subgenus *Lophopodomyia*							
	An. gilesi	15458	11244	1465	2108	648	NC037803
	An. pseudotibiamaculatus	15597	11242	1478	2122	768	NC037829
Outgroup							
	Cx. pipiens pallens	15617	11228	1482	2138	713	KT851543

**Anopheles medialis* (Harbach, 2018) = *Anopheles intermedius* (Peryassú, 1908).

Table 2 Organization of the *An. peditaeniatus* and *An. nitidus* mtgenomes.

Gene	Strand	Position (bp)		Length (bp)		Space(+)/overlap(-)		Start/Stop codon	
		punctulatus	*nitidus*	*punctulatus*	*nitidus*	*punctulatus*	*nitidus*	*punctulatus*	*nitidus*
trnI	J	1-68	1-68	68	68	0	0		
trnQ	N	66-134	66-134	69	69	-3	-3		
trnM	J	1134-202	134-202	69	69	-1	-1		
nad2	J	203-1228	203-1228	1026	1026	0	0	ATT/TAA	ATT/TAA
trnW	J	1227-1295	1227-1295	69	69	-2	-2		
trnC	N	1295-1358	1295-1358	64	64	-1	-1		
trnY	N	1360-1425	1360-1425	66	66	1	1		
cox1	J	1424-2960	1424-2965	1537	1542	-2	-2	TCG/T	TCG/TAA
trnL1	J	2961-3026	2961-3026	66	66	0	-5		
cox2	J	3028-3712	3028-3712	685	685	1	1	ATG/T	ATG/T
trnK	J	3713-3784	3713-3784	72	72	0	0		
trnD	J	3797-3865	3797-3865	69	69	12	12		
atp8	J	3866-4027	3866-4027	162	162	0	0	ATT/TAA	ATT/TAA
atp6	J	4021-4701	4021-4701	681	681	-7	-7	ATG/TAA	ATG/TAA
cox3	J	4701-5487	4701-5495	787	795	-1	-1	ATG/T	ATG/TAA
trnG	J	5488-5554	5488-5554	67	67	0	-8		
nad3	J	5555-5908	5555-5908	354	354	0	0	ATA/TAA	ATA/TAA
trnR	J	5907-5970	5907-5970	64	64	-2	-2		
trnA	J	5974-6038	5971-6036	65	66	3	0		
trnN	J	6039-6105	6037-6103	67	67	0	0		
trnS2	N	6106-6172	6104-6170	67	67	0	0		
trnE	J	6174-6239	6172-6237	66	66	1	1		
trnF	N	6238-6304	6236-6302	67	67	-2	-2		
nad5	N	6304-8046	6302-8017	1743	1766	-1	-1	GTG/TAA	ATT/TAA
trnH	N	8047-8110	8045-8109	64	65	0	27		
nad4	N	8111-9452	8113-9451	1342	1339	0	3	ATG/T	ATG/T
nad4L	N	9446-9745	9445-9744	300	300	-7	-7	ATG/TAA	ATG/TAA
trnT	J	9752-9816	9751-9815	65	65	6	6		
trnP	N	9817-9882	9816-9881	66	66	0	0		
nad6	J	9885-10409	9884-10408	525	525	2	2	ATT/TAA	ATT/TAA
cob	J	10409-11545	10408-11544	1137	1137	-1	-1	ATG/TAA	ATG/TAA
trnS1	J	11544-11609	11543-11608	66	66	-2	-2		
nad1	N	11628-12572	11629-12573	945	945	18	20	ATT/TAA	ATT/TAA
trnL2	N	12579-12644	12580-12645	66	66	6	6		
rrnL	N	12645-13972	12646-13973	1328	1328	0	0		
trnV	N	13973-14044	13974-14044	72	72	0	0		
rrnS	N	14045-14841	14045-14838	797	794	0	0		
CR		14842-15416	14839-15418	575	579	0	0		

Figures

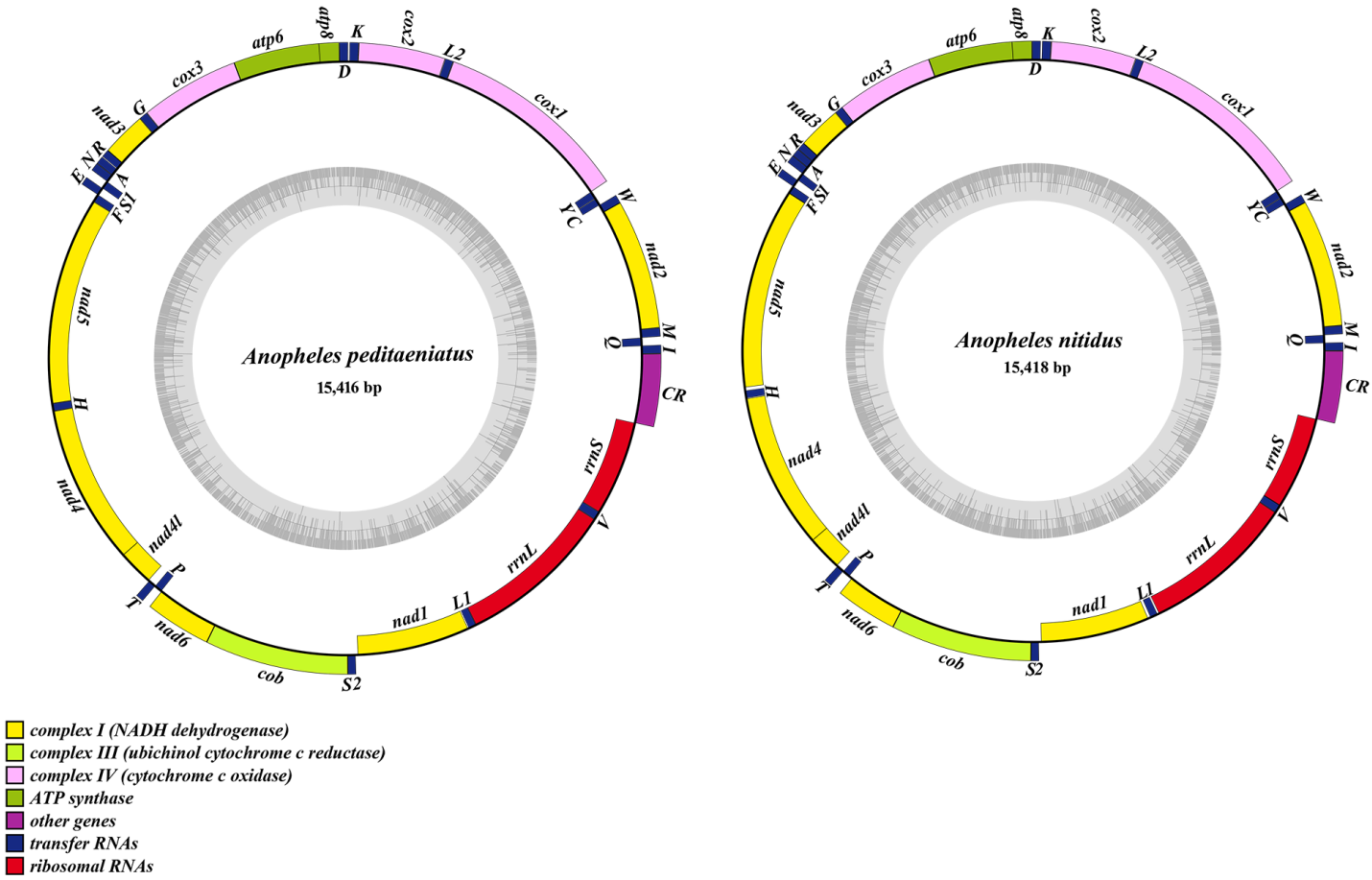

Figure 1

Mtgenome structure of An. peditaeniatus and An. nitidus.

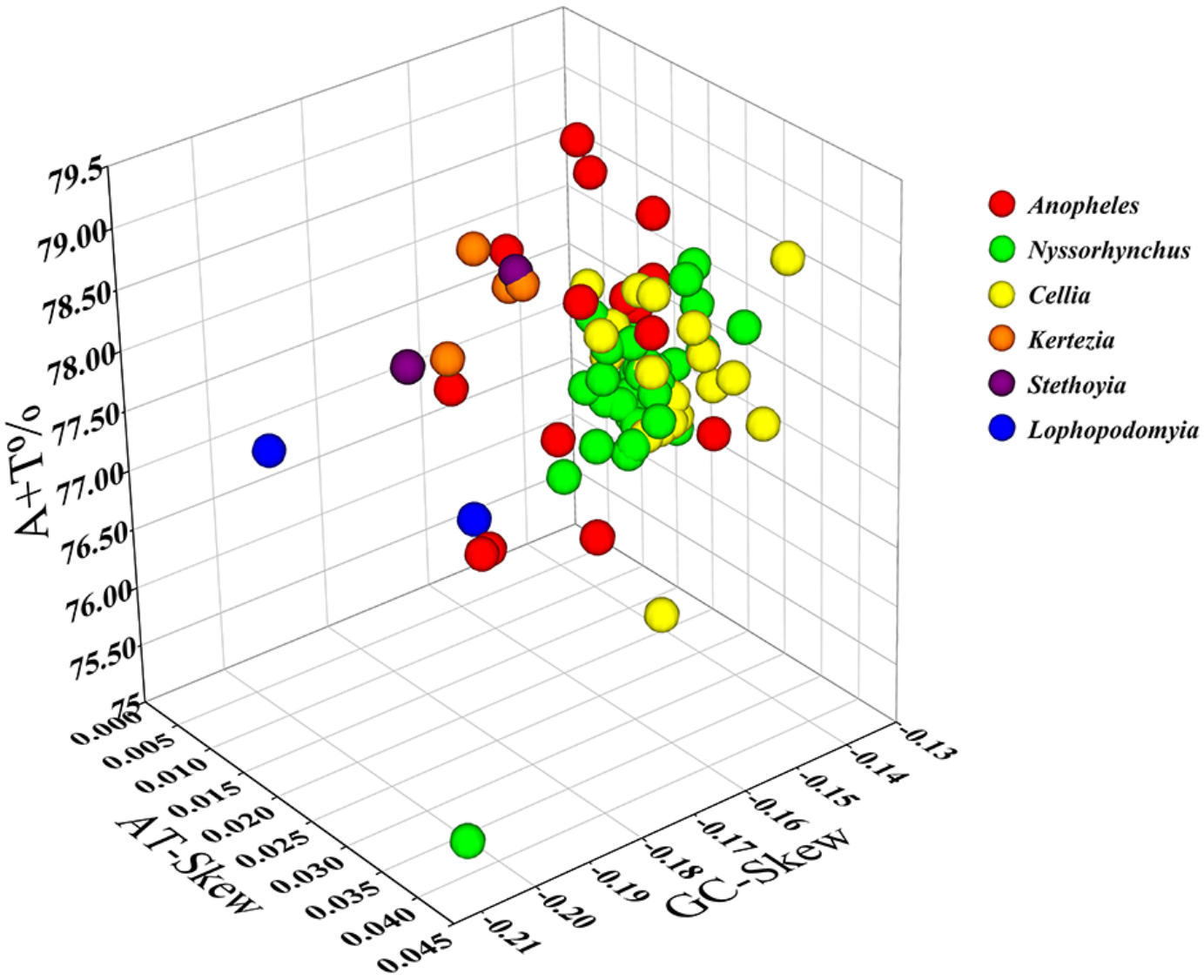

Figure 2

Three-dimensional scatter plot of the AT-Skew, GC-Skew and AT% of 76 mtgenome sequences in the genus Anopheles.

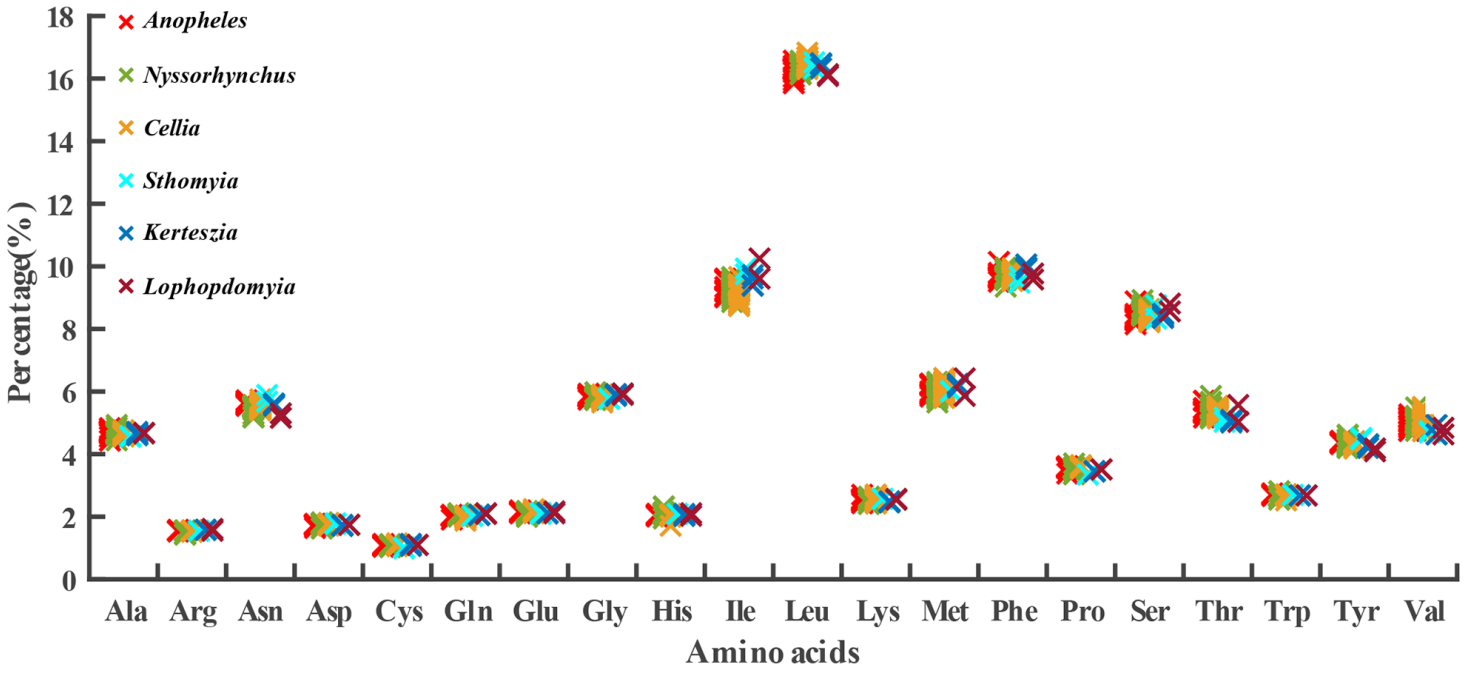

Figure 3

Frequency percentage of each of 20 coded amino acids in 76 mtgenome sequences in the genus Anopheles.

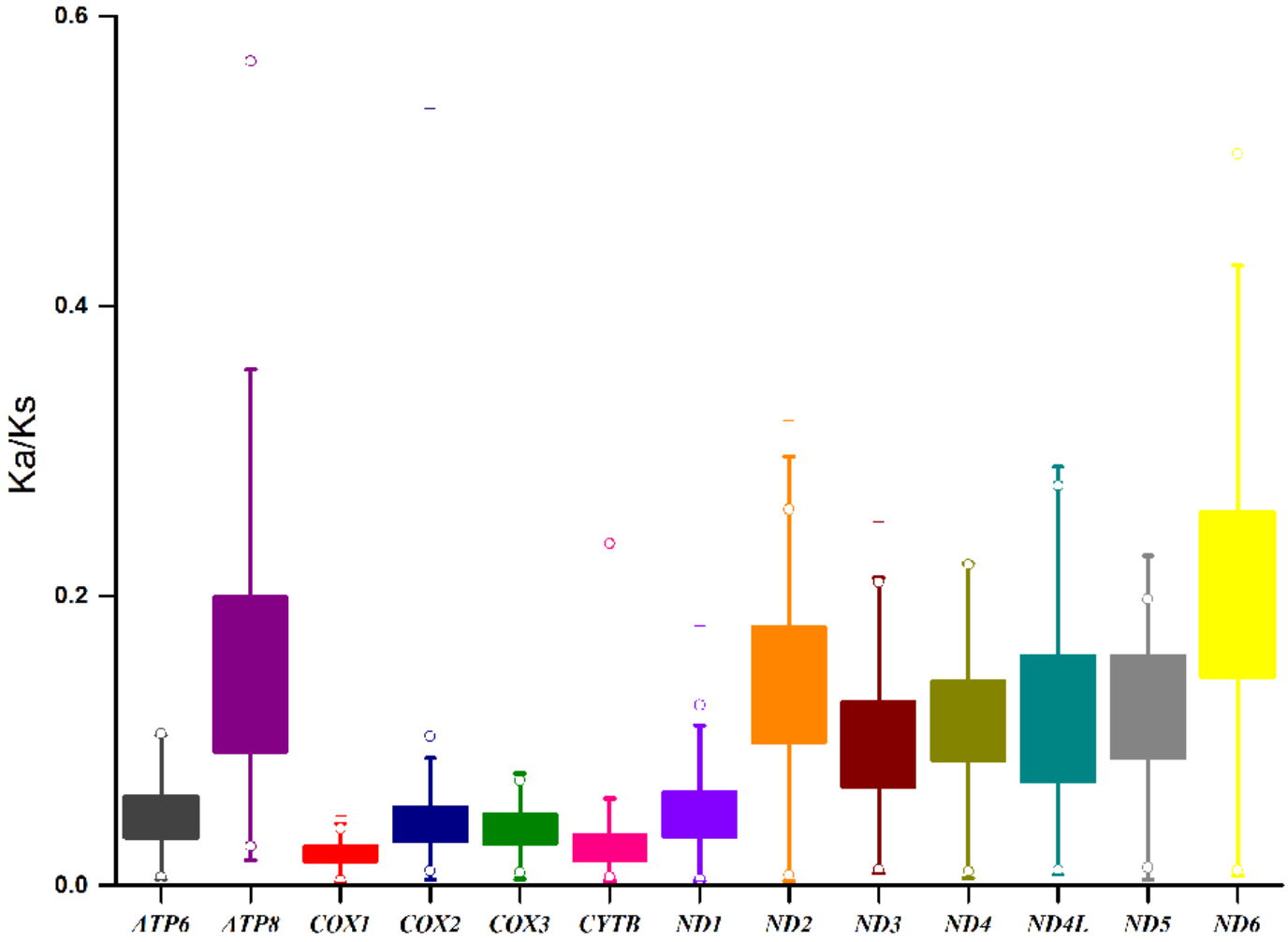

Figure 4

Evolutionary rates of 13 protein-coding genes (PCGs) within 76 mtgenomes in the genus Anopheles. Ka: Non-synonymous mutation rate; Ks: Synonymous mutation rate; Ka/Ks: The ratio of non-synonymous mutation rate to synonymous mutation rate. Neutral evolution (Ka/Ks=1), Purify selection (Ka/Ks<1), Positive selection (Ka/Ks>1).

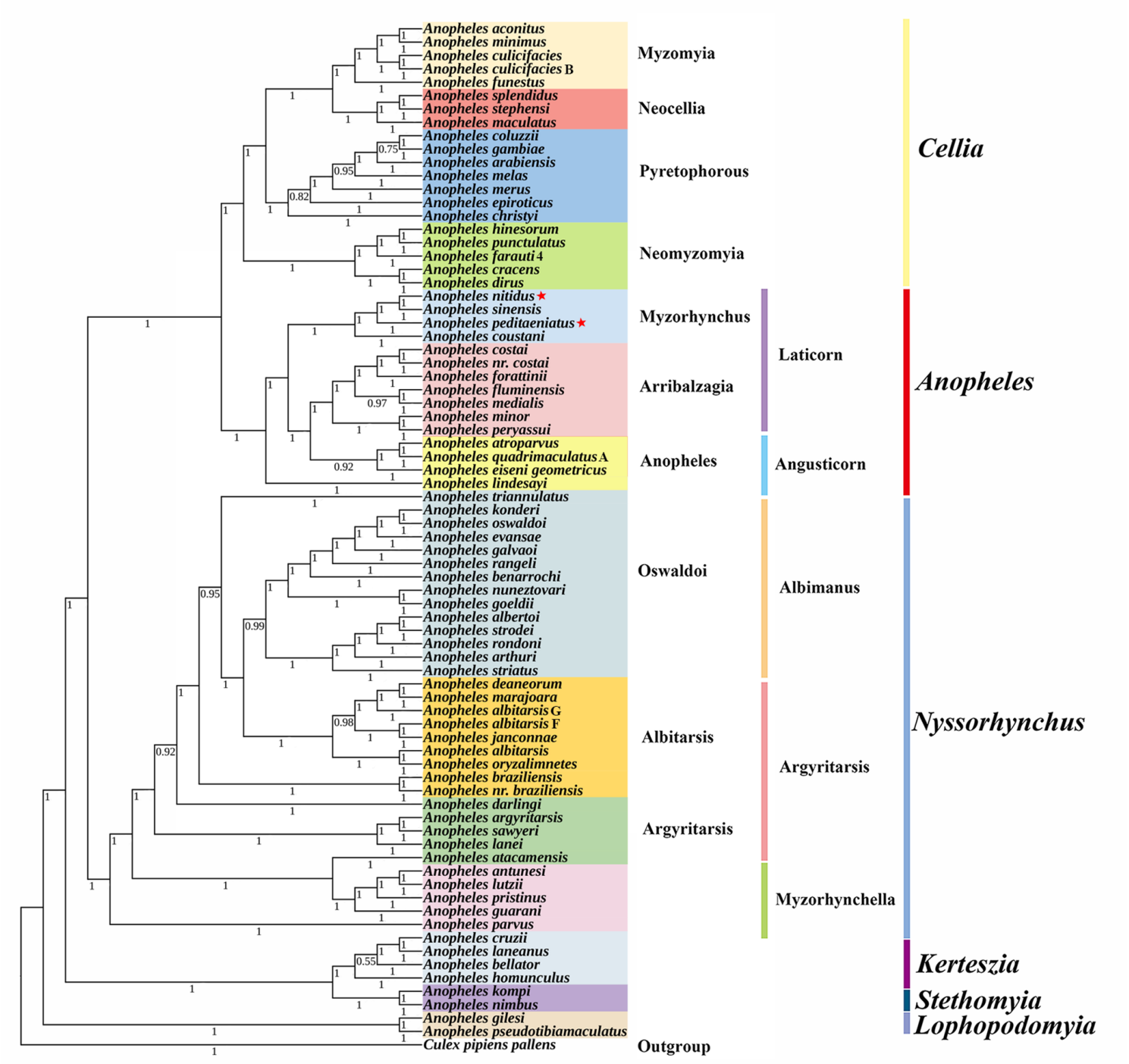

Figure 5

Phylogenetic relationships of 76 mtgenomes in the genus Anopheles. The phylogenetic tree was constructed based on nucleotide sequences of 13 protein-coding genes using MrBayes Infereence. The numbers at the nodes is Bayesian posterior probabilities. The mtgenomes of two species newly sequenced in this study are indicated by pentagrams. The GenBank accession numbers of the 76 mtgenome sequences are listed in Table 1.

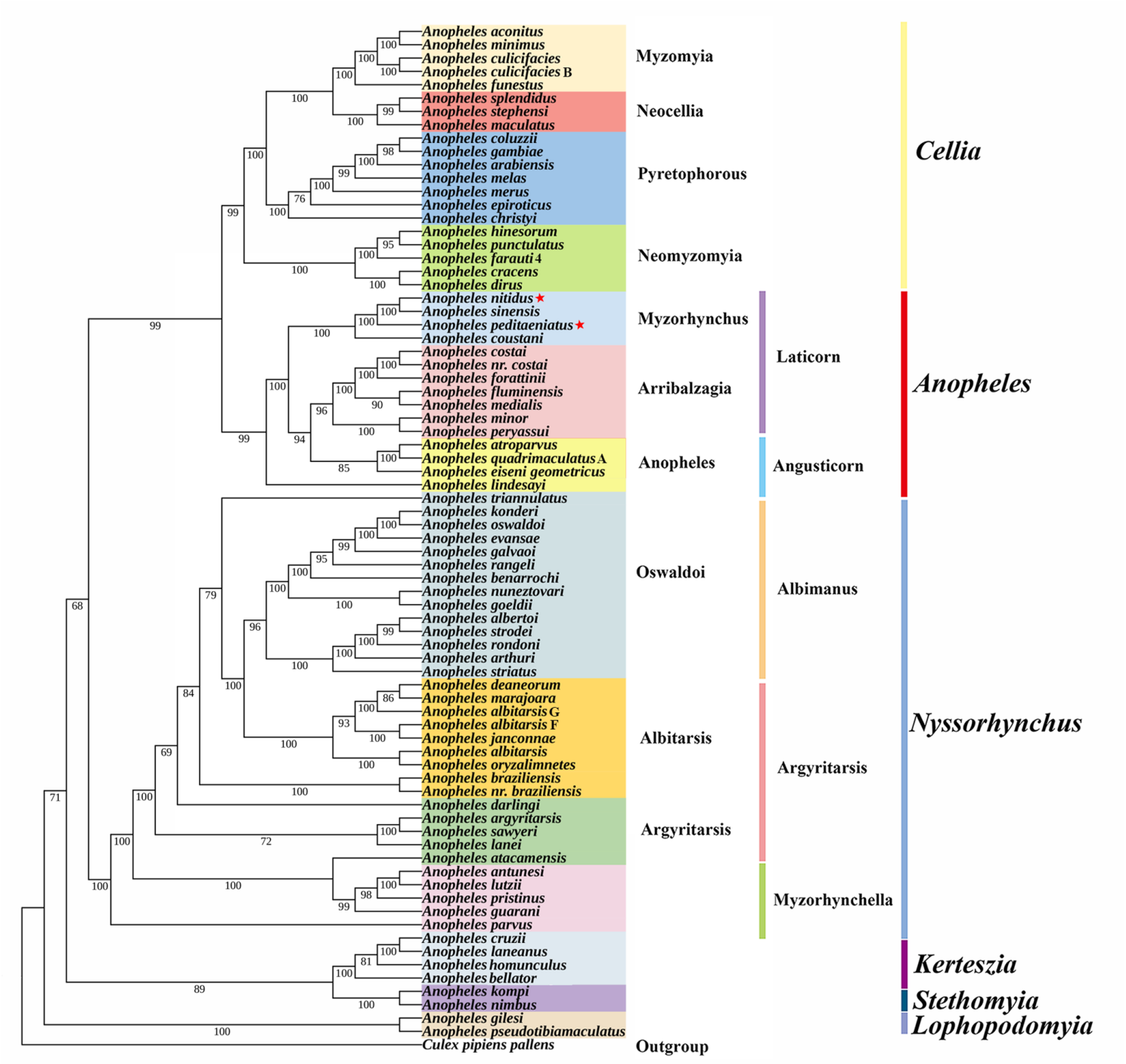

Figure 6

Phylogenetic relationships of 76 mtgenomes in the genus Anopheles. The phylogenetic tree was constructed based on nucleotide sequences of 13 protein-coding genes using Maximum Likelihood. The numbers at the nodes is bootstrap values. The mtgenomes of two species newly sequenced in this study are indicated by pentagrams. The GenBank accession numbers of the 76 mtgenome sequences are listed in Table 1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1.doc
- Additionalfile2TableS2.xls
- Additionalfile3FigureS1.doc
- Additionalfile4FigureS2.doc
- Graphicalabstract.tif