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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Research Article

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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, Stethomyia* and *Kerteszia* and *Lophopodomyia*. In *Nyssorhynchus*, 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 Cycloleppteron+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 *Kerteszia* [17, 18]. Generally, the monophyly of the subgenera *Anopheles, Nyssorhynchus, Cellia, Stethomyia, Kerteszia* 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, \textit{trnA} and \textit{trnR} of mosquitoes are rearranged to form “\textit{trnR-trnA}” arrangement [17, 18, 22].

In the present study, we sequenced and annotated the complete mitogenomes of \textit{An. peditaeniatus} and \textit{An. nitidus} in the genus \textit{Anopheles}, and comparatively analyzed the characteristics of 76 species of mtgenome sequences in the genus \textit{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 \textit{Anopheles}.

**Methods**

**Sample collection and DNA extraction**

Specimens of \textit{An. peditaeniatus} and \textit{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°C 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 throughput sequencing of mitochondrial genome in Shenzhen Huitong Biotechnology Co. Ltd..

**Mtgenomes assembly, annotation and characteristics analysis**

The mtgenomes of \textit{An. peditaeniatus} and \textit{An. nitidus} assembled and annotated using Mitos (http://mitos.bioinf.unileipzig.de/index.py) [30]. The annotation of 13 PCGs and two rRNA genes were 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 $AT\ skew = \frac{[A - T]}{[A + T]}$ and $GC\ skew = \frac{[G - C]}{[G + C]}$ [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 file 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 *Cellia*, 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 MI 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 ≥ 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 monophyletic, 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 necessity 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 *Drosophila yakuba* 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. lindesi                    | 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. ntidus                     | 15418          | 11168         | 1476           | 2122           | 580          | MW401801   |
|                 | An. peditaeniatus              | 15416          | 11224         | 1477           | 2125           | 575          | MT822295   |
|                 | An. sinensis                   | 15418          | 11224         | 1473           | 2125           | 577          | MF322628   |
| **Subgenus Nyssorhynchus** |                               |                |               |                |                |              |            |
| Genus                  | Species                | Accession Number | Other Accession Numbers |
|------------------------|------------------------|------------------|-------------------------|
| Albimanus/Oswaldoi     | *An. albertoi*         | NC037804         |                         |
|                        | *An. arthuri*          | NC037806         |                         |
|                        | *An. benarrochi*       | NC037787         |                         |
|                        | *An. evansae*          | NC037795         |                         |
|                        | *An. galvaoi*          | NC037814         |                         |
|                        | *An. goeldii*          | NC037810         |                         |
|                        | *An. konderi*          | NC037793         |                         |
|                        | *An. nuneztovari*      | NC037786         |                         |
|                        | *An. oswaldoi*         | NC037793         |                         |
|                        | *An. rangeli*          | NC037786         |                         |
|                        | *An. rondoni*          | NC037815         |                         |
|                        | *An. striatus*         | NC037801         |                         |
|                        | *An. strodei*          | NC037808         |                         |
|                        | *An. triannulatus*     | NC037800         |                         |
| Argyritarsis/Albitarsis| *An. albitarsis*       | NC020662         |                         |
|                        | *An. albitarsis F*     | NC030766         |                         |
|                        | *An. albitarsis G*     | NC030766         |                         |
|                        | *An. braziliensis*     | NC037791         |                         |
|                        | *An. nr. braziliensis* | MF381606         |                         |
|                        | *An. deaneorum*        | NC020663         |                         |
|                        | *An. janconnae*        | NC030766         |                         |
|                        | *An. marajoara*        | NC037788         |                         |
|                        | *An. oryzalimnetes*    | NC030765         |                         |
| Argyritarsis/Argyritarsis| *An. argyritarsis*   | NC037807         |                         |
|                        | *An. atacamensis*      | NC037792         |                         |
|                        | *An. darlingi*         | NC014275         |                         |
|                        | *An. lanei*            | NC037799         |                         |
|                        | *An. sawyeri*          | NC037798         |                         |
| Myzorhynchella/        | *An. antunesi*         | NC037817         |                         |
|                        | *An. guarani*          | NC037816         |                         |
|                        | *An. lutzii*           | NC037820         |                         |
|                        | *An. parvus*           | NC037805         |                         |
|                        | *An. pristinus*        | NC037824         |                         |
| Subgenus *Kerteszia*   | *An. bellator*         | NC030249         |                         |
|                        | *An. cruzii*           | NC024740         |                         |
|                        | *An. homunculus*       | NC030248         |                         |
|                        | *An. laneanus*         | NC030250         |                         |
| Subgenus *Stethomyia*  | *An. kompi*            | NC037827         |                         |
Subgenus *Lophopodomyia*

| Species               | Length (nt) | RefSeq Accession |
|-----------------------|-------------|------------------|
| *An. nimbus*          | 15476       | NC037811         |
| *An. gilesi*          | 15458       | NC037803         |
| *An. pseudotibiamaculatus* | 15597       | NC037829         |
| *Cx. pipiens pallens* | 15617       | KT851543         |

Outgroup

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

**Table 2** Organization of the *An. peditaeniatius* and *An. nitidus* mtgenomes.
| Gene | Strand | Position (bp) | Length (bp) | Space(+) / overlap(-) | Start/Stop codon |
|------|--------|--------------|-------------|-----------------------|-----------------|
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| trnI J | 1-68   | trnI J       | 68          | 0                     | ATT/TAA         |
| trnQ N | 66-134 | trnQ N       | 69          | -3                    | ATT/TAA         |
| trnM J | 1134-202 | trnM J       | 69          | -1                    | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| trnW J | 1227-1295 | trnW J       | 69          | -2                    | ATT/TAA         |
| trnC N | 1295-1358 | trnC N       | 64          | -1                    | ATT/TAA         |
| trnY N | 1360-1425 | trnY N       | 66          | 1                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| cox1 J | 1424-2965 | cox1 J       | 1537        | -2                    | TCG/T           |
| trnL1 J | 2961-3026 | trnL1 J      | 66          | 0                     | TCG/T           |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| atp8 J | 3866-4027 | atp8 J       | 162         | 0                     | ATT/TAA         |
| atp6 J | 4021-4701 | atp6 J       | 681         | -7                    | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad3 J | 5555-5908 | nad3 J       | 354         | 0                     | ATA/TAA         |
| trnR J | 5907-5970 | trnR J       | 64          | -2                    | ATA/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad5 N | 6304-8046 | nad5 N       | 1743        | -1                    | GTG/TAA         |
| trnH N | 8047-8110 | trnH N       | 64          | 27                    |                 |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad6 J | 9885-10409 | nad6 J      | 525         | 2                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad6L N | 9446-9745 | nad6L N     | 300         | -7                    | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| trnT J | 9752-9816 | trnT J      | 65          | 6                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| trnP N | 9817-9882 | trnP N      | 66          | 0                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad7 J | 11628-12572 | nad7 J    | 945         | 18                    | ATT/TAA         |
| trnL2 N | 12579-12644 | trnL2 N    | 66          | 6                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| cob J | 10409-11545 | cob J      | 1137        | -1                    | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| trnS1 J | 11544-11609 | trnS1 J    | 66          | -2                    | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| nad8 N | 11628-12572 | nad8 N     | 945         | 18                    | ATT/TAA         |
| trnV N | 13973-14044 | trnV N    | 72          | 0                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| rrnS N | 14045-14841 | rrnS N    | 797         | 0                     | ATT/TAA         |
|      |        |              |             |                       |                 |
|      |        | *punctulatus* |             |                       |                 |
|      |        | *nitidus*    |             |                       |                 |
| CR | 14842-15416 | CR        | 575         | 0                     | ATT/TAA         |
Figures

Anopheles pediaeniatus
15,416 bp

Anopheles nitidus
15,418 bp

Figure 1

Mtgenome structure of An. pediaeniatus 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 Inference. 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.

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