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Mitochondrial genome variation of mosquito species in the subgenus *Stegomyia* of the genus *Aedes* (Diptera: Culicidae)

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Abstract. Mosquitoes in the subgenus *Stegomyia* of the genus *Aedes* are vectors of a number of vertebrate viruses, including human arboviral fevers. Of particular interest is the study of the genetic characteristics of invasive populations of species in this group. We obtained, annotated and described the mitochondrial genomes of three *Stegomyia* mosquito species of the genus *Aedes*: *Ae. albopictus*, *Ae. flavopictus* and *Ae. sibiricus*. The mitochondrial genomes of *Ae. flavopictus* and *Ae. sibiricus* were obtained from mosquitoes from synanthropic populations in the Russian Far East. The mitochondrial genome of *Ae. sibiricus* is presented for the first time. The mitochondrial genome of *Ae. albopictus* was obtained for the C6/36 cell line. We selected three primer sets, for each mosquito species, that amplify the entire mitochondrial genome except for the control region and sequenced the genomes using the Sanger method. All three new genomes have an identical gene order. We identified 13 canonical protein-coding genes, 2 ribosomal RNA genes, and 22 transport RNA genes. Protein-coding genes have canonical start and stop codons with two exceptions. The canonical stop codon "TAA" is incomplete in the *cox*1 and *cox*2 genes. The *cox*1 gene lacks the canonical start codon for methionine. Nucleotide variability is mainly represented by point nucleotide substitutions. A phylogenetic analysis of the nucleotide sequences of complete mitochondrial genomes of all known mosquitoes species in the subgenus *Stegomyia* of the genus *Aedes* was performed. The data obtained made it possible to measure the ratio of synonymous to non-synonymous substitutions (Ka/Ks) in specific protein-coding genes.
Key words: invasive species; mitochondrial genome; phylogenetic analysis; mtDNA

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Характеристика трех митохондриальных геномов комаров рода *Aedes* (Diptera: Culicidae) подрода *Stegomyia*

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Аннотация. Комары рода *Aedes*, подрода *Stegomyia* являются переносчиками ряда вирусов позвоночных, в том числе возбудителей арбовирусных лихорадок человека. Особый интерес представляет изучение генетических особенностей синантропных популяций видов этой группы. Мы получили, аннотировали и описали митохондриальные геномы трех видов комаров рода *Aedes*, подрода *Stegomyia*: *Ae. albopictus*, *Ae. flavopictus* и *Ae. sibiricus*. Митохондриальные геномы *Ae. flavopictus* и *Ae. sibiricus* были получены от комаров из синантропных популяций с Дальнего Востока России. Митохондриальный геном *Ae. sibiricus* представлен нами впервые.

Митохондриальный геном *Ae. albopictus* был получен для клеточной линии C6/36. Мы подобрали три комплекта праймеров для каждого из видов комаров, которые амплифицируют весь митохондриальный геном, кроме контрольной области, и отсеквенировали геномы методом Сэнгера. Все три новых генома имеют одинаковый порядок расположения генов. Идентифицировано 13 канонических белок-кодирующих генов, 2 гена рибосомальной РНК, 22 гена транспортной РНК. Белок-кодирующие гены имеют канонические старт- и стоп-кодоны за двумя исключениями. Канонический стоп-кодон «TAA» неполный в генах *cox*1 и *cox*2. В гене *cox*1 отсутствует канонический старт-кодон для метионина. Нуклеотидная изменчивость представлена в основном точковыми нуклеотидными замещениями. Инсерции-делеции имеются в областях межгенных спейсеров. Проведен филогенетический анализ нуклеотидных последовательностей полных митохондриальных геномов всех известных видов комаров рода *Aedes*, подрода *Stegomyia*. Полученные данные позволили провести измерение соотношения синонимичных и несинонимичных замен (Ka/Ks) в конкретных белок-кодирующих генах.
Ключевые слова: инвазионный вид; митохондриальный геном; филогенетический анализ; мтДНК

Introduction

Mosquitoes of the genus *Aedes*, subgenus *Stegomyia*, are the main vectors of dengue, yellow fever and other arbovirus infections worldwide (Weetman et al., 2018). Of greatest interest and practical importance are the invasive mosquito species in this group that form dense synanthropic populations. *Aedes albopictusis* Skuse, 1894 is an invasive species native to Southeast Asia that has spread to all continents except Antarctica in the last 50 years (Medlock et al., 2012). In the Russian Federation, this species is found in the southern European part of the country. The study of its genetic variability in Russia is mainly based on the analysis of the barcode fragment of the mitochondrial gene *cox*1 (Fedorova et al., 2019; Bega et al., 2022).

The subgenus *Stegomyia* is represented in Russia by three other species of mosquitoes found in the Far East and Siberia – *Aedes flavopictus* Yamada, 1921, *Aedes sibiricus* Danilov & Filippova, 1978 and *Aedes galloisi* Yamada, 1921. These three species are considered native forest species. Comparative analyses of the genetic structure of *Ae. albopictus* and *Ae. flavopictus* populations from the Korean peninsula support this hypothesis (Shin, Jung, 2021). Previously, *Ae. flavopictus* and *Ae. sibiricus* did not form dense populations in the Far East and were only found as isolated specimens (Gutsevich et al., 1970). Recently there have been reports of sightings of these species in urban areas (Berlov, Kuberskaya, 2021; Berlov et al., 2021). We have obtained data on range expansion and the formation of dense synanthropic populations of *Ae. flavopictus* and *Ae. sibiricus* in the Russian Far East (Bega et al., 2022). This probably indicates the beginning of the formation of invasive populations of these species.

In this paper, we present the results of sequencing the mitochondrial genomes of representatives of potentially invasive populations of *Ae. flavopictus* and *Ae. sibiricus*, and the mitochondrial genome of the cell line *Ae. albopictus* C6/36, as well as the phylogenetic analysis of the obtained sequences.

The mitochondrial genome of *Ae. albopictus* is now well characterised, but some points remain controversial. The mitochondrial genomes of mosquitoes from the island of Taiwan, including the reference genome (ID NC_006817), have reading frame shifts and abnormal stop codons. This may be due to the fact that the sample was taken from an insular and presumably indigenous population. It may also be a consequence of the inclusion of nuclear copies of mitochondrial genes, or Numts, in the mitochondrial genome. Some sequences of the mitochondrial genome of *Ae. albopictus* represented in GenBank have deletions and poly(A) spacers (Battaglia et al., 2016; Ze-Ze et al., 2020). The features of the mitochondrial genome of *Ae. albopictus* cell culture have not been previously studied. C6/36 culture was obtained from mosquitoes, the place of capture of which is not precisely known (Singh, 1967). To date, the culture has been passaged in the laboratory for more than 50 years. Under cell culture conditions, with constant temperature and nutrient levels, the cells do not experience the selection factors that natural mosquito populations do. Obtaining the mitochondrial genome of a C6/36 cell culture is of interest because it shows which mitochondrial genes are under selection in natural populations. At the time of publication, only two mitochondrial genome sequences of *Ae. flavopictus*, NC_050044 and MT501510, from the southern part of the species range were available in NCBI GenBank. The genome we obtained represents a previously uncharacterised northern part of the range. The mitochondrial genome of *Ae. sibiricus* was obtained for the first time in this study. The NCBI GenBank had a mitochondrial genome for the closely related species *Ae. galloisi*. The sequences obtained in this study are of interest and can be used for further studies on the genetic characteristics of mosquitoes of the subgenus *Stegomyia*.

Materials and methods

Specimen collection and species identification. Mosquito samples were collected in the Russian Far East in the summer of 2020. We trapped *Ae. flavopictus* in Khabarovsk and *Ae. sibiricus* in Svobodny city, Amur region. *Aedes albopictus* clone C6/36 is a commercially available mosquito cell line isolated from larvae of this species (Singh, 1967). Species identification by morphological characters was carried out according to the keys in the identifiers (Gutsevich et al., 1970; Tanaka, 1979; Ree, 2003).

The taxonomic status of the mosquito we defined as *Ae. sibiricus* should be mentioned separately. Not all of the identifiers mentioned above include data on the separation of the species *Ae. sibiricus* from the previously described *Ae. galloisi* (Danilov, Filippova, 1978). We used keys to identify these species based on the colour of the legs and the struc-

ture of the hypopygium in males (Danilov, Filippova, 1978; Poltoratskaya, Mirzaeva, 2013). The species *Ae. sibiricus* is currently listed in the Mosquitoes of the World catalogue of blood-sucking mosquitoes (Wilkerson et al., 2021); however, the description of the species is only published in Russian and therefore *Ae. sibiricus* is not included in the GenBank taxonomic database.

DNA isolation and sequencing of the mitochondrial genome. Total DNA was isolated from individual adult mosquitoes. Each individual was homogenised in lysis solution. The composition of the lysis solution was as follows: 500 mM Tris-EDTA pH = 8.0, 100 μg/ml Proteinase K, 1 % Sodium N-lauroylsarcosinate, 100 mM NaCl. Lysis was performed at 50 °C for 3 hours. After lysis, the DNA was extracted with phenol. The phenol was in the upper layer. Two volumes of water were added to the resulting DNA solution, then the DNA was precipitated with isopropyl alcohol. After purification, the DNA was dissolved in deionised water.

Mitochondrial genomes were amplified using the Encyclo Plus PCR kit (Evrogen, Russia) and sequenced using the Sanger method. We selected the primers ourselves using Primer3 software (Rozen, Skaletsky, 2000) based on the *Ae. albopictus* mitochondrial genome published in the paper (Battaglia et al., 2016). PCR amplification for all primer pairs we selected was performed at an annealing temperature of 58 °C. The list of primers used is shown in Tables 1–3.

Bioinformatics analysis. Sequences were analysed using BLAST software to identify mitochondrial genes. Open reading frame start and stop codons were determined by comparison with start and stop codons of orthologous protein-coding genes in GenBank. Phylogenetic analysis was performed using the MEGA7 programme (Kumar et al., 2016). Sequences obtained from sequencing were aligned to sequences in the databases using NCBI resources (http://www.ncbi.nlm.nih.gov). We used the multiple sequence alignment algorithm Clustal W (Thompson et al., 1994). Visualisation of the mitochondrial genome ring was performed using Chloroplot software (Zheng et al., 2020). The algorithm for calculating the Ka/Ks ratio is described in the paper (Wang D. et al., 2011). We have carried out the calculation using the KaKs_Calculator software (Zhang Z. et al., 2006) using a simple substitution correction method (NG) (Nei, Gojobori, 1986). Suppose the length of the DNA sequence being compared is n and the number of substitutions between the sequences being compared is m. To calculate Ka and Ks, we need to count the number of synonymous (S) and non-synonymous (N) sites ($S + N = n$) and the number of synonymous (Sd) and non-synonymous (Nd) substitutions ($Sd + Nd = m$). Then, after correction for multiple substitutions, (Nd/N) and (Sd/S) can represent Ka and Ks, respectively. This is because the observed number of substitutions underestimates the true number of substitutions due to the divergence of sequences over time. Therefore, the calculation involved three steps: counting S and N, counting Sd and Nd, and correcting for multiple substitutions. Link to the programme distribution https://ngdc.cncb.ac.cn/biocode/tools/BT000001.

Results

Organisation of the derived mitochondrial genomes

The mitochondrial genomes of three mosquito species of the genus *Aedes*, subgenus *Stegomyia* (*Ae. albopictus*, *Ae. flavopictus* and *Ae. sibiricus*) are identical in gene order and similar in nucleotide sequence. The length of the mitochondrial genome excluding the length of control regions is as follows: *Ae. albopictus* 14,900 bp, *Ae. flavopictus* 14,893 bp, *Ae. sibiricus* 14,886 bp. Nucleotide variability is represented by point nucleotide substitutions. When comparing the mitochondrial genomes of *Ae. albopictus* and *Ae. flavopictus*, the degree of nucleotide divergence is minimal (5.74 %). The maximum degree of nucleotide divergence is observed when comparing the nucleotide sequences of the mitochondrial genomes of *Ae. albopictus* and *Ae. sibiricus* (7.51 %). The mitochondrial genomes of *Ae. flavopictus* and *Ae. sibiricus* differ by 6.62 %. All three mitochondrial genomes have a strong A + T = 78.4 % bias, which is characteristic of Diptera mitochondrial genomes.

We identified 13 canonical protein-coding genes (PCGs), 2 ribosomal RNA genes, and 22 transport RNA genes. All PCGs have canonical start and stop codons with two exceptions. The canonical stop codon "TAA" is incomplete in the *cox*1 and *cox*2 genes. It is thought that the missing base "A" is added during RNA processing. In addition, the canonical start codon for methionine is missing in the *cox*1 gene. The heavy (J) strand contains 22 genes, including 9 PCGs and 13 tRNA. The remaining 15 genes are encoded on the light strand (N-strand), including 4 PCGs, 2 rRNA and 9 tRNA. The ring genetic map of the mitochondrial genome of *Ae. sibiricus* is shown in Figure 1.

Phylogenetic analysis

The phylogenetic analysis of the nucleotide sequences of the mosquito mitochondrial genomes we obtained, using all available sequences of the mitochondrial genomes of *Ae. albopictus*, *Ae. flavopictus*, *Ae. aegypti* and *Ae. galloisi* registered in GenBank, is shown in Figure 2. The comparison region included the entire mitochondrial genome except for the control region.

Ae. albopictus, *Ae. aegypti* and *Ae. flavopictus* form independent clusters with high bootstrap support values. The mitochondrial genome of the C6/36 cell line clusters with the mitochondrial genomes of *Ae. albopictus* from the invasive part of the species' range. *Ae. sibiricus* and *Ae. galloisi* are clustered together.

PCGs variability analysis

We calculated the frequency ratio of point nucleotide substitutions leading to a change in the amino acid sequence (non-synonymous substitutions, Ka) or not leading to a change in the amino acid sequence of the protein (synonymous substitutions, Ks) Ka/Ks for PCGs in a pairwise comparison of the mitochondrial genomes obtained in this study: *Ae. albopictus* and *Ae. sibiricus*, *Ae. albopictus* and *Ae. flavopictus*, *Ae. flavopictus* and *Ae. sibiricus* (Fig. 3). The mosquito species we

Table 1. List of primers used to obtain the nucleotide sequence of the complete mitochondrial genome of C6/36 *Ae. albopictus* cell culture

Primer name	Sequence 5′→3′	Localisation on the GenBank sequence ID: OQ145430	PCR fragment length, bp
G1_18L	aatgaattgcctgataaaaagga	1–23	711
G1_18R	tgatttaatcctccaaatgc	711–692	
G4_5L	ttctataattattggggcatttgg	676–699	941
G4_5R	aaaagcatgagcagtaacaattaca	1617–1596	
G1_16L	ctggaatagtcggaacttcactaag	1505–1529	986
G1_16R	cggttaatcccccaactgta	2491–2472	
G1_15L	gccctgcacttttatgatcttt	2432–2453	919
G1_15R	tcattgatggccaataacttttt	3351–3330	
G1_14L	tggccatcaatgatattgaagtta	3339–3362	812
G1_14R	gaatcgattaggtattaatcaaaatgt	4160–4134	
G1_13L	ttggtcttttaattatcccatcaac	4111–4135	854
G1_13R	ttccccatcgtaatcctaatg	4965–4945	
G1_12L	tcgagaaggaacatttcaagg	4904–4924	895
G1_12R	ttggtaaaattaaagcaatttctacat	5799–5773	
G1_12-11L	tgtgacttccaatcacaagga	5528–5548	653
G1_12-11R	tgttgatcaagaaaaagctgcta	6181–6159	
G1_11L	tcatgaatgaaatcaaggagca	5885–5906	619
G1_11R	caagggtgaagagaatattttgg	6504–6482	
G1_10L	tttgaaactcttgcacatataatgaa	6423–6448	1027
G1_10R	tgctcctactcctgtttctgc	7450–7430	
G1_9L	gaatgaactaaagcagaaacagga	7418–7441	560
G1_9R	ttttattgaatgagaagttgtttcttt	7978–7952	
G1_9-8L	tcaccaattcgattagaaagagc	7631–7653	622
G1_9-8R	tcttcaggaagaagtcgagaattt	8253–8230	
G3_1L	aaaaattctcgacttcttcctga	8228–8250	715
G3_1R	ttgtgtatggtggttgcttttt	8943–8922	
G1_7L	agttgcctcaacatgagctt	8860–8879	858
G1_7R	gacgaaaacatcttctctgtacatt	9718–9694	
G1_6L	cagagaagatgttttcgtctagaaata	9700–9726	840
G1_6R	cccaataatgatccaaaatttca	10540–10562	
G1_5L	ttcagcctgatgaaattttgg	10530–10550	944
G1_5R	ggtcgagctccaattcatgt	11474–11455	
G1_4L	tgaattggagctcgacctgt	11458–11477	810
G1_4R	ggggtttatactgtaatagttgctgga	12268–12242	
G1_4-3L	ccttcagcaaaatcaaaagga	11994–12014	515
G1_4-3R	tcaaattcgtaaggggccta	12509–12490	
G3_2L	taggccccttacgaatttga	12490–12509	788
G3_2R	taaagggccgcagtattttg	13287–13268	
G1_2L	ctcattcaaccattcatacaagc	13204–13226	853
G1_2R	gaaaagaaatttgtgcaaatcaa	14057–14035	
G1_1L	tgatttgcacaaatttcttttca	14036–14058	679
G1_1R	ccagctaccgcggttataca	14715–14696	
G5_1L	ttgtataaccgcggtagctg	14695–14714	520
G5_1R	tgatgcttctaggaagaaatgaa	15215–15193	

Table 2. List of primers used to obtain the nucleotide sequence of the complete mitochondrial genome of *Ae. flavopictus*

Primer name	Sequence 5′→3′	Localisation on the GenBank sequence ID: OQ145431	PCR fragment length, bp
G1_18fL	aatgaaggccccgataaaaagga	1–23	710
G1_18fR	tggtttaatcctccaaatgc	710–691	
G1_17fL	ttactttctataattattggagcattt	670–696	898
G1_17fR	aaatatccctgaatgtctaagttcagt	1568–1542	
G1_16fL	ctggaatagtaggaacttctttaag	1504–1528	986
G1_16fR	cagttaatcctccaacgtta	2490–2471	
G1_15fL	gccctgctttattgtgatcttt	2431–2452	919
G1_15fR	tcattgatgcccaataaccttt	3350–3329	
G1_14fL	tgggcatcaatgatactgaagtta	3338–3361	812
G1_14fR	aaatcgattaggtattaatcagaatgt	4159–4133	
G3_14fL	ttggtcttttaattattccttcaaca	4110–4135	831
G3_3R	ggtcttcatacaatccccgt	4941–4922	
G3_3L	tcgagaaggaacatttcaagg	4903–4923	895
G1_12fR	taggtaaaattaaagcaatttctacat	5798–5772	
G1_12-11L	tgtgacttccaatcacaagga	5527–5547	656
G1_12-11R	tgttgatcaagaaaaagctgcta	6183–6161	
G1_11fL	ccatgaatgaaatcaaggagca	5884–5905	622
G1_11fR	caaggatgaagcgaatattttgg	6506–6484	
G1_10fL	tttgaaactcttggacatataatgaa	6425–6450	1027
G1_10fR	agcaccaacacctgtttctgc	7452–7432	
G1_9fL	gaatgaactaaagcagaaacaggt	7420–7443	513
G1_9fR	ttttattgaatgggaaattgtttcttt	7980–7954	
G1_9-8fL	tctacaattcgattagaaagagc	7633–7655	622
G1_9-8fR	tcttcaggaagagttcgggaattt	8255–8232	
G3_4fR	tcatatcattgacaccacaaatca	8106–8129	560
G3_4L	tctgttgctcatatgggtattgtt	8666–8643	
G4_6L	ttcgtcttcctattcgctca	8513–8532	794
G4_6R	gtttttggatttgtggtttaatttt	9307–9283	
G4_7L	aaaattaaaccacaaatccaaaaa	9283–9306	612
G4_7R	tttgggagttaatgaaaaggaa	9895–9874	
G4_8L	ttccttttcattaactcccaaag	9874–9896	817
G4_8R	tcgtaaaaatcaaccattatttacatc	10691–10665	
G1_5fL	ttcagcctgatgaaatttcgg	10532–10552	944
G1_5fR	ggtcgggctccaattcatgt	11476–11457	
G1_4fL	tgaattggagcccgacctgt	11460–11479	789
G1_4fR	ggggtttatactgtaatagttgctggg	12265–12239	
G1_4-3L	ccttcagcaaaatcaaaagga	11991–12011	515
G1_4-3fR	tcaaattcgtaaagggccaa	12506–12487	
G1_3fL	tgttccttagtaaataacttcacagca	12420–12446	809
G1_3R	tgaaggcttgtatgaatggttg	13229–13208	
G1_2L	ctcattcaaccattcatacaagc	13202–13224	829
G1_2R	gaaaagaaatttgtgcaaatcaa	14058–14036	
G1_1L	tgatttgcacaaatttcttttca	14037–14059	675
G1_1R	ccagctaccgcggttataca	14712–14693	
G5_2L	gctggcacaaattttaccaata	14708–14729	1000
G5_2R	cctatgggtcctaaatgaagaaaa	15684–15707	

Table 3. List of primers used to obtain the nucleotide sequence of the complete mitochondrial genome of *Ae. sibiricus*

Primer name	Sequence 5′→3′	Localisation on the GenBank sequence ID: OQ145432	PCR fragment length, bp
G1_18sL	aatgaattgcccgataaaaagga	1–23	706
G1_18R	tgatttaatcctccaaatgc	706–687	
G4_4L	tggagcatttggaggattaaa	683–703	599
G4_4R	caaatattttcagctttgaaggctat	1282–1257	
G3_5L	aactaatagccttcaaagctgaaa	1252–1275	417
G3_5R	tcaatttccaaatcctccaa	1669–1650	
G3_6L	ttcgaacagaacttagtcatccag	1536–1559	919
G3_6R	tcctaaagatcataaaagagcagga	2455–2431	
G1_15sL	gtcctgctctttttatgatcttt	2430–2451	919
G1_15sR	tcattgatgaccaataactttt	3349–3328	
G3_7L	tttgaacaattttaccagcaatta	3228–3251	881
G3_7R	agttgaaggaataattaaaagaccaa	4109–4134	
G3_8L	tgtatttgacccttcaactactatttttt	4050–4077	700
G3_8R	ctactaagtgaaaggggtgatttg	4750–4727	
G3_9L	gtcaacacacgcaaatcacc	4716–4735	810
G3_9R	tccttgtgattggaagtcacatatac	5546–5521	
G1_12-11L	tgtgacttccaatcacaagga	5526–5546	657
G1_12-11sR	tggtgatcaagaaaaagctgcta	6183–6161	
G1_11L	tcatgaatgaaatcaaggagca	5883–5904	614
G1_11sR	caaggatgaagagaatattttgg	6497–6475	
G3_10L	ctcttcatccttgatcaaattcc	6485–6507	959
G3_10R	cagcccctactcctgtttca	7444–7425	
G1_9sL	gaatgaactaaagctgaaacagga	7411–7434	560
G1_9sR	ttttattgaatgagaaattgtatcttt	7971–7945	
G1_9-8sL	tctccaatacgattagataaagc	7624–7646	622
G1_9-8sR	tcttcagggagaacccgagaattt	8246–8223	
G3_11L	aattctcgggttctccctga	8224–8243	897
G3_11R	ttttgaaagaagcttaattcctacatt	9121–9147	
G4_2L	ctgcttgtaaacgttcaggct	9074–9094	816
G4_2R	aactttgggagttaaagaaaaggaa	9890–9866	
G4_3L	cttccttttctttaactcccaaag	9865–9888	818
G4_3R	tcgtaaaaatcaaccattatttacatc	10683–10657	
G1_5sL	ttcagcttgatgaaattttgg	10524–10544	944
G1_5sR	ggtcgagctccaattcaggt	11468–11449	
G1_4sL	tgaattggagctcgaccagt	11452–11471	808
G1_4R	ggggtttatactgtaatagttgctgga	12260–12234	
G1_4-3sL	ccttcagcaaaatcaaaaggt	11986–12006	515
G1_4-3sR	tcaaattcggaaagggccta	12501–12482	
G1_3sL	tgttctttagtaaataacttcacagca	12415–12441	807
G1_3R	tgaaggcttgtatgaatggttg	13222–13201	
G3_12L	caaccattcatacaagccttca	13201–13222	849
G3_12R	gaaaagaaatttgtgcaaatcaa	14050–14028	
G1_1L	tgatttgcacaaatttcttttca	14029–14051	676
G1_1R	ccagctaccgcggttataca	14705–14686	
G5_3L	ttgtataaccgcggtagctg	14685–14704	358
G5_3R	ggggttatttttaataaggcaattt	15043–15019	

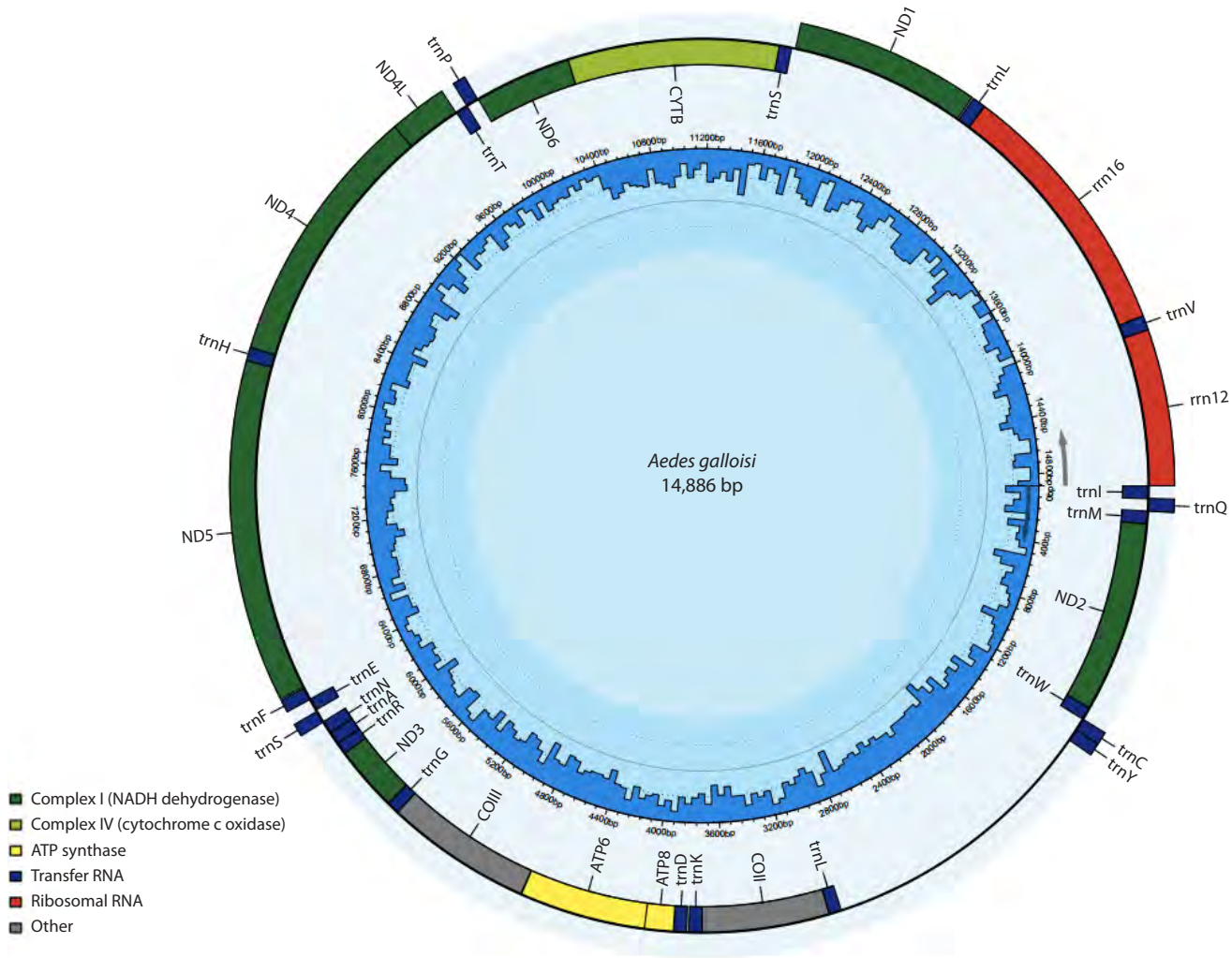

Fig. 1. Mitochondrial genome of *Ae. sibiricus* without the control site located between 12S rRNA and tRNA-Ile.

The nucleotide sequence has been deposited in the GenBank database under accession number OQ145432. The genome is registered as *Ae. galloisi* because the separation of the closely related species *Ae. galloisi* and *Ae. sibiricus* is not yet generally accepted, and the species *Ae. sibiricus* is not yet represented in the GenBank systematic database. We believe that the correct species name for the collected mosquitoes is *Ae. sibiricus*.

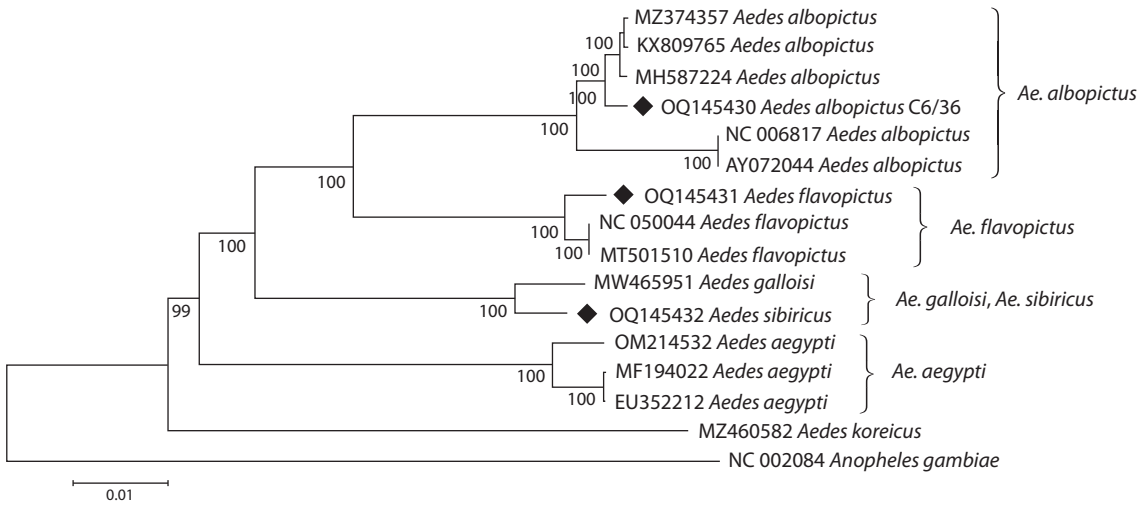

Fig. 2. NJ dendrogram of complete mitochondrial genomes.

The dendrogram is constructed using the maximum likelihood method. Branch lengths are expressed as the number of base substitutions per site. Bootstrap support values are shown next to the nodes (10,000 replicates). The complete mitochondrial genomes of *Ae. koreicus* and *Anopheles gambiae* were used as an external group. The mitochondrial genomes obtained in this study are marked with a diamond in the figure. The nucleotide sequences are registered in the GenBank database under the numbers OQ145430–OQ145432.

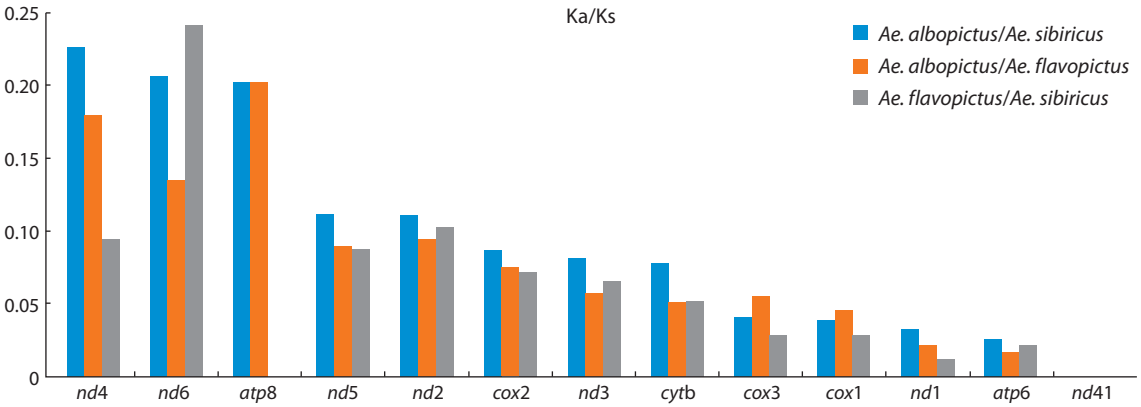

Fig. 3. Pairwise interspecies comparisons of the Ka/Ks ratio in protein-coding mitochondrial genes.

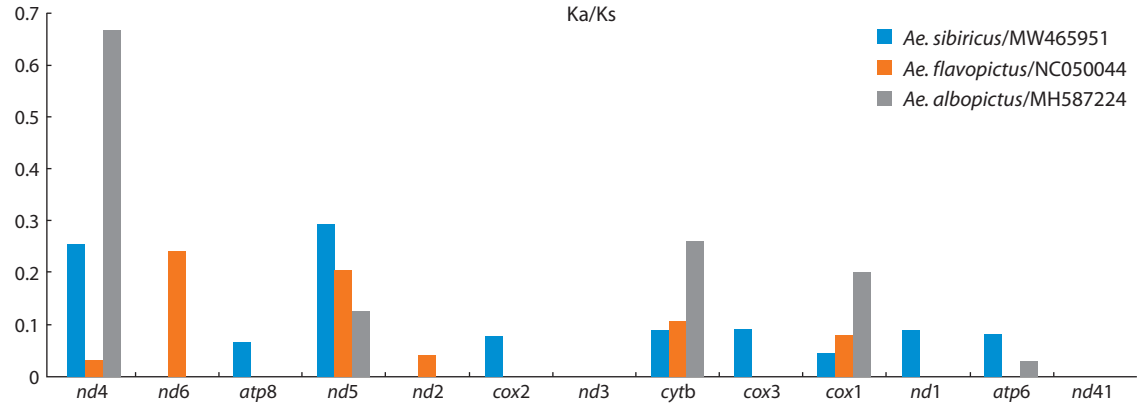

Fig. 4. Pairwise intraspecific comparisons of nucleotide variability in the magnitude of the Ka/Ks ratio of mitochondrial protein-coding genes.

studied are closely related, their habitats overlap slightly, but the centres of their ranges belong to different natural and climatic zones. *Ae. albopictus* is mainly restricted to tropical and subtropical climates, while *Ae. flavopictus* and *Ae. sibiricus* are restricted to temperate climates. At the same time, *Ae. flavopictus* predominates in zones with a monsoon climate, and *Ae. sibiricus*, in zones with a strongly continental climate. The pairwise comparison of PCGs was used to identify differences that may be adaptively relevant between the mosquito species. In Figure 3, Ka/Ks values are ranked in descending order based on the comparison of *Ae. albopictus* and *Ae. sibiricus*.

Ka/Ks ratios do not exceed 0.25 in all pairwise comparisons, indicating strong stabilising selection (Yang, Bielawski, 2000; Guo et al., 2021; Xing et al., 2022). The most variable genes between *Ae. albopictus*/*Ae. sibiricus* and *Ae. albopictus*/*Ae. flavopictus* are *nd*4, *nd*6 and *atp*8. The most conserved genes are *nd*1, *atp*6, *nd*4l and *cox*1.

In addition to interspecific comparisons, we performed intraspecific pairwise comparisons to assess the intraspecific variability of mitochondrial PCGs. The mitochondrial genome of *Ae. albopictus* cell culture C6/36 obtained in this study was compared with the genome of *Ae. albopictus* from China, GenBank ID MH587224. We compared the mitochondrial genome of *Ae. flavopictus* with the genome of *Ae. flavopictus* from Japan, GenBank ID NC050044, and the mitochondrial genome of *Ae. sibiricus* with the genome of *Ae. galloisi* from Japan, GenBank ID MW465951. The values of the Ka/Ks ratios are shown in Figure 4. The order of the genes is identical to the order of the gene rankings in Figure 3.

Within the *Ae. flavopictus* species, the highest Ka/Ks values were observed in the genes *nd*5, *nd*6, *cox*1, *cyt*b. The genes *atp*8, *cox*2, *nd*3, *cox*3, *nd*1, *atp*6, *nd*4l were conservative. When comparing the mitochondrial genomes of *Ae. albopictus* mosquitoes from the natural population and from C6/36 cell culture (Singh, 1967), the highest Ka/Ks ratio was observed in the genes *nd*4, *cyt*b, *cox*1, *nd*5. The most conserved genes were: *nd*6, *atp*8, *nd*2, *cox*2, *nd*3, *cox*3, *nd*1, *nd*4l. When comparing the mitochondrial PCGs of *Ae. sibiricus* and *Ae. galloisi*, the highest Ka/Ks values were observed in the genes *nd*4, *nd*5, *atp*8, *cox*2, *cyt*b, *cox*3, *nd*1, *cox*1, *atp*6. The following genes were conserved: *nd*6, *nd*2, *nd*3, *nd*4l.

Discussion

Organisation of the derived mitochondrial genomes

The nucleotide divergence values obtained in this study between the three closely related mosquito species are comparable and correspond to their geographical distribution in eastern Asia. *Ae. albopictus* is the most thermophilic species, characteristic of China and southern Asia. *Ae. sibiricus* is the most northerly. *Ae. flavopictus* occupies a middle position (Bega et al., 2022).

The use of molecular genetic markers to identify mosquito species is based on the use of a threshold of acceptable intraspecific variability of a given marker. This threshold is determined empirically for each marker and for each systematic group of insects (Zhang H.Z. et al., 2017). For example, for many insect groups, the threshold for intraspecific nucleotide variability of the BOLD fragment of the mitochondrial gene *cox*1 is 3 % (Hebert et al., 2003). Intraspecific variability of *Anopheles hyrcanus* s. l. mosquitoes in the Russian Far East ranged from 0.36 to 1.09 %, interspecific variability – from 2.34 to 4.50 % (Khrabrova et al., 2015). The average intraspecific variability of mosquitoes in China for the *cox*1 barcode fragment was 0.39 % (Wang G. et al., 2012). For complete mitochondrial genomes, much information has been accumulated, but there are no generally accepted quantitative generalisations.

Phylogenetic analysis

The *Ae. albopictus* mitochondrial genomes published to date can be divided into two groups. The first group was found on the island of Taiwan (presumably the native range of the species). Genomes from the second group were found in mosquitoes from the invasive part of the species range (Battaglia et al., 2016). The mitochondrial genome of the *Ae. albopictus* C6/36 cell line clustered with genomes belonging to the second group. The clustering obtained by analysing complete mitochondrial genomes is similar to that obtained in previous studies for the BOLD fragment of the *cox*1 gene (Bega et al., 2022).

PCGs variability analysis

The selection pressure on protein-coding genes can be assessed by determining their Ka/Ks ratio. We made such a comparison at the interspecific level by comparing the genomes obtained in this study. The highest Ka/Ks values for all PCGs, except *nd*6, *cox*1, *cox*3, were observed in the *Ae. albopictus*/*Ae. sibiricus* comparison. This result is in good agreement with the differences between species in terms of habitat ecology. The greater the differences in habitat between the species, the more significant the substitutions in the PCGs. The distribution of Ka/Ks values from higher to lower values within the PCGs was generally similar in all three pairwise comparisons, except for some peculiarities. For example, when comparing species from the same geographical area of the Russian Far East, *Ae. flavopictus*/*Ae. sibiricus*, the Ka/Ks ratio for the *nd*4 gene was significantly lower and no nucleotide substitutions were found at all for *atp*8. Calculating the frequency of substitutions normalised to one nucleotide, we can conclude that the *atp*8 gene in mosquitoes of the *Stegomyia* subgenus is characterised by a lower frequency of nucleotide substitutions than that in other protein-coding mitochondrial genes. A higher Ka/Ks ratio in the *atp*8 gene compared to other protein-coding mitochondrial genes was shown in a comparison of two Lepidoptera species of the genus *Gynaephora* living in different high mountain environments (Zhang B. et al., 2021), in parasitic wasp (Xing et al., 2022), and in mosquitoes of the *Anopheles* genus (Guo et al., 2021). This is probably due to the absence of strict constraints on the primary structure of the functional *atp*8 protein. In the *nd*1, *atp*6, *nd*4l and *cox*1 genes, the total frequency of nucleotide substitutions is comparable to that of other mitochondrial genes, but the Ka/Ks values are low, which confirms that these genes are under strong selective pressure.

In contrast to interspecific comparisons, the distribution of Ka/Ks between PCGs in intraspecific comparisons does not show clearly expressed general regularities, but characterises the specificity of variability accumulation for each species.

When comparing the mitochondrial genomes of *Ae. flavopictus*, the highest Ka/Ks values are observed for the *nd*5, *nd*6, *cox*1 and *cyt*b genes. This is due to the lower pressure of purifying selection. The pattern of intraspecific variability of these genes is similar to that found in the interspecific comparisons shown in Figure 3.

It is interesting to compare the mitochondrial genomes of *Ae. albopictus* mosquitoes from the natural population and from C6/36 cell culture. The highest Ka/Ks ratio is observed in the genes *nd*4, *cyt*b, *cox*1, *nd*5. The value of the Ka/Ks ratio in this case exceeds the values characteristic of both interspecific and intraspecific comparisons by a multitude, which allows us to conclude that selection in cell culture conditions is weak or absent. At the same time, the presence of fully conserved genes is observed: *nd*6, *atp*8, *nd*2, *cox*2, *nd*3, *cox*3, *nd*1, *nd*4l. This contrast in the variability of different genes of *Ae. albopictus* may be the result of the removal of a number of physiological constraints in cell culture conditions experienced by individuals in natural populations.

The variability observed when comparing the mitochondrial genomes of *Ae. sibiricus* and MW465951 mosquitoes generally corresponds to the level of interspecific variability in *Aedes* mosquitoes of the *Stegomyia* subgenus, with the exception of two abnormal genes: *nd*6 and *nd*5. The normally highly variable *nd*6 gene is monomorphic in this comparison, which may be due to the presence of stabilising selection. The *nd*5 gene, on the other hand, contains an abnormally high number of non-synonymous substitutions.

Conclusion

The study of the peculiarities of natural selection in invasive insect populations is still at the stage of accumulating material. One of the approaches used to detect the peculiarities of selection leading to the emergence of invasive populations in insects is to compare the mitochondrial genomes of native and invasive populations of the same species. Simultaneous coexistence of native and invasive populations is now known for many insect species, such as the Asian ladybird *Harmonia*

axyrid (Brown et al., 2011), the Japanese grape leafhopper *Arboridia kakogawana* (Piccinno et al., 2024), and several others. The study of the mitochondrial genomes of species that successfully synanthropise and form dense populations in urbanised areas is of interest for the discovery of mitochondrial genes involved in the genetic control of the increased viability trait characteristic of invasive insect populations.

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