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Comparative karyological analysis of four species of *Aedes* mosquitoes inhabiting the territory of the Republic of Kazakhstan

In the present study, karyotypic analysis of four species of *Aedes* mosquitoes (*Aedes vexans*, *Ae. caspius*, *Ae. cataphylla*, *Ae. subdiversus*) collected in the territory of the Republic of Kazakhstan was carried out. Chromosome length measurements were performed in each of the studied species, and C-staining, DAPI-fluorescence and fluorescence in situ hybridisation (FISH) methods were applied using 18S rDNA probe. C-staining and DAPI-fluorescence showed that regions of heterochromatin localized predominantly in the centromeric regions of chromosomes. FISH-hybridization results showed that in *Ae. cataphylla* the 18S rDNA loci are located on the first chromosome, whereas in *Aedes vexans*, *Ae. caspius* and *Ae. subdiversus* they were detected on the second chromosome.

Keywords: *Aedes*, bloodsucking mosquitoes, mitotic chromosomes, C-stain, DAPI, FISH, rDNA, heterochromatin.

Introduction

Mosquitoes of the *Culicidae* family represent the most numerous group of insects on the planet. These insects are widely distributed and can be found in both southern and northern regions. Mosquitoes are known carriers of a variety of infectious diseases caused by viral and parasitic pathogens, which significantly impact the health of both humans and animals. Many countries report imported cases of such diseases, while ongoing climate change contributes to the spread of disease vectors into previously unaffected areas. Among these expanding species, *Aedes* mosquitoes are commonly encountered [1–5].

The genus *Aedes* is characterized by high species diversity and includes numerous blood-feeding mosquito species [1]. One of the most invasive species of this genus, *Aedes koreicus*, was first identified in Kazakhstan in 2021 [6]. To effectively assess and mitigate potential epidemiological risks, it is essential to determine the precise species composition of local mosquito populations. However, species-level identification within the genus *Aedes* is often complicated due to morphological similarities among closely related taxa. As a result, the application of multiple diagnostic approaches is required to ensure accurate identification. Cytogenetic characteristics, such as the quantity and spatial organization of heterochromatin in chromosomes, are known to be species-specific in many animals and plants [7]. Among ribosomal genes, 18S rDNA is considered the most conserved, making it a valuable tool in taxonomic and phylogenetic research. In light of these considerations, a cytogenetic study was performed on selected *Aedes* species collected across Kazakhstan. The findings contribute to the development of additional taxonomic markers and offer insights into the evolutionary biology of *Aedes* mosquitoes.

Experimental

Fourth-instar larvae of *Aedes* mosquitoes were utilized in the present study. Field collections were carried out during spring 2024 in multiple regions of the Republic of Kazakhstan, including Karaganda, Kostanay, North Kazakhstan, and Akmola regions. The specimens represented various taxonomic groups: *Ae. vexans* (subgenus *Aedimorphus*), *Ae. caspius* (subgenus *Ochlerotatus*, caspius group), *Ae. cataphylla* (subgenus *Ochlerotatus*, communis group), and *Ae. subdiversus* (subgenus *Ochlerotatus*, rusticus group).

Sampling took place from March to May, with larvae collected from natural aquatic habitats and preserved in Carnoy's fixative (1 part glacial acetic acid to 3 parts 96 % ethanol). A taxonomic expert on blood-feeding mosquitoes performed morphological determinations using standardized identification protocols [8–10]. Analysis of larval characteristics was carried out with a Stemi 2000-C stereomicroscope (Carl Zeiss).

Cells obtained from the imaginal discs of *Aedes* larvae at the fourth instar stage were used to conduct cytogenetic analysis of four mosquito species: *Ae. vexans*, *Ae. caspius*, *Ae. cataphylla*, and *Ae. subdiversus*. This anatomical structure was selected due to the high density of cells undergoing metaphase, making it suitable for chromosome visualization. As in the majority of mosquito taxa, these species displayed a diploid chromosome number of $2n = 6$. The Chromosomes classified according their length.

For chromosomal analysis, the lactoacetoorcein technique was employed to stain preparations, allowing for measurement of chromosome length, estimation of centromeric index, and calculation of relative lengths. To examine constitutive heterochromatin, C-banding was applied, while DAPI staining was used to detect A-T rich heterochromatin regions. Fluorescence in situ hybridization (FISH) enabled localization and quantification of 18S rDNA loci across *Aedes* chromosomes from different subgenera [11–14].

Chromosomal imaging and analysis were conducted using Zeiss Axio Imager A1 and Z1 fluorescence microscopes (Zeiss, Germany). Comparative karyotype studies employing Giemsa C-banding and related techniques have proven valuable for revealing patterns of chromosomal differentiation. These studies highlight the variation in heterochromatin quantity and distribution as key elements in mosquito genome evolution, which show contrasting features among others mosquitoes lineages [15, 16].

Results and Discussion

Studies focused on *Aedes* reveal variability in overall chromosome lengths [17]. Chromosomes were stained using the lactoacetoorcein technique (Fig. 1), allowing for precise measurement of their size using the ImageJ software (Table 1). Based on this, the relative chromosome length ($Lr, \%$) and centromeric index ($Jc, \%$) were calculated (Table 2). Notably, chromosome 1 in all species is significantly shorter than chromosomes 2 and 3, which are nearly identical in length within each species.

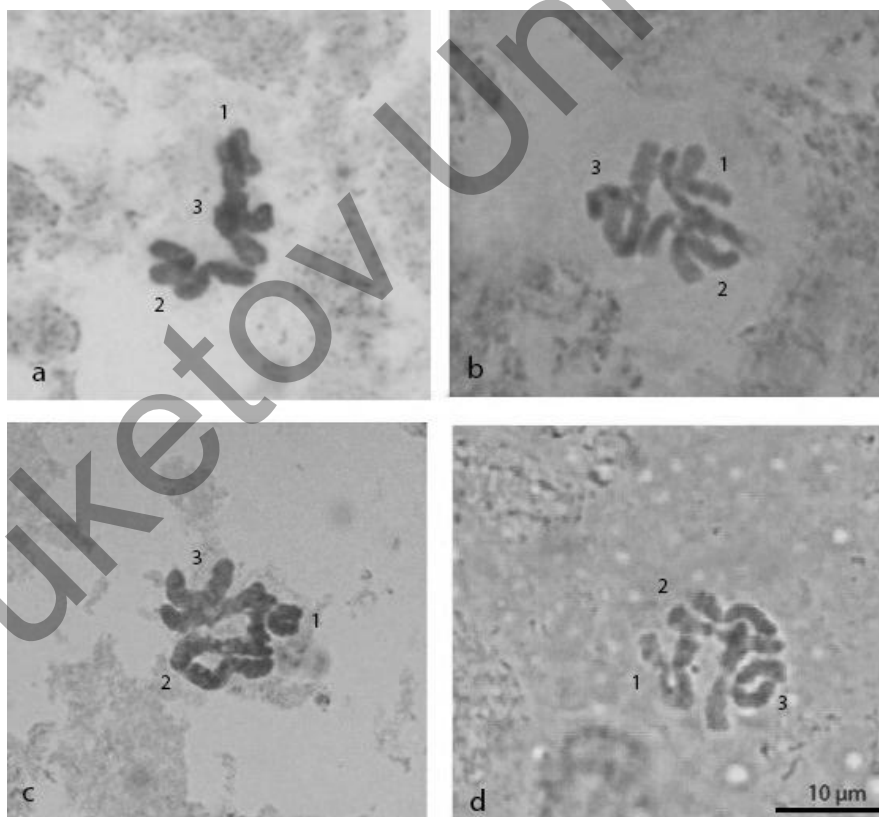


Figure 1. Lactoacetoorcein staining of chromosomes of *Ae. vexans* (a), *Ae. caspius* (b), *Ae. cataphylla* (c), *Ae. subdiversus* (d). 1,2,3 — chromosome numbers.

Table 1

Chromosome lengths of mosquitoes of the genus *Aedes*

Species	Chromosome 1, ±0,3 μm	Chromosome 2, ±0,3 μm	Chromosome 3, ±0,3 μm
<i>Ae.vexans</i>	5.4	9.01	8.22
<i>Ae.caspius</i>	6.26	9.99	9.03
<i>Ae.cataphylla</i>	6.5	10.34	9.7
<i>Ae.subdiversus</i>	6.46	10.35	9.78

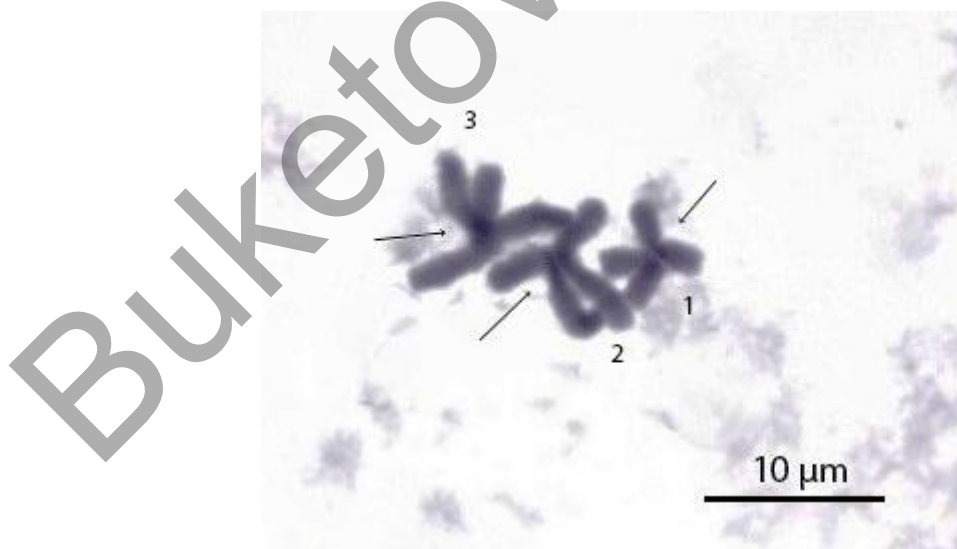
Centromeric index values were determined using the formula proposed in [18]. The observed relative lengths ranged from 24 % to 39 %, while centromeric indices fell between 45 % and 51 %, indicating that the chromosomes are metacentric, although chromosome 2 in some specimens approached submetacentric morphology.

Table 2

Centromeric index and relative length of chromosomes

Species	Chromosomes 1		Chromosomes 2		Chromosomes 3	
	L _r , %	J _c , %	L _r , %	J _c , %	L _r , %	J _c , %
<i>Ae.vexans</i>	24	48	39	50	36	49
<i>Ae.caspius</i>	25	48	39	46	35	48
<i>Ae.cataphylla</i>	24	48	38	47	36	47
<i>Ae.subdiversus</i>	24	48	39	45	36	47

C-banding analysis revealed the presence of constitutive heterochromatin in the centromeric areas of all examined species. As an illustration, Figure 2 shows heterochromatin patterns in *Ae. cataphylla*. Using DAPI staining, small A-T rich heterochromatin blocks were visualized, particularly in *Ae. vexans* and *Ae. subdiversus*. In contrast, these blocks were nearly undetectable in *Ae. caspius* and *Ae. cataphylla*. Figure 3 demonstrates the centromeric localization of DAPI-positive regions. These observations are consistent with previous works by Wasserlauf et al. (2018) and Alekseeva et al. (2020) [13, 14].

Figure 2. C-stained chromosomes in *Ae.cataphylla*.

Arrows indicate C-blocks of constitutive heterochromatin. The numbers indicate chromosome numbers.

Fluorescence in situ hybridization (FISH) was applied to all four studied species to map the 18S rDNA loci. All studied species have 1 loci 18S rDNA (Fig. 3).

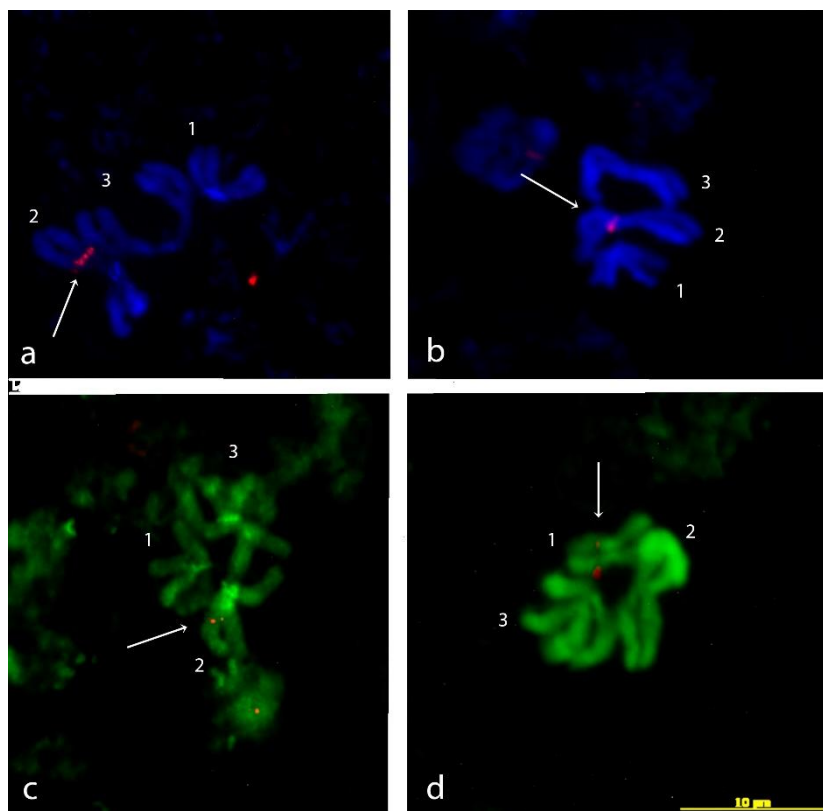


Figure 3. Localization of 18S rDNA on chromosomes of *Ae. vexans* (a), *Ae. caspius* (b) *Ae. subdiversus* (c) *Ae. cataphylla* (d). The arrows indicate the localization sites of 18S rDNA. The numbers indicate chromosome numbers. The chromosomes stained by DAPI. The colors of the chromosomes are artificial.

In *Ae. cataphylla*, the 18S rDNA cluster was located on chromosome 1, confirming earlier findings from specimens collected in the Tomsk region [19]. While *Ae. cataphylla* is part of the communis group within the *Ochlerotatus* subgenus — where 18S rDNA signals are typically observed on chromosome 2 [19] — our results show locus variability within this clade. *Ae. vexans* and *Ae. subdiversus*, which belong to different subgenera, exhibited 18S rDNA sites on chromosome 2 (Fig. 3).

Conclusions

The findings concerning chromosome size variation, as well as the distribution of heterochromatin and 18S rRNA gene loci, provide the additional cytogenetic markers for species differentiation within the *Aedes* genus and offer insights into their evolutionary history. The localization patterns of 18S rDNA in the species analyzed in this study (*Ae. vexans*, *Ae. caspius*, *Ae. subdiversus*, and *Ae. cataphylla*), and compared with earlier studies [19], reflect a common evolutionary pattern within groups of the *Ochlerotatus* subgenus. Observed differences in the chromosomal positioning of 18S rRNA genes — both between and within subgenera — suggest that evolutionary chromosomal rearrangements, such as translocations, have contributed to their relocation.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Alekseeva S.S.:** Methodology, Visualization, Investigation, Writing – original draft; **Andreeva Y.V.:** Conceptualization, Metodology, Investigation, Writing – original draft; **Sibataeva A.M.:** Mosquito collection; **Sibataev A.K.:** Conceptualization, Funding acquisition, Project administration, Supervision, mosquito collection.

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Қазақстан Республикасы аумағында таралған *Aedes* туысына жататын масалардың төрт түрінің салыстырмалы кариологиялық талдауы

Зерттеуде Қазақстан Республикасының аумағында жиналған масалардың *Aedes* туысының 4 түріне (*Aedes vexans*, *Ae. caspius*, *Ae. cataphylla*, *Ae. subdiversus*) кариологиялық талдау жүргізілді. Нәтижесінде әрбір түрдің хромосомаларының ұзындығы өлшенді, С- және DAPI бояуы және 18S rDNA зондымен хромосомалардың флуоресцентті гибридизациясы (FISH) жүргізілді. С- және DAPI бояулары

центромера аймағында гетерохроматиннің локализациясын көрсетті. FISH *Ae. cataphylla* 1-хромосомасында және *Aedes vexans*, *Ae. caspius* және *Ae. subdiversus* 2-хромосомада рДНК локустарын анықтады.

Кілт сөздер: *Aedes*, қансорғыш масалар, митоздық хромосомалар, С-бояғыш, DAPI, FISH, рДНК, гетерохроматин.

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Сравнительный кариологический анализ четырёх видов комаров рода *Aedes*, обитающих на территории Республики Казахстан

В настоящем исследовании проведён кариотипический анализ четырёх видов комаров рода *Aedes* (*Aedes vexans*, *Ae. caspius*, *Ae. cataphylla*, *Ae. subdiversus*), собранных на территории Республики Казахстан. В ходе работы были выполнены измерения длины хромосом у каждого из исследуемых видов, а также применены методы С-окрашивания, DAPI-флуоресценции и флуоресцентной гибридизации *in situ* (FISH) с использованием зонда 18S рДНК. С-окраска и DAPI-флуоресценция показали, что участки гетерохроматина локализуются преимущественно в центромерных зонах хромосом. По результатам FISH-гибридизации установлено, что у *Ae. cataphylla* локусы 18S рДНК располагаются на первой хромосоме, тогда как у *Aedes vexans*, *Ae. caspius* и *Ae. subdiversus* они выявлены на второй хромосоме.

Ключевые слова: *Aedes*, кровососущие комары, митотические хромосомы, С-окраска, DAPI, FISH, rDNA, гетерохроматин.

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