Received: 1 November 2022 | Revised: 17 August 2023 | Accepted: 24 August 2023

DOI: 10.1111/mec.17127

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

# Patterns of genetic differentiation imply distinct phylogeographic history of the mosquito species *Anopheles messeae* and *Anopheles daciae* in Eurasia

Ilya I. Brusentsov[1,2] | Mikhail I. Gordeev[3] | Andrey A. Yurchenko[1,2] | Dimitriy A. Karagodin[2] | Anton V. Moskaev[3] | James M. Hodge[1] | Vladimir A. Burlak[4] | Gleb N. Artemov[4] | Anuarbek K. Sibataev[5,6] | Norbert Becker[7,8] | Igor V. Sharakhov[1,4] | Elina M. Baricheva[2] | Maria V. Sharakhova[1,2]

[1]Department of Entomology, Virginia Polytechnic Institute and State University and Fralin Life Sciences Institute, Blacksburg, Virginia, USA

[2]Laboratory of Cell Differentiation Mechanisms, Institute of Cytology and Genetics, Novosibirsk, Russia

[3]State University of Education, Mytischi, Russia

[4]Laboratory of Ecology, Genetics, and Environmental Protection, Tomsk State University, Tomsk, Russia

[5]Department of General Biology and Genomics, L.N. Gumilyov Eurasian National University, Nur-Sultan, Kazakhstan

[6]Department of Agricultural Biology, Tomsk State University, Tomsk, Russia

[7]Center for Organismal Studies, University of Heidelberg, Heidelberg, Germany

[8]German Mosquito Control Association, Speyer, Germany

**Correspondence**
Maria V. Sharakhova, Department of Entomology, Fralin Life Sciences Institute, Virginia Tech, 360 West Campus Dr., Blacksburg, VA 24061, USA.
Email: msharakh@vt.edu

**Funding information**
Institute of Cytology and Genetics, Grant/Award Number: FWNR-2022-0015; Russian Science Foundation, Grant/Award Number: 19-14-00130, 21-14-00182 and 22-24-00183

**Handling Editor:** Sean Schoville

## Abstract

Detailed knowledge of phylogeography is important for control of mosquito species involved in the transmission of human infectious diseases. *Anopheles messeae* is a geographically widespread and genetically diverse dominant vector of malaria in Eurasia. A closely related species, *An. daciae*, was originally distinguished from *An. messeae* based on five nucleotide substitutions in its ribosomal DNA (rDNA). However, the patterns of phylogeographic history of these species in Eurasia remain poorly understood. Here, using internal transcribed spacer 2 (ITS2) of rDNA and karyotyping for the species identification we determined the composition of five *Anopheles* species in 28 locations in Eurasia. Based on the frequencies of 11 polymorphic chromosomal inversions used as genetic markers, a large-scale population genetics analysis was performed of 1932 mosquitoes identified as *An. messeae*, *An. daciae* and their hybrids. The largest genetic differences between the species were detected in the X sex chromosome suggesting a potential involvement of this chromosome in speciation. The frequencies of autosomal inversions in the same locations differed by 13%–45% between the species demonstrating a restricted gene flow between the species. Overall, *An. messeae* was identified as a diverse species with a more complex population structure than *An. daciae*. The clinal gradients in frequencies of chromosomal inversions were determined in both species implicating their possible involvement in climate adaptations. The frequencies of hybrids were low ~1% in northern Europe but high up to 50% in south-eastern populations. Thus, our study revealed critical differences in patterns of phylogeographic history between *An. messeae* and *An. daciae* in Eurasia. This knowledge will help to predict the potential of the malaria transmission in the northern territories of the continent.

**KEYWORDS**
*Anopheles*, clinal gradient, inversion polymorphism, mosquito, population genetics, population structure

## 1 | INTRODUCTION

Phylogeography is a rapidly developing discipline that analyses the genealogy of lineages within the context of their geographical distribution (Edwards et al., 2022). It is employed to better understand the natural histories of populations, subspecies and species. Application of different markers and large numbers of specimens from natural populations, together with modern analytical tools, provides important insights into the distribution of genetic diversity around the globe (Emerson & Hewitt, 2005). Chromosomal inversions play an important role in the evolution of eukaryotes (Ayala & Coluzzi, 2005; Kirkpatrick, 2010; Kirkpatrick & Barton, 2006). When inversion occurs, a piece of the chromosome flips 180 degrees and produces a reverse order of the genetic material. As a result, this part of the genome becomes protected from recombination during meiosis. By capturing different sets of alleles, inversions have effects on the ecological, behavioural and physiological adaptations of species to the natural environment (Ayala & Coluzzi, 2005). Reduced recombination in heterozygote inversions may promote ecological divergence leading to reproductive isolation and speciation (Hoffmann et al., 2004; Rieseberg, 2001). As a result of their functional importance, chromosomal inversions served as unique markers of population genetics and phylogenetics in species with well-developed polytene chromosomes, such as *Drosophila* (Krimbas & Powell, 1992) and malaria mosquitoes from the *Anopheles* genus (Coluzzi et al., 2002; Stegniy, 1991). Here, we argue that using polymorphic chromosomal inversions as genetic markers and molecular diagnostics of the species combined with modern statistical methods helped us to uncover the patterns of phylogeographic history of the two species of malaria mosquitoes in Eurasia.

Among different infectious diseases, malaria is traditionally considered the most dangerous disease transmitted to humans by mosquitoes (Rougeron et al., 2022). Despite extensive efforts to eradicate malaria, global climate change, human migration, political instability and the presence of competent malaria vectors have increased the risk of malaria importation and transmission in regions where it was previously eliminated (Chretien et al., 2015; Kulkarni et al., 2022; Rossati et al., 2016; Sainz-Elipe et al., 2010). Thus, understanding the phylogeographic history of malaria mosquitoes is important for the prediction of malaria propagation and for developing effective and adequate strategies for the eradication of this disease.

The most dangerous vectors of malaria in the Northern Hemisphere belong to the Maculipennis group (Sinka et al., 2010). The major vector of malaria in Europe, *Anopheles maculipennis*, Meigen, 1818, was originally considered as a single species until the paradox of 'anophelism without malaria' was observed (Hackett, 1937; Hackett & Missiroli, 1935). It was shown that in some parts of Europe, malaria was absent despite the presence of malaria vectors. This phenomenon led to the conclusion that *An. maculipennis* represents a complex of species with different abilities to transmit malaria. The presence of multiple species within the complex was supported by hybridization experiments (Kitzmiller et al., 1967; Stegniy, 1980; Stegniy et al., 1984) and by morphological differences of their egg chorion patterns (Gutsevich et al., 1970). Later, chromosomal analysis became a robust tool for species identification, distinguishing the taxonomic status of a species, and for population analysis. For example, fixed chromosomal differences discriminated *An. maculipennis* and *An. messeae* Falleroni, 1926 (Stegniy et al., 1973) and *An. sacharovi* Favre, 1903 and *An. martinius* Shingarev, 1926 (Stegniy, 1976). A new species from the Maculipennis group, *An. beklemishevi* Stegniy and Kabanova 1976, was specifically identified using fixed chromosome differences (Stegniy & Kabanova, 1978; Stegniy, Kabanova, & Novikov, 1976). Later, based on nucleotide differences in Internal Transcribed Spacer 2 (ITS2) of ribosomal DNA (rDNA) several new species were identified: *An. artemievi* Gordeyev, Zvantsov, Goryacheva, Shaikevich & Yezhov, 2005, *An. daciae* Linton, Nicolescu & Harbach, 2004, and *An. persiensis* Linton, Sedaghat & Harbach, 2003. Currently, the Maculipennis group comprises 11 Palearctic species, four of which, *An. atroparvus*, Van Thiel, 1972, *An. labranchiae*, Falleroni 1926, *An. sacharovi* and *An. messeae* are considered dominant vectors of malaria in Eurasia (Sinka et al., 2010).

*Anopheles messeae* is the most geographically widespread and genetically diverse species among the other dominant malaria vectors from the Maculipennis group, its extends from Ireland in the west to the Amur River region in the east and from Scandinavia and Yakutia in the north to Iran and northern China in the south (Gornostaeva & Danilov, 2002; Sinka et al., 2010; Zvantsov et al., 2014). Despite being largely zoophilic, *An. messeae* actively feeds on humans in cases of animal deficiency (Fyodorova et al., 2006) and it was the primary malaria vector of *Plasmodium vivax* malaria in Russia (Beklemishev, 1948; Daskova & Rasnicyn, 1982). As the most genetically diverse species among the Maculipennis subgroup, *An. messeae* carries five widely spread chromosomal inversions X1, X2, 2R1, 3R1 and 3L1, located on four chromosomal arms (Kabanova et al., 1972). The details of inversion polymorphism in natural populations of *An. messeae* were intensively investigated by a group of researchers led by V. Stegniy in 1970–1990s (Stegniy, 1991). These studies demonstrated a complex structure of *An. messeae* populations along the range of the species distribution. It was shown that inversions played an important role in climatic adaptation of the species in latitudinal and meridional directions (Stegniy, Kabanova, Novikov, et al., 1976). For example, a latitude gradient was described for the 2R1 inversion where the inverted variant was more abundant in northern populations, suggesting that this inversion could be involved in adaptation to cold temperatures and to successful overwintering. In contrast,

frequencies of the X1 and 3R1 inversions displayed a west–east longitude gradient with higher frequencies of the inverted variants found in eastern populations. Involvement of the chromosomal inversion in local adaptations of the species was also observed (Stegniy et al., 1978). For example, seasonal (Kabanova et al., 1973) and temporal dynamics (Pleshkova et al., 1978) in frequencies of the chromosomal inversions were shown in several geographical locations. Overall, these studies led to conclusions about the stable state of the inversion polymorphisms in natural populations of *An. messeae* (Stegniy, 1983a) and the dependence of chromosomal variability on landscape-climatic zones (Gordeev & Moskaev, 2016).

The hypothesis that combinations of different chromosomal inversions in natural populations of *An. messeae* represent two distinct chromosomal complexes was first introduced in 1979 by Novikov and Kabanova (1979). They showed that frequencies of some inversion combinations were higher or lower than expected from random mating, suggesting the presence of linkage disequilibrium between them. For example, the standard karyotype X0 was more frequently associated with standard karyotype variants 2R0, 3R0 and 3L0, whereas the inverted karyotypes X1 and X2 were associated with 2R1, 3R1 and 3L1 inversions (Gordeev & Stegniy, 1987; Novikov & Kabanova, 1979; Stegniy, 1983b). Later studies demonstrated that mosquitoes from different chromosomal complexes varied by female fecundity (Gordeev & Stegniy, 1987), larval diet (Gordeev & Troshkov, 1990) and sensitivity to the toxins of *Bacillus thuringiensis* (Gordeev & Burlak, 1991). It was also shown that adults and larvae belonging to different chromosomal complexes had different rate of survivorship under laboratory conditions (Gordeev & Perevozkin, 1995) and under selection pressure related to predators (Gordeev et al., 1997; Gordeev & Sibataev, 1995) and parasites (Burlak & Gordeev, 1998). In 2001, these chromosomal complexes were referred to as cryptic genetically isolated forms named 'A' and 'B' (Novikov & Shevchenko, 2001).

In 2004, independently of these studies, a new Maculipennis species, *An. daciae*, was differentiated from *An. messeae* based on five ITS2 nucleotide substitutions and morphological differences at the egg stage (Nicolescu et al., 2004). Later, sequencing of ITS2 indicated that chromosomal form 'A' of *An. messeae* is synonymous with *An. daciae* (Vaulin & Novikov, 2012). Additional geographical studies discovered *An. daciae* in Germany (Kampen et al., 2016; Kronefeld et al., 2014; Weitzel et al., 2012), the United Kingdom (Danabalan et al., 2014), Poland (Rydzanicz et al., 2017), the Czech Republic, Slovakia (Blažejová et al., 2018), Serbia (Kavran et al., 2018), Finland (Culverwell et al., 2020), Sweden (Lilja et al., 2020), Belgium (Smitz et al., 2021) and Italy (Calzolari et al., 2021). Significant differences in the frequencies of chromosomal inversions between *An. messeae* and *An. daciae* were determined from three populations from the Moscow region (Naumenko et al., 2020). This study also indicated that the inverted variant X1 was fixed in *An. messeae* populations while the standard X0 and inverted X1 karyotypes were segregated in *An. daciae* populations from the same locations. Whole-genome sequencing analyses demonstrated genome-wide differentiation between the species, which was especially pronounced on chromosome X. However, some authors doubt the separate species status of *An. messeae* and *An. daciae* and consider the genetic differences between them as polymorphism within *An. messeae* (Bertola et al., 2022; Bezzhonova & Goryacheva, 2008; Calzolari et al., 2021; Stegniy et al., 2016).

This study describes geographical distributions of *An. messeae*, *An. daciae* along with three other species from the Maculipennis group, *An. atroparvus*, *An. beklemishevi* and *An. maculipennis* in Eurasia. The species were identified using ITS2 length/sequences and karyotyping. For the first time, frequencies of chromosomal inversions were analysed independently in *An. messeae* and *An. daciae*. Using polymorphic inversions as genetic markers, the detailed statistical analyses of divergence, diversity and population structure of the two species in Eurasia were performed. In addition, we evaluated the potential role of chromosomal inversions in the speciation and climatic adaptations of the malaria vector species *An. messeae* and *An. daciae*. Overall, we think that the genetic differentiation found using chromosomal inversion analysis between *An. messeae* and *An. daciae* populations clearly evaluates them as separated evolutionary entities with distinct patterns of their phylogeographic history in Eurasia.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection and material preservation

A total of 3688 mosquito larvae were collected in 2017–2019 from 28 locations across 20 regions in Germany, Kazakhstan, Latvia and Russia (Figure 1, Table S1). The collections were carried out in late July–early August, when both species are abundant in the natural populations (Czajka et al., 2020). *Anopheles* larvae were collected using the dipping method (Benedict & Dotson, 2015). Each individual mosquito larva was numbered and dissected into two parts: head with thorax and abdomen. These dissected parts were placed into separate tubes for further analysis. Heads with thoraxes were kept in Carnoy's solution (1:3 acetic acid:ethanol), and abdomens were placed in 70% ethanol.

### 2.2 | Karyotyping

Salivary glands were dissected from the larval thorax for preparation of polytene chromosomes, and chromosome preparations were made by the standard lacto-aceto-orcein method (Kabanova et al., 1972). Polytene chromosomes were visualized using an Eclipse E200 light microscope (Nikon, BioVitrum, Moscow, Russia). Samples of *An. maculipennis*, *An. beklemishevi* were identified based on banding patterns of the polytene chromosomes (Artemov et al., 2018; Stegniy et al., 1973). Specimens of *An. messeae* and *An. daciae* were karyotyped using chromosome maps for the salivary glands (Artemov et al., 2021; Stegniy, Kabanova, & Novikov, 1976). A total of 11 chromosomal inversions (X1, X2, X3, X4, 2R1, 2R3, 2R4, 3R1, 3R2,

3L1 and 3L4) were identified and considered in this study (Table 1). The karyotypes of each specimen were described for the whole chromosomal complement. A total of 3133 samples were successfully karyotyped.

## 2.3 | Genotyping

For genotyping, the abdomen of each sample was homogenized in liquid nitrogen and genomic DNA was extracted using the standard

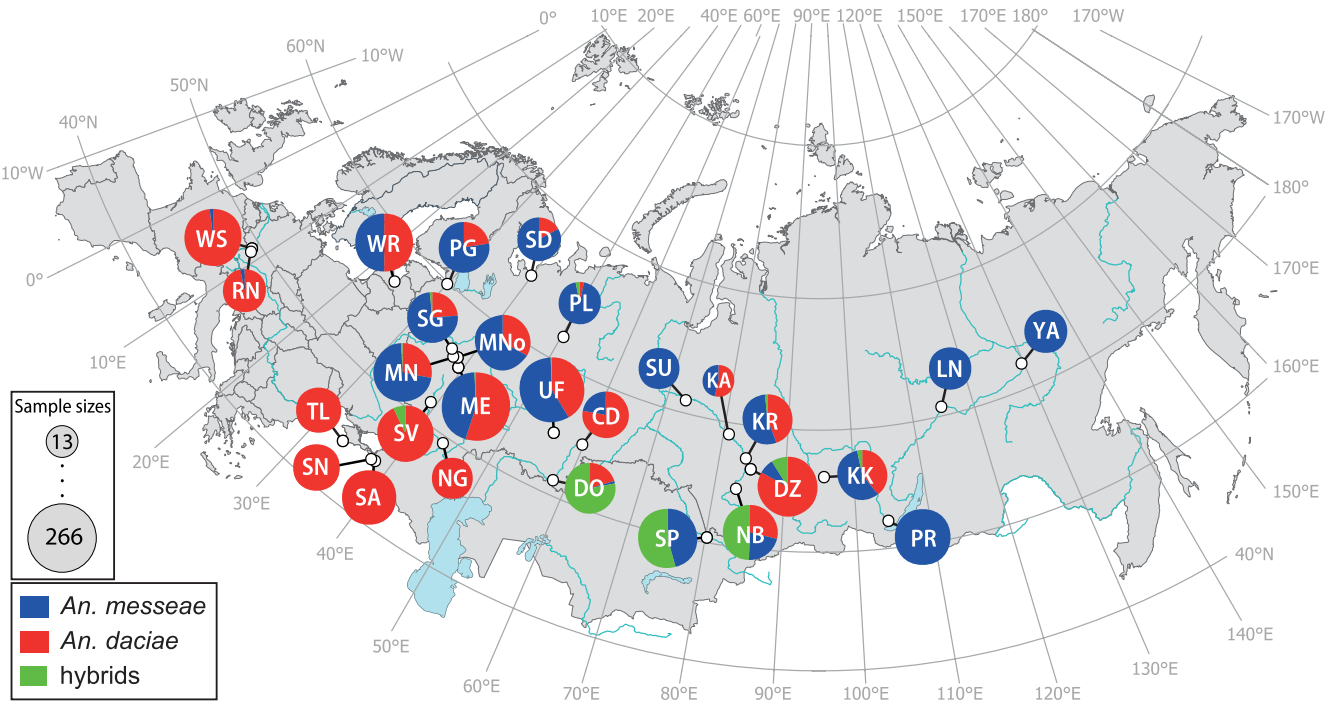


**FIGURE 1** Compositions of *Anopheles messeae*, *Anopheles daciae* and their hybrids in different locations in Eurasia. The proportions of *An. messeae*, *An. daciae* and their hybrids are shown as pie charts with different colours for each site. Sizes of the pie charts proportional to the logarithm of sample sizes in each location (from 13 to 266 specimens). Collection sites are indicated as: CD—Desyatiletie, DO—Dombarovka, DZ—Dzerzhinskoe, KA—Kargasok, KK—Kansk, KR—Krivosheino, LN—Lensk, ME—Yegoryevsk, MN—Noginsk, MNo—Novokosino, NB—Berdsk, NG—Novogrigoryevskaya, PG—Petergof, PL—Palevitsy, PR—Irkutsk, RN—Reinhausen, SA—Abinsk, SD—Severodvinsk, SG—Solnechnogorsk, SN—Novorossiysk, SP—Semey, SU—Surgut, SV—Voronezh, TL—Tylovoe, UF—Ufa, WR—Riga, WS—Speyer, YA—Yakutsk. Overall, *An. messeae* is considered as a north-eastern species and *An. daciae* as south-western species. Intriguingly, a high number of hybrids were found in some southern locations of the explored area.

**TABLE 1** Chromosomal inversions in *Anopheles messeae* and *Anopheles daciae*.

| | | | Frequencies (current study) | | |
|---|---|---|---|---|---|
| Inversion (citation) | Chromosomal location | Geographic distribution | *An. messeae* | *An. daciae* | Hybrid |
| X1 (a) | 2A-5B | Highly spread in Eurasia | 0.944 | 0.522 | 0.692 |
| X2 (a) | 1B-4B | Western Siberia | 0.026 | - | 0.097 |
| X3 (a) | 2A-3B | Latvia | 0.002 | 0.002 | - |
| X4 (b) | 1B-3B | The centre of the Russian Plain | 0.011 | - | 0.004 |
| 2R1 (a) | 7B/C-12C/13A | Forest zone of Eastern Europe, Western and Eastern Siberia | 0.262 | 0.005 | 0.008 |
| 2R3 (a) | 10B/11A-14A/14b | Latvia | - | 0.001 | - |
| 2R4 (b) | 6C/7A-10A/B | The centre of the Russian Plain (Meshcherskaya lowland) | - | 0.001 | - |
| 3R1 (a) | 23C/24A-26C/27A | Highly spread in Eurasia | 0.454 | 0.057 | 0.55 |
| 3R2 (a) | 23A/B-24C/25A | North of Western Siberia | 0.001 | - | - |
| 3L1 (a) | 34B/34C-37A/37B-38C/39A-39C/D | Highly spread in Eurasia except Western Europe | 0.169 | 0.028 | 0.377 |
| 3L4 (c) | 34B/34C-37A/37B | North-east of the Russian Plain | 0.001 | - | - |

*Note*: Inversions were first described in (a) Stegniy, Kabanova, and Novikov (1976); (b) Naumenko et al. (2020); and (c) this study.

protocol from the Qiagen DNeasy Blood and Tissue Kit (Qiagen). DNA elution was performed in 100 μL of water. ITS2 of the rDNA was amplified using the forward universal primer its2_mdir 5′-GCTCGTGGATCGATGAAGAC-3′, Tm = 57°C or its2_vdir 5′-TGTGA ACTGCAGGACACATG-3′ and the reverse primer its2_rev 5′-ATGCT TAAATTTAGGGGGTAGTC-3′, Tm = 54°C with modifications (Proft et al., 1999). The HotStarTaq Plus Master Mix Kit (Qiagen) was used for PCR amplification. The PCR mixture contained a total volume of 20 μL of ~40 ng of DNA, 0.5 μM of each forward and reverse primer and 10 μL of 2× HotStarTaq Plus reaction mix. PCR was performed using a thermal cycler (Eppendorf) with the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s and a final extension step at 72°C for 5 min. The reaction mix was then placed on hold at 4°C. Amplicons were visualized using gel electrophoresis in 2% agarose gel. The species diagnostics was based on the following PCR results: 477 bp length amplicon for *An. messeae* and *An. daciae*, 464 bp for *An. maculipennis*, 479 for *An. atroparvus* and >800 bp for *An. beklemishevi* (Kampen, 2005; Proft et al., 1999). Thus, *An. beklemishevi* was recognized based on the length of the PCR product of ITS2 but because the ITS2 length in *An. daciae*, *An. atroparvus* and *An. maculipennis* differ from that of *An. messeae* by 0, 2 and 13 bp, respectively (Kampen, 2005; Naumenko et al., 2020), these species were recognized by sequence analyses.

For DNA sequencing, amplicons were purified with a Wizard™ PCR Clean Up kit (Promega). PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) from suitable forward or reverse primers, and sequencing reactions were purified by chromatography on Sephadex G-50 (Sigma) and analysed in the SB RAS Genomics Core Facility (http://sequest.niboch.nsc.ru/). All specimens of *An. daciae*, *An. messeae* and *An. atroparvus* were genotyped by Sanger sequencing. Although species of *An. maculipennis* and *An. beklemishevi* were mostly identified by karyotyping (Artemov et al., 2018; Stegniy et al., 1973), ITS2 genotyping was additionally performed in 10–15 random individual mosquitoes from each species to confirm karyotyping results. In total, ~2600 specimens were successfully genotyped and analysed using SeqScape v 3.0 software (Thermo Fisher Scientific). Basecalling was performed using KB basecaller with mixed base identification enabled. Nucleotide positions in ITS2 sequences of *An. messeae* and *An. daciae* were compared with the reference sequence AY648982 in *An. messeae* (Nicolescu et al., 2004). The previously described SNPs, in positions 150, 211, 215, 217, 412 and 432 for distinguishing the ITS2 sequences of *An. messeae* from *An. daciae* (Naumenko et al., 2020) were investigated in detail. Data from basecalled.ab1 files were extracted by Perl script based on Bio::Trace::ABIF v 1.06 perl module (http://search.cpan.org/~vita/Bio-Trace-ABIF-1.06/). Peaks were visualized and analysed in Mathematica v 12 software (Wolfram Research) for base assignment using the Sign test of peak intensity and three background signal intensities from each side of the peak. To confirm the appropriateness of the nucleotide base assignment in these positions, all diagnostic SNPs were verified manually. Although *An. daciae* was originally described based on five chromosomal substitutions in positions 211, 215, 217, 412 and 432 (Nicolescu et al., 2004), we only considered nucleotide GG and AC 412 and 432 as species-specific for *An. messeae* and *An. daciae*, respectively, because heterogeneity was found in the first three nucleotide positions 211, 215, 217—W (A+T), W (A+T), Y (C+T) and additional position 150 M (A+C) (Naumenko et al., 2020). A total of 45 mosquitoes (2.28%) with unusual 412 and 432 base combinations were considered as unclassified and were excluded from the analysis.

## 2.4 | Statistical analysis of chromosomal inversion frequencies

A total of 1932 samples of *An. messeae* and *An. daciae*, which were genotyped and karyotyped (Table S2), were used for further statistical analysis. In addition to the samples collected in this study, we included data from three locations in the Moscow region (Noginsk, Novokosino and Yegoryevsk), which had been published earlier (Naumenko et al., 2020). Chromosomal inversions were utilized as genetic markers for statistical analysis using R packages (R Core Team, 2021). The statistical analysis included populations, in which the number of individuals was more than 10. Species and population differentiations were analysed using the 'poppr' package in R (Kamvar et al., 2014). Genotypic diversity was assessed by three parameters. First, the richness of multilocus genotypes (MLG) was assessed by the Menhinick index (Menhinick, 1964), demonstrating a combination of all chromosomal variants for each individual mosquito. Second, the Simpson Index was calculated to estimate the probability of two random mosquitoes having a different genotype (Simpson, 1949). For elimination of the error related to differences in sample size, an adjustment was made by multiplying them by $n/(n-1)$, where $n$ is the count of mosquitoes in the sample. Third, the Shannon–Wiener diversity index (Shannon, 1948) was used for quantifying the uncertainties associated with predicting the MLG in the next individual based on already identified MLGs.

Deviations from the Hardy–Weinberg equilibrium were assessed using the exact test of Hardy–Weinberg equilibrium (Wigginton et al., 2005) implemented in the Hardy–Weinberg R package (Graffelman, 2015) for each autosome for all populations of *An. messeae* and *An. daciae*. Genetic differentiation (Hedrick's G'st) between species by chromosomes was calculated using the 'mmod' R package (Winter, 2012).

Assessment of the genetic diversity in the studied species and population differentiation was performed using Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992; Michalakis & Excoffier, 1996). Countries or federal districts and sampling sites were used to partition the data into different stratifications. The pairwise $F_{ST}$ values (Weir & Hill, 2002) between the studied populations were calculated using the 'BEDASSLE' package (Gideon, 2014). To assess isolation by distance, a correlation test between pairwise $F_{ST}$ and the pairwise distance between populations was carried out. The distances were calculated using the formula:

$R_E \times \arccos\left(\sin\left(x_1\right)\sin\left(x_2\right) + \cos\left(y_1\right)\cos\left(y_2\right)\cos\left(\left|y_1 - y_2\right|\right)\right)$, where $(x, y)$ are the location coordinates in radians, and $R_E$—radius of Earth (6373 km). To exclude the influence of the mismatch of ranges, only locations where both species were present were included in the analysis.

Hierarchical clustering analysis and principal component analysis (PCA) were performed using the frequencies of chromosomal inversions on the population level to infer and visualize the relationship between the species and populations (Package for R 'stats'; R Core Team, 2021). For hierarchical cluster analysis, the Euclidean distances between samples were used and the WARD clustering method was chosen. Cluster number was performed by K-means method using the algorithm of Hartigan and Wong (1979).

## 3 | RESULTS

### 3.1 | Geographical distribution of the Maculipennis species in Eurasia

In this study, we analysed the composition of the Maculipennis species in 3688 samples from 28 locations in 20 regions in four countries in Eurasia (Figure 1, Table S1). Our study revealed the presence of five out of 11 species of the Palearctic members of the Maculipennis group in the investigated locations: *An. atroparvus*, *An. beklemishevi*, *An. daciae*, *An. maculipennis* and *An. messeae*. Two species: *An. beklemishevi* and *An. maculipennis* were mostly identified based on karyotyping (Artemov et al., 2018; Stegniy et al., 1973). Three other species *An. atroparvus*, *An. daciae* and *An. messeae* were diagnosed based on ITS2 sequence (Kampen, 2005; Naumenko et al., 2020).

Among all species, *An. messeae* and *An. daciae* were the most abundant in overall examined area. The representations of nucleotide variants in positions 150, 215, 217, 412 and 432 of ITS2 sequences, which were considered as diagnostic nucleotides for *An. messeae* and *An. daciae* (Naumenko et al., 2020; Nicolescu et al., 2004), in all samples are shown in Figure 2. For *An. messeae*, the main variant MTTCGG (95%) was observed earlier in the populations in the Moscow region (Naumenko et al., 2020). The second most common variant (3.7%) was without the heterogeneity at the nucleotide 150 (CTTCGG). Moreover, most of the samples, 18 out of 25, with this variant were found in the two eastern populations of Yakutsk and Lensk, where *An. messeae* was present without an admixture with other species. Two additional combinations containing a heterogeneous nucleotide K (T+G) at position 211 were found at low frequency of only 1.5% in three populations: Severodvinsk, Yakutsk and Krivosheino. Significantly greater diversity of 10 variants was determined in *An. daciae* samples in all diagnostic substitutions. As it was shown before (Kampen, 2005; Naumenko et al., 2020), they were associated with heterogeneous nucleotides in positions 211, 215 and 217, which contained various ratios of the major and additional nucleotides over a wide range. Although the major variant containing heterogeneous substitutions at all three positions was CWWYAC (81%), the variants containing one, two and no heterogeneous substitutions were also identified (Figure 2). Thus, we only considered nucleotide substitutions GG and AC in positions 412 and 432 as species-specific for *An. messeae* and *An. daciae*, respectively. Among all 1932 individual

```
                150          211 215 217        412         432
AF504204     gaccCattc   caccTtccTtCtctt   agagGtaca   tagcGgcgg
An. messeae
M1(OX360100) ....M....   ....T...T.C....   ....G....   ....G.... 635 (94.78%)
M2(OX359930) ....C....   ....T...T.C....   ....G....   ....G....  25 (3.73%)
M3(OX359931) ....M....   ....K...T.C....   ....G....   ....G....   7 (1.04%)
M4(OX359928) ....C....   ....K...T.C....   ....G....   ....G....   3 (0.45%)
An. daciae
D1(OX359328) ....C....   ....W...W.Y....   ....A....   ....C.... 653 (81.32%)
D2(OX359929) ....C....   ....A...W.Y....   ....A....   ....C....  92 (11.46%)
D3(OX360097) ....C....   ....A...A.T....   ....A....   ....C....  17 (2.12%)
D4(OX359473) ....C....   ....A...A.Y....   ....A....   ....C....  16 (1.99%)
D5(OX360092) ....C....   ....D...W.Y....   ....A....   ....C....  15 (1.87%)
D6(OX360089) ....C....   ....R...W.Y....   ....A....   ....C....   5 (0.62%)
D7(OX360087) ....C....   ....A...W.T....   ....A....   ....C....   2 (0.25%)
D8(OX360101) ....C....   ....W...A.Y....   ....A....   ....C....   1 (0.12%)
D9(OX360096) ....C....   ....R...A.T....   ....A....   ....C....   1 (0.12%)
D10(OX359327)....M....   ....W...W.Y....   ....A....   ....C....   1 (0.12%)
hybrids
H1(OX360090) ....C....   ....W...W.Y....   ....R....   ....S....  89 (50.00%)
H2(OX360091) ....M....   ....W...W.Y....   ....R....   ....S....  41 (23.03%)
H3(OX359324) ....C....   ....D...W.Y....   ....R....   ....S....  18 (10.11%)
H4(OX360098) ....M....   ....T...T.C....   ....R....   ....S....   9 (5.06%)
H5(OX359326) ....C....   ....W...T.Y....   ....R....   ....S....   6 (3.37%)
H6(OX359325) ....C....   ....T...T.C....   ....R....   ....S....   5 (2.81%)
H7(OX359329) ....C....   ....W...W.C....   ....R....   ....S....   3 (1.69%)
H8(OX360094) ....M....   ....W...T.Y....   ....R....   ....S....   2 (1.12%)
H9(OX360095) ....M....   ....T...W.Y....   ....R....   ....S....   1 (0.56%)
H10(OX359323)....C....   ....T...T.Y....   ....R....   ....S....   1 (0.56%)
H11(OX360088)....M....   ....W...W.C....   ....R....   ....S....   1 (0.56%)
H12(OX360093)....M....   ....W...T.C....   ....R....   ....S....   1 (0.56%)
H13(OX360099)....C....   ....D...W.C....   ....R....   ....S....   1 (0.56%)
```

**FIGURE 2** Variations in five diagnostic nucleotides of ITS2 sequences in *Anopheles messeae*, *Anopheles daciae* and their hybrids. The coordinates of the diagnostic nucleotides in the ITS2 sequence of *An. messeae* (AY648982) are shown above according to the primary description of the species (Nicolescu et al., 2004). The designation of variants of diagnostic nucleotides and their accession nos. obtained in this study are indicated on the left. Nucleotides corresponding to the reference ones are shown as dots, but other than the reference ones are shown as letters. The amount and frequencies of each variant inside a species are shown on the right. The number of variations was higher in *An. daciae* and hybrids than in *An. messeae*.

mosquitoes analysed based on ITS2 sequencing, 179 individuals, or 9.27%, were considered to be hybrids between *An. daciae* and *An. messeae*. Although hybrids have most diverse combinations of diagnostic nucleotides in positions 150, 215 and 217 (Figure 2), they were easily recognized by double peaks in the last two species-diagnostic positions 412 and 432.

*Anopheles messeae* and *An. daciae* accounted for 81.91% of all mosquito individuals in the studied regions and were recognized by ITS2 sequence data only. The geographical distribution of *An. messeae* and *An. daciae* widely overlapped in the central part of Eurasia: in Latvia and in the central part of European Russia, Urals and Western Siberia (Figure 1, Table S3). However, in the peripheral locations, the species composition varied from the complete absence of *An. messeae* in the southern regions of Russia (Abinsk, Novorossiysk, Voronezh, Novogrigoryevskaya, Dombarovka and Tylovoe) and Germany (Reinhausen, Speyer), to the complete absence of *An. daciae* in the northern regions of Russia (Palevitsy and Surgut), Eastern Siberia (Irkutsk, Lensk and Yakutsk) and Kazakhstan (Semey; Figure 1, Table S3). Although hybrids between *An. daciae* and *An. messeae* were found in 12 among 28 locations, hybrids represented only ~1% of the specimens in most places in northern Europe, but were abundant in south-eastern populations. The number of hybrids reached 53.54% in Semey, Kazakhstan, 78.13% in Dombarovka, Orenburg region, 49.43% in Berdsk and 8.89% in Dzerzhinskoe in the south of Western Siberia (Figure 1, Table S3). Moreover, in addition to these hybrids, only *An. messeae* or *An. daciae* were found in Semey and in Dombarovka, respectively.

In addition, three other species, *An. maculipennis*, *An. atroparvus* and *An. beklemishevi*, were found in several locations in Russia. *An. maculipennis* was widespread in two southern locations close to the Black Sea with 67% in Tylovoe and 36% in Novorossiysk. A total of 20% of this species were found in another European location in the Moscow region (Yegoryevsk). Another species, *An. beklemishevi* was present in the Tomsk region of Western Siberia at the level of 88% in Kargasok and 15% in Krivosheino, in Eastern Siberia at the level of 10% in Lensk and in the Northern European part of Russia at the level of 5% in Palevitsy. Only two specimens of *An. atroparvus* were found in Tylovoe, Crimea. Thus, we defined *An. beklemishevi* as a north-eastern species and *An. maculipennis* as a south-western species.

## 3.2 | Distinct patterns of inversion distribution in *An. messeae* and *An. daciae*

The inversion karyotyping was conducted on 670, 803 and 178 individuals of *An. messeae*, *An. daciae* and their hybrids, respectively. A polytene chromosomal complement in all *Anopheles* species is composed of three chromosomes (X, 2 and 3) comprising five chromosomal arms: X, 2R, 2L, 3R and 3L (Figure 3). Because one of the arms in the sex chromosome is absent in a polytene chromosome complement, we indicated it as X, but not as XL or XR (Artemov et al., 2021). The Y chromosome is heterochromatic and is absent in the polytene chromosome complement. Among all studied individual mosquitoes, karyotyping revealed presence of common inversions X1, 2R1, 3R1 and 3L1, inversions X2, X3, 2R3, 3R2, which have been previously described as endemic (Stegniy, Kabanova, & Novikov, 1976), rare inversions X4 and 2R4 (Naumenko et al., 2020) and

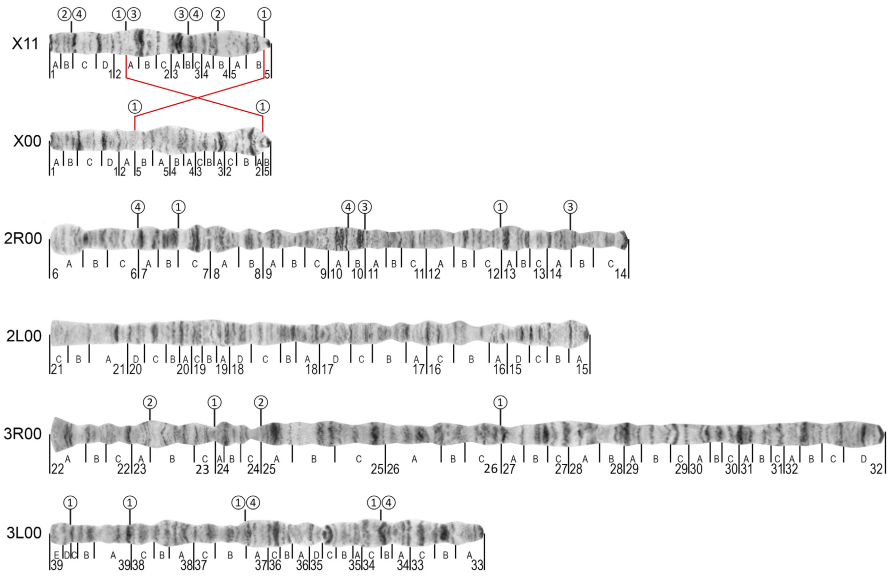


**FIGURE 3** Cytogenetic map of chromosomal inversions in *Anopheles messeae* and *Anopheles daciae*. Standard X00 and inverted X11 karyotypes are shown for the sex chromosome. Standard chromosome arrangements are shown for autosomal arms 2R00, 2L00, 3R00 and 3L00. Inversion breakpoints are indicated by numbers inside the circles above the chromosomes. Inversion X1 is additionally shown by red brackets. Numbered divisions and lettered subdivisions of the chromosomes are shown below the chromosomes. The order of the divisions on chromosome 1 is based on inverted arrangement X11 according to the previously published chromosomal map (Artemov et al., 2021). A total of 11 chromosomal inversions are shown. All chromosomal inversions on chromosome X are based on the inverted arrangement X11.

a newly described rare inversion 3L4 (Table 1). The positions of the breakpoints for these inversions are shown in Figure 3. According to the common inversion nomenclature for *An. messeae*, we referred to standard or 'not inverted' homokaryotypes as a 00 variant and inverted homokaryotypes as a 11 or 22 variant; heterokaryotypes were referred to as 01, 02, 03 or 04 variants. Inversions on the sex chromosome X in hemizygous males were referred as 0, 1, 2, 3 and 4 variants. Homokaryotypes 22, 33 and 44 were absent in this study.

Our analyses identified that 5 from 11 chromosomal inversions were species-specific and frequencies of other chromosomal inversions varied between *An. messeae*, *An. daciae* and their hybrids found in the same locations (Figure 4, Table 1). A standard karyotype X0 was determined in *An. messeae* at an extremely low frequency of 0.02, in most cases in hemizygote males or heterozygote females, whereas inverted variant X1 was the most prevalent in this species. In contrast, in *An. daciae*, the frequency of the X0 variant was 0.48. In hybrids, the frequency of this variant was 0.21. Inversion X2 was detected only in *An. messeae* populations in Western Siberia and Eastern Kazakhstan but was absent in European regions. The total frequency of this inversion was 0.03. Inversion X4 was rare with a frequency of 0.01, in total, and it was found only in *An. messeae* in Moscow populations. This chromosomal variant was observed only in hemizygote males and heterozygote females. In hybrids, inversions X2 and X4 were also found in Kazakhstan and in Moscow, respectively. The rare inversion X3 was only found in Latvia in both *An. messeae* and *An. daciae* with a frequency less than 0.01, in total, in both species (Figure S1). Moreover, in all cases, this inversion was present in hemizygote males in *An. messeae* but in heterozygote females in *An. daciae*. Autosomal inversion 2R1 was highly abundant in *An. messeae* (0.26) but found at extremely low frequency (0.01) in *An. daciae* and hybrids where it was found mostly as heterozygotes. The inverted autosomal variants 3R1 and 3L1 were present in both *An. messeae* and *An. daciae*, but their frequencies were significantly higher in *An. messeae* and hybrids (Table 1). In contrast, the rare autosomal inversions 2R4 and 2R3 were found as heterozygotes only in *An. daciae*.

Additionally to the differences identified in the frequencies of chromosomal variants between the species, our analysis also revealed distinctions between individual mosquito populations within each species (Figure S1). For example, the gradual increase in 2R1 chromosome inversion polymorphism in the *An. messeae* from south to north was observed. In southern populations, all mosquitoes carried the standard variant of chromosome 2R0, but with increasing latitude, the frequencies of the standard karyotype decreased and in some northern populations, such as Surgut in the north of Western Siberia and Palevitsy in the north of Europe, this variant was represented only in a small number of heterozygous individuals. In contrast, the inverted karyotype was prevalent in the north with a frequency of more than 90% suggesting an involvement of this inversion in adaptations for cold weather conditions and overwintering. Similarly, the gradual decrease in the diversity in chromosome 3 from east to west was determined. The proportions of standard karyotypes 3R0 and 3L0 steadily decreased as one goes eastward. A similar, but less pronounced, pattern of these inversion frequencies was found in *An. daciae*. Endemic inversions X2, X3 and X4 were found in Western Siberia, Latvia and the Moscow region, respectively. In addition, frequencies of the major inversions varied greatly among the geographical regions (Figure S1). To statistically identify the relationship between the frequencies of inversions and the geographical location of the mosquito collections, we constructed a correlation matrix (Table 2). A significant dependence was found for the latitude distribution of inversions on the 2R chromosome in

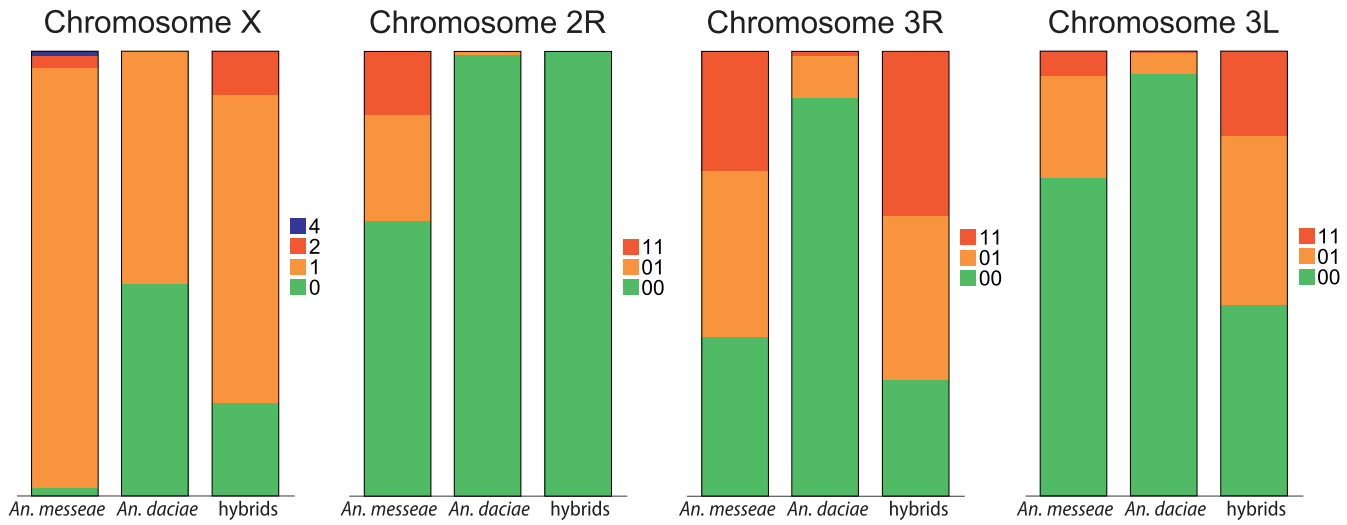


**FIGURE 4** Frequencies of the chromosomal inversion variants in *Anopheles messeae*, *Anopheles daciae* and their hybrids for all populations. To exclude differences related to the hemizygous males, frequencies of allelic chromosome variants are shown for the chromosome X but frequencies of diploid karyotypes are demonstrated for autosomes by different colours. Frequencies of rare inversions (samples count <5) are not shown. *An. messeae*, *An. daciae* and hybrids demonstrated significant differences in frequencies of chromosomal inversions.

*An. messeae*. The longitude dependence of inversion polymorphism was found for both arms of chromosome 3.

### 3.3 | Genetic differentiation and population structure of *An. messeae* and *An. daciae*

Hardy–Weinberg equilibrium within *An. messeae*, *An. daciae* and their hybrids was estimated separately. The statistical analysis demonstrated that the frequencies of homo- and heterokaryotypes, in general, did not deviate from the Hardy–Weinberg equilibrium suggesting random mating within the populations (Table S4). The exceptions were found in Petergof for the 2R and 3R inversions in the *An. messeae* population (deficiency of heterozygotes in both chromosomal arms), in Solnechnogorsk for the 2R inversion in the *An. daciae* population (absence of heterozygotes) and in hybrids for the 3R inversion from Berdsk (a significant excess of heterozygotes).

To clarify the genetic differentiation between *An. messeae* and *An. daciae*, the Hendrik's G'st indices were calculated (Table 3). Significant differentiations of 0.330 and 0.349 were found between *An. messeae* and *An. daciae* and between *An. daciae* and hybrids, respectively. At the same time, the differentiation within *An. messeae* and between *An. messeae* and hybrids was moderate (0.140). Chromosomal arms X, 2R and 3R contributed the most to interspecific differentiation ($F_{ST}$ were 0.409, 0.308 and 0.492, respectively) but the difference for the 3L arm was modest (0.128). Hybrids differed moderately from either species in the X chromosomes (<1.5). The 2R arm was the main contributor to the difference between *An. messeae* and hybrids (0.301) but not between *An. daciae* and hybrids (0). Chromosome arms 3R and 3L differed greatly in *An. daciae* and hybrids (0.639 and 0.430) but not between *An. messeae* and hybrids (0.033 and 0.162; Table 3).

Further statistics analysis demonstrated a higher genetic diversity in *An. messeae* and hybrids than in *An. daciae* (Table 4). For example, Menhinick indices were 3.23 in *An. messeae*, 4.04 in hybrids, but only 1.12 in *An. daciae*. Simpson indices were 0.96, 0.97 and 0.85 and Shannon indices were 3.65, 3.65 and 2.20 in *An. messeae*, hybrids and *An. daciae*, respectively. An AMOVA test revealed a clear population structure in *An. messeae* with differences between the populations and FDs/countries of 22.08% and 16.68%, respectively, but weak differences in *An. daciae* of 7.78% and 3.73%, respectively. The rest of the variance fell at the intrapopulation level of 61.23% in *An. messeae* and 88.49% in *An. daciae* (Table 5). In addition, we performed the isolation by distance test to estimate the genetic isolation between populations. The degree of differentiation between the populations of *An. messeae* was strongly depended on the distance between them ($r = .808$, $p < .001$; Figure 5). At the same time, this dependence among *An. daciae* was less than half weaker ($r = .381$, $p < .001$). PCA and hierarchical clustering based on the frequency of inversions in samples were performed to reliably separate the two species (Figure 6). This analysis indicated that populations of *An. messeae* did not form one distinct group. Based on K-means method, we assumed that the optimal number of clusters in the studied populations equals 4 (Figure S2), in which *An. messeae* populations formed three distinct group: European, Asian and North Eurasian (Figure 6). At the same time, *An. daciae* populations were grouped into one additional compact clade. The exception was the population from Dombarovka that grouped together with the clade of *An. messeae* from Asia. When PCA test was conducted separately for each species, chromosome 3 contributed equally to the diversity of both species (Figure S3A). High dispersion along the chromosome 2 axis was seen in *An. messeae* (Figure S3A), while in *An. daciae* high dispersion along the X chromosome axis was detected (Figure S3B). Thus, we determined significant differences between species at both the level of individual chromosomes and karyotypes and at the population level.

**TABLE 2** Clinal variability of inversion polymorphisms in *Anopheles messeae* and *Anopheles daciae*.

| | Latitude | | Longitude | |
|---|---|---|---|---|
| | *An. messeae* | *An. daciae* | *An. messeae* | *An. daciae* |
| X | −0.18** | −0.03 | −0.01 | −0.14** |
| 2R | −0.35** | −0.09* | 0.27** | −0.03 |
| 3R | 0.00 | −0.10* | −0.55** | −0.25** |
| 3L | −0.01 | 0.09* | −0.50** | −0.13** |

*$p$-value < .05; **$p$-value < .001.

**TABLE 3** Genetic differentiation (Hedrick's G'st) between *Anopheles messeae*, *Anopheles daciae* and hybrids.

| | *An. daciae*/*An. messeae* | *An. daciae*/ hybrids | *An. messeae*/ hybrids |
|---|---|---|---|
| X | 0.409* | 0.143* | 0.128* |
| 2R | 0.308* | 0.000 | 0.301* |
| 3R | 0.492* | 0.639* | 0.033* |
| 3L | 0.128* | 0.430* | 0.162* |
| Total | 0.330* | 0.349* | 0.140* |

*Significant difference ($p$-values < .05).

**TABLE 4** Genetic diversity in *Anopheles messeae*, *Anopheles daciae* and hybrids.

| Species | Number of sampled individuals | Number of multilocus genotypes | Menkhinik index | Corrected Simpson index ($\lambda \times \frac{n}{n-1}$) | Shannon–Wiener index |
|---|---|---|---|---|---|
| *An. messeae* | 830 | 93 | 3.23 | 0.96 | 3.65 |
| *An. daciae* | 923 | 34 | 1.12 | 0.85 | 2.20 |
| Hybrid | 179 | 54 | 4.04 | 0.97 | 3.65 |

| Levels | *An. messeae* (%) | *An. daciae* (%) |
|---|---|---|
| Variations between FDs/countries | 22.08* | 3.73* |
| Variations between populations within FDs/countries | 16.68* | 7.78* |
| Variations within populations | 61.23* | 88.49* |

*Significant difference ($p$-values < .05).

**TABLE 5** Assessment of genetic diversity and population differentiation in *Anopheles messeae* and *Anopheles daciae* based on Analysis of Molecular Variance.

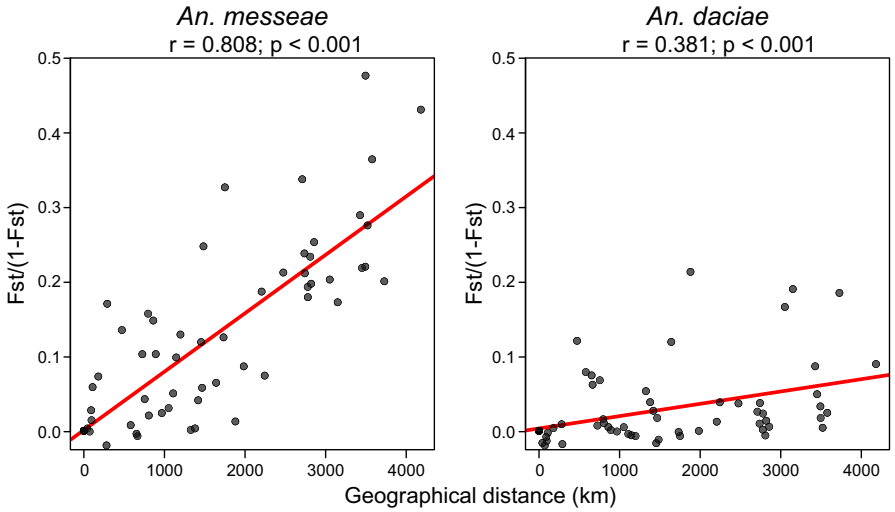


**FIGURE 5** Isolation by distance within populations of *Anopheles messeae* and *Anopheles daciae*. The graph of the linear regression model is shown in red; *r* stands for the correlation coefficient between the pairwise $F_{ST}$ and the pairwise distance between populations. The *x*-axis shows the distance between populations in kilometres. The *y*-axis indicates $F_{ST}/(1-F_{ST})$ parameter. The degree of differentiation between populations of *An. messeae* strongly depends on the distance between them, while this dependence is much weaker among *An. daciae* populations.

When analysing paired $F_{ST}$ between the populations, most of the *An. messeae* populations differed from each other, suggesting restriction of gene flow between them (Table S5). Interestingly, the three most northern populations of *An. messeae* from Europe (Palevitsy) and Asia (Surgut and Krivosheino) located 1200 km between each other, were slightly different from each other but significantly distinct from the rest of the populations of this species. A different picture was observed for *An. daciae*. Analysis of paired $F_{ST}$ within the species (Table S5) showed insignificant differences between populations throughout its range. The only exception was the population from Dombarovka (Orenburg region), which differed sharply from the rest of the populations ($F_{ST} > 0.25$). These data were consistent with the above results of hierarchical clustering analysis in which all mosquito populations clustered into 4 large groups.

In addition to *An. messeae* and *An. daciae*, our study revealed two distinct groups of hybrids: southern Eurasian (Semey/Dombarovka) and Asian (Berdsk/Tomsk) that were practically identical within the group (Figure 6). Interestingly, the southern Eurasian group of hybrids was clustered together with the Asian populations of *An. messeae*, whereas the Asian group of hybrids clustered together with *An. daciae* populations.

## 4 | DISCUSSION

### 4.1 | The role of the chromosomal inversions in mosquito evolution

In this study, we attempted to better understand the role of chromosomal inversions in the evolution of two closely related species from the Maculipennis group, *An. messeae* and *An. daciae*. For the first time, chromosomal inversions were analysed separately in the two species. Among 11 polymorphic inversions, the most indicative differences between *An. messeae* and *An. daciae* were associated with the X sex chromosome (Figure 3). The standard arrangement X0 was present with an extremely low frequency of 2%, in total, or was almost absent in the *An. messeae* populations, while the inverted arrangement X1 was found in very high frequency of 94% in the populations of this species (Table 1). In contrast, both standard X0 and inverted X1 arrangements were found in almost equal frequencies of 48% and 52%, respectively, along the *An. daciae* distribution. The frequencies of other inversions were significantly less abundant than X1 inversions in the *An. messeae* populations and almost absent in the *An. daciae* populations. The endemic Siberian X2 inversion and the rare X4 inversion were only found in *An. messeae* populations. The Latvian endemic X3 inversion (Figure S1) was found in low frequencies in both species (<1%) and was present in *An. messeae* only in males, while in *An. daciae* it was only found in heterozygous females. Interestingly, all chromosomal inversions found in *An. messeae* were formed based on the inverted X11 arrangement, suggesting that this karyotype is ancestral for *An. messeae* (Figure 3). Thus, we conclude that arrangement X1 was originally fixed in *An. messeae*, whereas the endemic or rare inversions on the X chromosome originated later after the two species split. Interestingly, in addition to five polymorphic inversions in the X chromosome of *An. messeae* and *An. daciae*, five fixed chromosomal inversions have been identified among the Palearctic species of the Maculipennis group (Stegniy, 1991) suggesting their potential role in the process of speciation.

All the widespread autosomal inversions identified by this study in natural populations of *An. messeae* and *An. daciae* were present in both species, indicating that their polymorphism is

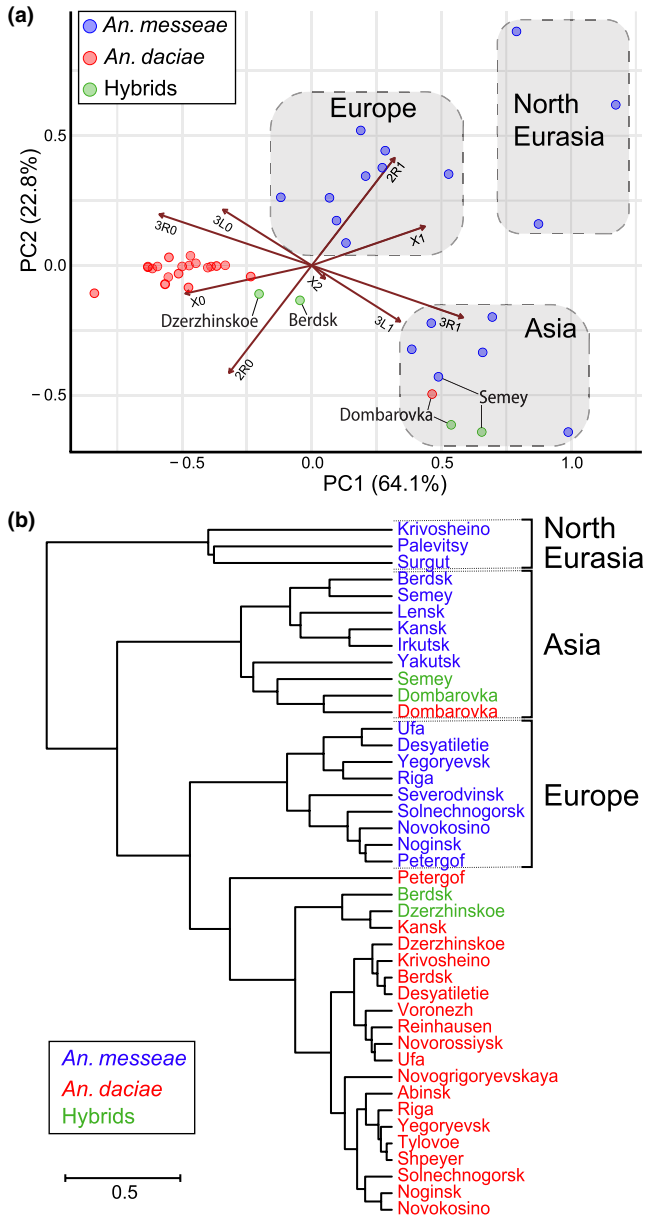


**FIGURE 6** Interpopulation PCA plot (a) and hierarchical clustering dendrogram (b) based on the frequencies of the chromosomal inversions in *Anopheles messeae*, *Anopheles daciae* and their hybrids. Species are indicated by different colours. Arrows on panel (a) indicate coordinate axes in space of frequencies of inversions X0, X1, X2, 2R0, 2R1, 3R0, 3R1, 3L0 and 3L1. Positions of populations from four locations Dzerzhinskoe, Berdsk, Semey and Dombarovka, where most of the hybrids were found, are indicated. Three major clades of *An. messeae*—North Eurasian, European and Asian—are shown by light grey colour in panel (a) and by brackets in panel (b). Based on statistical analysis, *An. messeae* is subdivided into three major clades, whereas *An. daciae* is represented by only a single population.

ancestral in both cases (Figure 3). However, the frequencies of the inverted variants 2R1, 3R1 and 3L1 were significantly higher in *An. messeae* versus *An. daciae* (0.26 vs. <0.01, 0.45 vs. 0.06, 0.17 vs. 0.03, respectively). The rare inversions 2R4 and 2R3 were found only in *An. daciae* from Riga and in the Moscow region (Yegoryevsk), respectively, and the 3R2 and 3L4 inversions were found in the *An. messeae* northern populations of Surgut and Severodvinsk, respectively (Figure S1). The presence of endemic chromosomal inversions suggests their involvement in local adaptations of mosquitoes. A latitude gradient was determined for 2R1 inversions with higher frequencies of inverted arrangements in northern populations (Figure S1). This result strongly supports the idea of the involvement of 2R1 inversion in adaptation to low temperatures and possibly in the development of diapause and overwintering under cold weather conditions in northern populations (Stegniy, Kabanova, Novikov, et al., 1976). Longitude gradient were determined for the inversions in the 3R and 3L arms. Gradients were more pronounced in populations of *An. messeae* than in *An. daciae*. Presence of large-scale geographical gradients of the autosomal inversions suggests their associations either with climatic adaptations of the mosquito populations or with a genetic drift. More studies are required to better understand the involvement of the chromosomal inversions in ecological and climate adaptations.

It is interesting to compare our data with the analyses of the chromosomal inversions conducted on African malaria mosquitoes from the *An. gambiae* complex. In this complex, seven species were differentiated by 10 fixed chromosomal inversions (Coluzzi et al., 2002) but unlike in *An. messeae* and *An. daciae*, five fixed chromosomal inversions located on the X chromosome in the *An. gambiae* complex have no polymorphic variants in any species within the complex (Coluzzi et al., 2002). Similar to the X1 inversion in *An. messeae* and *An. daciae*, an autosomal inversion 2La is highly polymorphic in *An. gambiae* but is fixed in its sister taxa *An. arabiensis* and *An. merus*. Detailed analyses demonstrated the ancestral state of the 2La inverted arrangement for this species complex (Kamali et al., 2012; Sharakhov et al., 2006). Intensive studies of inversion polymorphism within *An. gambiae* species identified five chromosomal forms related to the inversions in the 2R arm: Bamako, Savanna, Mopti, Forest and Bissau (Touré, 1989; Touré et al., 1994). In *An. gambiae*, the 2La inversion frequencies change depending on the aridity gradient of the environment (Ayala et al., 2017, 2019; Cheng et al., 2012). Autosomal chromosomal inversions have been shown to be involved in ecological specialization of the incipient species *An. gambiae* and *An. coluzzii* (Costantini et al., 2009; Simard et al., 2009) and play a role in ecotypic adaptation of four *Anopheles* species: *An. gambiae*, *An. coluzzii*, *An. arabiensis* and *An. funestus* (Ayala et al., 2017).

## 4.2 | Distinct phylogeographic history of *An. messeae* and *An. daciae*

The study provides some insights into phylogeographical differences between *An. messeae* and *An. daciae* in Eurasia. First of all, we determined distinct geographical distribution of the species with *An. messeae* absent in south Europe and *An. daciae* absent in eastern Siberia (Figure 1). Then, PCA and hierarchical cluster analysis, conducted based on the frequencies of the chromosomal

inversions, separated *An. messeae* and *An. daciae* into distinct clusters (Figure 6). Three additional clusters were identified within populations of *An. messeae*: Northern Eurasian, European and Asian. In contrast, populations of *An. daciae* clustered all together demonstrating no distinct population structure among locations in Eurasia. Moreover, the analysis of isolation by distance revealed a stronger correlation between the genetic differentiations of the populations and distances between them in *An. messeae* than in *An. daciae*. Because this analysis was carried out only within overlapping geographical ranges of the two species, this result suggests that *An. messeae* settled in this territory much earlier and was able to accumulate much stronger genetic differences between the populations while *An. daciae* colonized the studied area relatively recently and quickly. Indeed, fluctuations in frequencies in the X0 chromosomal variant (Stegniy et al., 2016), which is associated with *An. daciae*, indicate dramatic dynamics of the redistribution of the species in Eurasia during the last 40 years. In Western Siberia (Tomsk), frequencies of the X0 chromosome variant increased from 5% to 55% from 1974 to 2013. In Eastern Siberia (Krasnoyarsk), in 1974, the X0 variant was absent (0%) but in 2008 the frequency of this variant increased to 30%. At the same time, a tendency towards the increase in the frequency of the X0 chromosome variant was observed in a northern location (Syktyvkar). In southern Asia (Almaty), the X0 chromosome variant, which in 1974–1977 was absent (0%), had reached a frequency of 70% by 2014. In contrast, in Europe (Moscow) the X0 frequency declined from 50% (1975) to 10%. These data suggest a recent dramatic expansion of *An. daciae* in the northern, southern and eastern territories of Eurasia with some westward expansion of *An. messeae* in Europe.

According to our preliminary estimation, the split between *An. messeae* and *An. daciae* occurred ~2 Mya (Yurchenko et al., 2022) and thus overlapped with the glaciation period in Eurasia (Torsvik & Cocks, 2017). Thus, we hypothesize that the glaciation event can potentially result in a long period of disruption of an original population of the ancestral species into different southern refugia whereas one population of mosquitoes was isolated in the Asian location and another in the European location. As a potential outcome of such isolation, the two species accumulated genetic differences, which are especially pronounced in the chromosomal inversions and overall differentiation in the X chromosome (Naumenko et al., 2020). Later, when the climate became warmer, distribution of the species overlapped again but they became partially incompatible because they acquired postzygotic or prezygotic isolation barriers. In fact, the differences in acoustic signalling of adult mosquitoes were observed between mosquitoes with different chromosomal karyotypes potentially associated with *An. messeae* and *An. daciae* (Perevozkin et al., 2012). These differences may contribute to the reproductive isolation between the species.

Geographical or allopatric speciation is considered a classical form of speciation commonly present in nature (Mayr, 1963). The role of the climate oscillations in speciation and population genetic processes of European and North American species was highlighted in several reviews (Emerson & Hewitt, 2005; Hewitt, 2001, 2004). The influence of glaciation on species evolution has been shown for mammals (Berggren et al., 2005), fish (Aguilar et al., 2019), insects (Baird et al., 2021) and plants (Tzedakis et al., 2013). Among mosquitoes, the presence of two groups of *An. claviger* in France, which represent unclear genetic entities, was considered as allopatric divergence in southern refugia during the last glaciation period (Schaffner et al., 2003). In another study, data from the microsatellite analysis of populations of the West Nile vector mosquito *Culex tarsalis* suggest that this species underwent a range expansion across the western United States within the last 375,000–560,000 years, which may have been associated with the Pleistocene glaciation events that occurred in the Midwestern and Western United States between 350,000 and 1 Mya (Venkatesan et al., 2007).

In addition to the differences in population structure between *An. messeae* and *An. daciae*, we found high variations in the frequencies of their hybrids among the locations in areas where distributions of the two species were overlapped (Figure 1). The frequencies of hybrids were very low in northern Europe from 0% (Severodvinsk) to 1.59% (Solnechnogorsk) but higher, up to 7%, in southern Europe (Voronezh). In contrast, in two locations in south-western Siberia (Berdsk and Dzerzhinskoe), one Southern Urals location (Dombarovka) and one Kazakhstan location (Semey), the hybrid frequencies were very high (49.43%, 8.89%, 78.13% and 54.26%, respectively). Surprisingly, only *An. messeae*, in addition to the hybrids, was present in the Semey location and only *An. daciae*, in addition to the hybrids, was present in the Dombarovka location. These data could have multiple potential interpretations from just the simple presence of recombinants in rDNA sequences in one or in both species in some geographical areas to an existence of an additional cryptic taxon in Central Asia that is ancestral to both *An. messeae* and *An. daciae*. Southern Eurasia can also be considered as a hybrid zone between the two species. In the past, low frequencies of the heterozygote X01, which may represent potential hybrids between the two species, were always observed in the western part of the species distribution but were interpreted as disruptive selection that favours the X00 and X11 homozygotes at different times of the breeding and wintering seasons (Stegniy et al., 2016).

Hybridization between closely related species has been shown to be variable in other mosquitoes. For example, the number of hybrids between two incipient species, *An. gambiae* and *An. coluzzii*, from the *An. gambiae* complex varies among the locations from 0.2% in Coastal areas of Africa to 24% in Guinea-Bissau (Oliveira et al., 2008) and even up to 42% in some surrounding areas (Nwakanma et al., 2013). Patterns of interspecies hybridization vary in *Culex* mosquitoes. For example, massive hybrid zones were found between mosquitoes from the *Culex pipiens* complex. Diapausing in winter *Cx. pipiens* and nondiapausing *Cx. quinquefasciatus* form large hybrid zones where their distribution overlaps in North America, Africa and Asia (Aardema et al., 2022; Farajollahi et al., 2011). Two eco-physiological forms or subspecies, *Cx. p. pipiens* and *Cx. p. molestus*, are behaviourally divergent and truly isolated in northern Europe, but are gradually becoming well-mixed, intermediate populations in North Africa (Haba & McBride, 2022). A

number of studies suggest that *Cx. p. pipiens* and *Cx. p. molestus* represent different evolutionary entities (Fonseca et al., 2004; Gomes et al., 2015; Yurchenko et al., 2020).

We think that additional whole-genome sequencing approach of multiple individuals of *An. messeae*, *An. daciae* and their hybrids from different locations in Eurasia has to be considered for the future studies. This analysis will help to better understanding population structure, patterns of their diversification in Eurasia and the nature of hybrids between them. Similar studies were very productive for the African malaria mosquitoes (Anopheles gambiae 1000 Genomes Consortium, 2020). Another interesting future direction of this study is to explore the potential functional role of the chromosomal inversions in speciation, ecological and climatic adaptation of the *An. messeae*, *An. daciae* and other species from the Maculipennis group in Eurasia.

## 5 | CONCLUSIONS

This study evaluates the potential role of chromosomal inversions in genetic divergence of two closely related species *An. messeae* and *An. daciae* that were previously considered as a single species. Existence of species-specific inversions in the X chromosome in both species implies their potential involvement in speciation. The presence of longitude and latitude gradients in Eurasia associated with frequencies of the autosomal inversions suggests their role in adaptation to different climate conditions. We demonstrated that *An. messeae* and *An. daciae* represent distinct evolutionary entities, which differ from each other by their geographical distribution, population structure and patterns of their divergence in Eurasia. The study determined that *An. messeae* has much higher diversity and a more complex population structure, while *An. daciae* has low diversity and a more homogeneous structure of its populations. We hypothesize that glaciation events in Eurasia may have contributed to the original split between *An. messeae* and *An. daciae* and explain the higher genetic diversity within *An. messeae*. Our data suggest that *An. messeae* populated Eastern Eurasia earlier, whereas *An. daciae* spread in Asia more recently. Detailed knowledge of the population structure and phylogeographic history of the important malaria vectors *An. messeae* and *An. daciae* will help to predict the patterns of malaria transmission in Eurasia.

### AUTHOR CONTRIBUTIONS

Maria V. Sharakhova, Ilya I. Brusentsov and Mikhail I. Gordeev contributed to conceptualization; Ilya I. Brusentsov, Dimitriy A. Karagodin, Andrey A. Yurchenko and Anton V. Moskaev contributed to methodology; Ilya I. Brusentsov, Dimitriy A. Karagodin, James M. Hodge, Anton V. Moskaev and Mikhail I. Gordeev contributed to investigation; Maria V. Sharakhova, Ilya I. Brusentsov and Dimitriy A. Karagodin contributed to writing—original draft preparation; Maria V. Sharakhova, Ilya I. Brusentsov, Elina M. Baricheva, Igor V. Sharakhov and Dimitriy A. Karagodin contributed to writing—review and editing; Maria V. Sharakhova contributed to supervision; Elina M. Baricheva contributed to project administration; Maria V. Sharakhova, Elina M. Baricheva and Mikhail I. Gordeev contributed to funding acquisition; Ilya I. Brusentsov, Anton V. Moskaev, Mikhail I. Gordeev, Vladimir A. Burlak, Gleb N. Artemov, Anuarbek K. Sibataev, Norbert Becker, Igor V. Sharakhov and Maria V. Sharakhova contributed to mosquito collections. All authors have read and agreed to the published version of the manuscript.

### ACKNOWLEDGEMENTS

We thank the Russian Science Foundation for funding the ITS2 sequencing (grant no. 19-14-00130 to M.V.S.) and for supporting the inversion polymorphism analysis (grant 22-24-00183 to M.I.G). We also thank the Institute of Cytology and Genetics for funding the statistical analysis (FWNR-2022-0015 budget project) and Janet Webster for editing the text.

### FUNDING INFORMATION

Mosquito collections were supported by the Russian Science Foundation grant 19-14-00130 to M.V.S. ITS2 sequencing was funded by the Russian Science Foundation grant 21-14-00182 to I.V.S. Inversion polymorphism analysis was funded by the Russian Science Foundation grant 22-24-00183 to M.I.G. Statistical analysis was funded by the FWNR-2022-0015 project of the Institute of Cytology and Genetics.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

All the data are available in the text, figures and tables of this article. Consensus ITS2 sequences of all variants found in this study are available in GenBank under the accession nos. shown in Figure 2. The raw Sanger sequences data (ab1 files) of ITS2 for individual mosquitoes are available at https://doi.org/10.17632/f5n37yw87k.2.

### ETHICS STATEMENT

None declared.

### ORCID

*Andrey A. Yurchenko* https://orcid.org/0000-0002-2239-6902
*Maria V. Sharakhova* https://orcid.org/0000-0002-5790-3548

### REFERENCES

Aardema, M. L., Olatunji, S. K., & Fonseca, D. M. (2022). The enigmatic *Culex pipiens* (Diptera: Culicidae) species complex: Phylogenetic challenges and opportunities from a notoriously tricky mosquito group. *Annals of the Entomological Society of America*, *115*(1), 95–104. https://doi.org/10.1093/aesa/saab038

Aguilar, C., Miller, M. J., Loaiza, J. R., González, R., Krahe, R., & De León, L. F. (2019). Tempo and mode of allopatric divergence in the weakly electric fish *Sternopygus dariensis* in the Isthmus of Panama. *Scientific Reports*, *9*(1), 18828. https://doi.org/10.1038/S41598-019-55336-Y

Anopheles gambiae 1000 Genomes Consortium. (2020). Genome variation and population structure among 1142 mosquitoes of the African malaria vector species *Anopheles gambiae* and *Anopheles*

*coluzzii*. *Genome Research*, *30*(10), 1533–1546. https://doi.org/10.1101/gr.262790.120

Artemov, G. N., Fedorova, V. S., Karagodin, D. A., Brusentsov, I. I., Baricheva, E. M., Sharakhov, I. V., Gordeev, M. I., & Sharakhova, M. V. (2021). New cytogenetic photomap and molecular diagnostics for the cryptic species of the malaria mosquitoes *Anopheles messeae* and *Anopheles daciae* from Eurasia. *Insects*, *12*(9), 835. https://doi.org/10.3390/insects12090835

Artemov, G. N., Gordeev, M. I., Kokhanenko, A. A., Moskaev, A. V., Velichevskaya, A. I., Stegniy, V. N., Sharakhov, I. V., & Sharakhova, M. V. (2018). A standard photomap of ovarian nurse cell chromosomes and inversion polymorphism in *Anopheles beklemishevi*. *Parasites & Vectors*, *11*(1), 211. https://doi.org/10.1186/s13071-018-2657-3

Ayala, D., Acevedo, P., Pombi, M., Dia, I., Boccolini, D., Costantini, C., Simard, F., & Fontenille, D. (2017). Chromosome inversions and ecological plasticity in the main African malaria mosquitoes. *Evolution*, *71*(3), 686–701. https://doi.org/10.1111/evo.13176

Ayala, D., Zhang, S., Chateau, M., Fouet, C., Morlais, I., Costantini, C., Hahn, M. W., & Besansky, N. J. (2019). Association mapping desiccation resistance within chromosomal inversions in the African malaria vector *Anopheles gambiae*. *Molecular Ecology*, *28*(6), 1333–1342. https://doi.org/10.1111/mec.14880

Ayala, F. J., & Coluzzi, M. (2005). Chromosome speciation: Humans, *Drosophila*, and mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(Suppl), 6535–6542. https://doi.org/10.1073/pnas.0501847102

Baird, H. P., Shin, S., Oberprieler, R. G., Hullé, M., Vernon, P., Moon, K. L., Adams, R. H., McKenna, D. D., & Chown, S. L. (2021). Fifty million years of beetle evolution along the Antarctic Polar Front. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(24), e2017384118. https://doi.org/10.1073/pnas.2017384118

Beklemishev, V. N. (1948). *Anopheles* species in the USSR and neighboring Asian countries, their distribution and participation in the transmission of malaria (review). *Medical Parasitology and Parasitic Diseases*, *17*(3), 201–209.

Benedict, M., & Dotson, E. M. (2015). *Methods in Anopheles research* (p. 408). Retrieved from https://www.yumpu.com/en/document/view/49409322/methods-in-anopheles-research-mr4

Berggren, K. T., Ellegren, H., Hewitt, G. M., & Seddon, J. M. (2005). Understanding the phylogeographic patterns of European hedgehogs, *Erinaceus concolor* and *E. europaeus* using the MHC. *Heredity*, *95*, 84–90. https://doi.org/10.1038/sj.hdy.6800694

Bertola, M., Mazzucato, M., Pombi, M., & Montarsi, F. (2022). Updated occurrence and bionomics of potential malaria vectors in Europe: A systematic review (2000-2021). *Parasites & Vectors*, *15*(1), 88. https://doi.org/10.1186/s13071-022-05204-y

Bezzhonova, O. V., & Goryacheva, I. I. (2008). Intragenomic heterogeneity of rDNA internal transcribed spacer 2 in *Anopheles messeae* (Diptera: Culicidae). *Journal of Medical Entomology*, *45*(3), 337–341. https://doi.org/10.1603/0022-2585(2008)45[337:ihorit]2.0.co;2

Blažejová, H., Šebesta, O., Rettich, F., Mendel, J., Čabanová, V., Miterpáková, M., Betášová, L., Peško, J., Hubálek, Z., Kampen, H., & Rudolf, I. (2018). Cryptic species *Anopheles daciae* (Diptera: Culicidae) found in the Czech Republic and Slovakia. *Parasitology Research*, *117*(1), 315–321. https://doi.org/10.1007/s00436-017-5670-0

Burlak, V. A., & Gordeev, M. I. (1998). The effect of infection by the entomopathogenic bacterium *bacillus thuringiensis* on the spread of microsporidia in an inversion-polymorphic population of the malarial mosquito *Anopheles messeae* (Diptera: Culicidae). *Parazitologiia*, *32*(3), 264–267.

Calzolari, M., Desiato, R., Albieri, A., Bellavia, V., Bertola, M., Bonilauri, P., Callegari, E., Canziani, S., Lelli, D., Mosca, A., Mulatti, P., Peletto, S., Ravagnan, S., Roberto, P., Torri, D., Pombi, M., di Luca, M., & Montarsi, F. (2021). Mosquitoes of the Maculipennis complex in Northern Italy. *Scientific Reports*, *11*(1), 6421. https://doi.org/10.1038/s41598-021-85442-9

Cheng, C., White, B. J., Kamdem, G. C., Mockaitis, K., Costantini, C., Hahn, M. W., & Besansky, N. J. (2012). Ecological genomics of *Anopheles gambiae* along a latitudinal cline: A population-resequencing approach. *Genetics*, *190*(4), 1417–1432. https://doi.org/10.1534/genetics.111.137794

Chretien, J. P., Anyamba, A., Small, J., Britch, S., Sanchez, J. L., Halbach, A. C., Tucker, C., & Linthicum, K. J. (2015). Global climate anomalies and potential infectious disease risks: 2014-2015. *PLoS Currents*, *7*, 2014–2015. https://doi.org/10.1371/currents.outbreaks.95fbc4a8fb4695e049baabfc2fc8289f

Coluzzi, M., Sabatini, A., Della Torre, A., Di Deco, M. A., & Petrarca, V. (2002). A polytene chromosome analysis of the *Anopheles gambiae* species complex. *Science*, *298*(5597), 1415–1418. https://doi.org/10.1126/science.1077769

Costantini, C., Ayala, D., Guelbeogo, W. M., Pombi, M., Some, C. Y., Bassole, I. H., Ose, K., Fotsing, J. M., Sagnon, N.' F., Fontenille, D., Besansky, N. J., & Simard, F. (2009). Living at the edge: Biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. *BMC Ecology*, *9*, 16. https://doi.org/10.1186/1472-6785-9-16

Culverwell, C. L., Vapalahti, O. P., & Harbach, R. E. (2020). *Anopheles daciae*, a new country record for Finland. *Medical and Veterinary Entomology*, *34*(2), 145–150. https://doi.org/10.1111/mve.12431

Czajka, C., Weitzel, T., Kaiser, A., Pfitzner, W. P., & Becker, N. (2020). Species composition, geographical distribution and seasonal abundance of the *Anopheles maculipennis* complex along the Upper Rhine, Germany. *Parasitology Research*, *119*(1), 75–84. https://doi.org/10.1007/s00436-019-06551-z

Danabalan, R., Monaghan, M. T., Ponsonby, D. J., & Linton, Y.-M. (2014). Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. *Medical and Veterinary Entomology*, *28*(2), 169–178. https://doi.org/10.1111/mve.12023

Daskova, N. G., & Rasnicyn, S. P. (1982). Review of data on susceptibility of mosquitos in the USSR to imported strains of malaria parasites. *Bulletin of the World Health Organization*, *60*(6), 893–897.

Edwards, S. V., Robin, V. V., Ferrand, N., & Moritz, C. (2022). The evolution of comparative phylogeography: Putting the geography (and more) into comparative population genomics. *Genome Biology and Evolution*, *14*(1), evab176. https://doi.org/10.1093/gbe/evab176

Emerson, B. C., & Hewitt, G. M. (2005). Phylogeography. *Current Biology*, *15*(10), R367–R371. https://doi.org/10.1016/J.CUB.2005.05.016

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, *131*(2), 479–491.

Farajollahi, A., Fonseca, D. M., Kramer, L. D., & Marm Kilpatrick, A. (2011). "Bird biting" mosquitoes and human disease: A review of the role of *Culex pipiens* complex mosquitoes in epidemiology. *Infection, Genetics and Evolution*, *11*(7), 1577–1585. https://doi.org/10.1016/j.meegid.2011.08.013

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., Fleischer, R. C., & Wilkerson, R. C. (2004). Emerging vectors in the *Culex pipiens* complex. *Science*, *303*(5663), 1535–1538. https://doi.org/10.1126/science.1094247

Fyodorova, M. V., Savage, H. M., Lopatina, J. V., Bulgakova, T. A., Ivanitsky, A. V., Platonova, O. V., & Platonov, A. E. (2006). Evaluation of potential West Nile virus vectors in Volgograd Region, Russia, 2003 (Diptera: Culicidae): Species composition, bloodmeal host utilization, and virus infection rates of mosquitoes. *Journal of Medical Entomology*, *43*(3), 552–563. https://doi.org/10.1603/0022-2585(2006)43[552:EOPWNV]2.0.CO;2

Gideon, B. (2014). *BEDASSLE: Quantifies effects of geo/eco distance on genetic differentiation*. Retrieved from https://cran.r-project.org/package=BEDASSLE

Gomes, B., Wilding, C. S., Weetman, D., Sousa, C. A., Novo, M. T., Savage, H. M., Almeida, A. P. G., Pinto, J., & Donnelly, M. J. (2015). Limited genomic divergence between intraspecific forms of *Culex pipiens* under different ecological pressures. *BMC Evolutionary Biology*, *15*, 197. https://doi.org/10.1186/s12862-015-0477-z

Gordeev, M. I., & Burlak, V. A. (1991). Inversion polymorphism in malaria mosquito *Anopheles messeae*. Part X. Resistance of larvae with different genotypes to toxins of crystal-forming bacteria *Bacillus thuringiensis* subsp. *israelensis* (serovar H14). *Genetika*, *27*(2), 238–246.

Gordeev, M. I., & Moskaev, A. V. (2016). Chromosomal polymorphism in the populations of malaria mosquito *Anopheles messeae* (Diptera, Culicidae) in the Volga region. *Genetika*, *52*(6), 685–690.

Gordeev, M. I., & Perevozkin, V. P. (1995). Strategies for selection and stability to asphyxia in larvae of the malaria mosquito *Anopheles messeae* with various karyotypes. *Genetika*, *31*(2), 180–184.

Gordeev, M. I., Perevozkin, V. P., & Luk'yantsev, S. V. (1997). Genetic and ecological effects of *Argyroneta aquatica* hunting on *anopheles* and *Culex* mosquito larvae. *Genetica*, *33*(5), 704–709.

Gordeev, M. I., & Sibataev, A. K. (1995). Influence of the carnivorous plant bladderwort *Utricularia vulgaris* on selection processes in larvae of malarial mosquitoes. *Ecology*, *3*, 241–245.

Gordeev, M. I., & Stegniy, V. N. (1987). Inversion polymorphism in the malaria mosquito *Anopheles messeae*. VII. Fertility and the population genetics structure of the species. *Genetika*, *23*(12), 2169–2174.

Gordeev, M. I., & Troshkov, N. I. (1990). Inversion polymorphism of the malaria mosquito *Anopheles messeae*. IX. Cannibalism in larvae as a selection factor. *Genetika*, *26*(9), 1597–1603.

Gornostaeva, R. M., & Danilov, A. (2002). On distribution of malaria mosquitoes (Diptera, Culicidae: *Anopheles*) from Maculipennis complex in Russian territory. *Parasitology*, *26*, 33–47.

Graffelman, J. (2015). Exploring diallelic genetic markers: The HardyWeinberg package. *Journal of Statistical Software*, *64*(3), 1–23. https://doi.org/10.18637/jss.v064.i03

Gutsevich, A. V., Monchadsky, A. S., & Shtakelberg, A. A. (1970). *Fauna of the U.S.S.R. Diptera* (Vol. *3*). Nauka.

Haba, Y., & McBride, L. (2022). Origin and status of *Culex pipiens* mosquito ecotypes. *Current Biology*, *32*(5), R237–R246. https://doi.org/10.1016/j.cub.2022.01.062

Hackett, L. W. (1937). *Malaria in Europe: An ecological study* (p. 336). Oxford University Press.

Hackett, L. W., & Missiroli, A. (1935). The varieties of *Anopheles maculipennis* and their relation to the distribution of malaria in Europe. *Rivista di Malariologia*, *14*, 45–109.

Hartigan, J. A., & Wong, M. A. (1979). Algorithm AS 136: A K-means clustering algorithm. *Applied Statistics*, *28*(1), 100. https://doi.org/10.2307/2346830

Hewitt, G. M. (2001). Speciation, hybrid zones and phylogeography - or seeing genes in space and time. *Molecular Ecology*, *10*(3), 537–549. https://doi.org/10.1046/J.1365-294X.2001.01202.X

Hewitt, G. M. (2004). Biodiversity: A climate for colonization. *Heredity*, *92*(1), 1–2. https://doi.org/10.1038/SJ.HDY.6800365

Hoffmann, A. A., Sgrò, C. M., & Weeks, A. R. (2004). Chromosomal inversion polymorphisms and adaptation. *Trends in Ecology & Evolution*, *19*(9), 482–488. https://doi.org/10.1016/j.tree.2004.06.013

Kabanova, V. M., Kartashova, N. N., & Stegniy, V. N. (1972). Karyological study of natural populations of malarial mosquitoes in the Middle Ob river. I. Characteristics of the karyotype of *Anopheles maculipennis messeae*. *Tsitologiia*, *14*(5), 630–636.

Kabanova, V. M., Stegniy, V. N., & Luzhkova, A. G. (1973). Seasonal dynamics of inversion polymorphism in a natural population of the malarial mosquito *Anopheles messeae* (Diptera: Culicidae). *Genetika*, *9*(10), 78–82.

Kamali, M., Xia, A., Tu, Z., & Sharakhov, I. V. (2012). A new chromosomal phylogeny supports the repeated origin of vectorial capacity in malaria mosquitoes of the *Anopheles gambiae* complex. *PLoS Pathogens*, *8*(10), e1002960. https://doi.org/10.1371/journal.ppat.1002960

Kampen, H. (2005). Integration of *Anopheles beklemishevi* (Diptera: Culicidae) in a PCR assay diagnostic for palaearctic *Anopheles maculipennis* sibling species. *Parasitology Research*, *97*(2), 113–117. https://doi.org/10.1007/s00436-005-1392-9

Kampen, H., Schäfer, M., Zielke, D. E., & Walther, D. (2016). The *Anopheles maculipennis* complex (Diptera: Culicidae) in Germany: An update following recent monitoring activities. *Parasitology Research*, *115*(9), 3281–3294. https://doi.org/10.1007/s00436-016-5189-9

Kamvar, Z. N., Tabima, J. F., & Grünwald, N. J. (2014). Poppr: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ*, *2*, e281. https://doi.org/10.7717/peerj.281

Kavran, M., Zgomba, M., Weitzel, T., Petric, D., Manz, C., & Becker, N. (2018). Distribution of *Anopheles daciae* and other *Anopheles maculipennis* complex species in Serbia. *Parasitology Research*, *117*(10), 3277–3287. https://doi.org/10.1007/s00436-018-6028-y

Kirkpatrick, M. (2010). How and why chromosome inversions evolve. *PLoS Biology*, *8*(9), e1000501. https://doi.org/10.1371/journal.pbio.1000501

Kirkpatrick, M., & Barton, N. (2006). Chromosome inversions, local adaptation and speciation. *Genetics*, *173*(1), 419–434. https://doi.org/10.1534/genetics.105.047985

Kitzmiller, J. B., Frizzi, G., & Baker, R. (1967). Evolution and speciation within the Maculipennis complex of the genus *Anopheles*. In J. W. Wright (Ed.), *Genetics of insect vectors of disease* (pp. 151–210). Elsevier Publishing Company.

Krimbas, C. B., & Powell, J. R. (1992). *Drosophila inversion polymorphism*. CRC Press.

Kronefeld, M., Werner, D., & Kampen, H. (2014). PCR identification and distribution of *Anopheles daciae* (Diptera, Culicidae) in Germany. *Parasitology Research*, *113*(6), 2079–2086. https://doi.org/10.1007/s00436-014-3857-1

Kulkarni, M. A., Duguay, C., & Ost, K. (2022). Charting the evidence for climate change impacts on the global spread of malaria and dengue and adaptive responses: A scoping review of reviews. *Globalization and Health*, *18*(1), 1. https://doi.org/10.1186/s12992-021-00793-2

Lilja, T., Eklöf, D., Jaenson, T. G. T., Lindström, A., & Terenius, O. (2020). Single nucleotide polymorphism analysis of the ITS2 region of two sympatric malaria mosquito species in Sweden: *Anopheles daciae* and *Anopheles messeae*. *Medical and Veterinary Entomology*, *34*(3), 364–368. https://doi.org/10.1111/mve.12436

Mayr, E. (1963). *Animal species and evolution*. Harvard University Press.

Menhinick, E. F. (1964). A comparison of Some species-individuals diversity indices applied to samples of field insects. *Ecology*, *45*(4), 859–861. https://doi.org/10.2307/1934933

Michalakis, Y., & Excoffier, L. (1996). A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics*, *142*(3), 1061–1064.

Naumenko, A. N., Karagodin, D. A., Yurchenko, A. A., Moskaev, A. V., Martin, O. I., Baricheva, E. M., Sharakhov, I. V., Gordeev, M. I., & Sharakhova, M. V. (2020). Chromosome and genome divergence between the cryptic Eurasian malaria vector-species *Anopheles messeae* and *Anopheles daciae*. *Genes*, *11*(2), 165. https://doi.org/10.3390/genes11020165

Nicolescu, G., Linton, Y.-M., Vladimirescu, A., Howard, T. M., & Harbach, R. E. (2004). Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence. *Bulletin of Entomological Research*, *94*(6), 525–535. https://doi.org/10.1079/ber2004330

Novikov, Y. M., & Kabanova, V. M. (1979). Adaptive association of inversions in a natural population of the malaria mosquito *Anopheles messeae* Fall. *Genetika*, *15*(6), 1033–1045.

Novikov, Y. M., & Shevchenko, A. I. (2001). Inversion polymorphism and the divergence of two cryptic forms of *Anopheles messeae* (Diptera, Culicidae) at the level of genomic DNA repeats. *Russian Journal of Genetics*, *37*(7), 754–763. https://doi.org/10.1023/A:1016790724790

Nwakanma, D. C., Neafsey, D. E., Jawara, M., Adiamoh, M., Lund, E., Rodrigues, A., Loua, K. M., Konate, L., Sy, N., Dia, I., Awolola, T. S., Muskavitch, M. A. T., & Conway, D. J. (2013). Breakdown in the process of incipient speciation in *Anopheles gambiae*. *Genetics*, *193*(4), 1221–1231. https://doi.org/10.1534/genetics.112.148718

Oliveira, E., Salgueiro, P., Palsson, K., Vicente, J. L., Arez, A. P., Jaenson, T. G. T., Caccone, A., & Pinto, J. (2008). High levels of hybridization between molecular forms of *Anopheles gambiae* from Guinea Bissau. *Journal of Medical Entomology*, *45*(6), 1057–1063. https://doi.org/10.1603/0022-2585(2008)45[1057:hlohbm]2.0.co;2

Perevozkin, V. P., Printseva, A. A., Maslennikov, P. V., & Bondarchuk, S. S. (2012). Genetic aspects of sexual behavior in malaria mosquitoes on the basis of specific acoustic signals at mating. *Genetika*, *48*(6), 692–697.

Pleshkova, G. N., Stegniy, V. N., Novikov, Y. M., & Kabanova, V. M. (1978). Inversion polymorphism in the malarial mosquito, *Anopheles messeae*. III. The temporal dynamics of the inversion frequencies in the population of the center of a geographic range. *Genetika*, *14*, 2169–2176.

Proft, J., Maier, W. A., & Kampen, H. (1999). Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitology Research*, *85*(10), 837–843. https://doi.org/10.1007/s004360050642

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/

Rieseberg, L. H. (2001). Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution*, *16*(7), 351–358. https://doi.org/10.1016/s0169-5347(01)02187-5

Rossati, A., Bargiacchi, O., Kroumova, V., Zaramella, M., Caputo, A., & Garavelli, P. L. (2016). Climate, environment and transmission of malaria. *Le Infezioni in Medicina*, *24*(2), 93–104.

Rougeron, V., Boundenga, L., Arnathau, C., Durand, P., Renaud, F., & Prugnolle, F. (2022). A population genetic perspective on the origin, spread and adaptation of the human malaria agents *Plasmodium falciparum* and *Plasmodium vivax*. *FEMS Microbiology Reviews*, *46*(1), fuab047. https://doi.org/10.1093/FEMSRE/FUAB047

Rydzanicz, K., Czułowska, A., Manz, C., & Jawień, P. (2017). First record of *Anopheles daciae* (Linton, Nicolescu & Harbach, 2004) in Poland. *Journal of Vector Ecology*, *42*(1), 196–199. https://doi.org/10.1111/jvec.12257

Sainz-Elipe, S., Latorre, J. M., Escosa, R., Masià, M., Fuentes, M. V., Mas-Coma, S., & Bargues, M. D. (2010). Malaria resurgence risk in southern Europe: Climate assessment in an historically endemic area of rice fields at the Mediterranean shore of Spain. *Malaria Journal*, *9*, 221. https://doi.org/10.1186/1475-2875-9-221

Schaffner, F., Marquine, M., Pasteur, N., & Raymond, M. (2003). Genetic differentiation of *Anopheles claviger s.s.* in Europe. *Journal of Medical Entomology*, *40*(6), 865–875. https://doi.org/10.1603/0022-2585-40.6.865

Shannon, C. E. (1948). A mathematical theory of communication. *Bell System Technical Journal*, *27*(3), 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x

Sharakhov, I. V., White, B. J., Sharakhova, M. V., Kayondo, J., Lobo, N. F., Santolamazza, F., della Torre, A., Simard, F., Collins, F. H., & Besansky, N. J. (2006). Breakpoint structure reveals the unique origin of an interspecific chromosomal inversion (2La) in the *Anopheles gambiae* complex. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(16), 6258–6262. https://doi.org/10.1073/pnas.0509683103

Simard, F., Ayala, D., Kamdem, G. C., Pombi, M., Etouna, J., Ose, K., Fotsing, J. M., Fontenille, D., Besansky, N. J., & Costantini, C. (2009). Ecological niche partitioning between *Anopheles gambiae* molecular forms in Cameroon: The ecological side of speciation. *BMC Ecology*, *9*, 17. https://doi.org/10.1186/1472-6785-9-17

Simpson, E. H. (1949). Measurement of diversity. *Nature*, *163*(4148), 688. https://doi.org/10.1038/163688a0

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Kabaria, C. W., Okara, R. M., van Boeckel, T., Godfray, H. C. J., Harbach, R. E., & Hay, S. I. (2010). The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*, *3*, 117. https://doi.org/10.1186/1756-3305-3-117

Smitz, N., De Wolf, K., Gheysen, A., Deblauwe, I., Vanslembrouck, A., Meganck, K., De Witte, J., Schneider, A., Verlé, I., Dekoninck, W., Gombeer, S., Vanderheyden, A., De Meyer, M., Backeljau, T., Müller, R., & Van Bortel, W. (2021). DNA identification of species of the *Anopheles maculipennis* complex and first record of *An. daciae* in Belgium. *Medical and Veterinary Entomology*, *35*(3), 442–450. https://doi.org/10.1111/mve.12519

Stegniy, V. N. (1976). Revealing of chromosome races in the malaria mosquito *Anopheles sacharovi* (Diptera, Culicidae). *Tsitologia*, *18*, 1039–1041.

Stegniy, V. N. (1980). Reproductive interrelations of malarial mosquitoes of the complex *Anopheles maculipennis* (Diptera, Culicidae). *Zoologicheskii Zhurnal*, *59*, 1469–1475.

Stegniy, V. N. (1983a). Inversion polymorphism of the malarial mosquito *Anopheles messeae*. IV. The stability of the frequency distribution of the inversions by species area. *Genetika*, *19*, 466–473.

Stegniy, V. N. (1983b). Inversion polymorphism of the malarial mosquito *Anopheles messeae*. V. The interaction of different chromosomal inversions in the spatial area. *Genetika*, *19*, 474–482.

Stegniy, V. N. (1991). *Population genetics and evolution of malaria mosquitoes*. Tomsk State University Publisher.

Stegniy, V. N., & Kabanova, V. M. (1978). Cytoecological study of natural populations of malaria mosquitoes on the USSR territory. 1. Isolation of a new species of *Anopheles* in *Maculipennis* complex by the cytodiagnostic method. *Meditsinskaia Parazitologiia I Parazitarnye Bolezni*, *45*(2), 192–198.

Stegniy, V. N., Kabanova, V. M., & Novikov, Y. M. (1976). Study of the karyotype of the malaria mosquito. *Tsitologiia*, *18*(6), 760–766.

Stegniy, V. N., Kabanova, V. M., Novikov, Y. M., & Pleshkova, G. N. (1976). Inversion polymorphism in malaria mosquito *Anopheles messeae*. I. Distribution of the inversions in the species areal. *Genetika*, *12*, 47–55.

Stegniy, V. N., Novikov, Y. M., Pleshkova, G. N., & Kabanova, V. M. (1978). Inversion polymorphism in malaria mosquito *Anopheles messeae*. II. Interpopulation variablity of inversion frequncies. *Russian Journal of Genetics*, *14*, 1017–1023.

Stegniy, V. N., Pestryakova, T. S., & Kabanova, V. M. (1973). Cytological identification of sibling species of the malaria mosquitoes *Anopheles maculipennis* and *An. messeae*. *Zoologicheskii Zhurnal*, *52*, 1971–1976.

Stegniy, V. N., Pishchelko, A. O., Sibataev, A. K., & Abylkassymova, G. (2016). Spatial and temporal variations of the chromosomal inversion frequencies across the range of malaria mosquito *Anopheles messeae* Fall. (Culicidae) during the 40-year monitoring period. *Genetika*, *52*(6), 664–671.

Stegniy, V. N., Sichinava, S. G., & Sipovich, N. G. (1984). A hybridological analysis and biology of the malarial mosquitos of the complex *Anopheles-Maculipennis* (Diptera, Culicidae) in Western Georgia. *Zoologicheskii Zhurnal*, *63*, 300–303.

Torsvik, T. H., & Cocks, R. M. (2017). *Earth history and palaeogeogrphy*. Cambridge University Press.

Touré, Y. T. (1989). The current state of studies of malaria vectors and the antivectorial campaign in West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *83*(Suppl), 39–41. https://doi.org/10.1016/0035-9203(89)90602-0

Touré, Y. T., Petrarca, V., Traoré, S. F., Coulibaly, A., Maïga, H. M., Sankaré, O., Sow, M., di Deco, M. A., & Coluzzi, M. (1994). Ecological genetic studies in the chromosomal form Mopti of *Anopheles gambiae* s.str. in Mali, West Africa. *Genetica*, *94*(2–3), 213–223. https://doi.org/10.1007/BF01443435

Tzedakis, P. C., Emerson, B. C., & Hewitt, G. M. (2013). Cryptic or mystic? Glacial tree refugia in northern Europe. *Trends in Ecology & Evolution*, *28*(12), 696–704. https://doi.org/10.1016/J.TREE.2013.09.001

Vaulin, O. V., & Novikov, Y. M. (2012). Polymorphism and interspecific variability of cytochrome oxydase subunit I (COI) gene nucleotide sequence in sibling species of A and B *Anopheles messeae* and *An. beklemishevi* (Diptera: Culicidae). *Vavilov Journal of Genetics and Breeding*, *16*(2), 358–368.

Venkatesan, M., Westbrook, C. J., Hauer, M. C., & Rasgon, J. L. (2007). Evidence for a population expansion in the West Nile virus vector *Culex tarsalis*. *Molecular Biology and Evolution*, *24*(5), 1208–1218. https://doi.org/10.1093/MOLBEV/MSM040

Weir, B. S., & Hill, W. G. (2002). Estimating F-statistics. *Annual Review of Genetics*, *36*, 721–750. https://doi.org/10.1146/annurev.genet.36.050802.093940

Weitzel, T., Gauch, C., & Becker, N. (2012). Identification of *Anopheles daciae* in Germany through ITS2 sequencing. *Parasitology Research*, *111*(6), 2431–2438. https://doi.org/10.1007/s00436-012-3102-8

Wigginton, J. E., Cutler, D. J., & Abecasis, G. R. (2005). A note on exact tests of Hardy-Weinberg equilibrium. *American Journal of Human Genetics*, *76*(5), 887–893. https://doi.org/10.1086/429864

Winter, D. J. (2012). MMOD: An R library for the calculation of population differentiation statistics. *Molecular Ecology Resources*, *12*(6), 1158–1160. https://doi.org/10.1111/j.1755-0998.2012.03174.x

Yurchenko, A. A., Masri, R. A., Khrabrova, N. V., Sibataev, A. K., Fritz, M. L., & Sharakhova, M. V. (2020). Genomic differentiation and intercontinental population structure of mosquito vectors *Culex pipiens pipiens* and *Culex pipiens molestus*. *Scientific Reports*, *10*(1), 7504. https://doi.org/10.1038/s41598-020-63305-z

Yurchenko, A. A., Naumenko, A. N., Artemov, G. N., Karagodin, D. A., Hodge, J. M., Velichevskaya, A. I., Kokhanenko, A. A., Bondarenko, S. M., Abai, M. R., Kamali, M., Gordeev, M. I., Moskaev, A. V., Caputo, B., Aghayan, S. A., Baricheva, E. M., Stegniy, V. N., Sharakhova, M. V., & Sharakhov, I. V. (2022). Phylogenomics revealed migration routes and adaptive radiation timing of Holarctic malaria vectors of the Maculipennis group. *bioRxiv*. https://doi.org/10.1101/2022.08.10.503503

Zvantsov, A. B., Gordeev, M. I., Goryacheva, I. I., & Ezhov, M. N. (2014). The distribution of the mosquitoes of the *Anopheles maculipennis* complex (Diptera, Culicidae, Anophelinae) in Central Asia. *Meditsinskaia Parazitologiia I Parazitarnye Bolezni*, *4*, 19–23.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Brusentsov, I. I., Gordeev, M. I., Yurchenko, A. A., Karagodin, D. A., Moskaev, A. V., Hodge, J. M., Burlak, V. A., Artemov, G. N., Sibataev, A. K., Becker, N., Sharakhov, I. V., Baricheva, E. M., & Sharakhova, M. V. (2023). Patterns of genetic differentiation imply distinct phylogeographic history of the mosquito species *Anopheles messeae* and *Anopheles daciae* in Eurasia. *Molecular Ecology*, *32*, 5609–5625. https://doi.org/10.1111/mec.17127