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КЛЕТОЧНАЯ БИОЛОГИЯ И ГЕНЕТИКА

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Cryptic species of *Anopheles messeae* sensu lato (Diptera: Culicidae), their identification, features and nomenclature

The paper describes the change in perspective in the composition of the A. messeae taxonomic unit. Initially, based on the disequilibrium of natural populations, the species was differentiated into A and B forms using chromosomal inversions as markers. The positive assortative mating, as well as the ecological features and geographical distribution of these forms, made it possible to give them the status of species in statu nascendi. Later, we additionally investigated the EcoRI restriction fragments of the genomic DNA and the ITS2 nucleotide sequences in the A and B A. messeae species. Unambiguous differences between the species in the former marker and semi-quantitative differences in the latter one, alongside with the absence of hybrids in the populations studied, led us to conclude that A. messeae s.l. is comprised of two homosequential cryptic species with parallel chromosomal polymorphisms. Unequivocal parallels between A. lewisi Ludlow, 1920 and A. messeae B in regards to their features, as well as the identity of A. daciae Linton et al., 2004 to A. messeae A in its ITS2 sequence, and to A. messeae Fall. in diagnostic chromosomal inversions, allowed us to consider A. lewisi Ludlow, 1920 and A. messeae B as two names of the same biological species, and A. messeae Fall., 1926, A. messeae A, and A. daciae Linton et al., 2004 as three names of the other one. Both are members of the palaearctic group of the Maculipennis complex under the names Anopheles (Ano.) lewisi Ludlow, 1920 and Anopheles (Ano.) messeae Falleroni, 1926, respectively.

The paper contains 5 Figures, 4 Tables and 42 References.

Key words: *Anopheles;* cryptic species; ITS2; maculipennis; polymorphism; taxonprint.

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Introduction

Anopheles messeae Fall. belongs to the Palaearctic group of sibling species of the Maculipennis complex. Its range extends from the British Isles in the west to the Zeya-Bureya Plains in the east, and from the Arctic Circle to the Transcaucasia

and northern China [1, 2]. Its widespread occurrence creates conditions for a significant epidemiological threat. The study was motivated by the identification of two genetically and ecologically different cryptic species in *A. messeae* Fall. [3, 4], the isolation of a new species *A. daciae* [5] and the uncertainty of taxonomic relationships between *A. messeae* Fall. and the previously described *A. lewisi* [6]. Another incentive for this publication is the fact that a number of authors [7-13] adhere to an alternative point of view and study *A. messeae* as a single species even after the evidence on the complex nature of *A. messeae* s.l. taxon was obtained. We are sure that the complex composition of the *A. messeae* Fall. taxonomic unit and significant physiological differences between its cryptic species, which likely confer different vector abilities, call for its comprehensive analysis. The development of effective control measures depends on the accurate identification of vector species i.e. on whether the taxonomy appropriately reflects the biological reality. Precise species identification should help determine their potential roles in the transmission of pathogens, elucidate larval ecology and the behavior of adult insects, as well as assess their resistance to insecticides.

Materials and methods

This study is based on published research works [4, 14-26] and analysis of samples from Tomsk (08/18/1999) and Teguldet (07/08/2000), which is presented here for the first time. Preparation of chromosomes for analysis, cytogenetic investigation and calculations were carried out as described in these publications.

Results and discussion

The history of identification. *Anopheles messeae* was first described by Domenico Falleroni in 1926 [27], based on the egg coloring, in samples from central Italy. This feature varies slightly between and inside populations but differs drastically from that of other Palaearctic species of the Maculipennis complex. The Pontine Marshes located 40 km south-east of Rome are considered to be the typical locality for the species [28].

Cytogenetic analysis of polytene chromosomes is a technique, which revolutionized the field of mosquito sibling species analysis. Guido Frizzi in 1947 [29] was the first to investigate the chromosomes of the salivary glands of *Anopheles maculipennis* s.l. larvae and construct polytene chromosome maps for *A. maculipennis* var. *atroparvus* van Thiel that have become a standard in the field. Kabanova and co-workers [30] initiated the studies of the West Siberian populations of *A. messeae* in the USSR. Following the principles laid by Frizzi [29] and Kitzmiller et al. [31], chromosomes are numbered 1 to 3, in the ascending order of length; long arms are marked with the letter R and short ones are denoted with the letter L. The polytenic complex of *A. messeae* is comprised of five elements, namely 1L, 2R, 2L, 3R and 3L (the right arm of the chromosome 1 is

not polytenized), and is divided into 39 sections, with each section being further divided into two to four segments. Kabanova et al. [32] identified five paracentric inversions in chromosomes within natural mosquito populations. The inverted disk sequences are labeled as 1L1, 1L2, 2R1, 3R1, and 3L1 [2, 32]. Stegnii et al. [14, 15] created more practical photomaps of polytene chromosomes (Fig. 1) and investigated the geographical distribution of inversions [33].

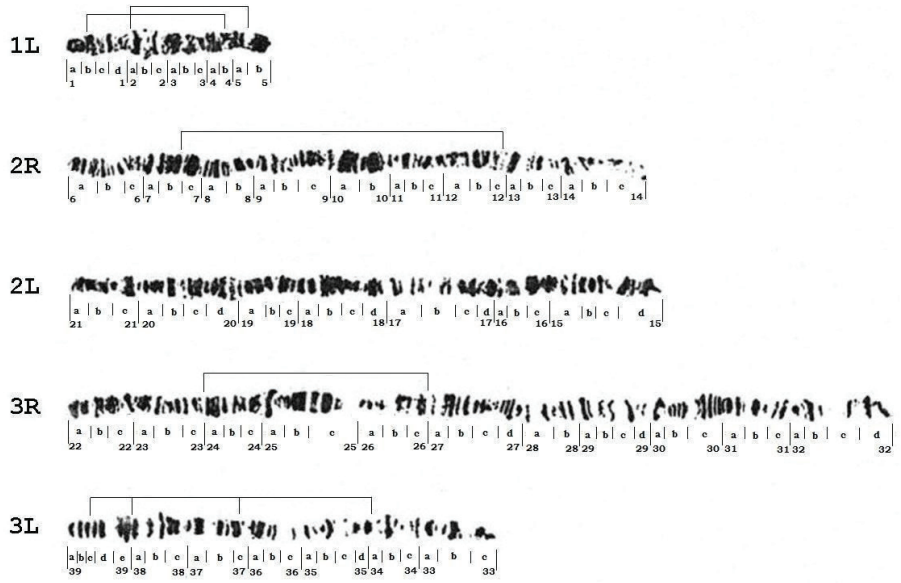

Fig. 1. Photomaps of polytene chromosomes of the salivary glands of *Anopheles messeae* s.l. larvae. Parentheses indicate chromosome regions involved in the inversions. From Stegnii et al. [14], with modifications

Polymorphisms in the sequences of all three chromosomes appeared to be widespread in the species natural populations. Alongside with the standard chromosomal variants, heterozygous and homozygous combinations of sequences that differ in the order of disks were identified. Six chromosome 1 variants (sex chromosome, heterozygotes: 1L/1L1, 1L/1L2, 1L1/1L2, and homozygotes: 1L/1L, 1L1/1L1, 1L2/1L2) were described in females, while males have only three hemizygous variants (1L/¬, 1L1/¬, 1L2/¬, where the symbol '¬' denotes chromosome Y). Variants of polymorphic arms of chromosomes 2 and 3 (autosomes, three of each) were also described: 2R/2R, 2R/2R1, 2R1/2R1; 3R/3R, 3R/3R1, 3R1/3R1; 3L/3L, 3L/3L1, 3L1/3L1 (Fig. 2).

Combinations of arm variants of different chromosomes in the processes of recombination and fertilization allow generating a large variety of karyotypes: 162 karyotypes for females (6×3×3×3) and 81 for males (3×3×3×3) [18]. To shorten karyotype descriptions, we have adopted numeric symbols, first used by Novikov

and Shevchenko [4]: sequence variants of arm 1L are denoted by *1, 2, 3*; standard and inverted sequence variants of arms 2R, 3R and 3L are denoted by *1* and *2*, respectively. Thus, we describe a female with the standard karyotype 1L/1L-2R/2R-3R/3R-3L/3L as *11'11'11'11*, a female heterozygous for four inversions *12'12'12'12*, and a male with the karyotype lL1/¬-2R/2R-3R1/3R1-3L/3L1 as *2¬'11'22'12*. We adopted the following numeric symbols for individual inversions and combinations that do not include all the elements of the complex: *.12..* for the 2R/2R1 heterozygote; *..22.* for the 3R1/3R1 homozygote; *.22'11.* for the 2R1/2R1-3R/3R combination. In the digital description of the karyotype, the dot indicates the degree of freedom. Therefore, dot can be preceded or followed by any variant of a polymorphic arm.

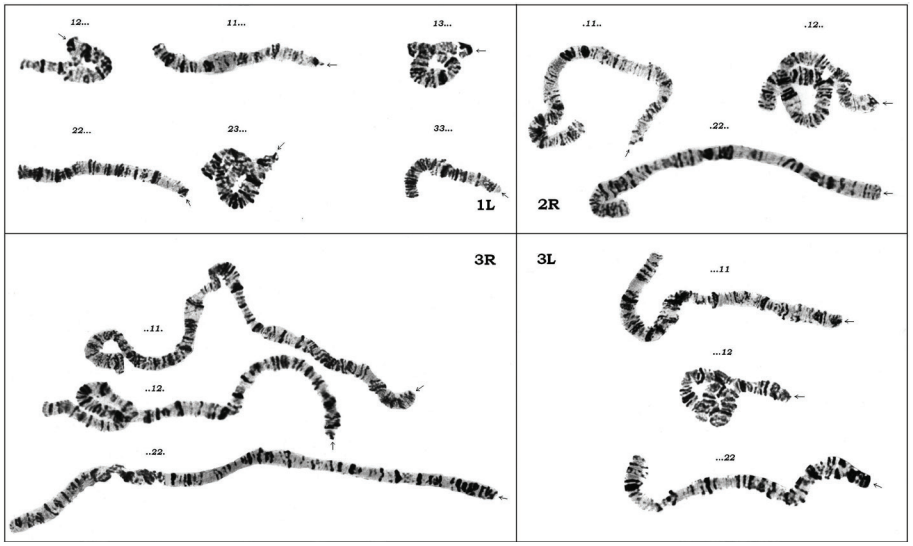

Fig. 2. Variants of polymorphic chromosome arms 1L, 2R, 3R and 3L. Arrows indicate the centromere areas. Numerical designation of variants is described in the text.

Analysis of mosquito populations using inversions as markers showed that neither the Hardy-Weinberg equilibrium nor equilibrium at the level of double-inversion combinations is typical of the central and western parts of the taxon range (Table 1).

In these areas, the karyotype pool appeared to be split into two associative groups of 'co-adapted' chromosomal variants, as it was proposed at the time (Fig. 3) [17, 18, 21]. The composition of the groups was as follows: the first is 1L/1L1, 1L1/1L1, 2R/2R, 3R1/3R1 and 3L/3L; the second is 1L/1L2, 1L2/1L2, 2R/2R1, 2R1/2R1, 3R/3R, 3L/3L1 and 3L1/3L1.

Variants of each group of karyotypes occurred together significantly more often than it is expected with random combinations. The degree of linkage disequilibrium (d) between different variants fluctuated significantly. As we subsequently

discovered, this fact and the fact that the 1L/1L and 3R/3R1 variants combined almost independently were statistically determined, namely by the degree of difference of cryptic species in the frequencies of the same inversions. Populations inhabiting the east of Krasnoyarsk, Altai region, and Kazakhstan with significant frequencies contained variants 1L/1L, 2R/2R and 3R/3R belonging to different associative groups, and most often were in equilibrium. At the next stage, we detected positive assortative mating of individuals in a natural population from the middle of the *A. messeae* range (Table 2) [18].

Table 1

Observed (n_o) and expected (n_e) numbers of variant combinations of the 2R and 3R chromosomal arms in the individual karyotypes in natural populations of Zelenodolsk (G, 55º51´ N, 48º31´ E; 07/08/1973), Kolarovo (K, 56º20´N; 84º56´E; 08/11/1975), Tomsk (Beloe Ozero; W, 56º50´N; 84º96´E; 08/18/1999) and Teguldet (T, 57º18´N; 88º10´E; 07/07/2000); G and K; by: Novikov [18])

Populations		Combinations of variants									Σ
		.11'11.	*.11'12.*	*.11'22.*	*.12'11.*	*.12'12.*	*.12'22.*	*.22'11.*	*.22'12.*	*.22'22.*	
G	n_o	13	25	13	15	29	7	5	9	2	118
	n_e	14.8	24.6	10.2	16.1	26.7	11.1	4.4	7.2	3.0	118.1
K	n_e	24	35	20	49	25	4	19	22	2	200
	n_o	30.8	31.0	7.8	42.8	43.1	10.9	14.9	15.0	3.8	200.1
T	n_e	9	24	55	1	2	5	92	144	66	398
	n_o	4.7	10.6	6.0	31.2	70.5	39.8	51.9	117.2	66.1	398.0
W	n_e	52	362	784	28	27	12	23	21	1	1310
	n_o	64.0	416.4	677.2	8.2	53.1	86.3	0.3	1.7	2.8	1310.0

Note: $d_G = 0.0133$; $d_K = 0.0311$; $d_T = 0.0533$; $d_W = 0.0273$; $p < 0.001$ for populations K, T, W; $p > 0.05$ for G; where d is a measure of linkage disequilibrium.

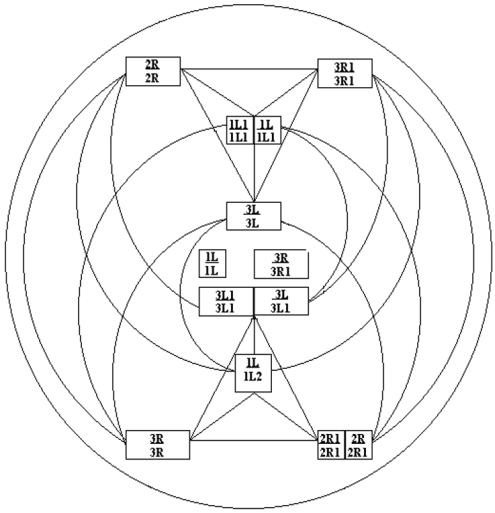

Fig. 3. Interactions of chromosome variants in the *A. messeae* populations from the central part of the range. Positive and negative interactions are indicated by straight lines and arcs, respectively. Variants of the first and the second associative group are presented at the top and at the bottom, correspondingly (from Novikov and Kabanova [21], with modifications)

The deviations of the observed frequencies of chromosome variant combinations from the expected frequencies assuming random pairing (Table 1) are in full agreement with the deviations from the random pair formation in the population (Table 2). In the Kolarovo population, 20 out of 200 individuals had the *.11'22.* combination of variants, whereas if combined independently, the number of carriers of such combination would be 7.8. In the same population, out of 122 pairs, 26 ♀*.11 ..* × ♂ *..22.* pairings were registered, while only 12.2 were expected in the panmictic population. Further, among the same number of pairs, 25 ♀ *..22.* × ♂ *.11..* pairings were observed, while only 15.0 were expected.

Table 2

Observed (n_o) and expected (n_e) numbers of combinations of the 2R and 3R variants in the Kolarovo population of *Anopheles messeae* during mating (by: Novikov [18])

♀♀		♂♂					
		.11..	*.12..*	*.22..*	*..11.*	..12.	..22.
.11..	n_o	44	7	4	10	19	26
	n_e	28.4	17.7	9.9	21.2	21.6	12.2
.12..	n_o	14	17	11	23	18	1
	n_e	21.7	12.7	7.6	16.2	16.5	9.3
.22..	n_o	5	13	7	14	11	2
	n_e	12.9	7.6	4.5	9.6	9.8	5.5
..11.	n_o	13	16	12	23	16	2
	n_e	21.2	12.4	7.4	15.8	16.1	9.1
..12.	n_o	25	19	8	19	23	10
	n_e	26.8	15.8	9.4	20.0	20.5	11.5
..22.	n_o	25	2	2	5	9	15
	n_e	15.0	8.8	5.2	11.2	11.4	6.4

Note: 122 pairs were analysed, df in all 4 cases is 8 ($p < 0.001$).

The above data indicate the lack of homogeneity within the *A. messeae* Fall. taxonomic unit, a phenomenon clearly associated with the sexual behavior.

Features of the 'forms' identified within the A. messeae Fall. Inversion chromosomal variants, which constitute the first and second associative groups, were found to be confined to different regions of the taxon range. Those in the first group demonstrated higher frequencies in the populations of the western part of the range, whereas those in the second associative group were encountered with high frequencies in Siberia, up to the Transbaikalia region, as well as in the Altai and in Kazakhstan. The widely spread 1L/1L, 2R/2R and 3R/3R1 variants did not comply with the predicted patterns. Despite some regularity in the distribution of chromosomal variants over the species range, there was no reason to isolate the 'northern' and 'southern' karyotypes proposed in many studies. For instance, the 'northern' heterozygotes 2R/2R1 and homozygotes 2R1/2R1 had increased frequencies not in the northern regions, but rather in humid floodplains of major rivers in the central part of the species range [3].

Analysis of the seasonal dynamics of associative groups of inversion chromosomal variants showed that in the Tomsk suburbs (Kolarovo village), the

frequency of carriers of the first group increased early in the breeding season, dropped after 10 days and increased again by mid-August. Carriers of the second group of variants were found to have several peculiar features: during August, they disappeared from the territory of the settlement, being absent among wintering females in the cattle barns, houses and other buildings, and also in the first fraction of overwintered females emerging in the cattle barns in late April. However, they made their appearance among overwintered females in early May. Such dynamics could only be explained by an assumption that mosquitoes within the second group of chromosomal variants overwinter in wild habitats. We managed to collect a small number of mosquitoes in abandoned bath-houses ('banyas') and cellars located outside houses in the settlement. All of them had variants of chromosomes of the second group in their karyotypes or belonged to *A. beklemishevi* [20]. It became obvious that *A. messeae* Fall. taxon is deeply differentiated, which is manifested in the genetic, environmental, physiological and behavioral features. Based on these facts, we came to a conclusion of the existence of two 'forms', or species A and B, *in statu nascendi* within *A. messeae* Fall. [3]. The relatively rapid increase in the proportion of the first species from 12% in 1974 to 98% in 1994 and the respective decline of the second one from 88% to 2% in Tomsk region due to global warming was one of the striking pieces evidence in favor of their ecological differentiation [34].

Coluzzi et al. [35], while assessing the significance of disequilibrium in the populations of *Anopheles gambiae* and being able to estimate the degree of assortative mating only using indirect data, confidently interpreted the situation as the initial stage of the speciation process. However, our findings on divergence and specification in *A. messeae* s.l., confirmed by molecular genetic studies, were hushed up for many years [7-13].

Molecular genetic analysis of the identified species was initiated in order to bring additional arguments in favor of the existence of the two species. With the aid of the diagnostic combinations of chromosomal variants, we selected individuals of both cryptic species from several geographically distant populations; in parallel, using the same means of selection, pure 'strains' of species (isofemale broods) were assembled. Samples from these populations and isofemale broods were analyzed by taxonomic typing of restriction fragments of genomic DNA [4, 23].

Three patterns were observed in *Eco*RI taxonprints of the *A. messeae* individuals examined (Fig. 4):

1) major fraction (MJ) 170 bp (lanes 1, 2, 5, 6, 7, 11, 12, 14, 15);

2) MJ 110 bp and MJ 65 bp, minor (MN) 170 bp (lanes 3, 4, 8, 9);

3) MJ 170 bp, MN 110 bp and MN 65 bp (lanes 10, 13).

The repeats detected in taxonprints are likely to represent an integral part of a bigger, more complex monomeric unit [23]. The first and second patterns are typical of the *A. messeae* B and *A. messeae* A, respectively. The absence of intermediate patterns in the co-habitat zones for these cryptic species can be considered as the proof of their reproductive isolation and of their genuine species status. In the

Altai and Kazakhstan populations, 85% of individuals had pattern 1, characteristic of *A. messeae* B, and 15% showed pattern 3, containing the major fraction typical of *A. messeae* B and two minor fractions were resembling repeats 110 bp and 65 bp of *A. messeae* A. *The monomorphism in the 170 bp fraction and the population equilibrium revealed using chromosomal inversions as markers justified the classification of both groups of individuals as belonging to A. messeae B.*

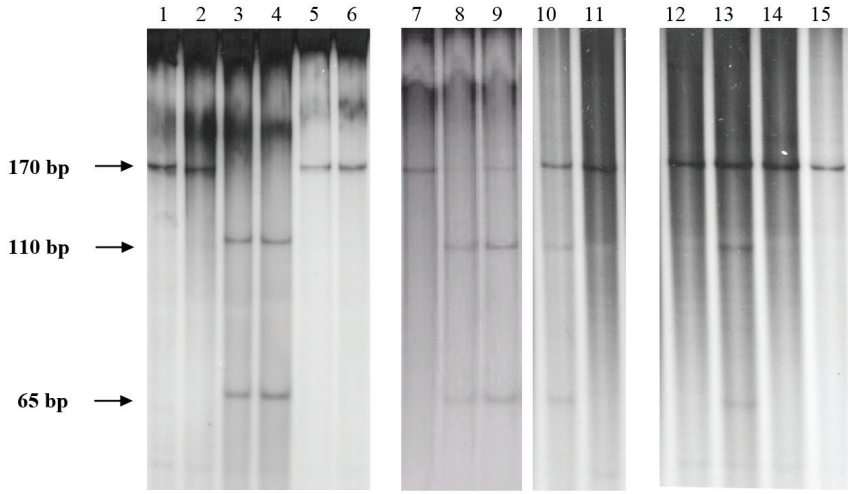

Fig. 4. Taxonprint of *Eco*RI DNA digests of *Anopheles messeae* individuals from natural populations: 1, 'Cherga'; 10, 11, 'Voevodskoe' (Altai); 2, 12-15, 'Pavlodar' (Kazakhstan); 3,'Kireevsk'; 4, 5, 'Chernilshchikovo'; 7-9, 'Teguldet' (Tomsk region); 6, 'Yakutsk'. Arrows indicate the positions of DNA size markers. From Shevchenko and Novikov [24]

Within both species, individuals with different combinations of inversion chromosome variants had identical electropherograms. On the other hand, a fraction of individuals featuring identical karyotypes belonged to different species [4, 23]. For example, females with a karyotype*11'11'11'11* (1L/1L-2R/2R-3R/3R-3L/3L) or males with a karyotype *1¬'11'22'11* (1L/¬-2R/2R-3R1/3R1-3L/3L) can equally belong to any of the two species. This finding indicates that isolation of 'northern' and/or 'southern' karyotypes is unsubstantiated. Consequently, the discrete differences of species A and B of the *A. messeae* Fall. taxon in repeated DNA sequences confirm their divergence at the genomic level but are not directly related to their inversion polymorphisms. Thus, even back in 2001, the taxonprint DNA analysis for the first time allowed to accurately identify the species for any *A. messeae* s.l. individual, regardless of its karyotype. This method strongly suggests that *A. messeae* A and *A. messeae* B represent cryptic homosequentical species characterized by parallel inversion polymorphisms but significantly different in the frequencies of inversion variants of chromosomes (Table 3).

Table 3

Frequencies of inversion chromosome variants in cryptic species A and B of the *Anopheles messeae* s.l. taxon

Variants	1L	1L1*	1L1†	1L2*	2R	2R1	3R	3R1	3L	3L1
A	0.6-0.9	0.4-0.1	-	-	0.9-1.0	0.1-0,0	0.1-0.5	0.9-0.5	0.9-1.0	0.1-0.0
B	0.8-1.0	-	0.2-0,0	0.2-0,0	0.2-1.0	0.8-0.0	0.4-1.0	0.6-0.0	0.4-0.9	0.6-0.1

Note: Approximate frequencies for all geographically distant populations studied are given; 1L1* and 1L2* are diagnostic sequences for A and B species, respectively; 1L1† is the sequence of *A. messeae* B often found in the Altai and Kazakhstan populations, which is syntypic to 1L1 *A. messeae* A.

In the European part of the Russian Federation, in the Urals and in Western Siberia, hetero- and homozygotes 1L/1L1 and 1L1/1L1 are considered diagnostic for the species A, especially in combination with the homozygote 3R1/3R1. In the Altai and Kazakhstan populations of *A. messeae* B, 1L1 sequence is very similar or identical in its structure to 1L1 of *A. messeae* A; however, it randomly combined with all other sequences, including 1L2. In these populations, the frequency of 3R1/3R1 homozygotes is typically relatively low. The syntypical sequences (1L1* and 1L1†) of different species are likely to belong to isolated genotypic environments. Notably, the more pronounced polymorphy of *A. messeae* B, as compared to its sibling [4, 23], is in line with its larger range and wider ecological potential with respect to abiotic factors. It has been suggested that the Altai and Kazakhstan populations of the species on one side and Siberian populations on the other side are somewhat isolated [23].

In order to expand the panel of genomic markers that allow identifying individuals of the A and B species, restriction analysis of nucleotide sequences of the PCR products of their ITS2 was carried out [22]. Digestion of the PCR products with 14 restriction enzymes revealed differences between the two species at 15 recognition sites of the ITS2 sequence. The A and B species were found to have 33 and 28 such sites, respectively. Among these, 23 sites were common for both species; while ten sites in A and five ones in B were specific. These finding has improved our understanding of the degree of genomic divergence between the cryptic species of *A. messeae* A and *A.* messeae B.

Sequencing of the ITS2 of the two species revealed differences in five positions (Figure 5a). DNA samples studied originated from the same populations as the samples selected for taxonomic typing analysis of restriction fragments of genomic DNA (Tomsk region, Altai, Yakutia, Kazakhstan). Each of the twenty DNA samples used for sequencing of ITS2 PCR products was previously analyzed using *Eco*RI taxonprint to establish its species identity. ITS2 samples from Kazakhstan, Yakutsk and some of Tomsk populations corresponded to sequences of individuals identified as *A. messeae* B; the remaining Tomsk samples belonged to *A. messeae* A. The ITS2 PCR product was absent in the samples from the Altai region populations, apparently due to a mutation in one of the primer sites, which was affixed in all copies of ITS2 as a result of a concerted evolution process. In all five diagnostic positions of the ITS2 PCR products of *A. messeae* B individuals,

the nucleotide electropherograms of sequencing reactions were defined unequivocally (a single peak detected in each position of all electropherograms) (Figure 5a, samples Ya-B, P-B, C-B).

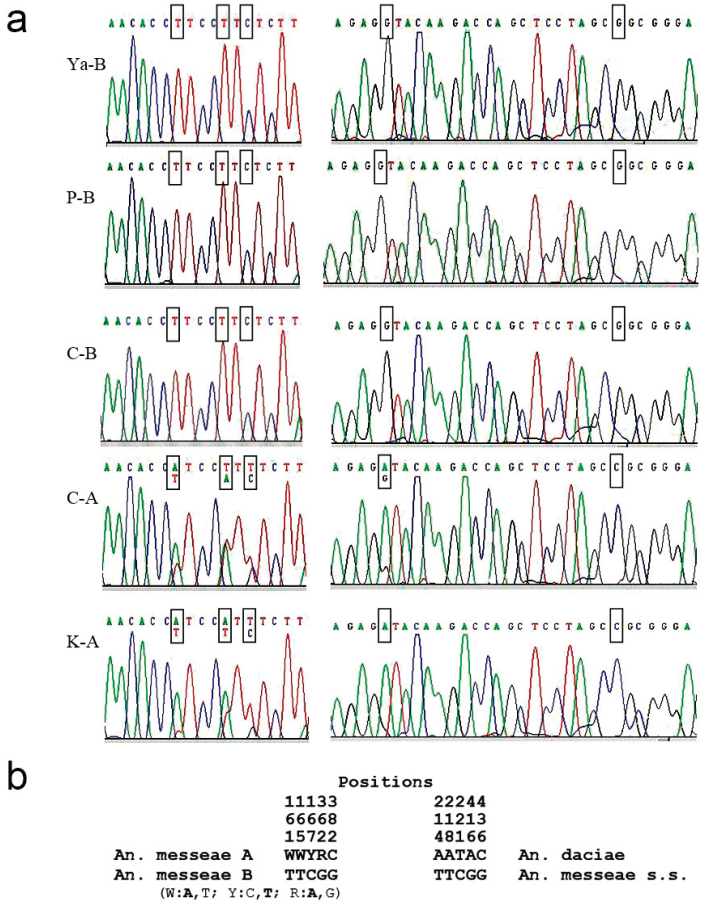

Fig. 5. Differences of ITS2 sequences within *Anopheles messeae* s.l. taxon: *a* – Fragments of electropherograms of the sequencing reactions for A and B *A. messeae* ITS2 sequence. Rectangles highlight the fragments used to establish differences between the two species. Results for *A. messeae* B: Ya-B (Yakutsk, MN481112), P-B (Pavlodar, MN481113), C-B (Chernilshchikovo, MN481111), and *A. messeae* A: K-A (Kireevsk, MN481110), C-A (Chernilshchikovo, MN481109) are shown in lanes 6, 2, 5 and 4, 3 of Figure 4, respectively; *b* – Summary of ITS2 sequence substitution described in A and B *A. messeae*, *A. messeae* s.s., and *A. daciae*

Consequently, almost all ITS2 copies in the *A. messeae* B genome are uniform. In contrast, in *A. messeae* A, in all ITS2 diagnostic positions, except the last one (i.e. 4 of 5), two peaks, major and minor, were revealed in each case (Fig. 5, *a*, samples C-A, K-A). The major peaks correspond to the nucleotides unique to

A. messeae A in these positions. This means that the major fraction of copies features the ITS2 pattern typical of this species. The minor peaks correspond to the nucleotides that are located in these ITS2 positions in *A. messeae* B. This means that the minor fraction of the ITS2 copies of *A. messeae* A corresponds to the ITS2 pattern of *A. messeae* B [23]. In other words, in four out of five diagnostic positions, the differences between the species A and B are *semi-quantitative* [24]. This circumstance led some authors to doubt the validity of isolating two distinct sibling species within the *A. messeae* s.l. taxon [36]. Another group of researchers [5] found the differences in the ITS2 sufficient to isolate the new species *A. daciae* Linton et al., 2004 despite the semi-quantitative nature of these differences (Fig. 5b). As an additional argument, the authors referred to the differences between the species in the color of the egg exochorion and the sequence of the mitochondrial marker COI. We made several attempts to find differences between the species in the color of exochorion on the single species strains, which were proven futile due to the interfamily and intrapopulation variability of this marker. The COI marker also proved impractical for the species identification [25, 37]. We believe that it is important to take the following into consideration: *is it possible to describe any traits in individuals of any species; however, will it be possible to identify individuals of this species in subsequent studies by analyzing these traits?* Thus, given the variability of the ITS2 sequence, it can be concluded that differences in its positions indicated above can only be considered as an additional evidence of the independence of the A and B *A. messeae* species. In addition to the genetic differences between the cryptic species *A. messeae* A and *A. messeae* B, as a result of observations and experiments carried out over many years, we have identified a deep differentiation between them in physiology, ecology and behavior (Table 4).

Table 4

Biological and ecological features of the cryptic species *Anopheles messeae* A and B

Species	*Anopheles messeae* A	*Anopheles messeae* B
Species range	From the British Isles and Atlantic coast of France to Krasnoyarsk; from Karelia to the Caucasus	From the British Isles and Atlantic coast of France to the Zeya-Bureya Plain; from the Arctic to Iran and northern China
Landscapes	From southern taiga to forest-steppe	From tundra to steppe
Connection with settlements	Very tight. Inhabits settlements and farms	Inhabits both settlements and wild habitats
Propensity for exophilia	Virtually absent	Clearly manifested.
Wintering places	Cattle barns, residential buildings and other above-ground buildings in settlements	Natural shelters on/in the ground, in villages – analogues of natural shelters and abandoned buildings.
Response to global warming	Extends the range to the north and east	See next position

Table 4 (end)

Species	*Anopheles messeae* A	*Anopheles messeae* B
Dynamics in the warming process	In regions of sympatry with B and *A. beklemishevi*, its frequency is rising	In regions of sympatry with A the frequencies of B and *A. beklemishevi* are declining
Potential epidemiological hazard	Hard to establish because of the widespread sympathy with B and/or *A. atroparvus*	High. Outbreaks of malaria are significant in the regions inhabited by species B only, or where its share is high as compared to species A
Aggressiveness	Moderate	High
Blood sucking	Female usually receives the full portion of blood from several shallow punctures	Female ordinarily makes one deep puncture and sucks the full portion of blood
Painfulness of bites	High	Moderate
Fecundity in culture (eggs per egg laying)	166.8	186.8
$n_{♂} / n_{♀}$ in overpopulated larvae cultures	0.6	0.4
Vitality in single species cultures	Larvae – A > B; adults (females) – B > A	
Duration of development	A > B	
Size of females grown up under the same conditions	A > B	
Larvae competitiveness in mixed cultures	*A. messeae* A > *A. beklemishevi* > *A. messeae* B	

Species nomenclature. Let us consider the situation with the nomenclature of the cryptic species of the *A. messeae* s.l. taxon. Given the major peaks in the ITS2 diagnostic positions of *A. messeae* A and the differences in alignments performed by Novikov et al. [22] and Nicolescu et al. [5], it is easy to verify that the sequences ITS2 *A. messeae* A (**AATAC**) and *A. daciae* are identical. The second marker haplotype (**TTCGG**) was attributed by Nicolescu et al. [5] to the second cryptic species, named *A. messeae* s.s. Let us analyze this confusing situation.

Comparison of the ITS2 sequences which we carried out for *A. messeae* A and *A. messeae* B with samples of *A. messeae* from the UK (AF452699, Italy (Z50105), AF452700), Greece (AF342711, AF342712), Iran (AY050639), China (AF305556), published in the DDBJ (http://www.ddbj.nig.ac.jp/), showed that the *ITS2 sample, defined as aligning to species A, is most similar to the sample from Italy* [22]. All other ITS2 samples were similar to the ITS2 of *A. messeae* B. Di Luca et al. [37] found that the two ITS2 haplotypes absent in the Balkan Peninsula and in Kazakhstan are frequent in Italian populations of *A. messeae*. Furthermore, structural chromosomal identity of *A. messeae* A (and therefore *A. daciae*) and *A. messeae* Fall., 1926 argues for their conspecificity. Cryptic species *A. messeae* A is characterized by chromosomal sequences 1L, 1L1, 2R, 3R1 and 3L. Moreover, everywhere except Kazakhstan and the Altai, where in the *A. messeae* B

populations an inversion formally similar to 1L1 of *A. messeae* A is present, the combination of 1L1 and 3R1, the frequencies of which are highest in the west of its range, is the diagnostic combination. The 3R1 sequence of *A. messeae* Fall. is inverted with respect to the disk order in the 3R chromosomal arm of *A. atroparvus*. Frizzi [29], having studied a sample of larvae from the Pavia province (north of Italy), described the polytene chromosomes of *A. messeae*, as compared to *A. atroparvus* van Thiel in the following way: *'... messeae has a small inversion in the X-chromosome and a large inversion in the right arm of chromosome III'*. Frizzi [38, 39] also described polymorphisms of these arms. Similarly, Stegnii [7] also described, the variants of chromosome arms 1L and 3R which are typical of western populations of *A. messeae* Fall., as compared to *A. atroparvus*. Thus, on the one hand, *A. messeae* A is identical to *A. messeae* Fall., 1926 based on the structure of chromosomes, while on the other hand, *A. messeae* A is identical to *A. daciae* Linton et al., 2004 based on the ITS2 nucleotide sequence. Therefore, *A. messeae* Fall., 1926 and *A. daciae* Linton et al., 2004 are identical in both chromosome structure and nucleotide sequence in the ITS2. In other words, we are dealing with three different names of a single biological species. In agreement with the results of Sharakhova et al. [40], chromosomal variants of *A. daciae* are identical to those of *A. messeae* A. Therefore, the authors of the above study, similar to Nicolescu et al., 2004, name the second cryptic species *A. messeae* s.s., recognizing its identity to the *A. messeae* B. In this case, there is no place for *A. messeae* s.s. in the *A. messeae* s.l. taxon, because the name *A. lewisi* (syn. *selengensis*) Ludlow, 1920 takes precedence over *A. messeae* Fall., 1926 [2, 41]. We note that Aitken knew the distribution of *A. messeae* Fall. but refrained from recognizing its synonymy with *A. lewisi* until more detailed information about the species became available [41].

Cryptic species of *A. messeae* s.l., whilst showing some variation of their features, do not differ morphologically at all stages of development. A Diptera expert A.V. Danilov did not find any differences between the larvae A and B species at the 4th instar we passed to him in 1986 (personal communication). Similar to Aitken [41], he did not find differences between the imago of *A. messeae* Fall. and *A. lewisi* Ludlow [2]. Analysis of over 500 individuals from a number of malaria mosquito populations of the Transbaikalia region [20] showed that, the geographical area of typical locality for *A. lewisi* (near the city of Ulan-Ude), is only inhabited by the cryptic species *A. messeae* B. Its presence in the extreme northeast region of the taxon range (Yakutsk) was confirmed by taxonprint analysis [4]. It is important to note the correspondence of the cytogenetic structure of the Yakut and Transbaikalia populations of *A. messeae* B. These facts and arguments allow us to consider the names *A. lewisi* and *A. messeae* B as belonging to a biological species other than *A. messeae* Fall. and to return *A. lewisi* from the *nomen oblitum* status [42] to the status of a valid (an existing) taxon. Thus, the *Anopheles messeae* s.l. taxon includes two homosequentical cryptic species, namely *Anopheles* (*Ano.*) *lewisi*, Ludlow, 1920 and *Anopheles* (*Ano.*) *messeae* Falleroni, 1926.

Conclusions

In this study, we state that *Anopheles messeae* s.l. taxon includes two homosequentical cryptic species with parallel chromosomal polymorphisms. In our research, we termed the cryptic species *A. messeae* A and *A. messeae* B. However, other names, particularly *A. lewisi* Ludlow, 1920; *A. messeae* s.s. Fall., 1926 and *A. daciae* Linton et al., 2004 are also known to refer to the species. Considering data on chromosomal polymorphisms in paracentric inversions, ecology divergence, geographic distribution and molecular markers, we concluded that *A. messeae* s.s. Fall., 1926, *A. messeae* A and *A. daciae* Linton et al., 2004 are synonyms for one of the cryptic species, while *A. lewisi* Ludlow, 1920 and *A. messeae* B are synonyms for the other one. It means that despite the current trend it is not correct to name the cryptic species *A. messeae* Fall., 1926 and *A. daciae* Linton et al., 2004, because in fact they are the same biological species. Thus, we propose to term the two homosequentical cryptic species as *Anopheles (Ano.) lewisi*, Ludlow, 1920 and *Anopheles (Ano.) messeae* Falleroni, 1926.

References

1. Beklemishev VN. Ekologiya malyariynogo komara [Ecology of the malaria mosquito]. Moscow: Medgiz Publ.; 1944. 299 p. In Russian
2. White GB. Systematic reappraisal of the *Anopheles maculipennis* complex. *Mosq. Syst.* 1978;10(1):13-44
3. Novikov YuM. *Anopheles messeae* Fall. (Diptera, Culicidae) – dva vida *in statu nascendi* [*Anopheles messeae* Fall. (Diptera, Culicidae) – two species *in statu nascendi*]. In: Makroevolyutsiya (Materialy I Vsesoyuznoy konferentsii po problemam evolyutsii) [Macroevolution]. Yanshin AL, editor. Moscow: Nauka Publ.; 1984. p. 13-14. In Russian
4. Novikov YuM, Shevchenko AI. Inversion polymorphism and the divergence of two cryptic forms of *Anopheles messeae* (Diptera, Culicidae) at the level of genomic DNA repeats. *Russ. J. Genet.* 2001;37(7):754-763. doi: 10.1023/A:1016790724790
5. Nicolescu G, Linton Y-M, Vladimirescu A, Howard TM, Harbach RE. 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. *Bull. Entomol. Res.* 2004;94(6):525-535. doi: 10.1079/ber2004330
6. Ludlow C. Siberian Anopheles. *Psyche*. 1920;27:74-78.
7. Stegniy VN. Populyatsionnaya genetika i evolyutsiya malyariynykh komarov [Population genetics and evolution of malaria mosquitoes]. Il'inskikh NN, editor. Tomsk: Tomsk State University Publ.; 1991. 137 p. In Russian
8. Stegniy VN, Sharakhova MV. Systemic reorganization of the polytene chromosomes architecture at ontogenic and phylogenetic levels in malarial mosquitoes – structural peculiarities of chromosomes attachment to nuclear-envelope. *Genetika*. 1991;27(5):828-835. In Russian
9. Stegnii VN, Sharakhov IV, Sharakhova MV, Sibataev A, Wasserlauf IE. Evolution-genetic analysis of the palearctic *Anopheles maculipennis* (Diptera: Culicidae) complex. In: *Proceedings of XX International Congress of Entomology* (Firenze, Italy, August 25-31, 1996). Italy: Congress Scientific Committee Publ.; 1996. p. 256.
10. Stegniy VN, Pishchelko AO, Sibataev AK, Abylkassymova G. 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. *Russ. J. Genet.* 2016;52(6):578-584. doi: 10.1134/S1022795416060132

11. Sharakhova MV, Stegniy VN, Braginets OP. Interspecific differences in structure of pericentromeric heterochromatin in ovarian trophocytes and evolution of malaria mosquitoes of the *Anopheles maculipennis* species complex. *Genetika.* 1997;33(12):1640-1648. In Russian
12. Gordeev MI, Bezzhonova OV, Moskaev AV. Chromosomal polymorphism in the populations of malaria vector mosquito *Anopheles messeae* at the south of Russian Plain. *Russ. J. Genet.* 2012;48(9):962-965. doi: 10.1134/S1022795412090037
13. Gordeev MI, Moskaev AV. Chromosomal Polymorphism in the populations of malaria mosquito *Anopheles messeae* (Diptera, Culicidae) in the Volga Region. *Russ. J. Genet.* 2016;52(6):597-602. doi: 10.1134/S1022795416040050
14. Stegnii VN, Kabanova VM, Novikov YuM. Study of karyotype of A*nopheles-messeae* Fall. *Tsitologiya.* 1976;18(6):760-766. In Russian
15. Stegnii VN, Kabanova VM, Novikov YuM, Pleshkova GN. Inversional polymorphism in *Anopheles-messeae*. 1. Distribution of inversions over area of species. *Genetika.* 1976;12(4):47-55. In Russian
16. Stegnii VN, Novikov YuM, Pleshkova GN, Kabanova VM. Inversional polymorphism in *Anopheles messeae*. 2. Interpopulational variability of inversion frequencies. *Genetika.* 1978;14(6):1017-1023. In Russian
17. Novikov YuM. Vliyanie assortativnogo skreshchivaniya na populyatsionnuyu strukturu malyariynogo komara *Anopheles messeae* [Effect of assortative mating on the population structure of malaria mosquito *Anopheles messeae*]. In: *Proceedings of XIV International Genetic Congress* (Moscow, USSR, August 21-30, 1978). Belyaev DK, editor. Moscow: Nauka Publ.; 1981. Vol. 1. p. 471. In Russian
18. Novikov YuM. Interaction between inversions in populations, karyotype pool differentiation, and mechanisms of maintaining the population-genetic homeostasis in malaria mosquito *Anopheles messeae* Fall. In: *New data on karyosistematics of Diptera*. Chubareva LA, editor. *Trudy Zoologicheskogo instituta Akademii nauk SSSR* [Proceedings of the Zoological Institute. Academy of Sciences of the USSR]. 1980;95:40-49. In Russian
19. Novikov YuM. Gomologiya struktury i analogiya funktsiy inversiy khromosom u kripticheskikh vidov *Anopheles messeae* [Structural homology and functional analogy of the chromosome inversions in the cryptic species of *Anopheles messeae*]. In: *Pervye Mezhdunarodnye Bekkerovskie chteniya* [First International Becker's Readings (Volgograd, Russia, May 27-29, 2010). Volgograd: Volgograd State University Publ; 2010. Vol. 2. pp. 142-144. In Russian
20. Novikov YuM. On the ecology and range of *Anopheles beklemishevi* (Diptera: Culicidae) with reference to the taxonomy of *A. lewisi*. *J Vector Ecol.* 2016;41(2):204-214. doi: 10.1111/jvec.12215
21. Novikov YuM, Kabanova VM. Adaptive association of inversions in the natural populations of malaria mosquito *Anopheles messeae* Fall. *Genetika* =. 1979;15(6):1033-1045. In Russian
22. Novikov YuM, Shevchenko AI, Vaulin OV. O molekulyarno-geneticheskoy divergentsii kripticheskikh vidov taksona *Anopheles messeae* (Diptera: Culicidae) i filogenii kompleksa Maculipennis [On molecular-genetic divergence of cryptic species of the *Anopheles messeae* taxon (Diptera, Culicidae) and phylogeny of the *Maculipennis* complex]. *Vestnik Tomskogo gosudarstvennogo universiteta = Tomsk State University J.* 2004;(10, Suppl.):69-77. In Russian
23. Shevchenko AI, Novikov YuM. Kripticheskie vidy *Anopheles messeae* (Diptera, Culicidae): khiatus i svyazi na molekulyarno-geneticheskom urovne [Cryptic species of *Anopheles messeae* (Diptera, Culicidae): Hiatus and relations at the molecular-genetic level]. In: *Genetika v XXI veke: sovremennoe sostoyanie i perspektivy razvitiya*. III s"ezd VOGiS [Genetics in the XXI century: Current state and development prospects. 3rd Congress of VOGiS (Moscow, Russia, June 6-12, 2004)]. Moscow: URSS Publ.; 2004. p. 230. In Russian

24. Vaulin OV, Shevchenko AI, Novikov YuM. Izmenchivost' markernykh genomnykh posledovatel'nostey ITS2 i COI vnutri kompleksa *Anopheles maculipennis* (Diptera, Culicidae) [Variability of marker genomic sequences ITS2 and COI within the *Anopheles maculipennis* complex (Diptera, Culicidae)]. In: *Genetika v XXI veke: sovremennoe sostoyanie i perspektivy razvitiya*. III s"ezd VOGiS [Genetics in the XXI century: Current state and development prospects. 3rd Congress of VOGiS (Moscow, Russia, June 6-12, 2004)]. Moscow: URSS Publ; 2004. p. 197. In Russian
25. Vaulin OV, Novikov YuM. Geograficheskaya izmenchivost' ITS2 rDNK i COI mtDNK i kripticheskie vidy malyariynogo komara *Anopheles messeae* Fall. (Diptera: Culicidae) [Geographical variability of ITS2 rDNA and COI mtDNA and cryptic species mosquito *Anopheles messeae* Fall. (Diptera: Culicidae)]. *Informatsionnyy vestnik VOGIS.* 2010;14(3):546-557. In Russian
26. Novikov YuM, Vaulin OV. Expansion of *Anopheles maculipennis* s.s. (Diptera: Culicidae) to northeastern Europe and northwestern Asia: Causes and Consequences. *Parasite Vector.* 2014;7:e389. doi: 10.1186/1756-3305-7-389
27. Falleroni D. Fauna anofelica italiana e suo 'habitat' (paludi, risaie, canali). Metodi di lotta contro la malaria. *Riv. Malariol.* 1926;5(5-6):553-593.
28. Bates M. The nomenclature and taxonomic status of the mosquitoes of the *Anopheles maculipennis* complex. *Ann. Entomol. Soc. Am.* 1940;33:343-356.
29. Frizzi G. Salivary gland chromosomes of Anopheles. *Nature.* 1947;160(4059):226-227. doi: 10.1038/160226a0
30. Kabanova VM, Kartashova NN, Stegnii VN. Karyological investigation of natural populations of malarial mosquitos in Middle Ob River. 1. Characteristics of the karyotype of *Anopheles maculipennis-messeae* Fall. *Tsitologiyia.* 1972;14(5):630-636. In Russian
31. Kitzmiller JB, Frizzi G, Baker RH. Evolution and speciation within the Maculipennis Complex of the genus *Anopheles*. In: *Genetics of Insect Vectors of Disease*. Wright JW, Pal R, editors. Amsterdam: Elsevier Publ.; 1967. pp. 151-210.
32. Kabanova VM, Kartashova NN, Stegnii VN. Karyological investigation of natural populations of malarial mosquitos in Middle Ob River. 2. Chromosome polymorphism of *Anopheles maculipennis-messeae*. *Tsitologiyia.* 1972;14(8):1027-1033. In Russian
33. Stegniyi VN, Kabanova VM. Inversional polymorphism in *Anopheles.* 6. Local selection and the spatial differentiation of inversional gene-complexes for fertility. *Genetika.* 1985;21(12):1970-1973. In Russian
34. Novikov YuM. Effekty global'nogo potepleniya: napravlennaya dinamika proportsiy vidov Anopheles i tsitogeneticheskoy struktury taksona Anopheles messeae Fall. v Zapadnoy Sibiri [Effects of global warming: Directed dynamics of Anopheles species proportions and cytogenetic structure of the taxon *Anopheles messeae* Fall. in Western Siberia]. In: *Problemy evolyutsionnoy tsitogenetiki, selektsii i introduktsii*. Materialy nauchnykh chteniy, posvyashchennykh 100-letiyu professora V.P. Chekhova. [Problems of evolutionary cytogenetics, selection and introduction. Materials of Scientific Readings dedicated to the 100th anniversary of Professor VP Chekhov (Tomsk, Russia, December 2-5, 1997). Tomsk: Tomsk Polytechnic University Publ.; 1997. pp. 39-41. In Russian
35. Coluzzi M, Petrarca V, Di Deco MA. Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. *B. Zool.* 1985;52(1-2):45-63. doi: 10.1080/11250008509440343
36. Bezzhonova, OV, Goryacheva II. Intragenomic heterogeneity of rDNA internal transcripbed spacer 2 in *Anopheles messeae* (Diptera: Culicidae). *J. Med. Entomol.* 2008;45(3):337-341. doi: 10.1603/0022-2585(2008)45[337:IHORIT]2.0.CO;2.short
37. Di Luca M, Boccolini D, Marinucci M, Romi R. Intrapopulation polymorphism in *Anopheles messeae* (*A. maculipennis* complex) inferred by molecular analysis. *J. Med. Entomol.* 2004;41(4):582-586. doi: 10.1603/0022-2585-41.4.582

38. Frizzi G. Dimorfismo cromosomico in *Anopheles maculipennis messeae*. *Sci. Genet.* 1951;4(1-2):79-93. In Italian
39. Frizzi G. Nuovi contributi e prospettive di ricerca nelgruppo *Anopheles maculipennis* in base allo studio del dimorfismo cromosomico (ordinamento ad X invertito e tipico) nel messeae. In: Italy, Centro di Genetica del Consiglio Nazionale delle Ricerche. Symposia genetic. Pavia. 1952. Vol. 3, p. 231. In Italian
40. Sharakhova MV, Naumenko AN, Karagodin DA, Yurchenko AI, Gordeev MI, Moskaev AV, Martin OI, Baricheva EM, Sharakhov IV. Genomic divergence and chromosomal differentiation in the malaria mosquito *Anopheles messeae* sensu lato. In: *Chromosome 2018*: International Conference (Novosibirsk, Russia, August 20-24, 2018). Abstracts. Novosibirsk: Institute of Molecular and Cellular Biology SB RAS; Novosibirsk State University Publ.; 2018. pp. 73-74.
41. Aitken THG. Studies of the anopheline complex of western America. *Univ. Calif. Publs Ent.* 1945;(7):273-364.
42. Knight KL, Stone A. A catalog of the mosquitoes of the world (Diptera: Culicidae). Thomas Say Found., 2nd edition. Vol. 6. Maryland: Entomological Society of America Publ.; 1977. xi+611 p.

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