ISSN 2079-0597, Russian Journal of Genetics: Applied Research, 2014, Vol. 4, No. 6, pp. 533–538. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © A.V. Ivannikov, O.V. Vaulin, Yu.A. Koromyslov, I.K. Zakharov, 2014, published in Vavilovskii Zhurnal Genetiki i Selektsii, 2014, Vol. 18, No. 2, pp. 308–314.

Comparative Molecular Genetic Study of the Asian Population of *Drosophila mercatorum* (Diptera; Drosophilidae) at the COI Gene Fragment and the ITS1–ITS2 Region of the rRNA Genes

A. V. Ivannikov, O. V. Vaulin, Yu. A. Koromyslov, and I. K. Zakharov

Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, 630090 Russia

e-mail: IvAn@bionet.nsc.ru

Received August 27, 2013; in final form, September 2, 2013

Abstract—*Drosophila mercatorum* is a species of neotropical origin. It is represented by two subspecies, *marcatorum* and *pararepleta*. Synanthropic subspecies *D. mercatorum mercatorum* spread into Eurasia in the mid-20th century, and to date, it is widespread over the territory of Europe and the former Soviet Union. The experiments were performed using the specimens of *D. m. mercatorum* from natural Asian populations, laboratory stock 2328 of *D. mercatorum* (displaying some morphological differences from Asian wild-type flies), the specimens of *D. busckii* and *D. virilis*, and the *COI* gene sequence of *D. mercatorum* from NCBI database, accession number, DQ471607. The specimens were investigated for the variability of the standard DNA sequences used for species identification (the cytochrome oxidase subunit I (COI) gene fragment and the ITS1–ITS2 region of rRNA genes). Sequences of the COI gene fragment from Asian *D. m. mercatorum* were identical between the specimens, but differed from the sequences of stock 2328, as well as from the NCBI sequence. Asian populations of *D. mercatorum* and stock 2328 were identical in the ITS1–ITS2 sequences. Low sequence divergence between the COI gene fragments, along with the absence of differences at the ITS1–ITS2 region of the rRNA genes indicate that the difference between the Asian populations of *D. m. mercatorum* and morphologically different stock 2328 are within the frames of intraspecies divergence.

Keywords: *Drosophila mercatorum*, invasive species, population, gene, COI, ITS1–ITS2

DOI: 10.1134/S2079059714060100

INTRODUCTION

Neotropical species *Drosophila* (*Drosophila*) *mercatorum* Patterson et Wheeler, 1942 within the genus *Drosophila* belongs to the subgenus of the same name, to the species group *repleta*, and within the group *repleta*, to the subgroup *mercatorum* (Wasserman, 1982). The species *Drosophila mercatorum* was first detected in Europe in 1953, in Barcelona, Spain (Prevosti, 1953). In the following two decades, *Drosophila mercatorum* in small amounts was found in the collections of Drosophilides from Spain and the Canary Islands (Monclús, 1964, 1976). In the early 1980s, distribution of the species in the Old World remained within the boundaries of the Western Mediterranean. Since the beginning of the 1990s, judging by the publications, there was an expansion of the species from the Western Mediterranean to northern Europe, the United Kingdom (Bennett et al., 1995), the Netherlands (Kraaijeveld, 1992), and to the east, inland, to Ukraine and Western Siberia (Ivannikov et al., 1993, 1998; Ivannikov and Zakharov, 1995). In 2000, the species *D. mercatorum* was for the first time described in the Russian Far East, in the city of Vladivostok by the entomologist, Vasily Sergeevich Sidorenko, who included it in the Key to the Insects of the Russian Far East (Sidorenko, 2001). Thus, during the last decade of the 20th century *Drosophila mercatorum* populated the whole territory of Northern Eurasia, from west to east. Over the past two decades, we collected the specimens of *D. mercatorum* in different populations of Western Siberia and Central Asia.

From the mid-1980s, the stock of the *Drosophila* lines of the Genetics of Populations Laboratory, Institute of Cytology and Genetics, Russian Academy of Sciences, contains the *Drosophila mercatorum* line 2328. This line was received by Prof. L.I. Korochkin from the *Drosophila* Stock Center, Bowling Green, United States. In our studies, we used this stock for morphological comparison and the performance of test crosses with the representatives of the new species (for the fauna of the former Soviet Union), detected in Ukraine, Central Asia, and Western Siberia (Novosibirsk). The test crosses in both directions and in all cases demonstrated complete reproductive compatibility, which in combination with the morphological criteria made it possible to define the new species as *Drosophila mercatorum*.

It should be noted that there is only one morphological difference between Asian populations of *Drosophila mercatorum* and the 2328 flies. It is the degree of

Table 1. Characteristics of the material examined

Geographical origin and the year of the line construction	Symbol, species subspecies	Data on the COI gene fragment	Data on the ITS1–ITS2 region of rRNA genes
Bishkek, 2004	D10 *D. m. mercatorum*	+	+
Bishkek, 2004	D12 *D. m. mercatorum*	+	–
Novosibirsk, 2005	D4 *D. m. mercatorum*	+	–
Novosibirsk, 2005	D7 *D. m. mercatorum*	+	–
Tomsk, 2006	D5 *D. m. mercatorum*	–	+
Tomsk, 2006	D6 *D. m. mercatorum*	+	–
Tomsk, 2006	D8 *D. m. mercatorum*	+	–
Tomsk, 2006	D9 *D. m. mercatorum*	+	–
Tomsk, 2006	D11 *D. m. mercatorum*	+	–
Tomsk, 2006	D13 *D. m. mercatorum*	+	–
Tomsk, 2006	D14 *D. m. mercatorum*	+	–
Tomsk, 2006	D15 *D. m. mercatorum*	+	–
Unknown	D1 *D. m. mercatorum*, stock 2328	+	+
Unknown	D2 *D. virilis*	–	+
Unknown	D18 *D. busckii*	+	+

cuticle pigmentation. The 2328 flies are darker, brown in color with black spots and bands, and with respect to the general chitin color are close to *Drosophila repleta* and *Drosophila virilis*. At the same time, Asian *D. mercatorum* flies are yellow and pale brown, with gray spots and bands. With respect to coloration, these flies are similar to *Drosophila busckii* and *Drosophila immigrans*.

Based on the fact that climatic conditions of the continental Palearctic (Western Siberia and Tien Shan), among all regions of the planetary distribution area of synanthropic *Drosopila*, are the most different from the climate of the neotropic Amazonian region, and due to some color differences between the Asian specimens of *D. mercatorum* from our collection and the 2328 flies, a working hypothesis was generated. According to this hypothesis, Asian lines of *D. mercatorum* and the stock line 2328 were treated as independent species or subspecies. The hypothesis was tested through identification of possible differences between the two groups of flies at the DNA level.

MATERIALS AND METHODS

The study was carried out using the *Drosophila mercatorum* laboratory stock 2328 and the lines based on the fertilized in the wild females of *Drosophila mercatorum* sequencing of the mtDNA COI gene fragment and of the ITS1–ITS2 region of rRNA genes was performed for these lines and for the representatives of some other *Drosophila* species (Table 1).

DNA was extracted from individual adult flies according to the standard technique (Bender et al., 1983). The polymerase chain reaction of the COI (cytochrome oxidase subunit 1) gene fragment was successfully performed using primers, designed earlier for the mosquitoes of the genus *Anopheles* (Vaulin and Novikov, 2012). The amplification primers were 5'-CGAGG-AATAG-TAGGA-ACTTC-3' (forward) and 5'-CTGTA-AATAT-GTGAT-GAGC-3' (reverse).

The reaction mixture for PCR of the COI gene fragment contained 1 × PCR buffer; 4 mM $MgCl_2$; 0.4 mM of each dNTP; and 1 unit of *Taq* polymerase. The reaction conditions included denaturation for 1 min at 94°C; annealing for 1 min at 50°C; extension for 1 min at 72°C; and final extension for 5 min at 72°C.

PCR and sequencing of the ITS1–ITS2 region was carried out using four primers designed based on the analysis of rRNA genes of *Drosophila melanogaster*, *D. virilis*, and *D. pseudoobscura* from the NCBI DNA database. The pair of primers (5'-AGGTG-AACTG-CGGAA-CGGAA-GGATC-3', forward and 5'-AGTCC-CATAT-GAGTT-GAGGT-TG-3', reverse) was used for the PCR of the ITS1 region. The pair of primers (5'-CTCTA-AGCGG-TGGAT-CACTC-3', forward and 5'-AGTCC-CATAT-GAGTT-GAGGT-TG-3', reverse) was used for the PCR of the ITS2 region. Forward primer targeting the ITS1 region and reverse primer targeting the ITS2 region were used for amplification of the whole ITS1–ITS2 region and for adequate sequencing of the 5.8S fragment. Composition of the reaction mixture for amplification of all three fragments, corresponding to the ITS1–ITS2 region, was the same as for amplification of the COI gene fragment, with the exclusion of primer composition. The PCR temperature conditions differed from those used for the PCR of the COI gene in the annealing temperature, which corresponded to 57°C. Sequencing was

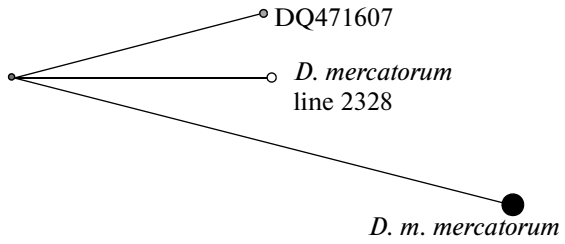

Fig. 1. Median-joining network for the COI gene sequences of *D. mercatorum*.

carried out using the resources of the Genomics Core Facility of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, http://sequest.niboch.nsc.ru. The sequences examined were deposited in the EMBL DNA database (http://www.ebi.ac.uk/ena/) under the access numbers ranging from HG798333 to HG798350. Based on the sequence data obtained and using the NETWORK 4.6.0.0. software program (Bandelt et al., 1990), a median-joining network of the COI sequences was constructed. Using the sequence data for the COI gene fragment and the fragment of rRNa genes, dendrograms were constructed as implemented in the MEGA5 software program (Tamura et al., 2011).

The sequence of the COI gene fragment of *Drosophila mercatorum* from the DNA database (accession number DQ471607) was also included in the analysis. The geographical origin of this database specimen is not specified. Since this specimen is designated as *Drosophila mercatorum*, it belongs to the nominative subspecies *Drosophila mercatorum mercatorum*. This is because when referring to nonnominative subspecies, its second subspecies name, other than the first species, is always mentioned.

RESULTS

For the COI gene in the examined specimens of *D. mercatorum* and *D. busckii*, gene fragments of 720 bp in size were obtained. No nucleotide deletions/insertions were identified.

For the representatives of the species *D. mercatorum*, a median-joining network of the COI sequences was constructed (Fig. 1). Based on the sequence data for the COI and rRNA gene fragments, the dendrograms were constructed using the maximum likelihood algorithm (Figs. 2, 3, 4).

We note that all the examined Asian specimens of *D. mercatorum* had identical sequences of the COI gene fragment analyzed. The database sequence and stock 2328 of *D. mercatorum* at our disposal differed from the Asian specimens by one amino acid substitution, and by two substitutions, from each other.

DISCUSSION

The COI gene and rRNA gene internally transcribed spacer sequences are widely used for species assignment of the groups of individuals. The mtDNA genes evolve relatively rapidly and are generally characterized by a higher gene drift effect compared to nuclear autosomal genes (Altukhov and Salmenkova, 2002; Altukhov, 2003). Because of this, the divergence of closely related species during some time interval should be accompanied by the divergence at the mitochondrial genes. In particular, the DNA barcoding technique implies that divergence of the individuals from two groups by Kimura's genetic distance (Kimura, 1980) of 0.02 at the COI gene is achieved in one million years of evolution. This is the time necessary and sufficient for segregation into individual species (Hebert et al., 2003). However, in the pairs of most closely related species, divergence at the mtDNA will not necessarily be observed. In particular, there is no

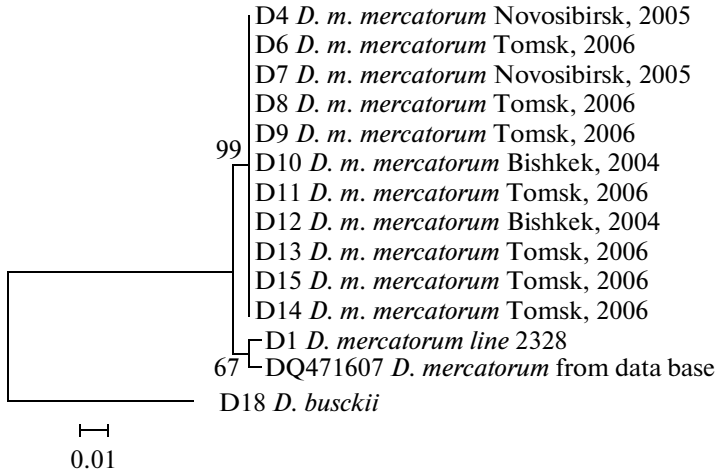

Fig. 2. Dendrogram constructed based on the sequence of the COI gene fragment and using the maximum likelihood method with 1000 iterations of bootstrapping for the specimens of *D. mercatorum*. The dendrogram was rooted on *D. busckii*.

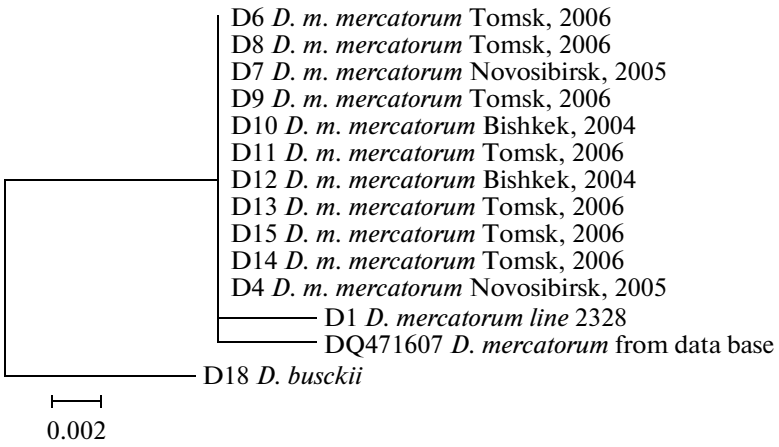

Fig. 3. Dendrogram constructed based on amino acid sequence corresponding to the COI gene fragment using the maximum likelihood technique for the specimens of *D. mercatorum*. The dendrogram was rooted on *D. busckii*. Due to the fact that most of the sequences were identical, while the others carried unique (not found in other specimens) amino acid substitutions, bootstrapping was not performed.

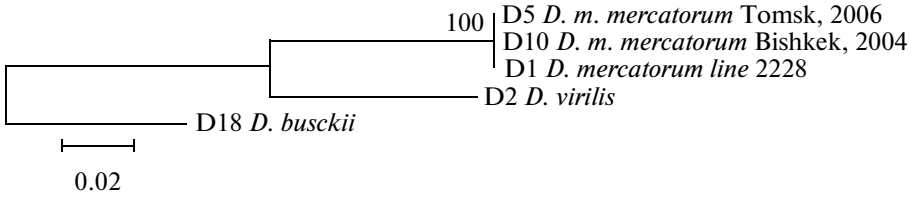

Fig. 4. Dendrogram constructed based on the ITS1–ITS2 sequence of rRNA genes and using the maximum likelihood method with 1000 iterations of bootstrapping for the specimens of *D. mercatorum*. The dendrogram was rooted on *D. virilis* and *D. busckii*.

differentiation at the mtDNA ND5 gene fragment between the chromosomally defined forms of *Anopheles funestus* (Michel et al., 2005). Similarly, there is no divergence at the COI gene in the cryptic species A and B *Anopheles messeae* (Vaulin and Novikov, 2010, 2012). At the same time, within a single species, there can be several individual clusters of mtDNA haplotypes, associated with different strains of *Wolbachia* endosymbiont (Hurst and Jiggins, 2003; Ilinsky and Zakharov, 2007). The values of Kimura's genetic distances between the specimens examined in the present study are demonstrated in Table 2. From the table it follows that differentiation at the COI for the specimens of *D. mercatorum* constituted from 0.006 to 0.008; i.e., it does not exceed the conditionally-species level of 0.02. At the same time, the genetic distance between different sequence variants of the COI gene fragment of *D. mercatorum* and the sequence of the COI gene fragment of *D. busckii* constitutes about 0.15; i.e., it considerably exceeds this threshold.

Variability at the rRNA gene cluster is to a high degree associated with the species assignment of the individuals. This is because the rRNA genes are represented by moderately repeated sequences, characterized by the effect of concerted repeat evolution (Dover and Flavell, 1984). In the general case, all individuals of the same species have an identical set of nucleotide substitutions in the rRNA gene cluster, or preserve in the genome several major sequence variants of these genes without the effects of genetic segregation in the populations (Beebe et al., 2001; Wilkerson et al., 2004). The closely resembling species A and B *Anopheles messeae* differ in five nucleotide substitutions in the rRNA gene ITS2 region (Novikov et al., 2004;

Table 2. Kimura's genetic distances in the COI gene sequences between the specimens examined

Specimens and their groups	Stock 2228 of *D. mercatorum*	Sequence DQ471607 of *D. mercatorum*	*D. busckii*
Asian specimens of *D. mercatorum*	0.008	0.008	0.149
Stock 2228 of *D. mercatorum*		0.006	0.153
Sequence DQ471607 of *D. mercatorum*			0.150

Vaulin and Novikov, 2010). It can be expected that if the specimens of *D. mercatorum* de facto represent different species, then the differences in the rRNA gene ITS1–ITS2 region would be observed. At the same time, in these specimens, no differences at this genomic region were identified. Thus, our working hypothesis that Asian *Drosophila mercatorum* could be treated as an independent species was not confirmed.

The mitochondrial COI gene sequence variation pattern in the examined Asian specimens of *D. mertcatorum* deserves special attention. Sequence uniformity at this genomic region observed in all Eurasian specimens examined can be interpreted in terms of the passage from the bottleneck of a group of migrants who entered Eurasia more than half a century ago. At the same time, the scarce data on the COI gene variability in non-Asian populations of *D. mercatorum* do not allow us to consider this conclusion as highly justified.

ACKNOWLEDGMENTS

This study was carried out within Base Budget Project no. VI.53.1.2.

REFERENCES

Altukhov, Yu.P., *Geneticheskie protsessy v populyatsiyakh* (TRANSLATION), Moscow: IKTs "Akademkniga", 2003.

Altukhov, Yu.P. and Salmenkova, E.A., DNA polymorphism in population genetics, *Russ. J. Genet.*, 2002, vol. 38, no. 9, pp. 989–1008.

Bächli, G. and Rocha, PiteM.T., Drosophilidae of the Palearctic region, in *The Genetics and Biology of Drosophila*, Ashburner, M., Carson, H.L., and Thompson, N., Jr., Eds., Acad. Press, 1981, vol. 3a, pp. 169–196.

Bandelt, H.-J., Forster, P., and Rohl, A., Median-joining networks for inferring intraspecific phylogenies, *Mol. Biol. Evol.,* 1999, vol. 16, no. 1, pp. 37–48.

Beebe, N.W., Maung, J., Hurk, A.F., Ellis, J.T., and Cooper, R.D., Ribosomal DNA spacer genotypes of the *Anopheles bancroftii* group (Diptera: Culicidae) from Australia and New Guinea, *Insect Mol. Biol.*, 2001, vol. 10, no. 5, pp. 407–414.

Bender, W., Spierer, P., Hognes, D.S., and Chambon, P., Chromosomal walking and jumping to isolate DNA from *Ace* and *rosy* loci of *bithorax* loci in *Drosophila melanogaster*, *J. Mol. Biol.*, 1983, vol. 168, no. 1, pp. 17–33.

Bennett, H.J., Jenkinsonm, L.S., and Davis, A.J., *Drosophila mercatorum* Patterson and Wheeler 1942 (Diptera: Drosophilidae): first record for the British isles, *Dros. Inform. Serv.*, 1995, vol. 76, pp. 94–95.

David, J.R. and Tsacas, L., Cosmopolitan, subcosmopolitan, and widespread species: different strategies with in the Drosophilid family (Diptera), *S. R. Soc. Biogeogr.,* 1980, vol. 57, no. 1, pp. 11–26.

Dobzhansky, Th. and Pavan, C., Studies on Brasilian species of *Drosophila*, Separata do Bol. Facul. Fil. Cien. e Letr. Univ. S. Paolo, no. 36, *Biologia Geral.*, 1943, no. 4.

Dover, G. and Flavell, R., Molecular coevolution: DNA divergence and the maintenance of function, *Cell*, 1984, vol. 38, no. 3, pp. 622–623.

Hebert, P.D.N., Cywinska, A., and Ball, S., Biological identifications through DNA barcodes, *Proc. R. Soc. London, Ser. B*, 2003, vol. 270, pp. 313–321.

Hurst, G.D.D. and Jiggins, F.M., Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts, *Proc. Roy. Soc. B: Biol. Sci.*, 2005, vol. 272, pp. 1525–1534.

Ilinsky, Yu.Yu. and Zakharov, I.K., Infection of the Uman' population of *Drosophila melanogaster* with the cytoplasmic endosymbiont *Wolbachia*, *Dokl. Biol. Sci.*, 2007, vol. 413, pp. 166–168.

Ivannikov, A.V. and Zakharov, I.K., Distribution of *Drosophila mercatorum* into Eurasian continent, *Dros. Inform. Serv.*, 1995, no. 76, p. 107.

Ivannikov, A.V., Zakharov, I.K., and Skibitsky, E.E., Synantropic *Drosophila species* in the Ukraine and Moldova, *Dros. Inform. Serv.*, 1993, no. 72, p. 87.

Ivannikov, A.V., Katokhin, A.V., and Sidorenko, V.S., List of Drosophilidae (Diptera) of West Siberia, *Far Eastern Entomol.*, 1998, no. 57, pp. 12–16.

Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, *J. Mol. Evol.*, 1980, vol. 16, pp. 111–120.

Kraaijeveld, A.R., First record of *Drosophila mercatorum* (Diptera: Drosophilidae) in northwestern Europe, *Entomologische Berichten*, Amsterdam, 1992, vol. 52, p. 32 (cited by: Bennett, H.J., Jenkinson, L.S., and Davis, A.J., 1995).

Michel, A.P., Guelbeogo, W.M., Grushko, O.B., et al., Molecular differentiation between chromosomally defined incipient species of *Anopheles funestus*, *Insect Mol. Biol.*, 2005, vol. 14, no. 4, pp. 375–387.

Monclús, M., Distribución y ecología de Drosophilidos en España. I. Especies de Drosophila de la region Catalana, *Genet. Iber.*, 1964, vol. 16, pp. 143–165.

Monclús, M., Distributión y ecología de Drosophilidos en España. II Especies de *Drosophila* de las Islas Canarias, con la description de una nueva especie, *Biol. R. Soc. Espanola Hist. Nat. (Biol.)*, 1976, vol. 74, pp. 197–213.

Monclús, M., Drosophilidae of Madeira, with the description of *Drosophila madeirensis* n. sp., *Soderdruckaus Z. f. zool. Systematik u. Evolutionstorschung*, 1984, vol. 22, no. 2, pp. 94–103.

Novikov, Yu.M., Shevchenko, A.I., and Vaulin, O.V., The molecular- genetic divergence of cryptic species of the taxon *Anopheles messeae* (Diptera: Culicidae) and the phylogeny of the complex *maculipennis*, *Vestn. Tomsk. Gos. Univ. (Suppl.)*, Tomsk: Tomsk. Gos. Univ., 2004, vol. 10, pp. 69–77.

Patterson, J.T. and Wheeler, M.R., Description of new species of the subgenera *Hirtodrosophila* and *Drosophila*, *Univ. Texas Publ.*, 1942, vol. 4213, pp. 67–109.

Prevosti, A., Two newly introduced species of drosophila found in Europe, *Dros. Inform. Serv.*, 1953, vol. 27, p. 110.

Sidorenko, V.S., Family Drosophilidae, in *Opredelitel' nasekomykh Dal'nego Vostoka Rossii* (Insects of Russian

Far East: An Identification Guide), Vladivostok: Dal'nauka, 2001, vol. 4, part 2, pp. 211–241.

Tamura, K., Peterson, D., Peterson, N., et al., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 2011, vol. 28, no. 10, pp. 2731–2739.

Vaulin, O.V. and Novikov, Yu.M., Geographic variation of ITS 2 rDNA and COI mtDNA in cryptic species of the malaria mosquito *Anopheles messeae* Fall. (Diptera: Culicidae), *Inform. Vestn. VOGiS*, 2010, vol. 14, no. 3, pp. 546–557.

Vaulin, O.V. and Novikov, Yu.M., YM Polymorphism and interspecific variability of the nucleotide sequence of the cytochrome oxidase subunit I (COI) gene from the sibling species A and B *Anopheles messeae* and *An. beklemishevi* (Diptera: Culicidae), *Vavilov. Zh. Genet. Selekts.,* 2012, vol. 16, no. 2, pp. 358–368.

Wasserman, M., Evolution of the *repleta* group, in *The Genetics and Biology of Drosophila*, Ashburner, M., Carson, H.L., and Thompson, J.N., Jr., Eds., Acad. Press, 1981, vol. 3b, pp. 61–139.

Wheeler, M.R., The Drosophilidae: a taxonomic overview, in *The Genetics and Biology of Drosophila*, Ashburner, M., Carson, H.L., and Thompson, J.N., Jr., Eds., Acad. Press, 1981, vol. 3a, pp. 1–97.

Wilkerson, R.C., Reinert, J.F., and Li, C., Ribosomal DNA ITS2 sequences differentiation six species in the *Anopheles crucians* complex (Diptera: Culicidae), *J. Med. Entomol.*, 2004, vol. 41, no. 3, pp. 392–401.

Translated by N. Maleeva