© *Comparative Cytogenetics, 2007. Vol. 1, No. 2, P. 129-138.* *ISSN 1993-0771 (Print), ISSN 1993-078X (Online)*

A study of the distribution of the *Culex pipiens* complex (Insecta: Diptera: Culicidae) mosquitoes in the European part of Russia by molecular methods of identification

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Abstract. Three members of the *Culex pipiens* complex mosquitoes, *Culex pipiens* L. (including the *pipiens* and *molestus* forms) and *Culex torrentium* Martini, occur in the European part of Russia. The geographical and biotopic distribution of these mosquitoes was studied using molecular-genetic methods for their identification. They included *Wolbachia* infection, sizes of ITS2 of rDNA, polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) of the cytochrome oxidase I (COI) gene of the mitochondrial DNA (both 3' and 5' ends of COI). In all, 40 collections of mosquitoes were made in 30 localities, and 1062 individuals were identified. The notion of the distribution of the sibling-species *C. pipiens* and *C. torrentium* was confirmed and enlarged. Homogeneous and heterogeneous populations consisting of *C. pipiens* (the *pipiens* form) and *C. torrentium* and those consisting of both forms of *C. pipiens* were revealed in overground and underground habitats. The possible mechanisms of isolation between these members of the *C. pipiens* complex are discussed.

Key words: *Culex pipiens*, *C. torrentium*, distribution, Russia, molecular diagnostics.

INTRODUCTION

Mosquitoes of the *Culex pipiens* complex are distributed all over the world and are of great fundamental, medical and veterinary importance as active bloodsuckers and vectors (Vinogradova, 1997, 2000). The *C. pipiens* complex includes five members, but only three of them occur in Russia. These are the northern house mosquito, *C. pipiens* Linnaeus, 1758, *C. torrentium* Martini, 1924 and *C. vagans* Wiedeman, 1828. *C. pipiens* is distributed over almost the entire territory of Russia. *C. vagans* is recorded in Siberia and Far East. *C. torrentium* is a primarily west Palaearctic species, occurring in Europe and some parts of Asia. *C. pipiens* and *C. torrentium* are considered as sibling species, and in a part of the distribution range they occur sympatrically.

C. pipiens is presented by two forms or ecotypes, the nominate *pipiens* form and the *molestus* form, the latter is known as urban mosquito. The *molestus* form described as distinct species *C. molestus* Forskal in 1775 from Egypt has been found since the 1920s in some European cities. Now it occurs in many cities in Europe, Asia including Japan and Near East, North and South America and Australia. In temperate zones this mosquito may develop throughout the year only in underground sites flooded by polluted waters, i.e. in the house basements, underground tunnels, etc., but in summer it may occasionally develop also in the above-ground water-bodies (Vinogradova, 2000).

The peculiarity of the *C. pipiens* complex is that there are significant ecophysiological differences between its members, which are relatively morphologically similar (Vinogradova, 2000, 2003). "The taxonomy of *pipiens* complex is enigma complicated by interpretational difficulties and controversy associated with number of bewildering morphological, behavioral/physiological, and genetic issues. An understanding of these issues is intimately related to the resolution of the *pipiens* complex" (Harbach et al., 1985, p. 19). Ecophysiological differences include autogeny, stenogamy versus eurygamy and reproductive diapause. Autogeny is the production of the first egg raft without taking a blood meal. Stenogamy is the ability to mate in confined space, without swarming, and the eurygamy is the need for a large space for mating. The *pipiens* form and *C. torrentium* are anautogenous, in most cases eurygamous and able to enter reproductive diapause, whereas the *molestus* form is autogenous, stenogamous and has no diapause.

C. pipiens attracts the attention of scientists because of its fundamental and applied importance. It is a vector of human lymphatic filariasis in the tropics and of many arboviral infections – Western equine, St. Louis, Japan encephalites, West Nile (WNF) and Rift valley fevers, and Ockelbo disease (Vinogradova, 2000; Petersen et al., 2002; Gratz, 2004). WNF outbreaks were registed in Volgograd, Astrakhan and some other provinces of Russia in 1999 (Platonov et al., 2001). The epidemiological role of *C. torrentium* is insufficiently studied.

In connection with this circumstance the correct identification of these mosquitoes, especially females, is very important. However there are objective difficulties in identification of two forms of *C. pipiens* and *C. torrentium* within the limits of traditional taxonomy. Almost all morphological features which are used in Russian (Gutsevich et al., 1970; Gornostaeva, Danilov, 1999) and foreign identification keys of culicids are variable and do not give accurate identification of these mosquitoes. Only males of *C. pipiens* and *C. torrentium* may be more or less satisfactorily differentiated by some structures of male genitalia, though they also may be variable (Service, 1968; Gutsevich et al., 1970; Dahl, 1988; Fedorova, Shaikevich, 2007). The great variation of the values of the siphonal index of the larvae in *C. pipiens* casts doubt on its diagnostic validity (Harbach et al., 1985), but the mean siphonal index may be used to differentiate populations of the two forms as it is usually 4.4 or less in the autogenous *molestus* form and 4.8 or more in the anautogenous *pipiens* form (Vinogradova, 2000).

This situation regarding the traditional taxonomy stimulated the development of molecular diagnostics of *C. pipiens* forms and *C. torrentium*, and we found such markers (Vinogradova et al., 2003; Vinogradova, Shaikevich, 2005, 2007; Shaikevich et al., 2005; Shaikevich, 2007). These are the *Wolbachia pipientis* Hertig infection, the transitions in nucleotide sequences of 3'and 5' ends of the cytochrome oxidase subunit I (COI) gene of mitochondrial DNA, the mtDNA fragments resulted by the restriction analysis and PCR fragments of ITS2 sequences of the ribosomal DNA. Other molecular markers for identification of the above mentioned mosquitoes are also known (Miller et al., 1996; Guillemaud et al., 1997; Kent et al., 2003; Smith, Fonseca, 2004; Fonseca et al., 2004; Khrabrova et al., 2005; Bahnk, Fonseca, 2006).

This paper is the first attempt to analyse the geographic and biotopic distribution of the *C. pipiens* complex mosquitoes in the European part of Russia using molecular methods of their identification.

MATERIAL AND METHODS

Fourth instar larvae of mosquitoes were collected in 2002-2006 in the flooded basements of

Table 1. Infection by *Wolbachia*, size of ITS2 and 3' COI mitotype in the geographical populations of mosquitoes of *C. pipiens* complex from underground habitats. Data: 4-8 (Vinogradova, Shaikevich, 2007). For a correlation between mitotype and species (the last column) see the text.

	Locality	**Coordinates, N/E**	**Number of specimens**	***Wolbachia* infection, %**	**ITS2 sizes (bp)**	**3' COI mitotype, species**
1	Petrozavodsk	62°47'/34°20'	53	72	466	M, *C. pipiens*
2	St. Petersburg	59°55'/30°15'	100	94	463	M, *C. pipiens*
3	Ekaterinburg	56°53'/60°35'	24	71	463	M, *C. pipiens*
4	Moscow	55°45'/37°35'	53	96	462	M, *C. pipiens*
5	N. Novgorod	56°19'/44°00'	40	100	466	M, *C. pipiens*
6	Volgograd	48°44'/44°25'	20	100	463	M, *C. pipiens*
7	Volzhsky	48°30'/45°10'	17	88	463	M, *C. pipiens*
8	Krasnodar	45°02'/39°00'	20	70	463	M, *C. pipiens*

dwelling houses in 9 cities and also in open aboveground water bodies in several settlements from different districts in the European part of Russia. The hibernating females were collected only in Iksha (Moscow Province). The larvae and adults were preserved in 96% alcohol for further molecular studies.

Mosquito DNA from individual larvae, pupae and adults, either native or preserved in 96% ethanol, was extracted using standard protocol DIAtom DNA Prep kit (Isogen, Russia). Amplification was carried out using 0.1 mg of the isolated DNA.

For the identification of the endosymbiotic bacteria *W. pipientis* in mosquitoes, primers specific for the wsp gene of *W. pipientis* were used (Braig et al., 1998). The 3'-end of the cytochrome oxidase I (COI) gene of mitochondrial DNA was amplified using UEA9 and UEA10 primers (Juan et al., 1996). Ribosomal DNA was amplified using primers complementary to the 5,8S and 28S rDNA (Porter, Collins, 1991). 5'-end of the COI gene of mtDNA was amplified using *Culex*COIF and *Culex*COIR primers (Shaikevich, 2007). PCR conditions were identical for all pairs of primers and consisted of primary denaturing at 95° C for 5 min; then 35 cycles of denaturing at 94° C for 30 sec, annealing at 55° C for 30 sec, extension at 72° C for 45 sec; and a final extension at 72° C for 10 min. PCR products were identified using electrophoresis in 1% agarose gel (Sigma, United States). Restriction enzyme digestions were performed in 30 µl volume. Digest master mix consisted of 5µl of the COI PCR product and 5 U of enzyme, 3 µl corresponded buffer, 0.3 µl BSA if it was not included in buffer and ddH_2O. For SspI restriction endonuclease reaction was incubated for 1 hour at 37° C. For HaeIII restriction endonuclease reaction was incubated for 1.5 hour at 37° C. For BclI restriction endonuclease reaction was incubated for 1 hour at 50° C. The digested products were visualized on 1% agarose gel.

RESULTS

Determination of *W. pipientis* in mosquitoes has been carried out using the *W. pipientis* wsp-specific primers. From 70 to 100% of *C. pipiens* larvae in both underground and above-ground habitats were found to be infected by *Wolbachia*,

Table 2. Infection by *Wolbachia,* size of ITS2 and 3' COI mitotype in the geographical populations of mosquitoes of *C. pipiens* complex from overground habitats. Data: 7, 8, 10-12 (Fedorova, Shaikevich 2007); 12 – hibernating females; 18 – adults; the other populations – larvae. For a correlation between mitotype and species (the last column) see the text.

No.	Locality	Coordinates, N/E	Number of specimens	Wolbachia, %	ITS2 sizes (bp)	3' COI mitotype (P, M), species
1	Karelia, Nilma	62°30'/33°05'	8	0	410	P, *C. torrentium*
2	Karelia, Mashozero	62°30'/34°00'	35	0	410	P, *C. torrentium*
3	Sverdlovsk Prov.,Taliza	60°15'/56°40'	12	100	460	M, *C. pipiens*
			24	0	410	P, *C. torrentium*
4	Leningrad Prov. Sosnovo	60°28'/30°15'	50	0	410	P, *C. torrentium*
5	Yaroslavl envir.	57°37'/39°42'	24	0	410	P, *C. torrentium*
6	Ivanovo Prov., Kokhma	56°55'/41°00'	20	0	410	P, *C. torrentium*
7	Moscow, Bitza	55°45'/37°35'	16	0	410	P, *C. torrentium*
8	Moscow, Ostankino	55°45'/37°35'	15	0	410	P, *C. torrentium*
			3	100	460	M, *C. pipiens*
9	Moscow Prov., Luzhki	55°20'/38°00'	20	90	460	M, *C. pipiens*
10	Moscow Prov., Chashnikovo	56°02'/37°07'	18	100	460	M, *C. pipiens*
			32	0	410	P, *C. torrentium*
11	Moscow Prov., Iksha	56°10'/37°30'	22	0	410	P, *C. torrentium*
12	Moscow Prov., Iksha	56°10'/37°30'	20	100	460	M, *C. pipiens*
13	Moscow Prov., Starikovo	56°10'/38°31'	20	100	460	M, *C. pipiens*
			24	0	410	P, *C. torrentium*
14	N. Novgor. Prov., Diveevo	55°10'/43°00'	18	0	410	P, *C. torrentium*
15	N. Novgor. Prov., Vyksa	55°18'/42°11'	24	0	410	P, *C. torrentium*
16	Saratov Prov.	51°32'/46°00'	9	0	410	P, *C. torrentium*
17	Volgograd Prov., Sarepta	48°30'/44°32'	20	100	460	M, *C. pipiens*
18	Krasnodar	45°02'/39°00'	20	98	460	M, *C. pipiens*

whereas no infection of *C. torrentium* larvae was revealed (Tables 1-2). The absence of infected *C. torrentium* even in the mixed mosquito populations including also *C. pipiens* testifies that horizontal transfer of *Wolbachia* in *C. pipiens* complex mosquitoes is either impossible or occurs rarely. It must be emphasized that not all individuals of *C. pipiens* were infected, therefore the *Wolbachia* infection not always is a reliable assay for the differentiation between these mosquito species.

The *Wolbachia* infection is known to correlate with mitochondrial DNA haplotype (mitotype M) occurring in *C. pipiens*. *C. torrentium*, never infected by *Wolbachia*, is characterized by mitotype P (Vinogradova, Shaikevich, 2007). The comparative analysis of the sequences of the 3'end of cytochrome oxidase I (COI) gene showed that the *C. pipiens* larvae from underground and above-ground populations are similar and, as a rule, differ from *C. torrentium* by six substitutions among 246 nucleotides. After SspI restriction two fragments 221 and 90 bp were obtained from *C. pipiens* DNA, whereas the studied region of COI gene of *C. torrentium* remained uncut. Thus, in this study this region of mtDNA was used as a DNA-marker for differentiation between *C. pipiens* and *C. torrentium* by restrictase analysis. During the analysis of COI gene two types of mtDNA, M and P, corresponding to *C. pipiens* and *C. torrentium*, respectively, were revealed (Tables 1-2).

In contrast with the conservative mtDNA, transcribed spacers of ribosomal DNA are highly variable. Comparison of PCR products of ITS2 in studied populations showed that ITS2 of *C. pipiens* exceeds that of *C. torrentium* approximately in 50bp, this difference arises from the numerous deletions scattered on all region. Autogenous and anautogenous forms (the *molestus* and *pipiens* forms) do not differ by this marker, that was recorded earlier (Vinogradova, Shaikevich, 2005, 2007).

Altogether 321 individuals of *C. torrentium* and 460 individuals of *C. pipiens* were identified using three mentioned markers. Ten homogeneous local populations of *C. torrentium*, 12 populations of *C. pipiens* and 4 heterogeneous populations were recorded (Tables 1-2). Heterogeneous populations were found in above-ground water in Moscow and environs and also in Sverdlovsk Prov. (Table 2, Nos 3, 8, 10, 13).

Polymorphism was noted between three studied members of *C. pipiens* complex in a 603 bp fragment of the 5'end of the mitochondrial COI gene. It was used as the basis for developing polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) assays for the differentiation of *C. pipiens* and *C. torrentium* by BclI restrictase, and anautogenous (*pipiens*) and autogenous (*molestus)* forms by HaeIII restrictase (Shaikevich, 2007). This method was used in the analysis of mosquito populations from Russia. After restriction with BclI of PCR products following the amplification with COIF, COIR primers three fragments (406, 118 and 79 bp) were obtained in autogenous and anautogenous *C. pipiens*, whereas only two fragments were obtained in *C. torrentium* (524 and 79 bp). After restriction of amplificates of DNA of COI gene with endonuclease HaeIII two fragments (206 and 397 bp) were recorded in anautogenous *C. pipiens*, whereas 603 amplicon from autogenous *C. pipiens* remained unchanged (Table 3). Altogether 123 individuals of the *molestus* form and 113 individuals of the *pipiens* form were identified using restrictases BclI and HaeIII.

Thus, the homogeneous larval populations of autogenous form were recorded in underground habitats in eight cities. A detailed study of the interrelation between autogenous and anautogenous forms of *C. pipiens* was carried out in Krasnodar, on the territory of Kuban Agronomic University. Here the autogenous *C. pipiens* inhabits the flooded basements of dwelling houses throughout the year (Table 3, No. 12). In summer it may tem-

Table 3. Fragment sizes after restriction of the 5' COI amplicon in geographical populations of mosquitoes of *C. pipiens* complex. Coordinates: Vladikavkaz - 43°05' N/44°40' E (22); Nal'chik - 43°30' N/43°30' E (19); Kizliyar – 43°50' N/46°30'E (20); the rest see in the Table 1; under – underground; over – overground habitats; No.13 – adults; the other cases – larvae.

No.	Locality	Biotope	Number of specimens	Restriction fragment sizes (bp)		Species, forms (f.)
				BclI	Hae III	
1	Petrozavodsk	under	10	406, 118, 79	603	f. *molestus*
2	St. Petersburg	under	10	406, 118, 79	603	f. *molestus*
3	St.Petersburg envir., Skachki	over	18	524, 79		*C. torrentium*
4	Moscow	under	10	406, 118, 79	603	f. *molestus*
5	Moscow Prov., Luzhki	over	10	406, 118, 79	397, 206	f. *pipiens*
6	Moscow Prov., Chashnikovo	over	10	524, 79		*C. torrentium*
			18	406, 118, 79	397, 206	f. *pipiens*
7	Moscow Prov., Starikovo	over	10	524, 79		*C. torrentium*
			19	406, 118, 79	397, 206	f. *pipiens*
8	N. Novgorod	under	10	406, 118, 79	603	f. *molestus*
9	N. Novgorod	over	3	524, 79		*C. torrentium*
			5	406, 118, 79	397, 206	f. *pipiens*
10	N. Novgorod, Gnilitsi	over	4	524, 79		*C. torrentium*
			5	406, 118, 79	397, 206	f. *pipiens*
11	Volgograd	under	30	406, 118, 79	603	f. *molestus*
12	Krasnodar	under	10	406, 118, 79	603	f. *molestus*
13	Krasnodar	over	31	406, 118, 79	603	f. *molestus*
14	Krasnodar	over	12	406, 118, 79	397, 206	f. *pipiens*
			1	406, 118, 79	603	f. *molestus*
15	Krasnodar	over	8	406, 118, 79	397, 206	f. *pipiens*
16	Krasnodar	over	8	406, 118, 79	603	f. *molestus*
17	Krasnodar	over	8	406, 118, 79	397, 206	f. *pipiens*
			2	406, 118, 79	603	f. *molestus*
18	Kabardino-Balkaria, Nal'chik	over	8	406, 118, 79	397, 206	f. *pipiens*
19	North Ossetiya, Kizliyar	over	8	406, 118, 79	397, 206	f. *pipiens*
20	Stavropol Krai, Georgievsk	over	7	406, 118, 79	397, 206	f. *pipiens*
21	Vladikavkaz	under	5	406, 118, 79	397, 206	f. *pipiens*
			1	406, 118, 79	603	f. *molestus*

porarily develop in above-ground water bodies (barrels) occurring as homogeneous populations (Table 3, No. 16) or combined with the anautogenous form (Table 3, No. 17). Such a heterogeneous population was also recorded in Krasnodar environs (15 km) in a barrel (Table 3, No. 14) located in a settlement with summer cottages (without basements), which suggested the possibility of native or artificial distribution of these mosquitoes from the city. Mass aggregation of autogenous adults was observed in a Krasnodar collector of rain-water, used by mosquitoes as day shelter (Table 3, No.13). By way of exception, a heterogeneous population of both forms developing in basements was revealed in summer in Vladikavkaz (Table 3, No. 21).

Mixed above-ground populations of *C. torrentium* and anautogenous *C. pipiens* were observed in the Moscow environs and in Nizhniy Novgorod and its environs (Table 3, Nos 6, 7, 9, 10). Homogeneous populations of anautogenous *C. pipiens* were found in above-ground water reservoirs in the Moscow environs, Krasnodar and in three cities in Northern Caucasus (Table 3, Nos 5, 15, 18, 19, 20).

DISCUSSION

The distribution of the anautogenous form of *C. pipiens* in the European part of Russia has been thoroughly studied using traditional methods. It was recorded in 10 of 41 European provinces (Vinogradova, 1997). This is a mass mosquito in the Northern Caucasus (the Rostov-na-Donu Province, Krasnodar Krai and Stavropol Krai, the Kabardino-Balkaria, North Ossetia and Dagestan), in the Udmurtia, in Astrakhan, Voronezh and Orenburg Provinces. This mosquito occurs in Moscow, Novgorod, Leningrad, and Sverdlovsk Provinces and in Karelia. The northern limit of its distribution nearly coincides with the Arctic Circle, and this mosquito is found in the southwestern part of Vologda Province and in the Komi Republic (south of the Arctic Circle). The autogenous mosquito (the *molestus* form) has been revealed in the majority of large cities of this part of Russia occurring exclusively in underground habitats (Markovich, Zarechnaya, 1992). Similarity of morphometric (the mean siphonal index of larvae), physiological (the autogeny), molecular-genetic (*Wolbachia* infection, mitotypes and genotypes) features was earlier revealed in ten geographically isolated city populations from the former USSR (Vinogradova, Shaikevich, 2007).

The distribution of *C. torrentium* is less well known, it is recorded in the Leningrad, Novgorod, Moscow (including Moscow and environs), Perm, Sverdlovsk, Penza, Saratov, Volgograd and Orenburg Provinces, and in the North Caucasus (Essentuki, Pyatigorsk) (Olenev, 1940; Vinogradova, 1997; Kunkova, Fedorova, 2003). However, the previous data on distribution of *C. pipiens* and *C. torrentium* may appear to be not quite correct, because their reliable identification is only possible when it is based on males, which was not always the case.

The study of the local populations of mosquitoes using molecular markers allowed to confirm the occurrence of *C. torrentium* in Leningrad Province (Sosnovo settlement), St. Petersburg environs (Skachki settlement), in Moscow environs and in Sverdlovsk Province (Tables 2-3). Homogeneous populations of this species were found for the first time in Karelia, in Yaroslavl environs, and in Ivanovo (Kokhma city), Saratov and Nizhniy Novgorod (Vyksa city, Diveevo settlement) Provinces.

Of some interest is the discovery of mixed populations including *C. torrentium* and anautogenous *C. pipiens*, and no hybrids between them were recorded. They were found in Moscow (Ostankino) and its environs (Chashnikovo and Starikovo settlements), and in Sverdlovsk Province (Talitsa city). In above-mentioned mixed populations 62% of larvae were *C. torrentium* and 38% – *C. pipiens* (a total of 165 specimens). In Russia such populations were recorded in Leningrad and its environs (Fedorov, 1983; Vinogradova, Oda, 1990).

Earlier the coexistence of these mosquito species confirmed by the structure of the male genitalia has been shown in the Botanical garden of Uppsala, Sweden (Raymond, 1995) and in southern England. In the latter case in 1964-1967 the proportion of C. torrentium in mixed populations from seven settlements was on the average 37% (Service, 1968), but in 1974-1975 in three other settlements – 80% (Gills, Gubbins, 1982). It is noteworthy that *C. torrentium* and anautogenous *C. pipiens* have similar biological characteristics, seasonal development and also common larval habitats including highly polluted water-bodies (Service, 1968; Ishii, Sohn, 1987; Dahl, 1988). The only difference concerns the swarming places of mosquitoes (Service, 1994). This raises the question of some isolating mechanisms permitting the coexistence of these sibling species. The cytoplasmic incompatibility resulting from crosses between *C. pipiens* infected by Wolbachia and uninfected *C. torrentium* is probably one of such mechanisms (Vinogradova et al., 2002). Such a situation was observed in experiments: only a unidirectional cross succeeded when males of *C. torrentium* from Almaty, Kazakhstan, readily mated with autogenous females of *C. pipiens*. Even then, the reproductive activity of the hybrid mosquitoes was considerably reduced as compared with that of parents (Vinogradova, 1997).

The mixed populations consisting of the autogenous and anautogenous *C. pipiens* were discovered in Krasnodar. Similar populations were earlier recorded in open water-bodies in Moscow, St. Petersburg, in the south of Russia (Novocherkassk) and also in Uzbekistan (Samarkand) and Kazakhstan (Almaty) (Vinogradova, 1997).

It is known that the level of reproductive isolation between these forms decreases from north to south of the species range (Farid et al., 1991; Byrne, Nichols, 1999). In the temperate belt autogenous and anautogenous *C. pipiens* are strongly isolated from one another in spite of their sympatric occurrence. This may be due to at least three isolating mechanisms: biotopical specialization (under- or above-ground water-bodies), the pattern of precopulation behaviour (stenogamy versus eurygamy) and the diapause inheritance type in crosses between *pipiens* and *molestus* forms (Vinogradova, 1997). Though there is no significant evidence of occurrence of hybrids between them in nature, however, in cages, where mosquitoes search and contact between partners are facilitated, crosses may be successful in one or both directions, depending on the geographical origin of mosquitoes, but the reproductive capacity and survival of hybrids are considerably reduced (Vinogradova, 1997).

In conclusion it may be said that molecular methods of diagnostics used, in spite of their being labour-intensive and expensive, are undoubtedly promising in the study of members of *C. pipiens* complex, whose identification presents difficulty with traditional taxonomy. Molecular markers allow refinement of the taxonomic rank of both species (*C. pipiens* and *C. torrentium*) and intraspecies (the *pipiens* and *molestus* forms) levels of any developmental stages of mosquitoes. These methods proved efficient in study of the geographical distribution and structure of local populations of *C. pipiens* complex mosquitoes in Russia.

Acknowledgements

We thank Natalia V. Kol for the assistance in carrying out some molecular assays. We are also grateful to the anonymous reviewers for their valuable comments to the manuscript. The study was supported by the Russian Foundation for Basic Research (Grant No. 05-04-49035) and the Leading Scientific Schools (Grant No. 827.2006.4).

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Received May 3, 2007.
Accepted by V.G. Kuznetsova, August 10, 2007.
Published December 28, 2007.