© Comparative Cytogenetics, 2010 . Vol. 4, No. 2, P. 161-174. ISSN 1993-0771 (Print), ISSN 1993-078X (Online)

Polymorphism of mitochondrial *COI* and nuclear ribosomal *ITS2* in the *Culex pipiens* complex and in *Culex torrentium* (Diptera: Culicidae)

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Abstract. Polymorphism of the mtDNA gene *COI* encoding cytochrome C oxidase subunit I was studied in the mosquitoes *Culex pipiens* Linnaeus, 1758 and *C. torrentium* Martini, 1925 from sixteen locations in Russia and in three laboratory strains of subtropical subspecies of the *C. pipiens* complex. Representatives of this complex are characterized by a high ecological plasticity and there are significant ecophysiological differences between its morphologically similar members. The full-size DNA sequence of the gene *COI* spans 1548 bp and has a total A+T content of 70.2 %. The TAA is a terminating codon in all studied representatives of the *C. pipiens* complex and *C. torrentium*. 64 variable nucleotide sites (4 %) were found, fifteen haplotypes were detected, and two heteroplasmic specimens of *C. torrentium* were recorded. *COI* haplotype diversity was low in *Wolbachia*–infected populations of the *C. pipiens* complex. Monomorphic haplotypes were found in *C. p. quinquefasciatus* and *C. p. pipiens* f. *molestus*. Three haplotypes were detected for the *C. p. pipiens,* but these haplotypes were not population-specific. On the other hand, each of the ten studied *Wolbachia*-uninfected *C. torrentium* individuals from three different populations had unique mitochondrial haplotypes. Polymorphism of the 478-bp *ITS2* nucleotide sequences was similar in infected *C. p. pipiens* and *C. p. pipiens* f. *molestus* and uninfected *C. torrentium* specimens. The *ITS2* genetic distance between *C. p. pipiens* and *C. torrentium* reached 12.5 %. Possible effects of *Wolbachia* invasion on *C. pipiens* populations are discussed.

Key words: *Culex pipens*, *C. torrentium*, *Wolbachia,* cytochrome C oxidase I, mtDNA, *ITS2*, polymorphism.

Introduction

Mosquitoes of the genus *Culex* Linnaeus, 1758 are active bloodsuckers and serve as vectors of many human and animal infections, such as West Nile fever, filariasis, encephalitis, etc. In this respect, the *Culex pipiens* species complex is of special interest, since the species show notable behavioral, morphological and physiological polymorphism in different parts of the inhabited area and have different epidemiological significances. The complex includes widely distributed species: *C. p. pipiens* Linnaeus, 1758, *C. p. quinquefasciatus* Say, 1823, *C. p. pallens* Coquillett, 1898 and *C. p. australicus* Dobrotworsky et Drummond 1953; a classification also exists in which the closely related species *C. torrentium* Martini, 1925 and *C. vagans* Wiedemann, 1828 are included in this complex (Vinogradova, 2000). No samples of *C. p. australicus* and *C. vagans* were available for this work. Though morphological variations between the species

and between the forms are negligible, notable ecophysiological difference does exist. *C. p. pipiens* includes two biotypes, these are *C. p. pipiens* and *C. p. pipiens* f. *molestus* Forskal, 1775. The mosquitoes of these two forms have different biological features. *C. p. pipiens* are non-autogenous (unable to lay the first portion of the eggs without taking a blood meal), diapausing (have a reproductive diapauses) and eurygamous mosquitoes (need large space for mating with swarming), while *C. p. pipiens* f. *molestus* are autogenous (lay the first portion of the eggs without preceding blood meal), non-diapausing (active all-year-round), and stenogamous (able to copulate within a limited space, without swarming). Within the temperate zone, these forms are biotopically isolated: *C. p. pipiens* mosquitoes inhabit the open water bodies, while *C. p. pipiens* f. *molestus* mosquitoes prefer underground water bodies (usually in basements of multistoried town buildings); on the other hand, both forms are found together in the subtropical zone. Both forms are active bloodsuckers and act as disease transmission vectors. *C. p. quinquefasciatus* and *C. p. pallens* are stenogamous, like *C. p. pipiens* f. *molestus,* and non-autogenous like *C. p. pipiens*. Diapause is absent in *C. p. quinquefasciatus*, but *C. p. pallens* may diapause.

C. torrentium and *C. p. pipiens* seem to be closely related species. Under laboratory condition, these two taxa can cross, but the hybrids are not viable (E.B. Vinogradova, personal communication). *C. torrentium* and *C. p. pipiens* have similar biological features: both are non-autogenous, diapausing and eurygamous. *C. torrentium* is widely distributed in the Palearctic, and both species often inhabit the same water bodies. Only minor morphological differences exist in their male genitalia. Similarities are often results in misidentification of these two species. However, genetic differences allow precise distinction of these species using the appropriate DNA markers (Fedorova, Shaikevich, 2007).

Mitochondrial DNA is often used in phylogenetic studies of insects, including mosquitoes (e.g., Morlais, Severson 2002; Rasgon et al., 2006; Cywinska et al., 2006; Kumar et al., 2007; Paudan, Ribolla, 2008). It is believed that nucleotide substitutions in mtDNA occur with about constant rate in the evolutionary time, and this allows one to estimate the time of a divergence (DeSalle et al., 1987). The gene encoding cytochrome oxydase I (*COI*) is the largest and the most conserved of the three mitochondrial genes coding for cytochrome oxydase subunits (Beard et al., 1993). The insect cells usually have one mitochondrial haplotype per individual, though heteroplasmia has been reported (White et al., 2008; Paudan, Ribolla 2008; Savamura et al., 2008).

It is known that *C. pipiens* are infected with intracellular symbiotic bacteria, *Wolbachia* Hertig, 1936. These bacteria, first discovered in mosquitoes of the genus *Culex* (Hertig, Wolbach, 1924), are maternally transmitted and may cause cytoplasmic incompatibility (e.g., Guillemaud et al., 1997; Sinkins et al., 2005; Walker et al., 2009). If the infected males are crossed with uninfected females, all the offspring die at the embryo stage, while a reciprocal cross produces normal progeny. Consequently, the infected females in mosquito populations possess reproductive advantage compared with uninfected females. Similar to mitochondria, the intracellular symbiotic bacteria are transmitted transovarily, while horizontal transmission of the symbionts is rare. Correlation of *Wolbachia* occurrence with a certain variant of mitochondrial haplotype was detected as “linkage disequilibrium” in some insect species (Montchamp-Moreau et al.,

Table 1. Sampling locations and *Wolbachia* infection in the studied mosquito populations.

	Form	Population origin	Abbreviation	Biotope	Latitude/ longitude	Provided by	Wolbachia Infection
1	*C. p. pipiens*	Russian, Iksha, Moscow Region	PIP1	Open, suburban	55°10' N/ 37°31' E	M. Fedorova .	100%
2	*C. p. pipiens*	Russian, Luzki, Moscow Region.	PIP2	Open, suburban	55°51' N/ 36°57' E	M. Fedorova	90%
3	*C. p. pipiens*	Russian, Sarepta, Volgograd Region.	PIP3	Open, suburban	48°31' N/ 44°30' E	M. Fedorova	100%
4	*C. p. pipiens*	Russian, Liteishik, Volgograd Region	PIP4	Open, suburban	48°45' N/ 44°42' E	M. Fedorova	100%
5	*C. p. pipiens f. molestus*	Russian, Moscow	MOL1	Basement, urban	55°55' N/ 37°20' E	E. Vinogradova	96%
6	*C. p. pipiens f. molestus*	Russian, Nizhniy Novgorod	MOL2	Basement, urban	56°20' N/ 44°00' E	E. Vinogradova	100%
7	*C. p. pipiens f. molestus*	Russian, Saint-Petersburg	MOL3	Basement, urban	59°56' N/ 30°15' E	E. Vinogradova	94%
8	*C. p. pipiens f. molestus*	Russian, Petrozavodsk	MOL4	Basement, urban	61°47' N/ 34°20' E	E. Vinogradova.	90%
9	*C. p. pipiens f. molestus*	Russian, Volgograd, ul Rokossovskogo	MOL5	Basement, urban	48°44' N/ 44°31' E	M. Fedorova	100%
10	*C. p. pipiens f. molestus*	Russian, Volgograd, ul. Aldanskaya	MOL6	Open, urban	48°31' N/ 44°30' E	M. Fedorova	100%
11	*C. p. quinquefasciatus*	India, Hyderabad	QUIN1	Lab culture	17°37' N/ 78°48' E	E. Vinogradova	80%
12	*C. p. quinquefasciatus*	India, Pondicherry	QUIN2	Lab culture	12°25' N/ 80°41' E	E. Vinogradova	42%
13	*C. p. pallens*	Japan, Ohno-cho, Hiroshima	PAL1	Lab culture	34°24' N/132°27'E	E. Vinogradova	90%
14	*C. torrentium*	Russian, St.69 km, Leningrad Region	TOR1	Open, suburban	60°49' N/ 30°10' E	E. Vinogradova	0%
15	*C. torrentium*	Russian, Chashnikovo, Moscow Region	TOR2	Open, suburban	56°01' N/ 37°10' E	M. Fedorova	0%
16	*C. torrentium*	Russian, Saratov Region	TOR3	Open, suburban	51°34' N/ 46°02' E	N. Polukonova	0%
17	*C. torrentium*	Russian, St. Skachki, Leningrad Region	TOR4	Open, suburban	59°45' N/ 30°07' E	E. Vinogradova	0%

1991; Schulenburg et al., 2002; Jiggins 2003; Rasgon et al., 2006). It was suggested that the lines of an insect host species formed after infection gain some competitive advantage(s). As a result of expansion of the special mtDNA variant, which is spread with the bacteria, polymorphism in the infected insect population will be lower than in the uninfected populations. This work aimed to analyze mtDNA variability basing on polymorphism study in the mtDNA gene *COI* in *Culex pipiens* complex and *C. torrentium* and to discuss evolutionary significance of *Wolbachia* infection in these mosquitoes.

Material and methods

The mosquitoes of the *C. pipiens* complex were collected in geographically distant areas (Table 1). The mosquitoes were identified by E.B. Vinogradova and M.V. Fedorova. DNA was isolated from ethanol-preserved mosquitoes using the DIAtom™ DNA

Prep Kit (Isogen, Moscow, Russia) and the polymerase chain reaction was run in the thermocycler GeneAmp^R PCR System 2700 (Applied Biosystems, Foster City, CA, USA) with the amplification kits GenePak™ PCR Core (Isogene, Moscow, Russia).

The mosquitoes were tested for *Wolbachia* infection using PCR with primers wsp81F and wsp691R, complementary to a gene *wsp* (Braig et al., 1998). The *ITS2* region was amplified using primers complementary to the 5.8S and 28S rRNA (Porter, Collins, 1991). The mtDNA gene *COI* was amplified using primers TY-J-1460 and UEA10 (Bernasconi et al., 2000) and CulexCOIF and Culex COIR (Shaikevich, 2007). The primers specially designed in this work for sequencing the full-size gene *COI*:

CulexCOIR1: 5'-TCTACTGAAGCTCC AGCATG-3', CulexCOIF2: 5'-GTAGTAAT TACTGCAGTTTTA-3', CulexCOIR2: 5'-CA AATAATGAAATTGTTCTACC-3'.

PCR amplification reactions were carried out in a final volume of 25 µl with PCR buffer (Isogen, Russia), 200µM of each dNTP, 2.5 mM MgCl2, one unit of Taq DNA polymerase, 5 pmol of each primer, and 0.1 µg of the isolated DNA. PCR conditions were the following: primary denaturing, 5 min at 94°C; 35 cycles: denaturing at 94°C, 30 s, annealing at 55°C, 40 s, elongation at 72°C, 40 s; final elongation at 72°C, 10 min. The amplicons were revealed by electrophoresis in 1% agarose gel (Sigma, St. Louis, Mo, USA). The amplified DNA fragments were isolated from the gel using JETQUICK Gel Extraction Spin Kit (Genomed, Löhne, Germany) for subsequent sequencing. Ten DNA samples were sequenced for each native population of *C. p. pipiens* and *C. p. pipiens* f. *molestus, C. torrentium,* and two DNA samples for laboratory cultures*: C. p. quinquefasciatus* and *C. p. pallens.* The sequencing was run from both primers on an ABI 310 automated sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The newly determined nucleotide sequences of the *COI* gene were submitted to GenBank under numbers FN395171-FN395206.

Table 2. Base composition in the *C. pipiens COI* gene at the three codon positions (%).

Position	A	T	C	G
1st	27.4	31	15.6	26.5
2nd	17.3	42	25.5	15.6
3rd	47.6	46	4.0	2.2
Avg.	30.7	39.5	15.0	14.8

The obtained sequences were analyzed using Chromas software (http://www.technelysium.com.au), phylogenetic analyses were conducted in MEGA4 (Tamura et. al. 2007). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of *Culex* complex. The evolutionary distances were computed using the Neighbor-Joining method with Maximum Composite Likelihood substitution model.

Results

Polymorphism of *COI* and *Wolbachia* infection in mosquitoes of the *C. pipiens* complex and *C. torrentium*

Not less than 20 mosquitoes from a population were analyzed to evaluate infection with *Wolbachia* using the PCR primers complementary to the gene *wsp* which encodes a *Wolbachia* cell wall protein. The mosquitoes from all studied populations of *C. p. pipiens* and *C. p. pipiens* f. *molestus*, *C. p. quinquefasciatus* and *C. p. pallens* were infected with *Wolbachia* (Table 1). The proportion of infected mosquitoes varied between the populations, being however always high (90-

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                                                    111111111111111111111111
                  12223333445555566666667777888999990000011111222222333345     34
          1156677015713560745780011578444824527899024559127781223790135692   2291
          2810369232059735405723689157147386542139207068624952893505450042   6720
PIP2-7    ACTTGAGACGGTTAGTTTATTTTGTCTTAGTAAGTATACATAAAATTTACTCATAAAATCTTTT   MVII
PIP2-16   G......................A........................................   ....
PIP2-19   G......................A........................................   ....
PIP1-3    ................................G...............................   ....
PIP1-4    ................................G...............................   ....
PIP1-6    ................................................................   ....
PIP1-7    ................................................................   ....
PIP1-8    ................................G...............................   ....
PIP3      ................................................................   ....
PIP4      ................................G...............................   ....
MOL1      ..........A...............................G.....................   ....
MOL2      ..........A...............................G.....................   ....
MOL3-10   ..........A...............................G.....................   ....
MOL3-36   ..........A...............................G.....................   ....
MOL4      ..........A...............................G.....................   ....
MOL5      ..........A...............................G.....................   ....
MOL6-1    ..........A...............................G.....................   ....
MOL6-2    ..........A...............................G.....................   ....
MOL6-3    ..........A...............................G.....................   ....
MOL6-4    ..........A...............................G.....................   ....
QUIN1-12  .............G.........................G........................   ....
QUIN1-23  .............G.........................G........................   ....
QUIN2-12  .............G.........................G........................   ....
QUIN2-20  .............G.........................G........................   ....
PAL-2     ................................................................   ....
PAL-4     ................................................................   ....
TOR1-1    .TCCACATT.ACA.ACACTCCGAACTCCT.CT.AA.CGTTCG.TG.CCG.CTGCGTTTCTCCCC   LIVA
TOR1-10   .TCCA.ATT.AC..A.A.TCCGAACTCCT.CT.AA.C.TTC..T.CCCGT.TGC.TTTCTCC.C   .IVA
TOR1-29   .TCCA.ATT.AC..A.A.TCCGAACT.CT.CT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
TOR2-14   .TCCA.ATT.AC..A.A.TCCGAACT.CTACT.AA.C.TTC..T.CCCG.C.G..TTTCTCCCC   .IVV
TOR2-13   .TCCA.ATT.AC..A.A.TCCGAACT.CTACT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
TOR2-27   .TCCA.ATTA.C..A.A.TCCGAACT.CTACT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
TOR2-28   .TCCA.ATT.AC..A.A.TCCGAACT.CTACT.AAGC.TTC..T.CCCG.C.G..TTTCTCC.C   .IVV
TOR3-4    .TCCA.ATT.AC..A.ACTCCGAACT.CTACT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
TOR3-5    GTCCA.ATT.AC..A.A.TCCGAACT.CTACT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
TOR3-7    .TCCA.ATT..C..A.A.TCCGAACT.CTACT.AA.C.TTC..T.CCCG.CTG..TTTCTCC.C   .IVV
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Fig. 1. Alignment showing 64 variable nucleotide sites and four variable amino acid sites of the gene *COI* from mosquitoes of the *C. pipiens* compex: *C. p. pipiens* (PIP), *C. p. pipiens* f. *molestus* (MOL), *C. p. quinquefasciatus* (QUIN), *C. p. pallens* (PAL) and *C. torrentium* (TOR). Numbers correspond to population identifiers in Table 1; sample numbers are also shown.

100%). The lowest percentage of infected individuals was in one of the studied laboratory cultures of *C. p. quinquefasciatus*. The DNA of all the uninfected individuals was positive for PCR amplification using primers for *COI*, indicating satisfactory DNA template quality. No infection was detected in *C. torrentium.*

We obtained the full-size DNA sequence of the gene *COI* encoding cytochrome oxydase subunit I in mosquitoes of the *C. pipiens* complex and *C. torrentium.* The gene *COI* is flanked by *tRNA-Tyrosine* at the 5' end and *by tRNA-Leucine* at the 3'end. Since amplification and subsequent DNA sequencing were run using the primer TY-J-1460 complementary to tRNA-Tyr located upstream of the *COI* gene, it was possible to identify the hexanucleotide ATTTAA, followed by the codon TCG which is the start codon for the gene *COI*. The primer UEA10 complementary to tRNA-Leu located downstream of the gene *COI* allowed us to determine the termination codon TAA. The

gene *COI* in the studied mosquitoes spans 1548 bp. DNA of this gene has a total A+T content of 70.2%. A and T are located mainly at the third codon positions (Table 2).

Polymorphism of the mtDNA was studied by comparison of the DNA structure of the *COI* gene in mosquitoes of the *Culex pipiens* complex and *C. torrentium*, collected in sixteen geographically distant areas (Table 1). In total, we found 64 variable nucleotide sites (4 %) in the 36 studied DNA sequences; among these 54 (3.5 %) were considered informative under the conditions of parsimony (Fig. 1).

The subspecies of *C. pipiens* have 7 variable sites in the gene *COI* (Fig. 1). All the detected nucleotide substitutions were transitions A↔G located at the third codon position and not changing the amino acid sequence. The mosquitoes of *C. p. pipiens* have three variable sites characterizing three haplotypes: A, B, and C. These haplotypes were not population-specific (Fig. 2). Haplotype A was found in mosquitoes from two Moscow Region populations, PIP1 (PIP1-6, PIP1-7) and PIP2 (PIP2-7), and from a Volgograd population PIP4 (PIP4-17); this haplotype was also detected in two studied individuals of *C. p. pallens.* Other mosquitoes from the population PIP2 (PIP2-16, PIP2-19) had haplotype B which differs from haplotype A by two transitions. Haplotype C was found in mosquitoes from PIP1, Moscow Region (PIP1-3, PIP1-4, PIP1-8) and from the PIP4 population, Volgograd (PIP4-13). All the studied mosquitoes *C. p. pipiens* f. *molestus* were identical and formed cluster D. The urban mosquitoes studied in this work were collected from geographically remote locations in Petozavodsk, St. Petersburg, Moscow, Nizhniy Novgorod, and Volgograd. All the *C. p. pipiens* f. *molestus* mosquitoes differ from *C. p. pipiens* by the two fixed nucleotide substitutions in positions 270 (G→A) and 1047 (A→G) (Fig. 1). The mosquitoes *C. p. quinquefasciatus* from the laboratory lines Hyderabad and Pondicherry (India) are identical and differ from the other subspecies by two transitions (A→G) in positions 357 and 999 (Fig. 1). They form haplotype cluster E.

The detected nucleotide substitutions for *C. p. pipiens* f. *molestus* and *C. p. quinquefasciatus* are strictly subspecies-specific. Consequently, the haplotype has no correlation with geographical coordinates of the area, being correlated with subspecies within the species complex.

The mosquitoes of the *Wolbachia* – uninfected species *C. torrentium* showed considerable polymorphism of the gene *COI.* Each of the ten studied individual mosquitoes from three populations: Leningrad Region (TOR1-1, TOR1-10, TOR1-29), Moscow Region (TOR2-13, TOR2-14, TOR2-27, TOR2-28), and Saratov Region (TOR3-4, TOR3-5, TOR3-7) have a different haplotype (F-O, Fig. 2). The DNA sequence of the gene *COI* for an individual *C. torrentium* differs by 1-13 nucleotide substitutions from *COI* sequence of other individual mosquitoes, both within the same population and between populations (Fig. 1). Among 21 variable sites, 19 nucleotide substitutions are at the third codon positions, one at the first and one at the second. Only one transition G→ A at position 744 is common for all three studied mosquitoes from population TOR1, Leningrad Region, and not detected in mosquitoes from the Moscow and Saratov Regions. Translation to amino acid sequence showed that *C. torrentium,* compared with subspecies *C. pipiens*, has amino acid substitutions at four sites. Two valine-isoleucine substitutions (Val↔Ile, Ile↔Val) are specific for all mosquitoes of this species. An additional amino acid substitution, valine for alanine (Val↔Ala) was found in two *C. torrentium* mosquitoes from the population

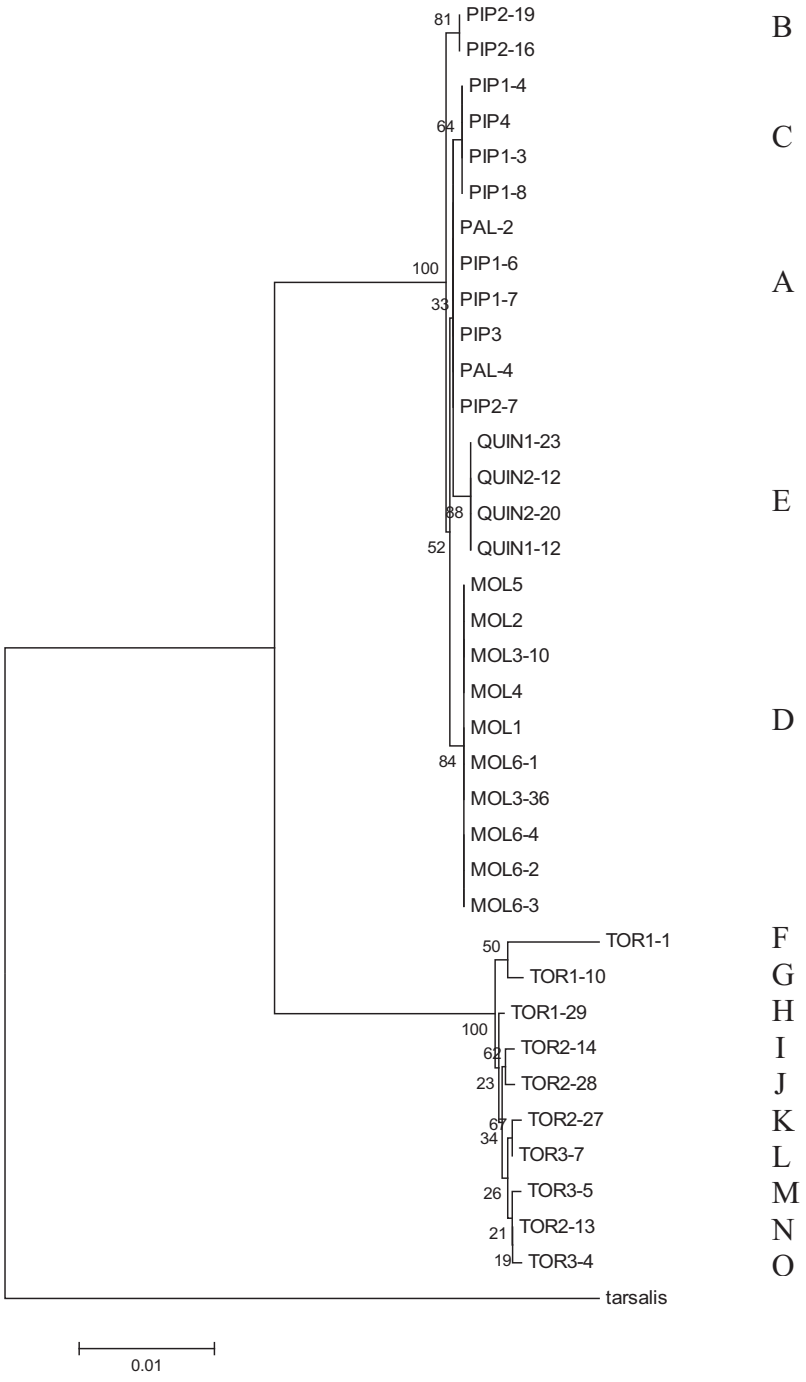

Fig. 2. The similarity dendrogram obtained for nucleotide sequences of the gene *COI* from mosquitoes of the *C. pipiens* complex. The Neighbor-Joining method was used. Numbers are bootstrap coefficients calculated for 1000 repeats. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The haplotypes are marked by letters on the right. The nucleotide sequence of homologous fragment of the gene *COI* from *C. tarsalis* (AF425847) is used as an external reference.

Table 3. Variable sites at the 5' region of the gene *COI* from *C. torrentium*.

Polimorfic sites	Nucleotide															Amino Acid			
sample/site	12	76	242	252	270	315	322	327	339	405	540	649	657	675	678	26	81	108	217
TOR2-13	A	A	T	G	A	C	A	A	T	T	T	T	T	T	A	M	V	T	F
TOR2-14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR2-27	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR2-28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR1-1	.	C	.	.	.	.	.	.	A	C	C	.	.	C	.	L	.	.	.
TOR1-10	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
TOR1-29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR3-4	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
TOR3-5	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR3-7	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR4-14-1	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.
TOR4-14-2	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TOR4-15-1	.	.	.	.	.	T	.	G	.	.	.	.	.	C	.	.	.	.	.
TOR4-15-2	.	.	C	.	G	.	.	.	.	.	.	A	C	.	G	.	A	.	I

TOR1. One more substitution of methionine with leucine (Met↔Leu) was found in mosquito TOR1-1 (Fig. 1).

Heteroplasmia in *C. torrentium*

Among mosquitoes collected in the Leningrad Region, two individuals with heteroplasmia were found. Both were *C. torrentium* collected at Station Skachki (TOR4). Using the PCR-RFLP method (Shaikevich 2007), two individual mosquitoes (TOR4-14 and TOR4-15) from *C. torrentium* populations were found to have more than one mtDNA sequence (Table 3). Treatment of the amplified DNA products with *Hae*III restriction endonuclease produced three fragments (725, 455, and 270 bp). Cloning of the PCR products showed that the *Hae*III restriction site was present in some clones and absent in other clones. After amplification and subsequent restriction, some clones were digested into two fragments, 455 and 270 bp, while other clones remained uncut (725 bp). Nucleotide sequences of the 5- end of the gene *COI* were obtained for clones from two individual mosquitoes, TOR4-14 and TOR4-15. We found 10 variable sites and 4 types of mtDNA gene *COI* sequence: clones TOR4-14-1 and TOR4-14-2, TOR4-15-1 and TOR4-15-2. Each individual mosquito has two types of sequences. Nine transitions were found: four G↔A and three T↔C at the third codon position, one G↔A at the first position, and one T↔C at the second position. One transversion A↔T is located at the first codon position. All substitutions at the third codon position do not affect the amino acid sequence. Transition G↔A at the first position results in Val substitution with Ala in TOR4-14-1. Transition T↔C at codon position 2 results in Thr substitution with Ala, and transversion A↔T substitutes Phe with Ile in TOR4-15-2 (Table 3).

Comparing the DNA sequences of the 5' ends of the *COI* gene of the two heteroplasmic mosquitoes from the population TOR4 and all the other studied *C. torrentium* specimens,

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[         1111111111111111122222222222222222222222223333333333333333333333333333
[         1111118888999999901111111222233333344444444000011111111112222222222333333
[         0245672679123456732345689256945678901234567678901234567890123456789012345
#PIP1-4   AGAGT-GTTTGTTCG-CGGTGCGACACGTGGACGACGACG---CGC-CACGT--CGTCGTCGTCACACGTTCG
#PIP2-7   .....-.........-.....................---...-.....--...................
#MOL4     .....-.........-.....................ACG...---...CA...................
#MOL5     .....-.........G.....................ACG...---...CA...................
#MOL2     .....-.........G.....................ACG...---...CA...................
#PIP3     .....-.........-.....................---...-.....--......---..........
#MOL3-10  .....-.........G.....................---...-.....--...................
#MOL1     .....-.........-.....................---...-.....--...................
#TOR2-14  GA.--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----
#TOR2-27  GAG--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----
#TOR1-1   GAG--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----
#TOR1-10  GAG--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----
#TOR3-4   GAG--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----
#TOR3-5   GAG--CTGCC-------A-----GTGACCTCC.--T....----------TCT---.G...T.GG.GT-----

         3333333333333333333333333333333333333333333333333444444444444444444444444]
         4444444555555566666666777777777888888899999999000000011111222222223337]
         3456789012567801245678123456789012348901356789012345701235012345680128]
#PIP1-4   ATCCGGCGTC-CGCGTACGCGTACAGAACAGAACAAAAC-CCGAGCATGCG-TACAACAGATAAACCC-T
#PIP2-7   ..........-..............................-...........-..............-.
#MOL4     ..........-..............................-.......-...C..............C.
#MOL5     ..........-..............................-.......-...C..............C.
#MOL2     ..........-..............................-.......-...-..............C.
#PIP3     ..........-..............................-.......-...-A.............-.
#MOL3-10  ..........-..............................-.......-...-..............C.
#MOL1     ..........-..............................-...........-..............C.
#TOR2-14  ----------CGCGTACGCGTCC.G-----------G.AATTATAT..A--GGGAGC.-------AA.C.
#TOR2-27  ----------CGCGTACGCGTCC.G-----------G.AATTATAT..ATAGG.-GC--------AA.C.
#TOR1-1   ----------CGCGTACGCGTCC.G-----------G.AATTATAT..ATAGG.---A-------AA.C.
#TOR1-10  ----------CGCGTACGCGTCC.G-----------G.AATTATAT..ATAGG.---A-------AA.C.
#TOR3-4   ----------CGCGTACGCGTCC.G-----------G.AATTAT----A.AGG.---.-------AA.C.
#TOR3-5   ----------CGCGTACGCGTCC.G-----------G.AATTATAT..A--GG.---.-------AA.C.
```

Fig. 3. Alignment showing variable nucleotide sites of the *ITS2* region from mosquitoes of the *C. pipiens* compex: *C. p. pipiens* (PIP), *C. p. pipiens* f. *molestus* (MOL) and *C. torrentium* (TOR). Numbers correspond to population identifiers in Table 1; sample numbers are also shown.

we found 15 variable sites and 11 types of the *COI* gene sequence. Variations in nucleotide composition for this fragment of the gene *COI* between mosquitoes from all studied populations are 0 – 0.6 %. The DNA sequences of mosquitoes TOR4-14 and TOR4-15 are different from DNA of other *C. torrentium* specimens by the higher frequency of amino acid substitutions. Comparison of the amino acid sequences of the 5' end of the gene *COI* shows that among studied individual mosquitoes of *C. torrentium* the main haplotype can be recorded (Table 3). A deviation from the main type (an amino acid substitution) was found only in one haplotype (TOR1). In two individuals from population TOR4, heteroplasmia results in two more variants of mtDNA, TOR4-15-2 with two amino acid substitutions and TOR4-14-1 with one.

Ribosomal DNA variability

The DNA variations between infected and uninfected mosquitoes were evaluated by comparison of 478bp nucleotide sequence of

the region of the second internal transcribed spacer (*ITS2*) in ribosomal RNA of *C. p. pipiens* (both forms) and *C. torrentium*. This region is variable and convenient for phylogenetic modeling, since a large set of data is available for the *ITS2* nucleotide sequences for the mosquitoes of the genus *Culex* from all over the world.

The mosquitoes *C. p. pipiens* from Petrozavodsk, Saint-Petersburg, Moscow, Nizhniy Novgorod and Volgograd urban underground populations on one hand, and from open suburban habitats from Moscow and Volgograd regions on the other hand differ in ACG, CGT and CA repeats, located in the downstream part of the studied fragment. Single nucleotide indels are also common for this fragment. The highest *ITS2* genetic distance between specimens of *C. p. pipiens* and *C. p. pipiens* f. *molestus* from various studied populations was found to be 2.3 % (Fig. 3). Such a high level of *ITS2* genetic variability is common for mosquitoes from both ecotypes and, contrary to mtDNA, *ITS2* cannot serve as a marker for detection of species and subspecies of *Wolbachia*-infected representatives of the complex. Similar high level within-species *ITS2* genetic distances (about 3%) was earlier found in C. *p. pipiens f. molestus* from four Russian towns (Vinogradova, Shaikevich, 2007). This fits well with the results obtained for American mosquitoes of the *C. pipiens* complex (Miller et. al. 1996).

The *ITS2* region from *C. torrentium* is 52-54 bp shorter than in *C. p. pipiens*; this difference is explained by multiple deletions in *C. torrentium*. The 3' end of the *ITS2* region in *C. torrentium* contains dinucleotide repeats in various combinations (Fedorova, Shaikevich, 2007). Variability of the *ITS2* region in the studied specimens of *C. torrentium* is 2 %, and the difference between *C. p. pipiens* and *C. torrentium* reaches 12.5 % (Fig. 3).

Discussion

For the first time we obtained the sequence of the complete mitochondrial gene *COI* from mosquitoes of the *C. pipiens* complex and *C. torrentium.* In earlier studies, the initiation codon TCG with the upstream ATTTAA sequence was detected in the gene *COI* from many species of Diptera, e.g. *Anopheles gambiae* Giles, 1902, *A. quadrimaculatus* Say, 1824 and *Aedes aegypti* (Linnaeus, 1762); however, the termination codon for these species was represented by single nucleotide T (Lunt et al., 1996; Bernasconi et al., 2000; Morlais, Severson, 2002). We have shown that according to the invertebrate mtDNA genetic code the TAA triplet is a terminating codon for the gene *COI* in all studied representatives of the *C. pipiens* complex and *C. torrentium*. The full-size gene *COI* spans 1548 bp and encodes 516 amino acids. Mutations are evenly distributed along the whole sequence, and more variable regions cannot be defined.

In 16 populations of the studied mosquito species 15 haplotypes were found (A-O), ten of these were detected in the *Wolbachia* – uninfected population of *C. torrentium*. Monomorphic haplotypes were found in *C. p. quinquefasciatus* (E) and *C. p. pipiens* f. *molestus* (D). Three haplotypes were detected for the *C. p. pipiens* (A, B, C); these haplotypes were not population-specific (Fig. 2). *C. p. pallens* was identical with the main haplotype of *C. p. pipiens* (A). Monomorphism in the studied samples of *C. p. quinquefasciatus,* as well as in *C. p. pallens* may result from the fact that we studied laboratory cultures and analyzed few individual mosquitoes. Polymorphism in populations of *C. p. pipiens* (the presence of three haplotypes) could arise after infection of the ancestral organisms by *Wolbachia.* Similarity of *C. p. pipiens* f. *molestus* suggests that the form *C. p. pipiens*,

inhabiting the underground environment and sometimes in summer in the south regions the open urban environment in Russia, originated rather recently; the populations inhabiting the vast area are of common origin.

Therefore, the intraspecific variability in nucleotide sequences on the mtDNA gene *COI* is low in *Wolbachia* – infected geographically remote mosquito populations of the *C. pipiens* complex. Earlier it was shown that the *Wolbachia*-infected *C. p. pipiens* and *C. p. quinquefasciatus* have considerably lower polymorphism of the mtDNA gene encoding NADH dehydrogenase subunit 4 (*ND4*), compared with uninfected population of *C. p. pipiens* from South Africa. The fragment of the gene *ND4* showed no difference for the infected *C. p. pipiens* and *C. p. quinquefasciatus* (Rasgon et al., 2006). In the studied gene *COI, C. p. pipiens* and *C. p. quinquefasciatus* differ by two fixed nucleotide substitutions.

Among *C. pipiens* subspecies, all detected substitutions were found to be A↔G transitions located at the third codon position and not affecting the amino acid sequence. It is known that the guanine bases are under maximal mutation pressure in human mtDNA, and the mutation spectra are shifted toward G→A transitions (Malyarchuk, 2005). This is probably also true for insects, and this explains why in our study the transitions A↔G are much more frequent than other mutations.

The Neighbor-Joining dendrogram demonstrating the relation of haplotypes within the studied *Culex* shows two main clusters: the first is formed by *Wolbachia*-infected subspecies of *C. pipiens,* and the second includes *Wolbachia* -uninfected *C. torrentium.* It should be noted that polymorphism of the mtDNA gene *COI* in *C. torrentium* includes not only nucleotide differences, but also some amino acid substitutions. Two cases of heterolasmia are found in this species. The presence of two distinct mtDNA gene sequences in one organism can be explained by amplification of nuclear pseudogenes, mutations in mtDNA in the cell or the presence of paternal mtDNA. The essential feature of a pseudogene is a stop-codon affecting translation into amino acid sequence. No stop codons were detected in the studied variants of the gene *COI* from *C. torrentium.* Multiple mtDNA differences in two individual *C. torrentium* mosquitoes with heteroplasmia (three and eight nucleotides) give evidence for two-parent mtDNA transfer in these cases. The presence of paternal mtDNA was recorded in the cells of *Drosophila simulans* Sturtevant, 1919 (Satta, Chigusa, 1991) and *Aedes aegypti* (Paudan, Ribolla, 2008).

Our data show considerable decrease of mtDNA polymorphism in *Wolbachia*–infected population of *C. pipiens* compared with uninfected *C. torrentium*. The number of haplotypes in *C. pipiens* was low, and same haplotypes were found in distant populations. At the same time *C. torrentium* has numerous haplotypes and each individual has a unique haplotype. These data correspond to the observations of Rasgon et al. (2006), though these authors compared more closely related forms of the genus *Culex*. Polymorphism in nuclear sequence (*ITS2*) was similar in infected *C. pipiens* and uninfected *C. torrentium*. It can be suggested that the reduction of mitochondrial polymorphism is more likely to be due to the selective sweep of the *Wolbachia*-infected haplotype than to a passage of *C. pipiens* through a bottleneck before the spread around the world. The relation between *Wolbachia* infection and decreased mtDNA polymorphism has been reported earlier (Montchamp-Moreau et al., 1991; Schulenburg et al., 2002; Jiggins 2003; Rasgon et al., 2006). It is considered that *Wolbachia* provides its host with some selective

advantage, and the infected individuals are favoured by natural selection. Consequently, the bacterium provides dissemination of a certain mtDNA haplotype. This phenomenon is called genetic hitchhiking (Barton, 2000).

The existing strict subspecies-specific pattern of haplotypes among *Wolbachia*-infected populations of mosquitoes *C. pipiens,* suggests that the infection of mosquitoes with the endosymbiotic bacteria occurred earlier than the divergence of subspecies. Probably, bacterial infection played a key role in subspecies formation, since it is known that *Wolbachia* induces cytoplasmic incompatibility in *C. pipiens.*

The hypothesis that the divergence of *C. pipiens* subspecies in physiological characters and biotopic preference occurred in post-glacial time after introduction of *Wolbachia* infection is also favored by the data on similarity of studied genes of *Wolbachia pipientis* in mosquitoes *C. pipiens.* Earlier we obtained the nucleotide sequence of the bacterial gene *wsp* from underground mosquitoes in Moscow, Petersburg, and Volgograd (Vinogradova et al., 2003). The GenBank contains 21 sequences of the gene *wsp* from endosymbionts of *C. p. pipiens* (both forms), *C. p. quinquefasciatus,* and *C. p. pallens* from geographically distant populations. The *wsp* gene was found to be identical for all studied bacteria, and these data correspond well with the results showing the absence of polymorphism in the other studied genes of *Wolbachia,* e.g. *fts*Z (Guillemaud et al., 1997) and 16S rRNA gene (Pidiyar et al., 2003). Only differences in the prophage-associated ankyrin repeat domain gene between *Wolbachia* from *C. p. pipiens* f. *molestus* and *C. p. quinquefasciatus* laboratory strains have been found (Walker et al., 2009). The absence of DNA polymorphism in bacteria from geographically remote mosquito populations indicates that *Wolbachia* infection occurred rather recently and spread rapidly after introduction by one or a few infected females.

In conclusion, our results suggest that *Wolbachia* probably could play an essential role in divergence of the two species: *C. pipiens* and *C. torrentium,* because of reproductive isolation of infected and uninfected species. Additional evidence is provided by the fact that viable hybrids of these closely related species can be obtained in the laboratory only if infected females of *C. pipiens* are crossed with uninfected males of *C. torrentium.* The reciprocal crosses produced no viable progeny (E.B. Vinogradova, personal communication). The hybrids were not detected in nature, though both species often inhabit the same areas.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (grant 08-04-01511-a) and by the Program of Basic Research of the Russian Academy of Sciences «Gene pool and the genetic diversity». The authors are grateful to E.B. Vinogradova (Zoological Institute, Russian Academy of Sciences) and to M.V. Fedorova (Moscow State University) who kindly provided material and for helpful advice and comments. We thank Dr. Ekaterina Gupalo for her assistance in preparation of the manuscript.

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Received June 17, 2010.
Accepted by V.A. Lukhtanov, November 6, 2010.
Published December 30, 2010.