

## PCR-RFLP of the COI gene reliably differentiates *Cx. pipiens*, *Cx. pipiens f. molestus* and *Cx. torrentium* of the Pipiens Complex

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### Abstract

*Culex pipiens* Linnaeus, *Culex pipiens f. molestus* Forskal and the closely related species *Culex torrentium* Martini are difficult to identify within the limits of traditional taxonomy. Polymorphism was noted between these species in a 603bp fragment of the 5' end of the mitochondrial cytochrome oxidase C subunit I gene and used as the basis for developing polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) assays for the differentiation of *Cx. pipiens* and *Cx. torrentium* by *BclI* restrictase, and *Cx. pipiens* and *Cx. pipiens f. molestus* by *HaeIII* restrictase. This method allows the identification of samples at any developmental stage.

**Keywords:** Pipiens Complex, Russia, COI, RFLP assay

### Introduction

In Russia, the mosquito *Culex pipiens* complex comprises the following species: *Cx. pipiens* Linnaeus and the autogenous *Cx. pipiens f. molestus* Forskal, *Cx. torrentium* Martini and *Cx. vagans* Wiedermann. *Culex pipiens f. molestus* is autogenous and can reproduce throughout the year in urban underground habitats (Vinogradova, 2000). Homogeneous or mixed populations of *Cx. pipiens* and *Cx. torrentium* often occur in sympatry in open water pools, where the proportion of *Cx. torrentium* may reach as high as 80% (Gilles & Gubbins, 1982, cited from Vinogradova, 1997). *Culex pipiens* and *Cx. torrentium* are similar in most morphological characters and can only be differentiated by characters of the male genitalia (Gutsevich, 1970; Dahl, 1988; Vinogradova, 2000; Fyodorova & Shaikevich, in press). They are most similar biologically, being anautogenous (unable to produce eggs without first taking a blood meal), eurygamous (unable to mate in confined spaces) and heterodynamic (may diapause in winter). In the contrast, *Cx. pipiens f. molestus* form is autogenous, eurygamous and homodynamic.

Members of the Pipiens Complex are competent vectors of many dangerous human and animal diseases, including West Nile virus, St. Louis and other related encephalitis, periodic lymphatic filariasis and avian malaria (Vinogradova, 2000; Turell *et al.*, 2001). West Nile virus (WNV) outbreaks among humans have been recorded in Morocco and Romania (1996), Italy (1998), Israel (1999-2000), USA (1999), France (2000) and in the Volgograd region of southern Russia

(Petersen & Roehrig, 2001; Petersen *et al.*, 2002). The virus was isolated from *Culex* mosquitoes in Italy (1998), France (2000), Morocco (2003) and southern Portugal (2004) (Esteves *et al.*, 2005). *Culex pipiens f. molestus* captured in Uzbekistan, was shown to be capable of transmitting WNV under laboratory conditions (Turell *et al.*, 2006) but investigations carried out during the outbreak in Volgograd and its vicinities suggested that the intense transmission of WNV to humans in urban areas may have been facilitated by the high abundance of *Cx. pipiens* breeding in multi-storied buildings (Fyodorova *et al.*, 2006). The role of the autogenous and anautogenous forms of *Cx. pipiens* mosquitoes in urban WNV transmission remains unclear. *Culex torrentium* is known as an ornithophilic mosquito, and is a vector of Sindbis virus in Africa, India, Malaysia, Philippines, Australia and Sweden, being more effective in the transmission of this virus than *Cx. pipiens* (Vinogradova, 1997).

In recent years, rapid progress has been made in the development of molecular approaches to the identifications of *Cx. pipiens* complex mosquitoes. DNA analysis has the advantage that differentiation can be undertaken at any developmental stage. Variable transcribed ribosomal DNA spacers ITS1 and ITS2 of fourteen *Culex* species have been investigated (Miller *et al.*, 1996). Structural differences were revealed in the A+T rich control region and in the mtDNA cytochrome oxidase II (COII) genes in laboratory strains of *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. torrentium* (Guillemaud *et al.*, 1997). Polymorphism of the

second intron of the acetylcholinesterase-2 (ace-2) gene was proposed as a marker for differentiation of the members of the *Cx. pipiens* complex (Smith & Fonseca, 2004) and microsatellite loci were shown to be useful for identification of the species and forms of the Pipiens Complex, as well as their hybrids (Fonseca *et al.*, 2004; Smith *et al.*, 2005; Bahnek & Fonseca, 2006). Earlier studies showed that *Cx. pipiens* and *Cx. pipiens f. molestus* are infected with an endosymbiotic bacterium Wolbachia and do not differ in the sequence of the 3' end region of the mitochondrial DNA gene for cytochrome oxidase I (COI) (Shaikevich *et al.*, 2005). *Culex torrentium* mosquitoes are not infected with this bacterium. *Culex pipiens* and *Cx. torrentium* differ in the nucleotide composition of the ITS2 region of ribosomal DNA and the 3' region of COI (Vinogradova & Shaikevich, 2005).

Herein we propose a simple, reliable method to differentiate individual specimens of the autogenous and anautogenous forms of *Cx. pipiens* and *Cx. torrentium* using HaeIII and BclI restriction of a 603bp portion of the 5' end of the mtDNA COI gene. Novel primers were designed to amplify this region.

#### Materials and methods

Bionomics and study sites of *Cx. pipiens*, *Cx. pipiens f. molestus* and *Cx. torrentium* populations included in the study are listed in Table 1. All mosquito populations were carefully characterized by their behavioral and taxonomic traits by the collectors (see Table 1). Portions of the collected larvae were reared in the laboratory and fed on yeast suspension. Emerged adults were supplied with 5% sucrose solution. Ten days after emergence, the ovaries of the females were dissected to determine the proportion of autogenous and anautogenous specimens per population. Identification of *Cx. torrentium* was based on examination of the male genitalia and ITS2 sequence analysis (data not shown) (Fyodorova & Shaikevich, 2007).

Mosquito DNA was extracted using DIAAtom™ DNA Prep kit (Isogen, Moscow). DNA samples were obtained from individual larvae, pupae, and imagoes, either native or preserved in 96% ethanol. Prior to DNA isolation from the alcohol-preserved material, alcohol was allowed to evaporate by heating at 65°C for 30-40 minutes. The *Culex* COIF 5'-TTGAGCTGGA-ATAGTTGGAAGCTT -3' and *Culex* COIR 5'-CCTCCAATTGGATCAAAGAATGA-3' primers were designed following alignment of available COI sequences.

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 MgCl<sub>2</sub>, one unit of Taq DNA polymerase, 0.2 µM of each primer, and 0.1 µg of the isolated DNA. Thermocycler conditions consisted of primary denaturation at 94°C for 5 min; then 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 40 sec, synthesis at 72°C for 40 sec; and a final synthesis at 72°C for 10 min. PCR products were identified by electrophoresis, using a 1% agarose gel (Sigma, United States). Amplified DNA fragments were isolated from the gel using JETQUICK Gel Extraction Spin Kit (Genomed, Germany) and then directly sequenced in both directions on an ABI PRISM 310 using the Applera (United States) reagents kit according to the instructions of the manufacturer. The DNA sequences were aligned using ClustalW and analysed using MEGA version 3.0 (Kumar *et al.*, 2004). COI sequences are available in GenBank under accession numbers AM403476, AM403477 & AM403492.

The amplicons were digested separately with HaeIII (Promega) and BclI (Promega) restriction endonucleases. Restriction enzyme digestions were performed in a 30 µl volume. The digest master mix consisted of 5 µl of the COI PCR product, 0.5 µl (5 U) of enzyme, 3 µl buffer C (Promega), 0.3 µl BSA (Promega) and 21.2 µl ddH<sub>2</sub>O. For HaeIII restriction, the endonuclease reaction was incubated for 1 hour at 37°C. For BclI restriction, the reaction mix was prepared as above, but the endonuclease reaction was incubated for 1 hour at 50°C. The digested products were visualized on a 1% agarose gel.

#### Results

A 603bp amplicon of the 5' region of the mtDNA COI gene was sequenced for *Cx. pipiens*, *Cx. pipiens f. molestus* and *Cx. torrentium* from populations in Russia. Twenty samples were sequenced from six populations (Table 1). Comparative analysis of the COI sequences showed that the autogenous underground populations of *Cx. pipiens f. molestus* differs from the overground, unautogenous *Cx. pipiens* by one fixed substitution A-G at 205 bp of the alignment (Figure 1). This difference could be detected following restriction of the COI amplicon with HaeIII (GG/CC), which is present in *Cx. pipiens*, but not in *Cx. pipiens f. molestus*. After restriction two fragments (206 & 397 bp) were obtained from *Cx. pipiens*, whereas *Cx. pipiens f. molestus* remains uncut (603bp). In all, 142 samples from 10 populations were assayed (Table 1). The 603bp amplicon from the 60 *Cx. pipiens f. molestus* samples after restriction with HaeIII remained unchanged. Eighty-two samples of *Cx. pipiens* showed the characteristic fragment sizes (206 and 397 bp) after restriction of the 603bp COI amplicon with HaeIII.

pipiens	<u>T TGA GCT GGA ATA GTT GGA ACT</u>	TCT TTA AGT TTA CTA ATT CGA GCA GAA TTA AGT CAA CCA GGT GTA TTT ATT GGA AAT
molestus	.....	.....
torrentium	.....	.....
	/BclI	.....
pipiens	<u>GAT CA</u>	ATT TAT TTA ACT GGT ATT GTA ACT GCT CAT GCT TTT ATT ATA ATT TTT TTA ATA CCA ATC ATA ATT GGA
molestus	.....	.....
torrentium	.....	.....
	/HaeIII	.....
pipiens	GGA TTT GGA AAT TGA TTA GTT CCT TTA ATG TTA GGA GCT CCA GAT <u>ATG GCC</u> TTT CCT CGA ATA AAT AAT ATA AGT TTT	.....
molestus	.....	.....
torrentium	.....	.....
pipiens	TGA ATA CTA CCT CCT TCA TTG ACA CTA CTA CTT TCA AGT AGT TTA GTA GAA AAT GGA GCT GGG ACT GGA TGA ACA GTG	.....
molestus	.....	.....
torrentium	.....	.....
pipiens	TAT CCC CCT CTT TCA TCT GGA ACA GCT CAT GCT GGA GCT TCA GTA GAC TTA GCT ATT TTT TCT TTA CAT TTA GCA GGA	.....
molestus	.....	.....
torrentium	.....	.....
pipiens	ATT TCA TCA ATT TTA GGT GCA GTA AAT TTT ATT ACA ACA GTA ATT AAT ATA CGA TCT TCA GGA ATT ACT CTT GAT CGA	.....
molestus	.....	.....
torrentium	.....	.....
	/BclI	.....
pipiens	ATA CCT TTA TTT <u>TGA TCA</u>	GTA GTA ATT ACT GCA GTT TTA TTA CTT TCT TTA CCT GTT TTA GCT GGT GCT ATT
molestus	.....	.....
torrentium	.....	.....
pipiens	ACT ATG TTA TTA ACA GAT CGA AAT TTA AAT ACT TCA <u>TTC TTT GAT CCA ATT GGA GG</u>	.....
molestus	.....	.....
torrentium	.....	.....

**Figure 1.** Nucleotide sequence variants of 603bp of the cytochrome oxidase I gene in *Cx. pipiens*, *Cx. pipiens* f. *molestus* and *Cx. torrentium*. Dots indicate homology to the *Cx. pipiens* sequence. Primers COIF and COIR are underlined. *HaeIII* (GG/CC) and *BclI* (T/GATCA) restriction endonuclease sites are in bold type and underlined.

Population	Stage	Habitat	Autogeny	Source	Fragment sizes after restriction (bp)		n=
					<i>HaeIII</i>	<i>BclII</i>	
<b><i>Culex pipiens f. molestus</i></b>							
Moscow	L,P	underground	*	EBV	603	406,118,79	10
St. Petersburg	L,P	underground	89%	EBV	603	406,118,79	10
Nizhniy Novgorod	L	underground	100%	EBV	603	406,118,79	10
Krasnodar	L	underground	90%	EBV	603	406,118,79	10
Volgograd	L	underground	87%			406,118,79	20
<b><i>Culex pipiens</i></b>							
Moscow region settlement Chashnikovo	L, P	overground	0%			406,118,79	18
Moscow region settlement Starikovo	L,P	overground	*			406,118,79	19
Moscow region settlement Luzhki	L	overground	0%			406,118,79	10
Moscow region settlement Iksha	A		Hibernating females			406,118,79	10
Volgograd region	L	overground	0%	MVF	206,397	406,118,79	25
<b>Total <i>Culex pipiens</i></b>							<b>142</b>
<b><i>Culex torrentium</i></b>							
Moscow region settlement Chashnikovo	L, A	overground	0%	MVF	603	524,79	10
Moscow region settlement Starikovo	L, A	overground	*	MVF	603	524,79	10
<b>Total <i>Culex torrentium</i></b>							<b>20</b>

**Table 1.** Ecology of mosquito populations used in this study showing the resulting fragment sizes after restriction of the 603bp amplicon with *HaeIII* and *BclII*. EBV = Dr. Elena B. Vinogradova, Department of Experimental Entomology, Zoological Institute, Russian Academy of Sciences, Russia. MVF = Dr. Marina V. Fyodorova, Department of Entomology, Faculty of Biology, Moscow State University, Russia. \*populations not tested for autogeny. Populations from Chashnikovo and Starikovo are mixed. *Cx. pipiens* and *Cx. torrentium* inhabit the same open water pools.

Comparison of the COI sequences of *Cx. torrentium* and *Cx. pipiens* revealed sixteen fixed nucleotide substitutions. The diagnostic adenosine (A) transition that separates *Cx. pipiens f. molestus* from *Cx. pipiens* is also shared by *Cx. torrentium*. Unfortunately the *HaeIII* recognition site is not present in *Cx. torrentium* (Figure 1). After *HaeIII* digestion, the COI product remains uncut in both *Cx. pipiens f. molestus* and *Cx. torrentium*. A second assay using *BclII* restrictase (T/GATCA) allowed the differentiation of *Cx. pipiens* and *Cx. pipiens f. molestus* and *Cx. torrentium*. The COI fragment of *Cx. pipiens* and *Cx. pipiens f. molestus* resulted in three fragments (406, 118 & 79 bp), whereas *Cx. torrentium* was cut only in two fragments (524 & 79 bp) (Figure 1). Analysis with restrictases *BclII* and *HaeIII* was carried out for 20 mosquitoes from two *Cx. torrentium*

populations of the Moscow region (Table 1). Thus, restriction analysis of the COI products with *BclII* restrictase makes it possible to identify a specimen as *Cx. torrentium* or *Cx. pipiens*. The second assay with *HaeIII* permits the differentiation of *Cx. pipiens* and *Cx. pipiens f. molestus*.

#### Discussion

Comparative analysis of the 5' end sequences of 603bp of the mtDNA COI gene shows that *Cx. pipiens* and *Cx. pipiens f. molestus* can be distinguished by a single fixed point mutation. This difference is maintained despite large geographical distances between the sampling sites. The substitution of guanine (G) by adenine (A) in the third position of the 68<sup>th</sup> codon of the COI gene fragment in underground mosquitoes is silent and does not affect either the amino acid

(ATA and ATG encode methionine) or the function of the COI gene.

The COI fragment of *Culex torrentium* differs by 16 substitutions (2.85 %) from *Cx. pipiens* (Figure 1). Only one substitution (guanine to adenine in the first position of the fifth codon in the primer area) is nonsynonymous, leading to the replacement of valine (GTT) with isoleucine (ATT). This is slightly higher than the 2.5 % differences between these mosquitoes using a portion of the 3' end of the COI gene shown previously (Vinogradova & Shaikovich, 2005).

The absence of nucleotide substitutions on the 3' end of COI and only one transition on the 5' end of COI suggest a recent divergence of *Cx. pipiens* and *Cx. pipiens* f. *molestus*. COI is the most slowly evolving region of all mitochondrial protein-coding genes (Bernasconi *et al.*, 2000). The rate of the evolution of synonymic sites is constant in closely related species, and time-proportional. The nucleotide sequence divergence in insect mtDNA is approximately 2% per million years (DeSalle *et al.*, 1987), which is mostly due to silent changes. Differences in the nucleotide composition of mtDNA of the COII gene between *Cx. pipiens* and *Cx. torrentium* is 2.1 % (Guillemaud *et al.*, 1997), corresponds to about one million years since the time of divergence. Our data on the polymorphism of the initial and terminal regions of the COI gene confirm the time of presumable divergence of *Cx. pipiens* and *Cx. torrentium*. The divergence of *Cx. pipiens* and *Cx. pipiens* f. *molestus* is 0.17%, which corresponds to 80,000 years, i.e., some 920,000 years later than the proposed divergence of *Cx. pipiens* and *Cx. torrentium*.

The RFLP assays herein allow the accurate identification of *Cx. pipiens* and *Cx. pipiens* f. *molestus* using HaeIII in all life stages (larva, pupa or adult). This will assist in the clarification of the role of *Cx. pipiens* and *Cx. pipiens* f. *molestus* in the transmission of diseases. Restriction analysis using BclI allows the identification of specimens of a closely related, morphologically hardly distinguishable sibling species *Cx. torrentium*. Given the role of *Cx. pipiens* complex members in many serious animal and human disease, this proposed method will be useful for characterising the taxonomic status of populations, particularly where more than one member occur sympatrically.

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