

# Assessment of distribution patterns of culex disease vectors by molecular assays

## Abstract

Study of distribution patterns of *Culex pipiens* forms and their sibling species *Culex torrentium* in Berlin and Hannover, Germany was done. The mosquitoes of the *Culex* genus have epidemiological significance as vectors of arboviruses and other pathogens of humans and animals. Based on molecular genetics methods it was shown that the park in Berlin in 2009 was inhabited by *Culex pipiens* form *pipiens*, while in the water container in Hannover coexistence of *Cx. pipiens* form *pipiens* and *Cx. torrentium* was noted. All females carrying sucked human blood and caught in the flats belong to *Cx. pipiens* form *molestus*. Molecular differentiation has revealed the coexistence of various *Culex* taxa in cities, which increases the probability of hybridization and the appearance of potential bridge vectors between birds and humans.

**Keywords:** *Culex pipiens*, *Culex torrentium*, PCR-RFLP, *COI*; *ace-2*, *Wolbachia*

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## Introduction

*Culex pipiens* complex taxa and sibling species *Cx. torrentium* are morphologically difficult to distinguish, but differ in behaviour and ecophysiology and, accordingly, in epidemiology. With a view of their epidemiological importance, knowledge of the distribution of morphologically similar mosquitoes in nature is important for the understanding of their vector-pathogen dynamics and for their control. Mosquitoes of the genus *Culex* are vectors of many dangerous human and animal infections, such as filariasis, avian malaria and arboviruses - West Nile virus, Japanese encephalitis, St. Louis encephalitis, Sindbis virus and others. Beside this, people attacked by mosquitoes suffer from itching and allergic reactions. Mosquitoes of the *Culex pipiens* complex are of special interest. The complex includes closely related ubiquitous species. Common in Europe are *Cx. pipiens* Linnaeus, 1758 and sibling species *Cx. torrentium* Martini, 1925. The subspecies *Cx. pipiens* includes two forms, the nominate *pipiens* form and the *molestus* form. Within the temperate zone, these forms are biotopically isolated and have somewhat opposite biological features. *C. pipiens* form *pipiens* are unautogenous (able to produce of the first egg raft only after taking a blood meal), diapausing (able to enter reproductive diapause) and eurygamous (they need a large space for mating) mosquitoes, inhabit the open water bodies. While *Cx. pipiens* form *molestus* are autogenous (produce of the first egg raft without taking a blood meal), non-diapausing, stenogamous (able to mate in confined space, without swarming), and usually inhabit the underground water bodies. *Cx. torrentium* is morphologically and ecologically very similar to *Cx. pipiens* form *pipiens*, the mosquitoes are unautogenous, diapausing and eurygamous.

Identification of *Cx. torrentium* cause considerable problems and *Cx. torrentium* is often confused with *Cx. pipiens*. Unautogenous form *pipiens* and *Cx. torrentium* inhabit the same terrestrial water basins forming 'pure' or mixed populations.<sup>1,2</sup> In W Europe, *Cx. torrentium* is spread from Scandinavian Peninsula to Portugal, including Great Britain.

It is hard and often impossible to identify the species for a wild larva or imago of the *Cx. pipiens* complex; the morphological distinguishing traits are not obvious, the main traits being the genital structure in adults and siphonal index in larvae. However, these traits

are variable, inducing problems in distinguishing between the two species.<sup>1,3</sup> Since the species of the complex show different physiology and behavior and are of different epidemiological significance, the methods for exact identification are being actively developed basing on DNA analysis (including PCR). Several methods have been developed to distinguish between *Cx. pipiens* and *Cx. torrentium*. One of the methods implies different lengths of the ITS2: 400 and 350 bp in *Cx. pipiens* and *Cx. torrentium*, respectively.<sup>4,5</sup> Another method suggested by Smith et al.<sup>6</sup> is based on polymorphism in intron 2 of the gene encoding acetylcholine esterase 2 (*ace-2*). Autogenous and anautogenous forms of *Cx. pipiens* differs in microsatellite signature (Bank & Fonseca, 2006) and with distinguished patterns of isoenzymes (Becker et al., 1999;<sup>7</sup> The aim of this work was to study species composition of mosquitoes from the genus *Culex* in Berlin and Hannover, Germany with molecular-genetics methods.

## Materials and methods

Mosquito larvae were collected in September 2009 from a non-functional fountain in the park Turmstrasse, 10559 Berlin. In Hannover, mosquito larvae were collected from a container with rain water in a country house area, Prussweg, 30165 Hannover. The females carrying sucked human blood were caught in the flats of multi-store buildings (Lubecker strasse, 10, 10559 Berlin and Prussweg 6, 30165 Hannover). The larvae and the imagoes were partly dried and partly fixed with 96% alcohol for subsequent analysis. The collected larvae and imagoes were morphologically characterized as *Cx. pipiens* s.l. by M.V. Fedorova and O.V. Bezzhonova.

DNA isolation, PCR and RFLP analyses were performed as described earlier.<sup>8</sup> The results were verified by analysis of the PCR products of the gene *ace-2*<sup>6</sup> using the primers ACEpip, ACETorr, and B1246s, suggested by the authors for Eurasia. *Wolbachia* infection was tested using PCR with primers wsp81F and wsp691R, complementary to a bacterial gene *wsp*.<sup>9</sup>

The results of the PCR-RFLP assay were confirmed by selective sequencing of the gene *COI*. DNA sequencing was performed using the ABI PRISM 310 sequencer and the Applera (USA) reagents kit according to the instructions of the manufacturer. The obtained sequences were analyzed using software Chromas (<http://www.technelysium.com.au>), polymorphism analyses were conducted in

MEGA6.<sup>10</sup> The sequences of the *COI* gene amplification products were submitted to the GenBank (accession numbers HM008665-HM008672).

## Results and discussion

As described earlier,<sup>8</sup> after the PCR with primers CulexCOIF and CulexCOIR and subsequent restriction with *Hae*III of the PCR products two fragments, 206 bp and 397 bp were obtained for the *Cx. pipiens* form *pipiens*. The fragment of the *Cx. pipiens* form *molestus* *COI* gene remained unchanged (603 bp). For mosquitoes *Cx. torrentium* intraspecific variation of *COI* gene nucleotide sequence is characteristic and in recognition sequence of *Hae*III endonucleases presence both adenine and guanine is possible: 60% -AGCC, 30% -GGCT (Shaikevich, 2009), therefore both variants of restriction are recorded. However, *Cx. torrentium* can be identified using *Bcl*I. After *Bcl*I restriction of the PCR products the DNA of *Cx. pipiens* of both

forms is cut in three fragments: 406bp, 118bp and 79bp. The DNA of *Cx. torrentium* is cut into only two fragments: 524bp and 79bp.

Typical 603-bp amplification products were obtained after amplification with primers CulexCOIF and CulexCOIR for all 35 studied mosquitoes. The restriction analysis using *Bcl*I showed that all mosquitoes from Berlin belonged to *Cx. pipiens*, while 4 of 14 mosquitoes from Hannover were identified as *Cx. torrentium* (Table 1). The amplicates of 29 samples were cut by *Hae*III into two fragments, 206 and 397 bp. In 6 samples only uncut 603-bp PCR products were present, these were obtained from all the blood-sucked females collected in the flats and from one individual collected from water container in Hannover characterized as *Cx. torrentium* using *Bcl*I (Table 1). Additional analysis of the PCR products of the gene *ace-2* confirmed that four individuals from Hannover population belong to *Cx. torrentium*. The other studied individuals belonged to *Cx. pipiens*.

**Table 1** The restriction analysis using *Bcl*I showed that all mosquitoes from Berlin belonged to *Cx. pipiens*, while 4 of 14 mosquitoes from Hannover were identified as *Cx. torrentium*

Sample Site	Sample Number	COI/ <i>Hae</i> III (bp)	COI/ <i>Bcl</i> I (bp)	ACE (bp)	COI GenBank AC number
Berlin, flat	B1	603	406, 118 and 79	610	HM008670
	B2	603	406, 118 and 79	610	HM008671
	B3	603	406, 118 and 79	610	
	B4	603	406, 118 and 79	610	
Berlin, park	B5	206 and 397	406, 118 and 79	610	HM008668
	B6	206 and 397	406, 118 and 79	610	HM008669
	B7	206 and 397	406, 118 and 79	610	
	B 8	206 and 397	406, 118 and 79	610	
	B 9	206 and 397	406, 118 and 79	610	
	B 10	206 and 397	406, 118 and 79	610	
	B 11	206 and 397	406, 118 and 79	610	
	B 12	206 and 397	406, 118 and 79	610	
	B 13	206 and 397	406, 118 and 79	610	
	B 14	206 and 397	406, 118 and 79	610	
	B 15	206 and 397	406, 118 and 79	610	
	B 16	206 and 397	406, 118 and 79	610	
	B 17	206 and 397	406, 118 and 79	610	
	B 18	206 and 397	406, 118 and 79	610	
	B 19	206 and 397	406, 118 and 79	610	
	B 20	206 and 397	406, 118 and 79	610	
	B 21	206 and 397	406, 118 and 79	610	
Hannover, water container	H1	206 and 397	406, 118 and 79	610	HM008665
	H 2	206 and 397	524 and 79.	416	
	H 3	206 and 397	524 and 79.	416	
	H 4	206 and 397	406, 118 and 79	610	HM008666
	H 5	206 and 397	406, 118 and 79	610	
	H 6	206 and 397	406, 118 and 79	610	
	H 7	603	524 and 79.	416	
	H 8	206 and 397	406, 118 and 79	610	
	H 9	206 and 397	406, 118 and 79	610	
	H 10	206 and 397	524 and 79.	416	HM008672
	H 11	206 and 397	406, 118 and 79	610	
	H 12	206 and 397	406, 118 and 79	610	
	H 13	206 and 397	406, 118 and 79	610	
Hannover, flat	H 14	603	406, 118 and 79	610	HM008667

Selective sequencing of amplicates (Figure 1) has shown that the DNA of *Cx. pipiens* mosquitoes of form *pipiens* differs from DNA of form *molestus* by single nucleotide substitution: position 119 of the studied fragment of the gene *COI*. The DNA of mosquitoes of form *molestus* has a characteristic substitution of Guanine within the *HaeIII* site (GG'CC) with Adenine (AGCC) with a loss of the respective restriction site. The DNA of *Cx. torrentium* in the studied fragment differs from DNA of *Cx. pipiens* by 13 nucleotide substitutions (2,5 %). These results are in agreement with earlier studies of the *Cx. pipiens* mosquitoes in Russia.<sup>2,8,9</sup>

[	112344444 445]
[	6161202355 660]
[	2942346125 780]
#pipBerlin5	CGTGTATTTT GTC
#pipBerlin6	.....
#pipHannover	1 ..... ..
#pipHannover4	.....
#pipMoscow	.....
#molBerlin1	.A.....
#molBerlin2	.A.....
#molHannover14	.A.....
#molVolgograd	.A.....
#torHannover10	TACAATCCGA ACT
#torSPetersburg	TACAATCCGA ACT

**Figure 1** Variable sites of the 517- bp 5'region of the *COI* gene.

The results show that the antropophylic females caught in multi-store buildings in Berlin and Hannover belong to *Cx. pipiens* form *molestus*. The non-functional park fountain in central Berlin is inhabited by *Cx. pipiens* form *pipiens*. Though the distance between the sampling sites is not long, populations of the form *pipiens* appear to exist separately, not mixing with individuals from multi-store buildings.

Of special interest is the mosquito population from the water container in Hannover, with coexistence of *Cx. pipiens* form *pipiens* and *Cx. torrentium*. Coexistence is not rare for these two species, earlier we recorded several mixed populations from Russia. *Cx. torrentium*, found in Russia was not infected by *Wolbachia*, contrary to *Cx. pipiens*, the populations of which contained up to 100% infected individuals (Vinogradova *et al.*, 2007). *Wolbachia* infections in *Culex pipiens* complex mosquitoes in Germany also were detected.<sup>11</sup> The PCR with primers for the gene *wsp* of *Wolbachia* showed no positive results for all four individuals of *Cx. torrentium* from Hannover. Though the number of the tested *Cx. torrentium* mosquitoes is low, it can be suggested that this member of the *Cx. pipiens* complex is not infected by these symbiotic bacteria in Germany. Probably, bacterial infection play a key role in reproductive isolation of *Cx. pipiens* and *Cx. torrentium*, since it is known that *Wolbachia* induces cytoplasmic incompatibility in *Culex* mosquitoes.

In conclusion, the method of species identification used in this work proved efficient for *Culex pipiens* mosquitoes populations from N Germany. Only one amplification reaction and two restriction reactions are sufficient for exact identification of both *Cx. pipiens*

forms, and also of *Cx. torrentium*. The analysis takes 2-3 days and is considerably less expensive that the direct sequencing of the PCR products. The DNA for the PCR-RELP analysis may be isolated from an individual or any part of an individual at any stage of development.<sup>12</sup>

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## Conflicts of Interest

None.

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