The distribution of *Wolbachia pipiensis* strains in natural populations of *Culex pipiens* mosquitoes (Diptera: Culicidae)

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Abstract. *Wolbachia* is a group of maternally inherited endosymbiotic bacteria that infect and induce cytoplasmic incompatibility in a wide range of arthropods. *Wolbachia* species are involved and play a significant role in some evolutionary processes, e.g. those of rapid speciation. We have investigated the distribution of *Wolbachia* strains based upon the polymorphism of transposable element Tr1 and pk1 gene, in natural populations of the *Cx. pipiens* complex mosquitoes from Russian Federation, Republic of Kazakhstan, Kyrgyz Republic and Republic of Belarus.

1. Introduction

The *Culex pipiens* complex includes some species of controversial taxonomic status which possess only minor morphological but great biological differences, including the ability to transmit human and animal diseases. Two *Cx. pipiens* forms, *pipiens* u *molestus* (hereinafter referred to as *pipiens* and *molestus*) without using a taxonomic names, which are controversial, have alternative biological characteristics. The *pipiens* requires a blood meal for egg development (anautogeny), prefers to feed on birds (ornitophily) and enters into diapause during the winter (heterodynamy). By contrast, the females of *molestus* typically do not require a blood meal to produce their first batch of eggs (autogeny), have a tendency to feed on mammals (mamophily) and has no winter diapause in life cycle (homodynamy). The *molestus* commonly breed in underground urban habitats and able to mate in confined spaces (stenogamy), whereas the ppiens colonizes above-ground habitats exclusively and mates in large, open areas (eurygamy). The strict association between physiological traits and ecology is observed in colder temperate climate regions [1, 2]. *Cx. torrentium* Martini 1924, is a sibling species of *Cx. pipiens*.

*Wolbachia* are common intracellular bacteria that are found in arthropods and nematodes. These α-proteobacteria endosymbionts are transmitted vertically through host eggs and alter host biology in diverse ways, including the induction of reproductive manipulations, such as feminization, parthenogenesis, male killing and sperm-egg incompatibility. Such manipulations enable *Wolbachia* to spread through arthropod populations and may drive arthropod evolution through their effects on host phenotypes [3].

Here we have investigated the distribution of *Wolbachia* strains based upon the polymorphism of transposable element *Tr1* [4] and *pk1* gene [5] in natural populations of the *Cx. pipiens* complex mosquitoes from Russian Federation, Republic of Kazakhstan, Kyrgyz Republic and Republic of Belarus.

2. Material and methods
Mosquitoes of *Culex pipiens* complex from both open and isolated, both natural and artificial habitats of Russian Federation (Tomsk, Novosibirsk, Altay, Krasnoyarsk and Krasnodar Regions), the Republic of Kazakhstan, Kyrgyz Republic and Republic of Belarus were collected as larvae or imago during the period 2000-2014. Molecular markers used to identify members of Cx. *pipiens* complex were as follows: SCAR-markers [6], second intron of acetylcholinesterase 2 gene (ACE2) [7], and RFLP-PCR of cytochrome oxidase I gene of mitochondrial DNA (3′- and 5′-ends, COI 3′ and COI 5′) [8, 9].

Primers 81F and 691R [10] were utilized to define infection by *Wolbachia*. Identification of *W. pipientis* variants was carried out by using assays based on *Trl* sequence according to Duron et al. [4] and *pkl* gene according to Dumas et al. [11].

3. Results and Discussion

2985 larvae and imago of *Culex pipiens* mosquito complex, including *Cx. torrentium* (1035 specimens), *pipiens* (1210 specimens), and *molestus* (740 specimens) from 87 natural populations has been collected and investigated. Both homogeneous and mixed populations have been revealed. *Cx. torrentium* is usual in the Tomsk Region (more than half of the total number of *Cx. torrentium* samples, most of which homogeneous). *Cx. torrentium* has also been found in the Novosibirsk, Altay and Krasnoyarsk regions, Republic of Kazakhstan, Kyrgyz Republic and Republic of Belarus. Specimens of *molestus* are abundant in all places studied. Mixed collections of mosquitoes are not rare and they were grouped into four types: a) *Cx. torrentium* and *pipiens* (the Tomsk, Novosibirsk, Altay and Krasnoyarsk regions, Republic of Kazakhstan, Republic of Belarus); b) *Cx. torrentium* and *molestus* (the Tomsk region); c) *pipiens* and *molestus* (the Novosibirsk, and Krasnodar Regions, Republic of Kazakhstan, Republic of Belarus); d) *Cx. torrentium*, *pipiens*, and *molestus* (the Tomsk Region, Kyrgyz Republic, Republic of Belarus).

We have shown that *Cx. torrentium* is uninfected by *Wolbachia*, which allows considering this feature at fixation in this species. 98.2 ± 0.5 % of *molestus* populations are infected (notably, uninfected *molestus* individuals are present only in Belarus populations). In *pipiens* populations from 34.5 % to 100 % individuals are infected (on the average 82.5 ± 1.13 %). It should be noted that most of *pipiens* mosquitoes had been collected on the territory of Belarus thus the analysis of these populations was performed apart from other populations. In Belarus populations 81.4 ± 1.6 % of *pipiens* mosquitoes are infected. The data are in accordance with those obtained by Vinogradova et al. [12] for the European part of Russian Federation: *Cx. torrentium* is not infected by *Wolbachia* also, and 70 – 90 % of *molestus* and *pipiens* mosquitoes are infected [12]. However, according to Duron et al. [4] 100% of *molestus* and *pipiens* in Portugal, Spain, France, Italy, Switzerland, Belgium, Great Britain, Netherlands, Greece, Turkey, Cyprus, Tunisia, and Australia are infected by *W. pipientis*.

Transposable element *Trl* polymorphism discovered by Duron et al. [4] consists in either presence or absence of the element as well as in sequence variations. Totally, five *W. pipientis* strains have been described: *wipip3*, which lacks *Tr1*; *wPip1*, *wPip2-A*, *wPip4*, which contain distinct *Tr1* alleles; *wPip2-B*, which contains the same *Tr1* sequence as *wPip2-A*, but at distinct locus [4].

In the populations studied two *Wolbachia* strains, *wPip3* и *wPip2-A* occur. All infected *molestus* mosquitoes carry the *wPip3* strain only, whereas *pipiens* carry both *wPip3* and *wPip2-A*. The *wPip2-A* strain has been found in Tomsk, Novosibirsk, Krasnodar and Altay Regions, and Republic of Belarus. The Kazakhstan and Kyrgyz populations of *pipiens* are infected by the *wPip3* strain only but this result may be due to low number of populations of *pipiens* studied in these regions. The *wPip3* strain is the most abundant in *pipiens* populations: 63.6 ± 1.58 % of all infected mosquitoes, but in different populations this value varies greatly: from 6.4 to 100 %; *wPip2-A* accounts for 36.4 ± 1.58 %. The *wPip2-A* strain is more common in Belarus *pipiens* populations (61.5 ± 2.3%).

Previously it has been demonstrated that *wPip3* found in Central and South America, Europe, Africa, Asia, and Oceania, but not in North America, *wPip3* is the most common *Wolbachia* strain in *pipiens*, *molestus*, and *C. p. quinquefasciatus* populations. The *wPip1* strain is prevalent in North America and overlaps with *wPip3* in Spain, Portugal, and South France populations. The *wPip2-A*
strain is present in Great Britain populations only, whereas wPip4 – in Cyprus’s only. It is noteworthy that in Great Britain molestus was infected by wPip3 and C. p. pipiens – by wPip2-A [4]. Our data indicate that molestus are infected by wPip3, while pipiens populations – by two Wolbachia strains – wPip2-A and wPip3, occurring sympatrically.

The wPip infections were genotyped using a series of specific PCR-RFLP (restriction fragment length polymorphism) assays based on pk1 Wolbachia marker, according to Dumas et al. [11]. 1637 specimens of pipiens (933 specimens), and molestus (704 specimens) infected by Wolbachia were included in pk1 analysis. We have been able to amplify pk1 alleles just for 417 specimens, including 321 pipiens and 96 molestus. We found the three known pk1 alleles: a (17.99 ± 1.88 %), c (58.99 ± 2.41 %) and d (23.02 ± 2.06 %). The molestus specimens carry the pk1 allele d only (100 %), whereas pipiens carry both a and c pk1 alleles. The pk1 allele c is the most abundant in pipiens populations (76.64 ± 2.36 %), it has been found in all studied regions. The pk1 allele a accounts for 23.36 ± 2.36 %, this allele was not detected in pipiens populations in Republic of Belarus despite the fact that almost half of pipiens specimens was collected in Republic of Belarus.

Therefore, a correlation between Wolbachia strain and Taxa was observed. The molestus populations carry the wPip3 strain (based on Tr1) and pk1 allele d only. Pipiens carry both wPip3 and wPip2-A (based on Tr1) and pk1 alleles a and c.

Reproductive isolation is one of the most important factors of speciation. Wolbachia may promote rapid speciation by causing reproductive incompatibility (CI) between populations [13], particularly when bidirectional incompatibility occurs between populations harbouring different bacteria [14].

There are many evidences that in moderate climate areas gene flow between local populations of the pipiens and molestus is restricted. The population genetic analyses in natural populations pipiens and molestus from Russia and Republic of Kazakhstan [15], England [16] and Germany [17, 18] revealed much higher genetic distances between local populations of pipiens and molestus compared to the low differentiation between populations within each taxon. The specific features of reproduction and development of the two forms has resulted in their spatial isolation in moderate climate areas, suggesting genetic isolation. The results of these investigations showed that in these regions the forms are genetically distinct, with no or poor gene flow between populations of different forms [15-18]. We have shown that molestus and pipiens are not biotopically isolated, on the contrary, their co-habitation is usual. On the other hand, our data about distribution of Wolbachia strains in natural populations of Cx. pipiens complex may suggest the existence of isolation mechanisms. It is important to note, that according to our data molestus is infected by wPipIV strain and pipiens is infected by wPipl and wPipII strains of Wolbachia. According to Atyame et al. [19] the wPipIV strain is incompatible (unidirectional and bidirectional incompatibility) with the most other strains, including wPipl and wPipII [19] and thus, Wolbachia may play the key role in genetic isolation of pipiens and molestus.

Our long-term studies (1999 - 2014) of species composition of Cx. pipiens complex mosquitoes indicates that at Asian part of Russian Federation Cx. torrentium is dominant species and in most habitats replace pipiens [20-22].

References
[1] Chevillon C, Rivet Y, Raymond M, Rousset F, Smouse P E and Pasteur N 1998 Migration/selection balance and ecotypic differentiation in the mosquito Culex pipiens Molecular Ecology 7 pp 197–208
[2] Vinogradova E B 1997 Mosquitoes of the Culex pipiens Complex in Russia (the Distribution, Taxonomy, Ecology, Physiology, Genetics, Applied Significance and the Control) (St. Petersburg: Zoological Institute of Russian Academy of Science) p 307
[3] Werren J H, Baldo L and Clark M E 2008 Wolbachia: master manipulators of invertebrate biology Nature Reviews Microbiology 6 pp 741–751
[4] Duron O, Lagnel J, Raymond M, Bourtzis K, Fort P and Weill M 2005 Transposable element polymorphism of Wolbachia in the mosquito Culex pipiens: evidence of genetic diversity, superinfection and recombination Molecular Ecology 14 pp 1561–73.
[5] Atyame C M, Duron O, Tortosa P, Pasteur N, Fort P and Weill M 2011 Multiple Wolbachia determinants control the evolution of cytoplasmic incompatibilities in Culex pipiens mosquito populations Mol. Ecol 20 (2) pp 286–298

[6] Khrabrova N V, Sibataev A K and Stegni V N 2006 Molecular markers in the identification of members of Culex pipiens (Diptera: Culicidae) Proc. of 1st All-Russian Conference on Bloodsucking Insects pp 211–213

[7] Smith J L and Fonseca D M 2004 Rapid assays for identification of members of the Culex (Culex) pipiens complex, their hybrids, and other sibling species (Diptera: Culicidae) American Journal of Tropical Medicine and Hygiene 70 pp 339–345

[8] Vinogradova E B and Shaikevich E V 2005 Differentiation between the urban mosquito Culex pipiens f. molestus and Culex torrentium (Diptera, Culicidae) by molecular methods Parasitologia 39 pp 574–576

[9] Shaikevich E V 2007 PCR-RFLP of the COI gene reliably differentiates Cx. pipiens, Cx. pipiens f. molestus and Cx. torrentium of the Pipiens Complex European Mosquito Bulletin 22 pp 25–30

[10] Zhou W, Rousset F and O’Neill S 1998 Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences Proc. R. Soc. Lond. B 265 pp 509–515

[11] Dumas E, Atyame C M, Milesi P, Fonseca D M, Shaikevich E V, Unal S, Makoundou P, Weill M and Duron O 2013 Population structure of Wolbachia and cytoplasmic introgression in a complex of mosquito species BMC Evol. Biol. 13 pp 181

[12] Vinogradova E B, Shaikevich E V and Ivanitsky A V 2007 A study of the distribution of the Culex pipiens complex (Insecta: Diptera: Culicidae) in the European part of Russia by the molecular methods of identification Comparative Cytogenetics 1 pp 129–138

[13] Breeuwer J A J and Werren J H 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species Nature 346 pp 511–518

[14] Werren J H, Zhang W and Guo L R 1995 Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods Proc. R. Soc. Lond. B 261 pp 55–71

[15] Lopatin O E 2000 Gnats Culex Pipiens: Electrophoretic Diversity of Enzymes. Sibirskiy Ekologicheskiy Zhurnal 4 pp 461–475

[16] Byrne K and Nichols R A 1999 Culex pipiens in London underground tunnels: differentiation between surface and subterranean populations Heredity 82 pp 7–15

[17] Weitzel T, Collado A, Jöst A, Pietsch K, Storch V and Becker N 2009 Genetic Differentiation of Populations within the Culex pipiens Complex and Phylogeny of Related Species J. Am. Mosq. Control Assoc. 25 (1) pp 6–17

[18] Becker N, Jöst A and Weitzel T 2012 The Culex pipiens complex in Europe J. Am. Mosq. Control Assoc. 4 pp 53–67

[19] Atyame C M, Labbé P, Dumas E, Milesi P, Charlat S, Fort P and Weill M 2014 Wolbachia Divergence and the Evolution of Cytoplasmic Incompatibility in Culex pipiens. PLoS One 9 (1): e87336. doi:10.1371/journal.pone.0087336

[20] Khrabrova N V, Sibataev A K and Stegni V N 2006 The distribution of mosquitoes of Culex pipiens complex (Diptera: Culicidae) in Tomsk region and Republic of Kazakhstan. Entomological studies in North Asia: VII interregional meeting of entomologists of Siberia and Russian Far East pp 434–435

[21] Khrabrova N V, Bukhanskaya E D and Sibataev A K 2008 The distribution of mosquitoes of Culex pipiens complex (Diptera: Culicidae) in Russian Federation and nearby countries // International Conference, Stavropol pp 385–390

[22] Khrabrova N V, Bukhanskaya E D, Sibataev A K and Volkova T V 2009 The distribution of strains of endosymbiotic bacteria Wolbachia pipientis in natural populations of Culex pipiens mosquitoes (Diptera: Culicidae) European Mosquito Bulletin 27 pp 18–22