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Wolbachia Infection and Mitochondrial DNA Comparisons among *Culex* Mosquitoes in South West Iran

A. Behbahani
Department of Medical Entomology, School of Health,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Abstract: The control of mosquito borne diseases needs new methods given widespread insecticide resistance in many mosquito species. The inherited endosymbiont *Wolbachia,* found in many arthropods, provides a biological system to reduce the transmission of these diseases and replace the population of vectors with non-vectors using cytoplasmic incompatibility. The aim of this study was to understand the rate of *Wolbachia* infection among *Culex* species in the region and to see the effect of *Wolbachia* infection on mitochondrial genome. In this study three species of *Culex* mosquitoes were collected from Shoushtar in south west of Iran and examined for *Wolbachia* infection by Polymerase Chain Reaction (PCR). All of the *C. quinquefasciatus* specimens were infected with *Wolbachia*, while *C. tritaeniorynchus* and *C. theileri* showed no infection with *Wolbachia.* The 340 bp of AT rich of mtDNA was sequenced from 30 individuals, 10 individuals of each species. Three sequence haplotypes were found in *C. tritaeniorynchus* and *C. theileri* while there was only one haplotype in *C. quinquefasciatus*. The reduction of haplotypes diversity may be result of a sweep of *Wolbachia* in this species.

Key words: AT rich, genetic control, shoushtar, sweep of *Wolbachia, C. quinquefasciatus*, *C. tritaeniorynchus*, *C. theileri*, inherited endosymbiont

INTRODUCTION

More than half of the population of the world lives in regions where mosquito borne diseases are widespread (TDR, 2009). The interruption of life cycle of the vector is the most effective method to control arthropod borne diseases. The extensive use of chemical insecticides against the vectors of diseases and harmful pests has resulted in the spread of insecticide resistance and relapse and recrudescence of these diseases (Gubler, 1998, 2009). Therefore, the investigation on the alternative methods which are safe in the environment is on the top of priorities.

Wolbachia is a group of bacteria belonged to alphaproteobacteria that live in the cytoplasm and transmit maternally to offspring of their hosts (O'Neill *et al*., 1992). About 66% of insect species are estimated to be *Wolbachia* infected (Hilgenboecker *et al*., 2008). These microbes manipulate the reproductive system of their hosts to increase their transmission probability within the infected population. Cytoplasmic incompatibility is the most widespread of these manipulation phenotypes of *Wolbachia* (Sinkins, 2004). On the basis of the above reason *Wolbachia* can be used as an important system to drive a suitable gene into the disease vectors population to disable them to transmit the disease agents, life-shortening or they can strongly inhibit the development of pathogens within the mosquito (McMeniman *et al*., 2009; Kambris *et al*., 2009, 2010; Moreira *et al*., 2009).

The biological control of mosquito in any area requires knowledge of field parameters such as the vector species, the parasites or related micro-organisms, the rate of natural infection and the interaction of the vector with the environment. Here field-collected *Culex* mosquitoes were examined for *Wolbachia* and in addition the diversity of mitochondrial DNA was examined in relation to *Wolbachia* infection.

MATERIALS AND METHODS

Culex specimens were collected from Shoushtar in Khouzestan province in South West of Iran during April 2001. The *Culex quinquefasciatus* and *Culex theileri* samples were caught by hand aspirator during the day time from two different places in one village (Abeed haji baba) and *Culex tritaeniorynchus* were caught by CDC light trap during the night from one animal house of another village (Shoaebiae). The samples were then killed with chloroform and individually preserved dry

in 1.5 mL microcentrifuge tubes under silica gel until identification in the laboratory. The samples were identified to species using morphological key of Zaim and Cranston (1986).

Total individual DNA was extracted from each single sample of *Culex quinquefasciatus*, *C. tritaeniorynchus* and *C. theileri* by the Livak DNA extraction procedure (Collins *et al*., 1987). They were examined for *Wolbachia* infection with PCR using the primers *wsp.* 81F (TGGTCCAATAAGTGATGAAGAAAC) and *wsp.* 691R (AAAAATTAAACGCTACTCCA) which were designed previously by Braig *et al.* (1998). The PCR conditions were as follows: 1X reaction buffer; 0.2 mM dNTPs mix; 5 mM $MgCl_2$; 0.2 μM forward primer; 0.2 μM reverse primer; 0.04 u μL^{-1} Taq DNA polymerase; nuclease-free water and template DNA. PCR was performed in a 100 μL reaction mixture. The first seven reaction components were combined in a 1.5 mL microcentrifuge tube and were mixed gently. Then, 4 μL DNA template was added in 96 μL of reaction mixture in 0.2 mL thin walled PCR tubes, these were mixed and transferred to the PCR cycler with the following conditions: 94°C, 5 min (1 cycle); 95°C, 30 sec; 48°C, 1 min; 62°C, 2.5 min (40 cycles) and 72°C for 5 min (1 cycle). The PCR reaction products were analyzed by agarose gel electrophoresis of a 5 μL aliquot from the total reaction. The products were observed by UV transillumination of the ethidium bromide-stained gel.

The A+T rich region in *Culex* spp. was amplified using general primer SR-J-14612 (Simon *et al*., 1994) as forward and CxAT3 (Guillemaud *et al*., 1997) as reverse primer. Specific new primers were designed to amplify this region in C. *tritaeniorynchus, C. quinquefasciatus* and *C. theileri*. The new designed primers as indicated in Table 1 were as TR-ATF and TR- ATR to amplify an A+T region in *C. tritaeniorynchus,* QU,TH-ATF, QU-ATR and TH-ATR to amplify the region in *C. quinquefasciatus* and *C. theileri*.

Table 1: Primers used to amplify A+T rich region sequence and the percent of GC content

Region	Sequence 5'-3'	T_m(°C)	GC content (%)
TR-ATF	TGTATAACCGCGGTAGCTGG	59.4	55.0
TR-ATR	TTATTTTTGATTGCGGGGAG	53.2	40.0
QU,TH-ATF	CGGTAGCTGGCACAAATTTTA	55.9	42.9
QU-ATR	GAGCAATGGGAAGGCTTACA	57.3	50.0
TH-ATR	TTTTTGAGCTACGGGAAGGA	55.3	45.0

RESULTS AND DISCUSION

Total 98 *Culex* spp. samples were collected in which 44 samples were identified as *C. quinquefasciatus*, 34 *C. tritaeniorynchus* and 20 *C. theileri*. All of the *C. quinquefasciatus* were infected with *Wolbachia*, based on PCR amplification of the *wsp* gene, while none of the *C. tritaeniorynchus* and *C. theileri* samples produced amplification of the *Wolbachia wsp* gene (Table 2).

The 340 bp of AT rich of mtDNA was sequenced from 30 individuals, 10 each of *C. quinquefasciatus*, *C. tritaeniorynchus* and *C. theileri*. The base composition of the mtDNA sequences as indicated in Table 3 is highly AT-biased with average of 89.1, 91.1 and 89.5% AT in the sequences of *C. quinquefasciatus, C. tritaeniorynchus* and *C. theileri*, respectively. Three sequence haplotypes were found in *C. tritaeniorynchus* and *C. theileri* while there was only one haplotype in *C. quinquefasciatus*.

Epidemics of the vector borne diseases such as malaria and arboviruses or re-emergence of them, following the economic and social changes after initial success, mean that these diseases still remains a major problem in many tropical or subtropical countries (Raghavendra *et al*., 2011; Gubler, 2009). The phenomenon of the cytoplasmic incompatibility of *Wolbachia* leading to spread in mosquito populations and its ability to induce inhibition of the development of viruses and parasites in the vector, plus the effects of lifespan-shortening this micro-organism made it a suitable way to prospect to control the vector borne diseases and malaria (McMeniman *et al*., 2009; Moreira *et al*., 2009; Kambris *et al*., 2009, 2010).

Study of *Wolbachia* infection in the field is an important step to evaluate the *Wolbachia* as a driving gene system (Turelli and Hoffmann, 1999). *Wolbachia* frequency and density in hosts vary significantly in nature compared to laboratory strains of the same species.

Table 2: The number of mosquito individuals, *Wolbachia* infection and the percent of *Wolbachia* infection in each species

Culex sp.	No.	*Wolbachia* infection	*Wolbachia* infection (%)
C. quinquefasciatus	44	Yes	100
C. tritaeniorynchus	34	No	0
C. theileri	20	No	0

Table 3: The number of mosquito individuals, sequence size (bp), the percent of AT, the number of mitochondrial haplotypes within each group of *Culex* species based on the AT-rich region sequence and the positions of nucleotide changes

Culex spp.	No.	Total size (bp)	AT content (%)	No of Haplotypes	The positions of nucleotide changes
C. quinquefasciatus	10	304	89.1	1	-
C. tritaeniorynchus	10	303	91.1	3	A-G (87), G-A (234)
C. theileri	10	304	89.5	3	T-C(80), A-T(232), C-T(274), C-T(280), A-G(282)

Part of these variation it is not only dependent on the genome of the host but environmental factors such as temperature can play a very important role in the varieties (Echaubard *et al*., 2010). In the collection region the temperature reaches more than 50°C in summer time and since high temperatures are known to be a cause of *Wolbachia* loss in laboratory studies, this could in theory reduce the incidence of *Wolbachia*. However, all 44 specimens of *C. quinquefasciatus* examined were positive for *Wolbachia*.

Wolbachia infection was not seen in any *C. tritaeniorynchus* samples and these negative results were similar with another study of *Wolbachia* incidence in *Culex* spp. in Thailand (Tiawsirisup *et al*., 2008). Also, positive amplification of the *Wolbachia* gene was not seen in *C. theileri*, a species which is considered as the main vector of *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal (Santa-Ana *et al*., 2006). No previous studies have examined *Wolbachia* presence or absence in this species.

In the current study, the AT region as part of the mtDNA genome was sequenced from individuals of the *Culex* species *C. quinquefasciatus*, *C. tritaeniorynchus* and *C. theileri*. Haplotype diversity was just one for the *Wolbachia* infected *C. quinquefasciatus* while three haplotypes were seen in the uninfected populations (*C. tritaeniorynchus* and *C. theileri*). This reduced mtDNA diversity seen in *C. quinquefasciatus* may have been the result of the spread of *Wolbachia* on a relatively recent evolutionary time scale (since both *Wolbachia* and *Mitochondria* are solely maternally inherited, there is complete linkage between them and thus spread of a mtDNA variant by hitchhiking is associated with the spread of a *Wolbachia* infection. The presented results are supported by previous studies of mitochondrial genes and *Wolbachia* infection (Turelli *et al*., 1992; Ballard and Kreitman, 1994; Rand *et al*., 1994; Ballard *et al*., 1996; Johnstone and Hurst, 1996; Rigaud *et al*., 1999; Shoemaker *et al*., 1999; Ballard, 2000). Low intraspecific mtDNA variation can also result from some demographic event affecting females such as recent bottlenecks, low effective population size or from a recent selective sweep of a favoured mtDNA variant (Johnstone and Hurst, 1996), further field and population genetic studies could be used to clarify these issues.

CONCLUSION

Our data, for the first time, showed that *Culex theileri* samples were negative for *Wolbachia*. While, the *Culex quinquefasciatus* samples were positive with only one haplotype of mitochondrial genome. Regarding these field data, conducting a local genetic control programme and laboratory experiments, to horizontal transfer of *Wolbachia* strains to main vectors of important vector borne disease will be considered.

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