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Short Communication

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Lack of Evidence for Natural *Wolbachia* Infections in *Aedes aegypti* (Diptera: Culicidae)

Andrea Gloria-Soria,[1–3] Tommaso G. Chiodo,[2] and Jeffrey R. Powell[2]

[1]Department of Environmental Sciences, The Connecticut Agricultural Experiment Station, 123 Huntington Street, PO Box 1106, New Haven, CT 06504, [2]Ecology and Evolutionary Biology Department, Yale University, New Haven, CT 06520, and [3]Corresponding author, e-mail: Andrea.Gloria-Soria@ct.gov

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Abstract

Wolbachia is a genus of endosymbiotic bacteria that infects 66% of all insect species. Its major impact on insects is in reproduction: sterility, production of one sex, and/or parthenogenesis. Another effect was discovered when the disease-transmitting mosquito, *Aedes aegypti* Linnaeus (Diptera: Culicidae), was infected with *Wolbachia* isolated from Drosophila: infected female mosquitoes became less capable of transmitting diseases such as dengue fever and chikungunya. This has led to releases of *Ae. aegypti* carrying *Wolbachia* in an attempt to control disease. An open question is whether there are natural *Wolbachia* infections of this mosquito. We assayed DNA from 2,663 *Ae. aegypti* from 27 countries on six continents, 230 from laboratory strains, and 72 *Aedes mascarensis* MacGregor (Diptera: Culicidae) for presence of *Wolbachia* DNA. Within the limits of our polymerase chain reaction–based assay, we found no evidence of *Wolbachia,* suggesting that natural infections of this endosymbiont are unlikely to occur throughout the worldwide distribution of *Ae. aegypti*.

Key words: *Wolbachia*, *Aedes aegypti*, *Aedes mascarensis*, endosymbiont, biological control

Wolbachia is a widespread bacterial endosymbiont found in a large number of arthropods, including 66% of all insect species, encompassing all orders (Hilgenboecker et al. 2008). Insects infected with *Wolbachia* often exhibit major disruptions in reproductive biology, including sterility, production of one sex, or parthenogenesis (Warren et al. 2008). A practical use of this endosymbiont–insect relationship was the discovery that when the disease-transmitting mosquito, *Aedes aegypti* Linnaeus (Diptera: Culicidae), is infected with a strain of *Wolbachia* isolated from *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), the mosquito is rendered much less capable of transmitting viruses causing diseases such as dengue, chikungunya, Zika, and yellow fever (Moreira et al. 2009; van den Hurk et al 2012; Aliota et al. 2016a,b). This has led to disease control programs using *Wolbachia*-infected *Ae. aegypti* releases in hopes of replacing the target *Wolbachia*-free mosquito population, thus reducing the risk of disease transmission by the vector.

One question that remains is whether there are any natural *Wolbachia* infections in *Ae. aegypti*. Surveys of natural populations of this mosquito for presence of *Wolbachia* have been limited (e.g., Kittayapong et al. 2000 in Thailand) and were negative. However, there is one report of three *Ae. aegypti* individuals from Florida positive for *Wolbachia* sequences (Coon et al. 2016). Another major disease vector mosquito, *Anopheles gambiae* Giles (Diptera: Culicidae), also thought not to be naturally infected with *Wolbachia*, was found to be infected in a limited geographic range in Burkina Faso (Baldini et al. 2014). Could wider sampling of *Ae. aegypti* reveal places that have populations naturally infected with *Wolbachia*? Over the last decade, we have accumulated a worldwide collection of more than 200 samples of *Ae. aegypti* from six continents and have stored genetic material from most of them. Here, we report results of a screen for the presence of *Wolbachia* DNA, conducted on 2,663 individual *Ae. aegypti* from 27 countries, as well as lab strains. We also assayed field and colony specimens of *Aedes mascarensis* MacGregor (Diptera: Culicidae), the closest relative to *Ae. aegypti* with which it can hybridize and produce fertile offspring (McClelland and Mamet 1962), thus potentially transferring endosymbionts.

Materials and Methods

In total 2, 663 *Ae. aegypti* mosquitoes from 63 populations representing 27 countries around the world, 230 from laboratory strains, and 32 field-collected *Ae. mascarensis* from Mauritius and 40 *Ae. mascarensis* from a colony we established in 2014 (~15 generations ago), were screened for the presence of *Wolbachia* DNA via polymerase chain reaction (PCR; Supplementary Table S1, Fig. 1).

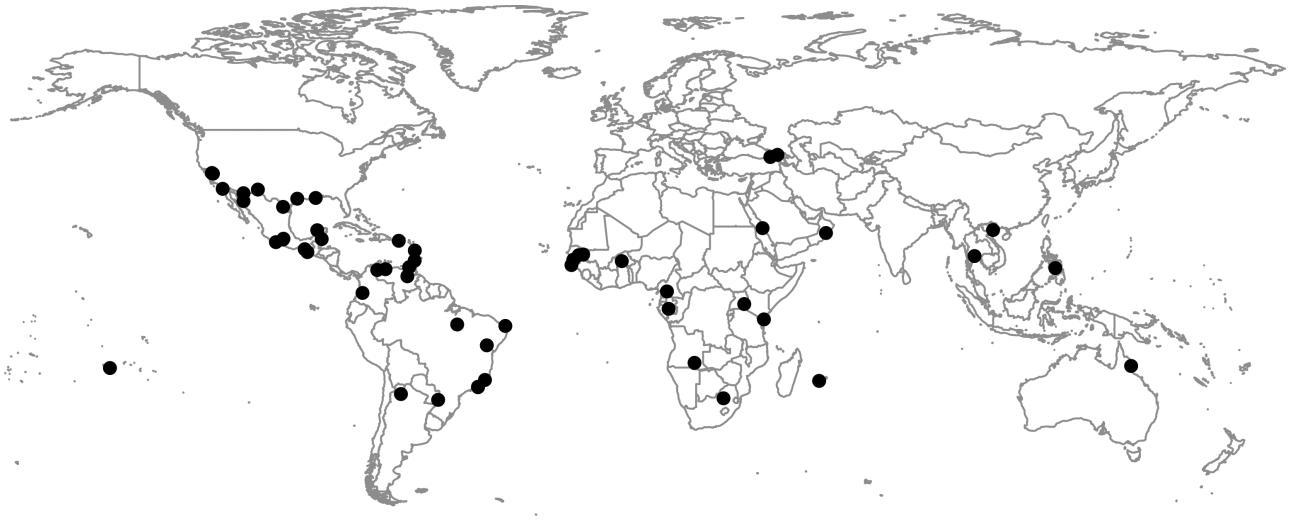

Fig. 1: World distribution of field sampling locations of *Aedes aegypti* and *Aedes mascarensis* screened for *Wolbachia* in this study.

With the exception of the laboratory strains, all specimens came either directly from the field or from F1–F2 generation in the laboratory. The screen targeted two genes, the *Wolbachia* outer surface protein gene *wsp* and the GroE operon containing the highly conserved bacterial heat shock proteins, GroES and GroEL. The *wsp* screen was performed with three different primer pairs: 1) *wsp*81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp*691R (5′-AAAAATTAAACGCTACTCCA-3′); found to amplify a 590–632 bp fragment of the *wsp* gene in different strains of *Wolbachia* groups A and B (Braig et al. 1998; Zhou et al. 1998). 2) *wspF* (5′-TGACTTCCGGAGTTACATCATAAC-3′) and *wsp*R (5′-AGTTGATGGTATTACCTATAA-3′); originally designed to amplify a 410 bp region of *wsp* locus of the tsetse fly (*Glossina morsitans morsitans* Wiedemann [Diptera: Glossinidae]) *Wolbachia* genome by the Aksoy laboratory at the Yale School of Public Health (unpublished) and determined to preferentially amplify *Wolbachia* group A based on sequence homology. 3) *wsp*81F (described above) and *wsp*522R (5′-ACCAGCTTTTGCTTGATA-3′); a combination of primers capable of discriminating the *Wolbachia* group B and that amplify a 442bp fragment of *wsp* (Zhou et al. 1998). The GroE screen was conducted with primers: *groEwF* (5′-GAAGAAAAACAAGGTGGAATTG-3′) and *groEwR* (5′-GTACCATCACCAACTTTGTC-3′) from Wiwatanaratanabutr et al. (2009), generating a 630 bp fragment. GoTaq DNA polymerase (Promega Corporation, WI) was used for most of the reactions following manufacturer instructions. Thermocycler conditions were: 95°C for 3 min; 30 × [95°C for 1 min, 57°C for 45 s, 72°C for 1 min]; 5 min at 72°C followed by a 4°C hold. Phusion High-Fidelity DNA polymerase (New England BioLabs Inc. MA) was used for the *wspF*/*wspR* amplifications following manufacturer instructions. Thermocycler conditions were: 98° for 30 s; 30 × [98°C for 10 s, 58°C for 30 s, 72°C for 30 s]; 5 min at 72°C followed by a 4°C hold. The following representative samples of group A and B *Wolbachia* strains (Zhou et al. 1998, Wiwatanaratanabutr et al. 2009) were used as positive controls: *Glossina morsitans* from a *Wolbachia*-infected colony (Alam et al. 2011); *Ae. aegypti* from areas in Cairns (AU) where *Wolbachia* releases have taken place for almost a decade (Hoffmann et al. 2011); field-collected *Aedes albopictus* Skuse (Diptera: Culicidae) from Mexico, Brazil, and the Black Sea; pinned specimens from *Mansonia titillans* Walker (Diptera: Culicidae) and *Mansonia dyari* Belkin, Heinemann & Page (Diptera: Culicidae); and *Culex pipiens* Linneaus (Diptera: Culicidae) and *Culex quinquefasciatus* Say (Diptera: Culicidae) from a colony at The Connecticut Agricultural Experiment Station. Negative controls (no template DNA) were run simultaneously to check for reagent contamination. Screening was performed on pools of up to 20 individuals and only individuals from positive pools were subsequently screened individually for confirmation. The ability to detect one infected individual within a pool was demonstrated using the *Ae. aegypti* and *G. morsitans* positive controls. The presence of *Wolbachia* DNA was visually scored on a 1.5% agarose gel after running 7 µl of the PCR product at 100 V for 30 min.

Results

With the exception of the *Ae. aegypti* Cairns (Australia) sample, where *Wolbachia*-infected mosquitoes have been released for several years (Hoffmann et al. 2011), no *Wolbachia* DNA was detected in any of the 2,893 *Ae. aegypti* mosquitoes (2,663 wild caught and 230 from laboratory colonies) screened for this endosymbiont, nor in *Ae. mascarensis*. These include mosquitoes from 63 populations from 27 countries (Fig. 1 and Supplementary Table S1). The ability of the screening primers to detect *Wolbachia* DNA was demonstrated by positive amplification of the tsetse fly (*G. morsitans*), *Ae. albopictus*, *D. melanogaster* (amplified from released individuals collected in Cairns, AU), *M. titillans*, *M. dyari*, *C. pipiens*, and *C. quinquefasciatus Wolbachia*. These controls yielded a positive result even when the known *Wolbachia*-positive samples (tsetse or mosquito) constituted 5% or less of the total DNA in the pool, supporting the feasibility of detecting even a single positive sample within our pools of 20 individuals.

Discussion

Our uniformly negative results contrast with one positive report based on larval collections in Jacksonville, FL (Coon et al. 2016). This study surveyed bacterial communities using bacterial 16S rRNA PCR primers. Both larvae and three adults reared from larvae were positive for two strains of *Wolbachia* related to strains colonizing *Ae. albopictus*. No *Wolbachia*-positive strain was established in the lab and resampling of the site with positive larvae a year later

did not detect *Wolbachia* in the *Ae. aegypti* sampled (M. Strand, personal communication).

Our *Ae. aegypti* sampling has been quite thorough across the species range, and the fact that we were unable to detect evidence of *Wolbachia* infections makes it unlikely that further sampling will reveal populations of *Ae. aegypti* with naturally established infections of this endosymbiont, at least within the limits of our PCR-based assay. Although our primers successfully detected natural infections in a variety of Diptera, belonging to *Wolbachia* groups A and B (Zhou et al. 1998, Wiwatanaratanabutr et al. 2009), it is conceivable that *Ae. aegypti* could be naturally infected with a strain(s) of *Wolbachia* divergent enough to be undetected. An NCBI BLAST search performed using these primers returned perfect matches for *Wolbachia* from other Dipterans, including mosquitoes (*Armigeres*, *Culex*) and sand flies (*Phlebotomus*), and non-Dipterans such as ants (*Technomyrmex*, *Solenopsis*), and beetles (*Deronectes*, *Hypothenemus*), among others (data not shown). These BLAST results together with our positive controls, suggest that our screen would have picked up a wide variety of *Wolbachia* strains, if they were present. We did not measure the limits of detection of this assay to the individual genome copies, so it is possible that a much lower abundance of *Wolbachia*, relative to our control species, may prevent detection by this method.

Had we found a *Wolbachia*-positive population of *Ae. aegypti*, it would have been interesting to explore whether the symbiotic relationship produced similar levels of refractoriness to transmission of pathogens in infected females and its impact on *Ae. aegypti* reproduction and life history traits. A naturally occurring vector-endosymbiont association, implying coadaptation, may have proved more stable than the artificial mosquito-Drosophila system currently being used for disease control, and may have circumvented the fitness deficits observed in the artificial system (Moreira et al. 2009), which likely limit the spread of infections after release (Schmidt et al. 2017). We were unable to find evidence of natural *Wolbachia* infections in *Ae. aegypti* anywhere in the world, to the extent represented in our collection. Thus, if such infections occur they are extremely rare.

Supplementary Material

Supplementary data are available at *Journal of Medical Entomology* online.

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Conflicts of interest

The authors declare no conflicts of interest.

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