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MOLECULAR AND ELECTRON MICROSCOPIC IDENTIFICATION OF *WOLBACHIA* IN *CULEX PIPIENS* COMPLEX POPULATIONS FROM THE UPPER RHINE VALLEY, GERMANY, AND CEBU CITY, PHILIPPINES

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ABSTRACT. We detected *Wolbachia* infections in *Culex pipiens* complex mosquitoes from the Upper Rhine Valley, Germany, and Cebu City, Philippines, with the use of polymerase chain reaction (PCR) amplification of the 16S rDNA of the bacteria and further confirmation by electron microscopy. Species of 5 different autogenous and anautogenous populations of *Culex pipiens* from Germany and 1 population of *Culex quinquefasciatus* from the Philippines were used in this study. Larvae were sampled from different localities and reared to the adult stage for further processing. Whole adult males and the heads of adult females were processed by PCR, whereas the abdomens of females were used for electron microscopic study when an infection was detected by PCR. All *Culex* populations assayed by PCR showed infection of *Wolbachia* at rates between 10 and 100%. Females from different populations exhibited higher infection rates than did the males, and, likewise, *Cx. pipiens* populations that were autogenous showed higher infection rates of *Wolbachia* than did anautogenous individuals. In *Cx. quinquefasciatus* populations, males were infected at 33.33% and females, only 10%. We studied the ultrastructure of *Wolbachia* in the ovaries of species belonging to *Cx. pipiens* complex by means of transmission electron microscopy. The bacteria exhibited typical morphology for *Wolbachia* with 3 enveloping membranes.

KEY WORDS *Wolbachia, Culex pipiens,* Germany, *Culex quinquefasciatus,* Philippines

INTRODUCTION

About 15–20% of all known insect genera are estimated to contain *Wolbachia* that are maternally inherited (Tai Min and Benzer 1997). *Wolbachia* are obligate intracellular symbionts that appear to be present in many invertebrates species, including species in the mosquito genera *Culex, Aedes,* and *Armigeres* (Clements 1992). *Wolbachia pipientis* in insects have been long implicated in the phenomenon of cytoplasmic incompatibility (CI), in which certain crosses between symbiont-infected individuals lead to embryonic death or sex ratio distortion (O'Neill et al. 1992). This phenomenon has been observed in many insect species including mosquitoes. Infection was first detected in the ovaries and testes of the mosquito *Culex pipiens* L. by light miscroscopy. It was named and described in the same insect by Hertig (1936). The ultrastructure of *Wolbachia* is typical of gram-negative bacteria. It has 3 plasma cell membranes, a cell wall, and an outermost cell membrane that is thought to be of host origin. Many ribosomes and fine strands of DNA are present within the cytoplasm (Clements 1992).

Wolbachia infections are mostly restricted to germ line cells, within which they are present in the cytoplasm. The bacteria occur in 2 different forms: rods and cocci (rounded cells). They are present in all life stages of mosquitoes. In recently laid eggs, *Wolbachia* is concentrated near the micropyle. As the embryo develops, *Wolbachia* infection becomes more restricted to the pole cells. During the 3rd and 4th larval stages, *Wolbachia* multiplies rapidly. It is transmitted transovarially to subsequent generations (Yen and Barr 1974, Yen 1975, Wright and Barr 1981, Clements 1992).

Our main objective in this study was to determine if *Wolbachia* is present among mosquito populations of *Culex pipiens* complex from the Upper Rhine Valley, Germany, and Cebu City, Philippines, in order to acquire information concerning the present status of *Wolbachia* infection among these mosquito populations that would be useful in other research. Another objective was to compare reproduction rates of infected and uninfected mosquitoes.

MATERIALS AND METHODS

Insect strains: Individuals from 5 populations of *Cx. pipiens* from the Upper Rhine Valley, Germany, and 1 population of *Culex quinquefasciatus* Say from Cebu City, Philippines, were used in this study. Larvae were collected from various breeding sites at different localities, reared to the adult stage in the laboratory, and coded. Samples were from the following locations: Promarkt, Mainzerstrasse, Wiesbaden, Germany (P), Theodor Heuss Ring, Wiesbaden, Germany (KL), Laubertsweg, Altenheim, Baden-Württemberg, Germany (L), Schwanau-Ottenheim, Baden-Württemberg, Germany (SO), Bergweg, Mainz-Bretzenheim, Rheinland-Pfalz, Germany (B), and Cebu City, Philippines (CP). Samples from P, L, and KL were from underground breeding sites, all others were from above surface breeding sites (Table 1). Rearing tests showed that all populations from underground breeding sites were autogenous whereas all populations from above surface breeding sites were unautogenous.

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Table 1. Percentages of *Wolbachia* infections in *Culex pipiens* complex populations from the Upper Rhine Valley, Germany, and Cebu City, Philippines.

Location[1]	Type of breeding site	No. males	No. females	No. infected males	No. infected females	% infected males	% infected females
P	Underground	10	10	5	10	50	100
L	Underground	10	10	2	6	20	60
KL	Underground	10	10	3	7	30	70
SO	Above surface	10	10	1	2	10	20
B	Above surface	5	10	1	1	20	10
CP	Above surface	30	30	7	3	23.33	10

[1] P, Promarkt, Mainzerstrasse, Wiesbaden, Germany; L, Laubertsweg, Altenheim, Baden-Württemberg, Germany; KL, Theodor Heuss Ring, Wiesbaden, Germany: SO, Schwanau-Ottenheim, Baden-Württemberg, Germany; B, Bergweg, Mainz-Bretzenheim, Rheinland-Pfalz, Germany; CP, Cebu City, Philippines. P, L, and KL were collected from underground breeding sites; all others, from above ground.

Cultured individuals of a laboratory *Drosophila melanogaster* strain (carrying the genetic markers of white, black cell, glaze eyes, and curly wings) that were infected with *Wolbachia* were used as a positive control, whereas water sterilized by ultraviolet (UV) radiation (UV water) was used as a negative control for the presence of *Wolbachia.*

Preparation of mosquito samples: Twenty to 60 adult male and female mosquitoes were used in the study. The whole adult males were used for polymerase chain reaction (PCR) assays whereas each adult female was sectioned into 2 parts. Adult female heads were used for PCR assays, and the abdomen from the same individual was used for electron microscopy when infection of the head was proven by PCR.

DNA extraction: Whole adult males and heads of adult females were placed in 1.5-ml Eppendorf tubes, ground in 100 μl extraction buffer (1 M Tris-HCl, pH 9.0, 0.5 M ethylenediaminetetraacetic acid, 20% sodium dodecyl sulfate, and 1% diethypyrocarbonate) and incubated at 70°C for 30 min. To each tube, 37.5 μL of 3 M KAc was added. Homogenized specimens were placed in ice for 30 min and centrifuged at 4°C (12,000–14,000 rpm) for 15 min. Supernatants were pipetted into new tubes and cellular debris were discarded. Two hundred microliters of ethanol (EtOH) was added to each tube, and the contents were gently mixed by overturning the tubes twice. Tubes were again centrifuged at 14,000 rpm for 5 min to pellet the DNA. Supernatants were carefully discarded while not disturbing the DNA pellets. Pellets were washed once with 100% EtOH, dried at 37°C for 1 h, and resuspended in 100 μl Tris ethylenediaminetetraacetic acid at 37°C for 15 min. DNA samples were stored at 4°C until used for PCR analysis.

Polymerase chain reaction assays: Polymerase chain reaction assays were conducted with 2 primers. Primer set 1 amplified a 0.9-kb fragment from *Wolbachia* 16S rDNA and is specific to *Wolbachia* (forward: 5′TTGTAGCCTGCTATGGTATAACT-3′, reverse: 5′GAATAGGTATGATTTTCATGT-3′). Primer set 2 amplified a 0.4-kb fragment from insect mitochondrial 12S rDNA (forward: 5′CTAGGATTAGATACCCTATT-3′, reverse: 5′AAGAGCGACGGGCGATG-3′). The 2nd primer set is universal for insect mitochondrial DNA and was used as a control to check the quality of each DNA extraction. The 2 primers were amplified simultaneously. Samples were amplified in an Eppendorf PCR Master Cycler machine by the following protocol: 1 cycle of 95°C for 5 min followed by 30 cycles each of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min. After amplification, samples were held at 72°C for 5 min and stored at 4°C until used.

Polymerase chain reaction products were separated with 1% agarose gel by electrophoresis, stained with ethidium bromide, and visualized under UV light. Gels were photographed for a permanent record of results.

Preparation of mosquito samples for electron microscopy: Abdomens of infected adult female mosquitoes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for a period of 2 h. Fixation was continued for a period of 3–4 h at 4°C. Tissues were dehydrated in increasing concentrations of alcohol (50, 70, 80, 96, and 100%). After dehydration, the tissues were infiltrated overnight in a 1:1 mixture of Spurr pur and absolute alcohol that was followed by 3:1 mixture of Spurr pur and absolute alcohol. Tissues were embedded in Spurr pur and polymerized at 60°C for 24 h. Ultrathin sections (60–90 μm) were cut with a Cambridge Huxley microtome and examined under Zeiss electron microscope.

Statistical analyses: Our samples were too small to permit meaningful statistical analyses of infection frequencies among the populations studied.

RESULTS

Polymerase chain reaction analysis confirmed that all *Cx. pipiens* populations from the Upper Rhine Valley, Germany, and the population of *Cx. quinquefasciatus* from Cebu City, Philippines, were infected with *Wolbachia* (Table 1). Individuals positive for *Wolbachia* infection exhibited bands of the *Wolbachia*-specific fragment at 0.9 kb (Fig. 1A, lanes 4, 6, and 7) and the insect mitochondrial

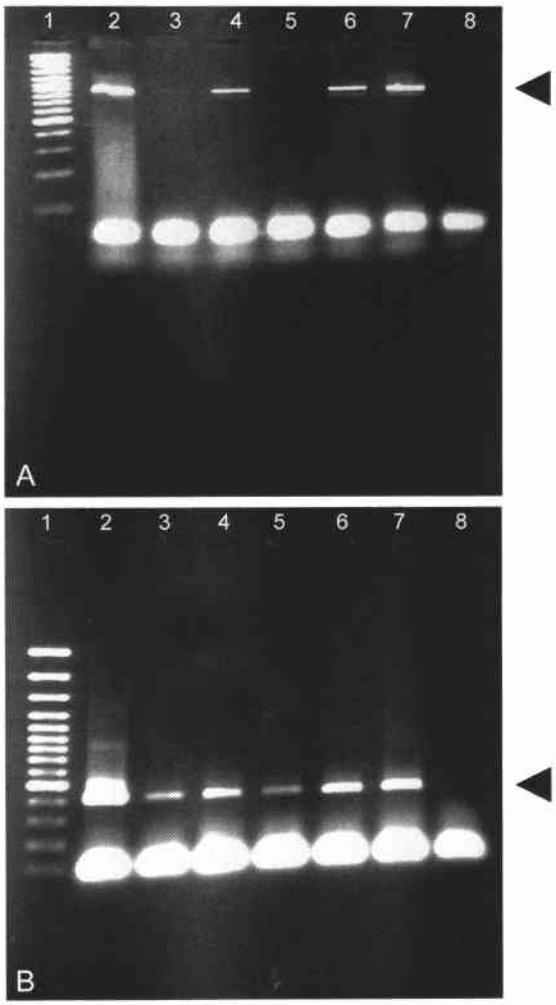

Fig. 1. (A) Example of PCR products. *Wolbachia*-specific bands are visible at 0.9 kb as indicated by the black arrow. Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3, *Wolbachia*-uninfected mosquito; 4, *Wolbachia*-infected mosquito; 5, *Wolbachia*-uninfected mosquito; 6, *Wolbachia*-infected mosquito; 7, *Wolbachia*-infected mosquito; 8, UV water negative control. (B) Insect control bands are visible at 0.4 kb mtDNA as indicated by the black arrow. Lanes: 1, DNA size markers; 2, *Drosophila*; 3–7, *Culex pipiens*; 8, UV water negative control.

DNA control fragment at 0.4 kb (Fig. 1B). For *Wolbachia*-negative individuals, only the mitochondrial DNA bands were detected.

Results showed that species from all populations of *Cx. pipiens* complex mosquitoes were infected with *Wolbachia* at different rates. The highest infection rate of 100% was observed in female populations designated P and was followed by an infection rate of 70% in KL females. Infection rates of 60, 20, and 10% were found in females of L, SO, and B populations, respectively.

The highest infection rate for males, 50%, was observed in males from population P. This rate was followed by 30% for KL males. Males of both L and B populations showed infection rates of 20%, whereas only 10% of SO males were infected.

Males of *Cx. quinquefasciatus* from Cebu City, Philippines, were infected at a rate of 23.33%; the infection rate for females was 10%.

Transmission electron photomicrographs of ovaries of infected *Cx. pipiens* complex individuals confirmed the presence of *Wolbachia*. The bacteria occurred predominantly in the oocytes (Fig. 2A). Two different forms of *Wolbachia* cells were observed, rods and cocci forms (Fig. 2B). The bacteria exhibited typical morphology for *Wolbachia* with 3 cell membranes: a plasma membrane, a cell wall, and an outermost membrane that is thought to be of host origin (Fig. 2C). Within the cytoplasm of the bacteria, various ribosomes and fine strands of DNA were present (Fig. 2D).

DISCUSSION

The results obtained showed that the underground populations exhibited high rates of *Wolbachia* infections. Under these extreme underground breeding conditions, only populations that are able to reproduce in high numbers will survive. The conditions in the underground breeding sites lead to a high selection pressure toward stenogamy, autogeny, and continuous reproduction without diapause. The advantages for the reproduction of *Culex* in underground breeding sites are evident: the regularly high content of organic materials in the breeding sites that favors larval development, reduced pressure by predators, and continuously favorable environmental conditions. On the other hand, the lack of blood meal hosts and the limited space for copulation favor both autogeny and stenogamy. Thus, we believe that infections with *Wolbachia* are consistent with selection pressures favoring evolutionary fitness because a female carrying *Wolbachia* can mate with either infected or uninfected males and produce viable eggs, all of which are infected with *Wolbachia*. A *Wolbachia* superinfection in *Aedes albopictus* (Skuse) is associated with both cytoplasmic incompatibility and increased host fecundity. Relative to uninfected females, infected females live longer, produce more eggs, and have higher hatching rates in compatible crosses (Dobson et al. 2002).

When an infected male mates with an uninfected female, the tainted sperm are not able to fertilize the eggs. Meanwhile, *Wolbachia* that are present in females produce an antidote that somehow restores the sperm to full viability (Zimmer 2001).

Experiments with two-spotted spider mites have shown that temperature has an effect on the abundance and spread of *Wolbachia*. In mites exposed to a temperature of 32±0.5°C, *Wolbachia* infections were eliminated after four generations (Opijnen Tbreeuwer 1999). This could explain the lower in-

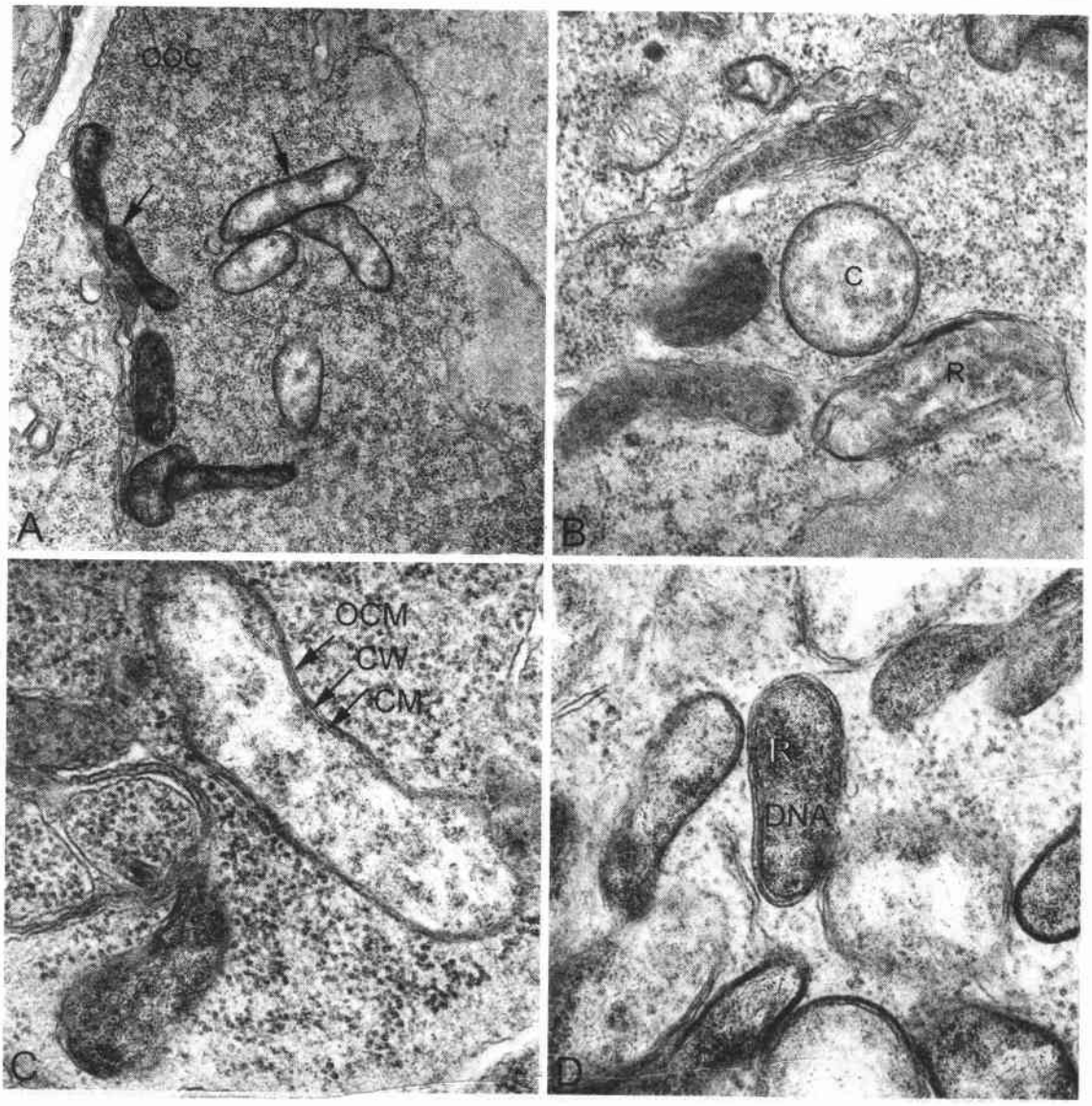

Fig. 2. Transmission electron micrographs of *Wolbachia* in ovaries of *Culex pipiens*. (A) The presence of *Wolbachia* (arrows) in an oocyte of *Cx. pipiens*.16,000×. (B) Two forms of *Wolbachia*, namely, rods (R) and cocci (C) in an oocyte of *Cx. pipiens*.12,000×. (C) *Wolbachia* showing 3 membranes, namely, cell membrane (CM), cell wall (CW), and an outermost cell membrane (OCM) that is thought to be of host origin. 40,000×. (D) Ribosomes (R) and strands of DNA in the cytoplasm of *Wolbachia*. 40,000×.

fection rate of *Wolbachia* obtained from species sampled in above surface breeding sites where temperatures may reach 30°C.

These studies confirm that PCR is a reliable method in detecting the presence of *Wolbachia* infections in *Cx. pipiens* complex mosquitoes. Infected and uninfected individuals were identified correctly, and no reaction failed to amplify the *Wolbachia*-specific DNA. Furthermore, transmission electron microscopy supported the results obtained by PCR and confirmed that the ultrastructure of *Wolbachia* was consistent with earlier published descriptions (Clements 1992).

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