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SHORT COMMUNICATION

No evidence of *Wolbachia* among Great Lakes area populations of *Daphnia pulex* (Crustacea: Cladocera)

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Wolbachia endobacteria are commonly associated with a variety of arthropod species as hosts and induce known changes in their hosts' life-history traits. Despite exhibiting several Wolbachia-like life-history traits, and despite being a common model organism, the zooplankton Daphnia pulex has not been formally tested for infection with Wolbachia. Among 203 isolates exhibiting a range of life-history phenotypes, we found no evidence of Wolbachia. This leaves the genes of D. pulex as the most likely cause of its own life-history traits.

INTRODUCTION

Intracellular bacteria of the genus *Wolbachia* are believed to be present in 20–76% of the world's arthropod species (Jeyaprakash and Hoy, 2000). It is known to induce several changes in its hosts' life histories, including female-biased sex ratios, parthenogenesis and male feminization (Stouthamer *et al*., 1999). Although *Wolbachia* has been found in other crustaceans (Cordaux *et al*., 2001), we know of no published study to have searched for it in species of *Daphnia*.

The life-history traits of the zooplankton *Daphnia pulex* (Leydig) overlap the life-history traits induced in hosts of *Wolbachia*. Female-biased sex ratios (Innes, 1997) and obligately parthenogenetic lines (Innes and Hebert, 1988; Hebert *et al*., 1989) are common, and its sex determination system is primarily environmentally based, making it conceivable for potential male offspring to be turned female by *Wolbachia*-induced hormonal changes. *Wolbachia*-induced host parthenogenesis is typically accomplished via automictic thelytoky, resulting in 100% homozygosity (Cook and Butcher, 1999). Clonal parthenogenesis via apomictic thelytoky maintains heterozygosity and has also been found to be induced by *Wolbachia* endosymbionts (Weeks and Breeuwer, 2001), resulting in a non-homozygous genome similar to that of *D. pulex* obligately parthenogenetic clones.

If *D. pulex* is infected with *Wolbachia*, then we must resolve the contributions of each to several life-history traits, as has been done recently with a species of spider mite (Vala *et al*., 2003). If *D. pulex* is not infected with *Wolbachia*, then its life-history traits can likely be attributed in large part to its own genes. This would increase the motivation behind finding the genes involved in meiosis suppression and average rate of male production in the soon-to-be sequenced *D. pulex* genome (http://daphnia.cgb.indiana.edu).

METHOD, RESULTS AND DISCUSSION

Daphnia pulex were sampled from several small ponds in the Great Lakes watershed (North America) in early May 2003, except the clone from pond LP8A which was sampled in Spring 2001 (Table I), using previously described methods (Innes, 1997). These populations represent a geographic cross-section of pond habitats

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Table I: Populations sampled, their method of reproduction and successful polymerase chain reaction (PCR) amplifications for Daphnia pulex p4m15 and Wolbachia wsp loci

Pond	Method of reproduction	*n* clones	*p4m15* amplified	*wsp* amplified
Disp	Mixed	28	28	0
LP8A	Cyclical parthenogenesis	1	1	0
LP8B	Cyclical parthenogenesis	21	21	0
LP9A	Cyclical parthenogenesis	18	18	0
Mar	Cyclical parthenogenesis	25	25	0
Morg	Obligate parthenogenesis	13	13	0
OjibDitch	Obligate parthenogenesis	16	16	0
Tex	Cyclical parthenogenesis	19	19	0
VBA	Obligate parthenogenesis	28	28	0
W3	Obligate parthenogenesis	15	15	0
War	Mixed	19	19	0
Total		203	203	0

Mixed, obligate parthenogenesis and cyclical parthenogenesis.

and locations. LP8A, LP8B and LP9A are near Long Point, Ontario (Innes, 1991). Mar, Morg, Tex and War are scattered near Ann Arbor, Michigan, being previously described as ponds 74, 69, 72 and 73 respectively (Hebert *et al.*, 1989). Windsor, Ontario, contains W3 (Hebert and Crease, 1983), OjibDitch which is near the previously described pond 5 (Hebert *et al.*, 1989) and Disp which is a newly described pond on Disputed Road. VBA is a newly sampled pond in the Village by the Arboretum, ~1.5 km south of Guelph University in Guelph, Ontario. These populations also represent a cross-section of phenotypes: obligately parthenogenetic and cyclically sexual lines (Innes *et al.*, 2000) (Table I) and lines that vary in their investment in males (Innes and Dunbrack, 1993).

Clones were kept in laboratory conditions as described previously (Innes and Dunbrack, 1993) until their DNA was extracted in early 2004. All *D. pulex* individuals have the ability to reproduce asexually, so clonal individuals were of the same genotype, with the exception of somatic-like mutations from mother to offspring. A total of 203 clones were sampled across 11 ponds, though some were likely clones of others in the same pond at the time of sampling and therefore redundant.

DNA was extracted from samples of approximately five large females per clone using a method modified from http://www.fruitfly.org/p_disrupt/inverse_pcr.html, as follows. Samples were ground in an Eppendorf tube using a pipet tip before 100 μL of Buffer A [100 mM Tris–HCl (pH 7.5), 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl and 0.5% SDS] was added. Tubes were incubated at 70°C for 35 min. Two hundred microliters of LiCl–KAc solution (one part 5 M KAc by volume with 2.5 parts 6 M LiCl) was added before tubes were incubated on ice for 15–20 min. Samples were spun at 13 700 *g* for 15 min. Supernatant was transferred into new tubes. One hundred and sixty microliters of cold (−20°C) isopropanol was added, and the sample was mixed and then spun for 15 min. We aspirated away the supernatant by vacuum, spun, and then aspirated the remaining liquid. Samples were washed twice with cold (4°C) 70% ethanol, being spun for 2 min before supernatant was aspirated away each time. DNA was resuspended in 35 μL of double-distilled water and left at 4°C overnight.

Polymerase chain reaction (PCR) was performed using the primer pair *wsp* 81F and *wsp* 691R, which amplifies an ~600 bp fragment of the *Wolbachia* surface protein gene *wsp* (Braig *et al.*, 1998). This primer pair has been used to detect a variety of *Wolbachia* strains in a range of crustacean (Cordaux *et al.*, 2001) and other arthropod hosts (Zhou *et al.*, 1998). It is considered the most sensitive of several primer pairs to *Wolbachia* DNA amplification (Hong *et al.*, 2002). A *D. pulex* nuclear microsatellite, *p4m15*, was also amplified for each DNA sample to ensure that the extraction procedure produced amplifiable DNA (primers: *p4m15F*, 5′-TCCACCTCCTTCCTCACCAA; *p4m15R*, 5′-GCGCGGCAGTGAAATAAATC; courtesy of J. K. Colbourne, Indiana University).

Reaction mixtures for both *wsp* and *p4m15* PCR contained 25 μL total volume: 2.5 μL of ×10 buffer (Fisher Scientific, Fairlawn, NJ, USA), 2.5 μL of 25 mM $MgCl_2$ (Fisher), 0.5 μL of 10 mM dNTPs, 0.25 μL of 10 μM forward and reverse primers, one unit of *Taq* (Fisher), 5 μL of DNA solution and 13.8 μL of water. Negative controls consisted of the same PCR mixtures but without the addition of DNA. *wsp* thermocycling conditions were modified from those described previously for crustaceans (Cordaux *et al.*, 2001), as follows: 94°C for 3 min; 36 cycles of 94°C for 30 sec, 53°C for 45 sec and 72°C for 1 min; and 72°C for 10 min. The annealing temperature was reduced from the original 55°C to 53°C to lower specificity and hence lower the possibility of false-negative results. Approximately 10 μL of PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination. Images of gels were taken with a digital camera and stored electronically.

DNA was also extracted from a single *Drosophila simulans* individual known to host *Wolbachia*, and this DNA was used as a positive control in each set of *wsp* PCR. All such positive controls amplified the expected ~600 bp band successfully, and all negative controls were free of bands.

If the *D. pulex* microsatellite PCR amplified the expected band for a sample, but the *wsp* PCR did not, then we concluded that the sample did not contain *Wolbachia*. The microsatellite was successfully amplified for all samples, while no samples amplified a band in the expected region for *wsp* PCRs.

A very faint band in the 375-bp region, however, was amplified in the majority of *wsp* PCR samples. We concluded that this amplified DNA was not of *Wolbachia* origin for three reasons. First, the product length of 375 bp is much smaller than the 590–632 bp products amplified from a variety of *Wolbachia* strains (Zhou *et al.*, 1998). Second, its amplification failed at the high annealing temperature of 56°C, while that of the positive control did not. Finally, upon sequencing, this product was determined to bear no greater resemblance to *Wolbachia* DNA than would be expected by chance beyond the primer regions.

It is possible that our methods failed to detect *Wolbachia* in samples for several reasons. Our primers, though standard among many *Wolbachia* studies (Schulenburg *et al.*, 2000; Hong *et al.*, 2002), may not amplify the *Wolbachia wsp* locus if mutations have occurred to render them not specific enough. There may also be inhibition of *Wolbachia* DNA amplification by something in the *D. pulex* DNA samples (Jeyaprakash and Hoy, 2000). Or, *Wolbachia* infection of individuals is localized and of low level, leaving insufficient template for amplification.

Though these concerns can never be completely alleviated, we decreased their likelihood of occurrence through several methods. We changed the annealing temperature of our *wsp* PCR to 45, 50, 54 and 56°C for subsets of samples. While some faint bands were amplified at 45°C, none were within 200 bp of the region expected of *wsp*. Each of these PCR conditions successfully amplified the ~600 bp band in the positive control. Lower temperatures decrease annealing specificity, which should have allowed amplification of *Wolbachia* DNA if its sequence were fairly similar to that of our primers or if there were some form of inhibition.

Inhibition was also addressed in a small subsample by successful amplification of small amounts of positive control DNA mixed with sample DNA. Low template level concerns were addressed by using concentrated DNA from many (~15) individuals of a clone, but still finding no *wsp* amplification. Although inhibition has been found in some studies (Jeyaprakash and Hoy, 2000), others have found very diluted DNA to still amplify without difficulty (Wenseleers *et al.*, 2002).

No evidence was found of *Wolbachia* infection among our samples, which represent a cross-section of *D. pulex* phenotypes. We therefore conclude that *Wolbachia* is not responsible for the life-history traits exhibited by the clones involved. Our results are complemented by those of S. West and D. Ebert (unpublished data), who also failed to find PCR-derived evidence of *Wolbachia* among three clones each of *Daphnia magna* Straus and *D. pulex* collected from separate populations in southern UK.

It is possible that infection with another parasite is responsible for the life-history traits of *D. pulex*, as is the case for several arthropods (Weeks *et al.*, 2002) including crustaceans (Kelly *et al.*, 2004), and more research is needed to test this possibility. The more likely explanation, however, is that the life-history traits of *D. pulex* are the result of its genotype and genotype × environment effects.

This conclusion supports previous evidence from *D. pulex* cross-breeding that both obligate parthenogenesis (Innes and Hebert, 1988) and the average rate of male production (Innes and Dunbrack, 1993) are traits inherited genetically. This study, therefore, provides increased support for the proposal to find the genes involved in meiosis suppression and rate of male production in the *D. pulex* genome.

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