

## Research Article

# Evidence against the Presence of *Wolbachia* in a Population of the Crayfish Species *Procambarus clarkii*

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*Wolbachia* is a genus of intracellular alpha-Proteobacteria that is maternally inherited and is capable of inducing a variety of reproductive alterations in host species. The host range of *Wolbachia* is not determined completely but is known to contain a number of arthropod taxa, including crustaceans. *Wolbachia* has not been reported in crayfish, but sampling has been limited to date. We examine a species of crayfish, *Procambarus clarkii*, for *Wolbachia* infection using a suite of *Wolbachia*-specific primers in PCR assays. All specimens yielded negative results for *Wolbachia* infection and mathematical analysis of sample size shows a near 100% probability of detection for populations with greater than 0.1% infection rate.

## 1. Introduction

There is much interest among evolutionary biologists in the genus *Wolbachia*, a maternally inherited intracellular bacterium within the order Rickettsiales [1–3]. This interest stems from *Wolbachia*'s large host range [4, 5] and from its effects on host reproductive biology, including cytoplasmic incompatibility, host feminization of genetic males, induction of parthenogenesis, and male killing (reviewed by [3]). Cytoplasmic incompatibility (CI) causes incompatibility between the sperm of infected males and the eggs of uninfected females [6]. Feminization is a *Wolbachia* induced phenotypic change observed in both insects [7–9] and isopods (Bouchon et al. 1998, [10–12]) that results in a genetic male host developing as a reproductively functional female. Parthenogenesis induction in hosts causes the asexual production of female offspring [13–15]. *Wolbachia* induced male killing occurs during embryogenesis and is theorized to give a fitness advantage to surviving female offspring [16–18]. Owing to the wide range of phenotypic alterations in *Wolbachia*-infected hosts, there has been much recent interest in the use of *Wolbachia* as biological control agents [19].

The host range for *Wolbachia* is not determined fully, but it has been estimated that in insects alone 1.69 to

5.07 million species are infected [4]. *Wolbachia* is also found in filarial nematodes [20], isopods [12], and mites [21]. Intertaxon transmission is consistent with aspects of *Wolbachia* phylogeny [22] and has been shown to occur between *Drosophila simulans* and the parasitoid *Leptopilina bouvardi* under lab conditions [23]. *Wolbachia* intertaxon transmission has also been implicated in Australian spiders [24] and a variety of crustacean hosts [25], suggesting that the host range of *Wolbachia* may be broader than is currently assumed. However, attempts to detect *Wolbachia* in other taxa have been met with mixed results. For example, Schilthuizen and Gittenberger's (1998) assay of 38 species of mollusks and Fitzsimmons (2004) assay of 203 *Daphnia pulex* failed to detect *Wolbachia*. Baldo et al. (2007) reported the presence of *Wolbachia* super group F in eleven species of South African scorpions. *Wolbachia* is common in filarial nematodes but remains unconfirmed in nonfilariid nematodes despite an in-depth study [26]. Intracellular *Rickettsia*-like bacteria have been found in some species of crayfish [27], but Vogt et al. [28] failed to detect *Wolbachia* in marbled crayfish using light microscopy.

Here we examine *Procambarus clarkii* (Decapoda: Astacidea) using *Wolbachia*-specific molecular markers on multiple tissue types. *P. clarkii* is the most commercially important

TABLE 1: Multiplex primers.

Primer	Primer sequence	Approximate fragment size	Source
<i>Wolbachia</i> specific forward	CATACCTATTCTGAAGGGATAG	450	[31]
<i>Wolbachia</i> specific reverse	AGCTTCGAGTGAAACCAATTC		
wsp_81F	TGGTCCAATAAGTGATGAAGAAAC	600	[32]
wsp_691R	AAAAATTAAACGCTACTCCA		
coxA_F1	TTGGRGCRTYAACTTTATAG	500	[33]
coxA_R1	CTAAAGACTTTKACRCCAGT		
ftsZ_F1	ATYATGGARCATATAAARGATAG	550	[33]
ftsZ_R1	TCRAGYAATGGATTRGATAT		

species of crayfish in North America and is a known vector for the European crayfish plague, *Aphanomyces astaci* [29].

## 2. Materials and Methods

**2.1. Husbandry and Sample Preparation.** We acquired 60 southern Louisiana wild caught *P. clarkii* from Atchafalaya Biological Supply Company of Raceland, Lafourche Parish, LA, USA. These crayfish were sampled from two or three natural or seminatural ponds and were not exposed to antibiotics or microbial inhibitors (Danny Kraemer, pers. comm.). At New Mexico State University, crayfish were kept alive in 55 liter tanks and fed Hikari Crab Cuisine for four days prior to sample extraction.

Gonad and heart tissue samples were isolated from 60 *P. clarkii*. DNA extractions were done using the Qiagen DNeasy® blood and tissue kit. DNA concentrations were measured via nanodrop and diluted to 20 ng/ $\mu$ L.

**2.2. Polymerase Chain Reaction Assay.** *P. clarkii* DNA isolates were tested for PCR-viable DNA using a universal invertebrate 16s primer pair: 16Sar-5'/16Sbr-3' [30]. PCR reactions totaling 12  $\mu$ L consisted of 6  $\mu$ L Promega® PCR master mix, 2  $\mu$ L DNA, and 4  $\mu$ L of 2.5  $\mu$ M primer mix. Negative controls consisted of nuclease free water run through the DNA extraction protocol replacing DNA template in the PCR mix. Cycling conditions were 60 seconds at 95°C; 35 cycles of 60 seconds at 94°C, 60 seconds at 42°C, and 90 seconds at 72°C; and 10 minutes at 72°C.

We used PCR amplification with a multiplex of *W*-spec, *wsp*, *coxA*, and *ftsZ* primers to test for the presence of *Wolbachia* in *P. clarkii* tissue samples that tested positive for PCR-viable DNA. PCR reactions totaling 19  $\mu$ L consisted of 9.5  $\mu$ L Promega® PCR master mix, 1.5  $\mu$ L DNA, and 8  $\mu$ L of multiplex primer mix. Multiplex primer mix consisted of 150 nm *coxA*\_F1, 150 nm *coxA*\_R1, 150 nm *ftsZ*\_F1, 150 nm *ftsZ*\_R1, 50 nm *wsp*\_F1, 50 nm *wsp*\_R1, 50 nm *W*-spec\_F1, and 50 nm *W*-spec\_R1 (Table 1). Multiplex PCR cycling conditions were 60 seconds at 95°C; 35 cycles of 60 seconds at 94°C, 60 seconds at 53°C, ramp of 20% per second to 72°C, and 60 seconds at 72°C; and 10 minutes at 72°C.

Three positive controls were used, containing DNA from the three lines of *Wolbachia*-infected *Drosophila simulans* known to have wMa, wAu, or wRi strains of *Wolbachia* [34]. The negative control was DNA isolated from an uninfected

strain of *D. simulans*. Concentration sensitivity of the multiplex was tested using a serial dilution of positive control DNA template. All samples and controls were then retested using an annealing temperature of 48°C. All PCR products were run on 1.5% agarose gels to verify the presence of amplified fragments; multiplex PCR products had to be run for a minimum of 45 minutes at 140 V to be able to visualize all four bands.

## 3. Results And Discussion

Of the 120 samples tested with the universal invertebrate 16s rDNA primer pair 116 tested positive, yielding fragments approximately 600 base pairs in length and indicating the presence of PCR-viable DNA in the heart and gonad isolates of 57 crayfish. The multiplex used on the isolates from the 57 crayfish failed to amplify any gene fragments. *Wolbachia*-infected *D. simulans* multiplex positive controls amplified appropriate sized gene fragments from all strains, including serial diluted template with a concentration of .02 ng/ $\mu$ L. The negative results from the multiplex assays of crayfish samples support the conclusion that *Wolbachia pipientis* is absent from this population of *P. clarkii*.

These results are consistent with a preliminary study of *Wolbachia* infection in *P. clarkia* and *Orconectes virilis* [35]. Using a different set of primers, we failed to detect *Wolbachia* in 15 individuals of *O. virilis* collected from the Rio Grande near Las Cruces, NM; we also failed to detect *Wolbachia* in 12 individuals of wild caught *P. clarkia* purchased from a Louisiana provider.

It is not possible to prove the negative, in this case, that *P. clarkii* lacks *Wolbachia*. It is possible, however, to assess the likelihood of a false negative given the experimental design. The probability of a false negative for *Wolbachia* infection assays can be determined by the number of individuals tested and the infection frequency of the population. The probability of observing at least one infected individual in a sample of  $n$  individuals taken from a population with an infection frequency of  $\mu$  is  $1 - (1 - \mu)^n$ . There is a near 100% probability of at least one positive result for an infection frequency of  $\mu = 0.1$  and a 95% chance for infection frequencies as low as  $\mu = 0.05$  (Figure 1). Consequently, we feel that our results provide robust evidence against the presence of *Wolbachia* in this population of *P. clarkii*.

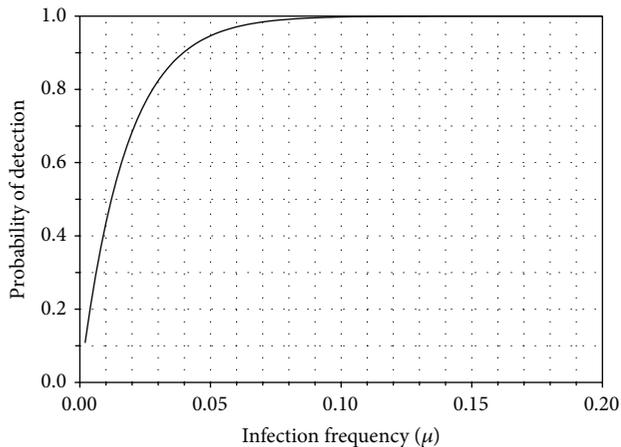


FIGURE 1: The probability that at least one individual out of 57 will test positive for *Wolbachia* as a function of the population infection frequency,  $\mu$ .

In determining the frequency of *Wolbachia* across species and within individual species, PCR assays are an invaluable tool. However, it must be recognized that *Wolbachia* infection rates within a species can exist at low frequency [5] and that *Wolbachia*-specific primers may not be effective on *Wolbachia* strains exhibiting high levels of sequence divergence [30]. In addition, *Wolbachia* infection studies testing small numbers of individuals have a high likelihood of false negatives and can lead to erroneously low estimates of species infection rates [5]. As investigation continues into *Wolbachia*'s host range, effort must be made to prevent false negatives where possible.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Acknowledgments

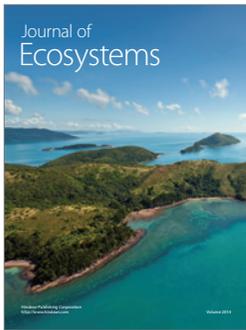
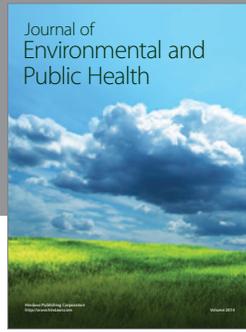
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