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# Optimization of Protocols for Wolbachia Detection Using PCR Methods

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by

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**Abstract**

*Wolbachia* is a genus of intracellular bacterium that is known to infect a wide range of arthropods. Species within this clade have the ability to modify the reproductive success of their hosts to promote their own distribution throughout host populations. *Wolbachia* cannot be cultured outside of the host and characterizing infection of gonadal cells might be time consuming. Thus, polymerase chain reaction (PCR) assays are a common method of *Wolbachia* DNA amplification from arthropods. I assessed the utility of 3 DNA sequence markers (16S, ITS, and 28S) paired with the *Wolbachia* surface protein (*wsp*) gene in various PCR protocols. The developed protocol was used on four species within the Cynipini tribe of gall wasps from central Illinois to detect *Wolbachia* infection. A PCR protocol was optimized using *wsp* gene and 28S primers to confirm DNA extraction, as well as the presence of *Wolbachia* strains in the horned oak gall wasp, *Callirhytis cornigera*. My results provided evidence for the potential of the combined use of 28S nuclear DNA gene and the *wsp* gene of *Wolbachia* for detection of *Wolbachia* infection in cynipid wasps, but further trials are needed to fine tune PCR protocol to yield consistent results, which is necessary for *Wolbachia* detection in oak gall wasp communities.

## Introduction

Bacterial symbionts belonging to the genus *Wolbachia* are found in a wide range of insect species (Werren et al., 1995). These alpha proteobacteria infect the reproductive tissues of their hosts causing different reproduction pathways such as cytoplasmic incompatibility, parthenogenesis induction, feminization of genetic males and male-killing (Werren et al. 1995, 2008). These modifications improve *Wolbachia* inheritance maternally from infected females to their progeny (Turelli, 1994). A meta-analysis of *Wolbachia* screenings in arthropods, mainly insect species, has estimated that there was a 66% infection rate (Hilgenboecker et al., 2008). This high of an infection rate and pandemic spread cannot be justified by vertical transmission alone.

The lateral transfer of DNA from *Wolbachia* to the host genome is widespread in some species (Reuter and Keller, 2003). Yang et al. (2013) showed that individual insects of different species within a gall community could be infected with multiple identical *Wolbachia* strains. This finding provided the first evidence of horizontal transmission within such communities. This type of transfer can occur through host-parasitoid association, blood contact, feeding relationship, and common usage of same plant tissues (Rigaurd and Juchault, 1995; Vavre et al, 1999; Mitsuhashi et al., 2002; Kittayapong et al., 2003). If the transferred genes are transcribed by the host nucleus, then proteins for the bacteria will be produced. Furthermore, the random insertion of *Wolbachia* genes into the host genome could cause deleterious effects of the gene and contribute to speciation (Baldo et al., 2002). By studying genomic recombination within *Wolbachia*, the mechanisms by which this organism can spread a desirable genotype through populations, such as the ability to hinder a vector species from transmitting a pathogen (Zabalou et al., 2004) can be better understood.

Oak gall wasps (Cynipini) include approximately 1000 species described within 25 genera worldwide (Rokas et al., 2001; Csoka et al., 2005; Abe et al., 2007). The Cynipini of North America are especially diverse, with about 700 species in 22 genera (Melika and Abrahamson, 2002). Although this clade contains the highest species diversity of gall wasps in North America, relatively little is known about their biology and their symbiosis with *Wolbachia* in particular. Examination of this group is difficult, because species within Cynipini are cyclically parthenogenetic, with both sexual and asexual generations, that differ according to gall structure, phenology, and adult size morphology (Abe, 1990).

In order to study this obligate intracellular bacterium, the presence of bacterial DNA within the host cells of the gall wasp should be verified using polymerase chain reaction (PCR) protocols. A second PCR should be conducted to detect the intensity of *Wolbachia* infection. Primers 16S rDNA, internal transcribed spacer (ITS), and 28S rDNA have been successfully used to amplify DNA from a wide variety of arthropods in various labs (Breeuwer et al., 1992; O'Neill et al., 1992; Stouthamer et al., 1993; Werren et al., 1995; Rokas et al., 2001; Ogden et al., 2009). In this study, I investigated protocols for a multiplex PCR method that will require a single PCR reaction to achieve two purposes – testing success of DNA extraction (control), and presence of *Wolbachia* (test) in cynipid gall wasps.

## Methods and Materials

### Study Species

Cynipid gall wasps were collected throughout Central Illinois. Galls of the horned oak gall wasp (*Callirhytis cornigera*) were found on Red Oak (*Quercus rubra*) located at the Green Creek Rest Area along Interstate 70 near Effingham, IL. The other gall inducers were collected

on their host plant, the white oak (*Quercus alba*): cherry gall wasp (*Cynips quercusfoli*) from Fox Ridge State Park (Charleston, IL), hedgehog gall wasp (*Acraspis erinacei*) from Lake Charleston (Charleston, IL), and oak acorn plum gall wasp (*Amphibolips quercusjuglans*) from the campus of Eastern Illinois University (Charleston, IL). They were compiled between April and September 2013. The dissected individuals were kept in 1.5 ml tubes and fixed with 100% ethanol and frozen at -20°C until ready for DNA extraction. The specimens were categorized based on their morphology, color, and presence or absence of bristles into three categories: adult, larva, and parasitoid (Table 1).

**DNA isolation**

The whole individual (>2 mm) was used for DNA extraction to accumulate enough tissue sample. The individuals immersed in ethanol were lightly dabbed onto a Kimwipe to wick away any ethanol and washed in sterile water to avoid any cross-contamination. The sample was transferred to a fresh 1.5 ml tube and macerated against the wall of the tube with the pestle. The final volume was brought to 500 µl with the addition of 480 µL of cell lysis buffer, 10 µl of 0.1 M dithiothreitol (DTT) and 10 µl of proteinase K (Promega). The solution was vortexed for 5-10 seconds and then incubated overnight at 55ºC (Innovative HL-2000 HybriLinker Hybridization Oven). For each species, DNA was extracted from individual wasps as described by Latch et al. (2008).

**Optimal molecular marker**

To identify the primer that provided the best amplification of DNA, three primers were tested on the same three DNA extraction samples from hedgehog gall wasps. For ITS rDna, the PCR reaction was conducted following methods from Rokas et al. (2001; genes and ingredients listed in Table 2). Primer, 16S rDNA, was tested following a PCR program developed by Zhou et

al. (1998). The PCR method for 28S rDNA was performed using a modified PCR protocol from Rokas et al. (2001; program specifications listed in Table 3).

***Wolbachia* screening**

A total of 48 gall wasps and 12 parasitoids were screened for the presence of *Wolbachia* strains. The primers 28S and *wsp* were used for each remaining DNA extraction samples. PCRs were performed in 25 µl volumes, comprising 1.5 µl of DNA sample, 2 µl *wsp* primer, 1 µl of 28S rDNA forward primer, 1 µl of 28S rDNA reverse primer, 12.5 µl GoTaq Green Master Mix, and nuclease-free water 7 µl. All polymerase chain reactions were performed in the PTC-100 Programmable Thermal Controller (MJ Research, Inc.; Foster City, CA).

**Gel-Electrophoresis**

Each PCR reaction for every individual was conducted on a 1% agarose gel. For visibility, 1 µL GelRed dye was added to the solution containing 90 µL Tris-Borate-EDTA and 2 g agarose gel (Fischer Chemicals; New Lawn, New Jersey). The gel was run at 100 V for 40 minutes. The completed gel was viewed under Bio-Rad Mini- PROTEAN 3 (Bio-Rad Laboratories, Hercules, CA)

## Results

The results of the screening show that the presence of *Wolbachia* infections in species of cynipid wasps, primarily oak gall wasps, exists in the central Illinois region. Of the four species, *Callirhytis cornigera* (horned oak gall wasp) was the only one to show possible infection (Table 4). The *wsp* gene was successfully amplified for three individuals of the horned oak gall wasps, providing evidence of *Wolbachia* infection (gel lanes 2, 3, and 4). The *wsp* gene fragments in the three individuals of the horned oak gall wasps were different in size (Table 5). DNA

amplification in lanes 5, 6, 7, and 8 indicate traces of DNA amplification, but not in size range of the targeted regions of either *wsp* or 28S. The distinct band of the last lane was in the size range of 28S gene (Fig. 1).

## Discussion

The size of target 28S fragment has a size of about 1073 base pairs (bp) in cynipid wasps (Rokas et al., 2001). The amplified fragment shown in lane 9 (cherry gall wasp; Fig. 1) has 1000 bp. This indicates that successful amplifications of the gene fragment is feasible for the cherry gall wasp (*Cynips quercusfoli*) and possibly other cynipid wasps as well. Further in the lane, there is a fragment past 300 bp, indicating non-specific amplification. To avoid non-specific primer binding, higher melting temperature ($T_m$) could be used in the future. Raising the $T_m$ for 28S may affect the amplification for *wsp*, however, which has lower $T_m$ of about 56°C. Because of this complication, one may have to separate the two procedures in PCR, instead of using multiplex PCR method for *Wolbachia* detection.

Both hedgehog gall wasp (*Acraspis erinacei*) and oak acorn plum gall wasp (*Amphibolips quercusjuglans*) did not have a *wsp* gene fragment. The faint bands around 2,000 bp might again indicate a lower $T_m$ than needed for the 28S rDNA. At low $T_m$, primers can bind to non-specific sites and amplify the non-target DNA fragments, which may exhaust or lower the amount of reagents available for target fragment amplification, resulting in the minimal amplification of the target genes. The bright bands with less than 300 bp may be from remaining RNA, because the DNA extraction protocol was chosen for the purpose of being cost-effective, but did not include procedures to actively remove RNA fragments. Alternatively, they could be primer-dimers, especially considering the fact that I lowered the $T_m$ of 28S by as much as 4°C in order for it to

also provide detection for the *wsp* gene of *Wolbachia.*

Tests of horned gall wasps were positive for *Wolbachia* infection. Lanes 3 and 4 have single bands, 500 bp and 600 bp respectively, falling near the normal size range of the *wsp* gene. While the single bright band of lane 2 (DNA from a gall wasp larva) has a size around 400 bp, although somewhat shorter than the normal size range of the gene, is still likely to be the *wsp* gene, thus suggesting a total of three *Wolbachia* strains. The *Wolbachia* DNA fragments surveyed by Zhou et al. (1998) were host species from areas outside the midwestern region of the United States. On the other hand, my sample size of gall wasps was relatively small and future studies with larger sample sizes might reveal higher diversity of *Wolbachia* associated with horned oak gall wasps.

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**Table 1**. The samples of gall wasps used in this study.

| Species | Common Name | Gall wasps | Parasitoids |
|---|---|---|---|
| *Callirhytis cornigera* | Horned oak gall wasp | 23 (3 larva) | 6 |
| C*ynips quercusfoli* | Cherry gall wasp | 9 | 2 (all larva) |
| *Acraspis erinacei* | Hedgehog gall wasp | 13 | 0 |
| *Amphibolips quercusjuglans* | Oak acorn plum gall wasp | 3 (all larva) | 4 (all larva) |

**Table 2.** List of genes and the primers used to amplify these genes. All primers are originally designed in the Wheeler/DeSalle Lab of the American Museum of Natural History and custom synthesized by Invitrogen Life Technologies (Carlsbad, CA).

| Gene Sequence | Template DNA | GoTaq Green Master Mix (Promega) | Nuclease-free water | Primers | Total Volume |
|---|---|---|---|---|---|
| 16S | 2 µl | 12 µl | 9 µl | 16S forward: 1µl<br>16S reverse: 1µl | 25 µl |
| ITS | 2 µl | 12 µl | 9 µl | ITS forward: 1µl<br>ITS reverse: 1µl | 25 µl |
| 28S | 2 µl | 12 µl | 9 µl | 28S forward: 1 µl<br>28S reverse: 1µl | 25 µl |

**Table 3.** PCR protocol for 16S were based on Zhou et al. PCR methods (1998). The PCR protocol used for 28S and 16S were from Rokas et al. (2001). Any alterations made to Rokas et al. (2001) 28S PCR protocol are indicated by bolded text.

| PCR Protocol | Denaturation | Primer Annealing/Extension | Elongation |
|---|---|---|---|
| 16S | 1 cycle 94°C for 3 minutes | 35 cycles: 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute | 5 minutes at 72°C |
| ITS | 1 cycle 94°C for 2 minutes | 35 cycles: 95°C for 1 minute, 55°C for 60 seconds, 72°C for 2 minute | 10 minutes at 72°C |
| 28S | 1 cycle 94°C for 5 minutes | **39 cycles**: 94°C for 1 minute, **58°C** for 1 minute, 72°C for 1 minute | **15 minutes** at 72°C |

**Table 4.** Possible presence of *Wolbachia* within the oak gall wasp species (Cynipini).

| Species | *Wolbachia* Infection |
|---|---|
| Horned oak gall wasp, *Callirhytis cornigera* | + |
| Oak acorn plum gall wasp, *Amphibolips quercusjuglans* | – |
| Hedgehog gall wasp, *Acraspis erinacei* | – |
| Cherry gall wasp, *Cynips quercusfoli* | – |

**Table 5.** Primer sequences and of each molecular marker tested in this study. Information was acquired from Promega (Madison, WI), Integrated DNA Technologies, INC (Coralville, Iowa), and Rokas et al. (2001).

| Locus | Sequence | length |
|---|---|---|
| *wsp* | Forward (5' - TGG TCC AAT AAG TGA TGA AGA AAC – 3') (24 nucleotides) | 590-632 bp |
| | Reverse (5' – AAA AAT TAA ACG CTA CTC CA – 3') (20 nucleotides) | |
| ITS | Forward (5' - GTT TCC GTA GGT GAA CCT GC – 3') (20 nucleotides) | 557 bp |
| | Reverse (5' – GAG AAC AGC AGG AAC ACA CAG AA - 3') (24 nucleotides) | |
| 16S | Forward (5' – CGC CTG TTT ATC AAA AAC AT – 3') (20 nucleotides) | 460 to 560 bp |
| | Reverse (5' - CTG CGG TTT GAA CTC AGA TCA – 3') (21 nucleotides) | |
| 28S | Forward (5' - GAC CCG TCT TGA AAC ACG GA – 3') (21 nucleotides) | 1073 bp |
| | Reverse (5' – TCG GAA GGA ACC AGC TAC TA – 3') (20 nucleotides) | |

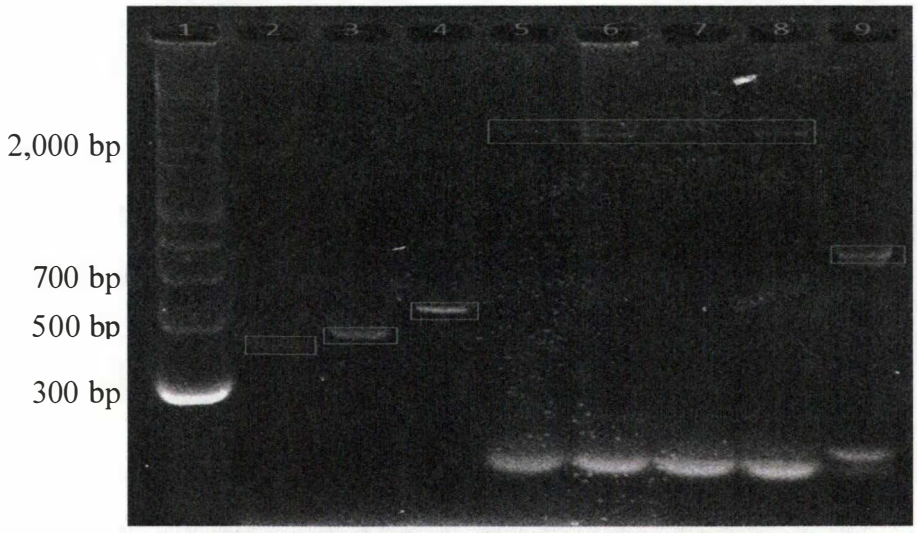


**Fig. 1.** A gel electrophoresis of the oak gall wasps (Hymenoptera: Cynipidae) with a DNA ladder in lane 1. In lanes 2-4 were samples from horned oak gall wasp (*Callirhytis cornigera*), whereas samples from oak acorn plum gall wasp (*Amphibolips quercusjuglan*) are in lanes 5 and 6, and samples from hedgehog gall wasp (*Acraspis erinacei*) occupied lanes 7 and 8. A sample from cherry gall wasp (*Cynips quercusfoli*) was in lane 9.