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Article

The presence of *Wolbachia* in Brood X cicadas

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SUMMARY

***Wolbachia* is an inherited bacterial symbiont that lives within the reproductive cells of approximately 60% of insect species. It manipulates sexual reproduction in insects in multiple ways that ultimately lead to an increased number of *Wolbachia*-infected females. It is not known if *Wolbachia* is a symbiont present in the Brood X seventeen-year periodical cicadas. Brood X cicadas have declining populations in Ohio and Indiana and factors that impact their declining numbers have not been identified. We hypothesized that Brood X cicadas would be infected with *Wolbachia*. Genomic DNA was isolated from one hundred cicadas from two species, 50 from *Magicicada septendecim* and 50 from the *Magicicada cassini*, collected in the greater Cincinnati, Ohio area. A polymerase chain reaction protocol screened the DNA samples and identified one *M. septendecim* cicada with a *Wolbachia*-specific 16S rRNA amplicon. A Basic Local Alignment Search Tool nucleotide (BLASTN) analysis of the 388 nucleotide sequence confirmed *Wolbachia* species which conformed to *Wolbachia* supergroup A. *Wolbachia* infection is low in Brood X *M. septendecim* cicadas and not identified in Brood X *M. cassini* cicadas. Efforts within the scientific community should continue the search for Wolbachia in cicadas, so the full ramifications of this symbiont on declining populations of Brood X cicadas can be uncovered.**

INTRODUCTION

Periodical organisms have a synchronized emergence of the adult at a particular location once in a fixed number of years, and it is an awe-inspiring event. One well known periodical insect in North America whose massive emergence creates a deafening chorus is the periodical cicada, genus *Magicicada*.

The species groups that live east of the Great Plains belong to the sap-sucking order hemiptera and include Decim, Cassini and Decula (1). Each species group of *Magicicada* includes one 13-year species and one 17-year species. According to their year of emergence, periodical cicadas are named as broods (2). Brood X was the most recent 17-year periodical cicada brood that emerged during the summer of 2021 in the Midwest and includes species *Magicicada septendecim*, *Magicicada cassini*, and *Magicicada septendecula*. While Brood X is the largest of the 17-year broods, its population has declined in Indiana and Ohio (3-6), sparking interest in the factors that impact these species' survival.

Wolbachia is an intracellular bacterium that lives in the reproductive organs of about 60% of known arthropods species (7). These endosymbionts infect the reproductive organs to enhance their own transmission through the female germ cells but can also impact host fitness and offspring health (8). *Wolbachia* host manipulation results in four reproductive phenotypes. The first is feminization, which occurs when male reproductive organs become female (9, 10). Second is cytoplasmic incompatibility, or the faulty formation of viable offspring between infected males and uninfected females (11, 12). The third is parthenogenesis, which is the formation of an embryo without the fertilization of the egg (13, 14). Lastly, male killing occurs when the bacterium actively kills infected males (15, 16).

Wolbachia infection has been identified in other species from the order hemiptera (17, 18); therefore, Brood X periodical cicadas that emerge in Southwestern Ohio were selected for investigation of the presence of *Wolbachia* infection. We hypothesized that Wolbachia infect Brood X *Magicicadas*. While the 17 years of subterranean existence make documentation of *Magicicadas* challenging, their mass emergence provides an abundance of specimens to obtain. We collected both *Magicicada septendecim* and *Magicicada cassini* Brood X species found in Cincinnati, Ohio during the 2021 emergence. We screened for evidence of *Wolbachia* infection using a PCR assay that amplifies the *Wolbachia*-specific region of the 16S rRNA gene.

Wolbachia positive samples were detected in 2.0% of the *M. septendecim* specimens and 0.0% of the *M. cassini* specimens. Phylogenetic analysis of the positive specimen's Wolbachia-specific 16S rRNA sequence suggested a close relationship to supergroup A. This project broadens the information known about Brood X cicadas and *Wolbachia* infection in arthropods.

RESULTS

Cicada Collection

During the emergence of the 17-year periodical cicadas in the spring of 2021 in Cincinnati, OH, 100 *Magicicadas* were collected. Using visual identification, 50 *Magicicada septendecim* and 50 *Magicicada cassini*, with an equal division of males and females, were identified and stored **(Figure 1)**. *M. septendecim* were collected from seven different locations,

Figure 1: Representative images of sampled Brood X periodical cicadas. Ventral views of representative A) female *Magicicada septendecim*, B) male *Magicicada septendecim*, C) female *Magicicada cassini*, and D) male *Magicicada cassini*. *Magicicada* are distinguished from other genera of cicada by their red eyes. *M. cassini* has a black abdomen without pattern and is smaller in size than *M. septendecim*. *M. septendecim* has broad orange stripes with more orange than black on its abdomen. Female cicadas are distinguished by the valvulae and ovipositor which give their lower abdomen a pointed shape. Male cicadas have a dome-shaped terminal segment and the tymbal organs are present on the upper abdomen.

and *M. cassini* were collected from five different locations across the greater Cincinnati area **(Figure 2)**. Because *Wolbachia* are concentrated in the reproductive cells of infected arthropods (7) and has been found in gut tissue (19), genomic DNA was isolated from abdominal tissue containing the gut and reproductive organs of each specimen.

Wolbachia Screening

The *Wolbachia* symbiont can be identified by the presence of its highly conserved 16S rRNA gene that can be detected by PCR amplification of the 438 bp product from the *Wolbachia*-specific region (20). To screen the cicada samples for *Wolbachia* 16S rRNA gene, a duplex PCR reaction containing both arthropod Cytochrome Oxidase 1 (CO1) primers and *Wolbachia*-specific 16S rRNA (Wspec) primers was performed on 100 cicada genomic DNA samples. *Wolbachia*-infected Drosophila control DNA yielded two amplicons: one corresponding to the arthropod CO1 gene and one corresponding to the *Wolbachia*-specific region of the16S rRNA gene. Uninfected Drosophila control DNA showed amplification of the arthropod CO1 gene, and no amplification of the *Wolbachia*-specific 16S rRNA gene fragment. During our duplex PCR screening of the cicada samples, two *M. septendecim* samples (one male in lane 5, one female in lane 12) **(Figure 3A)**, and three *M. cassini* samples, (two males, one female, agarose gel results not shown) contained a *Wolbachia*-specific16S rRNA amplicon of approximately 438 bp. However, after performing a single primer set PCR reaction containing only the *Wolbachia*-specific 16S rRNA PCR primers, only one male *M. septendecim* specimen produced an approximately 438 bp amplicon **(Figure 3B)**. Therefore, in our 100 cicada samples,

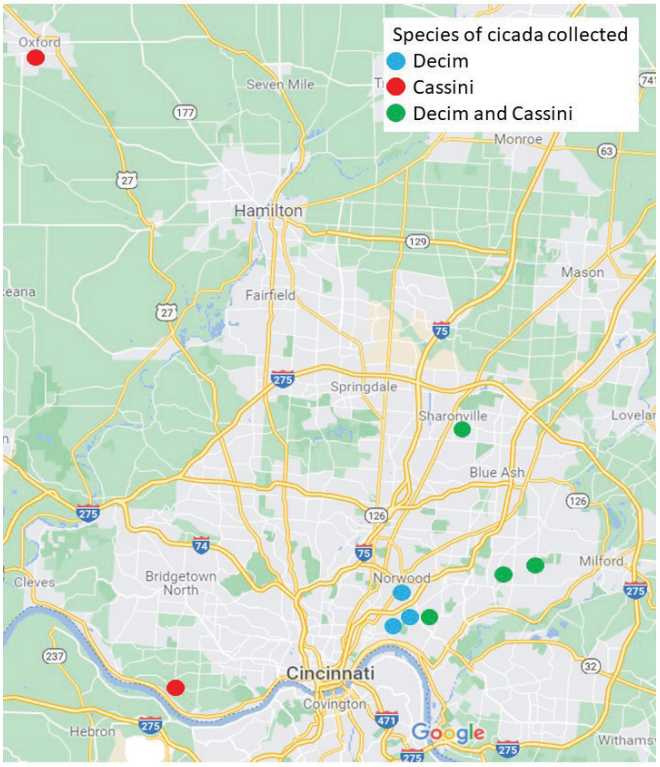

Figure 2: Map of cicada collection across greater Cincinnati area. Both *M. septendecim* and *M. cassini* were collected at four unique locations (green circles). *M. septendecim* specimens were collected at three additional locations (blue circles) and *M. cassini* specimens were collected at two additional locations (red circles).

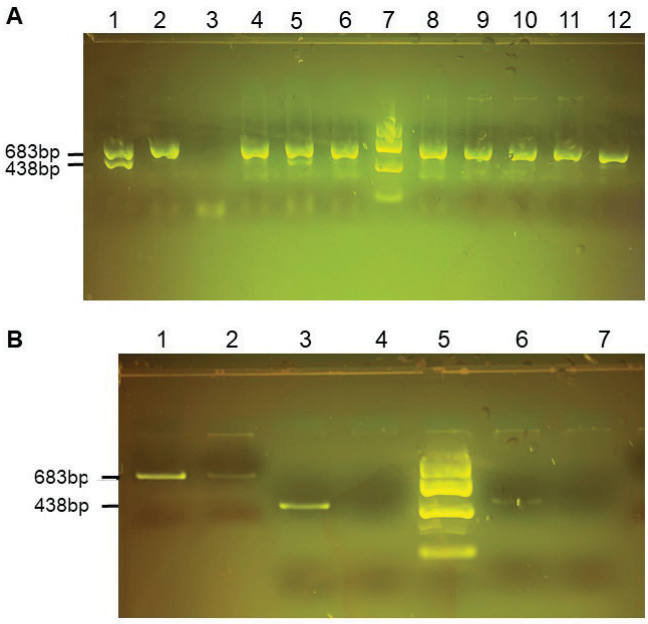

Figure 3: Agarose gel electrophoresis (2% agarose) of PCR products visualized with GelGreen. A) Dual CO1 and Wspec PCR screening reactions of select *M. septendecim* cicadas containing *Wolbachia* positive *Drosophila* control (Lane 1), *Wolbachia* negative *Drosophila* control (Lane 2), water control (Lane 3), Cicada 28 (Lane 4), Cicada 29 (Lane 5), Cicada 32 (Lane 6), pBR322/BstNI molecular weight markers (Lane 7), Cicada 33 (Lane 8), Cicada 34 (Lane 9), Cicada 35 (Lane 10), Cicada 36 (Lane 11), Cicada 37 (Lane 12). B) Single only primers set PCR reactions containing CO1 primers *Wolbachia* positive *Drosophila* control (Lane 1), CO1 primers *Wolbachia* negative *Drosophila* control (Lane 2), Wspec primers *Wolbachia* positive *Drosophila* control (Lane 3), Wspec primers *Wolbachia* negative *Drosophila* control (Lane 4), pBR322/BstNI MW markers (Lane 5), Cicada 29 Wspec primers (Lane 6), Cicada 37 Wspec primers (Lane 7).

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>Wolbachia pipientis strain wMel_I23 chromosome, complete genome
Sequence ID: CP042444.1 Length: 1269137
Range 1: 1170301 to 1170688

Score:717 bits(388), Expect:0.0,
Identities:388/388(100%),  Gaps:0/388(0%), Strand: Plus/Plus

Query  1        GTGTTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170301  GTGTTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC  1170360

Query  61       GCAACCCTCATCCTTAGTTACCATCAGGTAATGCTGGGGACTTTAAGGAAACTGCCAGTG  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170361  GCAACCCTCATCCTTAGTTACCATCAGGTAATGCTGGGGACTTTAAGGAAACTGCCAGTG  1170420

Query  121      ATAAACTGGAGGAAGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTAC  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170421  ATAAACTGGAGGAAGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTAC  1170480

Query  181      ACACGTGCTACAATGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTAAGCTAATCCCTT  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170481  ACACGTGCTACAATGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTAAGCTAATCCCTT  1170540

Query  241      AAAAGCCATCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTA  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170541  AAAAGCCATCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTA  1170600

Query  301      GTAATCGTGGATCAGCACGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGT  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170601  GTAATCGTGGATCAGCACGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGT  1170660

Query  361      CACGCCATGGGAATTGGTTTCACTCGAA  388
                |||||||||||||||||||||||||||
Sbjct  1170661  CACGCCATGGGAATTGGTTTCACTCGAA  1170688
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Figure 4: BLASTN alignment of the *Wolbachia*-specific 16S rRNA amplicon from Cicada 29. The 388 nucleotide sequence was compared with *Wolbachia pipientis* strain WMel_I23 chromosome (GenBank accession number CP042444).

2.0% of the *M. septendecim* samples (n=50) were positive for *Wolbachia* 16S rRNA gene, and 0.0% of *M. cassini* specimens (n=50) were positive for the *Wolbachia*-specific 16S rRNA gene.

Sequence Analysis and Phylogenetics

To verify the species of our arthropods and the positive *Wolbachia* PCR product, we analyzed the Sanger sequencing results of the amplicons using a Basic Local Alignment Search Tool nucleotide, BLASTN. Our analysis revealed that the sequences of the five randomly selected CO1 amplicons from the *M. septendecim* cicada specimens shared 100% identity with *Magicicada septendecim* reference sequence (data not shown) (21). The sequences of the five randomly selected CO1 amplicons from the *M. cassini* cicada specimens shared 99.85% identity with the *Magicicada cassini* reference sequence (data not shown) (21). There was less similarity between the *M. cassini* sequences and

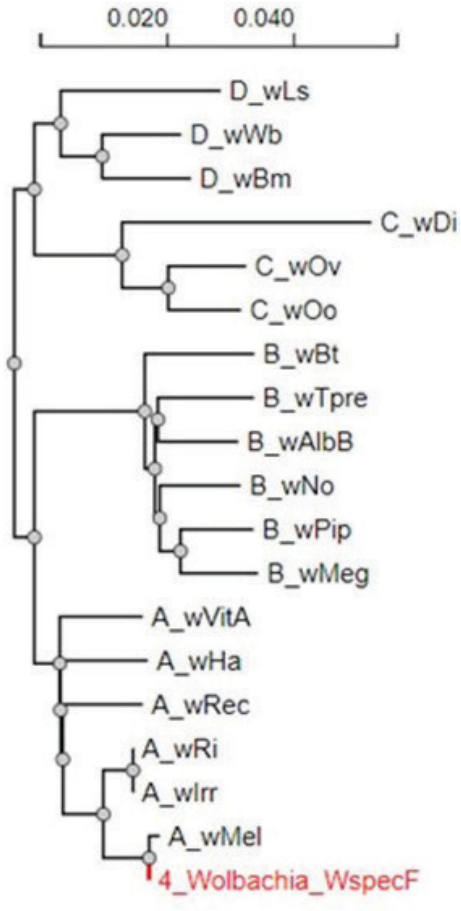

Figure 5: Phylogenic tree of *Wolbachia* including *Wolbachia* isolated from *Magicicada septendecim*. The tree was constructed with midpoint rooting between clades A/B and C/D. A phylogenic tree was built with FastMe tool using ngphylogeny.fr. The sequence from the *Wolbachia*-specific16S rRNA amplicon of Cicada 29 was aligned to the various representative *Wolbachia* supergroups taxa using MAFFT multiple sequence alignment tool. The scale bar refers to a phylogenetic distance of nucleotide substitutions per site.

the reference sequence, and this may be due to lower quality sequencing data from the amplicons. BLASTN analysis also confirmed the sequence of the *Wolbachia*-specific16S rRNA amplicon from the one *M. septendecim* cicada that screened positive for *Wolbachia*. The BLASTN alignment with a 388 nucleotide query length and 100% query cover aligned to *Wolbachia* with 100% identity **(Figure 4)**. This sequence indicates a putative supergroup classification of supergroup A since the *Wolbachia*-specific 16S rRNA amplicon sequence aligned with the A clade **(Figure 5)**.

DISCUSSION

This project set out to determine if *Wolbachia* symbionts can infect 17-year periodical *M. septendecim* and *M. cassini* cicadas. No published records have confirmed *Wolbachia* infection in periodical cicadas. Interest in *Wolbachia* infection persists due to its prevalence in many arthropods and the unusual effects it confers on host biology. Applied uses of *Wolbachia* infection may provide novel strategies to reduce the risk of infectious diseases (22, 23). Periodical cicadas have numerous, positive impacts on the ecology of a region, such as aerating the soil and being both a food and nutrient source. Understanding their role in an environment as well as how factors such as endosymbionts influence their population and interaction with others is crucial.

After screening one hundred 17-year periodical cicadas for the presence of *Wolbachia* infection using *Wolbachia*-specific16S rRNA PCR analysis followed by Sanger sequencing and BLASTN analysis, we determined that only 2.0% of our *M. septendecim* specimens and 0.0% of our *M. cassini* specimens were infected with *Wolbachia*. Therefore, it is possible that *Wolbachia* can infect cicadas; however, it appears uncommon in the Brood X cicadas surveyed in Cincinnati, Ohio during the 2021 emergence.

Individual organisms testing negative for *Wolbachia* does not mean the entire species is negative. Additional specimens in our collection could have been infected with *Wolbachia*, but if infection of the isolated reproductive tissue was low, the PCR may not have been sensitive enough to detect the presence of *Wolbachia*. The 16S rRNA gene is located within the mitochondria of this intracellular bacterium. If the mitochondrial DNA was not isolated efficiently, samples may yield false negative results. On the other hand, the sample testing positive for *Wolbachia* may be due to the presence of ectoparasitic mites which are known carriers of *Wolbachia* (24). Additionally, our research may have been limited by our small sample size confined to one major city in Brood X's range. We also only investigated two of the three species from Brood X since 17-year *M. decula* cicadas are rare in the locations screened.

We hope that our research inspires the scientific community to continue investigating *Wolbachia* in periodical cicadas. Additional studies on symbiotic bacteria of cicadas will elucidate the influence of endosymbionts on this insect population.

MATERIALS AND METHODS

Insect Collection

Magicicada septendecim and *Magicicada cassini* specimens were collected from trees in the Cincinnati, OH area **(Figure 2)** between May 2021 and June 2021. The cicada specimens were visually identified and catalogued **(Figure 1)**. *M. septendecim* were distinguished by broad orange stripes with more orange than black on the abdomen. *M. cassini* were distinguished by a black abdomen without pattern and are smaller in size than *M. septendecim*. Female cicadas were identified by the valvulae and ovipositor which give their lower abdomen a pointed shape. Male cicadas were identified by a dome-shaped terminal segment and the tymbal organs present on the upper abdomen. Insects were placed in cold 95% ethanol while alive and stored at -20°C until DNA was extracted.

DNA Isolation

Total genomic DNA was isolated from cicadas using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific). About 20 µg of lower abdominal tissue, which contains the gut and reproductive organs, from each *Magicicada* was placed into a 1.5 mL microcentrifuge tube and macerated with a pestle. Female cicadas had tissue removed from the abdomen behind sternite VII, tergite IX, and the valvulae; male cicadas had tissue removed from behind sternites V through VIII. Each tube containing tissue was incubated for at least one hour in a water bath at 56°C during the lysis step. The purified genomic DNA was stored at -20°C. Total genomic DNA from *Drosophila* infected with *Wolbachia* was used as a positive control. Total genomic DNA from *Drosophila* with no *Wolbachia* infection was used as a negative control.

DNA Amplification

A duplex PCR protocol was used with primers designed to amplify the insect CO1 gene fragment and the *Wolbachia*-specific 16S rRNA gene fragment: CO1_F 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3', CO1_R 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', Wspec-F 5'-CAT ACC TAT TCG AAG GGA TAG-3' and Wspec-R 5'-AGC TTC GAG TGA AAC CAA TTC-3' (20). The DNA amplification reactions were performed in 25 µL volumes consisting of 1 µL DNA template, 12.5 µL MiniOne Taq PCR Master Mix (2x) (MiniOne Systems), and 1 µL each of primer Wspec-F, Wspec-R, CO1_F, and CO1_R (5 µM). The thermo profile used with the MiniPCR mini16 thermal cycler for the duplex reaction was as follows: 120 seconds at 94°C followed by 30 cycles of 94°C for 10 seconds, 49°C for 20 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 5 min. To verify the presence of the *Wolbachia*-specific 16S rRNA amplicon in the duplex PCR reactions, the same genomic DNA sample is screened using a Wspec primers only PCR protocol (20). The thermo profile for the single reaction using Wspec primers was as follows: 120 seconds at 94°C followed by 35 cycles of

94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 5 min (25).

Agarose Gel Electrophoresis

A 2% agarose gel was prepared with TBE buffer. Electrophoresis was performed in the MiniPCR BlueGel electrophoresis system at 100 volts until the loading dye approached the end of the gel. PCR products were visualized with GelGreen, and 2 µL of the pBR322/BstNI Molecular Weight markers (Carolina Biological Supply) were used to determine the size of the amplified DNA products. In a single PCR reaction using the Wspec primers, one amplicon of approximately 438 bp is expected with an arthropod positive for *Wolbachia* infection (25). In a dual PCR reaction with both CO1 primers and Wspec 16S rRNA primers, two amplicons are expected for a *Wolbachia*-positive arthropod: 658 bp CO1 amplicon and 438 bp *Wolbachia*-specific 16S rRNA amplicon (20).

DNA Sequence Analysis

The amplicons were sequenced via Sanger Sequencing by Genewiz, Inc (South Plainfield, NJ). BLASTN analysis was used to identify the cicada and *Wolbachia* sequences (26). Sequence alignment of the *Wolbachia* 16S rRNA amplicon was performed with BLASTN and the amplicon was aligned to *Wolbachia pipientis* strain wMel_I23 (Accession number CP042444.1). Sequence alignments of *Magicicada* CO1 sequencing results were performed using SnapGene (27). The *M. septendecim* CO1 amplicons were aligned to *M. septendecim* reference sequence (21). The *M. cassini* CO1 amplicons were aligned to a *M. cassini* reference sequence (21). *Wolbachia* supergroup was identified using ngphylogeny.fr (28). The supergroup identification used representative *Wolbachia* supergroups taxa (29).

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