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Original Article

Detecting Natural *Wolbachia* Infection and Supergroup Identification of *Metochus uniguttatus* in North India

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ABSTRACT

Introduction: Insects, particularly arthropods, have evolved to tolerate a wide range of endosymbionts. *Wolbachia* is a widely prevalent bacterial endosymbiont that is gaining popularity in insect research due to its utility in pest and vector management. This study aimed to ascertain the spread of *Wolbachia* in a randomly sampled set of insects, identify any novel *Wolbachia* infections, and determine the *Wolbachia* infection's supergroup.

Materials and Methods: Field capture of insects was carried out manually with netting and gloves, and the insects were immediately stored in absolute ethanol to immobilize them. Phenol-chloroform-isoamyl alcohol was the method used to extract the insect DNA. Insect species identification was carried out via the CO1 mitochondrial gene using PCR amplification. PCR was utilized to identify the presence of *Wolbachia*, and 16S *Wspec* DNA was amplified to confirm its presence. The Sanger method was used to sequence the amplified CO1 and the positive samples for *Wspec*. Using NCBI blast, the sequences of the infected insects were compared with the database sequences. The obtained FASTAs were then aligned using Sequencher 5.4.6, and the chromatograms were examined to ensure contig quality and similarity.

Results: Among the 21 insects screened, one was found weakly positive (*Playpleura octoguttata*) and two were strongly positive (*Metochus uniguttatus* and *Velarifictorus micado*) with *Wolbachia*, which represented an infection rate of 14.29%. However, the individual infection rate in this limited sample size fell at the lower end compared to extensive surveys reporting rates between 20% and 76%. This study indicated the dissemination of *Wolbachia* in randomly screened insects. Moreover, this is one of the first records of *Metochus uniguttatus* being infected with *Wolbachia* in North India.

Conclusion: This study represents an initial exploration of insects not previously considered hosts of the *Wolbachia* endosymbiont. The results could be useful for future studies on insect biocontrol and pest management.

1. Introduction

Insects have one of the most successful lives on the planet, inhabiting a wide range of environments and adapting to a diverse diet. Associations with a range of symbionts, particularly endosymbionts, are key reasons for their ecological success.

Symbiosis is a subset of mutualism defined in 1879 by de Bary[1] as "*living together of unlike organisms*", and is mostly beneficial for one or both the parties involved. Endosymbiosis is a symbiosis between an organism (host) and organisms residing in that organism, named

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endosymbionts. These symbiotic relationships encompass various categories, including gut microbes, intra and extracellular symbionts, guest microbes, P (primary) and S (secondary) symbionts, facultative, and obligatory. Most endosymbionts can develop a mutualistic relationship with their host, while others can potentially have detrimental impacts on the partner insect's numerous biological activities[2]. They influence diet, behavior, reproduction and sex, and fecundity. Furthermore, endosymbionts may influence the host's ability to cause infections in humans, and remarkably, they can even impact the host's genetic material, affecting the DNA of the insect.

One major endosymbiont that is in an obligatory relationship with various insects is *Wolbachia pipientis. Wolbachia pipientis* was named after an internal Rickettsia-like organism (RLO) discovered in the gonad cells of the mosquito Culex pipiens[3]. Up to two-thirds of all insect species are infected by *Wolbachia*– arthropods[4], as well as a variety of mites, spiders, scorpions, and terrestrial crustaceans[5,6]. Furthermore, *Wolbachia* has been found in filarial worms and a plant-associated nematode[7,8]. Generally, the presence of *Wolbachia* ranges from 20% to 76%[9].

Despite being categorized as a mutualist, the intracellular endosymbiont *Wolbachia*, normally inherited maternally, has evolved several strategies to ensure its transmission over the fitness cost of the host.

These include feminization, leading to the conversion of genetic males into females, parthenogenesis, enabling the the production of diploid offspring without sexual reproduction, male killing, a phenomenon where infected males are selectively killed to benefit infected female siblings, and cytoplasmic incompatibility, resulting in the inability of infected males to successfully fertilize eggs from either uninfected females or females infected with different *Wolbachia* types. In addition to this reproductive parasitism, *Wolbachia* can influence other aspects of host fitness, including host longevity, fecundity, fertility, and host-parasitoid interactions[10]. They also demonstrate genetic hitchhiking and selective sweeps[11] and can vastly shape how an insect interacts with other insects and its environment. As a result, it is very insightful to study and analyze the presence of this endosymbiont in various insect populations.

With the exception of G and R, *Wolbachia* infections have been identified and categorized into 17 supergroups, from A to S, based on the last update in 2023[4,10].

As previously indicated, *Wolbachia* spreads among insects through various means while managing and influencing its host population. Leveraging this knowledge opens avenues for the development of natural endosymbiont-based insecticides, offering a promising strategy to combat agricultural pests, such as worms, locusts, and aphids. Moreover, the potential application extends to the realm of disease management agents. This strategy has been the focus of many studies so far[12,13]. Some even recommend increasing the screening of *Wolbachia* for possible pests or pest-interacting insects to find more potential solutions[14].

The aim of this study was thus to uncover more information about *Wolbachia* diversity in insects in North India. The screening results of 21 insects are presented in this article, along with the discovery of *Wolbachia* infection in the seed bug *Metochus uniguttatus*, a member of the Rhyparochromidae family, which Thunberg discovered and named in 1822.

2. Materials and Methods

2.1. Collection of insect samples

In two batches, 27 insects were captured and categorized into two sets, including samples 1 to 14, and samples A to M. The location and coordinates of the capture sites are depicted in Figure 1. *Nasonia vitripennis* grown in the lab served as a control.

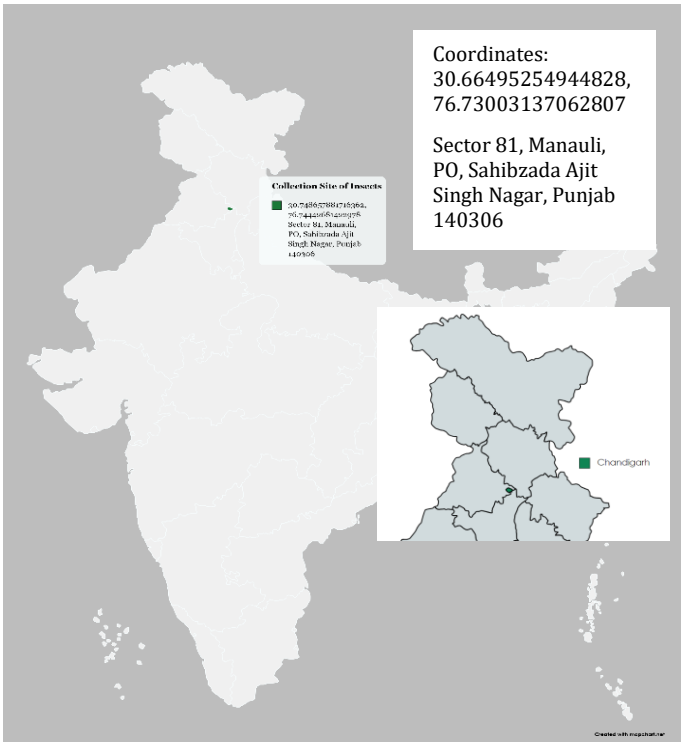

Figure 1. The collection site of insects used in the study

2.2. Insect whole genomic DNA extraction

2.2.1. Materials

In this phase of the study, microcentrifuge tube (MCTs), autoclaved distilled water, 5× Lysis buffer (0.5M tris HCl, 5M NaCl, 0.5M EDTA), 10% SDS, 22mg/mL proteinase K, pestles, water bath, phenol, CI (24:1 chloroform: isoamyl alcohol), chilled isopropanol, ethanol, and TE buffer were used.

A refrigerated tabletop centrifuge was utilized in the experimental procedures. All materials were purchased from Merk Co., Germany.

2.2.3. Methods

An appropriate volume of absolute ethanol was poured into Falcon conical screwcap centrifuge tubes. Insects (one to several of each species) were carefully collected while wearing gloves to avoid stings or bites. When the insects come into contact with ethanol, they become immersed, immobilized, and sterilized. This is a standard and painless procedure for killing insects. Captured insects were brought to the lab, photographed using a Leica M205C stereo microscope, and photo-identified.

Insects were treated, either whole or dissected (abdominal tissue for bigger insects), utilizing fresh 70% ethanol in MCTS, followed by autoclaved distilled water to remove the alcohol, following a previously confirmed method[15]. Lysis buffer was added in the ranges of- 200, 400, and 600 uL, according to the number and size of each specimen. Then, 10% SDS (or 1% of the amount of the buffer) and 2 uL of proteinase K were added. The insects were crushed with pestles and incubated in a water bath (6 hours overnight) at 37°C. Phenol was added. The MCTs were inverted to mix properly and centrifuged at 12700 rpm at 4°C for 5 minutes. The top aqueous layer was collected carefully in new MCTs. The sample was then treated with 24:1 Chloroform: isoamyl alcohol (same volume as the respective lysis buffer used), mixed, and again centrifuged at 12700 rpm at 4°C for 5 minutes. The top aqueous layer was collected. The addition of 150 µl (or 0.7 volume of the aqueous phase) of chilled isopropanol and 0.1 volume of sodium acetate (for finer DNA) was performed. Samples were incubated at room temperature for 30-40 minutes. Samples were centrifuged at maximum speed for 5 to 10 minutes at 4°C until a visible pellet was collected at the bottom. After washing with ethanol, the pellet was air-dried. Dissolved in 100µl TE by vortexing and spinning. The resulting solution was stored at -20°C[15].

2.3. DNA quantification

DNA purity was checked using Thermofisher NanoDrop One/OneC UV-Vis followed by 28s (IDT primers) rRNA housekeeping gene PCR to verify DNA integrity (Table 1).

Table 1. Reaction mixture for 28s rRNA PCR

Solution	Volume (uL)
Nuclease free H_2O	n×6.25
Buffer	n×1
dNTP	n×0.2
Forward Primer	n×0.25
Reverse Primer	n×0.25
Taq Polymerase	n×0.05
Total	8uL+ 2uL template DNA

The thermal cycler was set for a standard PCR cycle (35 cycles) with an annealing temperature of 56°C. The samples that showed ambiguity were discarded.

2.4. Wolbachia infection detection

DNA concentration of samples was adjusted to 40ng/uL. The reaction mixture scheme was similar to the 28s protocol. 16s *Wspec* IDT primers (Table 2) were used.

A standard PCR cycle of 39 cycles was performed with an annealing temperature of 56°C.

Table 2. The IDT forward and reverse primer sequences for 16s *Wspec* PCR

Primer	Band	Reference
Wspec Forward-CATACCTATTCGAAGGGATAG *Wspec* Reverse-AGCTTCGAGTGAAACCAATTC	500 bp	Werren and Windsor, (2000)[15]

2.5. ftsZ A and B PCR to verify Wolbachia infection in Nasonia to use as a positive control

Lab-raised lines of *Nasonia vitripennis* single A-infected, single B-infected, and double A B infected (wNvitA, *wNvitB, and w*NvitAB, collected from Punjab in 2015- NV-PU-14)[16] were used as *Wolbachia* infection positive control. For good measure, *ftsZ* PCR was performed on their extracted DNA to verify infection and supergroups A and B (Table 3).

Table 3. The IDT primer sequences and their annealing temperature for *ftsZ* A and *ftsZ* B PCR

Primer	Band	Reference
ftsZ A specific (53°) F-AAAGATAGTCATATGCTTTTC R-CATCGCTTTGCCCATCTCG *ftsZ* B specific (55°C) F-AAAGATAGCCATATGCTCTTT R-CATTGCTTTACCCATCTCA	435 bp	Baldo et al. (2006)[6]

The same reaction mixes and thermal cycler program as *Wspec* were used.

2.6. Insect species identification- DNA barcoding

Working solutions of 20ng/uL of the samples that showed positive *Wolbachia* infection were run for CO1 PCR (LCO and HCO IDT primers). Master mix and thermal cycle are similar to *Wspec,* with an annealing temperature of 51°C.

2.7. PCR product purification and sequencing

The materials included exonuclease, SAP, Exo buffer, CutSmart SAP buffer. The ExoSAP PCR "cleanup" procedure was employed. This process involved purifying the final samples intended for sequencing to eliminate primers, buffers, and all PCR products, retaining only the DNA for further analysis (Table 4).

The thermal cycler was set at 37°C for 1 hour, 90°C for 10 minutes, and 4°C until the sample was removed.

Table 4. Reaction mixture for ExoSAP (Exonuclease-Shrimp Alkaline Phosphatase) PCR Purification Protocol

Solutions	Volume (uL)
H_2O	n×2.85
SAP buffer cutsmart	n×0.3
EXO buffer	n×0.1
SAP enzyme	n×0.6
Exonuclease enzyme	n×0.15
Total	4uL + 6uL PCR product

2.8. Sequence analysis

The obtained sequence files contained FASTA and ab1. The NCBI Blast was performed using the FASTA, and the sequences of CO1 indicated the maximum identity from the database, while also closely resembling the morphological characteristics of the insects, were selected. Pairwise alignment files were saved. To check the *Wolbachia* super group, Blast was performed on the *Wspec* FASTA sequence. The ab1 files were opened in Sequencher 5.4.6, and contigs were made to check the quality of the sequenced samples, observe the base pairs to crosscheck the alignment, and verify the chromatogram of the sequences.

3. Results

3.1. Collection of insect samples: Photographs of Wolbachia-positive samples

Following ethics, the insects were treated with care and sacrificed in a painless manner. Only necessary insects were captured and used for further procedures.

3.2. DNA quantification

Out of 27 samples, four did not pass the nanodrop criteria for purity, and two samples did not show DNA band integrity. Hence, 21 samples were passed for further analysis.

3.3. ftsZ results of nasonia lab strains

The 16S Wspec analysis of the Nasonia sample, followed by ftsZ PCR, confirmed that these specimens carry *Wolbachia*. Therefore, they can be used as a positive control for checking the status of the rest of the insects.

3.4. Wolbachia infection status

Sample 10 (*Playpleura octoguttata*) indicated a weak positive result for *Wolbachia*, revealing a low titer infection (Figure 2). The corresponding 16s *Wspec* PCR indicated a very faint band upon normal. Conversely, Sample A (*Metochus uniguttatus*, Figure 3) and Sample K (*Velarifictorus micado*, Figure 4) were strongly positive.

Figure 2. Dorsal and lateral views of *Playpleura octoguttata*, a species of cicada

Figure 3. Lateral and side view of *Metochus uniguttatus*, seed bug from family Rhyparochromidae[17]

Figures 4. *Velarifictorus micado*, a species of cricket.

3.5. Sequence analysis

3.5.1. D.1 Insect identification-CO1 sequences

Most focus was given to Sample A due to the absence of any prior research documenting *Wolbachia* infection in the species. NCBI Blast was performed (Figure 5).

Sample A FASTA:

AGGGAAATTCTTTTGAATTTGTTGGACCACCCCCTCCTGTGGGGTCAAAGAATGAGGTATTAAAA
TTTCGGTCAGTTAATAATATTGTAATAGCTCCTGCTAATACTGGTAGTGAAAGAATTAAAAGTA
ACGCTGTAATTCCTACTGATCATACAAATAGTGGGATTTGTTCTGGGGATATTCCAGTGGGCCGT
ATATTAATGATTGTTGTAATGAAATTAATAGCTCCTAAAATTGAGGAAACTCCTGCTAAATGAA
GGGAAAAGATTGCTAAGTCTACTGAAGCCCCTCTATGGAATAATCTATTTGATAATGGAGGGTA
AACAGTTCATCCTGTCCCTGCTCCTGCTTCAACTATTCTTCTAGTCAATAATAGAGTTAAGGATG
GGGGTAAAAGTCAAAATCTTATATTATTTATTCGGGGAAATGCTATATCTGGTGCTCCAATTATT
AAAGGTACGAGTCAGTTTCCAAAACCTCCAATCATAATTGGTATAACCATAAAAAAAATTATAA
TAAATGCATGAGCAGTAACAATAACATTATAAATTTGGTCATCTCCAATAAATATCCCTGGTTGA
CCTAATTCAATACGGATAATTCATCTGAGAGATGATCCTACTATACCTGATCATATACCAAAGAT
AAAATACAGTGTTCCAATATCTTTATGATTTGTTGACCAAATT

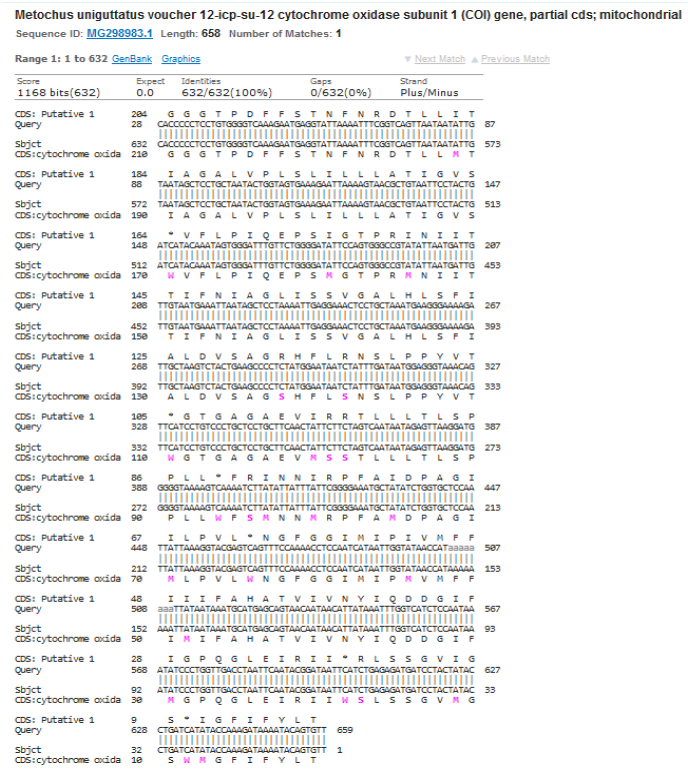

Figure 5. NCBI Blast of Sample A CO1 sequence showed 100% identity with *Metochus uniguttatus* SeqID: MG298983

Figure 6. The ab1 sequence file of CO1 sequence of *Metochus uniguttatus* visualized in Sequencher 5.4.6

Sequencher showed no ambiguity or mismatches of alignments in contig and chromatogram (Figure 6).

3.5.2. D.2 Wolbachia supergroup identification-Wspec sequences

NCBI Blast was performed on the Wspec FASTA (Figure 7).

Sample A *wspec* FASTA:
TTTGGTGCCAGCTTAACCTCAGGGTGTTATGCATGGGCTGTCGTCGAGCTCGATGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCCTTAGTTACCATCAGATAATGCTGGGGACT
TTAAGGAAACTGCTAGTGATAAACTGGAGGAAGGTGGGGATGATGTCAAGTCATCATGGCCCTTA
TGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAATGGGCTGCAAAGTCGCGAGACTAAGCC
AATCCCTTAAAAGCCATCTCAGTTCGGATTGTACTCTGCAACTCGAGTACATGAAGTTGGAATCG
CTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCAC
GCCATGGGAATTGGTTTCACTCGAAGCTAAG

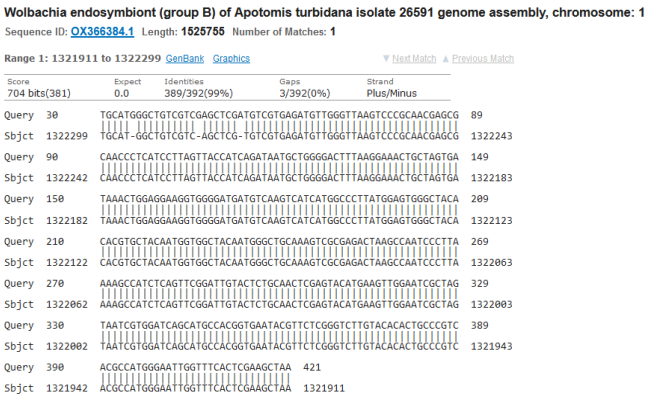

Figure 7. NCBI Blast of Sample A *Wspec* sequence showed an alignment match with *Wolbachia* endosymbiont supergroup B

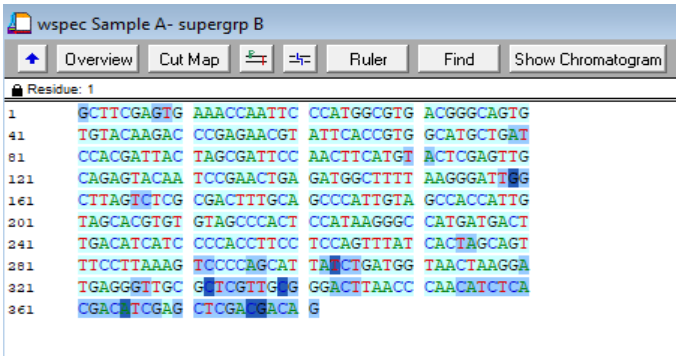

Figure 8. The ab1 *Wspec* sequence of *Metochus uniguttatus* file visualized in Sequencher 5.4.6

Sample A *Wspec* FASTA had 422 base pairs. It indicated a decent chromatogram, and apart from some confusing peaks, the rest of the sequence (Figure 8) was without any mismatches, gaps, or ambiguity. The extra overhangs from the 5' and 3' were deleted to get the clean sequence.

Similar analyses were performed on Sample 10 and Sample K.

3.6. Percent infection rate

Based on the obtained results, the percentage of infection in samples was calculated using formula 1.

Formula 1

$$\frac{\text{no. of infected insect samples} \times 100}{\text{total number of insects}}$$

$$(3\times100)/21 = 14.2857\%$$

Where, number of Wolbachia infected insects is 3 and total number of collected insects is 21

4. Discussion

It is generally tricky to screen for *Wolbachia* in a study with a small sample size as the infection frequency follows

a 'more-or-few' pattern[9]. This suggests that certain members may exhibit a notably high infection titer within the species, while determining positivity in others could be challenging. A similar case was found in this study, where Sample 10 Cicada (*Playpleura octoguttata)* only revealed a faint band despite repeated trials. In line with a study by Hasan et al.[18], this study is one of the few reports that have looked into the *Wolbachia* infection in Cicadidae.

Wolbachia infection can occasionally affect the integrity of mitochondrial genes, particularly the gene involved in insect identification in a study[19]. In this case, obtaining CO1 values for sample K (*Wolbachia* positive) proved to be challenging.

This could be a direct cause of the infection, as *Wolbachia* interferes with other maternally inherited genes, such as mitochondrial genes[19]. Much like this study, Sample K- *V. micado* has been previously found to be *Wolbachia* positive by Yue-Yuan Li et al.[20].

One of the primary objectives of this study was to assess the *Wolbachia* infection rates across a diverse selection of randomly chosen insect species. There is barely any information recorded about the seed insect *Metochus uniguttatus* or its distribution in India. This study is one of the first reports of *Wolbachia* infecting *Metochus uniguttatus* in North India (Coordinates: 30.66495254944828, 76.73003137062807). Furthermore, it clarified the previously unknown infection status of *Metochus uniguttatus*. No previous study has reported *Wolbachia* infecting this insect.

These findings contribute to the exploration of relationships between different species in insect ecosystems and food webs. As predatory insects[21], members of the Rhyparochromidae family of insects may carry endosymbionts from predator to prey, and an infection in one of these insects may indicate this horizontal transfer. Being plant feeders and potential pest threats, the insects under consideration open up opportunities for utilizing the biocontrol strategy of Wolbachia, given the newfound awareness of its infection in insects.

5. Conclusion

This paper briefly elaborated on how Wolbachia can be discovered in common arthropods in the surrounding environment. "Wolbachia is possibly the worst pandemic in the animal world," and it is demonstrable. Despite the surge in interest in this endosymbiont, there are still gaps in the information on Wolbachia in the scientific community. In a country like India, endowed with such a variety of insects, there has never been a sufficient survey of different species and their related holobionts. It is also necessary to develop better identification techniques, long PCRs, and tailor-made detection kits. As was said, Wolbachia has the potential to let the scientific community through previously closed doors. A few examples include the World Mosquito Program[22], use of Wolbachia to study the evolutionary fitness of hosts, and biological pest management. The surveying, sequencing, phylogenetic, and behavioral investigations pertaining to Wolbachia and their host insects must therefore continue.

In conclusion, Wolbachia is one of the perfect examples of how basic and cumbersome science can become something applied and translational for the public within a decade or so.

Declarations

Competing interests

There was no conflict of interest.

Authors' contributions

This article has a single author and all responsibilities are done by the author and also under her supervision.

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No funding information is to be declared.

Availability of data and materials

The manuscript contains the most important datasets generated and/or analyzed in the current study. For additional data (images and sequences), kindly contact the author.

Ethical considerations

The author checked for plagiarism and consented to the publishing of the article. The author has also checked the article for data fabrication, double publication, and redundancy.

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