

| **Discovery**~~iscovery~~ of a novel  
endosymbiont belonging to  
*Wolbachia* in A US Sandfly

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## **Abstract**

The sandfly is an important arthropod vector of many diseases affecting both human and animal health including *Leishmania*, an emerging disease in the United States that is endemic in primarily Southern Europe and Northern Africa. Sandflies from Laramier County, Colorado were analyzed by Next Generation deep sequencing for viruses, bacteria, phages, that may be present within their microbiome. Ultimately symbionts were found, which function similarly to rumen microbes in cattle. The project seeks to utilize Sanger sequencing to verify the sequences of a specific species of the midgut symbiote, and to explore the possible uses and significance of this finding in the perspective of control and public health. Methods utilized Sanger sequencing of PCR product from the symbionts, as well as reviewed of current literature on *Wolbachia* symbiont species. PCR product was compared to that of nucleic acid from sandflies from Texas which lacked the *Wolbachia* sequences. Results indicated a novel species of endosymbiont, which was demonstrated through a phylogenetic tree analysis. *Wolbachia* is useful for the suppression of controlling some arthropods that, like Sandflies, are often vectors for diseases with significant threats to human and animal health. The characterization of *Wolbachia* within Laramier County sandflies suggests possible methods of disease control should an emerging disease for which it is a vector spread to the region.

## **Introduction**

The sandfly, while not a commonly identified pest in the United States, carries with it a terrible reputation in subtropical regions of the world. An arthropod in the suborder *Nematocera*, the pest is related to black flies, midges, and mosquitoes [1]. It has life cycle of egg, larvae, pupae, and adult stages, and like their cousins, sandflies are hematophagous [2]. There are two types of sandflies, colloquially referred to as Old World and New World sandflies. “Old World”

sandflies are of the genus *Phlebotomus*, while “New World” sandflies are of the genus *Lutzomyia* [3]. Old world sandflies are the culprit for many diseases that are important to human and animal health and pose challenges to public health officials in areas around the Mediterranean especially. However, this is not to say that New world sandflies are not of importance. Studies have shown *Lutzomyia* species in Brazil carry some of the same diseases as Old world sandflies [4]. The particular disease that is pertinent to public health is the protozoal parasite, *Leishmania*, which causes ulcerative cutaneous lesions on humans and canines [2]. The disease is endemic in subtropical areas where sandflies are present, but in the United States it is still considered an emerging disease [5]. Control of sandfly vectors is considered the best way to control the spread of the parasite.

*Wolbachia* is a genus of small, obligate intracellular bacterium that resides in the cytoplasm of midgut cells of arthropods and some nematodes [1]. They are gram negative alphaproteobacteria in the order of *Rickettsiales*, which includes genera of *Anaplasma* and *Rickettsia*, other types of bacterium that are often found in ticks. *Wolbachia* are widespread and are thought to be found in 65% of insect species, according to a review in 2012 [6]. The bacterium is an endosymbiont and has a complicated relationship with its hosts. It induces host manipulations, particularly of the reproductive tract, which results in infertility or death of males [6,]. The bacteria are able to affect their hosts in 4 ways: by decreasing longevity, inducing cytoplasmic incompatibility, feminizing males, and parthenogenesis [6,7]. Utilization of the bacteria to induce host death or decreased fertility has been essential for the control of arboviral diseases, particularly those with *Aedes* mosquito vectors [6,8].

Researchers at the University of Wyoming captured sandflies, species *Lutzomyia anthophora*, and used deep sequencing in search of Vesicular Stomatitis Virus (VSV) RNA

sequences. Sandflies have been theorized as vectors of the virus, and their possible competency is concerning to Animal and Plant Health Inspection Service (APHIS) officials who are constantly on the lookout for a lookalike of the disease, foot and mouth disease [5]. While VSV was not detected, sequences from *Wolbachia* bacteria were detected. The finding of *Wolbachia* nucleic acid in a pool of Colorado sandflies after deep sequencing gives interesting insight into the potential future of sandfly control into the United States and poses questions of the utilization of biocontrol methods to prevent or control disease.

## Methods

A pool of 24 Laramer County, Colorado, sandflies (*Lutzomyia anthophora*) were analyzed for virus RNA sequences using the Illumina MiSeq system. The deep sequencing did not yield viral sequences, but over 300,000 base pairs of *Wolbachia* DNA were detected. Two contiguous (contigs) sequences were chosen for analysis and verification via Sanger sequencing. Specific regions within contigs of 27,000 and 28,000 bp (WO.CO.27k and WO.CO.28k) were amplified using Polymerase Chain Reaction (PCR). Briefly, the ingredients of the total reaction volume are detailed in Table 1. Thermocycle conditions were; one cycle of 95C for 5 minutes,

**Table 1.**

Reagent	Vol/rxn (μL)	# rxns	Final Volume
Water	8.5	3.5	29.75
2X Apex Master Mix	12.5	3.5	43.75
Forward Primer (10μM)	1	3.5	3.5
Reverse Primer (10μM)	1	3.5	3.5
<b>Subtotal</b> (MM per rxn)	23		
Template	2		
<b>Total reaction Volume</b>	<b>25</b>		

40x (95C 1 minute, 60 C 30 seconds, 72C 1 minute), one cycle of 72C for 5 minutes. The sequences were run in an agarose gel to verify presence of an amplicon of the expected size. Amplicons were run on a 1.5% gel, and column purified (Zymo DNA Clean and Concentrate

Kit, Irvine, CA). The amplicons were analyzed for quality and concentration of sample using a Nanodrop mass spectrophotometer, and submitted to GeneWhiz for Sanger sequencing per company directions (South Plainfield, New Jersey). The resulting sequences were analyzed using National Center for Biotechnology Information's BLAST (NCBI BLAST) search and used in phylogenetic estimation. The BLAST search distance tree and FigTree software were used to create a phylogenetic tree to visually evaluate the relatedness of our sequences with other *Wolbachia* in GenBank.

## Results

The gel analysis after amplification yielded bands of expected size. The WO.CO.27k amplicon length was expected to be 600bp, and the WO.CO.28k amplicon length was expected to be 553bp. Figure 1 is a picture of the gel. Lane 1 represents WO.CO.27k, lane 2 is the DNA ladder, lane 3 is no template control, lane 4 contained DNA of a *Wolbachia* negative sandfly pool from Texas, and lane 5 represents WO.CO.28K.

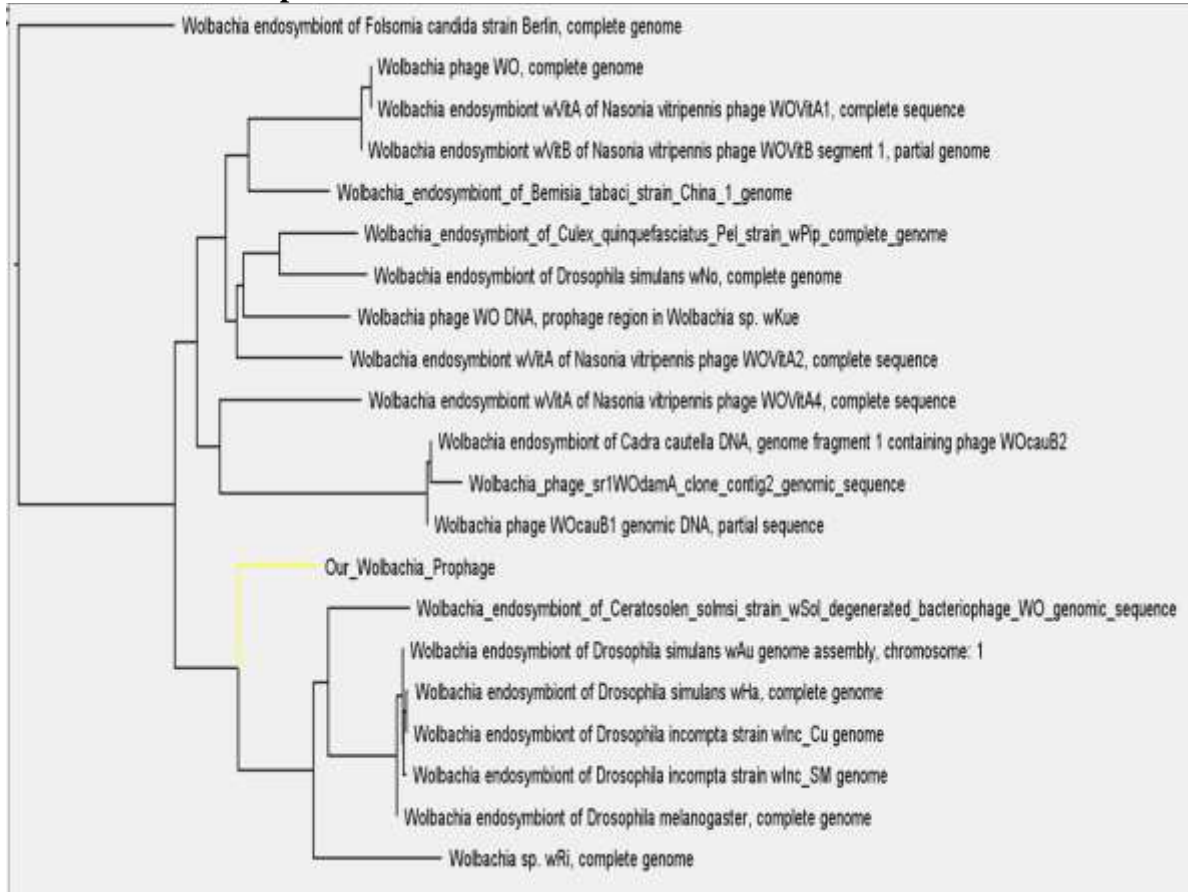
**Figure 1. Gel image of PCR amplicons using primers targeting sequences from Illumina MiSeq data assemblies.**



After the gel was viewed, the amplicon was analyzed for purity and quality of sample. The WO.CO.27k sample had a concentration of 46.3 ng/ $\mu$ L with a 260/280 ratio of 1.21. The WO.CO.28k sample had a concentration of 37.8ng/ $\mu$ L with a 260/280 ratio of 1.13. The

concentration allowed for calculation of the dilution of the PCR product into what was necessary for the Sanger sequencing mix, and the 260/280 ratio is a measure of purity of the sample. The results of the Sanger sequencing were inputted into NCBI BLAST, and the phylogenetic tree generated was utilized for this paper (Figure 2).

**Figure 2. Phylogenetic tree from NCBI BLAST search distance tree software. Yellow line denotes *Wolbachia* species**



## Discussion

Phylogenetic analysis shows that the *Wolbachia* DNA isolated from Larimer County, Colorado, sandflies is indicative of a novel species. Sanger sequencing confirmed the results of the next generation deep sequencing that gave us 300,000 base pairs.

While considered successful, there were project limitations. After PCR, the 260/280 values were lower than what is considered a high quality sample. The desired ratio is as close to

2 as possible, with a goal being around 1.8. The ratios of 1.21 and 1.13 are lower than that, however they were still adequate for Sanger sequencing, and resulted in 100% identity with the Illumina MiSeq generated data. Lower ratios and less purity can be attributed to lack of experience of the person who performed the gel purification. Another limitation was the sample sizes of the initial deep sequencing. The pool size of the Colorado sandflies was 24, while the pool size of the Texas sandflies was 4. With the difference in pool sizes, there is the possibility that the *Wolbachia* species identified in the Colorado sandflies is also in the Texas species, but was not detected.

The results of the identification of a novel *Wolbachia* species in Colorado sandflies may have tremendous impact on the control of *Lutzomyia anthophora* if the arthropod vector should ever become an issue. The bacterium is population-limiting for some arthropod vectors, and can serve as a biological control method for the diseases that they carry [8].

## **Conclusion**

Discovery of a novel species of *Wolbachia* in Colorado sandfly species *Lutzomyia anthophora* was confirmed via Sanger sequencing and phylogenetic analysis. Knowledge of unique *Wolbachia* species in local populations of sandfly is has implications for the control of the pest, as well as the diseases that they may act as a vector for. While public health officials in the United States are not yet concerned about sandfly populations and possible transmission of *Leishmania* to canines and humans, the sandfly is still of concern to the US government. Being able to control sandfly populations in areas of heavy US armed forces presence can assist in the quality of life of troops overseas. Additionally, arthropods and the diseases they carry will become more widespread as global temperatures rise and subtropical climates expand to encompass latitudes closer to the poles. The threat of this brings with it the threat of emerging

diseases to the United States, but if equipped with the proper knowledge of biocontrol tools, public health officials can stop outbreaks in their tracks.

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