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Survey of Mosquito Larvae for Potential Nematode Parasitic Biological Control in Alachua County

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Abstract

There is an obvious and considerable interest in developing novel methods to control mosquito-borne diseases due to their impacts on mammalian hosts. Biological control practices are sustainable alternatives to chemical control of mosquito populations. Some nematodes are efficient insect parasites and represent a potential source of novel biological control agents. Such beneficial nematodes are potent biological control agents that can remove harmful pests whilst also preserving non-target insect populations. One such nematode, *Romanomermis culicivorax*, is a parasite of mosquito larvae, inducing paralysis and eventually killing its host. The potential success of a biological control agent can be informed from the current prevalence of native populations. Thus, we conducted a survey of parasitic nematodes in mosquito larvae in Alachua County. Twenty-five samples were obtained from all around Alachua County, with assistance from the Gainesville Department of Public Works Mosquito Control Section. Subsequent visual inspection and PCR for *R. culicivorax* were conducted to detect the presence of mosquito and nematode DNA in each sample. Out of the twenty-five mosquito samples tested, only one sample tested positive for the presence of the beneficial nematode. This sample was found in an apartment complex in proximity to Devil's Millhopper Geological State Park. This work demonstrates the presence of mosquito parasitic nematodes in Alachua County, but at very low levels, potentially discouraging the use of such biological control practices. In addition, the proximity of the positive sample to the natural area may indicate the need to preserve such habitats for effective biological control measures.

Keywords: biological control, nematode, mosquito

Introduction

Mosquito-borne diseases have a lethal impact on humans and animals as they are vectors that spread diseases. These vectors can transmit bacteria, viruses, and parasites to mammalian hosts through mosquito feeding can result in serious infections. Approximately 390 million people are infected annually by dengue fever while hundreds of thousands of people are affected by the Zika virus, chikungunya, and yellow fever (World Mosquito Program, 2022). Other common diseases transmitted by mosquitoes include malaria and West Nile virus (Tolle, 2009). Mosquitoes inhabit most areas of the world and are commonly found in areas with high human population density, or areas such as forests, marshes, or tall grassy areas. Still, shallow aquatic environments are preferred habitats for mosquito larvae and pupae to thrive in as the surrounding soil is nutrient-rich (CDC, 2020).

Pesticides are utilized to control mosquito populations. Many of the pesticides that are commonly used to remove adult mosquito populations contain chemicals such as malathion and naled while synthetic pyrethroid chemicals such as prallethrin, etofenprox, pyrethrins, permethrin, resmethrin and sumithrin are also sprayed (EPA, n.d.). Given the toxicity of pesticides and insecticides on the environment, a natural biological control agent to control mosquito populations and prevent vector-borne diseases is in need.

Nematodes are one such potential biological control against harmful pests like mosquitoes with the ability to not target the insect populations that are generally beneficial to the ecosystem and other non-pest organisms in the same habitat. Nematodes can either be helpful or harmful as they can either be parasitic by feeding off of host plants or animals or they can be beneficial by distributing nutrients in soil for plant growth. One species of nematode that has been studied that is a parasite of mosquitoes and is potentially beneficial to preventing vector-borne diseases is *Romanomermis culicivorax*. This nematode can induce paralysis to the larvae and eventually kill the host. Given that the nematode already infects the mosquito at the larval stage, this prevents the mosquito from living its normal lifespan and reproducing.

Romanomermis culicivorax is a mermithid nematode that is parasitic during the developmental stage but free-living as adults. The life cycle development of *R. culicivorax* is only involved in the mosquito larval stage, as seen in Figure 2. Pre-parasitic juvenile nematodes penetrate into the hemocoel of the larvae, resulting in the death of the larvae between a 7-to-10-day period. The nematodes become post-parasitic juveniles and complete their development. The nematodes can molt, mate, and lay eggs within the larvae. *R. culicivorax* can use over 90 different mosquito species as a host (Bisen & Raghuvanshi, 2013).

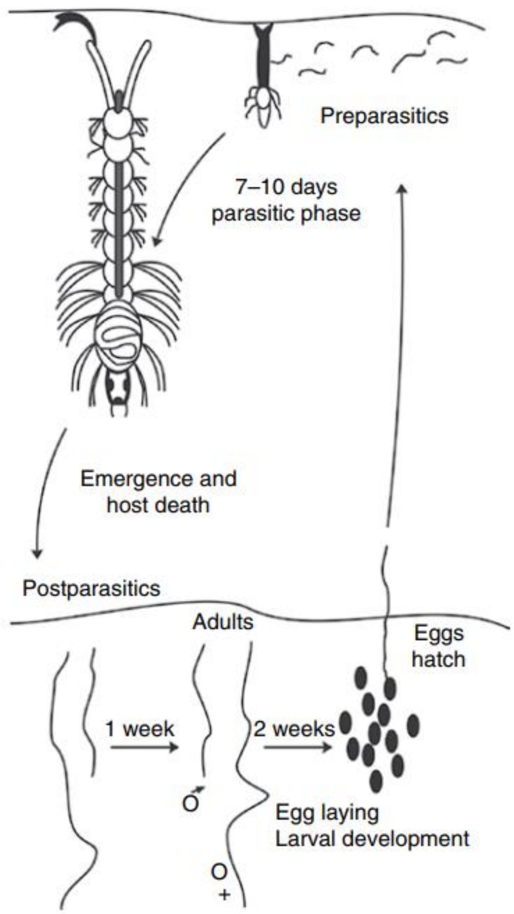

Figure 1. Parasitic *R. culicivorax* life cycle inside a host *Culex pipiens* mosquito (Bisen *et al.*, 2013)

The use of nematodes as biological control agents does pose many efficacy issues. Various environmental factors including temperature, pH, nutrient content, presence of organic matter, UV radiation and others can significantly impact the prevalence and persistence of nematodes in different habitats (Sharma and Gupta, 1976; Lacey and Georgis, 2012). To ensure the success of a nematode biological control agent, it is then important to understand the natural distribution of such nematodes and the environmental factors present in areas where they thrive. *R. culicivorax* has been implemented in biological control of mosquito population studies in southern parts of Florida (Levy and Miller 1977; Levy *et al.*, 1979), with varied success. Few studies have demonstrated successful and persistent *R. culicivorax* deployment (Platzer, 1981).

In this work, we sought to characterize the natural distribution of *R. culicivorax* in naturally occurring mosquito larval deposits. Using a combination of microscopy and molecular tools, we screen 25 unique sites for the presence of *R. culicivorax.* To the best of our knowledge, this is the first survey of its type in northcentral Florida.

Methodology

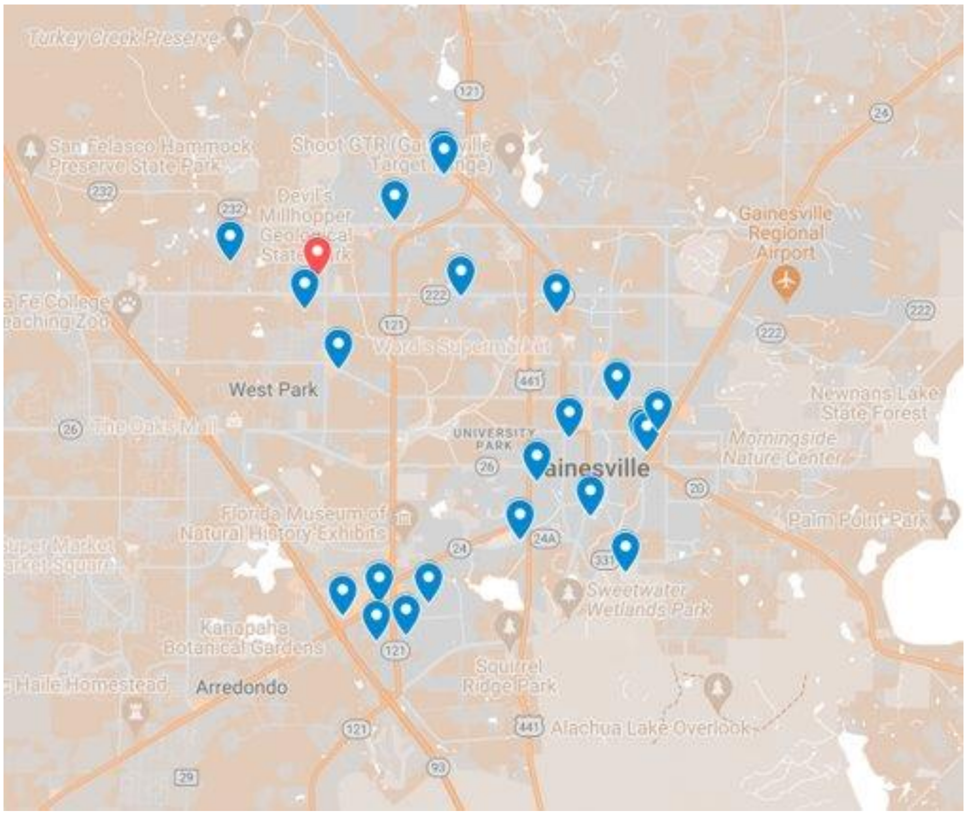

Figure 2. Map of mosquito larvae sampled in Alachua County. Red marker is positive for positive presence of nematode in larvae sample. Blue markers exhibit negative nematode presence in larvae.

Mosquito Larvae Sampling

Twenty-five mosquito larvae samples were collected around Alachua County in a nine-month period (Figure 2). Assistance to obtain these samples was provided by the Gainesville Department of Public Works Mosquito Control Section as they specialize in surveying and treating mosquito larvae-infested water. Typically, after substantial rains, collected samples were retrieved from Public Works after being cataloged by the Control Section and sent to lab for analysis. The larvae samples were stored at 4^{o}C once collected.

Nematode Detection by Microscopy

A fine sieve was used to filter the water that the mosquito larvae were in, and the larvae were observed under a dissecting microscope to observe if any parasitic nematodes were present. Each sample contained approximately 10 to 25 mosquito larvae and other organisms. As the nematode parasite could be in the same medium as the mosquito larval host as a pre-infective juvenile, or inside the larvae as a parasite, visual inspection alone is insufficient and time consuming to conclude nematode abundance.

Nematode Detection by PCR and Sequencing

Afterwards, DNA extraction of the mosquito larvae was conducted by grinding the larvae into a pulp with a mortar and pestle and using the Quick DNA Miniprep Plus Kit by Zymo Research(TM), following manufacture protocols The extracted DNA samples were checked for concentrations and purity by NanoDrop(TM), and the extractions were stored in the fridge. The DNA sample extracted was amplified by PCR with both mosquito and nematode primers. Mosquito primers used target COI and were derived from Monteiro and Pierce, 2001; (FWD: 5'-GGATCACCTGATATAGCATTCCC-3'; REV: 5'-CCCGGTAAAATTAAAATATAAACTTC). Novel primers were developed to detect the nematode parasite *R. culicivorax* based on the sequenced genome (Shiffer *et al.,* 2013) and COI loci; (FWD: 5'-AGGCTTCCTGTTTTAGCCGC-3'; REV: 5'-AGCTCACACAACACAGCCTAA). PCR reaction conditions contained 10uL 2x Taq Red (Apex Bioresearch), 5uL primer mix containing forward and reverse primers at a final concentration of 500nM and 5uL of sample DNA. Thermocycle conditions started with an initial denaturation step at 95^oC for 15 minutes followed by 30 cycles of 95^oC, 55^oC annealing and 72^oC extension, and ended with a final extension step at 72^oC for 30 minutes.

Gel electrophoresis of the resultant PCR products was then run to ensure viable mosquito DNA was present as well as the possibility of *R. culicivorax* DNA. Samples presumptively positive for *R. culicivorax* were purified from PCR reactions using Qiagen PCR Purification Kit and manufacturer's instructions and, along with the Forward primer, sent for sequencing with Eurofins Genomics. Resultant sequences were compared with target *R. culicivorax* loci by nucleotide BLAST.

Results and Discussion

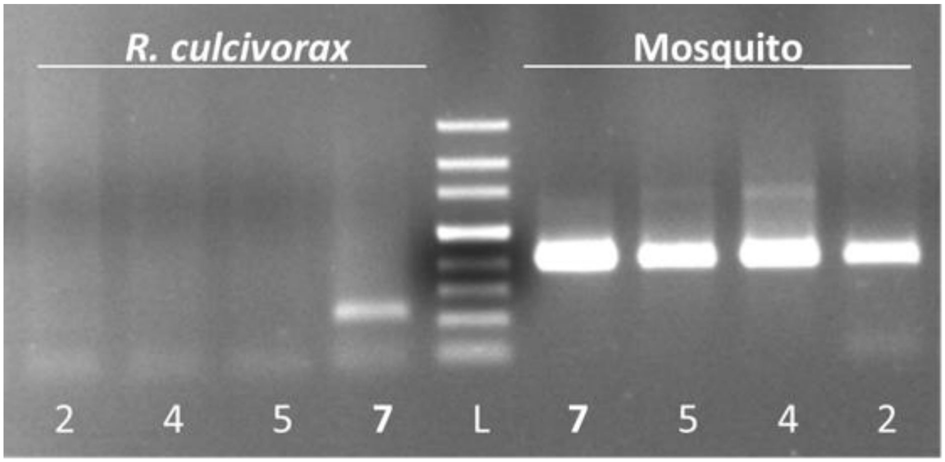

Figure 3. Gel results of mosquito and nematode DNA.

The mosquito larvae samples were collected within a seven-mile radius from University of Florida (UF) Gainesville campus (Figure 2). The sample locations within Alachau County are marked, including the sample presumptively positive for *R. culicivorax*. All the samples appeared to have been collected from areas with buildings and not from natural areas. This is due to the Department of Public Works Mosquito Control Section prioritizing areas with high human population densities. The larvae were found in floodwater locations due to excess rain, especially during hurricane season. These temporary pools of water created by the rain are where the majority of the larvae samples inhabit. Larvae samples were drawn from here and the water was subsequently treated by Gainesville Department of Public Works Mosquito Control Section, if needed, to control mosquito populations.

Of the twenty-five mosquito samples that were collected and DNA was extracted, only one sample, Sample #7, had a positive band on the gel electrophoresis indicating the presence of nematode DNA (Figure 3). Note, not all samples are shown for clarity. This larvae sample was found in an apartment complex. This is interesting given that nematodes are more likely to be found in soil-rich areas and not in areas of infrastructure. However, the location of the apartment complex is in proximity to Devil's Millhopper Geological State Park. As Alachua County is historically a swamp, this park is a natural sinkhole that contains water streams, nutrient-rich soil, and vegetation which are conditions that can allow for both mosquitoes and nematodes to thrive in.

However, subsequent sequencing of the PCR product derived from the nematode specific primers failed to align with the known target (data not shown). While it is not definitive, this result casts doubt on identifying *R. culicivorax* in the collected mosquito larvae samples. Other nematode parasites of mosquitoes are present in Florida, including *Agamermis pachysoma* on the mosquito host *Cx. pipiens* (Poinar, 1976). However, molecular information is not currently available in public databases for this nematode to compare with our sequences.

Conclusion

The survey of nematode-infected mosquitoes in Alachua County provides insight as to where these populations can be found. While the only presumptive positive sample was found in an apartment complex, understanding that Alachua County used to be a swampland shows that nematodes could be present in this county. Given that the apartment complex is located in proximity to Devil's Millhopper Geological State Park, potentially collecting mosquito larvae samples from the vicinity of the area to test for any presence of parasitic nematodes.

Given that the positive sample that was found cannot be exactly identified as *R. culicivorax*, further research needs to be done on what nematode species or other organism may have inhabited the host larvae. While *R. culicivorax* is a popular contender that infects mosquito larvae, there are other parasitic nematodes too such as the aforementioned *Agamermis pachysoma* (Poinar, 1976). Since molecular information is not available for all nematodes publicly, studying the molecular genetics of various nematodes may aid in identifying the exact organism that seems to have infected the larvae.

On the other hand, nematodes may not be able to persist in areas of Alachua County with high human population density. Mosquitoes can thrive in the presence of humans because they feed on blood. However, nematodes require natural areas that provide nutrient-rich soil and require organic matter to feed on as a fully developed adult. Therefore, it is understandable why nematodes are not found where these mosquito larvae samples were collected given that these samples come from areas with presence of human populations. As aforementioned, sampling larvae from natural areas with nutrient-rich soil and freshwater may yield different results.

Further research that could be conducted is studying the life cycle of infected mosquitoes. This way, by observing the physiological features of the mosquito, it could be understood how nematodes infect the larvae and understand the stages in the process of how the nematode kills the mosquito. By understanding what the nematode targets in the mosquito larvae that triggers the death mosquitoes, further research may allow for synthesis and activation of this natural chemical product that can make a significant impact. Considering that this chemical that triggers larval death may be non-toxic, environmentally friendly, and protect target insect populations from being victims of insecticide, this can be an organic larvicide that can be sprayed throughout nature to control mosquito populations.

Acknowledgements

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