Testing For Ryegrass And Potential Mutualistic Relationships In Arthropods From Longleaf Pine Savannas
Senior Project

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By

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Abstract

Invasive species like the red imported fire ant (*Solenopsis invicta*) are a common threat to protected areas such as longleaf pine savannas. A substantial part of *S. invicta*'s diet appears to be the invasive Rigid Ryegrass (*Lolium rigidum*), which does not occur in the burn-managed longleaf pine savannas. *Solenopsis invicta* could be consuming *L. rigidum* through a mutualistic relationship with honeydew-producing insects. Honeydew-producing insects could feed on *L. rigidum* in fields, then fly to the longleaf pine savannas where *S. invicta* tends them. Samples of *S. invicta*, honeydew insects and predatory arthropods went through DNA extraction, and commercial Sanger sequencing was performed on the amplified DNA (*ITS2* gene region). The samples had been stored in 70% ethanol since 2017. Half of the *S. invicta* samples and all honeydew and predatory insect samples were washed in diluted bleach for external decontamination. The results from the honeydew and predatory insects were inconclusive, possibly due to damage from the bleach solution and cross contamination with the positive control. The *S. invicta* predominately matched with the *Lolium* spp., supporting previous findings. Next steps would be to collect and reattempt testing honeydew insects. Native ants also should be examined to determine whether they are feeding from the same plants as does *S. invicta.*

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Introduction

Part of managing our natural resources and protected ecosystems is monitoring and understanding the spread of invasive species. One prevalent invasive arthropod in the southern United States is the red imported fire ant (*Solenopsis invicta*). For an invasive species to become established, it must be able to access resources to grow. *Solenopsis invicta* is an omnivorous species whose larvae primarly digest animal tissues while the adult workers subsist on a liquid diet (Helms and Vinson 2008).

One source for these liquids is from a mutualisitic relationship with honeydew producing insects from the order Hemiptera. *Solenopsis invicta* will tend these insects, sheltering them while gathering honeydew (Helms and Vinson 2002). Access to honeydew insects as well as plants and insect prey can stimulate colony growth (Helms and Vinson 2008). Mutualistic relationships between the invasive *S. invicta* and invasive hemiptera have been observed (Helms et al. 2011). The invasive hemiptera's populations are assisted through care by *S. invicta.* Often these hemiptera have a preference for certain host plants, which are sometimes also invasive. Notably, these mutualistic relationships have occurred between species whose native ranges do not overlap (Helms and Vinson 2002).

Solenopsis invicta has effectively colonized protected longleaf pine savanna habitats (*L. Kelly, unpublished data*). To examine the effect these invasive ants are having on the ecosystem, it is important to understand what they are eating. To determine the digested plant material in *S. invicta*, Sanger sequencing (also known as "chain

termination method") was used. Sanger sequencing detects the most abundant DNA using a single DNA template. This method may not be as inclusive as next-generation sequencing, which uses several DNA templates. Next-generation sequencing, however, is more expensive than Sanger sequencing and requires bioinformatics expertise. Previous studies, using Sanger sequencing of DNA extracted from whole bodies of *S. invicta* has revealed that ryegrass (specifically *Lolium rigidum* and *L. multiflorum*) is a major component of their diet (Kelly et al. 2021). *Lolium rigidum* and *L. multiflorum* are nonnative species, not found in longleaf pine savannas that are managed using prescribed fires. A possible explanation is that honeydew insects are feeding on ryegrass outside of the savannas then enter the savannas, and the ryegrass DNA found in fire ants originates from the honeydew they consume.

Methods

Specimens had been collected in 2017 via pitfall traps filled with propylene glycol. The samples were stored in 70% ethanol. Non-ant arthropods were sorted based upon whether the athropods produced honeydew or were predatory. Honeydew insects include sapsuckers and aphids. The "Honeydew" group was assigned for insects that were not classified further than that they were honeydew-producing. The specimens were from protected, longleaf pine savannas in the Green Swamp Preserve, Myrtle Head Savanna and Juniper Creek Game Land in Brunswick County, North Carolina. Fourteen samples of *Solenopsis invicta* (Red Imported Fire Ant) were selected, two of which were hand-collected samples. Two more samples from *S. invicta* colonies collected from the Green Swamp, that had previously tested positively for *Lolium rigidum,* were added. Seven samples of non-ant arthropods were selected for study.

Eight of the *S. invicta* samples were decontaminated in 1 mL of 3% bleach solution based upon a previous study (Greenstone et al. 2012). Another eight *S. invicta* samples were rinsed in 1 mL of distilled deionized water. The samples were rotated end over end for 40 minutes twice, with a break to replace the liquid in each sample with fresh liquid, while kept at 4°C. All bleached samples were rinsed three times with cold distilled deionized water to remove the bleach and other contaminants. Any remaining liquid was removed using pipette tips, and the samples were stored at -20°C.

Fire Ant DNA Extraction and Amplification

The DNA from the ant samples was extracted using lysis buffer and phenol/chloroform/isoamyl alcohol (PCI, Tris–HCl-saturated, pH 8) (Valles and Porter 2003). We added 150 µL of lysis buffer (50mM Tris-HCI pH 8; 4% SDS, 5% BME) to the samples (whole ants). Samples were ground using sterile micropestles for about 30 seconds. We added 200 µL of PCI (warmed to room temperature) to the samples and inverted five times. Samples were centrifuged for five minutes at room temperature and 13,000 rpm. The supernatant was removed, transferred to sterile 0.5-mL microcentrifuge tubes, and recentrifuged. We removed 50 µL of the supernatant from each tube and pipetted it into a fresh 1.5 mL centrifuge tube. We added 900 µL of room temperature isopropanol to the samples and inverted five times before centrifuging the samples for five minutes at 13,000 rpm. We removed the isopropanol, added 500 µL of 70% ethanol and centrifuged for five minutes at 13,000 rpm. We pipetted out all liquid from the tubes, avoiding the bottom pellet of nucleic acid. We rehydrated the pellets in 50 µL of nuclease free water, mixing it by vortex and stored the samples at -20°C.

The DNA extract was amplified using the polyamerase chain reaction (PCR) temperature protocol for UniPlant primers, which amplify minibarcodes for the *ITS2* gene region (Moorhouse-Gann et al. 2018). UniPlant primers were used to amplify short stretches of DNA and were applicable for amplifying DNA that has undergone some digestion. At this point we added two positive controls of plant DNA *(Hypericum hypericoides),* which had been sampled in 2019. We made 25-µL reactions of PCR master mix using Invitrogen™ Platinum™ Hot Start 2X Master Mix, prepared according manufacturer's instructions, except 0.5 µL of bovine serum albumin (BSA) (purified 100x 20mg/mL; BioLabs) replaced an equal volume of nuclease-free water, and DNA UniPlant primers (forward and reverse) had concentrations of 100 µM. We added 21 µL of PCR master mix and 4 µL of each DNA extract to sterile thermocycler tubes. We mixed the tubes by briefly vortexing and centrifuging. A drop of PCR-grade mineral oil was added to each tube, for the thermocycler (PTC 100 Programmable Thermal Controller) used did not have a heated lid. The conditions for the PCR were initial denaturation at 95°C for 15 min; 40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min; final extension of 72°C for 10 min. We transferred 20 µL of the PCR product from the bottom of each tube to a sterile 0.5 mL centrifuge tube. The products were then stored at -20°C.

Gel electrophoresis was run to determine if PCR products had any plant DNA bands. We visualized the bands on 1.5% agarose gels that contained 10 µL SYBR® Safe DNA gel stain per 50 mL of cooling agar. Gels were run at 120 V for about 45 minutes. Gels were removed and digitally photographed.

Non-ant Arthropod DNA Extraction and Amplification

Procedures for the predatory and honeydew insects samples were similar to the procedures used for the *S. invicta* samples. These samples were decontaminated for one 40 minute cycle in 3% bleach (Greenstone et al. 2012). Degradation was observed so a second wash in bleach solution was not attempted. Samples were stored at -20°C. The DNA was extracted using the same prodecure used for the *S. invicta* samples (Valles and Porter 2003). These samples were not ground, but there were no other deviations from the protocol. The DNA was amplified along side a positive control (PCR temperature protocol for UniPlant primers). We checked for bands of DNA using gel electrophoresis. The DNA concentration in the non-ant arthropod samples was measured by way of NanoDrop to determine whether there was enough DNA to warrant preparation for Sanger sequencing. All samples had similar concentrations (~ 500 ng/µL).

Preparation for Commercial Sanger Sequencing

We prepared all PCR products (*S. invicta*, predatory arthropods, and honeydew insects) for commercial lab Sanger testing (Genewiz). The PCR products were thawed and 10 µL were transferred into sterile 0.5 mL PCR tubes with 4 µL of ExoSAP-IT™ (Thermofisher Scientific) to enzymatically purify the products. PCR tubes were vortexed and centrifuged before placed in a thermocycler (PTC 100 Programmable Thermal Controller). The tubes were heated 15 minutes at 37°C, and then heated for 15 minutes at 80°C, and finally cooled at 4°C. We transferred 10 µL of each purified sample into strip 0.2 mL PCR tubes. We diluted forward UniPlant primer to be 5 µM. Samples were shipped overnight to Genewiz.

BLAST Searches

Once Sanger sequencing results were returned, each of the sequences was analyzed using the Basic Local Alignment Search Tool [BLAST] from the National Institutes of Health. BLAST searched for taxa having nucleotide sequences that most closely matched the nucleotides from the Sanger sequences. If matches were not detected under the program option for "highly similar sequences" (megablast), we ran a second search using the option for "somewhat similar sequences" (blastn). BLAST scores below a maximum score of 200 were likely to be less reliable and such samples were noted.

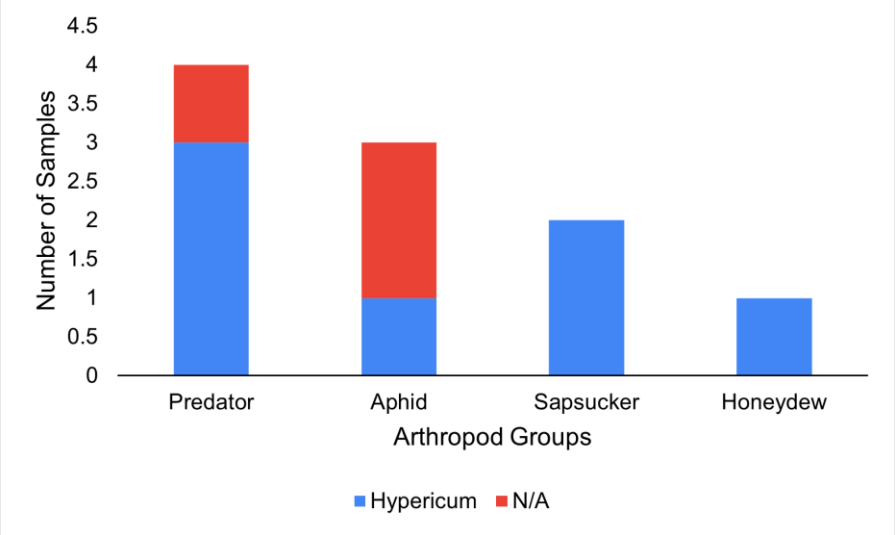
Results

Solenopsis invicta

Of the sixteen *S. invicta* samples, 43.75% matched with the ryegrass genus *Lolium*. The N/A group accounted for when a match could not be made. Matches to *Quercus, Aloidendron, Bonnetia,* and *Juglans* are likely less reliable as the maximum score was less than 200. One *S. invicta* sample matched with *Juglans hindsii* with a maximum score of 226. All matches to *Lolium* taxa had maximum scores well above 200.

Non-ant Arthropods

The predatory and honeydew insect samples predominantly matched with the *Hypericum* genus. Some samples were sequenced twice, which resulted in 12 Sanger results for our 7 predatory and honeydew insect samples. The N/A group accounted for whether a match could not be made or if the match was not for a plant genus. All but one match to the *Hypericum* taxa had a maximum score above 200, which would imply greater reliability. The majority (5 out of 9) of matches to *Hypericum* were above 400. Notably, the positive control samples (*Hypericum hypericoides)* all matched with max scores around 500.



Discussion

Understanding how invasive species like *Solenopsis invicta* are thriving beyond their native range is an important step when considering management of protected habitats. After understanding the main food sources, the next step would be to understand how *S. invicta* is accessing them. It is particularly important to understand how *S. invicta* could be accessing ryegrass, which is not found at the study sites. Results from this current study cannot reject nor support the hypothesis that the ryegrass DNA found in fire ants originates from honeydew.

The DNA from predatory and honeydew insects matched predominantly with the *Hypericum* genus. Some of the their DNA was resequenced due to the results being unclear or suspect. Notably, the majority (seven out of twelve sequences) matched with *Hypericum hypericoides*. *Hypericum hypericoides* is rather commonly found in longleaf pine savannas, and it cannot be ruled out that these non-ant arthopods fed on it. However, *H. hypericoides* was the species used for our positive plant DNA control. The non-ant arthropods were only washed once in diluted bleach rather than twice (as done for fire ants) due to the bleach solution causing loss of color and disintegration of the arthropods. Bleach can degrade DNA, which is why it was used for decontamination, so the consumed plant DNA was likely lost (Passi et al. 2012, Barthel et al. 2020). However, the NanoDrop results for the insect samples all had similar concentrations (~ 500 ng/µL). Such high similarity in concentrations is not usually seen across different samples. The chance of cross-contamination is therefore likely.

Across the sixteen *S. invicta* samples, 43.75% were matched with the ryegrass genus *Lolium*. This would support the results from previous DNA sequencing from *S. invicta* colonies in the study areas. Viable plant DNA could be found in the majority of these preserved specimens, despite initial collection in propylene glycol and permanent storage in ethanol. Only one of the samples lacked a BLAST match, and that sample had little liquid after the PCR process. Technical error, such as not adding master mix, could account for the lack of PCR product. This may also explain why one of the *S. invicta* samples matched with *H. hypercoides*, as both of these samples could not have gel electrophoresis run due to insufficient liquid. Our study information is important for any future testing based upon preserved specimens, and our study has evidence on the

integrity of the DNA within *S. invicta* after being preserved in 70% ethanol for about four years.

The next steps could be repeating the study after more honeydew insects have been sampled. Surface decontamination could potentially affect consumed plant DNA (Barthel et al. 2020). This does not seem to be an issue with the ant samples, but the difference in toughness of the exoskeletons between arthropods should be considered. The deterioration of the non-ant arthropods after a bleach wash would suggest that they may have softer exoskeletons than *S. invicta*. Another future step would be to examine ~~the~~ native ants for ingested plant DNA. *Solenopsis invicta* could be inhibiting the success of native ants by excluding access to resources such as honeydew insects (Wilder et al. 2013). Examining the ingested plant DNA in native ants found in these longleaf pine savannas can show whether the native ants are consuming the same plants as does *S. invicta*. This could indicate whether competition is present and give further incentive to do research into more targeted management practices.

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