Exploring the Correlation Between Leaf Miners and the Trees on Which They Were Found

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Abstract
The term 'leaf miner' is used for insects, such as beetles (*Coleoptera*), flies (*Diptera*), and moths (*Lepidoptera*) in the larvae stage. Leaf miners are a very small in size. Due to this, people never really see them, but they do see what they leave behind, which are tunnel-like paths. Leaf miners live up to their name. As a part of the phytophagous family, these insects live and feed off leaves. As they eat the leaf, they create tunnels (hence, leaf-miner). In New York City, there are many different ecosystems since there are so many animals and plants residing here [2]. We aimed to investigate the leaf miners within NYC's trees and find a correlation between the leaf miner species and the plant it was found on. The identification of these species could potentially help in the removal of them [1]. They could have also provided more information on whether the species of leaf miner is invasive to the plant/tree, which is especially important in an agricultural ecosystem, as pests are a major issue that farmers must confront when growing crops. Invasive species, just like any other species, are important to know about in order to understand how animals and plants interact with each other in an ecosystem.

Introduction
Leaf miners emerge from eggs laid by an insect. The term 'leaf miner' is used for insects, such as beetles (*Coleoptera*), flies (*Diptera*), and moths (*Lepidoptera*) in the larvae stage[4]. They can range from about ⅕" - ⅛" in size. As a part of the phytophagous family, these insects live and eat leaves. Of the many invasive species that exist in New York City [3, 4], leaf miners are species of insects that feed off the tissues of leaves, leaving behind tunnel-like paths, which destroys the plant over time. These tunnels eventually cause the leaves to become discolored and damaged. Though the tree or plant itself is not at risk, leaf miners can give insight on what type of pest resides in the area. This information could help reduce the pest and increase tree's/plant's leaf productivity. The identification of these species could potentially help in the removal of them because leaf miners eventually turn into grown insects that invade plants and trees [1]. Sometimes it is difficult to identify exactly which leaf miner resides in a certain plant based off the tracks left behind. Typically, the person studying the leaf miners would place the leaf in a bag and wait for the miner to emerge from the larvae left in the tracks in the leaf, but the miner sometimes dies before it can or has already left the leaf. In this case, DNA barcoding may be a better way to go about identifying a leaf miner from a plant as the sequencing could identify it [1]. One group has previously barcoded leaf miners using the leaf mines with partial success; they were able to identify the leaf miner using DNA barcoding 6-33% of the time, depending on how recently the leaf miner had been in the leaf [1].

Materials & Methods:
Gather materials: Gloves, leaves, Ziploc bags, freezer ,tweezers or scissors. Choose a location where leaves will be collected. (For our experiment we chose the sidewalks of Norwood, Bronx and the backyard of a group member's home in the East Village of Manhattan). When we collect the leaves, we intend to use the sample collection database from the Cold Spring Harbor Laboratory DNA Learning Center website [7]. When collecting the leaves gloves should be worn. Each leaf collected would be placed in a separate Ziploc Bag. Ziploc bags should then be labeled with where the leaves were found. The leaves are then to be stored in a freezer.

Each portion of each sample will be placed in separate tubes and labeled accordingly. After the samples are placed inside the tubes, 300 µL of lysis solution will be added to each tube. This is a solution that dissolves membranes, including the those of the nucleus, mitochondria, and chloroplasts. Using a clean plastic pestle for every sample to avoid contamination, grind the tissue firmly for about 2 minutes. This is necessary in order to break up cell walls and other tough material in the sample. After grinding the tissue, there should be a liquid with some tissue remaining. Once this is done, the tube is to be placed in a water bath for 10 minutes at 65° C. The tubes should be placed in a microcentrifuge and everything in the machine needs to be balanced out. The samples are to be centrifuged for one minute at maximum speed to separate the substances based on their density. This will form a liquid called a supernatant, which contains the DNA of what we are identifying and separates it from the plant debris. Transfer 150 µL of the supernatant to a new tube, making sure to not disturb the contents by shaking the tube. Add 3 µL of silica resin, a DNA binding matrix, to the tube. When the silica resin meets the lysis solution from before, it will bind to nucleic acids. Close the tube and flick the tube from the side to mix it. After this is done, the tubes are to be incubated again for five minutes at 57° C. The tubes are then to be placed in microcentrifuge again for thirty seconds. The process of centrifugation should turn the silica resin into a pellet, which is bound to the nucleic acid. This pellet should be at the bottom of the tube. After that, use the micropipette with a new tip for each sample to remove the liquid. Do not touch the white silica pellet when doing this step. Add 500 µL of the cold wash buffer and mix well. The buffer should wash the sample while the nucleic acids remain bound to the solid silica. Once the sample is put through the wash buffer, the samples should be placed in the microcentrifuge for thirty seconds again. After that, the micropipette should remove the liquid. The silica pellet should not be touched by the pipette. Once again, add 500 µL of ice-cold wash buffer to the pellet. The samples should be washed a second time and placed again in a microcentrifuge for thirty seconds. After that, pour out the supernatant. For 15 seconds, the tube should be spun in order to collect any remaining supernatant. After the spin, there may be about 50 µL of the supernatant remaining. Using a micropipette with a new tip, remove the liquid (supernatant), making sure the pellet at the bottom is not shaken. Add 100 µL of TE buffer, mix well and incubate once more at 57° C for 5 minutes. Centrifuge the tubes for 30 seconds at maximum speed to pellet the silica resin. Get new tubes and label them again to transfer the current samples; transfer 50 µL of the supernatant. Be sure to not disturb the pellet and discard the old tubes. The DNA samples should be stored in the TE buffer.

Materials & Methods (Part 2):
This buffer provides an environment that allows DNA and RNA nucleases to be less active. Samples are stored on ice or at -20° C until the next step [5].
Since we are identifying two different species, we will need to prepare two different solutions to place the samples in. One solution, to identify the leaf miner, will have a CO1 primer, while the other, to identify the plant, will have an RBCL primer. We will acquire PCR tubes containing PCR Beads consisting of Taq polymerase, nucleotides, and buffer. Make sure to label each tube using an identification number. We will then add 23 µL primer solution in each tube using a micropipette. For one minute, let the bead dissolve. It creates a mixture of components needed for a PCR reaction. After this, use a new micropipette tip and add 2 µL of the DNA (from the first part) into the PCR tube containing the primer and polymerase mixture. Once this is done, we will be ready to begin thermal cycling.

Thermal cycling is able to amplify DNA segments. We will place our PCR tubes into the machine and program it using specific protocols. Since we plan to use plant and invertebrate DNA, we will use both rbcL and COl primer protocols. For the plant DNA, the initial temperature should be 94°C and it will run for one minute. Then the plant samples will endure 35 cycles of following: 94°C for 15 seconds (denaturing DNA), 54°C for 15 seconds (annealing DNA), and 72°C for 30 seconds (extending DNA). For the invertebrate DNA, the initial temperature should be 94°C and will run for one minute. Then the invertebrate samples will endure 35 cycles of following: 95°C for 30 seconds (denaturing DNA), 50°C for 30 seconds (annealing DNA), and 72°C for 45 seconds (extending DNA). Once these processes are complete, we will store the amplified DNA on ice or -20°C until we are ready to continue the following step [5]

The next step after thermal cycling is gel electrophoresis. Gel electrophoresis is used to separate DNA fragments according to their size. To do this; the first would be to cover the ends of the gel-casting tray with tape and insert a well-forming comb. The 2% agarose solution containg GelGreen DNA stain should be poured into the tray and should stop being poured at one-third the height of the comb. Do not pour a thick overflowing gel onto the tray, if this happens it will make it much more difficult to see the DNA. Wait twenty minutes for the agarose gel to turn into a solid. Put the gel into the electrophoresis chamber and pour in enough TBE buffer that it covers the surface of the gel. Too much buffer would make the process run much longer. Take the comb out and add a TBE buffer to cover the gel and fill in the wells. It creates a smooth buffer surface. After this, use a micropipette with a new tip and load 5µl of 100bp ladder into the first well in each row and load 5µl of each sample into the other wells. And when doing this make sure the pipette doesn't go through the gel and touch the bottom. Put the remaining 20 µL of the PCR product on ice or at -20° C until the samples are submitted for sequencing. Make the gel run for thirty minutes at 130V. During this time separation has occurred because the cresol red should have removed at least 50 mm from the well. Then using UV or LED transillumination the gel can be viewed. We will be sure to take pictures of the system and results at the end [5]. PCR products will be sequenced. And then once we have acquired the sequence data from the PCR, we plan to use the DNA Subway database. This database can connect bioinformatics tools and other databases to create gene models, analyze DNA barcodes and work with phylogenetic trees.

Discussion
We had planned to collect all the necessary data and investigate further into the specific leaf miner and plant in order to find an explanation of why the leaf miner chose the plant it was found on. We also planned to research the certain (or multiple) leaf miner(s) based on the location of the plants (the actual city and state), as well as compare our results to the information that has already been found and recorded in databases about leaf miners in NYC to see if we have found any new species that haven't yet been discovered or studied. In addition, we planned to calculate a biodiversity index of each plant species that we discover by using the AMNH Biodiversity Index website [6]. To do so, we would have had to divide the total number of plants in the area (about 3x3 meters squared area) by the total number of individual plants we have observed.

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