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Study Methods

Bore wells

1. Within the 16 hectare ForestGeo Plot, 6 sites of different elevations were selected as the locations for 6 soil moisture probes and the future 6 bore wells. The sites were chosen for their gradients of elevation and moisture content. Sites 1E and 4C are at some of the highest elevations in the plot and assumedly the driest. Sites 2D and 1E are of average elevation and moisture. Sites 3A and 6B are at much lower elevation and are besides a lowland creek, making them the wettest locations.
2. These probes measure the volumetric water content, salinity, and temperature within the soil column. The probes reach 120 cm into the soil and take measurements at each increasing 10 cm in depth every 15 minutes. These soil moisture probes have been in operation for \_\_days from \_\_\_dates. The probes all connect via electrical wire to a central hub on the Plot.
3. Near the 6 soil posture probes, 6 bore wells were dug using a soil auger until no more soil could be extracted, meaning the soil was too wet to be compacted into the auger. The purpose of the bore wells was to measure the daily and long-term fluctuations of water within the water table.
4. At successional measured depths, (every 10 cm until 250 cm, then every 20 cm from 260 cm on), the auger was removed. The soil was removed from the canister and placed in a labeled gallon-size, freezer-safe plastic bag.
5. A thin slice of the soil was cut from the center of the sample, and placed in a labeled glass vial. The slices were cut from the center in the attempt to minimize contact with the sample along the sides of the borehole and avoid contamination. These slices would be used to test for oxygen isotopes throughout the soil column. Once the vial was capped (it ‘clicks’ when fully sealed), it was placed in a portable canister of liquid argon. The argon freezes the soil and the water inside of it to preserve the sample. The vial caps were sealed with parafilm and stored at -80C in the lab. In campaigns August 2018 onwards, vials were sealed with parafilm in the field and stored in an ice-cooler, then transferred to -80C in the lab (within a couple of hours of collection).
6. The process of auguring and sampling continued until the augur could bring up no more soil due to excessive water, rock barrier, or the accessible depth exceeded the maximum length of the auger (460 cm).
7. In place of the augur, an Atlantic Screen Inc. canister longer than the depth of the hole was placed in the borehole. The canister’s bottommost section has a meter (??) of ribbed slits and a conical closed end. The slits allow water to enter and exit the canister as the water table fluctuates. In order to reach the surface level, additional pipe pieces were screwed onto the slatted pipe. These pieces were not slatted, so as to prevent excess water from entering the canister. Pipes were added until the canister reached at least 30 cm above the top soil. The end of the pipe was covered by a screw top lid.
8. Fine grain sand (clean play sand) was added around the canister to fill the rest of the borehole made by the auger. This served the dual purpose of holding the canister steady and filtering out larger particles from entering the slats and giving false water table readings.
9. The top of the hole was secured by bentonite clay. The clay seals the canister in position and prevents water from flowing directly from the surface, through the sand, and into the canister.
10. A Diver unit (D1-801, vanEssen Instruments) was lowered via nylon string into the canister just until it was fully submerged into the water in the canister. The Diver recorded water temperature, barometric pressure, and water pressure data within the column every 5 minutes. Each borehole received its own programmed Diver unit. An additional Diver was placed in a protective box at the central hub for the soil moisture probes. This probe measured atmospheric barometric pressure and ambient temperature to serve as a comparison to the canister probes.
11. Back in the lab, the gallon-sized bags of soil were stored at 4℃ to preserve the roots and soil moisture.
12. An approximately 100 g piece of the soil samples from all of the depths were added to a labeled and tared paper sandwich bag. The bags were weighed to account for the initial weight of the soil and the water inside. The bags were then heated in an oven at 70℃ for 48 hours. This process removed all of the moisture from the soil subsamples until they were hard and rock-like. The dry samples were weighed and the difference between the weights was calculated as the water lost in the oven.
13. The remaining soil in the gallon-sized bags was weighed and returned to the 4℃ refrigerator until we were ready to sieve it for roots.

Sieving for Roots

1. Each sample was sieved using a series of 4 decreasing in size sieves [U.S.A Standard Testing Sieves by the Fisher Scientific Company, sizes No. 3 ½ (5.6 mm), No. 10 (2 mm), No. 18 (1 mm), and a solid bottom layer]. While sieving, roots roughly larger than 1 cm in length and 1 mm in width were collected and placed into labeled and tared paper envelopes.
2. Once all of the roots were collected, the samples were placed in a freezer at -80℃. The deep freeze was used to preserve the genetic integrity of the roots for DNA barcoding analysis.

Photography

1. The roots from each sample were photographed in a homemade photo booth using a sheet, some sheets of poster board, and a PVC pipe copy stand. They were photographed on a laminated 1 cm x 1 cm grid sheet with spaces for the plot, depth, number of roots, and the date of the photo. While the roots were awaiting photography, they were kept in a Styrofoam cooler filled with ice to keep them near frozen temperatures.
2. After being photographed, the roots were weighed to hundredths of grams.
3. The pictures were adjusted on the computer to improve the lighting.

Morphology

1. Using the photographs, morphology was roughly assessed based on root thickness, color, branching patterns, and texture (dry/fleshy). The 19 different morphologies were given color names (as shown below).

Table of Morphology Classes Based On Thickness, Color, Branding, and Texture

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Number | Color Name | Root Thickness | Root Color | Branching Pattern | Texture |
| 1 | Lilac | 3 | Dark | 3 | Dry |
| 2 | Light Yellow | 1 | Dark | 1 – 3 | Dry |
| 3 | Pink | 1 – 3 | Dark | 1 | Dry |
| 4 | Light Green | 2 or 3 | Dark | 4 | Dry |
| 5 | Light Gray | 3 – 4 | Light | 4 | Dry |
| 6 | White | 1 – 3 | Light | 5 | Dry |
| 7 | Blue | 2 or 3 | Light | 3 | Dry |
| 8 | Taupe | 2 – 4 | Tan | 3 | Fleshy |
| 9 | Asparagus | 4 or 5 | Gray | 2 or 3 | Fleshy |
| 10 | Dark Orange | 3 | Reddish | 3 | Dry/Fleshy |
| 11 | Dark Gray | 2 | Yellowish | 1 | Very Dry |
| 12 | Red | 4 | Black | 1 – 3 | Dry |
| 13 | Indigo | 5 | Dark | 1 – 5 | Dry |
| 14 | Light Orange | 3 or 4 (2 -3 cm length) | Dark | 1 | Dry/Fleshy |
| 15 | Green | 4 (2 cm length) | Dark | 1 | Dry |
| 16 | Neon Green | 4 (2 cm length) | Light | 1 | Dry |
| 17 | Magenta | Cambium stripped (Any) | Any | Any | Any |
| 18 | Canary | 4 | Dark | 3 | Cracked |
| 19 | Brown | 4 | Dark | Knobbed | Dry |
| \*Thickness based on scale of 1 – 5: 1 being very thin (1mm), 5 being very thick (<1cm) | | | | | |
| \*Color based on a light/dark scale unless a particular color hue was present | | | | | |
| \*Branching based on a scale of 1 – 5: 1 being no branches, 5 being abundant branches | | | | | |
| \*A green colored value indicates the critical trait of the morphology class. Lilac is the baseline. | | | | | |

1. For each photograph, all roots approximately greater than 3 cm x 1 mm, or 2 cm x 5 mm, were sorted into a morphology class.
2. From these selected roots, 96 samples were chosen for genetic identification.
   1. First and foremost, the depths of 10, 50, 100, 150, 200, 250, 300, 400, 460 (when appropriate) were chosen as starting points to represent the full range of the soil samples collected.
   2. At each of these points, one root of every morphology class present in the sample was selected for genetic analysis. If more than one root existed in a class, the largest of the roots was chosen as it would contain more material with which a genetic sample could be taken. This was done at all the appropriate 50 cm apart points.
   3. The rest of the samples were analyzed to find roots of every morphology found in that bore well, in hopes of capturing more diversity. These extra roots, were likewise chosen based on size compared to other roots of the same morphology class.

|  |  |  |  |
| --- | --- | --- | --- |
| Bore Well | Colors Present in the All Samples for Each Bore Well | Morphology Classes Present in the All Samples for Each Bore Well | Samples Selected for Genetic Analysis |
| 1E | 15 | Lilac, Light Green, Light Yellow, Light Orange, Taupe, Asparagus, Red, Indigo, Blue, Pink, Brown, Magenta, Neon Green, Green, Dark Orange | 26 |
| 2D | 12 | Lilac, Light Green, Light Yellow, Light Orange, Taupe, Asparagus, Red, Indigo, Blue, Pink, Magenta, Dark Gray | 22 |
| 4C | 12 | Lilac, Light Green, Light Yellow, Light Orange, Taupe, Asparagus, Red, Blue, Pink, Magenta, Light Gray, Dark Gray | 26 |
| 6B | 13 | Lilac, Light Green, Light Yellow, Light Orange, Taupe, Red, Indigo, Blue, Pink, Magenta, White, Light Gray, Canary | 22 |

1. Once the roots were chosen and marked in picture-form, the actual roots were found from the sample bags, and placed into individual, labeled plastic bags. They were then returned to the freezer.

Genetic Analysis

1. 50 ml of 2x CTAB buffer was mixed using 5 ml of 8.0 pH Tris HCl, 14 ml of NaCl, 4 ml of EDTA, 1 g of CTAB, and 27 ml of water. The buffer (0.2 ml) was then added to an empty 96 well plate.
2. The 96 roots were then grinded into powder using a mortar and pestle. In between roots, the mortar and pestle, spatulas, tweezers, and scissors were washed with soap and water, rinsed, wiped out with a clean paper towel, sprayed with ethanol, and left to air dry. A rotation of mortar and pestles was used.
3. The samples were added with the CTAB into the appropriate wells. This was done on ice, to keep the roots in cold temperatures.
4. Once the grinding process was finished, the plate was allowed to thaw. Once no longer completely frozen, a single metal ball was added to each well. The metal balls are used to liquefy the samples at high speed so that the DNA can be extracted.
5. The TissueLyser II (Qiagen) was used to shake the samples so that DNA can be extracted from the broken down larger particles.
   1. 20.0 Hz for 1 minute
   2. Switch sides, 20.0 Hz for 1 minute
   3. Switch sides, 20.0 Hz for 1 minute
6. Added 300 μL of RLT buffer to each well to lyse the cells and tissues to allow for extraction.
7. The well plate was then centrifuged for 5 minutes at 2000 x g to bring all of the larger fragments to the bottom of the plate.

DNA Extraction

1. DNA was extracted using the BioSprint 96. This piece of equipment uses a series of chemical baths and shaking processes to isolate DNA molecules. MagAttract Suspension G pellets are used to pick out, hold, and transport electrically charged DNA molecules through each of the 6 slots.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Slot | Name | Plate/Block | Solution in Well | Volume (μL) |
| 7 | Rod Cover | 96 Well Plate | 96 Rod Cover | - |
| 6 | Elution | 96 Well Plate | Distilled Water | 200 |
| 5 | Wash 4 | S-Block | Distilled Water w/ 0.02% Tween 20 | 500 |
| 4 | Wash 3 | S-Block | Ethanol (100%) | 500 |
| 3 | Wash 2 | S-Block | Ethanol (100)% | 500 |
| 2 | Wash 1 | S-Block | Buffer RPW | 500 |
| 1 | Lysate | S-Block | Lysate | 420 |

* 1. This was completed using the protocol detailed in the BioSprint DNA Plant Handbook (Qiagen, March 2005) using the BioSprint 96.

Summary Thus Far: Root samples (n = 96) were turned into powder with a mortar and pestle, given a lysis buffer, broken into sediment in a centrifuge, and added to a 96 well S-Block. The DNA was bound to the MagAttract Suspension G, washed with alcohol-containing buffers and ethanol, rapid rinsed, and eluted in water to produce pure DNA.

PCR

1. 20 μL of each sample where added to a working plate. The working plate allowed us to frequently use and refrigerate a small sample of the DNA material. The remaining sample was placed in the freezer incase more samples were needed.
2. A 14 unit test sample and then the official PCR were done. The official PCR was done with two 96 well plates, one with rbcL-1aF and rbcL-1aR and the other with psbA and trnH.
3. The PCR solution contained:
   1. 8 μL of water \* number of samples (n)
   2. 1.25 μL of the desired primers diluted 1:10 with distilled water \*n
   3. 12.5 μL of 2.0 x Taq RED Master Mix (Apex BioResearch Products) \* n
   4. 2 μL of DNA material from each sample
4. The PCR was done in a C1000 Touch Thermal Cycler by Bio-Rad. The settings were:
   1. (95℃ for 3 min, 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 1 min) \* 33 cycles, and then 72℃ for 10 min. Infinite hold was set for 12℃.
5. An electrophoresis gel test was ran with the 14 test samples and a 16 sample subset of the 192. The test used 3.0 μL of sample and the official used 5.0 μL of sample. The tests ran for 15 minutes and 20 minutes respectively.
6. A UVP Multi-Doctt Digital Imaging System was used to photograph the gels under UVB light.

ExoSAP

1. ExoSAP was added to the PCR product. ExoSAP is a reagent that cleans up the PCR product by removing excess primers and nucleotides.
2. Each sample received 2 μL of ExoSAP per 8 μL (1:4) of autoclaved (or sterilized) water.
3. The ExoSAP step was completed in the Thermal Cycler:
   1. 37℃ for 30 min, 80℃ for 20 min, 4℃ for infinite hold.
4. A gel was likewise ran for the ExoSAP product, with 3 μL of 12 samples for 20 minutes.

Sequencing

1. The sequencing step prepares the DNA to be analyzed by isolating samples for forward and reverse primers. So the two samples for the two primer pairs, have now been divided into four total well plates, each with one primer.
2. The sequencing solution contained:
   1. 2 μL of ExoSAP product (DNA) \* n
   2. 4.5 μL of autoclaved water \* n
   3. 0.5 μL of single primer \* n
   4. 2 μL of Big Dye Buffer \* n
   5. 1 μL of Big Dye \* n
3. Sequencing was completed in the Thermal Cycler:
   1. (96℃ for 1 min, 96℃ for 30 sec, 50℃ for 10 sec, 60℃ for 4 min) \* 29 cycles, 12℃ for infinite hold.

Filtration

1. Overnight, 1 unit of Sephedex was allowed to dissolve in 300 μL of water in a 96 well filtration plate.
2. The next morning, a closed 96-well plate was placed under the filter plate.
3. The two plates were put into the centrifuge at 910 \* g for 5 minutes.
4. The water in the closed plate was removed. The filtration plate was placed on top of the final well plate (that would be sent off for analysis). (The following step much be completed within 5 minutes so that the Sephedex would not dry out).
5. The sequencing product was pipetted on top of the Sephedex and returned to the centrifuge for a round at 910 \* g for 5 minutes and another at 1000 \* g for 2 minutes. This allowed the sequencing product to travel through the Sephedex and remove any remaining unwanted products.

Drying

1. The newly filtered plates were returned to the Thermal Cycler at 95℃ for 25 minutes. This time, the lid was left open so that excess liquid could escape by evaporation. The drying process enables the DNA product to be stored for the relative long-term and to be transported in a stable state.
2. The final four DNA product plates were stored the freezer to await transport to the Smithsonian Institution’s National Museum of Natural History.

Oxygen Isotope Analysis

1. In the lab, the vials containing the slices of soil were removed from the liquid argon container and the caps were wrapped tightly in parafilm. Then, they were placed in the -80℃ freezer to await testing.
   1. 16O is lighter and evaporates more quickly, meaning it is more common in the atmosphere. Thus, it is less common in the upper layers of the soil. However, as it is lighter, it sinks less.
   2. 18O is heavier and precipitates more quickly, meaning it is more common in rain. It is heavier and sinks deeper into the water column. However, as the 16O is taken out of the upper layers by evaporation and transpiration, the 18O fills that gap.

Dates of the Above Steps of the Process

|  |  |  |
| --- | --- | --- |
| Borehole | Step | Date(s) |
| 2D | Dug | 5/1/18, 5/2/18 |
|  | Pipes Installed | 5/4/18 |
|  | Diver Installed | 5/17/18 |
|  | Gravimetry | 5/1, 5/3/18 |
|  | Sieving | 5/4/18 |
|  | Oven Drying | 5/22-24/18 |
|  | Weighed  Root Photography/Weighing  Diver Removed to Reset  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample | 5/24/18  6/13/18  6/22/18  7/27/18  7/27/18 |
| 6B | Dug | 5/23/18 |
|  | Pipes Installed | 5/23/18 |
|  | Diver Installed | 5/25/18 |
|  | Gravimetry | 5/24/18 |
|  | Sieving | 5/25/18 |
|  | Oven Drying | 5/24-26/18 |
|  | Weighed  Root Photography/Weighing | 5/29/18  6/13/18 |
|  | Morphology Assessed  Pulled for Genetics  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample  “Wet” Gravimetry | 7/5/18  7/5/18  7/25/18  7/27/18  7/25/18 |
| 1E | Dug | 5/29/18 |
|  | Pipes Installed | 5/29/18 |
|  | Diver Installed | 5/30/18 |
|  | Gravimetry | 5/29/18 |
|  | Sieving | 5/31/18 |
|  | Oven Drying | 5/29-31/18 |
|  | Weighed  Root Photography/Weighing | 5/31/18  6/12/18 |
|  | Morphology Assessed  Pulled for Genetics  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample | 7/5/18  7/5/18  7/27/18  7/27/18 |
| 4C | Dug | 5/30/18 |
|  | Pipes Installed | 5/30/18 |
|  | Diver Installed | 5/30/18 |
|  | Gravimetry | 5/30/18 |
|  | Sieving | 6/1/18 |
|  | Oven Drying | 5/30-6/1/18 |
|  | Weighed  Root Photography/Weighing | 6/1/18  6/11/18 |
|  | Morphology Assessed  Pulled for Genetics  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample | 7/5/18  7/5/18  7/27/18  7/27/18 |
| 5F | Dug | 6/26/18 |
|  | Pipes Installed | 6/26/18 |
|  | Diver Installed | 6/29/18 |
|  | Gravimetry | 6/26/18 |
|  | Sieving | 6/28/18 |
|  | Oven Drying | 6/26-28/18 |
|  | Weighed  Root Photography/Weighing  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample | 7/2/18  6/29/18, 7/9/18  7/27/18  7/27/18 |
| 3A | Dug | 6/26/18 |
|  | Pipes Installed | 6/26/18 |
|  | Diver Installed | 6/29/18 |
|  | Gravimetry | 6/26/18 |
|  | Sieving | 6/26-28/18 |
|  | Oven Drying | 6/26-28/18 |
|  | Weighed  Root Photography/Weighing  Dug “Extreme Wet” Hole  Collected “Wet” Water Sample  “Wet” Gravimetry | 7/2/18  7/20/18  7/25/18  7/25/18  7/27/18 |

Dates that I Downloaded the Diver Data:

|  |  |
| --- | --- |
| Date | Divers Checked |
| 6/6/18 | 6B, 2D, 1E, 4C, BH |
| 6/18/18 | 6B, 2D, 1E, 4C, BH |
| 6/22/18 | Removed 2D to Reset/Later Returned |
| 7/19/18 | All 7 Divers |
| 7/30/18 | All 7 Divers |

**Genetics Dates**

Genetics grinding was done on the 6th, 7th, and 9th of 7/18 for bore wells 1E, 2D, 4C, and 6B.

TissueLyser, BioSprint 96, Test PCR, Test PCR Gel done on 7/10/2018.

Official PCR, Official PCR Gel Test, Exosap, Sephedex Prep, Exosap Test Gel, and Sequencing Reactions were done on 7/11/2018.

Filtration through Sephedex and Drying were done on 7/12/2018.

Samples were taken to NMNH on 7/17/2018

96-Well Plate Locations Chart

