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Hydraulic Traits as Determinants of Epiphyte Distribution in Mid-Elevation Rainforest in Puerto Rico.

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2015 Student Summer Scholarship Report

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Abstract

Ferns are the dominant epiphytic flora of the neotropical forests of Puerto Rico. The goal of this study is to identify anatomical features associated with species distribution among epiphytic ferns inhabiting the first three meters of a tree trunk, targeting eighteen of the most common species identified from a 2012 survey at El Yunque National Park, Puerto Rico. We collected an adult specimen of each species during our 2015 field work at El Verde Station, Luquillo LTER. At the field station, we dissected each specimen into leaf, stipe, and rhizome sections, photographed the largest leaf, measured the major dimensions (length and width) of each organ, and preserved them for transport to GVSU. To add statistical power to the 2012 distribution data, we also conducted a "blast" survey of the occurrence of the target (18) species on the first, second, or third meter of 300 trees. At GVSU, the preserved organ sections were macerated to separate conductive cells, permanent slides produced, and up to ten replicates of each cell type found were photographed for measurement of key hydraulic traits (e.g., conduit length and width, pit area, pit aperture, and pit frequency) that we suspect determine occurrence along the vertical moisture and exposure gradient on a tree. At the time of this report all species have been macerated at least once and approximately 60% of the 540 (anticipated) cell (including both type and replicate) have been successfully macerated, mounted on slides, and photographed. Some species have presented unexpected problems such as obstructive carbohydrate-protein clouding of slides and dehydration of the mounting medium (which is a product flaw). These problems were solved during Fall 2015 and we expect to complete the remainder of data collection during Winter 2015.

Introduction

Species distribution is a major focus in community ecology as it is a key element to understanding patterns of biodiversity. Biotic and abiotic factors are highly variable and ecologists attempt to tease apart the relative contributions of selection and stochastic ("Random") processes (Alonso 2006, Bell 2000, Hubbell 2001, 2005, MacArthur and Levins 1967, Silvertown 2004) as determinates of species distribution and community diversity. As modern day ecologists we recognize niche (selection) and neutrality as two ends of the same spectrum (Gravel *et al.* 2006; Vellend 2010). For example, a combination of niche and neutral models accurately predicted 94% of variation in the abundances of 30 plant species based on eight traits at 12 study sites (Shipley *et al.* 2006). The significance of the study is a realistic connection between community ecology, functional ecology, and biogeography on a measurable trait basis; however as the author states himself, the model needs more testing. Other models have proposed individual traits to reflect patterns of speciation and thus community diversity based on traits associated with dispersal (Swenson and Enquist 2009).

Epiphytic communities (plants that grow on, but generally do not harm trees) are particularly useful systems for studying the processes responsible for species distribution and community diversity. Trees are literally islands of suitable habitat for epiphytes that vary in size, age, (bark) texture, and isolation from other "islands" facilitating study of processes affecting dispersal. Likewise, a tree creates a complex vertical gradient that favors species with varying attributes in terms of growth rate versus drought tolerance. The base of a tree is distinguished by cool, low-illumination and wind, and moist conditions and increasingly warm, high-illumination and wind, and dry conditions with increasing trunk elevation. Because tree trunks retain only minute amounts of moisture compared to soil, the ability to efficiently capture, distribute, and

retain water is likely to be a primary determinant of epiphytic species distributions. Hydraulic traits are highly variable among all vascular plants as they have been evolving in response to numerous selective agents in different plant lineages since the origin of tracheophytes. In other words, there are a variety of different trait combinations that can – potentially – collectively can achieve the same abilities. Water conduction in plants is performed by tracheary cells that can be classified as tracheids, fiber tracheids, or vessel tube elements. Tracheids and fiber tracheids are thin cells that possess tapered ends forcing water to travel an inefficient pathway; however, their inefficiency is counter-balanced by a reduced likelihood of embolism (Brodersen *et al.* 2014; Pittermann *et al.* 2013; Vogel 2012). Embolisms occur when the water potential decreases to a threshold point in which evaporative pull exceeds the supply of water from roots, resulting in air seeding that breaks the water column and thus flow (Tyree and Sperry 1989). In contrast, vessel tube elements are typically wider and possess comparatively open end walls that facilitate more efficient, linear water transport, but are more likely to suffer embolisms during drought (Bailey 1953; Milburn 1979; Carlquist 1975). Increased conduit width allows for greater water conductance and in general increased vulnerability to embolisms (Woodhouse and Nobel, 1982;l Brodersen *et al.* 2014). Hydraulic efficiency measured by conduit area resistance to water conductance suggests increasing conduit width and length decreases resistance (Pittermann *et al.,* 2006). Likewise, large pit areas (holes on the sides of tracheary cells that facilitate lateral flow) allow for greater water conductance but increase the likelihood of embolism by lowering air seeding pressures (Brodersen *et al.,* 2014). At even an even higher microscopic scale, pit water conductance is also determined by the thickness and porosity of the pit membrane (Brodersen *et al.,* 2014; Pittermann *et al.,* 2013) with thicker membranes decreasing both flow and the ability to trap and break up embolisms. We predict then, that epiphytes with tracheary traits promoting

efficient water transport will be competitively dominant (i.e., be photosynthetically more active) near the cool, shady, moist base of a tree and species with traits promoting slow water transport (and thus low risk of embolism) will increasingly dominate as one travels vertically into warmer, brighter, drier elevations.

Stomates are a key feature to the control of water relations within a plant. Stomates are epidermal features composed of two guard cells that act as "mouths" that open and close to control transpiration (water loss) and carbon dioxide intake. Stomatal control minimizes the risk of embolism by closure during high transpiration stress (Broodrib and Jordan, 2008). In a recent study fern stomatal density and water conductance have shown to positively correlate with photosynthetic capacity (Zhang *et al.,* 2014). However, the ability of ferns to optimally manipulate their stomates is limited compared to seed plants. Fern stomates close passively in response to low internal water conditions, whereas the stomates of seed plants can open incrementally under water stress to alleviate oxidative stress associated high-oxygen levels produced from photosynthesis (Broodrib and Jordan, 2008). Together tracheary and stomatal traits control much of a plant's internal water balance. Although stomatal traits are not a focus of our S3 study, they are highly-relevant and are being pursued by a lab (GVSU undergraduate) collaborator.

The purpose of our study is to identify tracheary traits and trait combinations that are associated with vertical position within the basal three meters of a trunk. As stated above, we predict tracheary traits promoting water retention and minimizing the risk of embolism will be associated with higher positions on tree trunks.

Methods

Sample collection

The field component of the study is at mid elevation (500-650 meters) in the El Yunque National Forest, Puerto Rico. Epiphytic ferns were chosen as they dominate the epiphytic flora at the site. Eighteen target species were identified based on their frequency of occurrence in a previous 2012 survey of the epiphytic communities of 88 trees occurring in eight archipelagos composed of eleven trees.

In May 2015 we returned to Puerto Rico with two tasks: (1) conduct a blast survey that would strengthen the statistical power of the field distribution data and (2) (re)collect specimens for anatomical (tracheary cell) study at GVSU.

The blast survey was conducted by surveying three-hundred trees for occurrence of the eighteen target species on the first, second, or third meter of elevation on the tree trunk. To do so, we established a series of parallel, informal transects along an elevation gradient and identified a "center tree" at irregular intervals. The closest tree with a diameter-breast-height exceeding 10 cm in each of four compass quadrants was identified for survey, thus establishing a five-tree archipelago.

Specimen (re)collection was conducted at an area separate from the blast survey. Mature individuals were collected and separated into rhizome, stipe, leaf sections. Each section was photographed, measured for length and width dimensions suing calipers and tape measures, and then preserved in WardSafe for transport to GVSU.

Slide Preparation

A stock maceration (middle lamella digestion) solution was using a modified Grifford (1963) method. The solution was comprised of 5 parts glacial acetic acid, 4 parts DI water, 1 part 30% hydrogen peroxide. Each organ of the 18 species was macerated separately in a 15ml centrifuge tube with 10ml of maceration solution and placed in an oven at 56^0C. Samples were

left to soak until translucent and "soft" (i.e., digest the middle lamella that binds adjacent cells together) with maceration fluid changed every 3 to 4 days. Some samples, heavy in sclerenchyma, took much longer to soften. Once softened each sample was rinsed with DI water three times and placed back into centrifuge tube with 5ml of DI water. Next a couple drops of a 1:1 1% safranin and ethanol solution was added so that watercolor was blood red and left to sit for 24 hours. A Kendal SI-UC6250 jewelry sonicator was used to separate macerated cells by submerging each sample and sonicating. The sonication time necessary to fully macerate a sample varied, but was generally 2-3 minutes. Samples were then diluted with DI water and allowed to settle to the bottom. The diluted water was then pipetted out and then diluted with DI water again. This process was repeated until the water in the centrifuge tube was as clear as possible leaving only the red macerated material. After the first month one drop of a 10% phenol solution was added to prevent fungal and bacterial growth.

Permanent slides were produced for each organ by applying 2-4 drops of the resuspended maceration solution on a glass slide. The applied cells were diluted with DI water and spread across the slide using a plastic coverslip. Each glass slide was placed on a slide warmer at 45^0C to dry. Once dry, Sigma-Aldrich Clarion mounting medium was spread over the macerated cells, covered with a glass coverslip, and allowed to dry for at least 24 hours.

Microscopy

Slides of macerated tracheary cells were observed using a Nikon Eclipse DS-Fi2 coupled with NIS-Elements software. Our ultimate goal is to capture ten different examples (replicates) of each cell type each species possesses; the number of cell types will likely vary among species. Each cell has or will be photographed at various levels of magnification to capture and measure whole cell features (e.g., length and width) or subcelluar features (e.g., end walls and pits).

As macerating the entire tissue will yield tracheary elements, for a rhizome of leaf you can wait until the vascular skeleton (veins) is present and place in its own centrifuge tube with maceration fluid. For rhizomes you just need to pick away at the parenchyma before it is completely softened up. This way the new centrifuge tube is going to have very large concentrations of vascular tissue comparatively.

When sonicating do not worry about the ball of cells that commonly forms right after the material breaks apart, you can separate it later with light pipetting. I found this phenomenon to be very common, especially in thick tissues with a lot of parenchyma.

Notes on Technique

A simple reporting of the methods does not appropriately convey the nearly endless fine-tuning involved. Methods that successfully work on one species often do not work for others due to differences in species biochemistry and anatomy. Below is a list of the technical modifications I (Matt) developed during my S3 research.

1. If dealing with very large conduits, it is best to cut the pipette tip for a larger opening. This way you will pick up the bigger material and avoid breaking conduits.
2. Varying concentrations of a small, hydrophobic, opaque, granular (and yet unidentified) material were common. This clouding material not only obscured view, but it also adhered to the cells of interest. A variety of approaches were taken to eliminate the obscuring material. The most successful approach was to repeatedly rinse and thus dilute the material. To do so, I centrifuged the cells into a pellet. I then pipetted some of the concentrated cells into a new centrifuge tube with a fresh aliquot of 10 ml de-ionized water (DI). Once the material settled, it was gently pipetted into fresh DI the solution and gently shook, centrifuged. In repeating this process, the cells were removed from much of the obscuring material.

3. After applying the mounting solution it is helpful to obtain paper clips and pull the middle up and unfold it so that the point is facing down towards the base of the clip. In this way you can use the paperclip like a clamp that applies pressure to newly made slides to help push out and prevent air bubbles.
4. Slides may develop voids and air bubbles due to faulty mounting media; we used a product that is now unavailable from the manufacturer for this reason. To address this problem, in a fume hood apply heat to the underside of the slide from a reasonable distance with a lighter or some source of flame. The flame should not be directly touching the slide. Within a minute of applying heat you should see air masses forming and branching out under the coverslip. Once this happens to a large proportion of the coverslip use a tweezers or needle to push the coverslip off. There will be mounting residue left, but you can now apply a mounting medium with the same refraction properties and a new coverslip and allow to dry.

Results

A preliminary analysis was conducted for six species at the end of my S3 studies in August. An ordination that maps each species in tracheary-trait space was performed using principle components analysis (PCA). The first two PCA axes captured 96% of observed trait variance (Figure 1). When the six most influential traits of the PCA were correlated with mean tree height, the three most notable traits were longest tracheid length in the leaf (r^2=.943, p = 0.002), intermediate tracheid length in the leaf (r^2 = 0.943, p = 0.002), and intermediate tracheid length in the rhizome (r^2 = -0.714, p = 0.055) (Table 1).

Current Status (January 2015)

Two-hundered-thirty-nine of an anticipated 540 cells have been photographed (Table 2). From August 2015 to the present, we have been using a new, much higher-quality research grade

microscopes in Kindschi Hall. For purposes of consistency, all image capture and measurements had to restart. The new scopes facilitate much more accurate measurements.

Currently the most frequent cell type are tracheids (Figure 2c) as expected from literature on fern species other than those we are studying. To our surprise, vessel tube elements were observed in two species found at upper levels within our study, *Elaphoglossum glabellum* leaf (Figure 2a) and *Grammitis mollisma* (Figure 2b) as we expected to find this cell type primarily in species inhabiting low trunk positions. The most common pitting pattern was scalariform. Although distinctions between cell types can be difficult, pitting patterns are much easier to discern (Figure 2a-d). We are submitting with this report a flash drive containing all images captured at this time for OURS records; however, **these photographs cannot be shared publically under any circumstances as they are not yet copyrighted and are not yet incorporated into a publication.**

Publication Status

This report may be posted for public viewing; however, it should be clearly noted as a subcomponent of a much larger and yet incomplete study that will include field surveys and on-going anatomical work on stomates (for stomatal size, density and proximity to veins) and organ cross-sections (for parenchymatic path length). We expect that the maceration data for all species will be completed by end of Winter 2016 semester. We expect all elements of this enormous study to be completed and a manuscript ready for publication within two years.

Graduate Study

Matt is considering pursuing and expanding on his S3 work as a MS thesis research here in the Greer lab here at GVSU.

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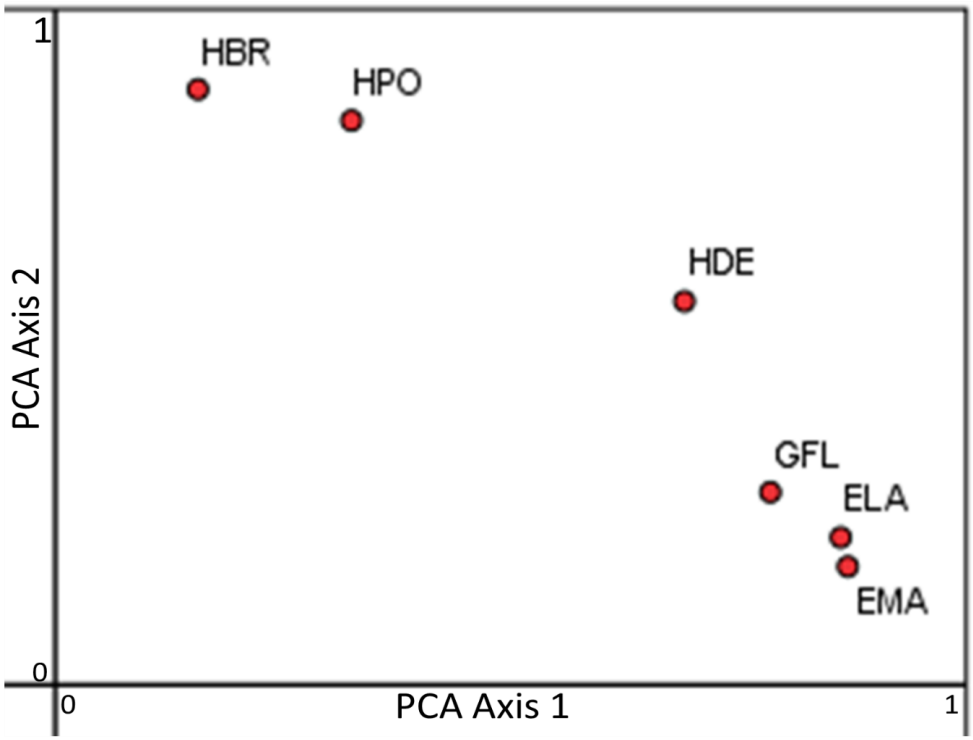

Figure 2. Ordination of six epiphytic fern species in traits space produced from principle components analysis (PCA). The first two axes captured a remarkable 96% of trait variance. Each axis is based on the following traits: **longest tracheid length in the leaf**, **intermediate tracheid length in the leaf**, shortest tracheid length in the leaf, longest tracheid length in the stipe, intermediate tracheid length in the stipe, shortest tracheid length in the stipe, longest tracheid length in the rhizome, **intermediate tracheid length in the rhizome**, shortest tracheid length in the rhizome. The most influential traits are bolded.

Table 2. Correlations between the three most influential traits from a PCA of six epiphytic ferns versus mean height occurrence on the basal three meters of eighty-eight trees surveyed in 2012 .

		Mean Plot Height
Longest tracheid length (LTL) Leaf	Correlation Coefficient	0.943
	Sig (1 tailed)	0.002
	N	6
Intermediate tracheid length (ITL) Leaf	Correlation Coefficient	0.943
	Sig (1 tailed)	0.002
	N	6
Intermediate tracheid length (ITL) Rhizome	Correlation Coefficient	-0.714
	Sig (1 tailed)	0.055
	N	6

Table 2. Tracheary cells successfully isolated and photographed for each species at the time of this report; numerous photographs of each cell at various magnifications were taken to capture specific subcellular features.

Species	**Code**	**Rhizome**	**Stipe**	**Leaf**
Cochlidium serrulata	CSE	0	0	0
Elaphoglossum crinitum	ECR	0	0	0
Elaphoglossum glabellum	EGL	10	4	5
Elaphoglossum latifolia	ELA	10	10	5
Elaphoglossum martinicense	EMA	6	4	7
Elaphoglossum maxonii	EMX	10	4	10
Elaphoglossum peltatum	EPE	0	3	0
Elaphoglossum rigidum	ERI	0	8	7
Grammitis mollisima	GMO	3	10	1
Grammitis flabelliformis	GFL	1	5	7
Hymenophyllum brevifrons	HBR	10	10	10
Hymenophyllum decurrens	HDE	7	10	4
Hymenophyllum polyanthos	HPO	4	4	5
Microgramma piloseloides	MPI	5	2	0
Nephrolepis cordifolia	NCO	5	6	8
Oleandra articulata	OAR	7	5	6
Trichomanes radicans	TRA	0	0	1
Trichomanes rigidium	TRI	0	0	0

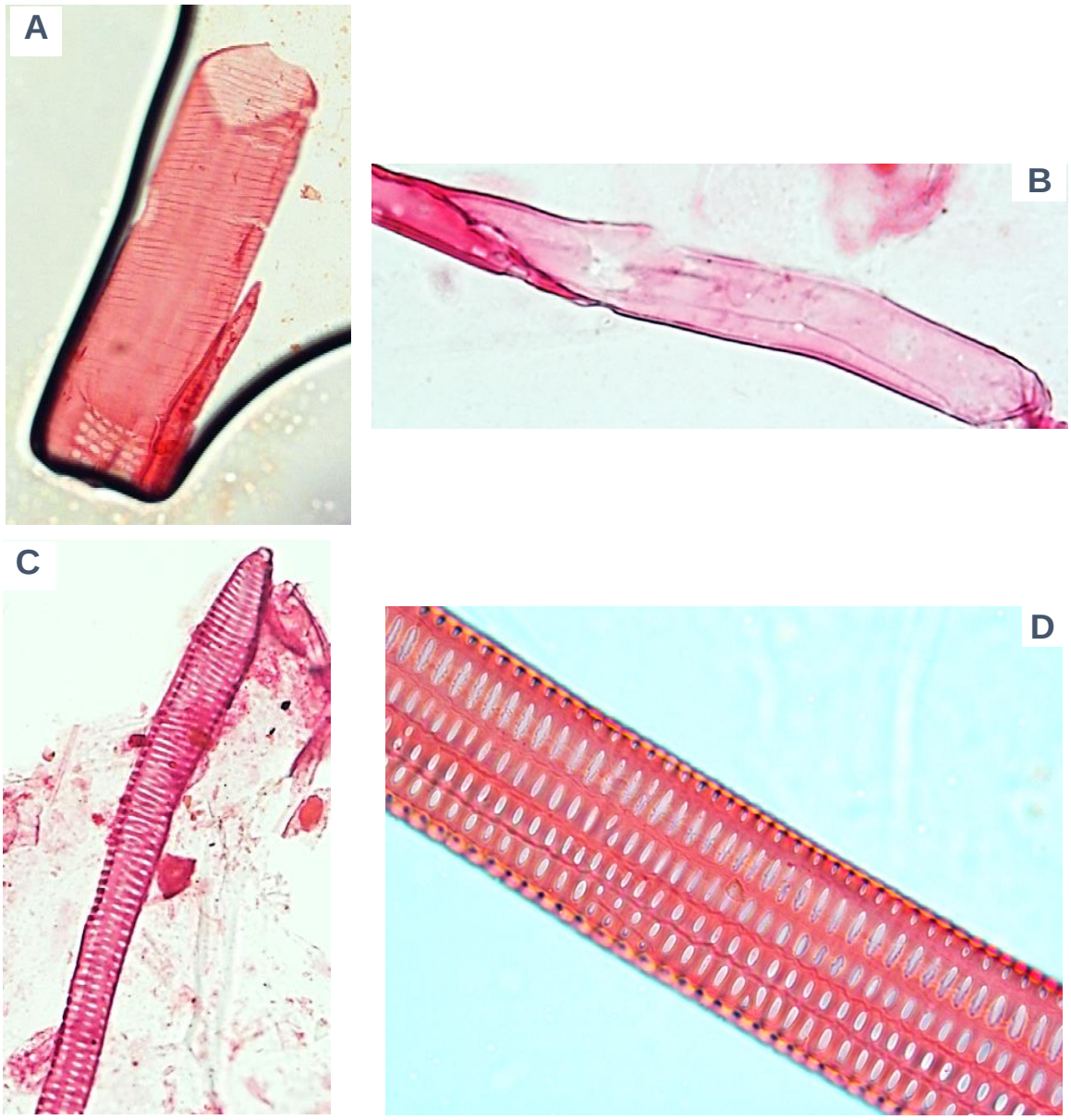

Figure 2. Examples of cell types and features captured during this study**:** a) rhizome vessel tube element of *Elaphoglossum glabellum*, b) rhizome vessel tube element of *Grammitis mollisma*, c) tracheid reticulate pitting of *Grammitis flabelliformis*, d) *Nephrolepis cordifolia* sclariform pitting. The entire image collection produced by this study have been deposited on a flash drive and submitted to the Office of Undergraduate Research.