Risky sex? Pollen and pollination in the grasses (Poaceae)

PROJECT DESCRIPTION

INTELLECTUAL MERIT

**Introduction and Objectives** (including Hypotheses)

Introduction

Imagine the remarkable and seemingly random journey a pollen grain must travel to accomplish pollination in wind pollinated plants. We expect the process of evolution to reduce inefficiencies in the risky act of anemophily. We propose the construction of virtual models of grass spikelets (the flower with associated bracts) for computational fluid dynamic (CFD) simulations and the imaging of pollen wall ultrastructure, with measurements of potential synapomorphies, to understand the evolutionary history of pollen and pollination in the grass family. The CFD simulations will provide a quantitative view into the aerodynamics of reproduction in the economically important grass family, Poaceae, and allow us to investigate to what extent spikelet morphology has been shaped by anemophily. Do variations in spikelet morphology, such as compression, presence or absence of awns, and stigma position at anthesis, affect pollination and are structural differences correlated to ecological niche? Grasses living in open habitats experience different wind speeds than those growing in forests, and the extensive modification of spikelet morphology among species may reflect alternate approaches to manipulating air currents around the spikelet to assist pollen capture by stigmas. Grass pollen is known to be relatively uniform at the scale of light microscopy, but the surface, or exine, of forest grass pollen is more highly ornamented when observed at higher magnification. Microchannels traversing the exine are assumed to be diagnostic for the grass family, but this trait has not been investigated in a phylogenetic context. Conducting a detailed analysis of pollen structure in combination with CFD simulations, while sampling informative clades in the phylogeny of Poaceae, will increase our understanding of the macroevolutionary history of grass spikelet morphology and pollen.

Wind pollination is ecologically common. This prevalence is in large part due to the ecological dominance of grasslands and their near global distribution. Temperate forests are also largely composed of anemophilous species. Despite the broad spatial distribution it is thought that anemophily evolved in only ~65 angiosperm lineages, whose members today comprise 10% of angiosperm species (Friedman & Barrett 2009). The transition to anemophily is not associated with high rates of speciation. Grasses are the unique exception, with ca. 12,000 species, making Poaceae the fifth most diverse plant family, and arguably the most successful lineage to evolve anemophily.

The family Cyperaceae, a distant relative within the Commelinid clade, is the closest wind pollinated family in terms of number of species (ca. 5,000) and possesses superficial similarities in floral design. Other significant wind pollinated clades include the Fagaceae and the conifers. It is worth noting that conifers and Gingko are the only exclusively wind-pollinated gymnosperms, whereas cycads and Gnetales are insect-pollinated (Peñalver et al. 2012). The diversification of the herbaceous Poaceae and Cyperaceae, which apparently converged on a bracteate floral structure, greatly exceeds their woody, catkin bearing counterparts.

Anemophily is a risky reproduction strategy in terms of pollination efficiency. Massive numbers of cheap gametophytes travel up to 20 km (Davis 2000) from their parent plant, and many of these pollen grains will fail to encounter a conspecific. The chaotic nature of pollen transport by wind has hindered quantitatively intensive analysis. Despite previous computational limitations researchers have built a theoretical framework for the conditions which allow anemophily to persist. These conditions include the presence of feathery stigmas, long stamen filaments absent or reduced nectaries fragrance perianth, inflorescence held away from vegetation. a high pollen:ovule ratio, pollen grains with a 10-50 µm diameter, slow terminal velocities of 2 – 6 cm/s (Adams et al. 1981), smooth surface, few and circular anulate apertures, low to moderate optimum wind speed habitat, low humidity habitat, infrequent precipitation habitat, open surrounding vegetation habitat, moderate to high plant density. (see bickel and freeman 1993 for plant geometry). Despite these hardships anemophily is still working.

Pollen in the Poaceae is monoporate (having one pore) and annulate (bearing an annulus, or thickened ring around the pore) (Fig. 1a). Under standard light microscopy, the exine (outer surface) of the pollen grain appears psilate (smooth) or nearly so; at Scanning Electron Microscopy (SEM) magnifications, the exine may appear psilate, or spinulose (spined) or scabrate (elements of any shape less than 1µm in any direction) sculpturing may be evident (Christensen et al., 1972; Zavada, 1983; Chaturvedi et al., 1998; Dórea et al., 2017, 2018). The pollen wall bears a thick footlayer (blue and purple bands in Fig 1b, c), and is tectate-columellate (Fig 1b,c) (Zavada, 1983). The single pore is generally operculate (having a sexine ectexine structure (Fig 1d right) covering part of the aperture, and which is isolated from the rest of the sexine (Fig. 1d).

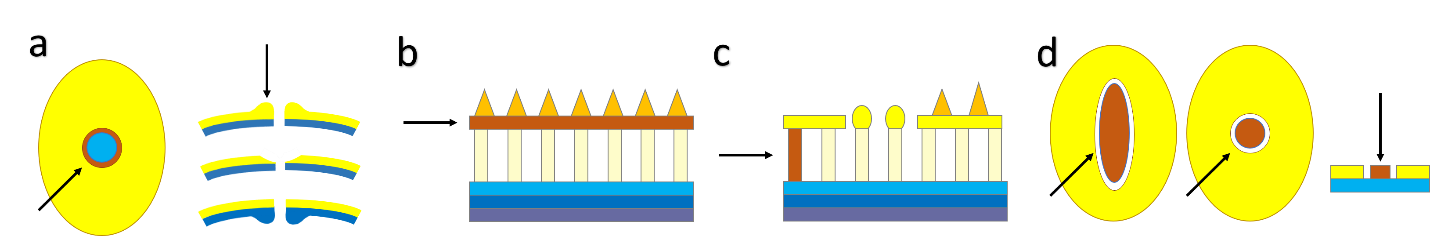


Figure 1: Key structures in a Poaceae pollen grain. In each of the following figures, dark orange highlights the structure being defined. a: Annulus, b: Tectum, c: Columellum, d: Operculum. Sexine shown in yellow in all but b. Cross-section in a right, b, c, and d far right. Polar view in a left and d left and center. Redrawn from Punt et al. (2007)

Intra-exinous channels are often noted in studies of grass pollen micromorphology (Fig 2) (Christensen et al., 1972; Christensen and Horner, 1974) or visible in Transmission Electron Microscopy (TEM) plates without being mentioned by the authors (c.f., Liu et al., 2004).

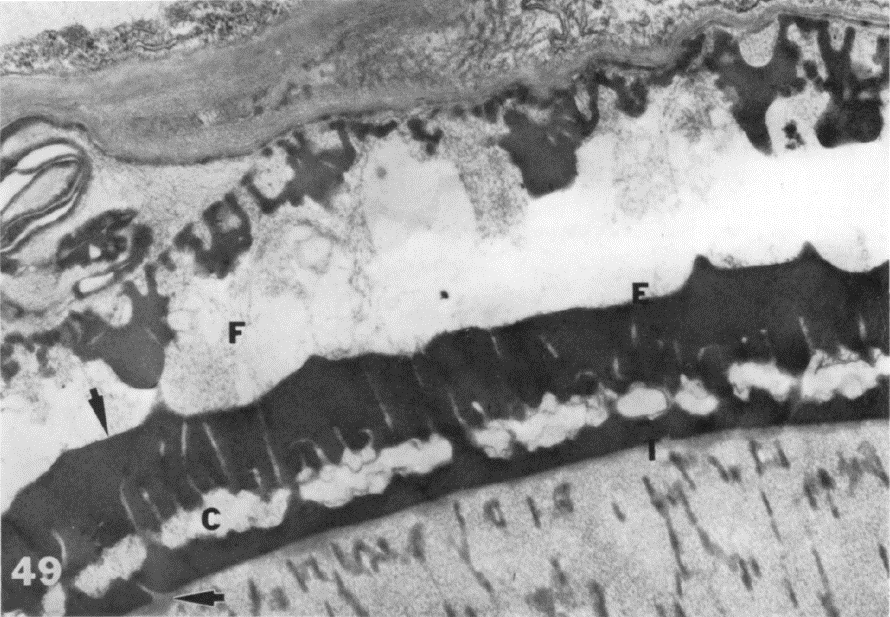


Figure 2: TEM image of mature grass pollen grain against anther wall. Intra-exinous channels are visible running perpendicular to exine axis (E) and above cavea (C). Image from Christensen and Horner (1974). Used with author permission.

Intra-exinous channels are sometimes listed as a ubiquitous pollen characteristic in the Poaceae (Zavada, 1983; Linder and Ferguson, 1985), and while this appears likely, the claim demands explicit testing. We therefore propose to survey pollen exine structure across the twelve subfamilies of the Poaceae and compare it to the exines in pollen from their close relatives in the Graminid clade: Ecdeiocoleaceae, Joinvilleaceae, and Flagellariaceae using TEM micrographs. Cyperaceae are excluded from this study, even though they also evolved peripheral pollen, as the unique pollen structures in this group are not known or suspected to include intra-exinous channels (see Halbritter et al., (2010)).

In addition to wind, pollen release, transport and deposition also depend on other abiotic factors such as humidity, rainfall and temperature.

Anemophily in grasses. Grass spikelet structure, bracteate, reduced flowers, feathery stigmas, versatile anthers. Grass pollen development (peripheral pollen). Grass pollen (uniporate, annulus, operculum, supposedly smooth walls, microchannels as a potential synapomorphy). Note similarities of Cyperaceae (bracteate spikelets, reduced flowers, independent evolution of peripheral pollen) but that these evolved independently. [Does peripheral pollen occur in any other wind-pollinated angiosperms, or in any angiosperms?]

However, insect visitation to grass inflorescences (and not just forest grasses) and those of a number of wind-pollinated plants have been documented. Ambophily vs. insect-assisted pollination. We have included some of the ambophilous grasses to test if the CFD results fall outside the range of expected behavior.

Objectives

-to examine the evolution of grass spikelets in the context of adaptation to wind pollination

-to examine the evolution of traits associated with air movement and pollen capture in the family in a phylogenetic context

-to examine the surface and ultrastructure of grass pollen in a phylogenetic framework

Hypotheses

*Spikelet/spikelet trait-related*

H0 A spikelet has the same pollen capture potential in all orientations

H1There are positions where pollen capture potential is greater than other positions

H0 A spikelet has the same pollen capture potential in all wind speeds

H1 A range of wind speeds increases the chance of pollen capture

H0 There is no correlation between habitat and pollen capture potential

H1 Spikelets differ in their aerodynamics (to a degree that influences pollination) that is specific to habitat, while also considering phylogenetic relatedness.

*Pollen-related*

H0 Intra-exinous channels are not a synapomorphy of the Poaceae

H1 Intra-exinous channels are a synapomorphy of the Poaceae

H0 Intra-exinous channel presence and/or structure is not taxonomically significant at the subfamily level in the Poaceae

H1 The presence and/or structure of intra-exinous channels is taxonomically significant at the subfamily level in the Poaceae

H2 Intra-exinous channels are a synapomorphy of the BOP + PACMAD clade

H0 The presence of intra-exinous channels does not correlate with habitat (forest-associated vs. grassland)

H1 The occurrence of intra-exinous channels differs between forest-associated taxa and grassland taxa

[might need to modify depending on what the literature search shows]

**Significance**

[If we include this section, we have to be careful not to be redundant with the Broader Impacts. But this would give us a chance to highlight the novelty of the virtual wind tunnel.]

**Proposed Work**

Pollination and Spikelet Evolution

Sampling

We propose 26 species (table #) for the 3D modeling component of this study, which are chosen with consideration of the availability of structurally uncompromised spikelet material, representation from open, margin, and forest habitats, and the strong presence of characters to be studied at the larger phylogenetic scale (see table #). It is feasible to use taxa from ten of the twelve subfamilies of Poaceae; only Pueloideae and Micrarioideae are avoided due to flowering material being difficult to obtain. *Anomochloa marantoidea*, *Streptocheata spicata*, and *Pharus latifolius* are chosen because they represent the earliest diverging lineages of Poaceae and fresh, flowering material can be obtained from specimens growing in the Pohl Conservatory at Iowa State University. Anomochloa and Streptocheata are both in the subfamily Anomochlooideae and are forest grasses. Pharus is in the subfamily Pharoideae and is considered to live in margins. The Panicoideae and Pooideae subfamilies contain locally available, con generic taxa with one species living in open habitat (*Dichanthelium oligosanthes*, *Elymus virginicus*, *Festuca paradoxa*, and *Poa pratensis*) and another species in forests (*Dichanthelium latifolium*, *Elymus hystrix*, *Festuca subverticillata*, and *Poa sylvestris*). These taxa form the core of our sampling strategy to contrast spikelets from open vs closed habitats. The additional species *Brachyeletrum erectum*, *Chasmanthium latifolium*, and *Diarrhena obovata* are added to represent grasses growing in forest margins, and *Centotheca lappacea* and *Zeugites pittieri* will represent additional forest grasses. The spikelets of *Panicum virgatum* exemplify an important variation in design where sterile lemmas accompany a single fertile floret. The spikelets of *Echinochloa crusgalli* share this design, but have a significant amount of ornamentation (awns and epidermal hairs) that will allow an interesting contrast among the open habitat Panicoideae. We propose sampling a species of Leersia and Ehrharta from within the Oryzoideae. The spikelets of *Leersia virginica* are an excellent example of lateral compression and *Ehrharta erecta* grows aggressively in our greenhouse and can easily provide fresh material at anthesis. *Phragmites australis* (Arundinoideae) is readily available and information gained about its reproductive behavior could be valuable because of its near global distribution and invasive tendencies. Two species of Bouteloua (Chloridoideae) will be modeled with the bonus opportunity of comparing the influence of spikelet packing. *Bouteloua curtipendula* has pendulant, solitary spikelets while the synflorescence of *Bouteloua gracilis* contains multiple closely spaced spikelets. *Aristida purpurea* (Aristidoideae) and *Danthonia spicata* (Danthonioideae) are both accessible and have characteristically large awns. *Raddia brasilliensis* and *Lithachne pauciflora* were chosen from the Bambusoideae because they are growing in the Pohl Conservatory at Iowa State University and flower regularly. Fresh material can be obtained from specimens growing in Iowa State Greenhouses or from local populations for all 26 grasses chosen for the modeling portion of this study.

Spikelet modeling

When the grasses on our sampling list reach the point of anthesis we will collect material for vouchers and reference photographs. A set of spikelets will be removed and fixed in scintillation viles of gFAA while other spikelets are dried to provide material for the light microscopy analysis of pollen. Fixed material will be grouped, dissected, and embedded into paraffin wax as described in the recent method paper (Klahs, Gallaher, and Clark 2018). Following the described procedure we will section spikelets and portions of spikelets to produce serial sections mounted on slides. Images of these sections are used to create 3D computer aided design (CAD) models using Cinema 4D software. The models consist of individual wireframe meshes for each spikelet bract. The meshes are sculpted using both the reference photographs of spikelets and the images of serial sections. The final 3D spikelet model accurately represents the biological structure and provides the virtual surface for computational fluid dynamic simulations. Examples of 3D models already created with this process can be viewed at <https://sketchfab.com/pklahs>.

Virtual wind tunnel experiments

After modeling is completed the virtual 3D spikelet is exported as a set of surface meshes. Computational fluid dynamics simulations predict airflow and pressure around the surface meshes by solving the Navier-Stokes equations. These simulations are computationally laborious and the number of vertices (and resulting polygons) can significantly increase calculation time. We use a script developed by a lab of mechanical engineers at Iowa State University (Ming-Chen Hsu citation) to solve the Navier-Stokes equations. The surface meshes are primed for simulation by a series of steps that involve positioning the spikelet, defining the space (a virtual wind tunnel), and initiating a directional flow (setting wind speed). The iterative calculation of wind speed and pressure converges to a quantitative portrait of the air moving around the grass spikelet. The results of a particular time step can be exported as a 3D matrix of pressure values and wind speed vectors. This matrix contains data for statistical comparisons between taxa and can be graphically represented (Fig #).

The visualization and quantitative measurement of air currents moving around the spikelet provides an unprecedented opportunity to study wind pollination. Our preliminary data from simulations of Panicum and both Festucas have already revealed interesting behavior in air current funneling. Air cannot pass through the surface meshes and is forced to move around the spikelet. This results in increased air speeds (above the speed produced by the simulation) and a change of pressure in specific regions adjacent to the spikelet. These regions,interestingly, are the same space occupied by the stigmas and may be evidence to support the claims of Carl Niklas (citation).

Pollen

Pollen Sampling

We selected thirty-six taxa from across the twelve subfamilies of the Poaceae, and four from the early-diverging Poales lineages (Flagellariaceae, Joinvilleaceae, and Ecdeiocoleaceae) to study pollen wall ultrastructure (Table). These taxa occur in a range of habitats, and represent forest grasses, margin dwellers, and open habitat species. Habitats span a range of conditions from xeric to wetland. The selected taxa and the habitats in which they reside will augment understanding about whether habitat correlates with the presence or absence of intra-exinous channels. Sampling is divided among C3 and C4 photosynthetic types. Taxonomic classifications follow the phylogenies of Soreng et al., (2015, 2017).

Previous work has confirmed the presence of intra-exinous channels in pollen from multiple members of the Pooideae (Linder and Ferguson, 1985; Peltre et al., 1987; Jewell et al., 1988; Marquez et al., 1997; Diethart et al., 2007), Panicoideae (Larson et al., 1962; Christensen and Horner, 1974; Peltre et al., 1987; Marquez et al., 1997), and Chloridoideae (Liu et al., 2004). Therefore, we focus the bulk of our sampling outside of these well-studied subfamilies. The exceptions are *Brachyelytrum erectum, Brachypodium distachyon, Diarrhena obovata, Glyceria striata*, and *Hesperostipa spartea* in the Pooideae; *Centotheca lappacea, Chasmanthium latifolium, Gynerium sagittatum, Echinochloa crusgalli, Panicum virgatum*, and *Danthoniopsis dinteri* in the Panicoideae; and *Centropodia glauca* and *Bouteloua curtipendula* in the Chloridoideae. These taxa are included to improve our understanding of pollen wall ultrastructure in the earliest-diverging members of their respective tribes and subtribes.

Twenty-three of the thirty-six proposed taxa are selected from subfamilies in which studies of pollen wall ultrastructure are rare or absent, or in which past work was inconclusive. The presence of intra-exinous channels has been confirmed in *Pariana stenolemma* pollen (Salgado-Labouriau et al., 1993), but no other bamboos have been sampled to date. We propose to study three additional members of the Bambusoideae for which pollen is readily available: *Arundinaria gigantea*, *Guadua angustifolia*, and *Lithachne pauciflora*.

Seminal work by Chanda and Rowley (1967) in the Flagellariaceae, Ecdeicoleaceae, and what is now known as the Joinvilleaceae, on pollen aperture morphology was inconclusive as to the presence of intra-exinous channels. *Joinvillea* was initially included in the Flagellariaceae, but the genus was subsequently separated into its own family, the Joinvilleaceae, by Tomlinson and Smith (1970). One species in the Joinvilleaceae (*Joinvillea ascendens*), one species in the Flagellariaceae (*Flagellaria indica*), and two species in the Ecdeicoleaceae (*Ecdeiocolea monostachya* and *Georgeantha hexandra*) will be sampled. Improved TEM technology will allow us to capture images in greater detail than was possible in the 1960s. The early-diverging Poales lineages have much to reveal about the evolution of pollen wall ultrastructure.

We propose to sample sixteen taxa in the remaining subfamilies of the Poaceae. This includes two each in the Anomochlooideae (*Anomochloa marantoidea* and *Streptochaeta spicata*), Aristidoideae (*Aristida purpurea* and *Stipagrostis hirtiglumis*), Arundinoideae (*Arundo donax* and *Phragmites australis*), Micrairoideae (*Eriachne sp.* and *Micraira sp.*), Pharoideae (*Leptaspis zeylanica* and *Pharus latifolius*), and Puelioideae (*Puelia sp.* and *Guaduella sp.*); three in the Oryzoideae (*Ehrharta erecta, Zizania palustris,* and *Streptogyna americana*); and one in the Danthonioideae (*Danthonia spicata*). This sampling scheme ensures that pollen wall ultrastructure of at least two members of each subfamily, distributed more or less evenly across tribes and subtribes, will be imaged either by our efforts or those of previous researchers.

Microscopy methods

Both fresh and dry pollen has been successfully employed in TEM studies of pollen wall ultrastructure by various researchers. Fresh material will be used whenever possible, and herbarium material will be used if no fresh pollen is available. Fresh pollen will be taken from mature anthers and immediately fixed in 70% ethanol for later use.

Herbarium pollen will be cleaned and rehydrated using the enzyme-based method of Schols et al., (2004). Enzymatic treatment of dry pollen provides sufficient cleaning and rehydration of grains intended for use in microscopy, without the risk of collapse incurred with standard acetolysis (Martin, 1969). Enzymatic treatment uses a 1:200 dilution of Agepon in distilled water to gently rehydrate whole anthers, followed by grinding through fine phosphor-bronze mesh into small test tubes. The solution is then centrifuged at 2800 rpm for 3 minutes, decanted, and the tubes refilled with a solution of distilled water, citrate buffer, cellulase, and pectinase. This mixture is shaken periodically over 24 hours, then centrifuged and decanted. The clean and rehydrated pollen grains are stored in 70% ethanol for later use. Pollen prepared in this manner is appropriate for SEM, TEM, and LM.

Pollen for SEM and TEM will be further prepared at Iowa State University’s Roy J. Carver High Resolution Microscopy Facility following their preferred protocols. One graduate student will receive training in SEM preparation methods and assist microscopy facility staff in processing and imaging pollen. The same graduate student will assist in capturing appropriate TEM images, but all preparations for TEM will be completed by microscopy facility staff.

Pollen intended for LM will be stained with safranin and mounted on slides in silicone oil (Dow Corning Corporation). Silicone oil is preferred because pollen grains are less prone to swelling in this medium, and grains can be easily rotated under the cover slip to capture images from multiple angles. Measurements will be made at 1000x magnification using oil immersion on a Nikon Ni-U upright microscope, and high-resolution images will be captured with a microscope-mounted Nikon camera.

**Preliminary Results**

Pollination and Spikelet Evolution

We have begun mapping spikelet characters that may influence pollination onto a recent, well supported phylogeny of the family (Saarela et al. 2018). This process informed the sampling strategy for this proposal and will be expanded to include more characters.

Number of Fertile florets, Number of Sterile Florets, Compress, Spikelet length, Disarticulation, Lower Glume length, Upper Glume length, Sterile Lemma length, Fertile Lemma length, Awns, Number of anthers per floret, Number of Stigmas, Stigma position

Fig #

A phylogeny of Poaceae with glume length as a ratio of total spikelet length mapped.

Pollen

Discuss what is known regarding the distribution of the intra-exinous channels

Include anything relevant about experience with processing pollen

**Timetable**

Year 1: Pollen sampling and microscopy; collection of spikelets and anthers. Year 2: Sectioning of spikelets and preparation of models; virtual wind tunnel experiments. Year 3:

BROADER IMPACTS

Educational: Grass spikelet models that are digitally dissectible.

Human Resources: Training of two Ph.D. students, one of whom is female and a first generation college and graduate school student; training of at least two undergraduates during the project.

Methods: Development of 3D methods and analyses

Other fields: potential impact on paleoecology/paleoclimatology

[work in BPMI?; Carver students?; SACNAS?]

**Results from Prior NSF Support**

Clark. DEB-1120750, $291,057, 2011-2016, COLLABORATIVE RESEARCH: **Ecological Diversification and Molecular Evolution of Grasses (Poaceae)***, with M. Duvall (DEB-1120761) and S. Kelchner (DEB-1120856)*.

DEB-1342787 to PI M. Ungerer (originally to J. Craine) at Kansas State University, $1,419,186 (**ISU/Clark subcontract** $207,121), 2013-2019, Dimensions/NASA: COLLABORATIVE RESEARCH: **The Biogeography and Evolution of Drought Tolerance in Grasses**, *with M. Duvall (DEB-1342782) and C. Still (DEB-1342703)*.

The questions, activities, and results of these two projects were complementary and overlapping. To simplify reporting, we integrated the results in the text below, with DEB-1120750 products labeled with an asterisk (\*) and DEB-1342787 labeled by a cross (†).To date, we have published 11 papers (9\* and 2\*†) and 2\*† are currently in review or revision. We have produced three permanent slide collections : 1) ca. 4,500 slides, representing sections in all three planes, of ca. 30 non-grass monocots and ca. 300 grass species; 2) leaf clearings of ca. 220 grass species, and 3) root cross sections of ca. 80 grass species. These are currently housed in the Clark lab at ISU (access available upon request), but we will select representative slides and images to be archived at the Cornell University Plant Anatomy Collection/Website.

*Intellectual Merit*

Major activities undertaken by the Clark lab were: 1) collection of critical taxa; 2) facilitation of plastome sequencing; 3) a family-wide leaf shape evolution analysis; 4) a survey of 3D leaf anatomy in the grasses; 5) a survey of grass root anatomy; 6) collection of root and leaf anatomical data as part of experimental drought tolerance and gene expression studies; and 7) analyses of leaf and root anatomy to test the evolutionary and ecological significance of climate and light. These activities produced several key findings that improved our understanding of grass evolution, as well as the genetic and functional bases of water and light relations.

Our plastome phylogenetic estimations confirmed the previous topology of three early-diverging grass lineages sister to two major clades (BOP + PACMAD), suggested Panicoideae as sister to ACMAD (instead of Aristidoideae), provided improved resolution of relationships within subfamilies, and revealed a new genus of bamboo (\*Attigala et al. 2014; \*Burke et al. 2014; \*Cotton et al. 2015; \*Saarela et al. 2015; \*Wysocki et al. 2015; \*†Attigala et al. 2016a,c; \*Burke et al. 2016b; \*Duvall et al. 2016). Our results date the origin of the grass family into the Early Cretaceous (\*†Burke et al. 2016a; \*†Gallaher et al., in revision), 20-30 My earlier than previous estimates.

Our studies of grass leaf shape evolution showed that strongly linear leaves occur in climates receiving <1,000 mm MAP, whereas in habitats with greater precipitation, all three shape categories (linear, intermediate, ovate) may be found. Ancestral grasses most likely had moderately broad leaves, and grew in mesic, partially shaded habitats. Strong leaf linearity evolved multiple times and preceded the evolution of C4 photosynthesis (\*†Gallaher et al., in revision). Anatomically, grass adaxial chlorenchyma cells are usually lobed in at least one plane, often the proximo-distal or lateral planes; the presence of lobes is plesiomorphic and general, with some reversions to the non-lobed condition. Five major morphotypes were identified; a species may have more than one, but we modeled the dominant adaxial cell shape adjacent to the sheaths of 1st order bundles. Using all data, adaxial chlorenchyma cell shape is significantly correlated with both photosynthetic pathway (PS) and light regime. Several descriptors (e.g., cell length:width, number of lobes in cross section) have significant phylogenetic signal, and some also show other significant associations (e.g., L:W with light regime and PS) (\*†Gallaher et al., in prep.).

We documented significant variation in transverse mature stelar root anatomy in grasses (\*†Cox et al., in prep.). Novel findings included internal (pith) phloem in two species and an apparent multiseriate endodermis (up to 7 layers) in ca. half of the species we sampled. Surprisingly, root anatomical variation showed no phylogenetic or photosynthetic pathway signal, and only a few, weak correlations with climatic variables.

*Broader Impacts*

Two postdoctoral fellows, 4 graduate students (3 Ph.D., 1 M.S.), 7 undergraduate students and 1 high school student (8 of whom were female) received training in aspects of Next Generation sequencing/assembly, plastome phylogenetics/bioinformatics, preparation and imaging of paraffin sections and leaf clearings, quantitative anatomy, 3D modeling/printing, and statistical analysis in R. We reported results in 8 (6\*, 2\*†) invited seminars, 10 (5\*, 5\*†) contributed talks and 3\* posters at national and international meetings/institutions. We are preparing animations and protocols for 3D printing are to be used in educational applications. Other products included an interactive software package for developing botanical keys (\*Attigala et al. 2016b).

**Details/Budget**

3-year project, with a start date as soon as possible in 2019 (but might have to be mid-semester in the spring or not until the start of summer semester)

12 months of RA support per year, plus benefits and tuition

Hourly for undergrads, plus benefits

Supplies for anatomical work for both components of the project

LM imaging, measurements, etc.

SEM training and beam time

TEM samples

Travel to meetings

Greenhouse supplies/costs

Publication costs

[likely no request for computer or software, but what about server time?]