PROJECT DESCRIPTION

**INTELLECTUAL MERIT**

**Introduction and Objectives**

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 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. It is the ~~prominent~~ dominant syndrome of reproduction in conifers and deciduous trees of temperate forests and employed by sedges in wetlands. The ecological prevalence of anemophily is in large part due to the ecological dominance of grasslands and their near global distribution. 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.

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 (Faegri & van der Pijl 1979). Predicted pollen characteristics include a high pollen:ovule ratio, pollen grains with a smooth surface, a diameter between 10-50 µm, and slow terminal velocities of 2 – 6 cm/s (cite). Wind pollinated angiosperms possess feathery stigmas and flowers held away from vegetation. They are often found in habitats with low humidity, infrequent precipitation, and with moderate to high conspecific density. Anemophily is a dangerous reproductive strategy unless selective pressures refine flowers and pollen to operate successfully and mitigate pollen lost in stochastic environments.

Flowers of Poaceae, even with their architectural diversity, conform to the predictions of the anemophilous syndrome. The flowers exhibit no fragrance, are relatively small, and not considered to be showy. A spikelet contains one to many flowers, each enclosed in bracts derived from petals and sepals. The set of flowers and bracts is again enclosed by two bracts known as glumes. The collection of bracts constitutes the exterior surface of the spikelet, and by extension manipulates the aerodynamics of the whole structure. Anthers and stigmas must extend beyond the aerodynamic boundary layer created by these bracts to interact with air currents (Friedman & Harder 2004). The release of pollen into suitable wind is controlled by the plant (cite). Every grass pollen develops while maintaining contact with the tapetum of the anther (peripheral pollen cite). The pollen grains swell rapidly under certain conditions to promote locular rupturing inside the anther (cite). This happens concurrently among conspecifics in a habitat, and is driven by humidity as a cue of potential rain (cite). The release of pollen from anthers is not random. The boundary layer and localized air currents are determined by the shape of the grass spikelet and therefore not random either.

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).

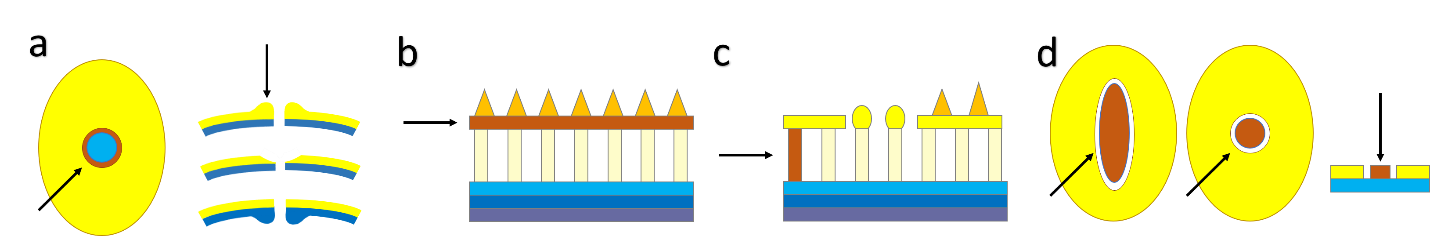


Figure 1: Key structures in a Poaceae pollen grain. In each of the following figures, dark orange highlights the structure being defined. a: Annulus, b: Tectum, c: Columellum, d: Operculum. Sexine shown in yellow in all but b. Cross-section in a right, b, c, and d far right. Polar view in a left and 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 & Horner, 1974) or visible in Transmission Electron Microscopy (TEM) plates without being mentioned by the authors (c.f., Liu et al., 2004). Intra-exinous channels are sometimes listed as a ubiquitous pollen characteristic in the Poaceae (Zavada, 1983; Linder & 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)].

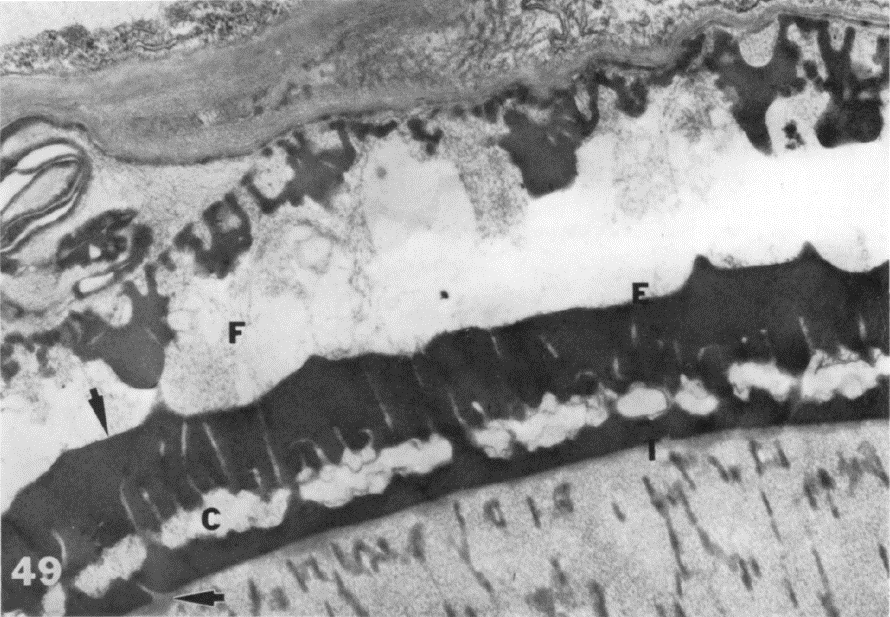


Figure 2: TEM image of mature grass pollen grain against anther wall. Intra-exinous channels are visible running perpendicular to exine axis (E) and above cavea (C). Image from Christensen & Horner (1974). Used with author permission.

The size and mass of most grass pollen conforms to the predictions for wind pollinated plants. These parameters result in the slow terminal velocity that allows pollen grains to be carried significant distances by air currents, and define the range of wind speeds suitable for that task. When stigmas of grass flowers encounter these suitable wind speeds there is the potential of capturing pollen by direct impaction. Pollen grains may also be carried by air currents into re-circling eddies where contact with stigmas may occur later on leeward surfaces. The conditions resulting in these interactions between stigmas and pollen is once again defined by the morphology of the spikelet and the resulting aerodynamics. We are interested in quantifying the probability of pollen interacting with stigmas in spikelets of different shape and comparing spikelet aerodynamics from grasses of habitats that experience different wind speeds.

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**

Explicitly testing something that has been assumed (Pollen)

[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**

Spikelet Modeling and Pollination

*Sampling*. We propose 27 grass species (Table 1) for the 3D modeling component of this study; for bisexual taxa, a representative spikelet will be modeled and for unisexual taxa, a representative female-fertile spikelet will be modeled. Taxa are chosen based on the availability of structurally uncompromised spikelet material (plants cultivated in the Pohl Conservatory at Iowa State University or from local populations); subfamilial coverage; representation from open, edge, and forest understory habitats; and the presence of representative character states for characters to be optimized on the plastome phylogeny (Table 2). Ten of the 12 subfamilies of Poaceae will be sampled, some for >1 taxon (e.g., Pooideae, Panicoideae); only Puelioideae and Micrairoideae are excluded due to the difficulty of obtaining fresh-fixed flowering material. Within the APP grade, *Anomochloa marantoidea*, *Streptocheata spicata*, and *Pharus latifolius* are chosen because they represent the earliest diverging lineages of Poaceae, they are forest-associated (the first two in understory and *Pharus* in edge and understory habitat), and fresh flowering material can be obtained from plants growing in the Pohl Conservatory. The “spikelets” of Anomochlooideae are bracteate and not readily comparable to the conventional spikelets seen in the rest of the family (the spikelet clade), and thus provide an interesting counterpoint to the other forest grasses in this study. *Pharus* represents the earliest diverging lineage of the spikelet clade, and it has unisexual spikelets. The Bambusoideae are a fundamentally forest-associated lineage, although some members occur in open habitats (Clark et al. 2015). We have excluded the woody bamboos from the spikelet modeling component, again due to the difficulty of obtaining appropriate flowering material given their long flowering cycles (Clark et al. 2015). However, the herbaceous bamboos are characteristic of the tropical forest understory, have sculptured pollen (Dórea et al., 2017, 2018) and flower regularly, so we include two species (*Lithachne pauciflora* and *Raddia brasiliensis*), both of which are in cultivation at the Pohl Conservatory (ISU). All herbaceous bamboos are at least functionally unisexual, but the female anthecia of *Lithachne* and *Raddia* differ significantly in shape and compression (Judziewicz et al. 1999). Oryzoideae are represented by *Ehrharta erecta* (two sterile lemmas, moderate lateral compression, cultivated) and *Leersia virginica* (no sterile lemmas, strong lateral compression, local).

The Pooideae and Panicoideae subfamilies include locally available, congeneric taxa with one species living in open habitat (*Elymus virginicus*, *Festuca paradoxa* and *Poa pratensis*/*Dichanthelium oligosanthes*) and another species in forests (*Elymus hystrix*, *Festuca subverticillata* and *Poa sylvestris*/*Dichanthelium latifolium*). These taxa form the core of our sampling strategy to contrast spikelets from open vs. closed habitats. The additional species *Brachyeletrum erectum* and *Diarrhena obovata* from the Pooideae are added to represent grasses growing in forest margins. In the Panicoideae, *Chasmanthium latifolium* represents edge habitat whereas *Centotheca lappacea* and *Zeugites pittieri* represent additional forest grasses. These three genera have laterally compressed spikelets with multiple florets, similar to the spikelets characteristic of many Pooideae (including *Festuca*), in contrast to the spikelets of *Dichanthelium* and *Panicum* *virgatum*, which exhibit the dorsally compressed spikelets with one fertile floret subtended by a sterile lemma and disarticulation below the glumes characteristic of the majority of the Panicoideae. The spikelets of *Echinochloa crusgalli* share this design, but have a significant amount of ornamentation (awns and epidermal hairs), which will allow an interesting contrast with the open habitat Panicoideae. *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 (ref). Two species of *Bouteloua* (Chloridoideae) will be modeled with the bonus opportunity of comparing the influence of spikelet packing. *Bouteloua curtipendula* has few spikelets per branch but many branches per synflorescence, while *B. gracilis* has multiple closely spaced spikelets per branch, with 1-3 branches per synflorescence. *Aristida purpurea* (Aristidoideae) and *Danthonia spicata* (Danthonioideae) are both accessible and have characteristically large awns, but *Aristida* has a single cylindrical floret per spikelet whereas *Danthonia* has laterally compressed spikelets with multiple florets.

*Spikelet modeling*. When the grasses on our sampling list reach anthesis, we will collect material for vouchers and take reference photographs. A set of spikelets will be removed and fixed in gFAA in scintillation vials, while fresh pollen from other spikelets will be fixed for pollen study (see below). Fixed material will be grouped, dissected, and embedded into paraffin wax as described in Klahs et al. (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 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 provide an unprecedented opportunity to study and quantify wind pollination, especially with respect to spikelet modifications for facilitating efficient pollen capture. 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 Karl Niklas (citation).

Pollen

*Sampling*. We selected a total of 44 taxa from across the 12 subfamilies of the Poaceae and four from its three sister families (Flagellariaceae, Joinvilleaceae, and Ecdeiocoleaceae) within the graminid clade of the Poales to study pollen morphology and wall ultrastructure (Table 1). 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. Sampling is also divided among C3 and C4 photosynthetic types. The selected taxa and their habitats and photosynthetic pathway will augment understanding about whether these correlate with the presence or absence of intra-exinous channels or other pollen traits.

[Here is probably the place to include a short statement on what TS brings—i.e., we have 36 taxa for which we propose to do both TEM and SEM, but we need to explain why both are needed to understand wall ultrastructure. Then, what else do we gain from SEM (re pollen morphology)? We have 27 taxa for which we proposed to do LM— this tracks the taxa for which spikelets are to be modeled, because Phil needs the size in order to plug this info into the simulations, no? This would also be the place to define SEM and LM if we end up not having done that in the Introduction.]

Thirty-six taxa will be evaluated using both Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). TEM is employed to study pollen wall ultrastructure in cross-section in extremely fine detail. This will allow us to measure the size and orientation of intra-exinous channels, if present. SEM is critical for describing exine ornamentation and making highly accurate measurements of surface structures. Twenty-seven taxa will also be evaluating using Light Microscopy (LM). LM is employed for general descriptions of pollen and making measurements of the shape and size of entire pollen grains. Information gleaned from LM, TEM, and SEM together will be used to create extremely detailed and realistic models of pollen grains for wind tunnel simulations.

Previous work has confirmed the presence of intra-exinous channels in pollen from multiple members of the Pooideae (Linder & Ferguson 1985; Peltre et al. 1987; Jewell et al. 1988; Marquez et al. 1997; Diethart et al. 2007), Panicoideae (Larson et al. 1962; Christensen & 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 subfamilies, tribes or subtribes, or to complement proposed spikelet modeling. Twenty-three of the 36 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*.

We also propose to sample 16 taxa in the remaining subfamilies of the Poaceae. This includes two each in the Anomochlooideae, Aristidoideae, Arundinoideae, Micrairoideae, Pharoideae, and Puelioideae; three in the Oryzoideae; and one in the Danthonioideae (Table 1). 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.

Seminal work by Chanda & 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. These families sister to the Poaceae have much to reveal about the evolution of pollen wall ultrastructure.

*Microscopy methods*. Both fresh and dry pollen have been successfully employed in pollen studies 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 specimen 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 sample preparation 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 (E.J. Cushing, *pers. comm.*). Measurements will be made at 1000x magnification using oil immersion on a compound microscope, and images will be captured with a microscope-mounted camera. ~~Nikon Ni-U upright microscope, and high-resolution images will be captured with a microscope-mounted Nikon camera~~.

Spikelet and pollen trait evolution

*Sampling*.

data set, characters, kew, explain that previous sampling is a subset into this larger. Expanding view to larger 150 sample

Taxonomic classifications follow Soreng et al. (2017); at the tribal level, all tribes in all subfamilies are represented, except for the Pooideae and Panicoideae, for which 11 of 15 and 11 of 13 tribes, respectively, are represented.

*Methods*.

phylogenies, bioclim

Table 2. Characters.

**Preliminary Results**

[Phil, we will want to have Fig. 3 showing the snapshot of the results of a simulation here—comparison of the two Festucas, I think? I think that the phylogeny with an optimization (Fig. 4) will also have to be placed here.]

**Timetable**

Year 1: Pollen sampling and microscopy; collection of spikelets and anthers; spikelet modeling; character scoring for optimizations. Year 2: Continue spikelet modeling; virtual wind tunnel experiments; character scoring for optimizations; preparation of manuscripts. Year 3: Virtual wind tunnel experiments; optimizations and related analyses; preparation of manuscripts.

**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

**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 (†).

*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 family-wide survey of 3D leaf anatomy; 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 potential functional bases of water and light relations.

Our plastome phylogenetic estimations confirmed the previous topology of three early-diverging grass lineages serially 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; \*†Saarela et al. 2018). 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 likely had moderately broad leaves, and grew in wet, partially shaded habitats. Strong leaf linearity evolved multiple times in association with invasion of open habitats and preceded the evolution of C4 photosynthesis, but direct transitions between linear and ovate leaves did not occur, highlighting the important evolutionary role of intermediate leaf shapes in forest margin habitats (\*†Gallaher et al., in revision). This leaf shape dataset was also analyzed in a study of persistent homology and leaf morphospace (\*†Li et al. 2018).

Most monocots have more or less spherical, unlobed adaxial chlorenchyma cells (a few exhibit lobing in one plane). 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 through modeling of adaxial cells adjacent to 1st order bundle sheaths. Adaxial chlorenchyma cell shape was significantly correlated with both photosynthetic pathway (PS) and light regime, with C4 grasses converging on the radiator shape. Several descriptors (e.g., cell width) had significant phylogenetic signal. Bundle sheath and chlorenchyma cell lengths were strongly correlated (\*†Gallaher et al., in prep.). Fusoid cells were determined to be homologous across the grass family (\*†Leandro et al. 2018).

We documented significant variation in transverse mature stelar root anatomy in grasses (\*†Cox et al., in prep.). Novel findings included an apparent multiseriate endodermis (up to 7 layers) in ca. half of the species sampled. Annual precipitation, available moisture, and mean-diurnal temperature range correlated with aspects of root anatomy, but few characters showed any phylogenetic or PS type signal.

*Broader Impacts*

Two postdoctoral fellows, 5 graduate students (4 Ph.D., 1 M.S.), 7 undergraduate students and 1 high school student (9 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 13 (8\*, 5\*†) invited seminars or symposium talks, 13 (4\*, 9\*†) contributed talks and 5 posters (3\*, 1\*†, 1†) at national and international meetings/institutions. Other products include an interactive software package for developing botanical keys (\*Attigala et al. 2016b) and a protocol for digitally modeling small plant structures using computer-aided design software (\*†Klahs et al. 2018).

*Publications* *and Other Products*

To date, we have published 15 papers (12\* and 3\*†) and 1\*† is currently in 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 are in the process of selecting representative slides and images to be archived at the Cornell University Plant Anatomy Collection/Website.