The evolutionary consequences of habitat change in grasses

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Abstract

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Grass dominated habitats cover more than 40% of Earth's total land surface. These ecosystems support diverse faunal communities, play a significant role in global nutrient cycling, and provide many other important ecosystem services. However, they are also at significant risk globally, and conserving these them requires a more complete understanding of their unique evolutionary histories.

The initial expansion of grassland habitats and subsequent assembly of modern grassland floras were among the most important ecological transitions of the past 66 million years. Despite an extensive body of work exploring the impacts of this transition (e.g., on mammalian faunas) few studies have examined the effects of this ecological shift on grasses themselves. Using a combination of modern and fossil data, I investigated how the transition from closed to open, grass dominated habitats helped drive the evolution of two key grass traits: herbivore defense and seed dispersal strategy.

Although a number of changes are apparent in the family (e.g., increased adoption of wind dispersal in more open habitats), the results demonstrate the complexity of responses, showing very little consistency across the family. This reinforces the need to consider both group (e.g., tribe) and context (e.g., North American grasslands) to understand the evolutionary changes experienced by grasses during the establishment and expansion of grassland ecosystems.

INTRODUCTION:

Grass dominated habitats cover more than 40% of Earth's total land surface (Shantz, 1954; Gibson, 2009). These ecosystems support diverse faunal communities, play a significant role in global nutrient cycling, and provide many other important ecosystem services (Street-Perrot and Barker, 2008; Gibson, 2009). Because of high soil fertility, grasslands are often converted for agricultural use (Shantz, 1954; Samson et al., 2004; Brown et al., 2005; Gibson, 2009), and, as a result, they possess a higher Conservation Risk Index than any other terrestrial biome (Gibson, 2009). Indeed, in North America, native tall-grass prairie has been reduced to approximately 10% of its original extent, with an overall 70% reduction in the native grasslands of the Great Plains (Samson et al., 2004). Conserving these critically important habitats requires a more complete understanding of their unique evolutionary histories, and the evolution and ecology of their constituent grass dominants.

Phylogenetic studies and fossil evidence support a Cretaceous age, and Gondwanan origin for grasses (Linder, 1987; Jacobs et al., 1999; Bremer, 2002; Prasad et al., 2005; Bouchenak-Khelladi et al., 2010). Nevertheless, the assembly of the first grassland ecosystems did not occur until the latter half of the Cenozoic, when grass dominated habitats spread across the continents starting in the late Oligocene-early Miocene (Strömberg, 2011; Strömberg et al., 2013). The initial expansion of habitats dominated by C_3 grasses, and the subsequent establishment of C_4 grasslands (e.g., Edwards et al., 2010; McInerney et al., 2011) were among the most important ecological transitions of the past 66 million years. Despite an extensive body of work exploring the impacts of this transition (e.g., on mammalian faunas; Webb, 1977; Stebbins, 1981; Strömberg, 2006) few studies have examined the effects of this ecological shift on grasses themselves. Using a combination of modern and fossil data, I investigated how the transition from closed to open, grass dominated habitats helped drive the evolution of two key traits: herbivore defense and seed dispersal strategy. Work consisted of three chapters which respectively comprised:

1) A reappraisal of the classic hypothesis (e.g., Solounias & Semprebon, 2002; Bouchenak-Khelladi et al., 2009) that grasses possessing the C_4 photosynthetic pathway evolved to accumulate high concentrations of biosilica as an herbivore deterrent, through coevolution with grazers in grassland environments.
2) An evaluation of the degree to which habitat type influenced the evolution of traits facilitating wind dispersal of seeds within the melicgrass clade. An additional aim was to establish a methodological approach for investigating the evolution of dispersal strategy, which could be applied to larger, more complex datasets.

3) An investigation of the evolution of dispersal strategy in response to transitions into grassland environments, evaluated across the grass family and incorporating both modern and fossil taxa.

Together the results of these studies demonstrate the complexity of responses to the occupation of grassland habitats, showing very little consistency of response across the family. Results reinforce the need to consider both groups (e.g., tribe) and context (e.g., North American grasslands) to develop a clearer picture of the evolutionary changes experienced by grasses during the establishment and expansion of grassland ecosystems.

CHAPTER 1: High silicon concentrations in grasses are linked to environmental conditions and not associated with C_4 photosynthesis

INTRODUCTION:

Phytoliths are microscopic bodies of solid, amorphous silica (SiO_2*nH_2O) that are deposited in plant tissues, and represent the major sink of silicon (Si) within most plants (Richmond and Sussman, 2003; Piperno, 2006). Members of all major plant lineages produce phytoliths, although the degree of deposition differs substantially between taxa (Hodson et al., 2005; Piperno, 2006; Katz, 2015; Trembath-Reichert et al., 2015; Strömberg et al., 2016). Several groups typically accumulate high concentrations of phytoliths, with grasses (Poaceae) being a particularly prominent and well-studied example (Jones and Beavers, 1964; Kaufman et al., 1985; Sangster et al., 2001; Hodson et al., 2005). Within grasses, silicon (deposited in phytoliths or as a bio-active element) has been linked to various functions, including increased pathogen resistance (Datnoff et al., 1997; Fauteux et al., 2005), as well as the alleviation of heavy metal toxicity (Sangster et al., 2001; Imtiaz et al., 2016), drought stress (Hattori et al., 2005; Kaya et al., 2006; Pei et al., 2010; Meunier et al., 2017), and salt stress (Imtiaz et al., 2016). However, it is the hypothesized role of phytoliths as an herbivore deterrent that has received the most attention.

A large body of work examining the relationship between grass phytolith deposition and herbivore feeding behavior has shown that grasses growing in heavily grazed areas usually have elevated silica concentrations (McNaughton and Tarrants, 1983; McNaughton et al., 1985; Cid et al., 1989). Defoliation by herbivores can also lead to the induction of silica deposition, although the effect is frequently delayed and sensitive to the type and amount of damage (Massey and Hartley, 2006; Reynolds et al., 2012; Soiniene et al., 2013; Huitu et al., 2014; Quigley and Anderson, 2014; Hartley and DeGabriel, 2016). Whether inducible or constitutive, high silica concentrations significantly alter feeding behavior in a variety of herbivores. Laboratory experiments have shown that insects and small mammalian herbivores select forage based upon silica content (Gali-Muhtasib et al., 1992; Massey and Hartley, 2006; Massey et al., 2007), and experience substantial negative fitness effects when fed a high silica diet (Massey et al., 2006; Massey and Hartley, 2006; Massey and Hartley, 2009; Reynolds et al., 2009; Huitu et al., 2014; Calandra et al., 2016).

The high degree of silicification in many grasses is an important and often underappreciated ecological aspect of the communities where they constitute the defining vegetative component (e.g., Street-Perrot and Barker, 2008; Song et al., 2017). Grasslands are an important component of the global Si cycle, and their Neogene expansion is thought to have caused important changes to its structure

(Kidder and Gierlowski-Kordesch, 2005; Street-Perrot and Barker, 2008; Conley and Carey, 2015). Over geologic timescales, Si cycling is closely linked to the global carbon (C) cycle via silicate weathering, drawing down CO_2 from the atmosphere, and ultimately incorporating it in carbonate minerals. This process plays a central role in controlling long term trends in the concentration of atmospheric CO_2 (Street-Perrot and Barker, 2008). Over shorter time scales, carbon sequestration by Si accumulating producers, like grasses, and their impact on Si fluxes from land and, thus weathering rates, are also key links between these two geochemical cycles (Conley, 2002; Street-Perrot and Barker, 2008; Conley and Carey, 2015; Song et al., 2017). Beyond their role in global nutrient cycles, grass-dominated habitats are ecologically, economically, and culturally important, covering more than 40% of Earth's land surface, supporting diverse and charismatic faunal communities, and providing many other important ecosystem services (Shantz, 1954; Gibson, 2009). Despite their importance, grasslands and savannas are frequently converted for anthropogenic use (e.g., agriculture). In North America, alone, native tall-grass prairie has been reduced to approximately 10% of its original extent, with an overall 70% reduction in the native grasslands of the Great Plains (Samson et al., 2004). These habitats consequently possess a higher Conservation Risk Index than any other terrestrial biome (Gibson, 2009). Nevertheless, their importance as conservation targets is often misunderstood (Griffith et al., 2017; Veldman et al., 2019. High silica concentrations in grasses have been invoked by hypotheses attempting to explain the predominance of hypsodonty (i.e., high crowned cheek teeth) in mammalian herbivores of modern, grass-dominated ecosystems. This pattern has often been interpreted as representing an adaptation for coping with increased levels of tooth-wear resulting from a grass-rich, and therefore silica-rich diet (Stirton, 1947; Stebbins, 1981; McNaughton and Tarrants, 1983; Flynn et al., 2003). However, recent paleontological evidence shows a temporal mismatch between the appearance of open grass-dominated habitats and the evolution of this putative adaptation for grazing, which has cast doubt upon the role of grass phytoliths in the evolution of hypsodonty, instead implicating other, exogenous abrasives (such as windblown dust; Jardine et al., 2012; Strömberg et al., 2013; Dunn et al., 2015; Semprebon et al., 2016, 2019). Silica in grasses also appears to be relatively ineffective at preventing feeding in large mammals in comparison to its deterrent effect on small mammals and insects (Cid et al., 1989; Massey et al., 2009; Hartley and DeGabriel, 2016; Strömberg et al., 2016).

Today, the most conspicuous habitats with abundant large grazers (e.g., Serengeti) are grasslands and savannas dominated by grasses possessing the C_4 photosynthetic pathway. C_4 photosynthesis describes a coordinated suite of anatomical and biochemical modifications to the ancestral C_3 photosynthetic pathway, that concentrate CO_2 around the key photosynthetic enzyme

RuBisCO. Doing so minimizes the energetically costly process of photorespiration, which is initiated when RuBisCO accepts O_2 (rather than CO_2) as a substrate (Sage and Monson, 1999; Gibson, 2009). C_4 photosynthesis, allows for greater photosynthetic efficiency under certain environmental conditions, most notably in habitats that are hot (RuBisCo's oxygen affinity is temperature dependent) and dry (plants are able to maintain a lower stomatal conductance for a given photosynthetic output, thus limiting water loss), though C4 plants are not restricted to these habitats (Ehleringer, 1978; Ehleringer et al., 1997; Sage and Monson, 1999; Gibson, 2009; Osborne and Freckleton, 2009; Watcharamongkol et al., 2018). Because C_4 grasses are dominant in many heavily grazed habitats, and because their dominance can be dependent upon grazers (Owen-Smith, 1989; Sage, 2001; Bouchenak-Khelladi et al., 2009), some authors have hypothesized that C_4 grasses have experienced stronger selection for this defense than C_3 lineages, resulting in higher silica concentrations in the former (Bouchenak-Khelladi et al., 2009). A number of observations are apparently consistent with this hypothesis (hereafter referred to as the 'C_4-grazer hypothesis'). Early studies investigating the role of silica in grass-herbivore dynamics did observe high silica concentrations in many of the C_4 grasses native to these habitats (e.g., McNaughton and Tarrants, 1983; McNaughton et al., 1985). Investigations of tooth wear in ungulates have also noted differences in wear patterns between grazers of C_4 dominated regions and those of other herbivores (Soulonias and Semprebon, 2002). The 'C_4-grazer hypothesis' was also likely influenced by the observation that C_4 grasses are generally less digestible than C_3 grasses, although this disparity is primarily related to differences in leaf anatomy, rather than silica concentrations (e.g., Caswell et al., 1973; Caswell and Reed, 1976; Wilson and Hattersley, 1983; Wilson et al., 1983; Heidorn and Joern, 1984; Bernays and Hamai, 1987). However, the few studies that have directly tested the 'C_4-grazer hypothesis' have returned mixed results. Bouchenak-Khelladi et al. (2009) recovered a trend towards higher silica concentrations among C_4 grass lineages, particularly within the Aristidoideae and Chloridoideae subfamilies, while McInerney et al. (2011) and Strömberg et al. (2016) found no differences between C_3 and C_4 lineages. To our knowledge, no other mechanism has been proposed to explain potential differences in silica content based upon photosynthetic pathway.

One drawback, shared by all of the studies testing the 'C_4-grazer hypothesis', is a lack of robust control for growth conditions (but see Hodson et al., 2005 and McInerney et al., 2011 for methods intended to correct for this). This is an important consideration, because environmental conditions such as soil type, nutrient availability, temperature, and water availability all impact silica uptake and deposition (Deren et al., 1992; Soininen et al., 2013; Hartley and DeGabriel, 2016; Quigley et al., 2020). Temperature and water availability are of particular interest because C_3 and C_4 grasses tend to dominate in regions that differ with respect to these two climatic variables, with C_4 grasses being more abundant in hot and (seasonally) dry areas (Teeri and Stowe, 1976; Ehleringer, 1978; Ehleringer et al., 1997; Sage and Monson, 1999; Edwards and Still, 2008; Gibson, 2009; Osborne and Freckleton, 2009). Previous work has suggested that drier conditions tend to promote silica deposition (Johnston et al., 1967; Blecker et al., 2006; Power et al., 2016; Issaharou-Matchi et al., 2016; Quigley et al., 2017), and temperature appears to have a positive relationship with silica deposition, although this relationship is not always consistent (Barber and Shone, 1966; Mitani and Ma, 2005; Johnson and Hartley, 2018; Johnson et al., 2019). However, neither of these two effects has been examined in more than a handful of species.

Using a new dataset compiled from grasses grown under controlled conditions in growth chambers (Atkinson et al., 2016), we examine whether **(1)** photosynthetic pathway influences the leaf concentration of Si (as a proxy for silica), with the prediction that C_4 grasses accumulate more Si than C_3 lineages. We also examine whether **(2)** adaptation to grassland habitats influences Si concentration (hereafter the 'grasslands hypothesis'), with the prediction that taxa typically occupying grassland and savanna habitats deposit more Si than grasses restricted to other habitat types (e.g., forest understory). This represents a generalization of the 'C_4-grazer hypothesis' that accommodates paleontological evidence that the habitat types in which many grazing ungulates emerged were dominated by C_3 rather than C_4 grasses for most of their histories (Strömberg, 2011). Finally, using plants grown under different temperature and watering regimes, we test whether **(3)** water availability and temperature affect the level of Si deposition, and whether the temperature and water conditions to which species are adapted impact these responses.

MATERIALS AND METHODS:

Sample Collection

Leaf material for use in elemental analyses was sourced from grasses grown in growth chambers at the University of Sheffield as part of the study by Atkinson et al., (2016), which was focused on differences in growth patterns between C_3 and C_4 taxa. Individuals sampled in this study were grown under three different treatments, hereafter referred to as standard, reduced temperature, and reduced watering treatments. Standard growth conditions were a 14-hour day with a daytime temperature of 30°C and a nighttime temperature of 25°C, comparable to growing season temperatures in some tropical savannas (e.g., Darwin, Australia or Campo Grande, Brazil; Global Climate Normals 1961-1990). In the reduced temperature treatment the daytime and nighttime temperatures were reduced to 20°C and 15°C respectively, conditions approximately equivalent to growing season temperatures in parts of northern Europe, albeit with a smaller range in daily temperature (Global Climate Normals 1961-1990). Under all but the reduced watering treatment, plants were given a non-limiting supply of water, with plants watered twice daily (Atkinson et al., 2016). Under the reduced watering treatment, this was reduced in frequency to twice a week. All plants were provided with a non-limiting nutrient supply, via the application of Long Ashton nutrient solution (Hewitt, 1966) two times per week. Plants were grown in a 90/10 vermiculite/sand mixture, which was the sole source of silicon available to plants (Atkinson et al., 2016). Though the nutrient solution did not provide any Si, Long Ashton is slightly acidic (~4.5), and pH does influence Si availability (Imtiaz et al., 2016; Quigley et al., 2017). However, this trait does not appear to influence the pH of the actual soil solution in the root zone of growing plants (Smith et al., 1983).The reduced temperature and watering treatments were conducted subsequent to the standard treatment using the same growth chambers. Unfortunately, because of the large number of plants and limited space in growth chambers (see Atkinson et al., 2016), it was not feasible to replicatethese two environmental treatments. Additional treamtments that examined the combined effects of temperature and water availability, or included longer growth periods were also infeasible for these reasons.. From here on we discuss the effects of temperature and water treatments, recognizing that other, unmeasured environmental variables may also have differed between them.

Plants were harvested, dried, and leaf material collected. Whenever possible, the entire lamina, or the laminae of multiple leaves, was collected. This ensured that concentration estimates were robust to problems associated with heterogeneity in phytolith distribution across the lamina (Sangster, 1977; Hodson and Sangster, 1988; O'Reagain and Mentis, 1989; de Melo et al., 2010), which may confound other methods of quantifying Si deposition (e.g., Bouchenak-Khelladi et al., 2009; Supporting Information Methods S1). From standard growth conditions, we sampled 274 individuals, representing 124 species, 69 genera and 20 tribes. This sample included at least six lineages that independently evolved C4 photosynthesis (Aliscioni et al., 2012; Spriggs et al., 2014), but excluded the subfamilies Arundinoideae, Bambusoideae, and the grade of early-diverging grasses (Anomochloideae, Pharoideae, Pueliodeae). However, as plants in these subfamilies all differ substantially in habit from the grasses that are the primary subject of the 'C4-grazer hypothesis', their exclusion is not seen as a major bias. A subset of the species in the standard treatment dataset was also sampled from the reduced temperature (82 individuals, 36 species), and reduced watering treatments (134 individuals, 53 species). Sample sizes were largely driven by what material was available in sufficient quantities for elemental analysis (i.e., ≥ 100 mg), but was comparable in number of taxa to previous studies, although differing slightly in coverage (Supporting Information Methods S1). In the reduced temperature treatment, three species (*Brachypodium distachyon* [L.] P. Beauv., *Poa arida* Vasey, and *Puccinellia distans* [Jacq.] Parl.) lacked counterparts from the standard dataset, while two species (*Brachypodium distachyon* and *Puccinellia distans*) from the reduced watering treatment lacked counterparts in the standard dataset. In both cases this was because sufficient material could not be collected from individuals grown under standard conditions.

Plants used in this study were harvested between one and five weeks after germination to allow for the determination of growth rates across the study species (the central focus of the original study; Atkinson et al., 2016). This setup, coupled with the sampling goals of our study (i.e., large number of species, multiple individuals per species) meant that we ultimately sampled leaves from plants of a variety of ages. Sampled individuals ranged in age from 10 to 60 days post germination (median 38), with the vast majority (83%) between 30 and 45 days (Supporting Information Table S1). There was also substantial variation in total dry mass of sampled plants, (0.03 to 9.71 grams, median 0.53), although only a small proportion of this variation could be attributed to differences in age (Supporting Information Methods S1).

Silicon Concentration Analysis

Estimates of bulk Si concentration were obtained using a portable X-ray fluorescence spectrometer (P-XRF) at the University of York, UK, following the methods of Reidinger et al. (2012). XRF provides accurate estimates of the concentration of elemental silicon (Reidinger et al., 2012), here used as a proxy for the concentration of silica within the leaf. While silicon may exist in other forms in plant tissues, it is primarily deposited as silica phytoliths (Richmond and Sussman, 2003; Hartley et al., 2015; Hartley and DeGabriel, 2016; Imtiaz et al., 2016), the concentration of which is strongly correlated with the abrasiveness of forage (Massey and Hartley, 2006; Massey et al., 2006; Massey et al., 2007; but also see Hartley et al., 2015). In addition, XRF provides comparable estimates, with the benefit of being faster, safer, and often more precise, than traditional gravimetric approaches for estimating silica concentration (Reidinger et al., 2012). Prior to analysis, dried leaf material was homogenized using a ballmill (Qiagen TissueLyser II), and pressed into 13 mm pellets using a manual hydraulic press (Atlas 15T; Reidinger et al., 2012) at 11 tons. To partially account for *u*-Drift in the instrument (i.e., variation in measured response between consecutive runs using identical parameters; Johnson, 2012; Reidinger et al., 2012), each pellet was analyzed twice, approximately 30 minutes apart, with a reading taken from each side of the pellet. Si concentrations for individuals were obtained by averaging these values. For species represented by more than one individual, intraspecific variation in Si concentration was quantified using the coefficient of variation (CV) of untransformed Si concentration values.

Classification of Variables

Species were classified as either C_3 or C_4 (Osborne et al., 2014). Of the 124 species from the standard conditions dataset, 34 were C_3, and 90 were C_4. In the reduced temperature dataset, there were 19 C_3 species and 17 C_4 species. The reduced watering dataset contained 20 C_3 species and 33 C_4. We also considered several variables related to each species' "selective environment" (i.e., the conditions in which each species typically occurs; Brandon, 1990). These were habitat type (grassland/savanna, other), habitat wetness (wet, dry, broad), and the mean annual precipitation (MAP_S) and mean annual temperature (MAT_S) averaged across each species' range. To record MAP_S and MAT_S, species occurrence data were downloaded from the Global Biodiversity Information Facility (GBIF.org) using the *rgbif* package (v. 1.3.0; Chamberlain et al., 2017) in the R software environment (v. 3.6.1; R Core Team, 2019). Records that were deemed unreliable were excluded from this dataset (Supporting Information Methods S2) using tests available in the R package *CoordinateCleaner* (v 2.0-11; Zizka et al., 2019). For each observation, MAT and MAP were extracted from WorldClim 2 climate surfaces (Fick and Hijmans, 2017) and then averaged to the species level. Classification of habitat type and habitat wetness was completed via a literature survey (Supporting Information Methods S2). Habitat wetness was based upon the local soil water conditions favored by each taxon; for example, a species could be classified as "wet" despite occupying a region with a low MAP (i.e., restricted to wetlands in a dry region).

Statistical Analysis

Statistical analysis was completed in the R software environment (v. 3.6.1; R Core Team, 2019). Silicon concentration was noticeably right hand skewed, and was therefore natural log-transformed prior to all analyses, unless stated otherwise. All phylogenetic comparative analyses employed the maximum credibility tree of Atkinson et al. (2016). The phylogenetic signal of Si concentration was measured with Pagel's λ (Pagel, 1999) in the package *phytools* (v. 0.6.99; Revell, 2012). The effects of all predictors were tested by fitting multivariate generalized linear mixed models (glmm) using Markov chain Monte Carlo (MCMC) sampling, as implemented in the package *MCMCglmm* (v. 2.29; Hadfield, 2010). Phylogenetic information was incorporated by including an inverted phylogenetic covariance matrix as a random effect in each model. All other variables were included as fixed effects. Models were given weakly informative priors, and, for each, a single chain was run for a total of 100,000 generations, sampling every tenth generation, and discarding the first 3,000 generations as a burn-in. Trace plots and effective sample sizes were inspected to ensure adequate convergence upon the posterior. For each model, the coefficient of determination of the fixed effects, and its 95% confidence interval, was obtained following the method of Nakagawa and Schielzeth (2013).

We tested whether plant age and/or mass (the latter natural log transformed) affected Si concentration in each dataset, and included them as covariates in subsequent models, where appropriate (see results). The effect of temperature treatment was tested in taxa grown under both standard and reduced temperature conditions, while the effect of watering treatment was tested in taxa grown under both standard and reduced watering conditions. We tested for interactions between temperature condition and MAT_S, and watering condition and both MAP_S and habitat wetness to assess whether the conditions to which species were adapted modified their response to changes in growth condition. The 'C_4-grazer' and 'grasslands' hypotheses were tested in all three growth treatments, with photosynthetic pathway and habitat type (respectively) as fixed effects. In all three treatment groups, we also tested a model including all five predictors (photosynthetic pathway, habitat type, MAT_S, MAP_S, habitat wetness) as fixed effects.

Character Evolution Model Fitting

Five models of character evolution were fit to species average Si concentration using the *OUwie* package (v. 1.5; Beaulieu and O'Meara, 2016). For this analysis, we treated C_3 and C_4 taxa as occupying separate evolutionary regimes (where possible). The models compared were a single rate Brownian Motion (BM) model (BM1), a multi-rate BM model (BMS) with different rates for C_3 and C_4 lineages, an Ornstein Uhlenbeck (OU) model with a single universal optimum (OU1), an OU model allowing for different optima for C_3 and C_4 lineages (OUM), and an OU model allowing for differences in optima and rate in C_3 and C_4 lineages (OUMV). These same models were fit using habitat type to define two evolutionary regimes (grassland/savanna, other). Note that, for the latter analysis, OUMV models were discarded as not all eigenvalues from the decomposition of the Hessian matrix were positive, indicating that parameter estimates were unreliable. For the same reason, OU models allowing the alpha parameter to vary between regimes (i.e., OUMA and OUMVA) were excluded from our analyses.

Prior to analysis, Si concentration values were shifted (i.e., Si% + 1) before natural log-transformation (excluding negative values increased model stability). Evolutionary regimes at internal nodes were inferred based on the results of ancestral state estimation of photosynthetic pathway and habitat type, which was completed via stochastic mapping (Supporting Information Methods S3;

Nielsen, 2002; Huelsenbeck et al., 2003) in the *phytools* package (v. 0.6.44; Revell, 2012). After models had been fit, the maximum likelihood parameter values were extracted and used to perform 1,000 parametric bootstrap replicates of each model. Weighted Akaike Information Criterion (AIC) values were calculated for models with the R package *MuMIn* (v. 1.42.1; Barton, 2018) and used to obtain the weighted average (and its standard deviation) for each model parameter from the bootstrap output for each of our predefined evolutionary regimes. These were subsequently used to compare regimes. Model fitting was only carried out using the largest of our three datasets (i.e., standard treatment) due to sample size limitations, OU models being especially sensitive to small sample sizes (Cooper et al., 2016).

RESULTS:

Patterns of Si Concentration

Silicon concentration estimates showed a large degree of variation under all three treatments, with several taxa showing very high concentrations, especially when considering the relatively young age of plants. Under standard conditions, Si concentration ranged from 0.2 to 5.2%, with a mean of 1.1%. The reduced watering treatment showed a similar range in values (0.3% to 4.6%) although the mean value was slightly higher (1.3%). In contrast, the higher Si concentrations observed under standard and reduced watering conditions were absent in the reduced temperature treatment (0.2 to 1.4%), which resulted in a lower mean concentration (0.7%). In general, taxa showing high Si concentrations under one treatment showed high Si concentrations under other treatments, although; this pattern was less pronounced in the reduced temperature treatment (Supporting Information Notes S1).

Sample Collection and Phylogenetic Signal

Under standard conditions, plant age and mass both showed a positive relationship with Si concentration (age: $p = 0.003$, mass: $p = 0.013$), although the combined effects only explained a small proportion of the variation ($R^2 = 0.026$ [95% CI: 0.008, 0.048]). The effect of plant mass was also significant under reduced temperature ($p = 0.032$), although this was only apparent when age ($p = 0.075$) was also included, with neither significant on its own (age: $p = 0.41$, mass: $p = 0.63$). Together, age and mass explained only a small proportion of the variation in Si concentration, although the coefficient of determination from the posterior sample varied quite a bit ($R^2 = 0.055$ [95% CI: << 0.001, 0.129]). Neither mass nor age were significant under reduced watering (age: $p = 0.86$, mass: $p = 0.63$).

Mass and age were therefore included as covariates in analyses using the standard and reduced temperature datasets, and discarded for those using the reduced watering dataset.

Among species grown under both standard and reduced temperature treatments (i.e., those for which the effect of temperature was assessed) only plant mass was significant (age: $p = 0.21$, mass: $p = 0.003$), although its effect disappeared when other predictors were included in the model. For species grown under both standard and low watering treatments, age ($p = 0.003$) and mass ($p = 0.017$) individually showed a significant effect, although the effect of mass was not significant when age was accounted for ($p = 0.33$). However, age on its own did not explain much of the variation in Si concentration ($R^2 = 0.013$ [95% CI: 0.013, 0.028]). Nevertheless, plant age was included as a covariate for analyses assessing the effects of watering changes; mass was discarded.

Tests of phylogenetic signal returned significant results under standard ($\lambda = 0.56$, $p < 0.001$) and reduced watering conditions ($\lambda = 0.93$, $p < 0.001$). Phylogenetic signal was strongest under reduced watering conditions, with a value of λ close to the expectation under a Brownian Motion model of evolution (i.e., $\lambda = 1$). Under reduced temperature conditions, however, the estimated value of λ was lower than in the other two datasets, and not significantly different from zero ($\lambda = 0.29$, $p = 0.07$).

Effect of Photosynthetic Pathway and Selective Environment on Si Concentration

Under standard conditions (Fig. 1), the effects of photosynthetic pathway ($p = 0.22$) and habitat type ($p = 0.29$) on Si concentration were not significant. However, in models including all of our predictors, the effect of MAT_S was significant ($p = 0.019$), with species from warmer regions having higher Si concentrations. A model including MAT_S as the only fixed effect (in addition to age and mass) supported the same effect ($p = 0.014$). However, in this model, the fixed effects explained only a small portion of the total variation in Si concentration ($R^2 = 0.043$ [95% CI: 0.014, 0.077]).

Under reduced temperature conditions, the effect of photosynthetic pathway was non-significant ($p = 0.65$), as was the effect of habitat type ($p = 0.93$). Results under reduced watering conditions were similar, with photosynthetic pathway ($p = 0.081$) and habitat type ($p = 0.111$) non-significant. Under neither reduced temperature nor reduced watering conditions was any of our five predictors (photosynthetic pathway, habitat type, MAT_S, MAP_S, habitat wetness) found to be significant.

Character Evolution Model Fitting

When evolutionary regimes were defined by photosynthetic pathway, three models contributed substantially to our final weighted parameter values, with OU models heavily favored over Brownian

Motion (weighted AIC values - BM1< 0.01, BMS< 0.01, OU1= 0.36, OUM= 0.30, OUMV= 0.34). Brownian Motion represents random evolution of a character while OU models modify this so that trait values are drawn to an "optimum" value. The better fit of OU models to our data, however, may be related to other factors (e.g., intraspecific variation) causing our data to deviate from a strict Brownian model, rather than evidence that Si concentration evolved following an OU model (Cooper et al., 2016). Theta (θ) represents the "optimum" evolutionary value of a trait in an OU model (i.e., the value toward which species are pulled), with the OUM and OUMV models allowing differences in this parameter between C_3 and C_4 regimes. Weighted θ values for C_3 and C_4 regimes were similar ($C_3 = 0.54 \pm 0.19$, $C_4 = 0.58 \pm 0.22$), with substantial overlap in the distribution of values between the two regimes (Fig. 2). Sigma squared (σ^2) values represent the rate of change in a trait; of the models applied here, the BMS and OUMV models allow variation in σ^2 values between regimes. Final, weighted σ^2 values were lower in C_4 taxa ($C_3 = 18.1 \pm 23.1$, $C_4 = 14.8 \pm 19.4$), although there was again substantial overlap in the distribution of values between regimes (Fig. 2). Alpha (α) represents the strength with which values are drawn towards the optimum in each regime. Because OUMA and OUMVA models were unstable (see Methods), final α values for the C_3 and C_4 regimes were identical ($C_3 = 123.1 \pm 145.3$, $C_4 = 123.1 \pm 145.3$).

When regimes were defined based on habitat type, the OU1 and OUM models were favored, with BM models again being rejected (BM1< 0.01, BMS< 0.01, OU1= 0.73, OUM= 0.27). However, in contrast to our photosynthetic pathway results, which did not favor any single model, we found that the OU1 model had weighted AIC values much higher than any other model. This model treats grassland and non-grassland taxa as belonging to a single, global regime. As a consequence, weighted θ (grassland = 0.50 ± 0.13, non-grassland = 0.51 ± 0.14), σ^2 (grassland = 30.5 ± 43.5, non-grassland = 30.5 ± 43.5), and α (grassland = 114.1 ± 139.4, non-grassland = 114.1 ± 139.4) parameter values were similar for both grassland and non-grassland taxa, with distributions again showing substantial overlap (Fig. 2).

Growth Conditions Effect on Si Concentration

Most species (31/36) grown in the reduced temperature treatment tended to have lower average Si concentrations ($p < 0.001$) than they had under standard conditions, with a median increase of 55% from reduced temperature to standard conditions (Fig. 3). The effects of temperature treatment explained a greater proportion of the total variation in Si concentration than plant age or photosynthetic pathway ($R^2 = 0.085$ [95% CI: 0.032, 0.146]). There was also a significant interaction between the effect of temperature treatment and MAT_S ($p = 0.015$), with larger Si concentration differences between standard and reduced temperature plants in species adapted to warmer climates (Fig. 4). In models including this interaction, the effect of MAT_S ($p < 0.001$) remained significant, although the effect of temperature treatment was no longer significant on its own ($p = 0.89$), suggesting that elevated concentration in species of warm regions under standard conditions were largely driving this effect. Fixed effects combined in this model to explain a considerable amount of the total variation in Si concentration ($R^2 = 0.194$ [95% CI: 0.082, 0.316]).

The reduced watering treatment was associated with higher Si concentrations in most of species (39/53), with a median increase of 22% in average Si concentration (Fig. 3). This effect was statistically significant ($p < 0.001$), although age and watering treatment explained only a small amount of the variation in Si concentration ($R^2 = 0.036$ [95% CI: 0.015, 0.061]). There was no significant interaction between watering treatment and MAP_S ($p = 0.66$). However, there was a weak interaction with habitat wetness (dry: $p = 0.044$; wet: $p = 0.071$), such that species adapted to dry habitats showed a slightly larger change in Si concentration under reduced watering than species of other habitats (Fig. 4). The effect of watering treatment on its own was not significant ($p = 0.101$) when accounting for this interaction. Fixed effects in this model explained a small proportion of the total variation in Si concentration ($R^2 = 0.049$ [95% CI: 0.022, 0.082]).

Intraspecific Variation in Si Concentration

In all three datasets, Si concentration varied substantially within species, with median species CV values of 17%, 19%, and 20% for the standard, reduced temperature, and reduced watering datasets respectively. Several species had intraspecific CV values comparable to, or exceeding the interspecific CV values of each dataset; however, these values are of questionable accuracy given the small sample sizes in many taxa. Small sample size promotes more extreme CV values, which don't accurately reflect the true level of variation within a species. However, the median values are unlikely to overestimate the level of variability in Si concentration, and may in fact underestimate it (Supporting Information Notes S2). There was relatively little overlap between datasets as to which species varied most in Si concentration. Only four species (*Avena sativa* L.*,* *Bothriochloa barbinodis* [Lag.] Herter, *Digitaria setigera* Roth, and *Setaria viridis* [L.] P. Beauv.) surpassed the 75th percentile in more than one dataset, and several taxa with high intraspecific CV values in one dataset fell below average in the others (e.g., *Eragrostis tef* [Zuccagni] Trotter, *Vulpia myuros* [L.] C.C. Gmel.).

DISCUSSION:

Evolution of Si Defenses in C_4 Grasses

The idea that C_4 grasses accumulate more silica than C_3 grasses appears to be relatively widely held, despite only mixed support in the literature (Kaufman et al., 1985; Bouchenak-Khelladi et al., 2009; McInerney et al., 2011; Strömberg et al., 2016). In this study, we did not find any strong evidence to suggest that Si concentration differed systematically between C_3 and C_4 grasses grown under common environmental conditions. While some C_4 taxa have high Si concentrations, this trait apparently evolved multiple times in both C_3 and C_4 lineages (Fig. 1). The idea central to the 'C_4-grazer hypothesis', that C_4 grasses experience disproportionately high grazing pressures, stems from the predominance of C_4 grasses in habitats currently sustaining large populations of mammalian grazers (e.g., McNaughton, 1976; Bouchenak-Khelladi et al., 2009; Hempson et al., 2015), where defoliation levels exceed those of most C_3 dominated habitats (Sage and Monson, 1999). However, current paleontological consensus indicates that the earliest grass-dominated habitats in many regions consisted primarily of C_3 grasses, which were only later replaced by C_4-dominated savannas and grasslands (Jacobs et al., 1999; Edwards et al., 2010; McInerney et al., 2011; Strömberg, 2011). These C_3-dominated habitats were also inhabited by diverse herbivore communities, including herding ungulates that likely fed on grasses (MacFadden, 1992; Janis et al., 2000; 2004; Mihlbachler et al., 2011; Semprebon et al., 2016; 2019). The modern association between C_4-dominance and abundant large grazers, therefore, does not reflect historic patterns (Strömberg, 2006; 2011). The emphasis that the 'C_4-grazer hypothesis' places on large mammals is also problematic. Compared to insects and small mammals, silica content does not consistently influence forage selection in large mammalian herbivores, nor does it clearly affect their fitness (Cid et al., 1989; Massey et al., 2009; Erickson, 2013; Hartley and DeGabriel, 2016; Strömberg et al., 2016). It is thus unlikely that grasses would respond to the proliferation of large grazers by increasing Si concentration.

Environmental conditions exerted a significant influence on Si concentration, with hot and dry environmental treatments promoting elevated Si concentrations. Given that C_4 taxa tend to be most abundant in hot, dry regions (Edwards and Still, 2008; Osborne and Freckleton, 2009), it is possible that previous reports of higher concentrations in C_4 grasses (Bouchenak-Khelladi et al., 2009) reflect phenotypic plasticity, instead of selection. Among C_4 grasses, Bouchenak-Khelladi et al. (2009) identified the Aristidoideae and Chloridoideae as showing pronounced increases in phytolith deposition. These subfamilies in particular are typical of hot and dry environments (Gibson, 2009; Edwards and Smith, 2010; Visser et al., 2012; Visser et al., 2014).

Overall, the available evidence leads us to reject the 'C_4-grazer hypothesis.' Even excluding the data presented here, it is consonant with neither recent ecological work nor current paleontological consensus. Given our results, we believe that climatic lineage partitioning may offer a more credible explanation for previous positive results.

The hypothesis that species occupying grass-dominated habitats may experience selection for stronger silica-based defenses (the 'grasslands hypothesis') cites the same mechanism of strong herbivore pressure in grassland environments as the 'C_4-grazer hypothesis', but relaxes the requirement that the targeted grasses possess the C_4 photosynthetic pathway. This hypothesis is therefore more consistent with recent work demonstrating that Earth's grasslands were C_3 dominated for most of their histories. The lineages that dominated early C_3 grasslands are also still important in many temperate grassland communities (Elias, 1942; Thomasson, 1985; Strömberg, 2005; 2011), potentially conserving the adaptive outcome of these selective pressures in the form of increased silica levels. However, our results did not provide any support for the 'grasslands hypothesis.'

Both the 'C_4-grazer' and 'grassland' hypotheses assume that grasses in grasslands and savannas experience higher herbivore pressure, and thus stronger selection for silica defenses, than grasses found in other ecosystems. While this seems intuitive, to our knowledge it has never been tested directly. Ecological evidence does suggest that more abundant or apparent plants (i.e., grasses surrounded by grasses) often experience higher herbivory, and are thus better defended (e.g., Root, 1973; McNaughton, 1978; Landa and Rabinowitz 1983; McLain and Shure, 1990). However, this is not always the case (e.g., Cates, 1981; Bach, 1988; Barbosa *et al.*, 2009). Furthermore, it is not sufficient that grasses experience higher herbivore pressure in grasslands; they must also experience it from herbivores for which silica is an effective defense. One potential explanation for our results is therefore that the assumption of higher herbivore pressure in grassland ecosystems (by silica-deterred herbivores), made by both hypotheses, is (and/or was) not met.

Impacts of Environmental Treatments on Si Deposition

When other factors are held constant, hot, dry conditions appear to promote the highest Si concentrations in most of the species included in this study. Previous work has shown both water availability and temperature to affect the uptake and deposition of Si (Barber and Shone, 1966; Johnston et al., 1967; Ma et al., 2002; Mitani and Ma, 2005; Blecker et al., 2006; Power et al., 2016; Issaharou-Matchi et al., 2016; Quigley et al., 2017; Johnson and Hartley, 2018; Quigley et al., 2020). These effects, however, are not always consistent. For example, Johnson and Hartley (2018) found mixed responses to an increase of 4 °C among eight Australian grasses, with only three showing a significant effect. The consistency of our results may derive from a larger temperature difference (10 °C) between treatments.

Several studies have demonstrated apparently temperature-dependent changes in the effectiveness of the machinery involved in active uptake of silicic acid (the form of Si taken up by plants) (although see Mitani et al., 2008), but using temperature changes far exceeding those employed here, with low temperatures near freezing (Barber and Shone, 1966; Ma et al., 2002; Mitani and Ma, 2005). It is unclear whether a 10 °C reduction in temperature, with a minimum temperature of 15 °C, would be enough to lower uptake rates through this mechanism alone. Temperature-driven changes in transpiration (Gates, 1968) may also contribute to differences in Si concentration. Although active processes are involved, previous work indicates that transpiration rate also impacts silica accumulation (Hartley, 2015; Kumar et al., 2017; McLarnon et al., 2017). Higher transpiration rates under hot conditions may help explain increased Si concentrations. Transpiration rates likely changed in response to watering treatment as well, although we expect them to be lower under reduced watering (where concentrations were higher), and thus are unlikely to explain the changes observed between treatments. Water availability may also affect the concentration of silicic acid in the soil solution by affecting leaching rates (with increased rates in wetter sites;Quigley et al., 2017). Plants outside our low watering treatment were watered liberally, and fertilizer did not contain a source of Si, making this an attractive explanation.

A final hypothesis worth considering is the ‘stress hypothesis’ of Johnson and Hartley (2018), which states that increases in Si concentration constitute a plastic response to stress, given silicon alleviates both temperature (Agarie et al., 1998; Wang et al., 2005; Soundararajan et al., 2014) and water stress (Ma, 2004; Hattori et al., 2005; Kaya et al., 2006; Pei et al., 2010; Chen et al., 2011; Meunier et al., 2017). The authors predict that this response would primarily benefit taxa of hot, arid environments (i.e., those most likely to experience these stressors). The observed relationship between $\mathrm{MAT_S}$ and Si concentration under standard conditions, and its dependence on temperature treatment are both explained by this hypothesis. Taxa adapted to warmer regions do show higher average concentrations, but this pattern disappears under cooler temperatures. The interaction between watering treatment and habitat wetness, where dry adapted species showed a more pronounced response to reduced watering, is also theoretically consistent with the ‘stress hypothesis’. However, this difference was slight (Fig. 4), and may not translate to a meaningful difference for mitigating drought stress.

Overall, it is difficult to determine with certainty the mechanisms driving the response of Si concentration to changes in environmental conditions. None are mutually exclusive, and several may be acting in concert. A number of the predictions made by the ‘stress hypothesis’ are, at least partially, supported by our data. However, it is unclear if treatments resulted in meaningful temperature and water stress in some species, and whether more extreme treatments should be explored. All of the mechanisms discussed here warrant further attention, but the possibility that the observed patterns may be explained by a stress mitigation strategy associated with the induction of Si deposition is particularly intriguing. Our results also raise the possibility that athropogenic modification of Earth's climate may lead to changes in the uptake and deposition of Si in grasses. Though predicted changes in temperature are unlikely to reach the magnitude of difference between our treatments, at least in the short-term, grasslands are found in regions likely to experience some of the largest increases in mean annual temperature (Collins et al., 2013). Changes to CO_2 concentrations also appear to directly impact Si deposition, but in the opposite direction, with elevated CO_2 associated with reduced Si accumulation (Johnson and Hartley, 2018). If pronounced, these climate-Si feedbacks may have meaningful ecological consequences, impacting community interactions or even Si cycling. However, how our results reflect patterns of Si uptake and deposition in natural communities remains to be established. Landscape scale variation in environmental and edaphic conditions contributes to spatial heterogeneity in Si deposition in grasses under natural conditions (e.g., Quigley et al., 2017). Changes in temperature and/or precipitation are, therefore likely to interact with the many other factors (e.g., grazing history, substrate type, taxonomic composition) that vary across natural landscapes and also influence Si concentration. While our findings reveal important information about the magnitude and phylogenetic scope of the effects that temperature and water availability can have on the deposition of Si in grasses, there is still much work to be done in understanding how these factors impact real communities.

Intraspecific Variation and Other Considerations

Our results suggest that Si concentration in grasses is quite variable within species, even when environmental conditions are controlled. Plant age and size contribute to this variation, although their effects appear to be weak under the conditions tested here. Indeed, of all possible pair wise comparisons of individuals within species, approximately half are consistent with a positive effect of age (215 of 423) or mass (240 of 417) on Si concentration. Developmental work indicates that phytolith deposition begins early in leaf development and may proceed rapidly in young leaves, before plateauing (Sangster, 1977; O'Reagain and Mentis, 1989; Piperno, 2006). Thus, for the plants considered here, leaves large enough for elemental analysis likely had similar opportunity for deposition as conspecifics, regardless of plant age or size. Whether this is also true of more mature plants, we cannot say. Indeed, some very high Si concentrations in the Poaceae appear to be the result of continual deposition and long leaf lifespans (Motomura et al., 2002). Overall, 95% of our species had CV values surpassing the approximate average intraspecific CV (5%) estimated by Simpson et al. (1960), although this was based upon zoological data. As another point of comparison, some species showed CV values comparable to or approaching those recorded for seed mass, which is generally considered a highly variable trait among plants (Jordano, 1984; Thompson, 1984; Michaels et al., 1988). High levels of intraspecific variation pose a significant sampling challenge, especially in large groups like the Poaceae. While many of our species were only represented by one or a few individuals, this is still an improvement over previous work (Supporting Information Methods S1). Additionally, one of the benefits of our mixed model approach is that we are able to consider every individual as a unique data point, and therefore better account for variation within species. Despite this advantage, we were fundamentally limited by the amount of material available for each species, and the effects of intraspecific variation, as well as age and/or size may become apparent as intraspecific sample sizes increase.

Taxon sampling is an important consideration in any comparative analysis, with small sampling differences potentially having major impacts on inferences about trait evolution (Ackerly, 2000; Salisbury and Kim, 2001; Finarelli and Flynn, 2006; Heath et al., 2008). How our results are affected is difficult to say, but the fact that they are concordant with those of Strömberg et al. (2016) and McInerney et al. (2011), despite sampling differences, is encouraging (Supporting Information Methods S1). Some of the variation in Si concentration is explained by phylogenetic history, although the strength of phylogenetic signal varies between datasets, and is strongest under the conditions promoting the highest Si concentrations (i.e., hot and dry). Phylogenetic signal may therefore reflect the Si-accumulating *potential* of species.

Phenotypic plasticity is another vital consideration for any comparative work on Si concentration. It may be accounted for by growing plants under controlled conditions. However, this comes with its own issues. When taxon sampling is broad, there may be little overlap in the preferred conditions of individual species, so many may be growing under temperatures and water regimes to which they are not well adapted. A multi-pronged approach with taxa grown under a variety of conditions, as taken here, may therefore prove the most valuable. Inference about broad trends in the evolution of Si concentration may additionally run into complications related to long-term changes in Earth's climate and atmosphere. Climatic conditions have changed substantially over the ~100 million years since grasses first evolved and began to diversify (Zachos et al., 2008; Strömberg, 2011; Gallaher et al., 2019), and it is possible that entirely non-analog conditions existed on Earth during this time (e.g., see discussion in Dunn et al., 2015). Consequently, inferences based on extant grasses growing under modern climatic regimes may not be wholly reliable for reconstructing patterns in the past. Unfortunately, as there is currently no way to make these inferences directly from the fossil record, comparative work on modern taxa is the only method available to us.

CONCLUSION:

Grassland ecosystems are of vast global importance. They provide many ecosystem services and are an important component of the global silicon cycle, which in turn is linked to global carbon cycling. Silicon also plays an important role in many of the ecological interactions in these ecosystems, notably as an important mechanical defense against grazing (in the form of silica phytoliths). We, tested the classic hypothesis that high grazing pressure in tropical grasslands and savannas led to the evolution of higher silica levels in C_4 grasses, but did not find any consistent difference in Si concentration between C_3 and C_4 grasses and thus rejected this hypothesis. The hypothesis that herbivore pressure in grass-dominated habitats more generally resulted in enhanced silica defenses in (C_3 or C_4) grassland taxa was not supported either. Growth conditions imparted a pronounced effect upon Si concentration, with hot and dry conditions promoting the highest Si concentrations in most of the sampled taxa. This pattern may ultimately explain why previous authors have found elevated concentrations in C_4 lineages. It also suggests a mechanism via which environmental conditions may influence grass-herbivore interactions. More broadly, these results add to our understanding of how global Si cycling may be altered by anthropogenic climate change.

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SUPPORTING INFORMATION:

Table S1- raw data for all analyzed samples, full caption given in Methods S1.

Methods S1 – discussion of sampling and data collection, with comparison to other recent work.

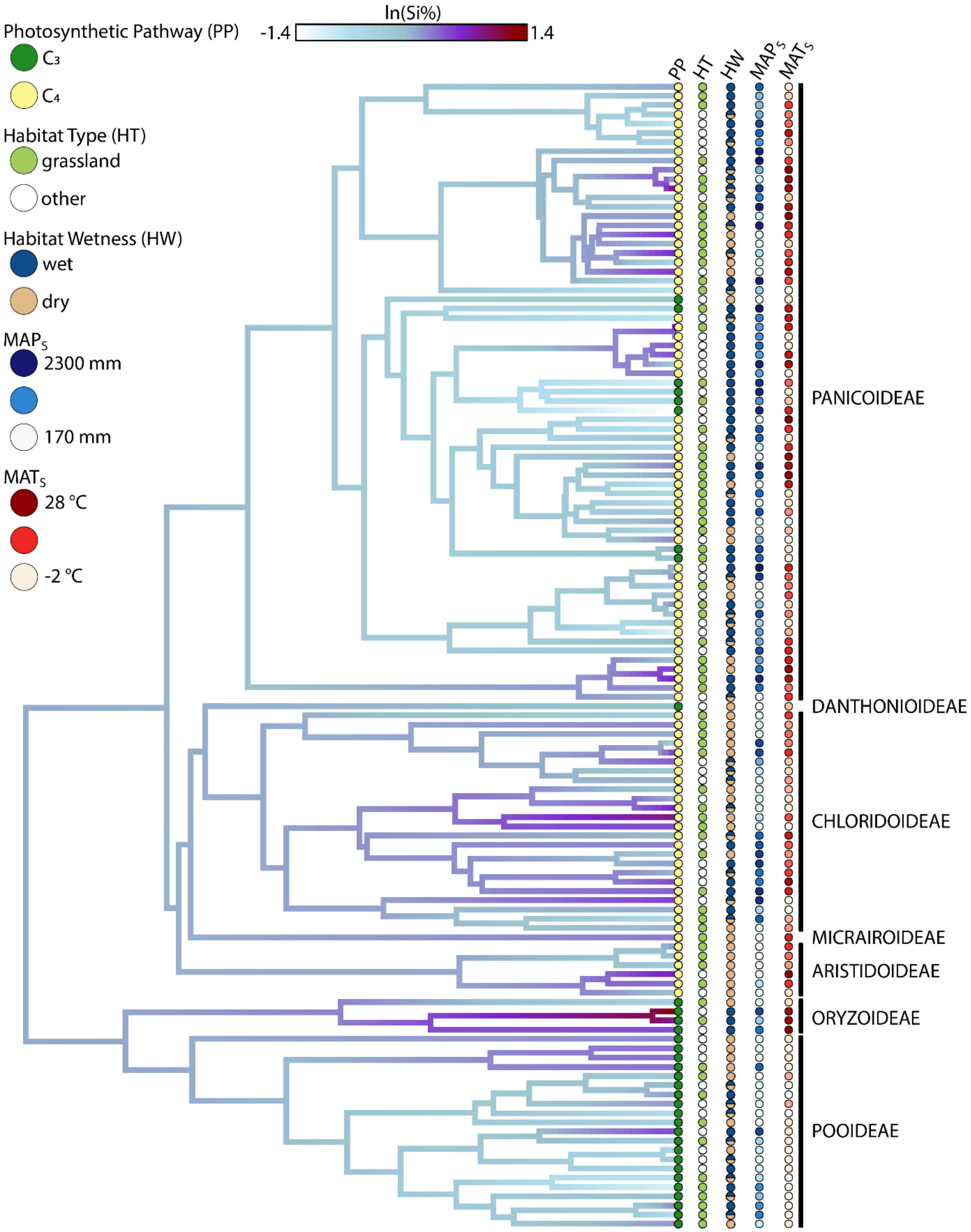
Methods S2 – elaboration on literature survey for habitat data, including excerpts from sources used to support all classification.

Methods S3 – elaboration on methods of ancestral state estimates used in character evolution models, Fig. S1, S2, S3, and S4.

Notes S1 – comparison of relative concentration within species included in multiple datasets

Notes S2 – elaboration on results related to intraspecific variation in Si concentration, and potential biases mentioned in the main text, Fig. S5, S6, and S7.

Fig. 1: Next page. Phylogenetic tree showing variation in silicon (Si) concentration for grasses grown under standard conditions, with subfamilies identified. Branch color represents ln(Si concentration), with coloring at interior nodes obtained via maximum likelihood ancestral state estimation (phytools v. 0.6.99; Revell, 2012). From left to right, colored labels at the tips represent photosynthetic pathway (PP; C_3 = green, C_4 = tan), habitat type (HT; grassland/savanna = green, other = white), habitat wetness (HW; wet = blue, dry = brown), and the mean annual precipitation (MAP_S) and mean annual temperature (MAT_S) averaged across each species' range.



Photosynthetic Pathway (PP)
C_3
C_4
Habitat Type (HT)
grassland
other
Habitat Wetness (HW)
wet
dry
MAP_S
2300 mm
170 mm
MAT_S
28 °C
-2 °C
ln(Si%)
-1.4
1.4
PP
HT
HW
MAP_S
MAT_S
PANICOIDEAE
DANTHONIOIDEAE
CHLORIDOIDEAE
MICRAIROIDEAE
ARISTIDOIDEAE
ORYZOIDEAE
POOIDEAE

Fig. 2 Comparison between predefined evolutionary regimes of weighted parameter values from the bootstrap output of the five-character evolution models tested, excluding parameters that were identical for both regimes. Error bars represent weighted standard deviation, based upon weighted Akaike information criterion scores.

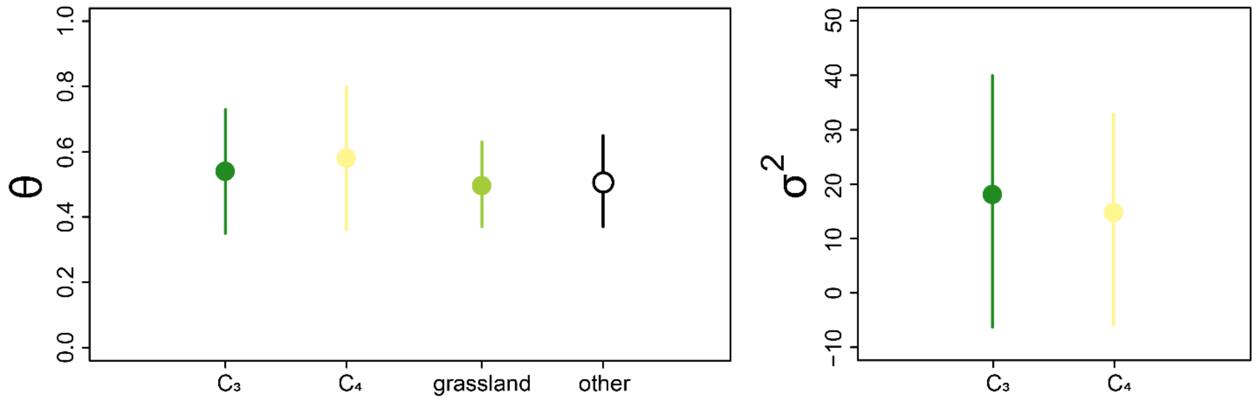


Fig. 3 Kernel density plots of silicon (Si) concentration under different growth conditions, showing only those species grown under both conditions. (a) Individuals of species grown under standard (red) and reduced temperature (blue) conditions, and (b) individuals of species grown under standard (green) and reduced watering (tan) conditions.

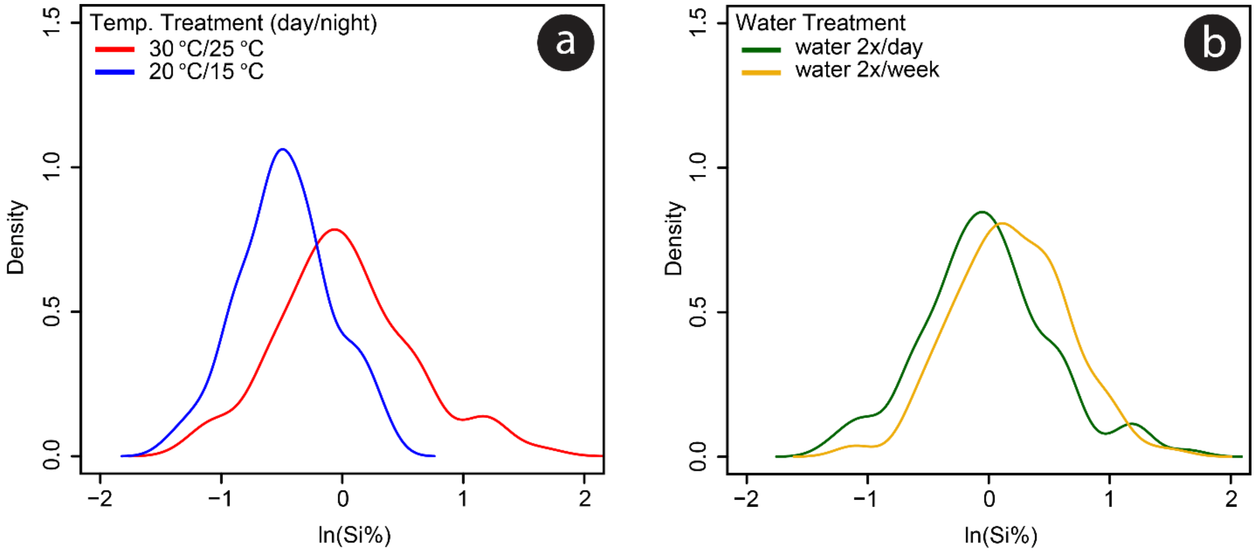
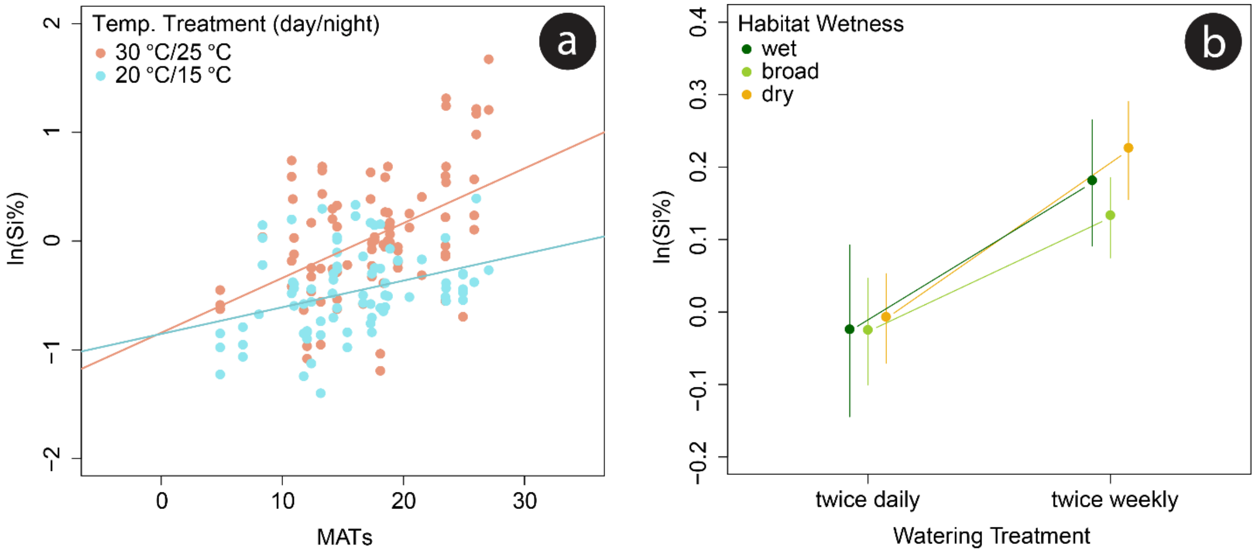


Fig. 4 Interactions between the effect of environmental treatment and species habitat preference on silicon (Si) concentration. (a) The relationship between mean annual temperature of species' range (MAT_S) and Si concentration in species grown under both standard (red) and reduced temperature (blue) treatments. (b) Average Si concentration of species adapted to wet (dark green), dry (tan), and broad (light green) habitats, when grown under standard (twice daily) and reduced watering (twice weekly) treatments. Error bars represent standard error.



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CHAPTER 2: Diverse drivers and treadeoffs shaped the evolution of wind dispersal in the melicgrass tribe (Poaceae: Meliceae)

INTRODUCTION:

Seed dispersal is an important process with broad impacts for plants and their communities. It determines the biotic and abiotic conditions that offspring encounter, and thus has major influence on seedling success (e.g., Janzen, 1970; Cheplick, 1993; Wenny, 2001; Comita *et al*., 2014). On larger scales, seed dispersal helps shape community composition, species distributions, and patterns of ecological succession (Willson, 1992; Dent and Estrada-Villegas, 2021). Plants have evolved many different strategies of dispersal, and the variety of biotic and abiotic agents they exploit has led to the great diversity of plant seed dispersal structures (diaspores), and many classic examples of adaptation (e.g., the floating fruits of *Cocos nucifera* or the "squirting" fruits of *Ecballium elaterium*; Ridley, 1930; van der Pijl, 1982). Dispersal strategy also has important conservation implications, with roles in shaping responses to anthropogenic changes (Nathan *et al*., 2011; Thurman *et al*., 2020), and mediating invasion patterns of exotic species (Junaedi *et al*., 2021; Sena *et al*., 2021). Human actions also frequently disrupt the function of dispersal networks (McConkey *et al*., 2012; Ruxton and Schaefer, 2012).

The traits that allow plants to move their seeds across the landscape have interested evolutionary biologists since the field's inception (e.g., Darwin, 1859; Stebbins, 1971; van der Pijl, 1982; Black, 2009). Nevertheless, the factors driving evolutionary shifts between dispersal strategies remain poorly understood. One factor commonly thought to strongly influence the dispersal strategy of a plant is its habitat (e.g., Stebbins, 1971; van der Pijl, 1982; Sorensen, 1986; Ozinga *et al*., 2004). This hypothesis stems from the observation that habitat types differ in both the number of seeds dispersed by a given vector (i.e., the biotic or abiotic transporting agent), and how common traits aiding in exploiting those vectors are in the flora (Harper *et al*., 1970; van der Pijl, 1982; Sorensen, 1986; Milton *et al*., 1990; Heinken *et al*., 2002; Ozinga *et al*., 2004; Iravani *et al*., 2011). The herbaceous flora of temperate forest understories, for example, contains many ant dispersed taxa (Handel *et al*., 1981; van der Pijl, 1982; Lengyel *et al*., 2010), whereas the woody flora of tropical forests is primarily dispersed by frugivorous vertebrates (Howe and Smallwood, 1982; Jordano, 2000). These patterns are often framed as being adaptive, the consequence of dispersal vectors being heterogeneously distributed between habitats (e.g., abundance of frugivores in tropical vs. temperate forests), which influences the number of seeds that are successfully dispersed (Stebbins, 1971; Janzen, 1982; Milton *et al*., 1990; Zelikova and Breed, 2008; Spasojevic *et al*., 2014). Selection for traits facilitating dispersal by more abundant or accessible vectors is expected as a result. Nevertheless, most communities include nearly the full complement of dispersal types (Ozinga *et al*., 2004), suggesting that a wide range of strategies can be successful in nearly any environment. Phylogenetic history also has a strong influence on dispersal traits and correlations between a given dispersal strategy and habitat type may result from processes other than selection (e.g., hitchhiking, lineage sorting) (Herrera, 1992; Spasojevic *et al*., 2014; Hulshof and Spasojevic, 2020; Kang *et al*., 2021). Trade-offs with other vegetative or reproductive traits may also play an important role (Nathan, 2001; Chen *et al*., 2020). The drivers of variation in dispersal strategy are therefore complex, and the abundance of a given dispersal syndrome in a particular habitat may be explained by various processes.

A major obstacle to understanding the evolution of dispersal strategy is the fact that strategy is often being difficult to define (Tackenberg *et al*., 2003; Green *et al*., 2021). Most commonly, it is summarized by assigning taxa to one of several dispersal syndromes corresponding to the dispersal vector (e.g., wind, water, vertebrates) via which they are presumed to most commonly disperse their seeds (e.g., Heleno and Vargas, 2015; Kang *et al*., 2021; Leo *et al*., 2021). Assignments may be based on observations of successful dispersal in the field, or interpretations of diaspore morphology (e.g., plants with fleshy fruit are gut dispersed). Although intuitive and easily applied, this method has many drawbacks. For instance, it makes no provision for the fact that dispersal potential can vary substantially between taxa assigned to the same dispersal syndrome (Augspurger, 1986; Augspurger and Franson, 1987; Matlack, 1987; Mouissie *et al*., 2005; Römermann *et al*., 2005; Tackenberg *et al*., 2006). Furthermore, species may disperse via multiple vectors, with taxa assigned to one syndrome posing high potential dispersal by other means (Ridley, 1930; Carlquist, 1967; van der Pijl, 1982; Fischer *et al*., 1996; Pakeman *et al*., 2002; Higgins *et al*., 2003; Vellend *et al*., 2003). In addition, this approach can be especially problematic for taxa whose diaspores do not clearly align with any traditional dispersal syndromes. These plants are often listed as having “unassisted” or “non-specialized” dispersal, and may make up the majority of a flora. However, many of these taxa possess high dispersal potentials, contrary to predictions based solely on their assigned syndrome (Heinken *et al*., 2002; Pakeman *et al*., 2002; Higgins *et al*., 2003; Myers *et al*., 2004; Calviño-Cancela *et al*., 2006; Green *et al*., 2021). There are a number of quantitative alternatives to this “syndrome-based” approach, which directly measure dispersal outcomes (e.g., Cremer *et al*., 2012) or plant traits known to influence dispersal distances (e.g., Will *et al*., 2007). Although these approaches may be limited by the difficulty with which data can be obtained (Rogers *et al*., 2019; Green *et al*., 2021), they solve many of the limitations described above. However, because of the struggle in acquiring data, there are relatively few studies of the evolution of seed dispersal that have adopted them (although see, e.g., Salazar-Tortosa *et al*., 2020).

In this study, we explore the hypothesized link between habitat type and dispersal strategy in plants, using a phylogenetic comparative approach and a quantitative framework for evaluating dispersal potential. Our model system is the ecologically diverse melicgrass tribe (Poaceae: Meliceae), which includes 158 species in seven genera (Soreng *et al*., 2015; Soreng *et al*., 2017). The Meliceae are most diverse in temperate regions (of both hemispheres) and occupy a wide range of habitats. These run the gamut from hydric to xeric and from full shade to full sun, including wetlands, forest understories, shrublands, grasslands, and disturbed habitats (Quattrochi, 2006; Barkworth *et al*., 2007). Diaspore morphology also varies considerably between species in the tribe, encompassing a wide range of size, shape, and structure, even including species with specialized appendages for attracting ants (elaiosomes; Sernander, 1906). The tribe, particularly the genus *Melica*, is also notable for including many apparent anemochores (taxa adapted to dispersing seeds via wind). Among these are species producing plumose diaspores (Russell and Ellis, 1982; Hensen and Müller, 1997), and species with enlarged, membranous glumes that form wing or parachute like structures (Sernander, 1906; Ridley, 1930; van der Pijl, 1982). These morphologies suggest that anemochory was acquired independently at least twice in the tribe and thus signify convergence on this strategy.

Anemochory is well studied and easily quantified, with simple models able to reasonably approximate dispersal distance from a few plant traits (Tackenberg *et al*., 2003; Katul *et al*., 2005; Thompson and Katul, 2008). This allows relative potential for wind dispersal to be reliably estimated, regardless of a taxon's putative dispersal syndrome. As with other dispersal modes, anemochory has traditionally been linked to particular habitat types. Previous authors have especially noted the prevalence of anemochores among communities occupying early successional or disturbed habitats (Ridley, 1930; van der Pijl, 1982; Schultz *et al*., 1991; Ozinga *et al*., 2004). Among plants with stature similar to melicgrasses, anemochory is also presumed to be a strategy exclusive to open environments (Stebbins, 1971; van der Pijl, 1982; Ozinga *et al*., 2004). In both environment types the relative paucity of surrounding vegetation to disrupt airflow is presumed to increase the effectiveness of dispersal by wind, particularly for low-growing plants (Stebbins, 1971; Ozinga *et al*., 2004). Selection is hypothesized to favour traits promoting this dispersal mode in taxa more frequently found in these types of habitats.

Given the variation in both diaspore morphology and habitat type in the Meliceae, the tribe provides an ideal test case for exploring the evolution of seed dispersal in response to the environment. Because there is existing evidence for convergence on anemochory within the tribe, and because this strategy readily accommodates a quantitative approach, we focus on the evolution of wind dispersal potential. Using a dataset of dispersal traits compiled for 24 Meliceae species, we test the hypothesis that **convergence on anemochory in the melicgrasses is associated with the occupation of open and/or disturbed habitats**. We do so by testing for correlation between proxies for dispersal distance and habitat occupation within a phylogenetic comparative framework. We also consider the range of morphologies used to achieve anemochory, their phylogenetic patterns, and the degree to which competing selective pressures and phylogenetic history influence wind dispersal potential.

METHODS:

Sampling

Diaspores were collected from 24 species of the Meliceae, including 13 species of *Melica,* 5 species of *Glyceria*, 3 species of *Pleuropogon*, and the species *Schizachne purpurascens* (Fig. 1). We also included *Brylkinia caudata*, which is sister to and occasionally included in the tribe (Soreng *et al*., 2017). Sampling of dispersal structures was limited to those species for which adequate sequence data were available to obtain a well resolved phylogenetic tree (see Methods - Phylogenetic Inference). Diaspores from each of the sampled species were collected from preserved specimens in the herbaria at the University of Washington (WTU), Missouri Botanical Garden (MO), and New York Botanical Garden (NY) (Supplementary Data Table S1). The structural unit constituting the diaspore (e.g., floret, spikelet) in each species was determined via a combination of personal observation and reference to Kew's GrassBASE online flora (Clayton *et al*., 2021). Diaspore structure was not uniform within species; therefore, we collected all structural units we thought likely to act as diaspores and treated each sample as separate data points in subsequent analyses (see Methods - Comparative Analyses). To control for diaspore maturity, we limited sampling to material that had already fallen, or fell readily, from the inflorescence and checked collection dates against published phenological data (e.g., Cope, 1982; Stubbendieck *et al*., 2017). For most species (n=19) we sampled several specimens, and multiple diaspores per specimen (Supplementary Data Table S1). This was done to account for variation in dispersal traits, both within and between individuals of the same species, which can be substantial and have a strong impact on patterns of dispersal (Teller *et al*., 2015; Snell *et al*., 2019; Wyse *et al*., 2019). However, due to limited availability of preserved material that met our sampling criteria this type of sampling was not feasible for all species. In total, we sampled 169 diaspores from 64 individuals, with an average of 7 (2-13) diaspores and 2.7 (1-6) individuals per species.

Dispersal Traits

Wind dispersal potential was estimated as a function of diaspore falling velocity and release height. Low falling velocities allow more time for wind to carry diaspores away from the parent plant and increase the likelihood of them being lifted by updrafts (Matlack, 1987; Tackenberg, 2003; Tackenberg *et al*., 2003; Soons *et al*., 2004; Katul *et al*., 2005). Greater release heights also prolong descent times and determine where diaspores are released relative to the surrounding vegetation, which influences the circulation conditions they encounter (Tackenberg *et al*., 2003; Soons *et al*., 2004; Katul *et al*., 2005). Falling velocity was measured from high-speed video (959.04 frames*s^{-1}; Sony RX100V Tokyo, Japan) of diaspores descending through an enclosed chamber (Supplementary Data Fig. S1), and calculated from the number of frames elapsed before diaspores travelled the vertical distance of the video frame, which was converted to real scale using a calibration video shot under identical conditions. Video recordings started after approximately one meter of descent and covered ~30 cm of vertical distance. Ten trials were completed for each diaspore, and the median (chosen for robustness to outliers) of these was used as an estimate of diaspore terminal velocity in subsequent analyses. Maximum average culm height, obtained from The Online World Grass Flora (Clayton *et al*., 2021), was used as an estimate of diaspore release height for each species.

From these data we estimated dispersal kernels in R (v. 4.0.5; R Core Team, 2021) using the Wald analytical long-distance dispersion (WALD) model (Katul *et al*., 2005; Thompson and Katul, 2008). Dispersal kernels are probability density functions that reflect the distance a diaspore is likely to travel from the parent given a set of environmental conditions. These were used to estimate the linear distance from the parent within which 95% of diaspores are expected to fall. We refer to this distance as the Wind Dispersal Range (WDR). The WALD model takes as its input diaspore terminal velocity, release height, and several environmental variables. The latter were kept constant for all samples, with the following values: $\bar{U}$ = 8.4 (average horizontal wind speed, m*s^{-1}), σ_w= 2.7 (standard deviation of vertical wind velocity, m*s^{-1}), h = 0.5 (surrounding vegetation height, m), κ = 0.6 (proportionality constant). These values were chosen from the literature (Katul *et al*., 2005; Thompson and Katul, 2008), and approximate a windy region with continuous herbaceous ground cover (i.e., conditions amenable to wind dispersal). Wind Dispersal Range was calculated from kernels using the *optimize* function to find the upper limit (u) of the interval, integrating over which returned a value of 0.95 (i.e., 95% of the area under the kernel).

In these analyses, we opted to control environmental model parameters, regardless of species' preferred habitat to avoid circularity in downstream models, which include habitat type as a predictor (see Methods – Comparative Analyses). As such, WDR does not necessarily reflect a prediction of expected dispersal distances under typical field conditions. Rather, it is intended to provide a measure of dispersal potential dependent only on the model parameters that are subject to direct selection (i.e., diaspore morphology and plant height). Differences in WDR between species occupying different habitats will thereby reflect differences in plant traits and, thus, the evolutionary rather than environmental changes likely to affect dispersal outcomes. However, to evaluate whether our interpretations were sensitive to variations in environmental conditions, we also present results under an alternative WALD parameterization reflecting more closed conditions ($\bar{U} = 1.55$, $\sigma_w = 0.61$, $h = 20$, $\kappa = 0.6$; Katul *et al.*, 2005; Thompson and Katul, 2008).

The terminal velocity of an object is correlated not just to its shape, but also to its mass, with more massive objects falling faster when other factors are equal. Seed mass is also a key functional trait that influences a variety of important reproductive functions (e.g., Smith and Fretwell, 1974; Jakobsson and Eriksson, 2000). To account for this as a possible influencing factor, we recorded diaspore mass using a Sartorius SE2 Ultra Micro (Göttingen, Germany) microbalance. The average of three repeated measurements for each diaspore was used as a variable in subsequent analyses.

Habitat Data

Habitat type data for each species were obtained using a geographic information system (GIS) approach. This was chosen because of the limited availability and variable quality of published habitat descriptions for many of the sampled taxa. Furthermore, because affinity for disturbed and open habitats is not binary in nature (Fensham et al., 1999; Valladares and Niinemets, 2008), the ability to treat environmental predictors quantitatively is an important benefit of the GIS approach. The volume of available GIS data and the need to reconcile contradictory published sources further informed our decision. The resulting habitat data are both quantitative, and more reproducible (and transferrable) than if we relied on investigator classification. Despite these benefits, GIS data do have a number of drawbacks (e.g., coarse spatial resolution, variable quality of records; Laris, 2005; Zizka et al., 2019). To "ground truth" our GIS data, we also completed an extensive literature survey of published habitat descriptions, and extensively compared both datasets (Supplementary Data Methods S1).

To test our hypotheses, we created three environmental variables using GIS data. Open habitat affinity was estimated from how frequently species were found in open habitats (e.g., grassland, shrub) relative to forests. Disturbed habitat affinity was estimated from species' relative association with fire prone and anthropogenically modified sites. Fire is a common and extremely important disturbance in many grass-dominated habitats (e.g., Bond and Keeley, 2005; Lehmann et al., 2014), and in

Mediterranean climate regions (e.g., Rundel et al., 2018) where melicgrasses are often found (Quattrochi et al., 2006). Although anthropogenic disturbance is unlikely to be a direct evolutionary driver for most disturbance associated traits in the Meliceae (given its stem age of ~40 Ma, Gallaher et al., 2019; Schubert et al., 2019), species commonly found at anthropogenic sites tend to be those with pre-existing adaptations to disturbed habitats, as appears to be the case for many herbaceous weeds (De Wet and Harlan, 1975; Radosevich et al., 2007; Supplementary Data Fig. S4; although see Mahaut et al., 2020).

Occurrence records for each of our species were downloaded from the Global Biodiversity Information Facility (GBIF; Supplementary Data Methods S1). Searches were manually limited to the native range of each species (with reference to standard handbooks; Supplementary Data Methods S1). Records were only kept if associated with relatively modern collections (arbitrarily set as 1950 - present), and we excluded observations associated with poor quality taxonomic or geospatial data using GBIF issue tags and tests available in the R package *CoordinateCleaner* (v 2.0-11; Zizka *et al*., 2019; Supplementary Data Methods S1). From the resulting dataset we used only unique locations for each species, to prevent sites with multiple individuals from disproportionately influencing habitat inferences (Reichgelt *et al*., 2018).

For each of our resulting occurrence records we extracted the land cover type it occurred in using v. 2.07 of the European Space Agency (ESA) Climate Change Initiative Land Cover (CCI-LC) maps (300-meter resolution, covering the years 1992 – 2015; ESA, 2021). These land cover data were extracted after matching occurrence records to the surface of the appropriate year, with records pre- or postdating this range assigned to the earliest and latest maps respectively. The UN Land Cover Classification System (LCCS) categories used by this scheme were combined to produce a more general classification system with classes: anthropogenic, forest/woodland, grassland/mosaic, hydric (e.g., wetlands), shrubland, and sparse/bare (e.g., tundra) (Supplementary Data Table S2). For all species, the proportion of occurrences found in each class was calculated and subsequently transformed using amalgamation (summed) log-ratio (SLR) transformations, because proportional data do not conform to the assumptions of most statistical tests (Aitchison, 1982; Supplementary Data Fig. S2). Class proportions equal to zero (undefined on log-ratio transformations) were changed to a value of 0.001 and values rescaled to ensure all proportions summed to one prior to transformation. This analysis was completed using the R package *easyCODA* (v. 0.34.3; Greenacre, 2018). We supplemented these data with information extracted from burned area occurrence maps also published by the ESA CCI Land Cover project (ESA, 2021). Weekly burn data from 2000 – 2012 were extracted for each occurrence, from which we calculated the proportion of records per species found at sites that burned at least once during the sample period. We discarded sites that we deemed as possessing an inadequate number of records (i.e., > 50% of weekly records absent), which amounted to approximately 20% of total occurrence records (0% - 39% per species). Proportions were then SLR transformed, with zeros first replaced with a value of 0.0001 (smaller value reflecting the difference in scale between burn and modified UN LCCS data; Supplementary Data Table S1). Raw occurrence data with extracted LC and climate data are presented in Supplementary Data Table S3.

Phylogenetic Inference

Sequence data for one nuclear (*ITS*) and 7 chloroplast markers (*matK*, *ndhF*, *psbA-trnH*, *rbcL*, *trnC-ycf6*, *trnK-rps16*, *trnL*) were retrieved from GenBank (Supplementary Data Table S4). We included data for 14 *Melica*, 4 *Pleuropogon*, and 4 *Glyceria* species, as well as *Schizachne purpurascens*, *Brylkinia caudata*, *Festuca rubra* and *Poa palustris*. Our sampling includes as much of the available sequence data for Meliceae while minimizing missing data. Sequences for several markers were concatenated, and for some species more than one concatenated sequence was included (Supplementary Data Table S4).

Maximum likelihood (ML) and Bayesian Inference analyses were conducted on the concatenated data matrix (36 chimeric concatenated sequences and 5,295 bp). Phylogenetic inference under ML was conducted with RaxML-HPC-HYBRID (Stamatakis, 2014) on the CIPRES Science Gateway. We used the parameter-rich GTRCAT model of substitution and defined each marker as a separate partition. Branch support was estimated using the automatic bootstrap convergence function (-autoMRE; Pattengale *et al*., 2010) RaxML was run as follows: raxmlHPC-HYBRID-AVX -T 4 -s infile -N autoMRE -n result -q part -k -f a -p 12345 -x 12345 -m GTRCAT. Bayesian inference was conducted in MrBayes v3.2.7 (Ronquist and Huelsenbeck, 2003), specifying each marker as a separate partition. We by-passed model selection and specified the parameter-rich GTR+G model of substitution. MrBayes was run with 2 runs, 4 chains, and 1 million generations, sampling every 1000 and a burnin fraction of 0.25. Convergence of chains was determined with Tracer v1.7.1 (ESS > 200 for all parameters; Rambaut *et al*., 2018; average deviation of split frequencies < 0.05). The posterior distribution of trees from the two runs was combined with LogCombiner and we summarized the resulting posterior distribution in a maximum clade credibility (MCC) tree using TreeAnnotator. All trees were rooted using the outgroups *Poa palustris* and *Festuca rubra*.

Comparative Analyses

To test whether the acquisition of traits associated with anemochory were specifically associated with the occupation of open and/or disturbed habitats, we fit phylogenetically informed Bayesian multilevel models using the R package *brms* (v. 2.15.0; Bürkner, 2017). Three environmental predictors were included, 1) the SLR transformed proportion of observations for each species found in forests and woodlands relative to sites classified as grassland, shrubland, or sparse vegetation cover, 2) the SLR transformed proportion of observations associated with burned sites, and 3) the transformed proportion of observations found in anthropogenically modified habitats (Supplementary Data Fig. S2). These three habitat predictors, along with log-transformed diaspore mass, were included as fixed effects in a model with falling velocity and WDR as joint outcome variables. A lognormal response distribution with an identity link was used for both. Phylogenetic information was incorporated by treating species as a grouping factor over which the model intercept was assumed to vary, with effects correlated via a phylogenetic covariance matrix computed from the maximum clade credibility tree (*ape* v. 5.4; Paradis and Schliep, 2019). Correlations between the phylogenetic group level effects on each response variable were also included, to account for covariation between these traits. Four chains of 8,000 generations were run, with every other generation sampled and the first 2,000 discarded as burnin. Weakly informative priors were assigned to all model parameters (Supplementary Data Table S5). Following model fitting, we used the *performance* package (v. 0.7.3; Lüdecke *et al*., 2021) to obtain a sample of Bayesian R^2 estimates for our final model (Gelman *et al*., 2019). We also tested for the presence of phylogenetic signal in individual dispersal traits using Pagel's λ (Pagel, 1999) estimated for species average values using the *phytools* package (v. 0.7 - 70; Revell, 2012).

RESULTS:

Phylogenetic Inference

Phylogenetic reconstructions using Bayesian inference and ML analyses were concordant, differing only in branches with weak support (Fig.2; Supplementary Data Fig. S3). The monophyly of *Melica* was strongly supported and relationships recovered (notably the paraphyly of *Glyceria*, and intergeneric relationships) were consistent with recent phylogenetic treatments of the tribe (Schubert *et al*., 2019). Most branches were recovered with strong support, excepting several within *Melica* (Fig. 2; Supplementary Data Fig. S3).

Habitat & Dispersal Data

For our 24 sampled species we obtained 8,428 georeferenced records that met our sampling criteria (of

389,897 downloaded). The number of usable records varied substantially between taxa (16 to 1,639), with all but our most poorly sampled species (*Melica virgata*) having at least 40, and most exceeding 100 records. Habitat data extracted from georeferenced occurrence records were consistent with those published in standard handbooks (Supplementary Data Results S1, Table S6, Fig. S4). Our data suggest that the sampled species most frequently occupy temperate woodlands but show a considerable range in their affinity for more open sites. They are also common in anthropogenically modified sites, particularly in sites classified as agricultural (Fig. 3). Occurrences were more prevalent in evergreen, conifer-dominated forests than broadleaved deciduous or mixed forests. Among open habitats, occurrences were most frequent in grasslands and grassland mosaics, with only a handful of taxa showing affinities for shrublands (e.g., *Melica bulbosa*). Burn rates were generally low for most of the sampled taxa. Of the 24 species, nine were found exclusively at sites that did not burn between the years 2000 and 2012. Although exposure to fire was low among the remaining taxa, a handful did experience burns with moderate frequency (e.g., *Melica racemosa* with 21% of occurrences at sites with ≥ 1 fire from 2000 – 2012; Supplementary Data Fig. S2).

Falling velocity and estimated Wind Dispersal Range (WDR) varied substantially among sampled diaspores. Recorded falling velocities varied by nearly an order of magnitude, from a minimum of 0.4 $m*s^{-1}$ (*Melica transsilvanica*) to a maximum of 3.7 $m*s^{-1}$ (*Pleuropogon californicus*). Estimated WDR varied from a minimum of 3.6 m (*P. californicus*) to a maximum of 70.3 m (*M. transsilvanica*). Among the sampled taxa, *Melica ciliata* is likely the best documented anemochore (Hensen and Müller, 1997). We measured this taxon to possess diaspore falling velocities ranging between 0.5 and 1.0 $m*s^{-1}$, corresponding to variation in WDR from 32 to 60 m. Nine other species in our sample produced at least some diaspores with falling velocities within this range, and seven taxa showed comparable WDR (Supplementary Data Table S1).

Comparative Analysis

Results suggested adequate convergence and sufficient sampling of the posterior in multilevel models, with effective sample sizes exceeding the recommended 100 samples per chain and Rhat values equalling the convergence expectation of 1.0 for each model parameter. Full model outputs and posterior densities of slope estimates are presented in the Supplementary Data (Tables S7 & S8; Fig. S5). Diaspore falling velocity was correlated with proxies for both disturbance and habitat openness. Habitat openness showed the clearest relationship, with taxa more likely to occupy open habitats possessing reduced diaspore falling velocities in both models including (slope estimate: 0.14, 95% posterior

Credibility Interval: 0.06, 0.22) and excluding (estimate: 0.12, CI: 0.04, 0.21) mass as a covariate (Fig. 4f). Increased likelihood of occupying anthropogenically modified habitats was also associated with lower falling velocities. However, this effect was only recovered in models that did not include mass as a covariate (estimate: -0.13, CI: -0.24, -0.02 vs. estimate: -0.04, CI: -0.15, 0.07), suggesting the effect is explained by a reduction in diaspore size (all else equal lower mass results in lower falling velocity) (Fig. 4d). Although models suggest that species occupying more frequently burned sites also possess lower falling velocities, the effect was sensitive to the inclusion of mass, and 95% Credibility Intervals for slope parameters overlapped with zero (i.e., no effect) (Fig. 4e). Despite lower falling velocities, open habitat occupation was not strongly correlated with a greater WDR under any model condition (Fig. 4c). The same was also true of burn frequency (Fig. 4b). In contrast, species more likely to occupy anthropogenically modified habitats were found to have higher WDR. This effect was only recovered when mass was excluded as a model predictor (estimate: 0.26, CI: 0.09, 0.44 vs. estimate: 0.14, CI: -0.04, 0.31) (Fig. 4a). Mass was positively correlated with falling velocity (estimate: 0.67, CI: 0.49, 0.85) and negatively correlated with WDR (estimate: -0.99, CI: -1.23, -0.76). Models excluding mass (i.e., with only environmental predictors) had estimated Bayesian R^2 values of 0.68 (± 0.03) when predicting falling velocity, and 0.74 (± 0.03) when predicting WDR. These values dropped respectively to 0.29 (± 0.11) and 0.13 (± 0.12) when phylogenetic (i.e., group level) effects were excluded (Supplementary Data Fig. S6). Because of the direct effect of mass on falling velocity, R^2 values were higher for models including mass with values of 0.76 (± 0.02) when predicting falling velocity, and 0.82 (± 0.02) when predicting WDR. These dropped respectively to 0.39 (± 0.11) and 0.41 (± 0.21) when phylogenetic (i.e., group level) effects were excluded (Supplementary Data Fig. S6). Among species average dispersal traits, phylogenetic signal was only apparent in diaspore mass ($\lambda = 0.89$, $p = 0.01$), with results for culm height ($\lambda = 0.76$, p = 0.07) and falling velocity ($\lambda = 0.43$, $p = 0.24$) not significantly different from models with no phylogenetic structure (i.e., $\lambda = 0$).

Results presented above and those in Fig. 4 exclude *Pleuropogon sabinei* because it was a strong outlier in both habitat and growth habit. This species has extremely small stature (5-20 cm; Clayton *et al*., 2021), and is found exclusively at high latitudes, typically in marshy areas without much additional vegetation cover (e.g., thawing tundra; Supplementary Data Methods S1). Although results are largely congruent regardless of whether the taxon is included, its position at extreme ends of both environmental and morphological spectra make interpretation of patterns within the remaining taxa more difficult. We present results from analyses including this taxon in the Supplementary Data (Supplementary Data Fig. S7). Results from models where WDR was parameterized to reflect circulation conditions more typical of forest understory returned similar results to those described above. However, the inclusion of mass did not weaken the relationship between WDR and anthropogenic site occupation as much as in main model results, reflecting a stronger influence of plant height under these circulation conditions (Supplementary Data Fig. S8).

DISCUSSION:

Convergence of Diaspore Traits

Diaspore morphology varied substantially among sampled species (Fig. 1). Those achieving low diaspore falling velocities (i.e., comparable to known anemochores like *M. ciliata)* fell into four general categories. These were, 1) species with plumose diaspores (*M. ciliata*, *M. racemosa, M. transsilvanica*), 2) species with large, winglike glumes (*M. altissima, M. stricta*), 3) species with loosely compacted and laterally compressed spikelets (*M. scabrosa*), 4) species without diaspore traits overtly linked to anemochory (*G. grandis, G. maxima, G. striata, M. subulata*) (Fig. 1). Although not limited to these taxa, all members of the final group did produce autorotating diaspores (up to ~60 revolutions*s^{-1}). Autorotation can reduce falling velocity by generating lift (Norberg, 1973). It is common in anemochorous trees (e.g., *Acer, Fraxinus)*, but rarely described among herbaceous plants (Ridley, 1930; Norberg, 1973; van der Pijl, 1982). Within Meliceae, autorotation was observed across a range of morphologies with both high and low falling velocities. In some species, its occurrence was inconsistent, with only a portion of sampled diaspores showing autorotation (see Supplementary Data Results S2; Video S1; Video S2; Figs. S9 – S11).

The existence of four different morphological solutions for achieving low falling velocities (Figs. 1 & 3) suggests that diaspore traits facilitating wind dispersal have evolved at least four times within the tribe. This is almost certainly an underestimate considering the limited proportion of species in the tribe that were sampled. Furthermore, in some cases, diaspore morphology within groups is likely to be homoplastic. For example, although the diaspores of *M. stricta* and *M. altissima* both retain large wing-like glumes, the former is nested within a clade where diaspores are shed without these bracts, suggesting that their retention evolved independently (Figs. 1 & 2). Although ambiguity in the interspecific relationships within *Melica* (Fig. 2) makes homology difficult to establish with certainty, the variety of distinct diaspore morphologies achieving low falling velocities indicates there has been even more extensive convergence than previously suspected.

The Role of Open Habitats and Disturbance

In addition to revealing patterns of wind dispersal potential across the melicgrasses, our results shed light on potential environmental drivers of convergence. Proxies for both habitat openness and disturbance frequency were associated with traits conferring greater wind dispersal potential. However, the pathways through which openness and disturbance appear to have influenced the evolution of anemochory differ substantially.

Open habitats. - The diaspores of taxa more frequently found in open habitats typically showed lower falling velocities than forest adapted taxa (Fig. 4f), consistent with our prediction, and in line with the hypotheses of previous authors (Stebbins, 1971; Ozinga *et al*., 2004). Open habitats typically have stronger horizontal air currents and thermal updrafts, in addition to providing fewer barriers to movement (Stebbins, 1971; Tackenberg *et al*., 2003; Ozinga *et al*., 2004; Nathan *et al*., 2008). As a consequence, individuals in these habitats with dispersal traits that aid in exploiting wind should disperse their seeds further (and more evenly). Evolution toward lower falling velocities in open habitat lineages is thus consistent with selection for diaspore traits exploiting wind where it is likely to be a more effective dispersal vector. However, increases in dispersal distance conferred by diaspore traits seem to be offset by reductions in plant height in these environments (Supplementary Data Fig. S12). When comparing predicted dispersal distances under the same environmental conditions, open habitat taxa do not show a clear advantage over their forest occupying relatives (Fig. 4c), a pattern that holds regardless of habitat type (Supplementary Data Fig. S8). Although the more amenable circulation conditions experienced by open-adapted species should typically translate to greater wind dispersal distances than forest dwellers (Supplementary Data Fig. S8, Tackenberg et al., 2003), our results suggest that this is merely a consequence of environment, not evolution toward greater overall wind dispersal potential.

One possible explanation for our counterintuitive results is a trade-off between dispersal potential and hydraulic safety. Average plant height is known to covary with water availability. Taller plants are more susceptible to embolism because traits related to hydraulic safety scale with height (e.g., conduit width) (Moles *et al*., 2009; Olson *et al*., 2018; Liu *et al*., 2019; Vijayaraghavareddy *et al*., 2020). Open habitats are most frequently found in dry (or seasonally dry) regions and foster local conditions (e.g., increased light exposure and air temperatures) that promote water loss (Sankaran *et al*., 2005; Hirota *et al*., 2011; Siegert and Levia, 2011; Staver *et al*., 2011; Karki and Goodman, 2015). It is possible that the benefit of enhanced hydraulic safety may outweigh the advantages of increased dispersal distances that come with larger stature in these habitats. Similar trade-offs have been demonstrated in anemochorous forbs, where reduced plant heigh under water stress has been shown to limit dispersal potential (Zhu *et al*., 2021). A similar trade-off could arise from selection for shorter plants to limit the likelihood of lodging (i.e., culm displacement/breakage from strong winds and/or extreme weather), because wind speeds in open habitats tend to be higher (e.g., Pinthus, 1974; Baker et al., 1998). Although at present we cannot confirm the existence of either trade-off in the Meliceae, both warrant further investigation.

Disturbance. - Fire was an infrequent source of disturbance for most of our species, with many found exclusively at sites with no recorded burns and fire exposure generally low among the remainder. Nevertheless, fire frequency did show a weak correlation with diaspore falling velocity in models excluding mass, although this was not replicated for wind dispersal range (Fig. 4b,e). Previous work indicates that conditions following fires are likely to benefit anemochores by removing standing vegetation, increasing local wind speeds, and intensifying thermal updrafts (Monty *et al*., 2013; Keith *et al*., 2020). However, the lack of a clear relationship with either dispersal traits and the narrow range of sampled fire frequencies suggests that exposure to fire in the melicgrasses is likely too infrequent to impose any strong selective pressure on dispersal in these taxa. Although use of GIS based fire maps is common (e.g., Abad et al., 2013; Archibald et al., 2013; Adagbasa et al., 2020), the short period of time over which these data are recorded and relatively small number of fires they capture suggest some caution should be used when interpreting our results. Although, infrequent exposure to fires appears consistent with the mesic forest habitats many taxa occupy (Archibald et al., 2013; Supplementary Data Methods S1), the data may fail to capture smaller scale fires (Laris, 2005). Furthermore, human suppression of fire in many regions may artificially condense the observed range of variation. Although the borderline results we observe hint at an the importance of fire (see e.g., Supplementary Data Fig. S8, S11), further work with more reliable predictors of fire exposure are ultimately required.

In contrast, species that more frequently experienced anthropogenic disturbances showed a clear propensity toward greater wind dispersal potential. Unlike the results for open habitat taxa, this effect was expressed as both lower diaspore falling velocity and greater wind dispersal range (WDR). Contributing to the latter, culm heights were generally greater in species occupying anthropogenically modified habitats. It is possible that greater stature functions to increase dispersal performance, as has been observed in some herb lineages (Williams et al., 2016), and is hypothesized in some groups of early land plants (Boyce et al., 2017). However, other functions may explain variation in culm height, such as the intensity of light competition which can also be high in herb rich, anthropogenically disturbed sites (Givnish, 1982). Although the height data used herein reflect species average traits, culm height is extremely plastic (e.g., Bello et al., 2017; Mu et al., 2021). As such, individual responses to their environment may further modify these relationships.

The effect of anthropogenic disturbance on falling velocity and WDR was also highly dependent on whether diaspore mass was included as a model predictor, suggesting that increases in wind dispersal performance are largely explained by the smaller size of diaspores in these habitats (Fig. 4a,d; Supplementary Data Fig. S13).Other factors being equal, smaller diaspores will achieve lower falling velocities and thus disperse farther. However, diaspore mass also influences a variety of other processes. Seed size plays an important role in recruitment success and seedling competitive ability, both of which are typically improved in large-seeded taxa (Turnbull *et al*., 1999; Jakobsson and Eriksson, 2000; Leishman, 2001). Usually, seedlings derived from large seeds are also better able to cope with a variety of environmental stresses (e.g., drought, high temperatures, shade) (Bierzychudek, 1982; Westoby *et al*., 1992; Leishman *et al*., 2000; Khurana and Singh, 2004). On the other hand, increased size typically comes at the cost of the number of seeds a plant is able to produce, thus reducing the number of dispersal opportunities in addition to (in our case) the dispersal capacity of individual diaspores (Smith and Fretwell, 1974; Jakobsson and Eriksson, 2000; Leishman, 2001; Thomson *et al*., 2011; but see also Gnan *et al*., 2014). Whether patterns observed in the Meliceae reflect selection for anemochory per se, or whether they resulted as a consequence of selection for a greater number of small diaspores is unclear.

A reproductive strategy including production of many small diaspores would put disturbance adapted melicgrasses in line with the strategies adopted by many other ruderals. (Salisbury, 1942; Tautenhahn *et al*., 2008; Wu *et al*., 2015). In these taxa, high diaspore production maximizes the chance of encountering new sites when they are unpredictably distributed in space and time. Indeed, habitat patchiness has been shown to promote evolution favouring increased dispersal capacity in many cases (Hof *et al*., 2012; Williams *et al*., 2016; Parvinen *et al*., 2020). However, not all disturbance adapted plants adopt this reproductive strategy (Grime, 1977; Blais and Lechowicz, 1989; Dalling and Hubbell, 2002; Johnson *et al*., 2003). For example, plants may instead prioritise producing diaspores that remain viable until conditions favour germination (Whelan, 1986; Chen *et al*., 2020). Such a strategy might also lead to smaller diaspores, because large seeds are better provisioned for immediate germination and possess proportionately weaker mechanical defences than small seeds, making long periods of dormancy riskier (Rees, 1996; Wu *et al*., 2019; Chen *et al*., 2020). Although our data are consistent with a hypothesized evolutionary link between disturbance and anemochory, there are several alternate interpretations. Further work focused on the total reproductive investment and seed dormancy of these taxa is necessary to fully evaluate the factors driving the evolution of dispersal syndromes.

Additional Caveats and Considerations for Future Work

Although the quantitative framework we developed for this study presents a number of major advantages over alternative approaches, it nevertheless makes a number of simplifications. We consider several of the more important ones in more depth below.

The Meliceae is notable among herbs for including both autorotating and non-autorotating diaspores, which achieve low velocity in fundamentally different ways (lift vs. drag) (Augspurger, 1986; Ortega-Jimenez et al., 2019; Sohn and Im, 2022). While plumose diaspores quickly approach terminal velocity, autorotating diaspores experience a period of rapid descent prior to the initiation of rotation, after which velocity quickly decreases to reach its "terminal" value (here the equilibrium velocity during autorotation) (Norberg, 1973; Guries and Nordheim, 1984; Sohn and Im, 2022). In still air, the consequence is that mean descent velocity may exceed "terminal" velocity in autorotating diaspores but will be below that value in plumose diaspores (Supplementary Data Fig. S9). However, when exposed to wind, autorotation initiates sooner (Norberg, 1973; Ortega-Jimenez et al., 2019), and because force is required to separate diaspores from the mother, still conditions are likely rarely encountered by autorotating diaspores in the field (Augspurger and Franson, 1987; Green and Johnson, 1990). Consequently, recording methods which capture mean velocity may systematically underestimate of dispersal distances of autorotating taxa. Although this is an important consideration, several observations lead us to believe it is a minor concern in the present study. First, our recordings deliberately exclude the initial period of descent and capture only ~30 cm near the bottom of travel (Supporting Information Methods S1, Fig. S9). Furthermore, most of our diaspores initiated autorotation very quickly, due in part to their small size (initial fall distance is negatively correlated with wing length; Guries and Nordheim, 1984). Second, the speed of rotation appeared to be consistent within individual trials, suggesting diaspores were recorded at "terminal" velocity, which is reached as rotation speeds stabilise (Sohn and Im, 2022). We also note that ground truthing for the WALD model used in this study included a variety of both autorotating and non-autorotating taxa (Katul et al., 2005), and other work has shown that autorotating taxa conform to the predictions made by other simple dispersal models (Green and Johnson, 1990); although this work is entirely on woody plants. As a final check we reran analyses using minimum and maximum (rather than median) velocity for autorotating specimens, to gauge the sensitivity of our results to recorded values for these specimens. However, neither analysis substantially differed from our primary results (Supplementary Data Fig. S11). We therefore conclude that differences between autorotating and non-autorotating taxa are unlikely to bias our overall conclusions.

Although we have found evidence consistent with habitat playing some role in the evolution of dispersal strategy, much of the variation in our dispersal traits remains unexplained. This is evidenced by the generally low and uncertain Bayesian R^2 estimates, particularly when phylogenetic effects are excluded (Supplementary Data Fig. S6). These results also suggest that phylogenetic history plays an important role in the observed patterns of dispersal traits. Nevertheless, the strength of the phylogenetic signal in individual dispersal traits varied, with only diaspore mass showing clear phylogenetic structuring. A high degree of intraspecific variation in dispersal traits is also likely to contribute to the uncertainty in our results. Observed intraspecific variation was a primary driver in the decision to employ a multilevel modelling approach, as these methods are better able to incorporate this variation than most commonly used phylogenetic comparative methods (PCMs). The quality of inferred phylogenies is another source of uncertainty, because, as with all PCMs, results depend on the inferred phylogenetic relationships, which were not all well supported (Fig. 2). Trees were also unfortunately based on partial sampling of the tribe, and were inferred from a relatively small number of primarily plastid loci. As such, sampling could be expanded to better represent the diversity within the tribe, particularly within the genus *Melica*. In addition, the generation of larger nuclear datasets would allow for the inference of a robust species-tree hypothesis below the genus level.

To this point, we have primarily framed our discussion of dispersal traits in context of potential adaptive changes, consistent with the classic hypotheses we are testing. However, other processes can also explain the observed patterns, and traits improving dispersal performance may not be adaptive in the strict sense. Indeed, our observation that autorotation is widespread, potentially ancestral, and not restricted to anemochores in the Meliceae (Supplementary Data Fig. S10) suggests at least some of the traits associated with wind dispersal might have originally evolved for a different reason (i.e., be exaptive; Gould and Lewontin, 1979). Similar patterns have been observed in maples, where the initiation of autorotation appears to be largely independent of the aerodynamic forces acting on the wing (Varshney et al., 2012). Interpreting observed correlations as adaptive also assumes that current distributions reflect historic habitat preferences. Even if this assumption is true, it is possible that traits enhancing anemochory facilitated colonization of these habitats rather than the other way around (i.e., lineage sorting). It is also important to note that greater dispersal distance does not necessarily correspond to increased fitness, and in some circumstances "successful" dispersal may be that which limits the distance diaspores travel from the parent plant (Cody and Overton, 1996; Parvinen et al., 2020).

Finally, although plant height and diaspore morphology are the most obvious, they are not the only plant traits that influence dispersal distance (Green and Johnson, 1990). For example, changes to the force required for abscission, how long fruits take to mature, and the time of year when they mature all influence the circulation conditions under which dispersal occurs, and thus likely outcomes (Green and Johnson, 1990). Furthermore, these traits are all at least partly heritable (Lin et al., 2007; Yu and Kellogg, 2018). Unfortunately, they are also poorly studied in the focal group. Although our approach attempts to achieve more biologically realistic results by including vegetative traits like plant height, it is still ultimately a simplified view of seed dispersal. A priority of future research should be understanding how suites of dispersal relevant traits coevolve.

Summary

Our study shows that the melicgrass tribe includes many taxa suited to dispersing their seeds by wind, and that many of them likely acquired this capability convergently. Although much of the observed variation in wind dispersal performance remains unexplained, our results are consistent with the hypothesis that open and disturbed habitats both select for traits associated with this dispersal strategy. However, results also hint at the complexity of these relationships, suggesting that acquisition of traits influencing the effectiveness and nature of seed dispersal is limited by both reproductive (e.g., establishment success) and non-reproductive (e.g., drought resistance) processes. Although our results may help answer several important questions about the evolution of dispersal, they raise many additional ones (e.g., whether traits covary with reproductive investment). Although our approach inevitably simplifies a complex system, we believe that it may serve as a blueprint for future studies exploring seed dispersal in more realistic evolutionary contexts.

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Fig. 1. Next page. Representative images of diaspores for each of the sampled species. Relationships in the stylized evolutionary tree are based on the maximum clade credibility tree shown in Fig. 2. Taxa are 1 - *Brylkinia caudata*, 2 - *Schizachne purpurascens*, 3 - *Glyceria striata*, 4 - *Glyceria maxima*, 5 - *Glyceria grandis*, 6 - *Pleuropogon refractus*, 7 - *Pleuropogon californicus*, 8 - *Pleuropogon sabinei*, 9 - *Glyceria borealis*, 10 - *Glyceria septentrionalis*, 11 - *Melica ciliata*, 12 - *Melica transsilvanica*, 13 - *Melica racemosa*, 14 - *Melica scabrosa*, 15 - *Melica virgata*, 16 - *Melica onoei*, 17 - *Melica mutica*, 18 - *Melica subulata*, 19 - *Melica stricta*, 20 - *Melica bulbosa*, 21 - *Melica californica*, 22 - *Melica turczaninowiana*, 23 - *Melica nutans*, 24 - *Melica altissima*.


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6
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3
2
1
5 mm

Fig. 2. Next page. Maximum clade credibility tree summarizing the posterior tree distribution from Bayesian phylogenetic analyses. Posterior probability and bootstrap support values are shown above branches. Values > 0.7 posterior probability are shown. Tree was rooted using *Poa palustris* and *Festuca rubra* as outgroups.

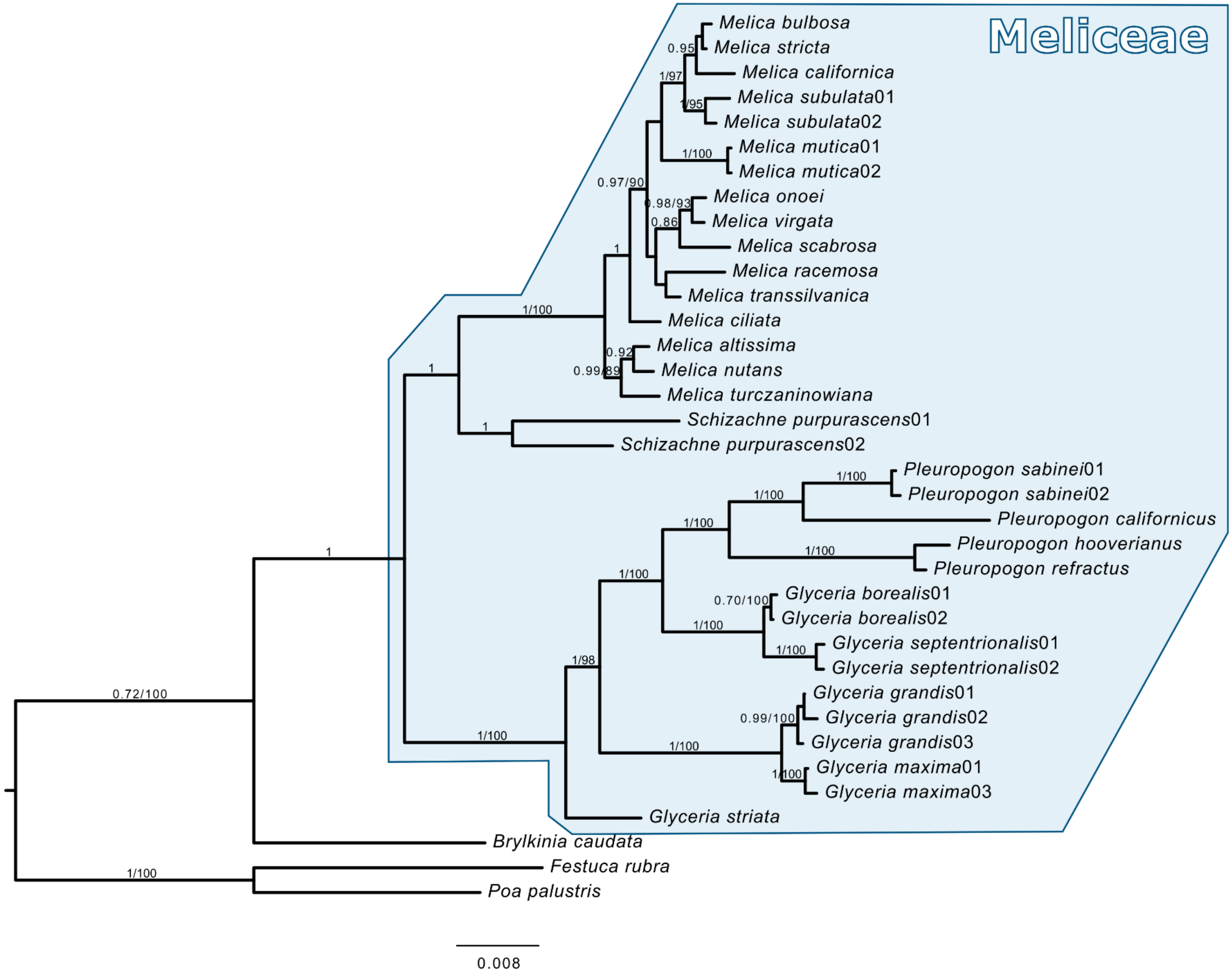

Meliceae
Melica bulbosa
0.95
Melica stricta
1/97
Melica californica
1/95
Melica subulata01
Melica subulata02
1/100
Melica mutica01
Melica mutica02
0.97/90
0.98/93
Melica onoei
0.86
Melica virgata
Melica scabrosa
1
Melica racemosa
Melica transsilvanica
1/100
Melica ciliata
0.92
Melica altissima
0.99/89
1
Melica nutans
Melica turczaninowiana
1
Schizachne purpurascens01
Schizachne purpurascens02
1/100
Pleuropogon sabinei01
1/100
Pleuropogon sabinei02
1/100
Pleuropogon californicus
1/100
Pleuropogon hooverianus
Pleuropogon refractus
1/100
0.70/100
Glyceria borealis01
1
Glyceria borealis02
1/100
1/100
Glyceria septentrionalis01
Glyceria septentrionalis02
1/98
Glyceria grandis01
0.99/100
Glyceria grandis02
1/100
1/100
Glyceria grandis03
Glyceria maxima01
1/100
Glyceria maxima03
Glyceria striata
0.72/100
Brylkinia caudata
1/100
Festuca rubra
Poa palustris
0.008

Fig. 3. Next page. Phylogenetic trees showing variation in diaspore falling velocity and habitat variables, reconstructions at interior nodes were obtained via maximum likelihood ancestral state estimation (*phytools* v. 0.7-70; Revell, 2012). For a) branch colour represents log transformed mean diaspore falling velocity. Diaspore silhouettes are shown for each species to approximate scale at the tips (Fig. 1). The different morphologies putatively associated with convergent anemochory are also indicated (see Discussion: Convergence of Diaspore Traits). For the remaining trees, branch colour represents b) SLR transformed proportion of occurrences in anthropogenic sites c) SLR transformed proportion of occurrences in previously burned sites, and d) proportion of occurrences in forests relative to open habitat types.

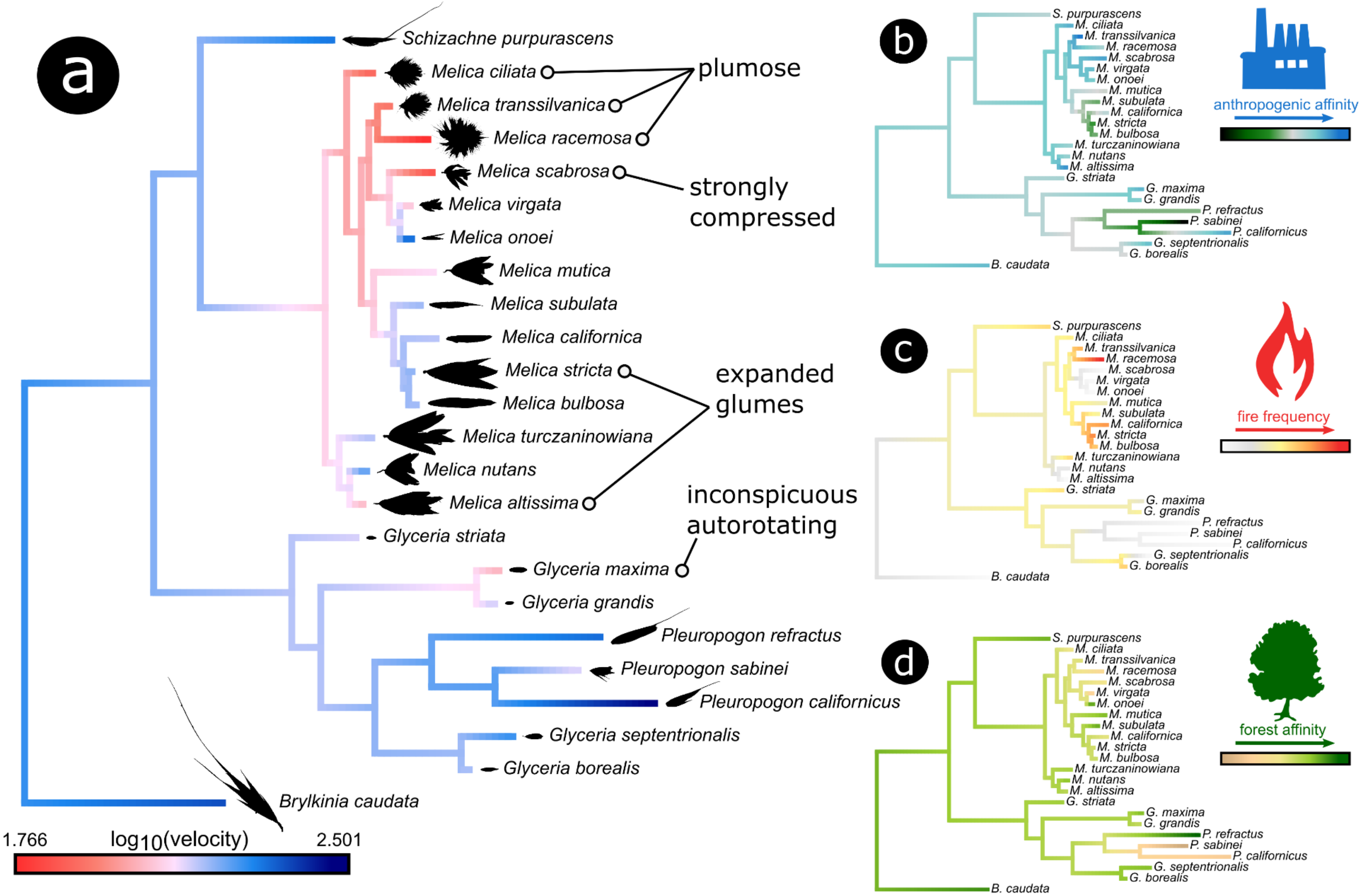
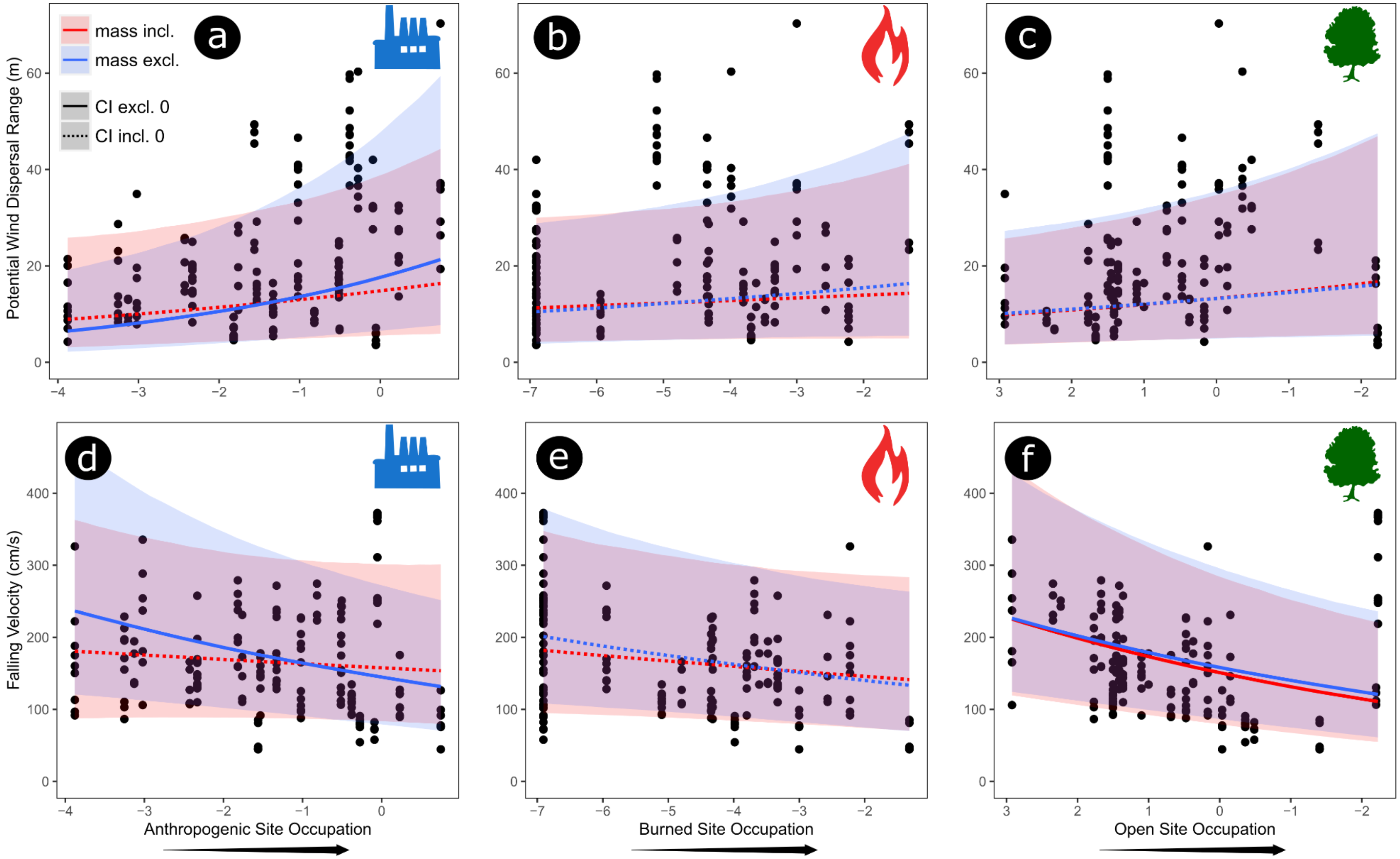

a
Schizachne purpurascens
Melica ciliata
Melica transsilvanica
Melica racemosa
Melica scabrosa
Melica virgata
Melica onoei
Melica mutica
Melica subulata
Melica californica
Melica stricta
Melica bulbosa
Melica turczaninowiana
Melica nutans
Melica altissima
Glyceria striata
Glyceria maxima
Glyceria grandis
Pleuropogon refractus
Pleuropogon sabinei
Pleuropogon californicus
Glyceria septentrionalis
Glyceria borealis
Brylkinia caudata
plumose
strongly compressed
expanded glumes
inconspicuous autorotating
1.766
log10(velocity)
2.501
b
anthropogenic affinity
c
fire frequency
d
forest affinity
S. purpurascens
M. ciliata
M. transsilvanica
M. racemosa
M. scabrosa
M. virgata
M. onoei
M. mutica
M. subulata
M. californica
M. stricta
M. bulbosa
M. turczaninowiana
M. nutans
M. altissima
G. striata
G. maxima
G. grandis
P. refractus
P. sabinei
P. californicus
G. septentrionalis
G. borealis
B. caudata

Fig. 4. Next page. Conditional effects of models predicting Wind Dispersal Range (WDR) and diaspore falling velocity. Results are shown for models including (red) and excluding (blue) diaspore mass as a predictor. Dashed lines represent effects where posterior credibility intervals (CI) for the slope overlap with zero (i.e., no effect), solid lines indicate the opposite. Results are shown for the effects of a, d) anthropogenic habit occupation, b, e) burn frequency, and c, f) open habitat occupation. Note that the direction of the horizontal axes in sections c and f are reversed so the direction of predicted effects is the same for all three habitat variables.



mass incl.
mass excl.
CI excl. 0
CI incl. 0
a
b
c
d
e
f
Potential Wind Dispersal Range (m)
0
20
40
60
Falling Velocity (cm/s)
0
100
200
300
400
−4
−3
−2
−1
0
−7
−6
−5
−4
−3
−2
3
2
1
0
−1
−2
Anthropogenic Site Occupation
Burned Site Occupation
Open Site Occupation

References:

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CHAPTER 3: The role of grasslands in the evolution of seed dispersal strategy in Poaceae

INTRODUCTION:

Grasslands cover approximately 40% of Earth's land surface (Shantz, 1954; Gibson, 2009) and provide several important ecological, economic, and cultural services (Street-Perrott and Barker, 2008; Gibson, 2009). Although grasslands are widespread, and most extant grasses are adapted to open habitats (Gallaher et al., 2019), these conditions did not characterize most of the family's evolutionary history. Current estimates date the origination of the grass family (Poaceae) to the early Cretaceous (Prasad et al., 2011; Gallaher et al., 2019), whereas strong evidence for grasslands does not appear until the Late Oligocene – Early Miocene (Strömberg, 2005; Strömberg, 2011). What's more, these first grasslands likely represented mosaic landscapes with considerable woody components and many other notable differences (e.g., taxonomic, physiologic) to modern grasslands in the same region (Edwards et al., 2010; McInerney et al., 2011; Strömberg, 2011). In most regions, structurally (e.g., largely treeless) and compositionally modern grasslands didn't appear for another ~15 Ma, in the Late Miocene and Early Pliocene (Thomasson, 1990; Edwards et al., 2010; Strömberg et al, 2011).

In many groups, the evolutionary and ecological consequences of the grassland expansion are well studied, particularly fossil mammals (e.g., Stirton, 1947; Webb, 1977; Stebbins, 1981; Strömberg, 2006; Hopkins, 2007). However, little is known about the evolutionary changes experienced by grasses during this transition, despite defining the habitat type. In part, this is because grasses have a notoriously difficult fossil record, with most common fossil types rare, taxonomically ambiguous, or both (Elias, 1942; Thomasson, 1980; Jacobs et al., 1999; Strömberg, 2011). Consequently, what little is known has largely relied on inference from modern data (e.g., leaf shape, Gallaher et al., 2019; herbivore defense, Strömberg et al., 2016; Brightly et al., 2020). One important aspect of plant biology hypothesized to have changed during this transition is seed dispersal strategy. Seed dispersal is an important process which plants rely on to reach new suitable sites where the next generation can establish (Janzen, 1970; Cheplick, 1993; Comita et al., 2014). Because they are sessile, plants (with rare exception) rely upon external vectors to disperse their seeds. This has a strong influence on plant reproductive morphology, and a diverse array of forms have evolved to exploit a correspondingly wide range of potential vectors (e.g., wind, water, birds, ants) (Ridley, 1930; van der Pijl, 1982). Large changes to vegetation structure, faunal community composition, and abiotic conditions as grasslands expanded (Webb, 1977; Retallack, 2001; Strömberg, 2011; Hyland et al., 2019) would have had a pronounced effect on existing dispersal networks. Consistent with this hypothesis, the suite of observed dispersal strategies, generally differs between modern grasslands and other communities (Stebbins, 1971; van der Pijl, 1982). Strong evidence has also been found for the important role that changing habitat has in driving evolution in dispersal traits in a number of plant lineages (Sorensen, 1986; Ozinga et al., 2004, Dunn et al., 2007; Salazar-Tortosa et al., 2019; Brightly et al., in prep.).

The diversity of dispersal strategy in grasses has attracted considerable attention (e.g., van der Pijl,1983), resulting in a wealth of hypotheses based upon the dispersal characters commonly observed in grasses of different ecosystems. For example, the basal APP grade grasses (subfamilies Anomochloideae, Pueliodeae, and Pharoideae), which retains the family’s ancestral forest understory habitat (Gallaher et al., 2019), typically possess dispersal traits facilitating adhesive dispersal (epizoochory; van der Pijl, 1982). Several taxa convergently adapted to these habitats show similar dispersal traits, suggesting this dispersal strategy may be favored in forests (van der Pijl, 1982; Sorensen, 1986). Among the core grasses (i.e., non-APP grade) traits facilitating other dispersal types are also common. Despite lacking fleshy fruits, transport in the gut of herbivores has been described for many grasses (endozoochory; Welch, 1985; Gardener et al., 1993, Anderson et al., 2013), often facilitated by incidental consumption of seeds by grazers. This dispersal mode inspired Janzen’s (1984) “foliage is the fruit” hypothesis, where vegetative parts serve an attractive function analogous to that of fleshy fruits.

The rise of grazing faunas following the expansion of grasslands (Webb, 1977; Stebbins, 1981; Strömberg, 2006), may have increased the opportunity for this type of dispersal to occur. Wind is the most widespread abiotic vector via which grasses spread their seeds (anemochory), and several authors have hypothesized that opening of habitats (e.g., as grassland expanded) also helped facilitate adoption of this strategy (Stebbins, 1971; van der Pijl, 1982; Ozinga et al., 2004; Brightly et al., in prep). Although these observations are all suggestive of interesting evolutionary patterns, few studies have gone beyond this exploratory stage in an attempt to better characterize the evolution of dispersal strategy within the family.

Although modern comparative work sheds some light on grasses' evolutionary responses to the expansion of grassland habitats, relying exclusively on extant taxa to make inferences is fraught with potential difficulties. Absence (or exclusion) of fossil evidence can lead to biased impressions of evolutionary patterns (Finarelli and Flynn, 2006; Wood et al., 2013, Weaver et al., 2022). Unfortunately, the difficult fossil record of Poaceae, often prevents integrating modern and fossil data to test such hypotheses. However, one underutilized record of grass fossils holds promise for informing questions about the evolution of dispersal strategy in the family, namely assemblages of silicified anthoecia (the hull formed by the palea and lemma, two bracts subtending the grass fruit), which are found in large quantities at several Cenozoic sites in the western United States, and which are dominated by grasses of the tribe Stipeae (e.g., Elias, 1942; Thomasson, 1978a, 1985, 2005). This record is unique for preserving macroscopic remains, with cellular detail, in high abundance, and taxonomic fidelity (Elias, 1942; Thomasson, 1980a; Jacobs et al., 1999; Thomasson, 2005; Strömberg, 2011). Furthermore, these structures are functionally important, as the anthoecium preserves the external morphology of the seed dispersal unit (diaspore) (Thomasson, 1985). The record of fossil anthoecia spans approximately 20 Ma, beginning around the Oligo-Miocene boundary (Elias, 1942; Thomasson, 1985). It thus preserves the morphology of dispersal structures produced by grasses during the period of initial grassland assembly in the Great Plains Region (Strömberg, 2005, 2011).

Here we construct a large comparative dataset with which to explore the evolution of dispersal mode across the grass family in relation to habitat. **We test the hypothesis that transitions into grassland habitats facilitated the evolution of traits conferring greater potential for wind dispersal and endozoochory, while forest habitats supported the evolution of adhesive dispersal.** We then compile a preliminary dataset of Oligocene-Miocene fossils and integrate them with the modern data. This allows us to discuss likely dispersal modes for fossil taxa, the range of observed morphologies in fossil and modern grasslands, and the taphonomic biases most relevant to reconstructing fossil dispersal ecology and further integration of modern and fossil records.

METHODS:

Modern Sampling

Diaspores were collected from 270 species, including 264 grasses, *Flagellaria indica* (outgroup) and 5 non-grass reference taxa (Table 1). Sampling attempted to maximize phylogenetic breadth, and the range of included dispersal morphologies and preferred habitats. Samples were primarily collected from preserved specimens in the collections at the University of Washington, Missouri Botanical Garden, US National, and Steere herbaria (Supplementary Data Table S1), supplemented by a small amount of material obtained from the field collections, greenhouse plants, and the US National Plant Germplasm (Supplementary Data Table S1). The structural unit constituting the diaspore (e.g., floret, spikelet) in each species was determined via a combination of personal observation by WHB and reference to Kew's GrassBASE online flora (Clayton *et al*., 2021). Diaspore structure was not uniform within species; therefore, we collected all structures we thought likely to disperse as a cohesive unit. To control for diaspore maturity, we limited sampling to material that had already fallen, or fell readily. For all species we attempted to sample several specimens, and multiple diaspores per specimen (Supplementary Data Table S1) to account for variation in dispersal traits which can be substantial and have a strong impact on dispersal outcomes (Teller *et al*., 2015; Snell *et al*., 2019; Wyse *et al*., 2019). In total, we sampled 923 diaspores from 431 individuals, with an average of 2 (1-11) diaspores and 1.6 (1-6) individuals per species.

Fossil Sampling and Vegetational Context

Fossil samples were obtained through our own field collections and material borrowed from the Sternberg Museum of Natural History at Fort Hayes State University. Physical specimens of high enough quality to include were obtained from four localities from which fossil anthoecia had previously been collected (e.g., Thomasson, 1982, 1985). These were the Aphelops (UWBM P?), Merychippus (UWBM P?), and Pliohippus (UWBM P?) Draw sites, located north of Mitchell, Nebraska and assigned to the Sheep Creek Formation (Hemingfordian, 20.4 – 16.3 Ma) (Skinner, 1977; Thomasson, 1982, 1985; Alroy, 2000), and a roadcut (UWBM P?) assigned to the Monroe Creek Formation or Harrison Formation (which unfortunately cannot be distinguished at the site), located just outside of Broadwater, Nebraska. This context suggests a Harrisonian or Monroecreekian stages of the Arikareean (26.3 – 20.6 Ma) (Thomasson, 1985; Alroy, 2000). Additional notes on site location and the stratigraphic context of fossils are provided in the Supporting Information (Methods S1). A total of nine fossil taxa were incorporated into our dataset using published descriptions and images (Table 1; Supporting Information Table S2).

To provide independent vegetational context for the fossil seed taxa, sediment samples for phytolith extraction were collected from as many localities from which physical specimens were obtained as possible. Standard procedures for phytolith extraction and phytolith assemblage analysis were conducted for each sample (Strömberg, 2005 and thereafter) and used to reconstruct the vegetation at each site. Where feasible, phytolith assemblage analysis was conducted on samples taken from the same levels where fossil anthoecia were collected, although variation in the quality of phytolith preservation meant that this was not always possible. See Supplementary Data (Methods S1) for further details, including stratigraphic context of analyzed phytolith assemblages.

Phylogenetic Inference

Sequence data for one nuclear (*ITS*) and three chloroplast markers (*ndhF*, *trnK-matK*, *rbcL*) were retrieved from the NCBI nucleotide database (Supplementary Data Table S3). These were obtained from a query for all grasses for which we collected diaspore samples, as well as their congeners and the outgroup taxa *Flagellaria*, *Joinvillea*, and *Ecdeiocolea*. Sequences from chloroplast markers were extracted either as individual gene entries, or from complete chloroplast genomes, when available. For the latter, BLASTN v2.9.0 (Camacho et al. 2008) and BEDTools (Quinlan and Hall 2010) were used to respectively identify the plastome regions containing the markers and extract their sequences. The resulting dataset included sequence data for 519 taxa. Unfortunately, for 21 species in our dispersal dataset lacked previously published sequence data for any of these four markers.

For the phylogenetic analyses, the markers were individually aligned using MAFFT v7.427 (Katoh and Standley 2013) and subsequently concatenated in a single supermatrix (alignment length = 19,649 bp). A maximum likelihood (ML) phylogeny was then estimated using RAxML (Stamatakis, 2014) on the CIPRES Science Gateway. We used the parameter-rich GTRCAT model of substitution, and 100 bootstrap pseudoreplicates to evaluate branch support. RAxML was run as follows: raxmlHPC-HYBRID -T 4 -s infile.fa -n result -c 25 -m GTRCAT -p 12345 -f a -N 100 -x 12345. To evaluate the effect of missing data, an additional ML analysis was conducted after trimming the original supermatrix using trimAl v1.4 (Capella-Gutiérrez et al. 2009) to remove sites with more than 90% missing data (alignment length = 13,891 bp). We then computed the maximum agreement subtree (MAST) between these two topologies (*phangorn* v 2.8.1; Schliep, 2011), to obtain the tree preserving only those relationships robust to the inclusion or exclusion of sites with considerable missing data. Subsequent comparative analyses were conducted using the MAST topology in addition to that from the full complement of sequence data.

Modern Dispersal Traits

To evaluate potential for dispersal by the three hypothesized mechanisms, we first compiled a list of traits known to facilitate dispersal by wind or through attachment to or consumption by animals following the dispersal modes outlined in the introduction. To facilitate integration of fossil data, we focused on traits which could be measured or reasonably estimated from fossils (see Methods – Fossil Dispersal Traits).

We used diaspore velocity as a proxy for wind dispersal potential. Low falling velocity allows more time for wind to carry diaspores away from the parent plant and increase the likelihood of being lifted by updrafts (Matlack, 1987; Tackenberg, 2003; Tackenberg et al., 2003; Soons et al., 2004; Katul et al., 2005). Falling velocity was measured from high-speed video (959.04 frames*s-1; Sony RX100V Tokyo, Japan) of diaspores descending through an enclosed chamber (Supplementary Data Fig. S4) following protocols established in Brightly et al. (in prep). The median of ten trials was used in subsequent analyses.

Potential for endozoochory was evaluated via several traits known to influence rate of gut passage and likelihood of seed survival. Diaspore mass is the trait best associated with incidental endozoochory, and there is a substantial body of evidence suggesting that smaller diaspores are more likely to survive, and germinate following gut passage (Heinken et al., 2002; Pakeman et al., 2002; Mouissie et al., 2005; Rosas et al., 2008; Brochet et al., 2010; Iravani et al., 2011, although see Bruun and Poschlod, 2006). We recorded diaspore mass using a Sartorius SE2 Ultra Micro (Göttingen, Germany) microbalance, taking the average of three measurements for each diaspore. Because harder diaspores are also known to survive gut passage at higher rates (Neto et al., 1987; Gardener et al., 1993; Quinn et al., 1994; Lovas-Kiss et al., 2019) we recorded whether the bract enclosing the caryopsis (grass fruit) was membranous (thin, flexible), coriaceous (thick, leathery), or indurate (hardened, stiff) using the Online World Grass Flora (Clayton et al., 2021) and our own observations. In addition, we recorded whether diaspores possessed a sharp callus, awns, or similar structure (e.g., the sharp projections *Cenchrus* burs), as these can often injure grazers or otherwise influence the diaspore's likelihood of being consumed (Thomasson, 1985; Quattrochi, 2006; Stubbendieck et al., 2016). These data were compiled from our own observations with additional reference to the Online World Grass Flora (Clayton et al., 2021).

Epizoochorous potential was evaluated on the basis of traits influencing performance in two phases of the dispersal process 1.) becoming attached to passing animals and 2.) retention once attached. The first is strongly influenced by diaspore surface structure and exposure on the plant, with rougher and more exposed diaspores more likely to become attached (Fischer et al., 1996; Will et al., 2007). The second phase is largely a function of the diaspore mass, with smaller diapores less likely to become dislodged and thus more likely to be carried longer distances (Römermann et al., 2005; Tackenberg et al., 2006). Surface roughness was measured from diaspore outlines (tracing tool, ImageJ; Schneider et al., 2012), which were taken from high macro images (Sony RX100V Tokyo, Japan) following the protocol of Will et al. (2007). To ensure the entire margin of diaspores was in focus we used z-projected images computed with Zerene stacker (Zerene Systems LLC Richland, USA). Diaspore exposure was classified as exposed, partially enclosed, or enclosed following the criteria of Will et al. (2007) (see also Supporting Information Methods S2). Roughness and exposure were used to predict the proportion of seeds likely to become attached to sheep wool using the regression models of Will et al. (2007).

In addition to the diaspore traits described above, plant stature is strongly linked with dispersal strategy (Thomson et al., 2011; Albert et al., 2015; Williams et al., 2016). Taller grasses have an advantage for wind dispersal (increased time of descent, access to more favorable circulation conditions; Tackenberg et al., 2003; Soons et al., 2004) and are more likely to disperse their seeds epizoochorously (greater likelihood of contacting large passing animals; Fischer et al., 1996; Albert et al., 2015). Growth form also has a strong influence on the presentation of the inflorescence relative to the foliage, with mat forming grasses often holding reproductive and vegetative parts in closer association, potentially impacting their likelihood of consumption (Dinerstein, 1989; Anderson et al., 2013). We broadly classified plant stature using growth habit, with taxa classified as erect/caespitose or decumbent/mat-forming based upon a survey of published data (Clayton et al., 2021; Supporting Information Table S1).

To aid in interpretation of the dispersal traits of both modern and fossil taxa, we included in our sample 20 grasses and herbs described in the literature as dispersing via one of the three focal mechanisms (Table 1).

Fossil Dispersal Data

Fossil traits were recorded using a combination of direct measurements, and informed estimates. Both surface roughness and callus morphology were recorded directly from complete or near complete specimens (Supporting Information Table S2). Lemma induration, diaspore enclosure, and awn length were all estimated using fossil traits with reference to nearest living relatives. To estimate falling velocity, we constructed a regression model using data from extant stipoid grasses (i.e., nearest living relatives of most fossil taxa) included in the dataset. This model predicted falling velocity from the morphological traits described above, with the addition of standard shape measures (circularity, aspect ratio, solidity) obtained from diaspore outlines in ImageJ. Using these variables, we tested several predictor combinations and evaluated model fit using the Akaiki Information Criterion (AIC). The best model was then used to predict falling velocities for fossil taxa.

Habitat Data

Habitat data for each species were obtained using a combination of published habitat descriptions, and georeferenced occurrence records. These data were used to create three different variables recording affinity for grasslands.

Georeferenced occurrence records were used to extract information from landcover maps constructed from satellite images. These data have the benefit of being quantitative, and thus confer greater biological realism when habitat preferences do not fall into discrete categories (e.g., Fensham et al., 1999; Valladares and Niinemets, 2008). Occurrence records were downloaded from the Global Biodiversity Information Facility (GBIF; Gbif.org, 2022a; 2022b), for all preserved specimens of the Poaceae and three outgroup genera (*Flagellaria*, *Joinvillea*, *Ecdeiocolea*). We restricted records to those associated with preserved specimens to minimize the uncertainty caused by misidentification. Records were only kept if associated with relatively modern collections (arbitrarily set as 1950 - present), and we also excluded those associated with poor quality taxonomic or geospatial data, identified using a combination of GBIF issue tags and tests available in the R package CoordinateCleaner (v 2.0-11; Zizka et al., 2019; Supplementary Data Methods S3). For each species, we kept a single record per location, to prevent well collected sites from skewing results (Reichgelt et al., 2018). Taxa represented by fewer than ten occurrences were dropped from the final dataset (101 species, ~20% of those in phylogeny).

For each occurrence record we extracted percent local tree cover (250m scale) using v.6 of the MOD44B Vegetation Continuous Fields (VCF) maps published by the National Aeronautics and Space Administration (NASA) (DiMiceli et al., 2021). For each occurrence we recorded the median coverage value within a 1km radius (median uncertainty among downloaded GBIF records) and then averaged yearly values over the 21 year recording period (2000 – 2020). Data were extracted using Google Earth Engine (Gorelick et al., 2017), with script provided in the Supporting Information (Methods S3). We additionally extracted the proportion of species occurrences found in grassland regions using Dixon et al.'s (2014) map of world grasslands. We thus obtained two complimentary GIS habitat predictors. Tree cover data are resolved to a higher spatial resolution, but measure openness rather than grasslands per se (which can vary in degree of woody cover; Oliveira and Marquis, 2002; Sankaran et al., 2005). By contrast, grassland region data reflect the distribution of grasslands more directly, but are resolved to a coarser spatial resolution. We expect that grassland adapted taxa will generally occur in grassland regions at sites with low tree cover, although having them separate will allow us to tease apart potential drivers (e.g., whether grassland occupation, or openness more generally is the more likely driver).

Our final metric identified grassland adapted clades using an ancestral state reconstruction (ASR), which combined information from both GIS based measures (tree cover, grassland region) and published descriptions of species ecology. We compiled a list of grasses in our dataset unambiguously associated with grasslands via a literature survey to identify taxa considered ecologically dominant within a global sample of grassland ecosystems (Supplementary Data Methods S3). This was supplemented by a list of taxa unambiguously absent from grasslands, on the basis of restriction to forest habitats (Supplementary Data Methods S3). The result was a list of 90 reference taxa, 66 occupying grasslands and 24 absent from them. Using these taxa in conjunction with GIS habitat predictors, we defined a prior probability of grassland adaptation for each species in our dataset, which was incorporated into ASR. Priors were constructed by rescaling each GIS predictor so taxa with values comparable to known grassland dominants were considered more likely to be grassland adapted, while those closer to forest taxa were considered less likely to be grassland adapted. Maximum and minimum values were set 0.9 and 0.1 respectively (Fig. 1). Reference taxa were given prior probability values of 0 (non-grassland) or 1 (grassland), and species without any habitat data were assumed to have equal likelihood of occupying either habitat type (i.e., 0.5). The latter were only kept if associated with dispersal data (26 taxa). To make full use of available data, we defined priors for all taxa in the phylogeny with habitat information (359 species), regardless of whether they had dispersal data. This allowed an additional ~100 taxa (compared to the dispersal dataset) to inform habitat reconstructions.

Priors were incorporated into ASR of grassland occupation, completed using stochastic mapping (Huelsenbeck et al., 2003) as applied in the R package phytools (v 1.0-1, Revell, 2012). Models were fit under a “symmetrical” rates model and ran for 250 simulated character mappings. Membership within major clades reconstructed as ancestrally grassland adapted was incorporated into subsequent analyses.

As shorthand, we refer to this classification as “grassland radiation” below. For all taxa reconstructed as grassland adapted, we also extracted the grassland type in which they were most frequently found (e.g., temperate, tropical montane, etc), following the coarsest classification scheme of Dixon et al. (2014). We refer to this classification as “grassland formation” or “type” below.

Comparative Analysis

Ancestral State Reconstruction and Morphospace Visualization – For each of our habitat predictors and dispersal traits we ran ancestral state reconstructions (ASR) using functions available in the R package *phytools* (v 1.0-1, Revell, 2012). For categorical traits we employed stochastic mapping (Huelsenbeck et al., 2003) with character histories reconstructed based on 250 simulated character mappings. Rates models for these reconstructions were selected on the basis of Akaike Information Criterion (AIC) values. Continuous traits were reconstructed using the *contMap* function (*phytools* v 1.0-1, Revell, 2012).

To visualize the dispersal morphospace we compiled all traits scored for both fossil and modern taxa (attachment potential, falling velocity, lemma induration, callus type, awn length) and ordinated them using Factor Analysis of Mixed Data (FAMD; Pagès, 2004), as applied by the *FactoMineR* package (v 2.6, Lê et al., 2008). This method was chosen to accommodate the combination of continuous and categorical traits in our dataset. To incorporate reconstructions of past trait states we included node states from trait specific ASRs in the ordination (Guillerme et al., 2020). Resulting FAMD coordinate values were then visualized using the *phylomorphospace* function (*phytools* v 1.0-1, Revell, 2012). Because diaspore mass could not be measured in fossils, surface area was used as a substitute, since it was strongly correlated with mass in extant taxa ($p << 0.001$, $R^2 = 0.64$).

Phylogenetic Multilevel Models – To test whether the evolution of traits associated with anemochory, endozoochory, and epizoochory were correlated with grassland predictors we fit phylogenetically informed Bayesian multilevel models using the R package *brms* (v 2.15.0; Bürkner, 2017). Four sets of models were fit, corresponding to the following four responses: **1)** falling velocity, modelled as lognormal distribution, **2)** multivariate response of AtP and diaspore roughness modelled, respectively, as skew-normal and lognormally distributed, **3)** the presence or absence of all three categorical endozoochory traits (diaspores non-membranous, with blunt calli, and without awns) modelled as a Bernoulli distribution, and **4)** mass modelled as lognormal distribution. Default link functions (i.e., function defining relationship between response distribution and the linear predictor) were used in all models. For all models, percent tree cover, proportion of occurrences in grassland regions, and grassland radiation or type were included as predictors. For the latter, we avoided including both grassland radiation and type in a single model due to their strong collinearity. In addition, we tested for potential interactions between habitat predictors and plant growth habit and diaspore mass (except in mass response models), given the ability these traits to influence dispersal outcomes (Albert et al., 2015; Brightly et al., in prep.). Phylogenetic information was incorporated by treating species as a grouping factor over which the model intercept was allowed to vary, with this effect correlated between taxa via a phylogenetic covariance matrix computed from our phylogenetic reconstruction (*ape* v. 5.4; Paradis and Schliep, 2019). Four chains of 4,000 generations were run, with every other generation sampled and the first 1,000 discarded as burnin. Weakly informative priors were assigned to all model parameters (Supplementary Data Tables S5-S8).

RESULTS:

Habitat Data

From the original 6.7 million records obtained from GBIF, there were 470,910 from 3,044 species (82% of species with dispersal data) that met our sampling criteria. Number of records per species varied from 10 – 4319 (median 44). The percent of species' occurrences found in known grassland regions varied from 0-100% (median 30%). The average value for grassland reference taxa was substantially higher than non-grassland reference taxa (51% ± 5 versus 10% ± 6). Percent tree cover ranged from 0 – 75% (median 17%), with grassland reference taxa lower (14% ± 2) than non-grassland reference taxa (50% ± 5). A number of extracted MOD44 data points were flagged as potentially being of lower quality (e.g., due to cloud cover). Excluding these points resulted in a small number of taxa (~4%) being dropped from the dataset, but otherwise did not substantially change observed patterns or model results (see Results: Comparative Analysis). GIS data with extracted habitat variables are included with the Supporting Information (Table S4).

Ancestral state reconstructions based upon rescaled GIS predictors and grassland and non-grassland reference taxa identified five major radiations of grassland adapted taxa, along with several smaller groups of grassland adapted taxa. In order size in our sample these were 1) the Chloridoideae, 2) the majority of the Andropogoneae, 3) the Aristidoideae, 4) the Triticeae, and 5) the Paspaleae (Fig. 1). The Paspaleae also included a small group which transitioned out of grassland ecosystems, that we classified with the remainder of the tribe due to their persistence in relatively open habitats (Fig. 2). Likewise, although only a few of the sampled extant members of the Stipeae showed strong association with grassland environments (Fig. 1), we classified them with the other five radiations owing to their historic importance in early grasslands (Elias, 1942; Thomasson, 1987; Strömberg, 2005) and general proclivity for open habitats (Fig. 2). Most of the species we reconstructed as grassland adapted occupied temperate (32 species) or lowland tropical grasslands (29), with a smaller number classified a warm-arid (8 species). Although several other grassland types (e.g., tropical montane) were found, they were all grouped with one of these larger groups to avoid prohibitively small sample sizes in subsequent analyses.

At fossil sites, we were able to obtain useable phytolith data from eight sedimentary layers, representing five diaspore sites, which spanned the temporal range of the diaspore record (Oligo-Miocene boundary to Late Miocene; Supporting Information Results S1). Phytolith assemblages at all sites were interpreted as grass-dominated based on the preponderance of morphotypes produced by open habitat grasses (Strömberg, 2005 Strömberg and McInerney, 2011; Strömberg et al., 2018). The sole exception was the oldest site (Monroe Creek/Harrison Fm, Table 1), which is reconstructed as closed canopy forest, due to abundant forest indicator phytoliths, including from bamboos and/or other forest grasses (Strömberg, 2005, Gallaher et al., 2020). At grass-dominated sites, phytolith assemblages suggest grass communities were predominantly composed of stipoid grasses, suggesting that domination of the diaspore record by this tribe reflects a true pattern of relative abundance of those grasses. This reconstruction is consistent with previous work in the region (Strömberg, 2005; Strömberg and McInerney, 2011). Full assemblage results for each analyzed site are included in the Supporting Information (Results S1).

Comparative Analysis

Maximum likelihood topologies from both the full and trimmed matrixes, with support values are provided in the Supporting Information (Figs. S7-S8). The maximum agreement subtree (MAST) topology preserved the vast majority of relationships recovered in the full matrix analysis (> 96% of relationships conserved). Reconstructions of grassland adaptation using this topology, and all other ancestral state reconstructions (ASR) for dispersal traits not presented below are presented in the Supporting Information (Fig. S9-S14). Multilevel models showed convergence of chains and adequate sampling of the posterior distribution, with all effective sample sizes exceeding 500 per chain and Rhat values of 1.0 (the expectation at convergence) for all model parameters. Results reported below are separated by model response. Values are from models returning the strongest results for the relevant predictor. Full outputs, including those using alternate MOD44 tree cover data and MAST topology are included in the Supporting Information (Results S2 – S5).

Falling Velocity – Velocity showed a positive correlation with both percentage of tree cover (slope estimate 0.89, 95% posterior credibility interval 0.25 – 1.54) and grassland region (0.43, CI: 0.14 –

0.71; Fig. 3). However, the latter disappeared when mass was included as a model predictor (0.13, CI: -0.08 – 0.36). Grassland radiation did not show consistent correlation (Supporting Information Results S2), but formation type did, with grasses of warm-arid grasslands showing higher falling velocities (0.52, CI: 0.18 – 0.85), although this effect disappeared in models including mass (0.13, CI: -0.14 – 0.39). None of our models supported the existence of interactions between habitat, diaspore size, or growth habit(Supporting Information Results S2). Using trimmed tree cover data and MAST topology, resulted in only minor changes that did not meaningfully affect interpretations (Results S2).

Mass – Diaspore mass showed positive correlation only with formation type, with warm-arid grassland taxa tending to be more massive (0.80, CI: 0.01 – 1.58; Fig. 4). Percent tree cover (0.12, CI: -1.04 – 1.29), grassland region (0.54, CI: -0.11 – 1.20), and radiation (Supporting Information Results S3) did not show any pronounced effect. In addition, we found no support for interactions between any of these predictors or with growth habit. Using trimmed tree cover data and MAST topology returned similar, albeit slightly weaker results for all parameters (Results S3).

Endozoochory – Categorical traits associated with endozoochory did not show any direct correlation with either degree of tree cover (135.76, CI: -98.03 – 403.95) or grassland region (38.11, CI: -87.51 – 173.44). Formation type showed weak correlation, with warm-arid grassland taxa more likely to possess endozoochory related traits, although credibility intervals (CI) for this effect overlapped zero (-292.32, CI: -687.01 – 2.10). Radiation showed a clear relationship, with none of the grassland Andropogoneae possessing all three traits, and the entire Paspaleae having the full complement (Supporting Information Figs. S10-S12). We additionally recovered an interaction between grassland region and radiation, which showed stipoid grasses in grassland regions as less likely to possess all three endozoochory traits than other groups, although this effect had high uncertainty as only a handful of these taxa possessed all three traits (Supporting Information Fig. S15). Using trimmed tree cover data and MAST topology had no impact on results (Results S4).

Epizoochory – Models predicting diaspore roughness and attachment potential revealed the most complex relationships. Note that roughness is expressed on a counterintuitive scale, with lower values indicating rougher diaspores. Below, we refer to diaspores as rougher (i.e., low roughness value) or less rough (i.e., high value) for ease of interpretation. Degree of tree cover did not show clear association with attachment potential (AtP; - 0.16, CI: -0.88 – 0.56; Fig. 5). Although it did show a weak relationship with roughness (suggesting rougher diaspores in more open areas) the CI for this effect overlapped with zero (0.27, CI: -0.05 – 0.60). The opposite pattern was observed with grassland region, which showed no clear correlation with roughness (-0.07, CI: -0.22 – 0.07), but a slight positive correlation with AtP (0.33, CI: -0.04 – 0.69). Radiation did not show any correlation with either response, although grassland type did – with warm-arid grassland taxa tending to have less rough diaspores (0.22, CI: 0.03 – 0.40). However, AtP in species of warm-arid grasslands was not different from other grasses (0.37, CI: -0.20 – 0.92), being offset by increased exposure. We found evidence for a number of two way and three-way interactions between diaspore mass, formation type, radiation, and grassland region which affected both diaspore roughness and AtP. These suggested that as taxa became more restricted to temperate grassland regions, their diaspores became less rough, with more pronounced effects in taxa with more massive diaspores (Fig. 5). By contrast, small seeded, warm-arid grassland taxa tended to have rougher diaspores the more restricted to those regions they became, while large seeded taxa showed the opposite relationship. Consistent with roughness patterns, temperate grassland taxa with larger seeds tended to have lower AtP as they became more restricted to those environments (Fig. 5). Counterintuitively, however this relationship was reversed in smaller seeded taxa, suggesting these also displayed increased exposure. Analysis using trimmed tree cover data and MAST topology recovered the same major relationships, although these were strengthened in most cases, particularly the trend of rougher diaspores in open areas, and differences between grassland types (Results S5).

Reference Taxa – Taxa described in the literature as displaying anemochory, endozoochory, or epizoochory showed predictably strong values for variables relevant to each of those dispersal modes (Table 1). Taxa were also recovered as distinct occupants of our reconstructed morphospace (Fig. 6). The degree of variation was highest among endozoochorous reference taxa, although they did not overlap with the two remaining groups.

Fossil Reconstructions

In total, we obtained dispersal data for 150 fossils representing 14 species from all known fossil genera (Table 1). Lemma texture was scored as indurate for all included fossil taxa except *Archaeoleersia*. The manner of preservation suggests a high degree of silicification in living bracts, implying an indurate morphology (Elias, 1942; Thomasson, 1985). This is consistent with restriction of the recovered taxa to clades that evolved indurate reproductive bracts. *Archeoleersia* was coded as coriaceous, matching all sampled extant *Leersia* (Supporting Information Fig. S10). Awns of all fossil taxa were interpreted to be absent (e.g., *Panicum elegans*, *Archaeoleersia nebraskensis*) or deciduous (*Berriochloa* spp., *Paleoeriocoma hitchcockii*, *Nassella reynoldsii*). The former was on the basis of their absence from extant *Panicum* and *Leersia* (Clayton et al., 2021), and the latter was based on the tendency for many extant stipoid grasses to lose their awns, and the total absence of awns from all fossil specimens (Elias, 1942; Thomasson, 1985; Clayton et al., 2021). All stipoid grasses (*Berriochloa* spp., *Paleoeriocoma hitchcockii*, *Nassella reynoldsii*) were coded as enclosed, since the glumes of all extant Stipeae are persistent and reach or exceed the apex of the lemma (Clayton et al., 2021). Although the awns of many extant Stipeae are exposed beyond the apex of these glumes, the likely deciduous awns of fossil taxa preclude them from being coded as either partially or entirely exposed (although see Discussion: Taphonomic Biases). As all extant *Leersia* have exposed diaspores (Fig. 5), *A. nebraskensis* was coded thusly. *Panicum elegans* was coded as enclosed, although this interpretation relies on the acceptance that the floret, rather than the spikelet was the dispersal structure, and thus was obscured by the sterile floret and glumes (see discussion in Supporting Information Results S6). In addition to generally low exposure, fossils were less rough than most extant taxa, which together contributed to low predicted attachment potentials (Table 1).

Regression models predicting falling velocity found several traits strongly correlated with falling velocity. The best-fit model (see Supporting Information Results S6 for full model comparisons) included diaspore roughness, floret solidity (i.e., ratio of object area to area of its convex hull) , log-transformed floret area, square root of awn size, and degree of lemma induration as predictors (solidity and area measures both excluded awns). All predictors were significant and the model explained 51% of the variation in falling velocity, which decreased in taxa with smaller florets (excluding awn), larger awns, less indurate bracts, and more complex diaspore shapes or surface structure (Fig. 6b,c). Falling velocities of fossil taxa, predicted from this model, were higher than the average observed among extant grasses (Table 1). The combined dispersal traits of fossil taxa resulted in their being restricted to a small, peripheral portion of dispersal morphospace (Fig. 6d –f),adjacent to extant stipoid grasses, and species with indurate lemmas (Fig. 6e,f). *Archaeoleersia* was a slight exception, falling nearer to the center of the area of morphospace occupied by extant taxa.

DISCUSSION

Patterns Among Modern Grasses

Our results reveal a complicated pattern of dispersal evolution in grasses with respect to habitat. Support for the prediction that traits associated with endozoochory and anemochory are primarily found in lineages with higher grassland affinity was mixed. Similarly, while tree cover showed some positive association with epizoochorous traits, the hypothesis that their evolution is primarily favored in forests was not strongly supported. Instead, results suggest that dispersal evolution tends to be lineage and habitat specific, with grasslands playing a variable role.

Our study indicates that key aspects of habitat structure may in many cases be more important than grass-dominance per se. This is exemplified by wind dispersal results, which showed a stronger correlation with tree cover than with occupying grassland regions or membership in a grassland adapted clade (Fig. 3). Correlation with openness is intuitive, since open habitats typically have circulation conditions more conducive to wind dispersal (e.g., stronger updrafts) and fewer barriers to movement (Stebbins, 1971; Tackenberg et al., 2003; Ozinga et al., 2004; Nathan et al., 2008). Diaspore traits facilitating anemochory would thus have the greatest impact on dispersal outcome, and thus fitness, in open regions. Although grasslands tend to be open, the degree of canopy cover varies considerably between regions and through time (Oliveira and Marquis, 2002; Sankaran et al., 2005; Strömberg, 2011), and not all open habitats are grass-dominated (Dixon et al., 2014; Dunn et al., 2015). Consistent with our interpretation, recent work has uncovered a similar correlation with openness in the melicgrasses, which are not strongly associated with grasslands (Fig. 1; Brightly et al., in prep.).

Grassland type appears to influence dispersal evolution, with warm-arid grassland species, in particular, recovered as distinct due to their typically more massive diaspores. This trait also helps explain high diaspore falling velocities among these taxa. Wind dispersal potential may be even further reduced, because plants of arid regions are more likely to be short (Brightly et al., in prep). Although compromising wind dispersal potential, seedlings sprouting from larger seeds are often better able to cope with drought conditions, which has led to selection for higher seed mass in some dry-adapted lineages (Leishman and Westoby, 1994; Moles and Westoby, 2004; Salazar-Tortosa et al., 2019, although see Westoby et al., 1992). Consequently, the unique response in warm-arid grasslands may result from dispersal limitations imposed by traits promoting establishment success. Water availability is known to impose dispersal limitations in a number of groups (Tabarelli et al., 2003; Hulshof and Spasojevic, 2020). Warm arid grassland species also appear marginally more likely to possess the endozoochory promoting traits of greater bract induration and absence of long awns or sharp calluses. However, the high uncertainty of this result and the trend of these taxa away from the optimal endozoochory promoting trait of smaller daispore size suggests there is unlikely to be strong selection promoting endozoochory in these habitats. In this regard at least, warm arid grasslands appear to not differ from other grassland types.

The most complex effects of grassland type appear among epizoochory related traits. Warm-arid taxa again were distinct, generally possessing less rough diaspores, although this did not translate to lower predicted Attachment Potential (AtP; e.g., due concurrent increases in exposure; Fig. 5). More broadly, the influence of grassland type varied substantially with both the strength of species' association with grassland regions and the size of the diaspores they produced. Among temperate grassland species, greater restriction to those habitats correlates with less rough diaspores, particularly when they are also heavy (i.e., with poor retention potential; Tackenberg et al., 2006). By contrast, as small seeded taxa become more restricted to temperate grasslands, their predicted AtP increases, apparently as a consequence of greater diaspore exposure since they do not become notably rougher (Fig. 5).Small seeded taxa are also more likely to be retained on animals for longer periods, and thus dispersed further (Römerman et al., 2005; Tackenberg et al., 2006). Warm arid grassland taxa showed a similar pattern with roughness, whereby small seeded taxa acquired traits more consistent with epizoochorous dispersal as they increasingly entered grassland regions, while large seeded taxa showed the opposite pattern (Fig. 5); note, however, that these results were not mirrored in predicted AtP. In contrast, species from lowland tropical grasslands and savannas showed no such patterns. Thus, it appears in some grassland types, evolution of diaspore traits consistent with adhesive dispersal is promoted, while in others it is not. Furthermore, effects are strongest in taxa that can be successful in both attachment and retention of diaspores, whereas species with more massive diaspores, which are less likely to be retained for extended periods, tend to adopt traits that make attachment less likely.

Relationships within epizoochory traits demonstrate another feature of our results, namely that phylogenetic history has a major impact on patterns of dispersal traits. As described above, this is exemplified by the role that diaspore mass has in altering the relationship between habitat and adhesive dispersal traits. Exposure also has a high phylogenetic signal since it is largely dependent on the structure forming the diaspore, which is highly conserved (Supporting Information Figs. S13). The Stipeae for example retain their glumes on the parent, which consequently cover a portion of the floret which serves as the diaspore. Our three discrete endozoochory promoting traits were also phylogenetically conserved (Supporting Information Figs. S10-S12), with three of the identified grassland radiations always (Paspaleae) or never (Aristidoideae, Andropogoneae) possessing all three traits. The apparent adaptation of the Paspaleae to this dispersal mode is unsurprising, given that the tribe contains two of our endozoochorous reference taxa (Table 1). Furthermore, while grasses as a whole showed no clear relationship between grassland occupation and the presence of these traits, they were apparently disfavored in grassland regions among the stipoid grasses. Thus, although we were unable to recover strong evidence that grasslands consistently promoted the evolution of endozoochory traits, our results do suggest that group phylogenetic history is a critical determinant of whether that dispersal mode can be successfully realized.

In summary, the role of grasslands in dispersal evolution is complex. While the increased openness they often provide might promote the evolution of wind dispersal traits, other habitats (e.g., shrubland) likely do the same. Our original hypotheses regarding biotic vectors were also not strongly supported. Epizoochory appears to be a strategy which can be successful in both open and closed habitats. Potential dispersers are abundant in many grassland environments, and there are multiple reports of successful epizoochorous dispersal in them (e.g., Fischer et al., 1996; Rosas et al., 2008). It thus makes sense that evolution would favor traits facilitating this type of dispersal in a range of environments, although a variety of factors may influence the likelihood of species adopting this strategy. The absence of any clear patterns among endozoochory promoting traits may be partly methodological since the traits facilitating this are the least well understood, as evidenced by our reference taxa not always possessing the full complement of purported endozoochory traits (Table 1). While seeds undoubtedly disperse following this method, there is debate about the degree to which there is evolution for traits promoting it (Collins and Uno, 1985; Dinnerstein et al., 1989; Bruun and Poschlod, 2006). Our failure to find any consistent pattern of adoption of these traits in habitats where exposure to potential endozoochory vectors is highest, is consistent with much of the criticism of the "foliage is the fruit" hypothesis (Janzen, 1984). While some grassland taxa may fit this type (e.g., Quinn et al., 1994), they are likely the exception rather than the rule.

Fossil Grasses:

Reconstructed Dispersal Ecology - Fossils grasses display an interesting suite of characters, which largely place them at the periphery of the functional morphospace of extant grasses. Our results suggest that they were unlikely to have been well suited to anemochory, in large part due to their lack of persistent awns and possession of well indurated (i.e., heavier) lemmas (Fig. 6b, 6c; Table 1). Deciduous awns and the large (relative to the lemma) persistent glumes in all extant (and presumably fossil) Stipeae also ensured a low degree of diaspore exposure, suggesting poor attachment potential, although the typically small diaspore size may have facilitated long retention times once attached (Table 1; Römermann et al., 2005; Tackenberg et al., 2006). The absence of any taxa without a sharp callus until the latter half of the Miocene (Table 1, Fig. 6a) also suggests that during the initial expansion of grasslands most taxa were poorly adapted to endozoochorous dispersal. That said, the small size of many *Berriochloa* (Table 1, Fig. 6a) may have meant the negative impact of a sharp callus was mitigated (i.e., less likely to be injurious and thus avoided by consumers). The more recent, *Paleoeriocoma, Nassella,* and *Panicum,* by comparison, all possessed the full complement of traits conducive to endozoochory. The suite of traits in *Berriochloa*, however, make it difficult to assign it to any dispersal mode with any degree of certainty.

One additional dispersal vector worth considering for fossil grasses is ants (myrmecochory). Potential for this type of dispersal is suggested by the aggregation of fossil anthoecia in lenses which have been interpreted as fossilized ant nests (Smith et al., 2011). However, ant dispersal mutualisms are traditionally associated with lipid rich appendages (eliasomes), which are consumed while seeds are discarded and thus escape predation (van der Pijl, 1982; Edwards et al., 2006; Lengyel et al., 2010). These appendages are unknown from modern stipoid grasses and too labile for preservation in the fossil record. It is thus difficult to discern to what extent plants benefitted this relationship (i.e., whether it was purely predatory), and thus whether there might be selection for traits promoting it. Commonly, ants that cache large numbers of seeds inside their nests, as observed in the fossil record, are specialized granivores, and thus poor-quality dispersers (Giladi and Larsson, 2006). Unfortunately, outside the eliasome, there are few diaspore traits clearly associated with a myrmecochorous habit (van der Pijl, 1982), making it even more difficult to discern the true nature of this relationship. A number of extant Stipeae are known to interact with ant seed predators which sometimes facilitate dispersal (Schöning et al., 2004). Although little is currently known about coevolution of Stipeae with ants, these taxa may hold the key to disentangling the nature of ant-grass relationships during the Miocene.

Interpretations of the dispersal ecology of fossil Stipeae hinge partially on the assumption that the awns were completely and rapidly deciduous. This seems intuitive given their complete absence from the fossil record and the presence abscising awns among extant members of the tribe (Elias, 1942; Cialdell and Giussani, 2002; Clayton et al., 2021). However, the ease with which supposedly deciduous awns become detached varies (van der Pijl, 1982, pers. obs.), and some taxa have tardily deciduous awns facilitating epizoochorous dispersal. In *Heteropogon contortus*, the awns of adjacent diaspores become entangled allowing them to remain attached to the plant even after spikelets are abscised.

Spikelets are consequently exserted, facilitating attachment to passing animals. A weak connection between the awn and the spikelet means that contact with a passing animal is likely to result in attachment of only the main body of the spikelet, now much smaller and more likely to be carried a long distance (van der Pijl, 1982). There are reports that some stipoid grasses (e.g., *Hesperostipa* spp., hypothesized nearest living relative of *Berriochloa*, Thomasson, 1978) disperse following a similar pattern (van der Pijl, 1982), although no thorough study has been conducted.

Our phytolith data suggest that the earliest *Berriochloa* were found in habitats with features consistent with closed canopy forests (Fig. 6a; Supporting Information Results S1). The remaining genera (with the exception of *Archaeoleersia* which, like its living relatives was associated with bodies of water; Thomasson, 1980b) are all exclusively recovered from sites reconstructed as being grass-dominated (e.g., open woodland, grassland mosaic; Supporting Information Fig. S6). Given the small sample sizes involved, and presence of *Berriochloa* in both habitat types it is difficult to draw any strong conclusions from temporal patterns. Nevertheless, the counterintuitive suite of traits present in *Berriochloa* may partially be explained by their origination in closed sites (whether understory or margin) and subsequent transition into non-analog early grasslands (Strömberg, 2011). Per our modern results, poor wind dispersal capability may be tied to ancestral occupation of closed habitats, and the relatively greater woody cover of the early grass-dominated habitats which replaced them (Thomasson, 1990; Strömberg, 2011). *Berriochloa* also appears in the fossil record before the establishment of a highly specialized grazing fauna (Strömberg, 2006), which may have impacted the likelihood of diaspore consumption, and thus whether endozoochory promoting traits provided any fitness advantage. The appearance of small, awnless, and well indurated diaspores without sharp calluses only in the latter half of the Miocene, and the derived status of most of these traits within the tribe (Cialdella and Giussani, 2002; Supporting Information Figs. S10-S12) suggests that any selection for endozoochory would have only occurred more recently. In general, later Miocene sites are functionally more diverse than Early Miocene sites, almost entirely the result of increased generic diversity (Thomasson, 1985; Fig. 6).

Taphonomy – Regardless of their putative dispersal strategy, it is clear that the fossil taxa included here occupy only a limited proportion of the available functional dispersal space (Fig. 6). The difference is especially stark in comparison with the ecologically dominant taxa of extant grassland communities, which typically include a range of morphologies more comparable to that observed in the family as a whole (Fig. 6, see also Supporting Information Figs. S16-S19). Fossil communities don't approach this, even if the entire ~20Ma year record is condensed to a single anachronistic community (Fig. 6). Two non-competing hypotheses can be used to explain this pattern. The first is that early grass-dominated habitats possessed a non-analogue range of dispersal ecologies, perhaps due to a different distribution of dispersal vectors (e.g., greater canopy coverage, paucity of grazers), as discussed above. The second is that our view of the range of dispersal ecologies is biased by the type of grasses which are preserved in the fossil record.

The current best hypothesis is that fossil anthoecia are preserved, largely as a result of plants depositing silica in the palea and lemma when alive, making the tissues more resistant and thus less likely to be lost (Elias, 1942, Thomasson, 1985). With the exception of *Archaeoleersia*, all recovered taxa belong to lineages which have at least some well indurated members (Supporting Information Fig. S10). However, because the known record of *Archaeoleersia* is restricted to nine samples from a single level at a single site (Thomasson, 1980b), it appears to be the exception rather than the rule. Interpretation of the fossil record as comprising only the indurate component of the grass flora, may explain the narrow range of observed dispersal ecologies, since extant species with indurate bracts occupy a restricted region of morphospace, that fossils overlap (Fig. 6e). The complete or nearly complete dominance of stipoid grasses through the Miocene (Fig. 6; Elias, 1942, Thomasson, 1990), also suggests the record is biased, since extant grasslands are typically much more diverse (Supporting Information Methods S3).

Although phytolith records appear to corroborate this dominance at some sites, several other open-habitat grass lineages were also present (e.g., Chloridoideae), if not always in high abundance (Strömberg, 2005; Supporting Information Fig. S6). Furthermore, although the majority of stipoid grasses have high levels of bract induration, membranous lemmas are a feature of several genera (e.g., *Jarava*), particularly those with high wind dispersal potential (e.g., *Jarava;* Fig 6b,c; Supporting Information Fig. S20). Thus, even if communities were entirely composed of stipoid grasses, it does not rule out the possibility of that a portion of the community is missing from the record.

Adding to the biases described above, there is also an apparent size bias in the record, resulting from extreme fragility of most recovered anthoecia. Unfortunately, integration with our modern dataset requires more or less complete specimens, resulting in a likely bias toward smaller taxa, which more frequently were found complete. Thomasson (1985), describes a general trend of increased size in the genus *Berriochloa* through the Miocene, which is likely to be poorly reflected in our results because of this bias (Table 1).

Finally, the association of fossil anthoecia with ant nests raises the potential that the record may suffer from (non-human) collector bias. Although anthoecia associated with nests are by no means the only ones preserved, the relative ease in recovering these fossils means they provide the vast majority of specimens. Unfortunately, it is at present unclear whether any morphological or taxonomic differences exist between anthoecia preserved in and out of association with fossil ant nests. Several logistical issues (e.g., low fossil concentration, fragility precluding techniques like screenwashing) also make collection techniques that would address these issues difficult (e.g., census collections). Ultimately, however, our understanding of the record, the biases inherent in it, and the insights which can be gleaned from suffer most from a lack of study. Despite the biases, this record is one of the most useful sources for advancing our understanding of grass evolution, covering as it does an important period of transition for the family in great taxonomic detail.

Conclusion:

Overall, available data suggest that the evolution of dispersal strategy in grasses in response to the spread grassland environments was complex. Few family-wide patterns can be drawn, and while transition into and out of grasslands was likely consequential, the nature of effects we context dependent (e.g., to taxonomic group, climate). Although there are a number of challenges to integrating fossil data into our comparative framework, initial efforts suggest fossil taxa possessed range of dispersal ecologies poorly captured by modern species. This result reinforces the importance of fossil data to developing an accurate understanding of the evolution of dispersal strategy within the family.

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Figure 1: Combined geographic information system (GIS) and literature based ancestral state reconstruction of grassland affinity for all grasses with either GIS data or dispersal data. Panel **a)** shows range of values from MODIS tree cover variable, with reference taxa identified (green – forest, tan – grassland). Averages for each reference group, with 95% confidence intervals, are shown at the right, along with the rescaled prior probability values passed to ancestral state reconstructions (see Methods – Habitat Data). Panel **b)** shows ancestral state reconstruction of grassland adaptation with external and interior nodes inferred as grassland adapted shown in green, and non-grassland adapted nodes shown in grey. Labels represent major groups (tribe or subfamily). Abbreviations are given for the Anomochlooideae-Puelioideae-Pharoideae grade (APP), Arundinoideae (Aru.), Aristidoideae (Ari.), Bambusoideae (Bm.), Danthonioideae (Dan.), Micrairoideae (Micrair.), Oryzoideae (Oryz.), Paspaleae (Paspal.), and Triticeae (Trit.).

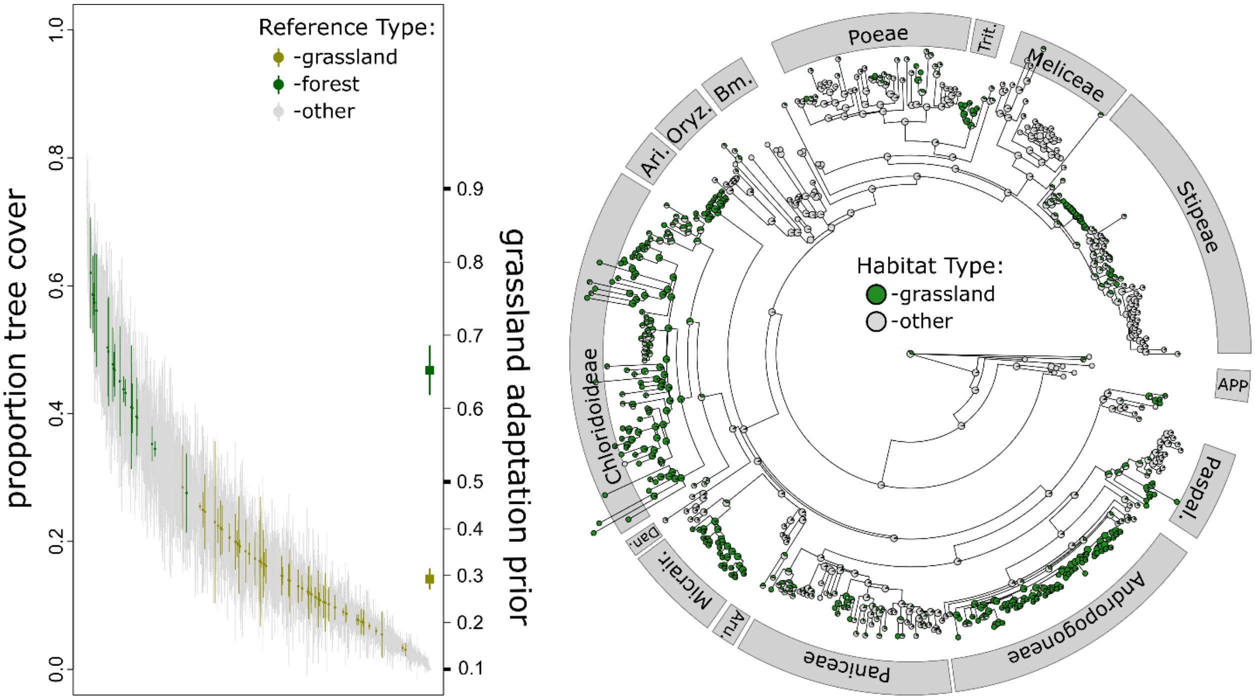


Figure 2: Ancestral state reconstructions of **a)** proportion of species' occurrences found in grassland regions, and **b)** reconstruction of percent tree cover. Both are overlayed with inference of grassland adaptation taken from Fig. 1b. Labels in both trees represent major groups (tribe or subfamily). Abbreviations are given for the Anomochlooideae-Puelioideae-Pharoideae grade (APP), Arundinoideae (Ar.), Aristidoideae (Ari.), Bambusoideae (Bm.), Danthonioideae (D.), Micrairoideae (Micrair.), Oryzoideae (Oryz.), and Triticeae (Tri.).

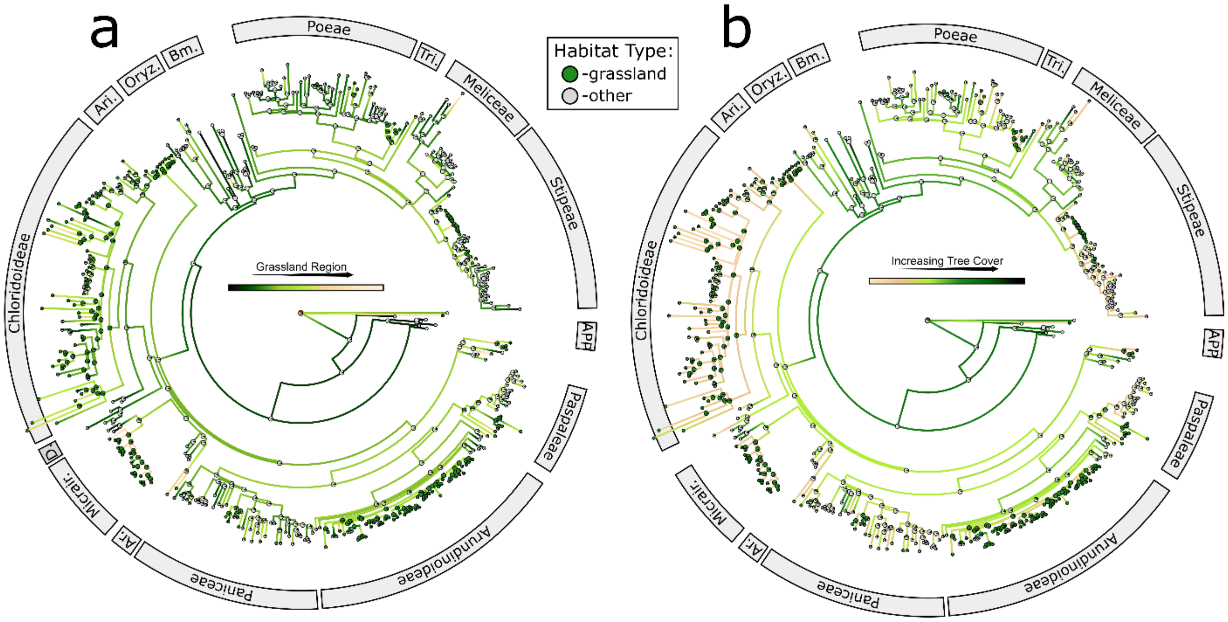


Figure 3: Evolution of diaspore falling velocity within the Poaceae, showing **a)** ancestral state reconstruction of falling velocity. Abbreviations are given for the Anomochlooideae-Puelioideae-Pharoideae grade (APP), Arundinoideae (Aru.), Aristidoideae (Ari.), Bambusoideae (Bm.), Danthonioideae (D.), Micrairoideae (M.), Oryzoideae (Oryz.), and Triticeae (Tri.). Panel **b)** shows posterior estimates for the effect sizes of grassland region occupation and degree of tree cover on the evolution of falling velocity. Mean estimate values and their 95% credibility intervals (CI) are also given. Panel **c)** shows posterior estimates (with mean and 95% CI) of the effect of different grassland types with non-grassland taxa as reference (i.e., zero). All effects are from models without interactions (see Results) and without mass as a predictor. Note the positive relationship of both GIS predictors with falling velocity despite their opposite sign.

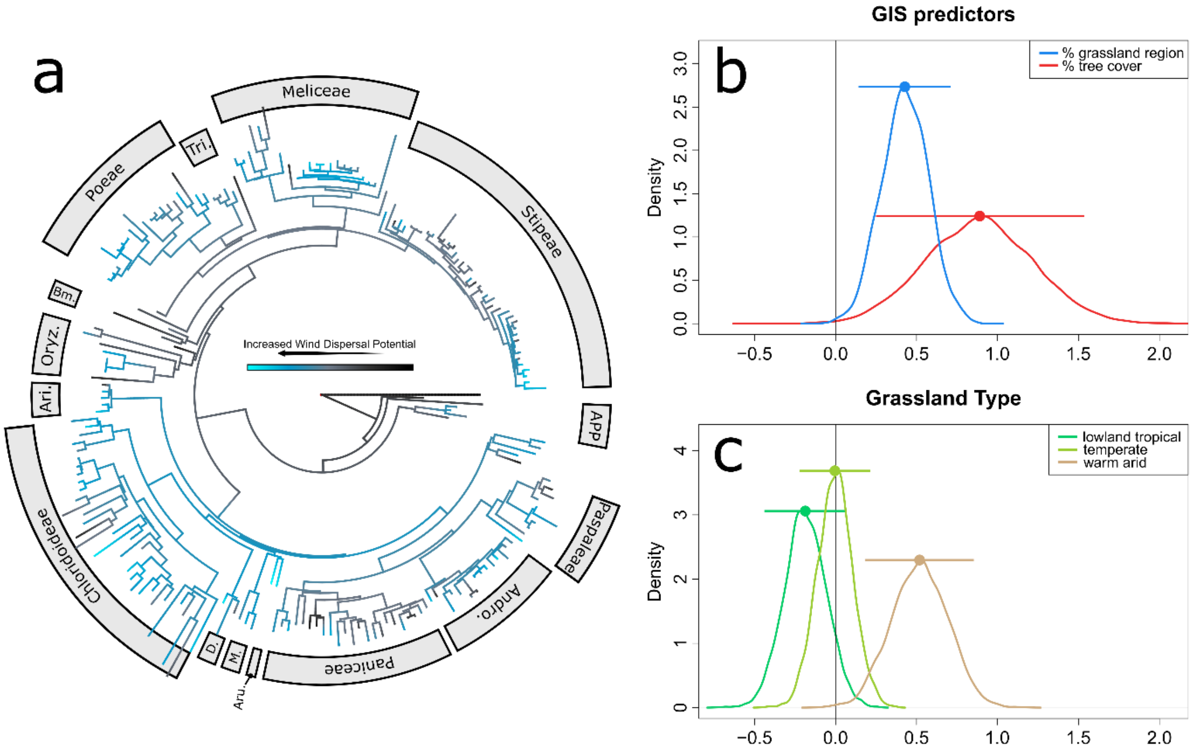


Figure 4: Evolution of diaspore mass within the Poaceae. Panel **a)** shows ancestral state reconstruction of log-transformed mass. Abbreviations are given for the Anomochlooideae-Puelioideae-Pharoideae grade (APP), Arundinoideae (Aru.), Aristidoideae (Ari.), Bambusoideae (Bm.), Danthonioideae (D.), Micrairoideae (M.), Oryzoideae (Oryz.), and Triticeae (Tri.). Panel **b)** shows posterior estimates for the effect sizes of grassland region occupation and degree of tree cover on the evolution of mass. Panel **c)** shows posterior estimates of the effect of different grassland types with non-grasslands reference (i.e., zero). The 95% credibility intervals for effect size overlapped with zero for all predictors, with the exception of warm-arid grassland.

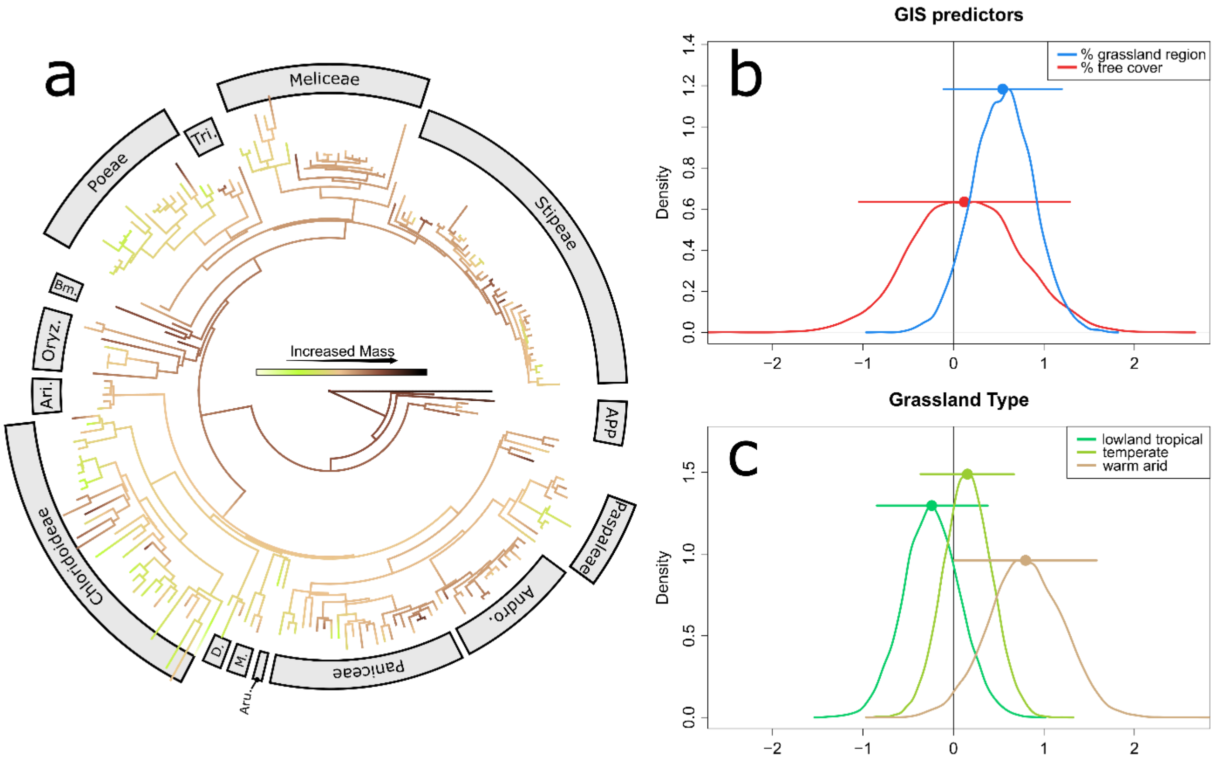


Figure 5: Evolution of adhesive dispersal traits within the Poaceae. Panel **a)** shows ancestral state reconstruction of predicted attachment potential within the family, with reconstruction of the degree of diaspore exposure shown at interior and external nodes. Abbreviations are given for the Anomochlooideae-Puelioideae-Pharoideae grade (APP), Arundinoideae (Aru.), Aristidoideae (Ari.), Bambusoideae (Bm.), Danthonioideae (D.), Micrairoideae (M.), Oryzoideae (Oryz.), and Triticeae (Tri.). Panel **b)** shows conditional effects for the relationship between diaspore roughness and degree of grassland region occupation, with effects broken down by grassland formation type. The three individual panels correspond to effects conditioned at smaller than average (0.2 g), average (2.3 g), and larger than average (9.2 g) diaspore mass. Note that higher roughness values correspond to smoother diaspores. Panel **c)** shows the same effects, but with predicted attachment potential as the response.

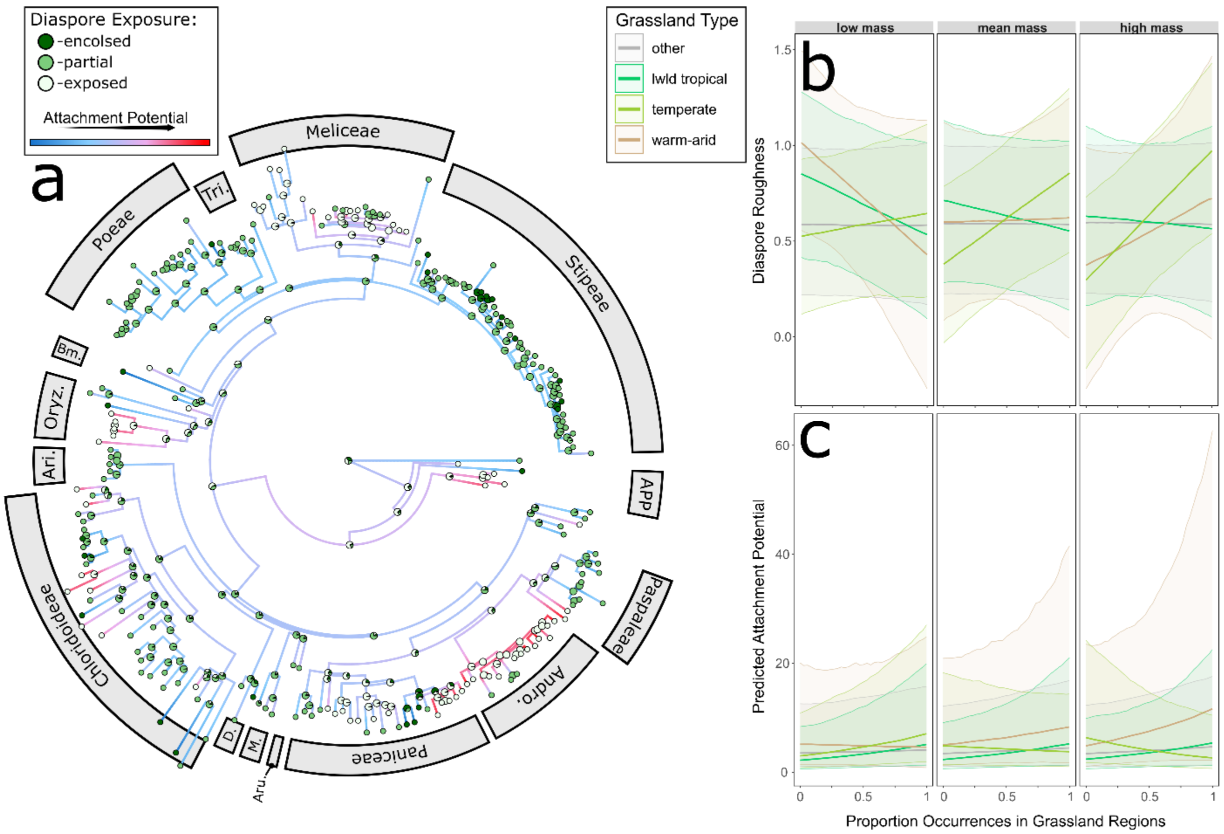
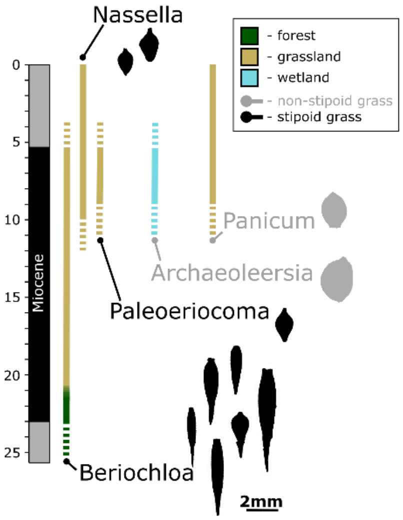
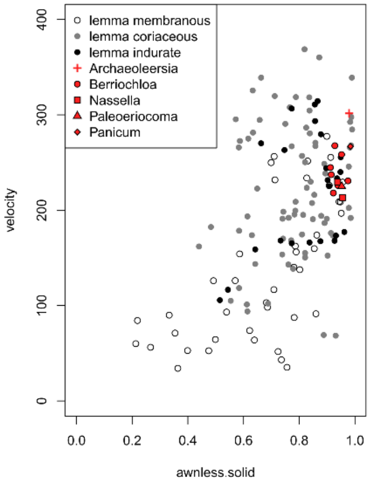


Figure 6: Reconstructions of fossil dispersal ecology. Panel **a)** shows approximate temporal ranges and habitat preferences of all five “seed” genera represented in the fossil record. Green reflects apparent affinity for forested habitats, while tan reflects affinities for grassland habitats, as reconstructed by phytolith assemblage analysis at sites preserving fossil anthoecia. Panel **b)** shows relationship between two important predictors included in our falling velocity calibration models (solidity of the floret without the awn, lemma induration), with predicted placement of fossil species shown (red). Panel **c)** shows also the effect of awn presence or absence in this model. Panels **d – f)** show the first two axes of the reconstructed phylomorphospace of modern taxa with fossil species overlayed in red. In each, the radiation of stipoid grasses is also indicated in green. Panel **d)** identifies the location of reference dispersal taxa, **e)** identifies the location of taxa with high bract induration, and **f)** identifies the approximate range of morphologies found among ecologically dominant members of the extant North American Great Plains, with the degree of lemma induration (and thus preservation potential) identified.


Nassella
0
5
10
15
20
25
Miocene
Paleoeriocoma
Beriochloa
Panicum
Archaeoleersia
2mm
- forest
- grassland
- wetland
- non-stipoid grass
- stipoid grass


lemma membranous
lemma coriaceous
lemma indurate
Archaeoleersia
Berriochloa
Nassella
Paleoeriocoma
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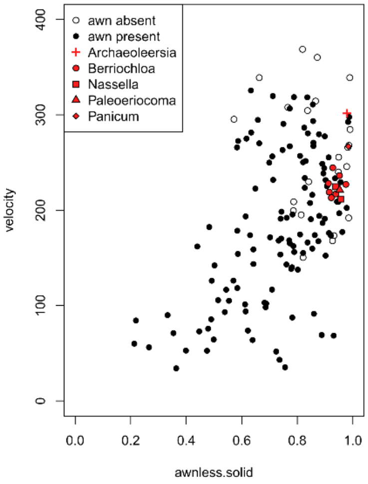
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Paleoeriocoma
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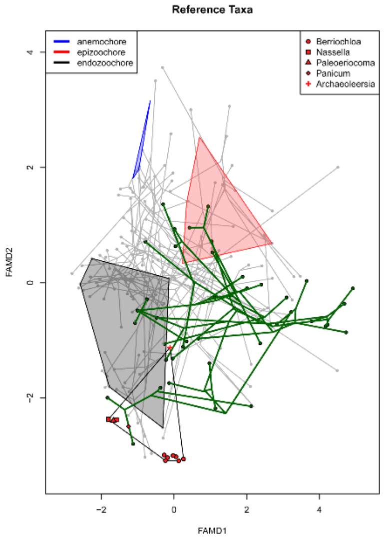
Reference Taxa
anemochore
epizoochore
endozoochore
Berriochloa
Nassella
Paleoeriocoma
Panicum
Archaeoleersia
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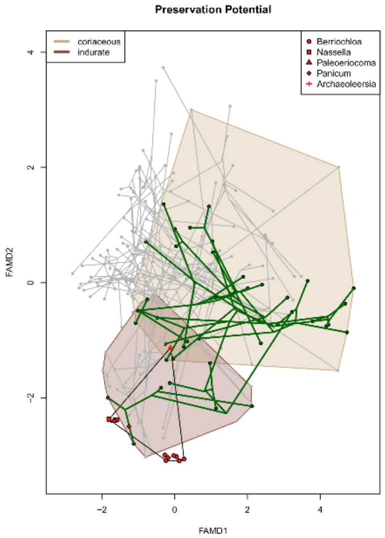
Preservation Potential
coriaceous
indurate
Berriochloa
Nassella
Paleoeriocoma
Panicum
Archaeoleersia
FAMD2
-2
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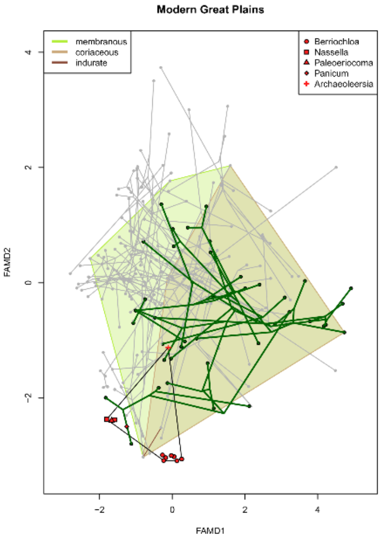
Modern Great Plains
membranous
coriaceous
indurate
Berriochloa
Nassella
Paleoeriocoma
Panicum
Archaeoleersia
FAMD2
-2
0
2
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FAMD1

Table 1: Next page. Table showing measured dispersal traits for reference taxa with known to successfully by epizoochory, endozoochory or anemochory and reconstructions for fossil taxa. Shown traits are those used directly, or indirectly in comparative models, with the exception of diaspore mass, which could not be directly estimated for fossil taxa. For modern taxa, references refer to the publications providing evidence for its dispersal type. Stratigraphic range data for fossil taxa was compiled from Elias (1942) and Thomasson (1978a, 1978b, 1980a, 1980b, 1982, 1985, 1987, 2005). Temporal range is given in North American Land Mammal Ages. These approximately correspond to absolute time as follows: Arikareean (26.3 – 20.6 Ma), Hemingfordian (20.6 – 16.3 Ma), Clarendonian (13.6 – 10.3 Ma), Hemphillian (10.3 – 4.9 Ma).

tip	**type**	**rough**	**exposure**	**AtP**	**velocity (cm/s)**	**lemma**	**callus**	**reference**
dataset average	-	0.54	partial	5.3	179.3	membranous	blunt	-
Cenchrus_echinatus	adhesive	0.18	exposed	18.27	279	coriaceous	sharp	Ridley (1930)
Cenchrus_longispinus	adhesive	0.18	exposed	18.06	308.66	coriaceous	sharp	Ridley (1930)
Circaea_lutetiana	adhesive	0.23	exposed	16.54	217.02	coriaceous	blunt	Will et al. (2007)
Crepis_atrabarba	adhesive	0.18	exposed	18.29	133.92	coriaceous	blunt	Will et al. (2007)
Geum_urbanum	adhesive	0.46	exposed	10.53	256.65	coriaceous	blunt	Will et al. (2007)
Agrostis_capillaris	internal	0.87	enclosed	0.93	92.1	membranous	blunt	Welch (1985)
Axonopus_fissifolius	internal	0.61	partial	2.62	171.22	indurate	blunt	Gardener et al. (1993)
Bouteloua_dactyloides	internal	0.55	enclosed	1.34	344.41	indurate	blunt	Quinn et al. (1994)
Bouteloua_gracilis	internal	0.26	partial	4.07	142.02	coriaceous	blunt	Quinn et al. (1994)
Cenchrus_clandestinus	internal	0.47	enclosed	1.44	232.69	membranous	blunt	Gardener et al. (1993)
Cynodon_dactylon	internal	0.82	partial	1.9	150.11	coriaceous	blunt	Anderson et al. (2013)
Panicum_coloratum	internal	0.8	exposed	4.08	111.98	indurate	blunt	Anderson et al. (2013)
Paspalum_notatum	internal	0.87	partial	1.73	188.17	indurate	blunt	Gardener et al. (1993)
Poa_annua	internal	0.66	partial	2.42	186.83	membranous	blunt	Mouisse et al. (2005)
Poa_pratensis	internal	0.59	partial	2.7	86.54	membranous	blunt	Welch (1985)
Cortaderia_selloana	wind	0.14	partial	4.63	28.82	membranous	blunt	Ridley (1930)
Phragmites_australis	wind	0.26	partial	4.08	17.51	membranous	blunt	Ridley (1930)
Sonchus_oleraceus	wind	0.18	partial	4.42	31.62	membranous	blunt	Ridley (1930)
Taraxacum_officinale	wind	0.2	exposed	17.72	69.85	membranous	blunt	Ridley (1930)
Archaeoleersia nebraskensis	fossil	0.92	exposed	2.39	301.55	coriaceous	blunt	
Berriochloa communis	fossil	0.92	enclosed	0.88	258.44	indurate	sharp	
Berriochloa dawsensis	fossil	0.92	enclosed	0.88	229.72	indurate	sharp	
Berriochloa gabeli	fossil	0.91	enclosed	0.89	244.82	indurate	sharp	
Berriochloa hirsuta	fossil	0.9	enclosed	0.9	264.86	indurate	sharp	
Berriochloa huletti	fossil	0.82	enclosed	0.99	294.38	indurate	sharp	
Berriochloa intermedia	fossil	0.87	enclosed	0.93	301.1	indurate	sharp	
Berriochloa minima	fossil	0.88	enclosed	0.92	237.31	indurate	sharp	
Berriochloa minuta	fossil	0.93	enclosed	0.86	230.83	indurate	sharp	
Berriochloa primaeva	fossil	0.9	enclosed	0.9	218.09	indurate	sharp	
Nassella indet	fossil	0.91	enclosed	0.89	213.01	indurate	blunt	
Nassella reynoldsii	fossil	0.92	enclosed	0.87	229.74	indurate	blunt	
Paleoeriocoma hitchcockii	fossil	0.93	enclosed	0.87	224.97	indurate	blunt	
Panicum elegans	fossil	0.94	enclosed?	0.85	266.99	indurate	blunt	

CONCLUSION:

Results of these combined studies showed a complex pattern of responses. While grasslands are widespread and of great global significance, grasslands in different regions diverge in a number of important ways (e.g., degree of canopy cover). This, in addition to the history of the region and group in question, appears to modulate how species responded to transitions into grasslands.

In Chapter 1, we tested the classic hypothesis that high grazing pressure in tropical grasslands and savannas led to the evolution of higher silica levels in C_4 grasses, but did not find any consistent difference in Si concentration between C_3 and C_4 grasses and thus rejected this hypothesis. The hypothesis that herbivore pressure in grass-dominated habitats more generally resulted in enhanced silica defenses in (C_3 or C_4) grassland taxa was not supported either. However, we did find that growth conditions imparted a pronounced effect upon Si concentration, with hot and dry conditions promoting the highest Si concentrations in most of the sampled taxa. This pattern may ultimately explain why previous authors have found elevated concentrations in C_4 lineages. It also suggests a mechanism via which environmental conditions may influence grass–herbivore interactions.

In Chapter 2, we found evidence consistent with the hypothesis that open and disturbed habitats both select for traits associated with wind dispersal in melicgrasses. However, results also hinted at the complexity of these relationships, suggesting that traits influencing how effectively and by what means seeds are dispersed are limited by several reproductive and non-reproductive processes. Our interpretation that wind dispersal potential is limited in open habitats by a tradeoff with hydraulic safety again suggested that climate (e.g., degree of rainfall) played an important role in shaping evolutionary patterns.

In Chapter 3, we expanded our dispersal dataset to cover the entire grass family and additional dispersal traits. This more thorough look at the evolution of dispersal strategy in grasses, again revealed complex patterns. Although transitions in and out of grasslands appear to have been consequential, the nature of effects were highly dependent on context (e.g., taxonomic group, climate). Compounding this complexity, we found that fossil taxa possessed a range of dispersal ecologies that was poorly captured by modern species, which may be explained by the non-analogue, early grassland habitats which they occupied (e.g., lack of specialized grazers, higher tree cover).

In synthesis, it appears that there was no unifying evolutionary response to grassland expansion among the grasses occupying them. This result mirrors recent reevaluation of likely causes of grassland expansion, which also seem to vary in space and time (e.g., Gibson, 2009; Strömberg, 2011; Lehmann et al., 2013; Strömberg and Staver, 2022). Moving forward, a focus on region and group specific patterns is likely to be the most promising strategy for developing a more complete understanding of the consequences of this major transition.

Conclusions References:

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