Annals of Botany **120**: 725–738, 2017
doi:10.1093/aob/mcx058, available online at www.academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

PART OF A SPECIAL ISSUE ON MORPHOLOGY AND ADAPTATION

Polyphyly of Arundinoideae (Poaceae) and evolution of the twisted geniculate lemma awn

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Received: 27 October 2016 Returned for revision: 28 November 2016 Editorial decision: 29 March 2017 Accepted: 19 April 2017
Published electronically: 21 June 2017

• **Background and Aims** Subfamily Arundinoideae represents one of the last unsolved taxonomic mysteries in the grass family (Poaceae) due to the narrow and remote distributions of many of its 19 morphologically and ecologically heterogeneous genera. Resolving the phylogenetic relationships of these genera could have substantial implications for understanding character evolution in the grasses, for example the twisted geniculate awn – a hygroscopic awn that has been shown to be important in seed germination for some grass species. In this study, the phylogenetic positions of most arundinoid genera were determined using DNA from herbarium specimens, and their placement affects interpretation of this ecologically important trait.
• **Methods** A phylogenetic analysis was conducted on a matrix of full-plastome sequences from 123 species in 107 genera representing all grass subfamilies, with 15 of the 19 genera in subfamily Arundinoideae. Parsimony and maximum likelihood mapping approaches were used to estimate ancestral states for presence of a geniculate lemma awn with a twisted column across Poaceae. Lastly, anatomical characters were examined for former arundinoid taxa using light microscopy and scanning electron microscopy.
• **Key Results** Four genera traditionally included in Arundinoideae fell outside the subfamily in the plastome phylogeny, with the remaining 11 genera forming Arundinoideae *sensu stricto*. The twisted geniculate awn has originated independently at least five times in the PACMAD grasses, in the subfamilies Panicoideae, Danthonioideae/Chloridoideae and Arundinoideae. Morphological and anatomical characters support the new positions of the misplaced arundinoid genera in the phylogeny, but also highlight convergent and parallel evolution in the grasses.
• **Conclusions** In placing the majority of arundinoid genera in a phylogenetic framework, our study answers one of the last remaining big questions in grass taxonomy while highlighting examples of convergent evolution in an ecologically important trait, the hygroscopic, twisted geniculate awn.

Key words: Arundinoideae, awns, Crinipes group, Poaceae, plastome phylogenomics, PACMAD grasses.

INTRODUCTION

The grass family, Poaceae, has a remarkably stable, phylogenetically based classification as recently summarized by Soreng *et al.* (2015) and Kellogg (2015). Of the 12 subfamilies, the limits of 11 are largely resolved. Only one subfamily, Arundinoideae, remains an apparent catch-all group, with a history of including heterogeneous and unrelated taxa (summarized in Table 1).

Most authors were aware that Arundinoideae was an artificial group. Watson and Dallwitz (1992) called the subfamily 'an unsatisfactory assemblage of convenience, which is not amenable to anything approaching a diagnostic description, and is probably polyphyletic'. Kellogg and Campbell (1987) were the first to identify the Arundinoideae as polyphyletic using an explicitly cladistic approach, and argued that the subfamily 'should be interpreted as an assemblage of basal groups and evolutionary dead ends'.

Molecular phylogenetic studies confirmed polyphyly of Arundinoideae and removed many disparate elements, notably the subfamily Danthonioideae. These studies included those using sequences of the chloroplast genes *rbcL* (Barker *et al.*, 1995), *ndhF* (Clark *et al.*, 1995) and *rpoC2* (Barker *et al.*, 1998), the nuclear gene phytochrome B (Mathews *et al.*, 2000) and combined molecular data (Grass Phylogeny Working Group, 2001; Grass Phylogeny Working Group II, 2012; Cotton *et al.*, 2015; Duvall *et al.*, 2017). A few studies (e.g. Linder *et al.*, 1997; Grass Phylogeny Working Group, 2001) included morphological phylogenetics as well. A late addition to the shrinking Arundinoideae was made by Ingram *et al.* (2011), who added the misnamed '*Eragrostis*' *walteri* on the basis of two chloroplast genes and internal transcribed sequence (ITS), solving the mystery of this C_3 species in a C_4 genus in the mostly C_4 subfamily Chloridoideae.

Based on available phylogenetic data, Soreng *et al.* (2015) and Kellogg (2015) now recognize 17 or 19 genera in Arundinoideae, respectively (Table 1). Soreng *et al.* (2015) remove *Alloeochaete* and *Phaenanthoecium* in Danthonioideae on the basis of 'well developed, flattened, coiled, geniculate awns diverging between relatively slender lateral lobes, typical of Danthonieae, but not found in Arundinoideae'. These genera were historically associated with the tribe Danthonieae (e.g. Watson and Dallwitz, 1992; Clayton and Renvoize, 1999),

TABLE 1. *Recent classifications of Arundinoideae showing included genera*

Clayton and Renvoize (1986)	Grass Phylogeny Working Group (2001)	Soreng (2015)	Kellogg (2015)
Alloeochaete	–	Danthonioideae	*Alloeochaete*
Amphipogon	*Amphipogon*	*Amphipogon*	*Amphipogon*
Arundo	*Arundo*	*Arundo*	*Arundo*
Crinipes	*Crinipes*	*Crinipes*	*Crinipes*
Danthonidium	–	Danthonioideae	Danthonidium
Dichaetaria	*Dichaetaria*	*Dichaetaria*	*Dichaetaria*
Diplopogon	–	–	–
Dregeochloa	*Dregeochloa*	*Dregeochloa*	*Dregeochloa*
Elytrophorus	*Elytrophorus*	*Elytrophorus*	*Elytrophorus*
Chloridoideae	Chloridoideae	*'Eragrostis' walteri*	*'Eragrostis' walteri*
Hakonechloa	*Hakonechloa*	*Hakonechloa*	*Hakonechloa*
Leptagrostis	*Leptagrostis*	*Leptagrostis*	*Leptagrostis*
Molinia	*Molinia*	*Molinia*	*Molinia*
–	*Moliniopsis*	*Moliniopsis*	*Moliniopsis*
Monachather	–	*Monachather*	*Monachather*
Nematopoa	*Nematopoa*	*Nematopoa*	*Nematopoa*
Phaenanthoecium	–	Danthonioideae	*Phaenanthoecium*
Phragmites	*Phragmites*	*Phragmites*	*Phragmites*
Piptophyllum	*Piptophyllum*	*Piptophyllum*	*Piptophyllum*
Styppeiochloa	*Steyppeiochloa*	*Styppeiochloa*	*Styppeiochloa*
Zenkeria	*Zenkeria*	*Zenkeria*	*Zenkeria*
Anisopogon	Pooideae	Pooideae	Pooideae
Centropodia	Chloridoideae	Chloridoideae	Chloridoideae
Chaetobromus	–	Danthonioideae	Danthonioideae
Chionochloa	Danthonioideae	Danthonioideae	Danthonioideae
Cortaderia	Danthonioideae	Danthonioideae	Danthonioideae
Danthonia	Danthonioideae	Danthonioideae	Danthonioideae
Gynerium	*Incertae sedis*	Panicoideae	Panicoideae
Lamprothyrsus	Danthonioideae	Danthonioideae	Danthonioideae
Notochloe	Danthonioideae	Danthonioideae	Danthonioideae
Pentameris	Danthonioideae	Danthonioideae	Danthonioideae
Pentaschistis	Danthonioideae	Danthonioideae	Danthonioideae
Plinthanthesis	Danthonioideae	Danthonioideae	Danthonioideae
Poagrostis	–	Danthonioideae	Danthonioideae
Prionanthium	Danthonioideae	Danthonioideae	Danthonioideae
Pseudopentameris	Danthonioideae	Danthonioideae	Danthonioideae
Pyrrhanthera	–	Danthonioideae	Danthonioideae
Rytidosperma	Danthonioideae	Danthonioideae	Danthonioideae
Schismus	Danthonioideae	Danthonioideae	Danthonioideae
Spartochloa	–	Panicoideae	Panicoideae
Tribolium	Danthonioideae	Danthonioideae	Danthonioideae
Urochloa	–	Panicoideae	Panicoideae
Thysanolaena	Centothecoideae	Panicoideae	Panicoideae
Micraira	Micrairoideae	Micrairoideae	Micrairoideae
Aristida	Aristidoideae	Aristidoideae	Aristidoideae
Sartidia	Aristidoideae	Aristidoideae	Aristidoideae
Stipagrostis	Aristidoideae	Aristidoideae	Aristidoideae

For other earlier classifications, see Barker et al. (1995, 1998).

which became the basis of subfamily Danthonioideae (Grass Phylogeny Working Group, 2001). However, they are not mentioned in the revision of Danthonioideae by Linder *et al.* (2010). Soreng *et al.* (2015) divide their Arundinoideae into tribes, whereas Kellogg (2015) does not.

Following the circumscription of Kellogg (2015; Table 1), Arundinoideae *sensu lato* (*s.l.*) is the smallest subfamily in the PACMAD clade (the large clade that includes Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae), comprising only 50 species in 19 genera. These species are morphologically and ecologically diverse, suggesting that the subfamily may still be polyphyletic. Arundinoideae *s.l.* thus represents a significant obstacle to inferring character evolution in the PACMAD clade because it contains a heterogeneous group of species of uncertain phylogenetic placement. Some species have characters similar to those found in other distantly related subfamilies, so that their misplacement within Arundinoideae could artificially increase estimates of how many times such characters have evolved independently.

One such trait whose evolutionary interpretation is potentially affected by phylogenetic placement of Arundinoideae *s.l.* is the presence of a flattened geniculate awn with a twisted basal column at or near the apex of the lemma, which characterizes many members of Danthonioideae (Conert, 1987). This structure is presumed to influence the microsite and orientation in which the grass diaspore is buried through hygroscopic uncoiling and recoiling pushing the diaspore across the ground (Peart, 1979; Garnier and Dajoz, 2001; Johnson and Baruch, 2014). Actively assisted burial by hygroscopic awns has been shown to affect germination rates in grasses in some habitats, suggesting that the trait may be under strong selection in certain lineages (Simpson, 1952; Peart 1979, 1981; Peart and Clifford, 1987).

A hygroscopic awn with a basal twisted column is part of the suite of characters defining the typical 'danthonioid' lemma in which the medial awn is flattened and arises from a sinus between two prominent apical lobes (De Wet, 1956; Kabuye and Renvoize, 1975). Humphreys *et al.* (2010) explored the evolution of awns and other lemma characters in Danthonioideae. Their analysis found that the presence of hygroscopic awns is probably ancestral in Danthonioideae, and only two genera in the subfamily, *Tribolium* and *Schismus*, have diversified with an awnless lemma. Losses of awns across the subfamily are typically accompanied by a suite of changes in lemma characters, suggesting that the hygroscopic awn is an important component of a burial syndrome under potentially significant selection pressures.

A twisted geniculate awn is found in several grass clades (Davidse, 1987). The presence of this kind of awn in the Aveneae caused early classifications to include *Danthonia* and its relatives in the former tribe; analyses of anatomy and cytology showed that the two groups are not closely related (Hubbard, 1948), and thus the twisted geniculate awn must have developed in parallel between them (De Wet, 1956). Similar awns also appear in the Panicoideae, although in most cases the base of the awn is not broadly flattened as it is in Danthonioideae. In addition, members of Panicoideae typically have distinctive two-flowered spikelets, so were not considered closely related to the Danthonieae (Grass Phylogeny Working Group, 2001). Arundinoideae *s.l.* has four genera with 'danthonioid' awns: *Alloeochaete*, *Phaenanthoecium*, *Dregeochloa* and *Amphipogon*. Only *Dregeochloa* and *Amphipogon* have been included in molecular phylogenies (Barker, 1997; Barker *et al.*, 1998; Grass Phylogeny Working Group, 2001; Grass Phylogeny Working Group II, 2012). Placing all four of these genera is necessary to assess the scale of the convergence in this important trait across grasses, and to enable subsequent studies on its ecological and evolutionary significance to be put in a robust phylogenetic framework.

The main historic obstacle to working with the Arundinoideae, namely the distribution of its geographically scattered members in remote areas of the world, remains

unchanged. Herbarium specimens are the only sources of morphological, anatomical and genetic information for many genera in the subfamily, and DNA extracted from these specimens is often highly degraded, making PCR amplification and Sanger sequencing difficult or impossible (i.e. Särkinen *et al*., 2012). In such cases, genome survey sequencing (GSS) can be a powerful tool (e.g. Jankowiak *et al*., 2005; Besnard *et al*., 2014). The small fragments used in this type of sequencing (≤500 bp) are potentially well suited to the degraded DNA found in herbarium specimens, and the enormous amount of sequence data generated means that rigorous quality control can be used to remove any contaminants or poor-quality fragments.

In this study, we resolve long-standing questions regarding the phylogenetic placement of 15 genera included in the Arundinoideae *s.l.* using a new phylogeny of Poaceae based on full chloroplast genomes. In addition to this problematic subfamily, we generate and include several plastomes from the subfamily Micrairoideae, which has been identified as sister to the Arundinoideae (Grass Phylogeny Working Group, 2001; Grass Phylogeny Working Group II, 2012; Cotton *et al*., 2015; Duvall *et al*., 2017) and has a taxonomic history almost as complicated (reviewed in Sànchez-Ken *et al*., 2007). The 32 newly generated sequences include five genera not part of any previous molecular phylogenetic analysis: *Alloeochaete*, *Crinipes*, *Dichaetaria*, *Nematopoa* and *Phaenanthoecium*. To test the polyphyly of Arundinoideae *s.l.*, we also sample published plastomes from all other subfamilies in Poaceae. Using this phylogenetic framework, we then reconstruct the evolutionary history of the 'danthonioid' awn to see how placement of arundinoid taxa affects interpretations of this distinctive character. Finally, we survey leaf cross-sectional and epidermal anatomy for taxa that are misplaced in the Arundinoideae *s.l.* to assess compatibility with subfamilial limits based on these traits.

MATERIALS AND METHODS

Taxon sampling

Representatives of 15 of the 19 genera currently assigned to Arundinoideae were sampled, including multiple species within a genus wherever possible (Supplementary Data Table S1). To test possible polyphyly of the subfamily, we also included a broad sample of published plastomes from all other PACMAD subfamilies. We considered the possibility that some 'arundinoid' taxa might actually belong in other subfamilies. To test this, we included taxa previously identified as sister to the remaining taxa of each subfamily so we could be confident that placement was not an artefact of limited sampling. Published plastomes for 23 BOP (Bambusoideae, Oryzoideae, Pooideae) clade taxa as well as samples from the basal grade of Anomochlooideae, Pharoideae and Puelioideae were included to test congruence of this larger sample with previously published phylogenies of the family. Sequence data for *Eragrostis tef* (SRR1463402) were downloaded from the Sequence Read Archive (SRA) to provide another '*Eragrostis*' species. All other plastomes were taken from GenBank, except for *Danthoniopsis dinteri* assembled from Washburn *et al*. (2015), and *Chasmanthium laxum*, which was assembled from whole-genome shotgun data (Kellogg lab, unpubl. data). In total, 131 full plastomes from 123 species representing all subfamilies in Poaceae were included in the phylogenetic analysis.

DNA isolation and sequencing

Plant material was obtained from either field-dried collections or herbarium specimens, and ground using a mortar and pestle with sterilized sand. Total DNA was extracted using the QIAGEN EasyDNA Plant Mini Kit, a modified cetyltrimethylammonium bromide (CTAB) protocol (Cota-Sánchez *et al*., 2006), or a combination of the two in which QIAGEN columns were used to clean and isolate the extracted DNA. Sample DNA was sheared using a Covaris S220 sonicator with peak power of 175 and duty factor of 5·0 for 200 cycles for 30 s with a target size of 500 bp. Libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs, Inc.) according to the manufacturer's instructions. Fragments were size selected to 400–500 bp, purified using Agencourt AMPure XP Beads (Beckman Coulter, Inc.) and sequenced using an Illumina 2 × 250 HiSeq 2500 paired-end Rapid Run at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center.

Plastome assembly and phylogenetics

Raw paired-end reads were cleaned using Trimmomatic v. 0.32 (Bolger *et al*., 2014) for TruSeq3-PE adaptors with one mismatch, a palindrome clip threshold of 30 and a simple clip threshold of 10. After adaptor trimming, reads were quality trimmed in Trimmomatic using a sliding window of 10 bp with a minimum average phred score of 20, keeping reads with a minimum length of 40. Plastome-like reads were identified by mapping filtered reads to a set of existing grass chloroplast genomes with bowtie2 v. 2.2.6 under the 'very-sensitive-local' parameter set (Langmead and Salzberg, 2012). Mapped reads were assembled with SPAdes v. 3.1.0 using kmers of 55, 87 and 121 and the 'only-assembler' option (Bankevich *et al*., 2012). SPAdes contigs were then meta-assembled in afin (http://github.com/mrmckain/Fast-Plast/tree/master/afin) using the full trimmed read data set under the following parameters: a stop extension value of 0.1, an initial trim of 100 bp, a maximum extension of 100 bp per loop and 50 search loops. afin trims the ends of starting contigs, extends their lengths using matching trimmed reads, attempts to fuse all contigs if the threshold of 10 % mismatch is met for contig overlap and iterates these steps 50 times. Contigs generated by afin were manually assembled into complete plastomes in Sequencher version 5.3 (Gene Codes Corporation) by identifying inverted repeat (IR) boundaries through sequence similarity and, where necessary, searching trimmed reads to connect any remaining fragments through *in silico* genome walking. Contigs were scaffolded to *Schizachyrium scoparium*, and gaps in the final assembly were filled with Ns. Some variation was found between the IR regions in some samples, but read lengths were not long enough to phase single nucleotide polymorphisms (SNPs); therefore the inverted repeat B (IRB) region was duplicated and inverted to serve as IRA. A coverage analysis (https://github.com/mrmckain/Fast-Plast) of a 25 bp sliding window was used to check completed plastome assemblies for

accuracy, with further modifications to the assemblies made when dips in coverage were identified. Plastome sequences were oriented to start near *psbA* of the large single copy (LSC) and end with IRA. Annotations and Circos graphs of finished plastomes were made in Verdant (McKain *et al*., 2017; verdant.iplantcollaborative.org). Full plastome sequences are deposited in both Verdant and GenBank (accession numbers MF035966-MF035997).

Finished plastomes were divided into the IRB, small single copy (SSC) and LSC regions; each region was aligned using MAFFT v. 7.029b with default parameters (Katoh, 2013) and the three alignments were concatenated into a single alignment and trimmed with Gblocks version 0.91b (Castresana, 2000). Three treatments of gaps were used to create edited alignments: (1) all sites with gaps excluded (no gaps): (2) all sites with gaps in less than half of the sampled taxa included (less than half gaps); and (3) all sites included (all gaps). GTR + I + gamma was identified as the best model of base pair substitution based on the Aikake Information Criterion (AIC) using jModelTest2 (Guindon and Gascuel, 2003; Darriba *et al*., 2012). All four alignments – untrimmed, no gaps, less than half gaps and all gaps – were analysed using maximum likelihood (ML) with RAxML v. 8.0.22 under the GTR + I + gamma model with 500 bootstrap replicates (Stamatakis, 2014). The three early-diverging grass subfamilies – Anomochlooideae, Pharoideae and Puelioideae – were used as outgroups. Alternative topologies were tested using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) in RAxML. The all gaps alignment with *Anomochloa* as an outgroup was selected for subsequent analyses as a compromise between selecting the least ambiguous alignment while including the maximum amount of data. This alignment was analysed using Bayesian inference in MrBayes v. 3.2.6 (Ronquist *et al*., 2012). For this analysis, two simultaneous independent MCMCMC runs were conducted with four chains – one cold and three heated – for 1 million generations using a GTR + I + gamma evolutionary model with five rate categories to approximate the continuous gamma distribution. Trees were visualized using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Morphological character coding and ancestral state reconstruction

The presence or absence of a geniculate lemma awn with a twisted basal column was recorded from herbarium specimens for all genera in Arundinoideae *s.l.* and combined with data taken from the literature for other taxa (Watson and Dallwitz, 1992; Clayton and Renvoize, 1999; Clayton *et al*., 2006 onwards) (Supplementary Data Table S2). For the purposes of this study, we did not distinguish between awns in which the column was flattened ('danthonioid') and those in which the column was terete. Species with a geniculate awn lacking the twisted column, such as *Monachather paradoxus*, were coded as lacking this character (0). Other traits associated with the diaspore burial syndrome of Humphreys *et al*. (2010), such as the presence of a hairy callus and hairs on the lemma body, were not included due to the difficulty of assigning character states across the range of diversity in Poaceae. Additionally, many species in the current study are polymorphic for these characters, and some clades that appear monomorphic in our sample have alternative character states in other unsampled taxa (e.g. Danthonioideae and *Amphipogon*, both of which have taxa with and without geniculate awns). Thus, a comprehensive study of these important characters is beyond the scope of the current project and would probably be more appropriately addressed at a smaller phylogenetic scale. However, the distribution of a relatively unambiguous character, such as the geniculate and twisted lemma awn, across the phylogeny can help provide context for future evolutionary studies of burial syndromes and suggest taxa on which such studies would be most profitably conducted.

Ancestral state reconstruction was performed on the ML phylogeny with two exceptions: the three outgroup subfamilies were excluded to facilitate visualization of the results, and duplicate species were reduced to a single sample in the phylogeny for trait analysis to prevent bias in the ancestral state reconstruction. Character histories were analysed with parsimony, which ignores branch lengths in the phylogeny, using Mesquite version 3.2 (Maddison and Maddison, 2015) and with ML, which assumes a Brownian motion model, using the R package corHMM (Beaulieu *et al*., 2013). Two different models of trait evolution were used for likelihood analyses: (1) the equal rates model (ER), which assumes equal rates of change between all character states; and (2) the all rates different model (ARD), which assigns a different rate to each transition, including reversals (Paradis *et al*., 2004).

Divergence date estimation with BEAST

BEAST v. 1.8.3 (Drummond *et al*., 2012) was used with the 'all gaps' plastome alignment to test the effect of our increased sampling on divergence dates within the PACMAD clade. BEAUti v. 1.8.3 was used to set parameters. The ML tree from RAxML was used as a starting tree after being transformed using the chronopl function in the R package ape with a lambda of 1 and four fossil ages (see below under BEAST priors) used as minimum age constraints (Paradis *et al*., 2004). Five separate identical runs of 100 million generations each were run on the CIPRES Gateway (Miller *et al*., 2010), sampling trees every 1000 generations using an uncorrelated relaxed clock model with a lognormal relaxed distribution and with a Yule process model of speciation as a tree prior. A GTR + I + Gamma nucleotide substitution model with four gamma categories was used, with base pair frequencies estimated from the alignment. Four fossil calibrations were specified as stem calibrations with lognormal distributions, with a mean of zero, s.d. of one, an offset from zero equal to the estimated age of the fossil and an initial value of the fossil age. These fossils were assigned positions in the phylogeny according to Vicentini *et al*. (2008) as follows: 7 million years ago (Mya) for the most recent common ancestor of *Setaria* and *Panicum* (Elias, 1942); 19 Mya for stem Chloridoideae (Strömberg, 2005); 35 Mya for the ancestor of BOP + PACMAD (Strömberg, 2005); and 55 Mya for all grass subfamilies excluding Anomochlooideae (Crepet and Feldman, 1991). Each clade involved in the fossil calibrations was also constrained to be monophyletic in the dating analysis to reduce computational effort slightly. LogCombiner v. 1.8.3 was used to combine the last 1000 trees from each of the ten BEAST

runs, yielding effective sample sizes (ESSs) >200 for all parameters, and the concatenated tree file was annotated in TreeAnnotator v. 1.8.3.

Alignments, trees, and BEAST control and output files are available in Dryad (http://dx.doi.org/10.5061/dryad.v7m05).

Leaf anatomy

Cross-sections were made from near the middle of mature leaves taken from herbarium specimens, rehydrated in Pohl's Wetting Solution (Pohl, 1965), fixed in FAA, dehydrated in an ethanol series, infiltrated with paraffin using *tert*-butanol as an intermediate solvent and embedded in paraffin according to the method in Ruzin (1999). Sections of 10 µm were made using a Microm HM 355 S rotary microtome (Microm International GmbH, Walldorf, Germany) and were stained with the Safranin–Fast Green protocol given in Sass (1951). Sections were photographed using an Olympus BX53 light microscope with a DP25 digital camera attachment (Olympus Corporation, Center Valley, PA, USA).

Leaf fragments were also taken from herbarium specimens to examine epidermal anatomy. These leaf pieces were first rehydrated and dehydrated as above, then transitioned into 100 % xylene solution and sonicated for 10 min each to remove epicuticular wax. The dewaxed fragments were then moved back into 100 % ethanol, dried in a Tousimis Samdri-780a Critical Point Dryer, coated with gold–palladium in a Tousimis Samsputter-2a Sputter Coater (Tousimis, Rockville, MD, USA) and viewed with a Hitachi S-2600H (Hitachi High Technologies America, Inc., Dallas, TX, USA) scanning electron microscope. Critical point drying, sputter coating and scanning electron microscopy (SEM) work was performed at The Research Center for Auditory and Vestibular Studies at Washington University in St. Louis.

Leaf cross-sections and epidermal SEM images were generated for the four misplaced 'arundinoids'. A full morphological and anatomical analysis of Arundinoideae *sensu stricto* (*s.s.*) is in preparation.

RESULTS

Plastome assembly, annotation and alignment

Average coverage for each of the 32 plastomes generated by this study ranged from 31× to 452×, with total lengths of 133 327–141 203 bp (Table S1). Lengths of the LSC, SSC and IR ranged from 79 379 to 82 836 bp, 12 246 to 12 759 bp and 20 048 to 22 762 bp, respectively. Annotations of the plastomes showed conservation of genes in all newly assembled plastomes, with two exceptions. The LSC copy of *rpl23* is pseudogenized in sampled *Crinipes* species, *Elytrophorus globularis*, '*Eragrostis*' *walteri* and both accessions of *Monochather paradoxus*. The IRA copy of *rps19* is pseudogenized in *Coelachne africana* due to a shift of the IR boundary. Although *Hakonechloa macra* (GenBank KJ920232.1) was reported to have lost the *rpl16* gene and pseudogenized *rpl14* (Cotton *et al.*, 2015), the two individuals sampled in this study both have functional versions of these genes.

The alignments range from 77 882 bp when all gaps are excluded to 172 824 bp without trimming, demonstrating the considerable extent of gaps in the full alignment. Part of the reason for this is inclusion of *Anomochloa*, which differs from the characteristic grass plastome structure in several aspects, such as the absence of an *rpoC1* intron and a 39 bp sub-repeat in the *rpoC2* insert instead of the 21 bp sub-repeat found in the rest of the grasses (Morris and Duvall, 2010). Use of *Pharus* as an outgroup reduces the number of ambiguous regions in the alignment because *Pharus* has the dominant grass plastome architecture, but does not affect inferred phylogenetic relationships.

Phylogenetic analysis

The ML tree in Fig. 2 was the result of analysis of the most inclusive Gblocks-edited alignment (made with the all gaps option) using *Anomochloa marantoidea* as an outgroup. The tree topology was robust to outgroup sampling and alignment trimming, except that the placement of Aristidoideae changed between two different positions. Bootstrap support for the placement of this subfamily ranged from 51 to 81 %, with alignments with fewer gaps favouring Aristidoideae as sister to the rest of PACMAD and those with more gaps favouring Panicoideae in this position (Table 2). Despite higher bootstrap values in phylogenetic estimates of the alignments with more gaps, the two topologies were not significantly different according to S–H tests (Shimodaira and Hasegawa, 1999). The Bayesian analysis of the all-gaps alignment yielded an identical topology to the ML tree, with a posterior probability of 0·94 for the placement of Aristidoideae sister to the rest of PACMAD.

Monophyly of Arundinoideae *s.l.* was not supported in any tree, with four genera consistently falling into other subfamilies in all analyses. The Zimbabwean monospecific genus *Nematopoa* groups with members of Chloridoideae, the Ethiopian monospecific genus *Phaenanthoecium* groups with Danthonioideae, and *Dichaetaria* and *Alloeochaete* form a clade sister to Panicoideae. These placements have 100 % bootstrap support and posterior probabilities of 1·0 under all variations of tree estimation, as does monophyly of the remaining Arundinoideae. The latter clade, referred to hereafter as Arundinoideae *s.s.*, includes the cosmopolitan reeds *Arundo* and *Phragmites*; the temperate genera *Molinia* and *Hakonechloa*; the African *Crinipes*, *Styppeiochloa*, *Dregeochloa* plus the misnamed '*Eragrostis*' *walteri*; the Australian genera *Amphipogon* and *Monachather*; and the African–Australian–Asian genus *Elytrophorus*. Relationships among genera in this subfamily are strongly supported, with all nodes receiving >95 % bootstrap support.

Molecular dating analysis

BEAST recovered a maximum clade credibility tree (Supplementary Data Fig. S1) with identical topology and similar support values to the ML tree in Fig. 2. Aristidoideae is sister to the rest of PACMAD with a posterior probability of 0·91. Despite the large number of generations in the combined analysis, ESS values for many parameters were well below the recommended 200. For example, the common ancestor of Arundinoideae and Micrairoideae is estimated to have lived

between approx. 10 and 26 Mya based on the 95 % highest probability density (HPD). Similarly, the BOP and PACMAD clades are estimated to share a common ancestor that lived between approx. 28 and 47 Mya.

Phylogenetic distribution of the twisted geniculate lemma awn

A geniculate lemma awn with a twisted basal column is estimated to have originated at least five times independently across the PACMAD grasses according to both ML (Fig. 3) and parsimony analyses (Supplementary Data Fig. S2), with at least two separate origins in the BOP clade. The best fit model of evolution under the ML 'all rates different' scenario estimates gains of twisted geniculate awns to be almost three times as frequent as losses (AIC = 80·90), but this fit is not a significant improvement over the simpler 'equal rates' model (AIC = 80·62). With the current taxonomic sampling, the presence of twisted geniculate awns is estimated to be ancestral in the Danthonioideae as well as the Andropogoneae + Arundinelleae in subfamily Panicoideae. All members of Danthonioideae in our sample have a flattened geniculate awn with a twisted column, though many members of that subfamily have lost the trait (Humphreys *et al.*, 2010). Based on our sampling in the Panicoideae, geniculate awns with twisted columns have evolved at least three times independently in the subfamily, with members of the Andropogoneae experiencing at least three separate subsequent losses of awns.

Leaf anatomy

Leaf cross-sections were generated for three of the four genera falling outside Arundinoideae *s.s.* *Dichaetaria* specimens did not yield satisfactory sections. Both *Phaenanthoecium* and *Alloeochaete* exhibit C_3 leaf anatomy, with the veins separated

FIG. 1. Sample of the diversity of floret form in grasses. From left to right: awnless *Isachne arundinacea* (Harris 12487); straight-awned *Eriachne pallescens* (Beaman 10813); and twisted and geniculately awned *Tenaxia californica* (Schlechter 9499). Florets were photographed individually with focus stacking using a Nikon D90 camera on an Infinity K2/SC™ Long-Distance Microscope with either a CF4 or CF3 objective. Stacked images were assembled using Helicon Focus, and the resulting compiled images were edited in GIMP (http://gimp.org/). Floret images were extracted individually, size-adjusted to be at the same scale and combined onto a solid black background. No other adjustments were made. Abbreviations: A, awn; L, lemma; C, callus; H, hair on lemma body. Scale bar = 1 mm.

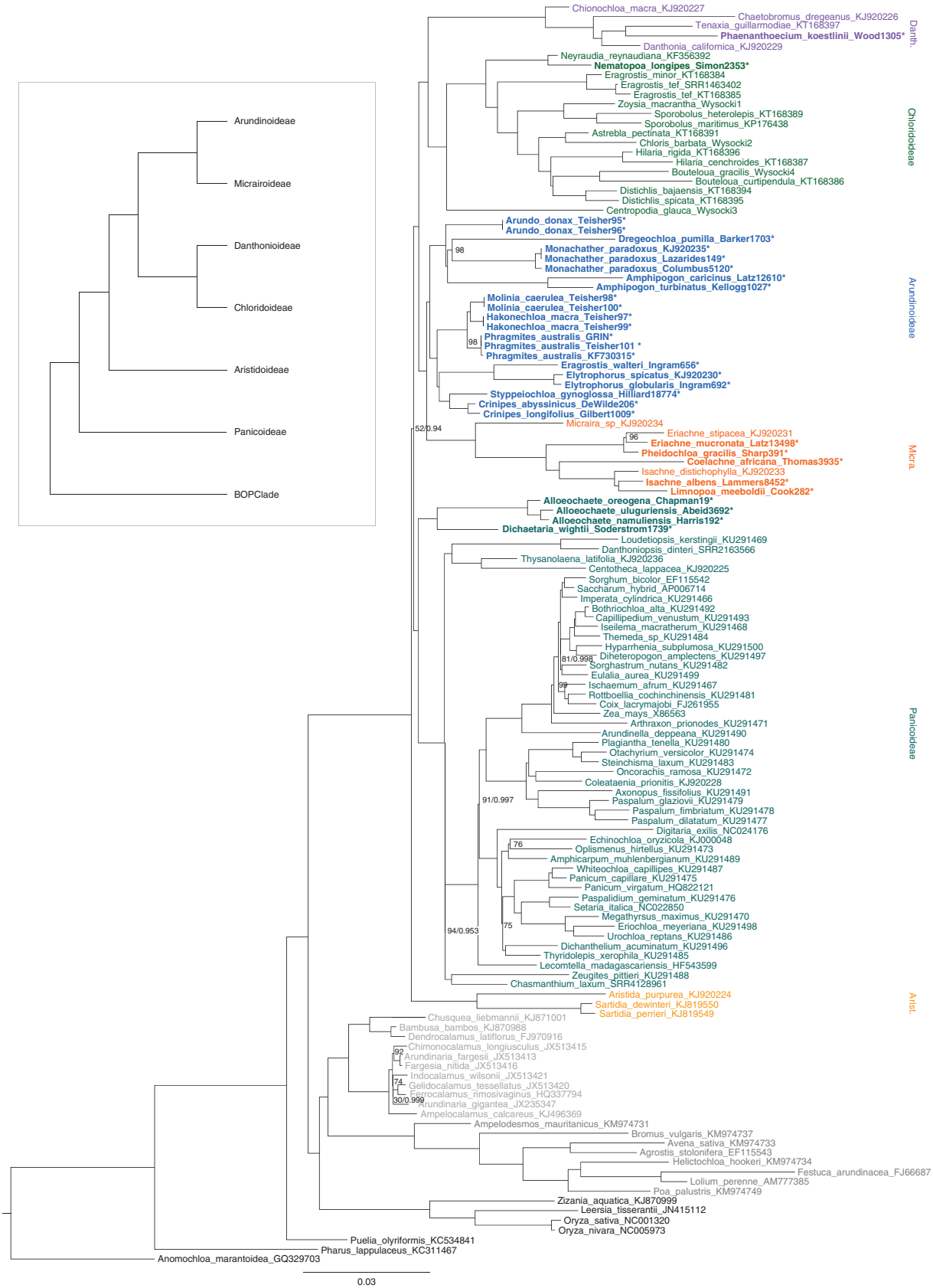

FIG. 2. Maximum likelihood phylogeny of 131 full plastomes across the grass family, with *Anomochloa* used as an outgroup. Bootstrap values <100 are shown above nodes, followed by posterior probabilities <1·0 from Bayesian analysis. Subfamilies in PACMAD are grouped by colour, while those in BOP are shades of grey. Samples in bold with asterisks were generated for the current study. The insert shows alternative topology for PACMAD relationships in which Panicoideae is sister to the rest of the clade.

TABLE 2. *Alternative phylogenetic analyses and their effects on the placement of Aristidoideae (A*) within PACMAD as well as the bootstrap support for that placement*

Alignment	Topology supported	Bootstrap
Untrimmed Anomochloa Out	A*(P(CMAD))	51
AllGaps Anomochloa Out	A*(P(CMAD))	52
HalfGaps Anomochloa Out	P(A*(CMAD))	57
NoGaps Anomochloa Out	P(A*(CMAD))	81

by more than two mesophyll cells (Fig. 4A, B). In contrast, *Nematopoa longipes* has Kranz anatomy (Fig. 4C), consistent with its placement in the largely C_4 Chloridoideae in the plastome phylogeny. *Nematopoa* also has an unusual distribution of sclerenchyma, which extends the entire width of the leaf just inside the abaxial epidermis and forms a layer 2–4 cells thick. Leaves of all three genera are curled adaxially and exhibit prominent ribs on the adaxial side. *Phaenanthoecium* and *Alloeochaete* have large bulliform cells, and *Alloeochaete* and *Nematopoa* exhibit macrohairs.

Adaxial and abaxial epidermal SEM images for representatives of all four misplaced genera are shown in Fig. 5. All sections are oriented with the long axis of the leaf horizontal and the apex to the left. Some features used in previous grass classifications, such as microhairs, macrohairs, prickles and silica bodies, are labelled and discussed below.

DISCUSSION

Our analyses show that Arundinoideae *s.l.* is polyphyletic. This finding has significant consequences for evolutionary inferences of morphology and anatomy across the PACMAD clade. In particular, the geniculate awn with a twisted column appears to be more homoplasious than previously thought, with two additional independent origins of the trait inferred by parsimony and ML reconstruction on the chloroplast tree. The new placements of the four pseudo-arundinoid genera are surprising in the case of *Alloeochaete* and *Dichaetaria*, anatomically sensible in the case of *Nematopoa*, and morphologically corroborated in the case of *Phaenanthoecium*.

The twisted geniculate awn

Arundinoideae *s.s.* contains two taxa, *Dregeochloa* and *Amphipogon*, with a geniculate awn with a twisted basal column. Within *Amphipogon*, only *A. setaceus* has such an awn; the species is not sampled here but morphologically otherwise is a 'good' *Amphipogon*. The two taxa fall in a clade with *Monachather*, which has spikelets that are similar to those of *Dregeochloa* and awns that are geniculate, but not twisted into a column. *Dregeochloa* was segregated from *Danthonia* (Conert, 1966) and was previously considered to be a member of Danthonieae on the basis of its lemmas (e.g. Renvoize, 1981; Clayton and Renvoize, 1986; Watson and Dallwitz, 1992), though Barker *et al.* (1998) found some moderate support for its placement in the Arundineae based on *rpoC2* sequence data. *Monachather* was similarly thought to be danthonioid until the analysis of *rbcL* sequence data by Linder *et al.* (1997) placed it with *Arundo*.

Like *Dregeochloa*, *Phaenanthoecium* was removed from *Danthonia* (Hubbard *et al.*, 1936), but retained in the tribe Danthonieae by most authors (e.g. Kabuye and Renvoize, 1975; Watson and Dallwitz, 1992). In this case, morphology of the spikelet is concordant with the phylogenetic history of the plastome. *Phaenanthoecium* is placed sister to *Tenaxia guillarmodiae* in our phylogeny, but our sample of Danthonioideae is too sparse to assess the implications of this result for the classification of this subfamily.

Alloeochaete was also based on a former *Danthonia*, *Danthonia andongensis* Rendle (Hubbard, 1940). Kabuye and Renvoize (1975) considered whether the genus belonged in tribe Danthonieae, concluding that despite being unique among the danthonioid genera in several morphological attributes (i.e. the male lowest floret, five-nerved lemmas and florets longer than glumes), 'the similarity in lemma morphology clearly demonstrates that *Alloeochaete* is closely related to *Danthonia* sensu lato'. The placement of this genus as sister to the Panicoideae (Fig. 2) provides another example of parallel development in lemma characters in grasses (Fig. 3).

In this study, we have not incorporated development in coding character states for the awn, largely because little is known about awn development and genetics (Kellogg, 2015). The twisted geniculate awns in various subfamilies may arise from different developmental pathways using different gene networks. This seems likely to be the case in the Aveneae and Danthonieae given that awns arise from the back of the lemma in the former and from a sinus at the apex of the lemma in the latter (De Wet, 1956). *Dregeochloa* and *Monachather* are closely related according to the chloroplast tree, and both have geniculate awns, though only the former has a twisted column. The developmental changes involved in producing this difference are unknown, as is the ecological significance of a coil as opposed to a hygroscopic kink.

The multiple independent origins of the twisted geniculate awn suggest that it is not a particularly difficult structure to evolve. Furthermore, Humphreys *et al.* (2010) showed that lineages in Danthonioideae lose these awns less frequently than might be expected by chance, and in only two cases of loss did the awnless state persist through subsequent diversification, suggesting selection for the awned lemma in most of the subfamily. A similarly interesting evolutionary situation may be present in the Andropogoneae (Panicoideae), which appear to be characterized by multiple losses of the twisted geniculate awn. Expanded sampling in this clade would potentially provide a valuable parallel to the study by Humphreys *et al.* (2010) to understand the evolutionary role of hygroscopic awns in grass adaptation and diversification.

A pointed, hairy callus and tufts of hairs frequently in rows across the back of the lemma together with the twisted geniculate awn form the 'active burial syndrome' characterizing most Danthonoideae (Humphreys *et al.*, 2010). These traits also appear together in non-danthonioids such as *Alloeochaete* and *Dregeochloa*, suggesting either strong selection for all three operating in unison in distantly related taxa or a shared developmental pathway, or both. Ecological experiments on germination, such as those done by Peart (1979, 1981, 1984) and Peart and Clifford (1987) in Australian grasses, and developmental

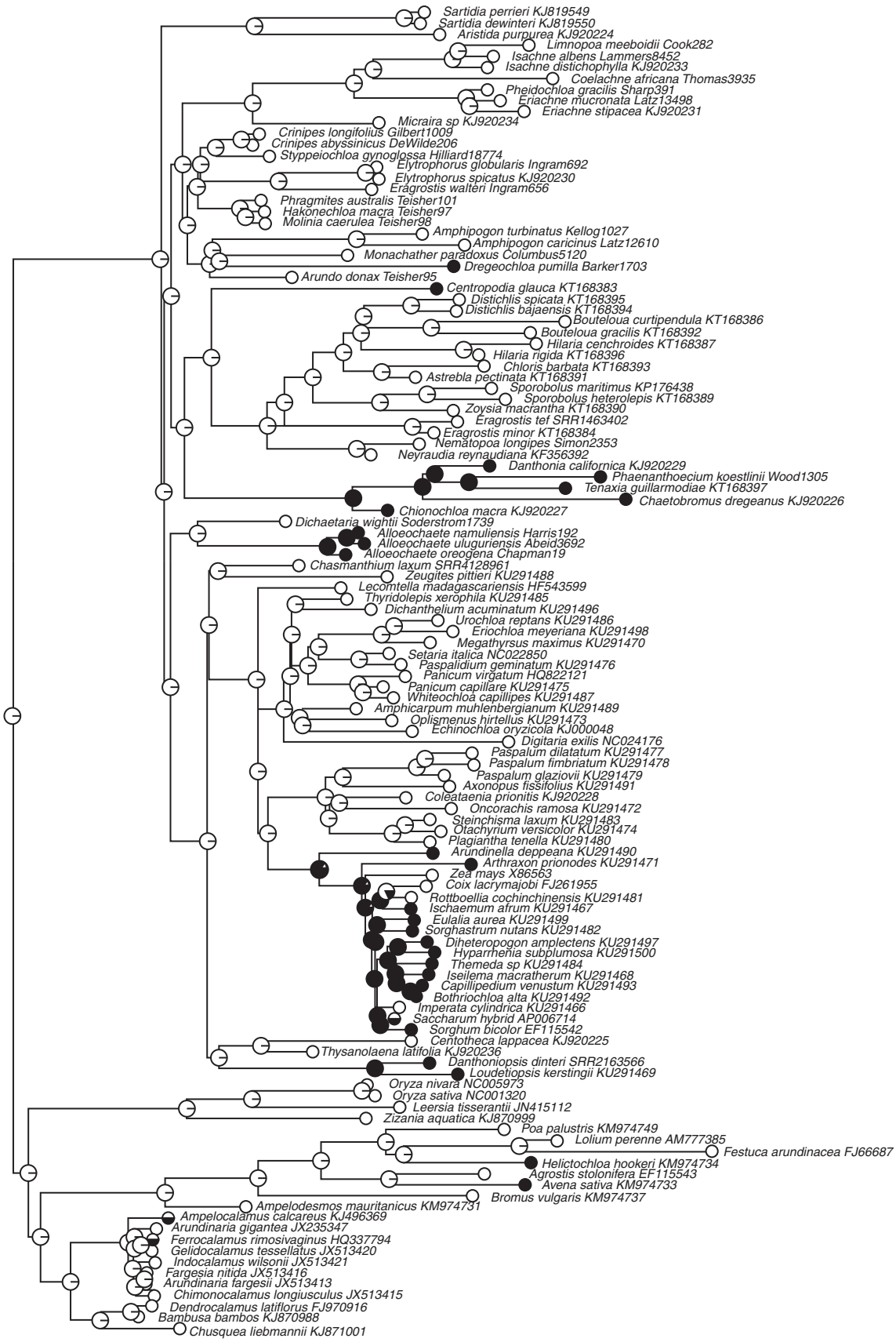

FIG. 3. Maximum likelihood ancestral states for a geniculate awn with a twisted basal column across the BOP and PACMAD clades under the equal rates model of trait evolution using the R package corHMM. Filled circles, presence of the trait; open circles, absence of the trait.

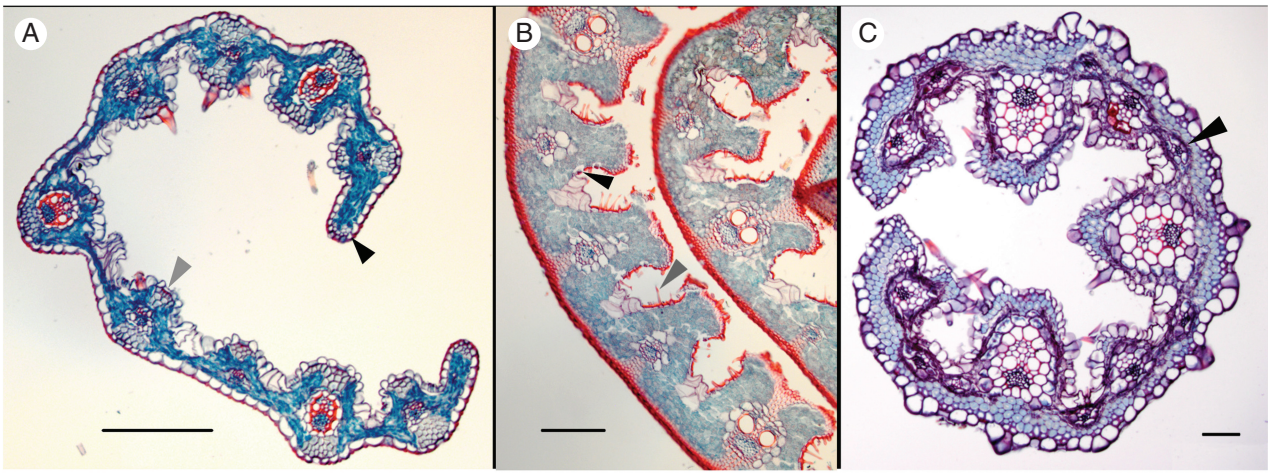

FIG. 4. Cross-sections of species from three of the four genera misplaced in Arundinoideae *s.l.* Black scale bars are approx. 500 µm. (A) *Phaenanthoecium koestlinii* (Wood 1305) showing schlerenchyma around the vascular bundles (grey arrow) and forming a cap at the leaf margins (black arrow). (B) *Alloeochaete andongensis* (Gossweiler 11810) has well-defined ribs around vascular bundles that form deep pits containing stomata (black arrow) and macrohair-like prickles (grey arrow, also in Fig. 5B). (C) *Nematopoa longipes* (Simon 2353) appears to be C_4 with small tertiary vascular bundles (black arrow) limiting the distance between bundle sheath cells.

genetic investigations into the genetic architecture underlying lemma formation will be necessary to understand fully the evolution of grass awns. Further study is needed to analyse awn states, as described here, with other 'active burial syndrome' lemma features.

Anatomy of arundinoid imposters

Nematopoa longipes belongs in the Chloridoideae based on plastome sequence data and leaf anatomy. The monotypic genus was separated from the chloridoid *Triraphis* by Hubbard (1957*a*), but the similarity between the two seems likely to be due to shared ancestry. Leaf cross-sectional anatomy (Fig. 4C) shows *Nematopoa* to be C_4, with close vein spacing and double bundle sheaths with chlorenchyma arranged radially in the outer sheath, supporting placement of the genus in the Chloridoideae. Curiously, both Renvoize (1981) and Linder *et al.* (1997; Fig. 5C) concluded that this genus is C_3 on the basis of leaf anatomy. The specimen used in the current study was identified and examined by Linder *et al.* for their morphological analysis, so we do not think misidentification is the cause of the discrepancy. More probably, the difference in interpretation may be because the tertiary vascular bundles shown in Fig. 4C (black arrow) are quite small. Depending on the stain used and the orientation and quality of the section, they could easily be missed, causing the leaf to appear to have widely spaced vascular bundles consistent with the C_3 pathway. In addition, the extensive sclerenchyma just inside the abaxial epidermis is in the position occupied by chlorenchyma in many C_3 species, so could easily be misinterpreted. Sclerenchyma is more commonly associated with veins, as observed here in *Phaenanthoecium* and *Alloeochaete*, and the distribution in *Nematopoa* is unusual.

Our specimen of *Nematopoa* also has short and relatively squat microhairs with the apical cell approximately the same length as the basal cell like those found in Chloridoideae (Fig. 5F), though the apical cell does not appear obviously spherical as in the rest of this subfamily (e.g. Palmer *et al.*, 1985). The longitudinally oriented dumb-bell and cruciform silica bodies found on the adaxial (not shown) and abaxial leaf surface in this specimen are similarly consistent with structures found in Chloridoideae; however, these characters are also found among other subfamilies (Metcalfe, 1960; Renvoize, 1981, Reimer and Cota-Sànchez, 2007).

Phaenanthoecium koestlinii is the only member of its genus (Watson and Dallwitz, 1992), and has a lemma similar to those found in Danthonioideae. Anatomical justification for the placement of *Phaenanthoecium* in this subfamily is difficult, since there are no obvious leaf anatomical synapomorphies for Danthonioideae or any substantial sub-set thereof. However, the anatomical features found in *Phaenanthoecium* can at least be shown to be compatible with those found across the subfamily. In cross-section, *Phaenanthoecium* has non-Kranz anatomy, with midveins indistinguishable from other primary vascular bundles, evenly distributed chlorenchyma cells, sclerenchyma bands on the abaxial and adaxial sides of the vascular bundles and small sclerenchyma caps along the margins of the leaf. Similar traits are found in the danthonioid genus *Chaetobromus* (*C. involucratus*; Ellis, 1988*b*) and some species of *Pentameris* (*P. thuarii* and *P. dregeana*; Ellis, 1985*a*, 1986). In addition, the leaf margins are distinctly rounded rather than reaching a point, a character that is similar to that seen in *Pentaschistis dentata* and *P. ecklonii* (formerly placed in *Prionanthium*; Ellis, 1989). The microhairs in Fig. 5G and macrohair and macrohair-like prickles in Fig. 5H resemble those found in species of *Danthonia* (Reimer and Cota-Sànchez, 2007) and *Urochlaena* (Ellis, 1988*a*), though similar traits can be found in other subfamilies (e.g. Palmer and Tucker, 1981, 1983; Palmer *et al.*, 1985; Palmer and Gerbeth-Jones, 1986, 1988). Verboom *et al.* (1994) found that haustorial synergids in the embryo sac may be a synapomorphy for the Danthonioideae, but this trait requires ample living material to assess and has thus been sampled for a relatively small sub-set of the subfamily.

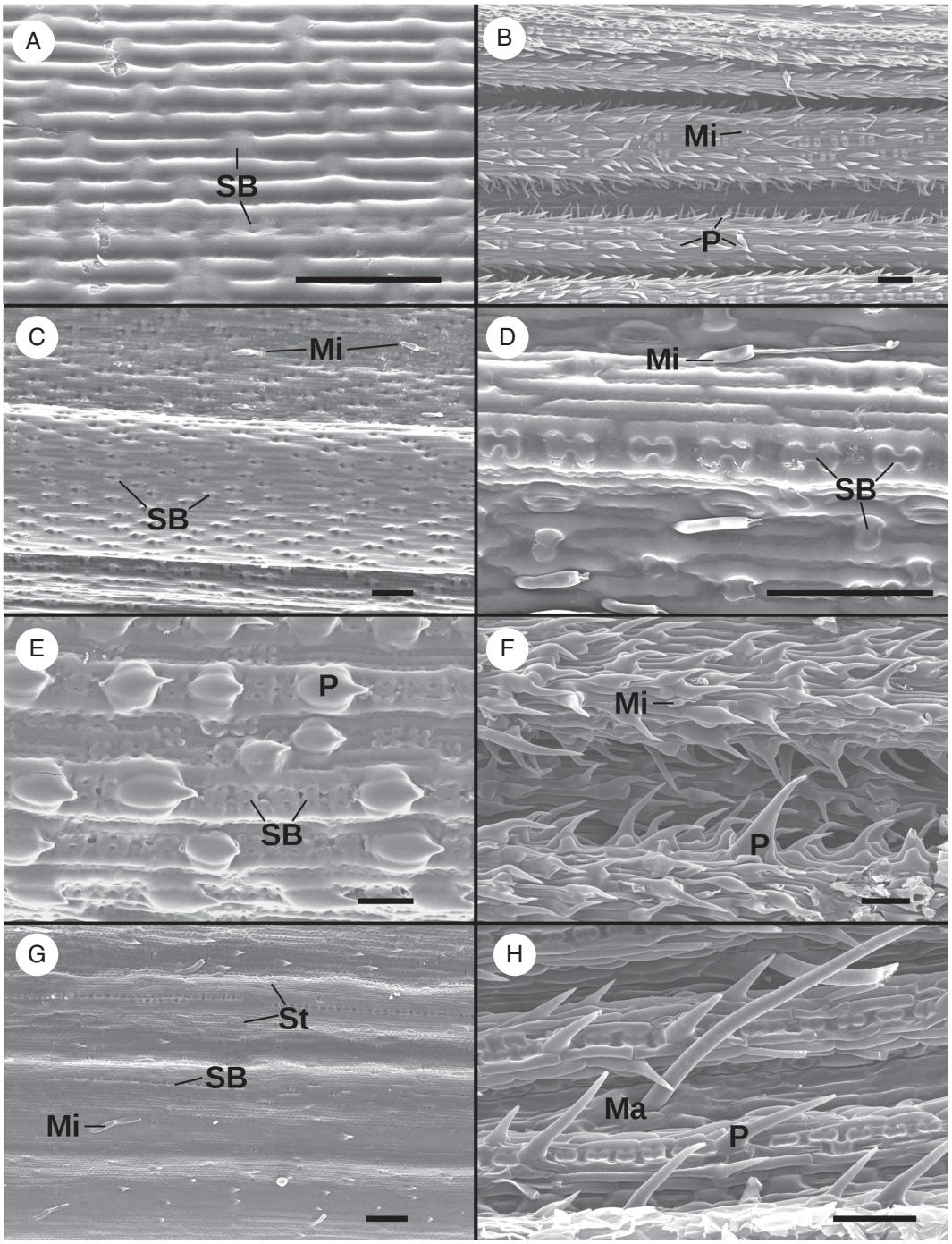

FIG. 5. Epidermal SEM images from the abaxial (A, C, E, G) and adaxial (B, D, F, H) sides of the middle sections of mature leaves from herbarium specimens. (A, B) *Alloeochaete andongensis* (Gossweiler 11810), (C, D) *Dichaetaria wightii* (Soderstrom 1739), (E, F) *Nematopoa longipes* (Simon 2353) and (G, H) *Phaenanthoecium koestlinii* (Wood 1305). Selected features are marked as follows: Mi, microhair; Ma, macrohair; P, prickle; SB, silica body; St, stomate. Black and white scale bars are approx. 50 μm.

Dichaetaria and *Alloeochaete* form a clade sister to the Panicoideae, despite morphological similarity to members of Arundinoideae and Danthonioideae. The other early-diverging members of Panicoideae are highly heterogeneous, explaining in part why this affiliation was not previously identified. Anatomically, the samples from these two genera in our analysis share a few epidermal features. Both have microhairs on the costal zones that are narrow and have apical cells twice as long as the basal cells (Fig. 5B, D) and a mix of dumb-bell- and saddle-shaped silica bodies (Fig. 5A, D). In cross-section, *Alloeochaete andongensis* (Fig. 4B) strongly resembles *A. gracillima* and *A. uluguruensis* (Linder *et al.*, 1997).

Alloeochaete has broad adaxial ribs that are nearly square on the adaxial side. The major veins have sclerenchyma girders on both sides, whereas minor veins lack adaxial girders. The overall shape of the ribs is reminiscent of that in the danthonioid *Pseudopentameris brachyphylla*, although *P. brachyphylla* has adaxial and abaxial sclerenchyma girders associated with all veins (Ellis, 1985*b*). Anatomical evidence is thus apparently at odds with the phylogenetic placement of *Alloeochaete*.

Arundinoideae sensu stricto

Resolution of relationships within Arundinoideae *s.s.* in our study is significantly improved over phylogenies based on individual chloroplast genes (e.g. Grass Phylogeny Working Group II, 2012) and those based on full plastome sequences but with only a few arundinoid genera (e.g. Cotton *et al.*, 2015; Duvall *et al.*, 2017). The genera of Arundinoideae *s.s.* still form a morphologically and ecologically heterogeneous assemblage. The genera form two clades, one with glumes shorter than the spikelet and the other with glumes as long as or longer than the spikelet. The 'short glumes' group consists of *Phragmites*, *Hakonechloa* and *Molinia*, as well as another clade of mostly African genera. '*Eragrostis*' *walteri*, formerly thought to be a unique example of reversion from C_4 to C_3 photosynthesis (Ingram *et al.*, 2011), falls in this clade and is sufficiently distinct from its sister taxon *Elytrophorus* that it should be assigned its own genus. The other two taxa in the 'short glumes' clade, *Styppeiochloa* and *Crinipes*, are sister taxa, as suggested by their taxonomic history (the type species of *Styppeiochloa* was segregated from *Crinipes*) and supported by their similar preference for seasonally wet, rocky habitats, their one-nerved glumes and their often two-flowered spikelets. The 'long glumes' clade of Arundinoideae contains, in addition to *Arundo*, the Australian genera *Amphipogon* and *Monachather*, and the South African genus *Dregeochloa*.

Four genera possibly belonging in the Arundinoideae are not included in this study. The monotypic African genera *Leptagrostis* and *Piptophyllum* have insufficient collected material to conduct destructive sampling. Herbarium samples of the Indian genera *Danthonidium* and *Zenkeria* yielded DNA that was too degraded to be sequenced. None of these taxa has unambiguous synapomorphies to support their placement in the current phylogeny. Linder *et al.* (1997) placed *Leptagrostis*, *Piptophyllum* and *Zenkeria* in the 'crinipoid' group, whose other members are placed in the plastome tree in the Arundinoideae, Chloridoideae and at the base of the Panicoideae (see above). Like *Nematopoa*, *Piptophyllum* was considered intermediate between *Triraphis* and *Crinipes* (Hubbard, 1957*b*), but leaf anatomical data for this monotypic genus are lacking. Interestingly, and unlike *Nematopoa*, *Triraphis* or *Crinipes*, the lemma awn in *Piptophyllum* is slightly twisted at the base, suggesting a possible additional independent origin of this trait. The spikelets of *Danthonidium* have lemmas similar to *Dregeochloa* and *Monachather*, but also to several taxa in subfamily Danthonioideae and Panicoideae. Soreng *et al.* (2015) treat *Danthonidium* as *incertae sedis* in the Danthonioideae along with *Alloeochaete* and *Phaenanthoecium*, which are recovered in very different clades in our analysis.

Micrairoideae

Our analysis places *Limnopoa* within the large genus *Isachne*, and Duvall *et al.* (2017) found that *Hubbardia* is sister to *Limnopoa*, suggesting that generic limits in these taxa need to be revised. *Isachne* is reported to have almost 100 species (Kellogg, 2015), but the most comprehensive revision of its members included only the 23 species from Malesia (Iskandar and Veldkamp, 2003). *Limnopoa meeboldii* is the sole species of *Limnopoa*, and *Hubbardia heptaneuron* is the only species of *Hubbardia*, so transferring these names to *Isachne* would not be difficult.

Two monotypic genera in Micrairoideae, *Heteranthoecia* and *Sphaerocaryum*, have not been sampled for molecular sequence data, and only one species of *Coelachne* is included in our phylogeny, so relationships within the tribes Hubbardieae and Isachneae *sensu* Soreng *et al.* (2015) are difficult to assess. Given the size, morphological heterogeneity and geographic distribution of *Isachne*, especially if *Hubbardia* and *Limnopoa* are included, it may be best to assign these genera to a single tribe, Isachneae, pending further phylogenetic sampling. Alternatively, Kellogg (2015) suggests that segregation of genera into tribes in this relatively small subfamily is unnecessary.

Phylogenetic position of Aristidoideae

Many previous phylogenies have placed Aristidoideae sister to the remainder of the PACMAD clade (e.g. Clark *et al.*, 1995; Grass Phylogeny Working Group II, 2012). In contrast, some more recent studies using whole plastomes have put Panicoideae in that position, with Aristidoideae sister to the CMAD group (Cotton *et al.* 2015; Burke *et al.*, 2016; Fig. 2, inset), although data from these studies could not reject the possibility of Aristidoideae being sister to a clade comprising the remainder of PACMAD. Here we find that placement of Aristidoideae is sensitive to inclusion of gaps in the alignment. This sensitivity means that either regions with high insertion/deletion rates contain phylogenetic signal and thus the more gap-inclusive alignments more closely approximate the 'true' plastome phylogenetic history, or these gap-filled regions introduce more phylogenetic noise into the data. Given the large number of taxa sampled and the fact that full chloroplast genomes were used in the analysis, it seems unlikely that the question of the correct placement of Aristidoideae will be resolved using plastome sequences.

Divergence date estimates

Ages inferred in the current analysis are younger than previously published estimates. Vicentini *et al.* (2008) reported age estimates for the ancestor of BOP and PACMAD ranging from 48 to 85 Mya, while Christin *et al.* (2014) reported ages for the same divergence of 20–62 Mya from plastid and 51–63 Mya from nuclear sequence data across four different dating analyses. Our analysis yielded a median age of approx. 33 Mya for this clade, with a 95 % HPD range of approx. 28–47 Mya, falling within the range estimated by Christin *et al.* (2014). Accurate absolute dating for events in grasses may not be

possible given the family's sparse fossil record and heterogeneous molecular evolutionary rates.

CONCLUSION

This study represents the largest plastome phylogeny of the grass family to date as well as the most complete sampling of genera in the taxonomically difficult subfamily Arundinoideae. Resolving the polyphyly of this poorly studied group has substantial implications for ancestral trait estimations across the PACMAD clade as shown by our new understanding of the phylogenetic distribution of the 'danthonioid' awn. Four genera are removed from the Arundinoideae *s.s.* on the basis of the plastome phylogeny: *Alloeochaete* and *Dichaetaria* are sister to Panicoideae; *Phaenanthoecium* is placed in Danthonioideae; and *Nematopoa* appears to belong in Chloridoideae. These new placements have some support from morphological and anatomical traits, but are equally representative of the notorious tendency for parallel evolution in the Poaceae. Herbarium specimens were vital for resolving these long-standing issues in grass classification and will continue to be an essential resource for phylogenetics of taxa for which field collections are not practical.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: concatenated tree file from 1 million trees taken from BEAST analysis. Figure S2: parsimony ancestral state reconstruction of twisted geniculate awns on the phylogeny from Fig. 2 using Mesquite. Table S1: plastomes included in the phylogenetic analysis, with assembly statistics for newly generated sequences. Table S2: character states for presence/absence of a twisted geniculate lemma awn in species sampled in the plastome phylogeny.

ACKNOWLEDGEMENTS

The authors thank Maria Vorontsova at Kew for assistance in sampling specimens, Gerrit Davidse for help with character coding, Jim Solomon for co-ordinating herbarium loans, and Paul Calloman for help with Fig. 1, and the editors and two anonymous reviewers for helpful suggestions on an earlier version of the manuscript. This work was supported by the National Science Foundation grant DEB-1457748 to E.A.K., Washington University in St. Louis, and the Research Center for Auditory and Vestibular Studies is supported by the National Institutes of Health NIDCD Grant P30DC04665. J.K.T., E.A.K. and M.R.M. contributed to the conception and design of the study. J.K.T. and M.R.M. performed the research, and acquired and analysed the data. All authors contributed to interpretation of the data and writing of the manuscript.

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