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PHYLOGENOMICS AND PLASTOME EVOLUTION OF THE CHLORIDOID GRASSES (CHLORIDOIDEAE: POACEAE)

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Premise of research. Studies of complete plastomes have proven informative for our understanding of the molecular evolution and phylogenomics of grasses, but subfamily Chloridoideae has not been included in this research. In previous multilocus studies, specific deep branches, as in the large clade corresponding to Cynodonteae, are not uniformly well supported.

Methodology. In this study, a plastome phylogenomic analysis sampled 14 species representing 4 tribes and 10 genera of Chloridoideae. One species was Sanger sequenced, and 14 other species, including outgroups, were sequenced with next-generation sequencing-by-synthesis methods. Plastomes from next-generation sequences were assembled by de novo methods, and the unambiguously aligned coding and noncoding sequences of the entire plastomes were analyzed phylogenetically.

Pivotal results. Complete plastomes showed rare genomic changes in *Distichlis*, *Centropodia*, and *Eragrostis tef* that were of potential phylogenomic significance. Phylogenomic analyses showed uniformly strong support for all ingroup relationships except one node in Cynodonteae in which a short internal branch connected long terminal branches. Resolution within this clade was found to be taxon dependent and possibly subject to long-branch attraction artifacts.

Conclusions. Our study indicates that the increase in phylogenetic information in sequences of entire plastomes well resolves and strongly supports relationships among tribes and genera of chloridoid grasses. Sampling more species, especially in the *Centropodia* + *Ellisochloa* clade and Cynodonteae, will further address relationships in these groups and clarify the evolutionary origins of the subfamily.

Keywords: Chloridoideae, Cynodonteae, phylogenomics, plastid genome, plastome.

Online enhancements: appendix figure and tables.

The grasses (Poaceae) are an ecologically and evolutionarily complex family that have been the subject of many systematic and phylogenetic studies. Phylogenetic studies of the family have found evidence of hybridization, polyploid speciation, intergenome transfer, and evolutionary convergence (e.g., GPWG I 2001; Saarela et al. 2015). Subfamilies of Poaceae have been delimited on the basis of phylogenetic analyses of molecular and morphological characters (e.g., GPWG I 2001; Sánchez-Ken et al. 2007; Sánchez-Ken and Clark 2010; GPWG II 2012). A major radiation of six of these grass subfamilies is designated as the PACMAD (Panicoideae, Aristidoideae, Chloridoideae, Micraroideae, Arundinoideae, Danthonioideae; Duvall et al. 2007; GPWG II 2012) clade. Relationships among the PACMAD lineages have been difficult to resolve, with several recent studies finding different branching orders (Vicentini et al. 2008; Bouchenak-Khelladi et al. 2010; GPWG II 2012; Cotton et al. 2015). PACMAD grass lineages apparently radiated at an estimated time of 32–44 Ma (Christin et al. 2008; Bouchenak-Khelladi et al. 2010; Cotton et al. 2015).

One of the largest and most morphologically diverse of the PACMAD subfamilies is Chloridoideae. Chloridoideae comprise ca. 1600 species and are second in number of species among the PACMAD subfamilies only to Panicoideae (ca. 3300 species). Chloridoid grasses are of economic value as cultivated grains (*Eleusine coracana*, *Eragrostis tef*), turf (*Bouteloua* spp., *Cynodon dactylon*, *Zoysia* spp.), forage plants (*Bouteloua* spp., *Eragrostis* spp., and others), potential sources of biofuel (*Spartina pectinata*, *Sporobolus maritimus*), and oilseed crops (*Halopyrum mucronatum*; Zeid et al. 2011; Boscaiu and Vicente 2013; Abideen et al. 2014). Chloridoid species play fundamental

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ecosystem roles in diverse habitats including salt marshes, vernal pools, and arid grasslands (de Carvalho et al. 2013).

Chloridoid grasses encompass considerable morphological diversity with few invariant or consistently varying synapomorphies, which presents challenges for understanding relationships at different taxonomic levels. Most chloridoids have bicellular microhairs that are club shaped with a turbinate basal cell and hemispheric distal cell (Tateoka et al. 1959; Jacobs 1987; Liu et al. 2010), but are otherwise diverse in terms of vegetative and reproductive macro- and microstructural features. Depending on how the subfamily is circumscribed, most or all species exhibit C_4 photosynthesis (GPWG I 2001). Moreover, several independent transitions between C_4 subtypes in the subfamily have been suggested (Christin et al. 2007, 2008; Roalson 2011). Peterson et al. (2011) expanded Chloridoideae to include the *Centropodia* + *Ellisochloa* clade in what they recognized as Centropodieae. Species of *Ellisochloa* have non-Kranz leaf anatomy (Ellis 1982; Tomlinson 1985; Christin et al. 2013) and exhibit isotopic evidence of C_3 physiology (Peterson et al. 2011). However, more study is needed to ascertain relationships among these species. In this article we adopt a conservative position of leaving the taxonomic status of the *Centropodia* + *Ellisochloa* clade as unresolved.

Despite their morphological variability, in taxonomically dense phylogenetic studies with moderate amounts of plastid sequence data, Chloridoideae are strongly supported as monophyletic and sister to the *Centropodia* + *Ellisochloa* clade; this group is in turn sister to Danthonioideae (Bouchenak-Kellahdi et al. 2008; Christin et al. 2008; GPWG II 2012). However, phylogenetic relationships within the subfamily are not uniformly well supported, especially among the deep branches in the large clade corresponding to Cynodonteae (Columbus et al. 2007; Peterson et al. 2010). Obtaining more phylogenetically informative characters might help address these problematic branches. We chose this subfamily for a plastid phylogenomic study to explore the utility of complete plastid genome (plastome) sequences to resolve systematic issues.

Sequencing and analysis of complete plastid genomes have the potential to increase the number of informative characters in phylogenetic studies over those of typical multilocus studies by several orders of magnitude. Due to improvements in DNA sequencing technology, entire grass plastomes can be routinely assembled directly from next-generation read pools, and this is reflected in the recent published literature on grass plastome phylogenomics (Zhang et al. 2011; Wu and Ge 2012; Besnard et al. 2013, 2014; Burke et al. 2014; Jones et al. 2014; Cotton et al. 2015; Saarela et al. 2015; Wysocki et al. 2015). In part, this is made possible by the large number of existing grass plastomes that can be used to identify highly conserved regions to serve as reference points for scaffolding contigs. The utility of this approach is documented in several plastome phylogenomic studies. Morris and Duvall (2010) and Jones et al. (2014) addressed evolutionary questions of the most deeply diverging subfamilies of grasses, Anomochlooideae, Pharoideae, and Puelioideae, with analyses of complete plastomes. Burke et al. (2012, 2014) related divergences among New World Bambusoideae with specific biogeographic and paleoclimatic events using a phylogenomic approach. Phylogenetic relationships of other groups of bambusoids have also been investigated with these techniques (Wu et al. 2009; Zhang et al. 2011; Wysocki et al. 2015), as well as relationships among Bambusoideae, Pooideae, and Oryzoideae (as Ehrhartoideae; Wu and Ge 2012).

Previous studies of complete plastomes have investigated partitioning the sequence data to remove signal that conflicts with the dominant phylogenetic signal. These studies failed to show clear advantages when restricting the plastome data to selected partitions, such as coding sequences or major plastome subregions (Zhang et al. 2011; Burke et al. 2012; Ma et al. 2014; Cotton et al. 2015; Saarela et al. 2015). The use of all unambiguously aligned coding and noncoding sequences from the entire plastome in these studies substantially increased phylogenetic information and raised support values to their maximum levels at most nodes.

Chloridoids have been poorly sampled in plastid phylogenomic studies of grasses; the only completely sequenced chloridoid plastomes are those of *Neyraudia reynaudiana* (Wysocki et al. 2014), a species in the deeply diverging tribe Triraphideae, and *Spartina maritima*, which is classified in Zoysieae (Rousseau-Gueutin et al. 2015).

Here we investigated the complete plastomes of 14 representative Chloridoideae species (13 new in this article) and analyzed them with 12 PACMAD outgroups (2 new) to address the following four objectives: (1) We examined the efficacy of plastid phylogenomics in finding better support for hypotheses of relationships within the subfamily. (2) We investigated the impact of taxon sampling on topology and support values with taxon-removal experiments in Cynodonteae. (3) We tested whether alternative phylogenetic hypotheses suggested by the taxon-removal experiments provided equivalent explanations of the data. (4) We observed molecular evolutionary events, particularly those that could be considered rare genomic changes, in a plastome phylogenomic context.

Material and Methods

Sampling in this study included representative species of four chloridoid tribes: Triraphideae, Eragrostideae, Zoysieae, and Cynodonteae (Soreng et al. 2015). Within Cynodonteae, sampling emphasized the species-rich subtribe Eleusininae. We also included *Centropodia glauca*. Previously published plastid sequences were available for the other five PACMAD subfamilies to use as outgroups. One additional danthonioid species was sequenced in this study for better representation of the outgroup subfamily previously identified as the sister to the chloridoids + *Centropodia*. Other nonchloridoid taxa included Aristidoideae (*Aristida purpurea*), Danthonioideae (*Chaetobromus involucratus*, *Chionochloa macra*, *Danthonia californica*, and *Merxmuellera guillarmodae*), Micraioideae (*Eriachne stipacea*, *Isachne distichophylla*, and *Micraira spiciformis*), Arundinoideae (*Phragmites australis*), and Panicoideae (*Panicum virgatum* and *Setaria italica*; table 1). DNA was extracted from silica-dried leaf samples with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) after homogenization of the tissue in liquid nitrogen. Herbarium vouchers were collected for all samples (table 1).

Sequencing of the *Eragrostis tef* plastome used the Sanger method. PCR reactions were performed using primers specific to the inverted repeat (IR; Dhingra and Folta 2005) and single-

Table 1

Taxon Sampling, Herbarium Vouchers, and GenBank Accession Numbers for the Grass Plastomes Analyzed in This Study

Subfamily/tribe and species	GenBank accessions, vouchers
Chloridoideae:	
Cynodonteae:	
Astrebla pectinata (Lindl.) F.Muell. ex Benth[a]	KT168391, USDA PI 238232
Bouteloua curtipendula (Michx.) Torr.[a]	KT168386, S. Burke 27 (DEK)
Bouteloua gracilis (Kunth) Lag. Ex Griffiths[a]	KT168392, J. T. Columbus 3219 (RSA)
Chloris barbata Sw.[a]	KT168393, USDA PI 308556
Distichlis bajaensis H. L. Bell[a]	KT168394, Bell 458 (RSA)
Distichlis spicata (L.) Greene var. *stricta* (Torr.) Scribn.[a]	KT168395, Saarela 677 (CAN)
Hilaria cenchroides Kunth[a]	KT168387, J. T. Columbus 5049 (RSA)
Hilaria rigida (Thurb.) Benth. ex Scribn.[a]	KT168396, J. T. Columbus 3588 (RSA)
Triraphideae:	
Neyraudia reynaudiana	NC_024262, J. T. Columbus 5302 (RSA)
Eragrostideae:	
Eragrostis minor Host[a]	KT168384, L. Clark 1333 (ISC)
Eragrostis tef (Zuccagni) Trotter[a]	KT168385, A. Ingram 544 (WAB)
Zoysieae:	
Spartina pectinata Bosc ex Link[a]	KT168388, P. Peterson 20865 (CAN)
Sporobolus heterolepis (Gray) A. Gray[a]	KT168389, M. Duvall s. n. (DEK)
Zoysia macrantha Desv.[a]	KT168390, J. T. Columbus 5049 (RSA)
Aristidoideae:	
Aristida purpurea	NC_025228
Arundinoideae:	
Phragmites australis	NC_022958
Danthonioideae:	
Chaetobromus involucratus	KJ920226
Chionochloa macra	NC_025230
Danthonia californica	NC_025232
Merxmuellera guillarmodae Conert[a]	KT168397, N. Barker 1009 (BOL)
Micrairoideae:	
Eriachne stipacea	NC_025234
Isachne distichophylla	NC_025236
Micraira spiciformis	KJ920234
Panicoideae:	
Panicum virgatum	NC_015990
Setaria italica	NC_022850
Unplaced:	
Centropodia glauca (Nees) T. A. Cope[a]	KT168383, Linder 5410 (BOL)

[a] This article.

copy regions of the grass plastome (Leseberg and Duvall 2009). All four IR boundaries and one complete copy of the IR were sequenced. Touchdown PCR using "round I" conditions was performed for all amplifications (Dhingra and Folta 2005). In the case of amplification failures, the methods of Morris and Duvall (2010) were followed, including the design of species-specific primers (table A1; tables A1–A4 available online). The Wizard SV PCR Clean-Up System (Promega, Madison, WI) was used to prepare amplifications for automated sequencing (Macrogen, Seoul, South Korea). A complete plastid chromosome was assembled by identifying overlapping forward, reverse, and adjacent segments in Geneious Pro v8.0.2 (Biomatters, Auckland, New Zealand).

Several next-generation-sequencing (NGS) library preparation approaches were used to accommodate different starting quantities of DNA and sequencing goals. DNA extracts from *Distichlis spicata* var. *stricta* and *Hilaria cenchroides* were used to prepare libraries with the TruSeq low-throughput protocol and sequenced as described in Wysocki et al. (2014). DNA extracts from *Astrebla pectinata*, *Bouteloua curtipendula*, *Chloris barbata*, *C. glauca*, *Eragrostis minor*, *M. guillarmodae*, *Spartina pectinata*, *Sporobolus heterolepis*, and *Zoysia macrantha* were used to prepare libraries with the Nextera protocol (Illumina, San Diego, CA) and sequenced as described in Burke et al. (2014). Sequencing of these species was performed at the core DNA facility at Iowa State University, Ames.

Additionally, plastomes of *Bouteloua gracilis*, *Distichlis bajaensis*, and *Hilaria rigida* were sequenced in a skim of the genome that contained selectively amplified nuclear loci, high-copy elements of the nuclear genome, and both organellar genomes. The enrichment was performed for another project on low-copy nuclear loci, but complete plastomes were present in these read pools. Library construction followed MYbaits (MYcroarray, Ann Arbor, MI) and Illumina protocols as described in Tennessen et al. (2013), except indexed adaptors (Bioo Scientific, Austin, TX) were ligated to genomic fragments

and the libraries were ×24 multiplexed for 101 base paired-end sequencing on an Illumina Hi-Seq 2000 at the Center for Genome Research and Biocomputing at Oregon State University, Corvallis.

NGS Plastome Assembly and Verification

Illumina reads were assembled into complete plastid chromosomes with de novo assembly methods. The plastome assembly methods are fully described in Wysocki et al. (2014). Briefly, these included iterative use of the Velvet software package (Zerbino et al. 2008), loading contigs from the previous step into the assembler multiple times with kmer lengths specified to increase in steps of six from 19 to 85 bp. Contigs were scaffolded with the anchored conserved region extension method, which queries contig sets for segments that are invariant across Poaceae. Any remaining gaps between contigs in the scaffolds were resolved with contigs or reads by locating perfectly overlapping regions of at least 20 bp on the end of an incomplete assembly. Ambiguities and assembly artifacts were identified and corrected by searches of these regions in the original read files. A final verification step was performed by mapping reads to the assembled plastome to detect inconsistencies in base composition and overlap. Read depths were determined by mapping the quality-trimmed read pool against the de novo assembly in Geneious Pro. Numbers of reads, assembled contigs, and mean coverage values were recorded.

Plastome Annotation and Analysis

Fully assembled plastomes were annotated by aligning to a previously published and annotated plastome, *Neyraudia reynaudiana* (NC_024262), in Geneious Pro. Reference annotations were transferred to the new plastome when the annotation shared a minimum similarity of 70%. Coding sequences for each species were examined, and adjustments were made to correctly position boundaries, to preserve reading frames, and in the case of tRNAs and rRNAs to preserve full lengths. The endpoints of the large IR were located using the methods from Burke et al. (2012). BLASTn searches (Altschul et al. 1997) were conducted on selected sequence regions to compare identities with banked sequences.

Phylogenomic Analyses

A plastome-scale DNA matrix was assembled for 14 species of chloridoid grasses plus 12 other PACMAD species (table 1). The matrix of complete plastomes, excluding IR region "A" (IRa), was aligned in Geneious Pro with the MAFFT v6.814b plug-in (Katoh et al. 2005) using the auto function for the algorithm and other default settings. The IRa sequence was excluded from the data matrix to avoid double representation of this large repeat region. Characters for which gaps were introduced in one or more sequences by the alignments were excluded from the data matrix prior to phylogenetic analyses. The two panicoid species were selected as a monophyletic outgroup.

A maximum likelihood (ML) analysis (Felsenstein 1981) was conducted with the GTR+G+I substitution model selected by jModelTest v2.1.3 (Guindon and Gascuel 2003; Darriba et al. 2012) under the Akaike information criterion (Akaike 1974). The ML analysis was implemented in RAxML-HPC2 v7.2.8 (Stamatakis et al. 2008) to determine the ML tree and bootstrap values (BV) from 1000 bootstrap pseudoreplicates. Bootstrap consensus trees were generated using the Consense function of the Phylip software package (Felsenstein 2005). Bayesian inference (BI) analysis was performed using MrBayes on XSEDE v3.1.2 (Ronquist and Huelsenbeck 2003) at the CIPRES Science Gateway (Miller et al. 2010) using the same substitution model as in RAxML (invgamma; nst = 6). Two independent MrBayes analyses were performed with four chains and 2 million generations each, at which point the average standard deviation of split frequencies was <0.000001. The first 25% of the samples were discarded as burn-in. Other parameters were set at defaults. Branch and bound maximum parsimony (MP; Fitch 1977) and MP bootstrap (Felsenstein 1985) analyses with 1000 branch and bound pseudoreplicates were performed on the chloridoid alignment in PAUP v4.0b10 (Swofford 2003).

A broader, second analysis following the same analytical methods was also performed except that the MP analysis was performed as a heuristic search with 100 random-addition sequence replicates. In this analysis a total of 53 complete plastomes, which included those previously analyzed in Cotton et al. (2015) and Besnard et al. (2014), were analyzed.

Taxon-Removal Experiments

The eight sampled members of the Cynodonteae were selected to test the effects of systematic taxon exclusion. Matrices that included every possible (256) combination of these taxa were generated using a Python script, aligned using MAFFT, and all positions with introduced gaps were removed using TrimAl v.1.2rev59 (Capella-Gutiérrez et al. 2009). The ML phylogeny and bootstrap support values were then estimated for each tree using RAxML as above. All trees were then visually inspected to locate differences in topology and bootstrap support consistent with the inclusion or exclusion of particular taxa.

Tests of Alternative Topologies

Shimodaira-Hasegawa (SH) tests (Shimodaira and Hasegawa 1999) were used to assess the log-likelihood difference between the RAxML ML estimate tree and two alternative topologies suggested by the taxon-removal experiments. The full data matrix was used to estimate two constraint trees in RAxML altering the topology of Cynodonteae from the best tree (fig. 1; *Hilaria* sister to *Bouteloua* + *Distichlis*) to *Hilaria* + *Bouteloua* and *Hilaria* + *Distichlis*, respectively. SH tests that resampled estimated log likelihoods were conducted in PAUP* v. 4.0b10 (Swofford 2003) with a GTR+G+I substitution model, full optimization, and 1000 bootstrap replicates. Other likelihood parameters were estimated (basefreq = estim shape = estim rmatrix = estim pinvar = estim).

Results

Plastome Features

Complete plastome sequences were newly determined from 13 species of Chloridoideae, *Centropodia glauca*, and *Merx-*

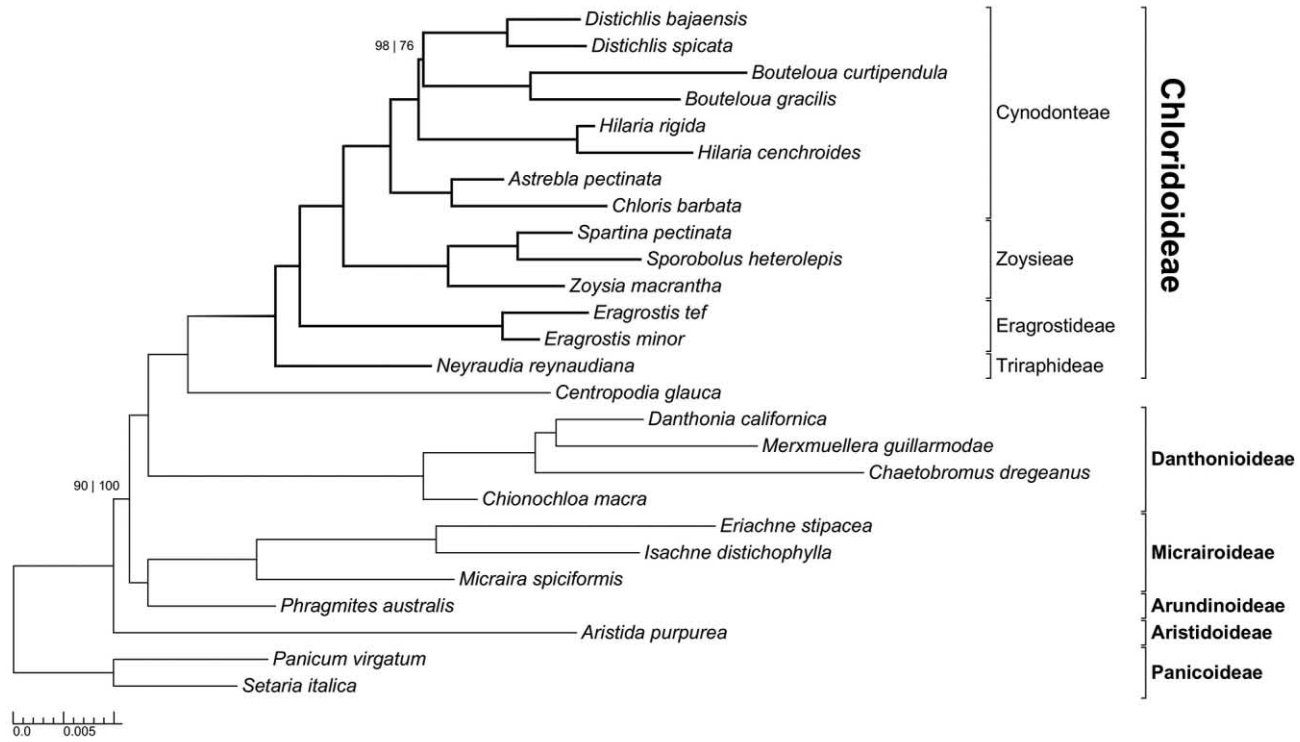

Fig. 1 Maximum likelihood phylogram (i.e., the "best tree") inferred from 26 complete plastomes. Branch lengths are proportional to the number of substitutions per site along the branch. The chloridoid subtree is emphasized with darker lines. Subfamilies and tribes are indicated. Note that maximum parsimony (MP) and Bayesian inference (BI) analyses produced topologies that were identical to the maximum likelihood (ML) topology. Nodes are supported with 100% bootstrap values except where noted at two nodes (ML | MP). In the BI analysis, all nodes were supported with posterior probabilities of 1.0.

muellera guillarmodae (Danthonioideae), totaling over 2 million bases of nucleotide sequence. Numbers of reads, assembled contigs, and mean coverage values for plastomes produced by next-generation methods are reported (table A2). These plastomes ranged from 133,742 to 137,616 bp in length (table 2). The overall plastome structure in the PACMAD species was highly conserved and largely reflected the gene content, intron-exon structure, and gene order of the plastomes of other crown grasses. Within this conserved framework, several rare genomic changes were observed and are especially striking in coding and noncoding regions of the plastomes of *Distichlis* spp. (table A3).

Notable variation was observed in the length of the *rpl16* intron. The lengths of *rpl16* introns for most chloridoid species in this study spanned a narrow range of 1021–1102 bases (mean: 1064; table 2). However, the *rpl16* intron was unusually short in *C. glauca* at only 868 bases. A BLASTn search of the *C. glauca rpl16* intron showed that the greatest similarity was to those of three arundinoid grasses (*Hakonechloa macra*, *Phragmites australis*, and *Monachather paradoxus*; 100% coverage; 89%–91% identity). The *rpl16* intron was unusually long in the plastomes of *Distichlis* spp. (2961 and 3137 bases for *Distichlis bajaensis* and *Distichlis spicata*, respectively). The upstream 180-base region and downstream 770-base region of this intron in *Distichlis* resembles typical chloridoid *rpl16* intron sequences (minimum 87% identity), but internal sequences of the *D. spicata rpl16* intron (but not that of *D. bajaensis*) ranging in length from 56 to 174 bases have 84% or more identity to *Distichlis* IR sequences. These inserted intron sequences were not found in any of the other chloridoid plastomes, nor did they show significant similarities to other sequences in BLASTn searches.

Phylogenomic Analyses

The plastome alignment length for 14 chloridoids plus 12 other taxa was 94,837 bases after removal of the IRa sequences and all nucleotide sites with at least one introduced gap. Excluding these sites removed inversions, indel regions, and ambiguously aligned portions of the plastomes. The aligned data matrix and associated tree files are available at the TreeBase repository (http://purl.org/phylo/treebase/phylows/study/TB2:S18944).

Topologies produced by analyses of this matrix with the ML, BI, and MP methods were identical. ML analysis produced a tree with a $-\ln L = 289{,}408.64$ (fig. 1). ML BV were 100% for all but two nodes: (1) that uniting the two species of *Bouteloua* with two species of *Distichlis* (ML BV = 98) and (2) the deep node uniting the CMAD (Chloridoideae, Micrairoideae, Arundinoideae, and Danthonioideae) taxa and *Aristida purpurea* (ML BV = 90). In the BI analysis, all posterior probability (PP) values were at maximum (PP = 1.0; results not shown). Parsimony analysis produced a single MP tree of 24,690 steps, an ensemble consistency index of 0.6274 (exclud-

Table 2

Complete Plastome and Subregion Lengths (Nucleotides) for Taxa in This Study

	Total	LSC	SSC	IR	*rpl16* intron
Astrebla pectinata	135,737	81,045	12,658	21,017	1070
Bouteloua curtipendula	133,866	79,309	12,607	20,975	1071
Bouteloua gracilis	133,742	79,315	12,389	21,019	1038
Chloris barbata	135,372	80,819	12,529	21,012	1102
Distichlis bajaensis	135,452	83,456	12,746	19,625	2961
Distichlis spicata var. *stricta*	138,504	83,374	12,679	21,225	3137
Hilaria cenchroides	135,825	80,238	13,423	21,082	1021
Hilaria rigida	135,313	80,576	12,605	21,066	1046
Neyraudia reynaudiana	135,367	80,616	12,695	21,028	1051
Centropodia glauca	134,594	80,093	12,475	21,013	868
Eragrostis minor	135,091	80,382	12,577	21,066	1097
Eragrostis tef	134,435	79,803	12,580	21,026	1095
Spartina pectinata	135,618	80,923	12,725	20,985	1066
Sporobolus heterolepis	135,106	81,214	12,750	20,571	1032
Zoysia macrantha	136,285	80,211	12,722	21,676	1076
Aristida purpurea	138,423	80,437	12,540	22,723	1024
Phragmites australis	137,561	82,327	12,740	21,247	1071
Chaetobromus involucratus	132,576	78,798	11,934	20,922	957
Chionochloa macra	135,720	80,888	12,518	21,157	959
Danthonia californica	134,191	79,647	12,346	21,099	963
Merxmuellera guillarmodae	132,895	79,023	11,348	21,262	977
Eriachne stipacea	134,746	79,849	12,573	21,162	1027
Isachne distichophylla	135,782	81,006	12,650	21,063	1027
Micraira spiciformis	135,318	80,073	12,721	21,262	1028
Panicum virgatum	139,677	81,729	12,538	22,705	1080
Setaria italica	135,516	79,894	12,024	21,799	842

Note. LSC = large single copy; SSC = small single copy; IR = inverted repeat.

ing uninformative characters), and a retention index of 0.7611. The only MP BV <100% was on the very short branch uniting *Bouteloua* and *Distichlis* (MP BV = 76%; see fig. 1).

The topological results reported here will emphasize relationships among chloridoid taxa. Fourteen chloridoid species were sister to *C. glauca*, and this group was in turn sister to Danthonioideae in all analyses. The next successively diverging lineages were *Neyraudia reynaudiana*, Eragrostideae, and Zoysieae + Cynodonteae. Within Cynodonteae, *Astrebla pectinata* + *Chloris barbata* (Eleusininae) are sister to the remaining species, then two *Hilaria* spp. (Hilariinae), which are in turn sister to *Bouteloua* (Boutelouinae) + *Distichlis* (Monanthochloinae; fig. 1).

The results of the 53-taxon analysis confirm those of the more focused analysis (fig. A1, available online) and that of Cotton et al. (2015). That is, the same topology was retrieved for Chloridoideae (all support values at maximum except the MP BV for the node uniting *Bouteloua* and *Distichlis* was 88%). Furthermore, Panicoideae are sister to ACMAD species with moderate to high support (ML BV = 94% | MP BV = 82% | BI PP = 1.0).

Taxon-Removal Analyses

The taxon-removal analyses focused on the relationships between *Bouteloua*, *Distichlis*, and *Hilaria*, which was the only portion of the tree topology that varied. Of the 256 trees produced in the taxon-removal analysis, 108 had at least one representative species of each of these three genera. Within these 108 trees, three topologies (summarized in fig. 2) were recovered. (1) The majority topology retrieved from analyses of 88 taxon subsets reflects that of the complete analysis (fig. 1) in which representative *Hilaria* spp. are sister to *Distichlis* + *Bouteloua*. In these topologies, the ML BV at all ingroup nodes are uniformly 100% except for the node uniting *Bouteloua* spp. and *Distichlis* spp., where ML BV range from 44% to 100% with a mean of 79%. (2) Analyses of 13 of the 108 subsets produced a topology in which *Distichlis* spp. are sister to *Bouteloua* + *Hilaria*. Again, the only ingroup node without 100% support was that uniting *Bouteloua* with *Hilaria*, where ML BV ranged from 48% to 86% (mean ML BV = 61.5%). (3) A second minority topology was retrieved in the analyses of 7 of the 108 subsets in which *Bouteloua* spp. are sister to *Distichlis* + *Hilaria*. ML BV were at maximum for all ingroup nodes except for that uniting *Distichlis* and *Hilaria*, where values of 47% to 88% were obtained (mean ML BV = 63%).

Alternative Hypothesis Testing

When tree searches using the full data set and a GTR+I+G model were constrained to force monophyly of either (1) *Bouteloua* + *Hilaria* or (2) *Distichlis* + *Hilaria*, tree likelihoods were significantly decreased ($P < 0.05$; table 3) compared to that of the ML topology of the full data set (fig. 1).

Discussion

In this study, 13 complete chloridoid grass plastomes were sequenced using genome-skimming techniques. Three some-

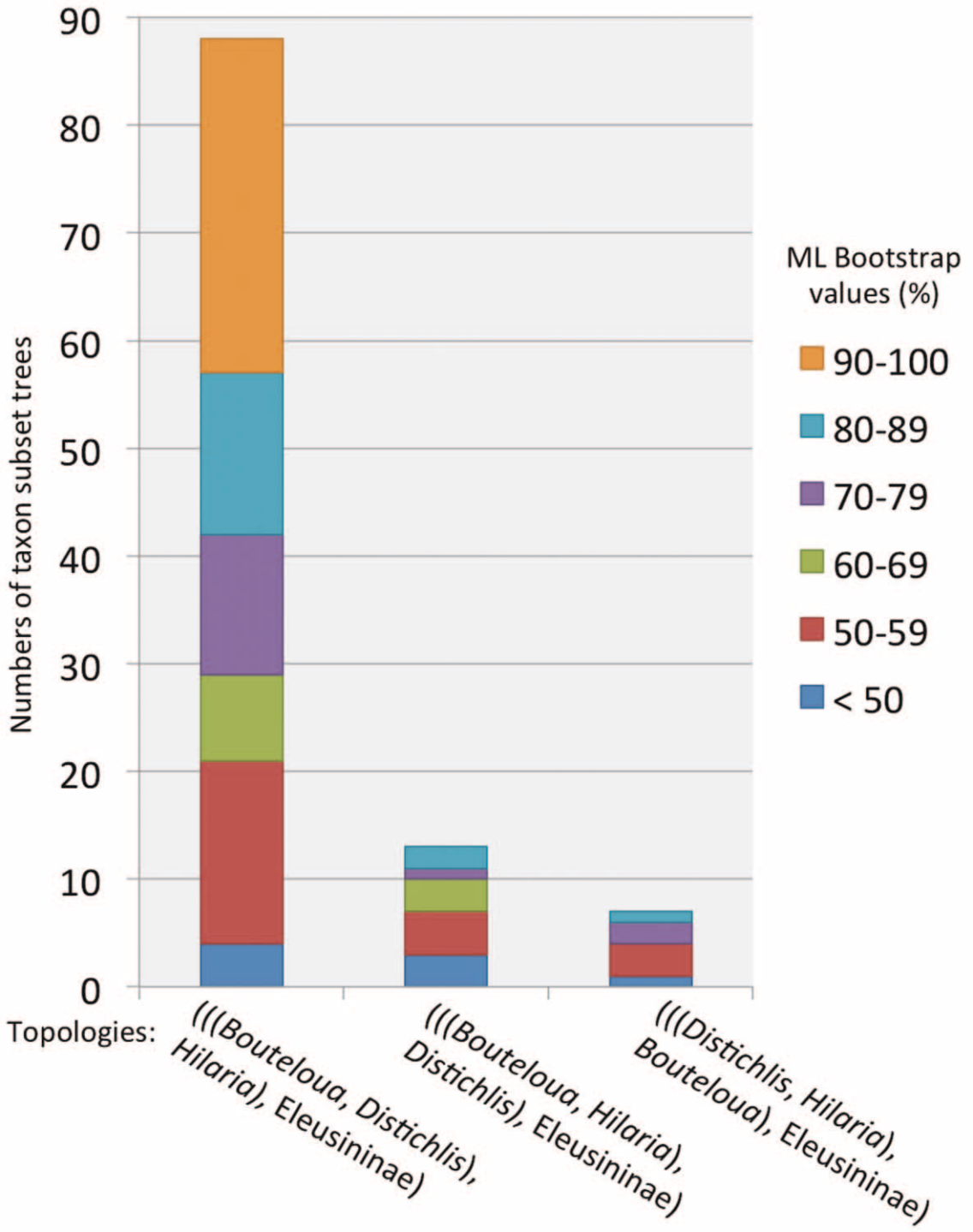

Fig. 2 Results from the taxon-removal experiments. The relevant portions of the topologies that vary for the three results are indicated in Newick format. Colors in the histograms correspond to maximum likelihood (ML) bootstrap value for a single node in each topology, resolving the relationship of one or both species of (1) *Bouteloua*, *Distichlis*, (2) *Bouteloua*, *Hilaria*, or (3) *Distichlis*, *Hilaria*.

what different methods of library preparation produced sequence files ranging from 3.5 to 14.4 million reads. De novo assemblies using the methods of Wysocki et al. (2014) produced a mean of 4.5 contigs per plastome, and for one species, *Bouteloua curtipendula*, the complete plastome was obtained as a single contig (table A2). Assembly software is most likely to fail in repetitive sequence regions such as the boundaries of the large-plastome IRs. However, unambiguous

Table 3

Shimodaira-Hasegawa Test Results

Topology	−lnL	Difference, −lnL values	*P* value
Constraint: ((*Distichlis, Hilaria*), *Bouteloua*)	289,425.4	16.6	.016
Constraint: ((*Bouteloua, Hilaria*), *Distichlis*)	289,425.2	16.4	.012
Majority: ((*Bouteloua, Distichlis*), *Hilaria*)	289,408.8	(Best)	...

Note. Underlining denotes significant *P* values.

resolution of these areas was possible by using quality-trimmed reads to extend the termini of contigs until high intercontig coverage was obtained.

Plastome Features

Plastomes are well characterized in grasses with a considerably greater number sequenced from Poaceae than from any other angiosperm family (see http://www.ncbi.nlm.nih.gov/genome/browse/). However, the only publicly available complete plastomes for Chloridoideae are those for *Neyraudia reynaudiana* and *Spartina maritima* (GenBank accessions NC_024262 and NC_027650).

Here we add data for 13 species of chloridoids to investigate the sequence, structure, and phylogenomic information for plastomes in this subfamily. Chloridoid plastomes contain some unusual features within the standard framework of the quadripartite structure, gene content, and gene order found in plastomes of other Poaceae (Shimada et al. 1990; Saski et al. 2007).

The two species of *Distichlis* showed similar but not identical rare genomic changes at two loci (table A3). In the *rpoA* coding sequence there are inserts of 90 bp (*Distichlis bajaensis*) and 159 bp (*Distichlis spicata*). Although these inserts do not introduce a frame shift in the *rpoA* gene, they do add 30 and 53 amino acids, respectively, which would follow codon 240 in the *rpoA* message. This can be compared to another plastid RNA polymerase gene in grasses, *rpoC2*, which has large internal inserted regions (Cummings et al. 1994). However, the mechanism responsible for the *rpoA* insertions in *Distichlis* is unclear, as there are no associated tandem repeats evident that would suggest slipped-strand insertions. The inserts may represent separate mutation events in the two species of *Distichlis* as suggested by their different sizes. However, the fact that they are found in closely related species, that the inserts have 82% nucleotide identity over the span of their shared sequence, and that no other chloridoids or even outgroup species in our analysis have inserts of any size at this position in *rpoA* could support an interpretation of a single insertion event with subsequent modification, possibly with degradation in *D. bajaensis*.

A second distinctive feature of the two species of *Distichlis* is the size of the *rpl16* intron. In both species the intron is over 1500 bases longer than in other chloridoids. The terminal regions flanking the exons show high identities to sequences of other chloridoid *rpl16* introns, possibly because of constraints imposed by intron processing. However, the central portions may be under relaxed functional constraint, because they are not similar to the *rpl16* intron sequences of other PACMAD species and have only 55% nucleotide identity to each other. Again, since the *D. spicata rpl16* intron differs in length and sequence from that of *D. bajaensis*, their unique features could be the result of independent molecular evolutionary histories, but the close relationship of the taxa and similarity in intron sizes may indicate common ancestry with subsequent mutation. Further sampling will be required to distinguish among these scenarios.

Phylogenomics

Plastid phylogenomic analyses with moderate taxon sampling typically show a high ratio of informative characters per node in phylogenetic trees. When only unambiguously aligned characters are analyzed, this has resulted in uniformly high bootstrap support values in the phylogenies of other groups of grasses (Zhang et al. 2011; Burke et al. 2012, 2014; Wu and Ge 2012; Jones et al. 2014; Cotton et al. 2015; Saarela et al. 2015; Wysocki et al. 2015). In this study, a total of over 8200 parsimony-informative characters were analyzed to resolve relationships at 24 nodes for 26 species of grasses.

Subclades of the PACMAD clade, which correspond to recognized taxa (e.g., Soreng et al. 2015) that were sampled with two or more species in our analyses, were all supported as monophyletic at the maximum BV = 100%. Included among these monophyletic taxa were three subfamilies: Chloridoideae (14 spp.), Danthonioideae (4 spp.), and Micraroideae (3 spp.). Within Chloridoideae, monophyletic groups included two tribes: Cynodonteae (8 spp.) and Zoysieae (3 spp.); two subtribes: Eleusininae and Sporobolinae (2 spp. each); and two species each of *Bouteloua*, *Distichlis*, *Eragrostis*, and *Hilaria*. Relationships among these monophyletic taxa were supported at BV = 100% with one exception (see below). At the subfamily level, our PACMAD taxa largely corroborate the relationships retrieved by GPWG II (2012), but a recent alternative hypothesis guided our choice of Panicoideae, rather than Aristidoideae, as the sister group and outgroup to the remaining ACMAD taxa (Besnard et al. 2014; Cotton et al. 2015).

The phylogenetic signal from our full plastome data supports *Centropodia glauca* as sister to Chloridoideae. In this context, the insertions in *trnG-trnM* and the short *rpl16* intron in *C. glauca* could be interpreted as symplesiomorphic with nonchloridoid PACMAD taxa. However, the sequence similarities between *C. glauca* and Arundinoideae in *trnG-trnM* and *rpl16* intron sequences is somewhat unexpected. Danthonioideae are strongly supported as sister to *Centropodia* + Chloridoideae with plastome data, whereas Arundinoideae are strongly supported as sister to Micraroideae (fig. 1; GPWG II 2012; Cotton et al. 2015). The high nucleotide simi-

larity of these loci to arundinoid rather than danthonioid taxa may be a result of unequal representation of published PACMAD plastomes or may be due to convergent processes or undocumented recombination events among PACMAD lineages. Whatever the cause, the unique features and phylogenomics of *Centropodia* suggest that further research, perhaps exploiting nuclear phylogenomics, may be productive.

The chloridoid lineages that diverge after *C. glauca* in our full plastome phylogenies (fig. 1) correspond to the tribes Triraphideae (*N. reynaudiana*), Eragrostideae (two species of *Eragrostis*), Zoysieae (three species), and Cynodonteae (eight species), consistent with earlier studies with similar generic sampling (Columbus et al. 2007; Peterson et al. 2010, 2014). The eight sampled members of Cynodonteae formed a monophyletic group (BV = 100%). *Astrebla pectinata* and *Chloris barbata* are members of Eleusininae and formed a sister clade to the remaining Cynodonteae. Among these six species, the two species of *Hilaria* formed a clade that was sister to the sampled species of *Bouteloua* and *Distichlis* (fig. 1).

Taxon-Removal Experiments

Three different results were retrieved here when selected members of Cynodonteae were excluded from 108 ML analyses with otherwise identical topologies (fig. 2; table A4, available as a PDF). These 108 ML trees were inferred from subset matrices with at least one representative each of *Bouteloua*, *Distichlis*, and *Hilaria*, and the tree topologies varied only with respect to the relationships among these three genera. In 81.5% of these trees, relationships among the three genera matched the analyses of the complete data in which *Bouteloua* was sister to *Distichlis* (i.e., the majority topology). Two minority topologies, in which *Hilaria* was the immediate sister to either *Bouteloua* (12% of trees) or *Distichlis* (6.5%), were also retrieved, although both alternative trees were demonstrated to be significantly less likely by SH tests. GPWG II (2012) retrieved the second of these minority topologies with weak support PP = 0.66 and ML BV = 19%, although this may be related to differences in taxonomic sampling. Both the topological and bootstrap results were observed to depend on which species of *Bouteloua* was included in the analysis. *Bouteloua* (i.e., *Bouteloua gracilis*) was sister to *Hilaria* only in those taxon subsets that excluded *B. curtipendula*, although *B. curtipendula* was also excluded in 20 other subsets in which the majority topology was retrieved (table A4). Note that *B. curtipendula* is on the longest branch in the Cynodonteae subtree (fig. 1). Likewise, *Distichlis* was sister to *Hilaria* only when *B. gracilis* and either *Hilaria cenchroides* or *Hilaria rigida* were excluded, although these conditions also retrieved one or the other alternative topologies in 17 other instances (table A4). Moreover, the node uniting species in the two genera to the exclusion of the third was supported at a ML BV exceeding 96% only when both species of *Bouteloua* were included in the subset. However, inclusion of both species did not guarantee a ML BV above 96%. Beyond this, no particular subsets of Cynodonteae taxa were apparently required to retrieve the majority topology. The overall results suggest that the dominant signal in the plastome supports a sister relationship between *Bouteloua* and *Distichlis*.

There are a number of possible explanations for the results obtained in the taxon-removal analysis. One relates to batch effects, which occur when plastome sequences generated by different library preparation and sequencing methods are combined in the same analysis and bias the results (Leek et al. 2010). However, if there was a significant batch effect in our phylogeny, then we would expect the three plastomes from the selectively amplified genome skim (*B. gracilis*, *D. bajaensis*, *H. rigida*) to form a clade, but that is not the case (fig. 1). When only the single-end samples were included, then the majority topology was recovered. When only the genome skim samples were included, then *Distichlis* was sister to *Hilaria* + *B. gracilis*. Given these results, batch effects seem unlikely to explain our results.

Other explanations relate to branch lengths. Note that in the majority topology, the longest branches in the Cynodonteae subtree are those leading to *B. curtipendula*, the *Hilaria* clade, and *C. barbata*, while the branch uniting *Bouteloua* and *Distichlis* is the shortest branch in the tree (fig. 1). Excluding one species of *Bouteloua* or *Hilaria* has the effect of shifting the balance of branch lengths in the Cynodonteae subtree by failing to break certain long branches. Two examples serve to illustrate this point. First, excluding *B. curtipendula* from the analysis causes the longest branch to become that subtending *B. gracilis*, which then can be attracted to the long branch to *Hilaria*. Second, excluding *B. gracilis* from the analysis increases the length of the already long branch leading to *B. curtipendula*, which is then sometimes attracted to *C. barbata* when it is also in that subset or to Zoysieae/Eragrostideae when it is not. Either way, this leaves *Distichlis* sister to *Hilaria* rather than *B. curtipendula*. In both cases, the removal of one taxon fails to break a long branch not found in the full data set and so sometimes retrieves another topology or reduces the BV of one node.

A third possible explanation of the taxon dependency of the inferred Cynodonteae subtree topology also relates to branch length. The shortest branch in the majority topology, which was the only node in the chloridoid subtree with less than maximum support (ML BV = 98; MP BV = 76), united two species of *Bouteloua* with two species of *Distichlis*. This short branch is consistent with a rapid radiation among these closely related species and is consistent with the pattern of accelerated diversifications in recently diverged sublineages of PACMAD grasses (Spriggs et al. 2014). A short internal branch, with relatively few phylogenetically informative characters to support the node, surrounded by long-branch taxa contributes to the topological instability of this portion of our plastome phylogeny and may partly explain our results for the taxon-removal analyses. Whatever the cause, the suggestion that the minority topologies are artifactual is consistent with the results of our SH tests.

Conclusions

The addition of 13 complete plastome sequences for Chloridoideae demonstrated that species across this subfamily exhibit a mixture of conserved and rare plastome features. Rare genomic changes were observed in *C. glauca* and have unexpected similarities to Arundinoideae. This suggests that further comparative study of the plastomes of *Centropodia* and

Ellisochloa is warranted to investigate the evolutionary significance of these distinctive plastome characters. A complementary nuclear phylogenomic study would clarify the phylogenetic position of *Centropodia* + *Ellisochloa*, but in this study the plastid signal strongly positions *C. glauca* as sister to sampled Chloridoideae. The two sampled *Distichlis* species were found to have large insertions in *rpoA* and the *rpl16* intron, and additional sampling in the genus (11 spp. total) may elucidate whether these changes are independent or derived from a single mutation. Taxon-removal analyses have been key in testing other phylogenies (Kelchner and Bamboo Phylogeny Group 2012; Parks et al. 2012; Wickett et al. 2014). Our taxon-removal analyses among Cynodonteae emphasize the synergistic effects of the included long-branch taxa. Note that ML analyses of taxon subsets missing only one or two Cynodonteae sometimes produced a minority topology with a significantly decreased likelihood score. On the other hand, analyses of some of those subsets in which fully half of the Cynodonteae were excluded retrieved the majority topology (table A4). This issue is clearly not simply one of the total number of included taxa. Sampling in clades that may have experienced a rapid radiation, producing a pattern of short internal and long terminal branches in phylogenetic trees, requires particular care. Our analyses of complete plastomes for 14 chloridoid species provides a well-supported set of relationships that can be tested with further sampling of complete plastomes from other genera and congeneric species, especially within the Cynodonteae tribe. This work will likely yield additional insights into the molecular evolution and phylogenomics of Chloridoideae.

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