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Chloroplast DNA inversions and the origin of the grass family (Poaceae)

JEFF J. DOYLE*, JERROLD I. DAVIS, ROBERT J. SORENG, DAVID GARVIN, AND MARNI J. ANDERSON

L. H. Bailey Hortorium, Cornell University, Ithaca, NY 14853

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ABSTRACT **The phylogenetic affinities of the grass family (Poaceae) have long been debated. The chloroplast genomes of at least some grasses have been known to possess three inversions relative to the typical gene arrangement found in most flowering plants. We have surveyed for the presence of these inversions in grasses and other monocots by polymerase chain reaction amplification with primers constructed from sequences flanking the inversion end points. Amplification phenotypes diagnostic for the largest inversion (28 kilobase pairs) were found in genera representing all grass subfamilies, and in the nongrass families Restionaceae, Ecdeiocoleaceae, and Joinvilleaceae, but not in any other monocots—notably, Flagellariaceae, Anarthriaceae, Cyperaceae, or Juncaceae. This finding is consistent with one of the two principal views of grass phylogeny in suggesting that Poaceae and Cyperaceae (sedges) are not closest relatives. A second (≈6 kilobases) inversion appears to occur in a subset of the families possessing the 28-kilobase inversion and links Joinvilleaceae and Poaceae, while the smallest inversion appears unique to grasses. These inversions thus provide a nested set of phylogenetic characters, indicating a hierarchy of relationships in the grasses and allies, with Joinvilleaceae identified as the likely sister group to the Poaceae.**

The origin and phylogenetic affinities of the economically important grass family (Poaceae) have long been controversial, in part because the family is unique morphologically and anatomically. This controversy has been reflected in the diversity of taxonomic treatments suggested for the family and other putatively related groups. For example, the familiar system of Cronquist (1), following one traditional view (2), places the grasses in an order with the sedges (Cyperaceae), another grass-like group of monocots with minute, predominantly wind-pollinated flowers. An alternative view, also with a long history (3, 4), is that grasses are most closely related to other families, particularly those of a predominantly Southern Hemisphere group that includes Restionaceae, Flagellariaceae, and relatives. The latter view is supported by the recent analyses of Dahlgren *et al.* (5). However, relationships among the several families within this group have not been resolved by morphological and anatomical data (5–7).

The ≈134-kilobase (kb) chloroplast genome of grasses possesses three inversions relative to the chloroplast DNA gene arrangement found in most other flowering plants, including the small number of nongrass monocots sampled to date (8–12). The relative timing of two of the inversions is known: the ≈6-kb inversion spans one end point of the 28-kb inversion and thus must have originated at a later time (11); the relative time of origin of the smallest inversion is unknown, as it lies wholly within the 28-kb inversion and outside the 6-kb inversion (see Fig. 1). Here we report the occurrence of two of the inversions in families other than grasses and discuss the molecular and phylogenetic implications of their taxonomic distribution.

MATERIALS AND METHODS

DNA was isolated from small amounts of either fresh leaf tissue or dried herbarium specimens by the method of Doyle and Doyle (13). Representatives of eight of the nine monocot superorders of Dahlgren *et al.* (5) (all but Triuridiflorae) were surveyed (Table 1). PCR (14) amplification primers were designed against evolutionarily conserved sequences (mostly tRNA genes) flanking the end points of regions involved in each of the three grass inversions, using information from the completely sequenced chloroplast genomes of *Oryza sativa* (rice) (11), *Nicotiana tabacum* (tobacco) (15), and the liverwort *Marchantia polymorpha* (16) (Fig. 1; Table 2).

Double-stranded PCR amplifications were carried out with a Hybrid thermal cycler (National Labnet, Woodbridge, NJ); standard conditions for reactions were 35 cycles (using the tube control option) of 94°C for 15 sec (denaturation), 55°C–62°C for 1 min (primer annealing), and 72°C for 2 min (extension). One hundred microliter reaction mixtures used manufacturer-supplied (Promega) 10× reaction buffer (containing 15 mM $MgCl_2$) and included deoxynucleotide triphosphates (1.25 mM each), primers (0.5 μM each), 2.5 units of *Taq* polymerase (Promega), and 0.1–1.0 μg of template DNA. Products were electrophoretically separated in 1.5% Tris borate/EDTA agarose gels and visualized by staining with ethidium bromide under ultraviolet light. Reactions with no DNA added to the mixture were run as controls.

Both double-stranded amplification products using the *trnR–rps14* primer pair, and single-stranded secondary amplification products of that product using only the *trnR* primer, were sequenced with the Pharmacia T7 sequencing kit with modifications (17). The *rps14* amplification primer was used as the sequencing primer.

RESULTS

No results are reported here for any taxon unless its DNA was capable of being amplified, as indicated by a "universal" primer set (*trnfM* + *rps14*; see below). Several taxa, notably some from which only herbarium material was available, did not meet this criterion. For taxa with "amplifiable" DNA, an inversion was considered present if (*i*) a strong amplification product was observed with a primer pair specific for at least one of the two end points of that inversion, and (*ii*) no product was observed with primers diagnostic for the uninverted condition at that same end.

Inversion 1 (28 kb). The presence/absence of this inversion was surveyed primarily at the *rps14* end of the inversion, by amplification experiments using *rps14* with either *trnR* or *trnG*-GCC (Fig. 1; Table 2). The primer pair *trnR* + *trnfM*-r was used as further confirmation of the inversion in some taxa. It proved difficult to obtain unambiguous results for the

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*To whom reprint requests should be addressed.

Table 1. Distribution of inversions in monocots

Taxon	Inversion 1	Inversion 2	Inversion 3
Liliiflorae			
Liliales: Orchidaceae			
Govenia capitata	1, [2]	6	10, [11]
Ariflorae			
Arales: Araceae			
Spathicarpa sagittifolia	1, [2]	6, [7]	10, [11]
Arales: Lemnaceae			
Lemna minor	1		
Alismatiflorae			
Alismatales: Alismataceae			
Sagittaria sp.	1, [2]		10, [11]
Bromeliiflorae			
Bromeliales: Bromeliaceae			
Catopsis sp.	1, [2]		
Typhales: Typhaceae			
Typha latifolia	1, [2]		10, [11]
Zingiberiflorae			
Zingiberales: Musaceae			
Musa sp.	1, [2]		10, [11]
Zingiberales: Zingiberaceae			
Hedychium coronarium	1, [2]		
Commeliniflorae			
Commelinales			
Commelinaceae			
Commelinantia sp.	1, [2]		10, [11]
Hadrodemas warszewiczianum	1, [2]	6, [7]	10, [11]
Zebrina sp.	1, [2]	6, [7]	10, [11]
Xyridaceae			
Xyris ambigua	3, [4, 5]	6, [7]	10, [11]
Eriocaulaceae			
Eriocaulon sp.	1, [2]	6, [7]	10, [11]
Cyperales			
Juncaceae			
Juncus balticus	3, [4, 5]	8, [9]	10, [11]
Cyperaceae			
Carex debilis	3, [4, 5]	8, [9]	10, [11]
Cyperus papyrus	3, [4, 5]	6, [7]	
Poales			
Anarthriaceae			
Anarthria scabra	3, [4, 5]	8, [9]	10, [11]
Ecdeiocoleaceae			
Ecdeiocolea monostachya	**2, [1] 4, 5, [3]**		10, [11]
Flagellariaceae			
Flagellaria indica	1, [2]	6, [7]	10, [11]
Joinvilleaceae			
Joinvillea ascendens	**2, [1] 4, 5, [3]**	**7, [6]**	10, [11]
Restionaceae			
Chondropetalum andreaeanum	**2, [1]**	6, [7]	10, [11]
Rhodocoma giganteus	**2, [1]**	6, [7]	
Poaceae			
Bambusoideae			
Bambusa vulgaris	**2**		**11, [10]**
Brachyelytrum erectum	**2, [1]**		
Lithachne humilis	**2, [1] 4, 5, [3]**	**7, [6]**	**11, [10]**
Pharus latifolius	**2, [1]**		**11, [10]**
Streptochaeta angustifolia	**2**		**11, [10]**
Pooideae			
Poa pratensis	**2, [1]**		
Centothecoideae			
Chasmanthium latifolium	**2, [1]**	**7, [6]**	**11, [10]**
Arundinoideae			
Arundo donax	**2**	**7, [6]**	**11, [10]**
Chionochloa rigida	**2**		**11, [10]**
Cortaderia selloana	**2, [1]**	**7, [6]**	**11, [10]**
Molinia litoralis	**2, [1]**		
Thysanolaena maxima	**2, [1]**	**7, [6]**	**11, [10]**
Phragmites communis	**2**		**11, [10]**
Chloridoideae			
Eragrostis sp.	**2**	**7**	**11**
Panicoideae			
Oplismenus sp.	**2, [1]**	**7, [6]**	**11, [10]**
Cyclanthiflorae			
Cyclanthales: Cyclanthaceae	1, [2]		
Carludovica drudei	1, [2]	6, [7]	10, [11]
Areciflorae			
Arecales: Arecaceae			
Bismarckia nobilis	1, [2]	6	10
Pandaniflorae			
Pandanales: Pandanaceae			
Pandanus veitchii	1, [2]	6, [7]	10, [11]

The presence of an inversion is denoted by boldface type for an experimental result. Negative results are shown in brackets and are only given when confirming a positive result. Experimental results are primer pairs (see Table 2 and Fig. 1) numbered as follows: 1, *trnG*-GCC + *rps14*; 2, *trnR* + *rps14*; 3, *trnR* + *trnG*-UCC 3′ exon; 4, *trnfM* + *trnG*-UCC 3′ exon; 5, *trnR* + *trnfM*-r; 6, *trnS* + *trnG*-UCC 5′ exon; 7, *trnS* + *psbD*; 8, *trnT* + *psbD*; 9, *trnT* + *trnG*-UCC 5′ exon; 10, *trnE* + *trnT*-r; 11, *trnE* + *trnT*-r.
†Voucher information is available on request.

trnG-UCC end of the 28-kb inversion. Despite increasing stringency by raising annealing temperatures, faint amplification products often appeared in control reactions (e.g., faint products in grasses using the primer pair *trnG*-UCC 3′ exon + *trnR*, or in dicot controls using *trnG*-UCC 3′ exon + *trnfM*). Results of experiments at this end are therefore reported only for taxa in which no amplification was observed with either pair of primers at the *rps14* end of the inversion and are considered weaker evidence than results involving *rps14*.

PCR phenotypes indicative of the presence of the 28-kb inversion were not observed outside the Commeliniflorae [sensu Dahlgren *et al.* (5)], the superorder to which grasses belong (Table 1). All grasses sampled possessed amplification phenotypes consistent with the presence of the inversion, including representatives of all six subfamilies (18) and multiple genera of Arundinoideae and Bambusoideae, the two subfamilies considered by various authors to be particularly heterogeneous or basal within the family (19, 20) (Table 1). PCR amplification phenotypes consistent with the presence of the 28-kb inversion also were found in representatives of three nongrass families of the superorder Commeliniflorae, order Poales: the monogeneric families Joinvilleaceae and Ecdeiocoleaceae and two genera of the large family Restionaceae (Table 1; Fig. 2*A*). For the first two of these three families, conclusive data were obtained from both ends of the inversion, while for two Restionaceae only the *rps14* end of the inversion gave consistent results.

Three other nongrass members of the order Poales also were surveyed (Table 1). *Flagellaria indica* (Flagellariaceae) possessed a PCR phenotype with *rps14* indicating the absence of the 28-kb inversion. Amplification products were observed for *Anarthria scabra* (Anarthriaceae) using *rps14* + *trnfM* (the universal set), thus indicating the ability of its

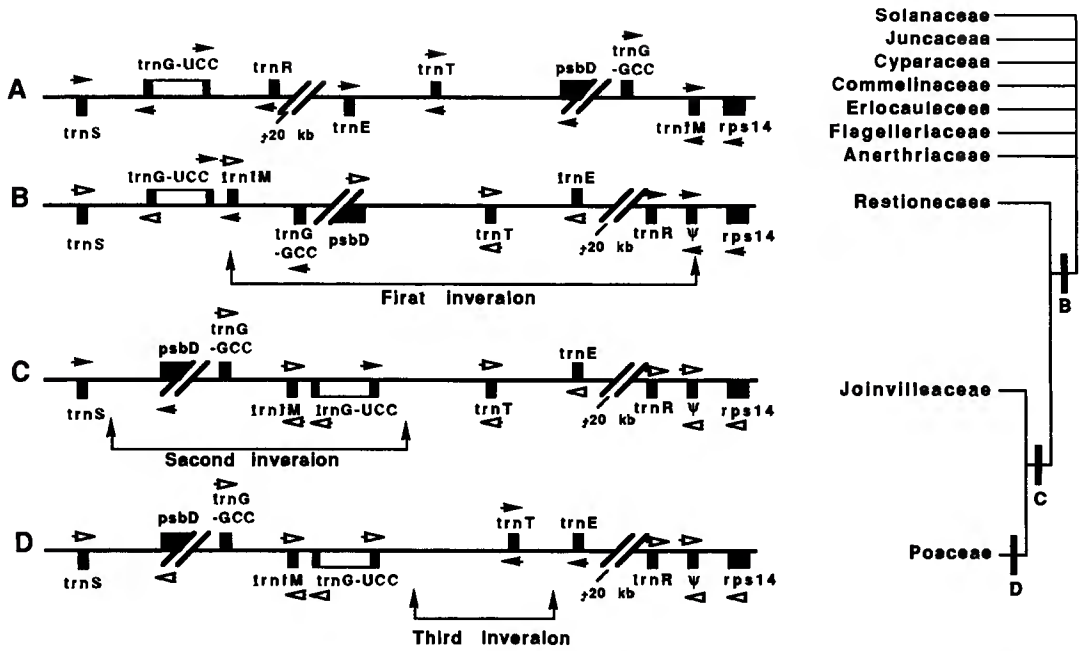

FIG. 1. Chloroplast DNA inversions in grasses and allies. (*A–D*) Linearized representations (not to scale) of portions of circular chloroplast genomes, showing locations of genes used in the construction of inversion-diagnostic PCR primers (see Table 2). Genes above the line are transcribed from left to right. (*A*) Gene order in tobacco (15), which lacks all three inversions, inferred for several monocot families reported here. Position and orientation of solid arrows indicate direction and strand of each primer used in this study. (*B*) Inferred gene order of chloroplast genomes having only the first (28 kb) inversion. Primers used for diagnosis of the first inversion and the presence of the chimeric pseudogene (ψ) are shown as solid horizontal arrows. (*C*) Inferred gene order of chloroplast genomes having both the first and second inversion. The extent of the second (≈6 kb) inversion is indicated, with diagnostic primers as in *B*. (*D*) Gene order of chloroplast genomes such as that of rice (11), possessing all three inversions. Listed on the right of each of these gene maps are plant families having these various conditions. The phylogenetic relationships among these families are represented as a cladogram, with the presence of the inversions (listed as *B*, *C*, and *D*) shown as derived characters. The uninverted condition is present in many plants (12), and only the dicotyledonous family Solanaceae (which includes tobacco) and a few monocotyledonous families are listed. The grouping Restionaceae/Joinvilleaceae/Poaceae is thus supported by the first inversion in chloroplast genomes of these taxa (*B*); Joinvilleaceae/Poaceae is supported by the second inversion (*C*) and the third inversion (*D*) is unique to grasses and serves as a character uniting the many members of that family.

DNA to be amplified. However, no results were obtained when *rps14* was used with either of the primers diagnostic for the *rps14* end point of the inversion. *A. scabra* produced strong amplifications with *trnG*-UCC 3′ exon + *trnR* and no priming above control levels with either *trnG*-UCC 3′ + *trnfM* or *trnR* + *trnfM*-r, suggesting the absence of the 28-kb inversion. Samples of species from several genera of Centrolepidaceae (*Brizula*, *Centrolepis*, and *Gaimardia*) did not prime with any primers, including the universal set, and in fact inhibited control amplifications.

No evidence of the 28-kb inversion was observed in two other orders of Commeliniflorae (Commelinales and Cyperales), and no representatives of the remaining order (Hydatellales) were available. Strong amplifications with *rps14* + *trnG*-GCC were obtained from two families of Commelinales (Commelinaceae and Eriocaulaceae). Samples from a third family of this order, Xyridaceae, produced results similar to those observed for *Anarthria*, in which no amplification was observed with primers from the *rps14* end point, but results consistent with the absence of the inversion were obtained by using primers from the *trnG*-UCC end. This same composite phenotype also was obtained for species of the two families of Cyperales surveyed (Juncaceae and Cyperaceae).

Evidence that a chimeric pseudogene associated with the 28-kb inversion in rice (11) is also present in all taxa having this inversion was obtained by using the primer combination

Table 2. Primer sequences used in screening for inversions

Primer sequence	Gene	Coordinates
5′-TGACCTCAAGGTTATGAGCC-3′	*trnfM*	12,863–12,882
	trnfM/G	36,098–36,118
5′-GGCTCATAACCTTGAGGTCA-3′	*trnfM*-r	12,882–12,863
	trnfM/G	36,098–36,118
5′-CTATCCGGACACATACTTCG-3′	*rps14*	36,380–36,361
5′-GTCCTATCCATTAGACAATGG-3′	*trnR*	35,914–35,933
5′-TTGCCAAGGAGAAGAT(A/G)CG-3′	*trnG*-GCC	12,361–12,379
5′-TACCAAGGGCTATAGTCAT-3′	*psbD*	8,918–8,900
5′-TACAACGGATTAGCAATCC-3′	*trnS*	7,869–7,887
5′-ATACCACTAAACTATACCC-3′	*trnG*-UCC 5′	13,732–13,750
5′-CAAGCTAACGATGCGGGTTC-3′	*trnG*-UCC 3′	13,039–13,020
5′-GCCCTTTTAACTCAGTGGTA-3′	*trnT*	15,060–15,079
5′-TACCACTGAGTTAAAAGGGC-3′	*trnT*-r	15,079–15,060
5′-GCCTCCTTGAAAGAGAGATG-3′	*trnE*	15,694–15,675

Coordinates are those of the rice chloroplast genome (11).

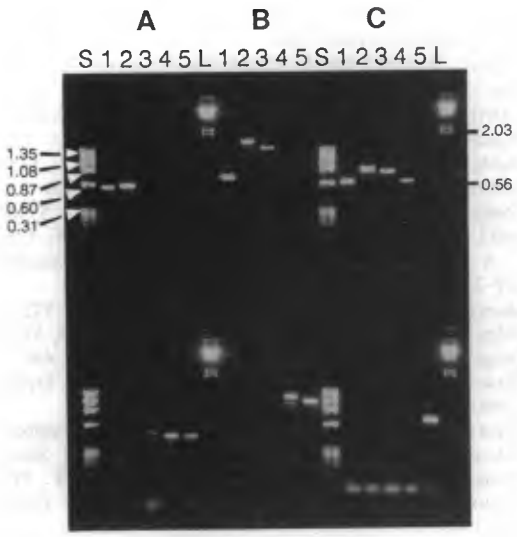

FIG. 2. Representative PCR amplifications with primers diagnostic for the three grass chloroplast DNA inversions. A series of five samples is shown for each set of diagnostic primers, separated by size standards (S, ϕX174 bacteriophage digested with *Hae* III; L, λ bacteriophage digested with *Hin*dIII; sizes of some fragments, in kb, are shown on the left or right of the gel). Lanes: 1, *Solanum albidum*, a relative of tobacco, which represents the uninverted condition (15); 2, *F. indica* (Flagellariaceae); 3, *C. andreaeanum* (Restionaceae); 4, *J. ascendens* (Joinvilleaceae); 5, *L. humilis*, a grass related to rice, which possesses all three inversions (11). (*A*) Experiments testing for the absence (*Upper*; primers *rps14* + *trnG*) or presence (*Lower*; primers *rps14* + *trnR*) of the 28-kb inversion. (*B*) Test for the absence (*Upper*; primers *trnS* + *trnG*-UCC 5′ exon) or presence (*Lower*; primers *trnS* + *psbD*) of the second (≈6 kb) inversion. (*C*) Test for the absence (*Upper*; primers *trnE* + *trnT*-r) or presence (*Lower*; primers *trnE* + *trnT*) of the third (smallest) inversion. Interpretation of results is shown in Fig. 1.

trnfM + *rps14*. This combination produced an ≈300-base-pair product in all taxa, including those with the 28-kb inversion, where no product should be amplified unless the pseudogene is present (Fig. 1). To confirm the presence of the pseudogene in nongrass taxa possessing the 28-kb inversion PCR phenotype, the *trnR* + *rps14* amplification products of *Chondropetalum andreaeanum* (Restionaceae) and *Joinvillea ascendens* (Joinvilleaceae) were sequenced through the region predicted to contain pseudo-*trnfM/G*; comparison of these sequences with grass *trnfM* pseudogene sequences (11, 21, 22) (Fig. 3) revealed considerable nucleotide similarity, consistent with amplification results.

```
ory ATGCATATATCATAAAGAAGGAATATGGAGCGGGTAGTGGGAATCGAACCC
tri .....................G........C..........G.......
zea ..A..............................A......CA.T.........
joi ..............----.........C........G...............
cho ..A.....................................A..T.........

ory GCAACCCCACGGTTATGAGCCTTGTCAGCTACCAAACTGTTCTA-TCCTG-
tri .........A.............A.G.............C....--TACTC
zea .T.......A..........T....G.............C..C.--...TC
joi .T.......A....C..........G.............C....C...ACC
cho .T.......A-.....A...T....G...........A.C....--...TC

ory TTAAACTAAAGAGAGGGGAACTAGTGGATAAAAGGGGGGTTGAATACGCCC
tri .............-...T................A-......G........
zea ...GC---------...A.......----------.......G...G....
joi ............ACT........A.........AA-A.....G........
cho ............C..A..................-......G........
```

FIG. 3. Nucleotide sequences of a portion of the *trnR* + *rps14* PCR amplification product in the nongrasses *J. ascendens* (joi; Joinvilleaceae) and *C. andreaeanum* (cho; Restionaceae), compared with chimeric pseudogene (*trnfM/G*) sequences of the grasses *O. sativa* (ory) (11), *Triticum aestivum* (tri) (21), and *Zea mays* (zea) (22). The chimeric *trnfM/G* pseudogene is indicated in boldface type. Dots indicate shared nucleotides; gaps, indicated by dashes, have been inserted by inspection to maximize similarity.

Inversion 2 (≈6 kb). Screening for this inversion primarily used the primer sets *trnS* + either *psbD* (presence) or *trnG*-UCC 5′ exon (absence), since the *trnT* end of the inversion is complicated by the third inversion (Fig. 1). Amplification phenotypes predicted for chloroplast genomes possessing the second inversion were found only in a subset of the groups that possessed the 28-kb inversion (Fig. 2*B*), consistent with the inversion chronology suggested by the observation that this inversion includes the *trnG*-UCC end point of the 28-kb inversion (11) (Fig. 1). All grasses surveyed showed evidence of this inversion, as did *J. ascendens* (Joinvilleaceae). In contrast, DNAs of Restionaceae genera amplified only with the noninversion primer pair (*trnS* + *trnG*-UCC 5′ exon). *Ecdeiocolea monostachya* did not amplify with any of the four primer sets diagnostic for the presence or absence of the second inversion, and thus its condition remains unclear.

Inversion 3 (*trnT*). Evidence for the presence of the smallest of the three inversions (amplification with *trnE* + *trnT*) was found only in grasses, where it occurred in all genera surveyed (Table 1). *J. ascendens*, along with *E. monostachya*, genera of Restionaceae, and all other plants studied, amplified only with the noninversion primer pair (*trnE* + *trnT*-r; Fig. 2*C*).

DISCUSSION

Inversions and other chloroplast genome rearrangements are relatively uncommon among land plants (12). The rarity of such mutations facilitates homology determination and has made them useful phylogenetic markers in several plant groups (12). This same rarity, however, limits the utility of each rearrangement to providing only a broad picture of relationships. The presence of three chloroplast DNA inversions in grasses thus affords a powerful set of tools for elucidating phylogenetic relationships of grasses and their allies.

Our results indicate that these grass chloroplast DNA inversions are limited to superorder Commeliniflorae of Dahlgren *et al.* (5), a finding consistent with the limited number of previous studies of nongrass monocot chloroplast genomes (12). All three inversions previously have been reported to occur in a handful of grasses from subfamilies Pooideae, Panicoideae, and Bambusoideae (8–12); our results confirm their presence throughout the family.

The 28-kb inversion unites grasses with the monogeneric families Joinvilleaceae and Ecdeiocoleaceae and with at least some members of the relatively large family Restionaceae. These families are thus separated from other Commeliniflorae, none of which gives evidence of having the inversion. The ≈6-kb inversion links grasses with Joinvilleaceae, separating these two families from Restionaceae; unfortunately, the position of *Ecdeiocolea* could not be resolved in our experiments. The *trnT* inversion appears to be confined to grasses and thus is another of the many unique features that unite that distinctive family (6).

This distribution of the 28-kb inversion supports the view that grasses are most closely related to a predominantly Southern Hemisphere group comprising the relatively large family Restionaceae and several smaller families. This is consistent with the cladistic studies of Dahlgren *et al.* (5), whose results identify a monophyletic group (classified as order Poales) that includes Poaceae plus Centrolepidaceae, Restionaceae (including its segregates Ecdeiocoleaceae and Anarthriaceae), Joinvilleaceae, and Flagellariaceae; Cyperaceae and Juncaceae form part of a second clade that is sister to this group. The absence of this inversion from Flagellariaceae is noteworthy. Dahlgren *et al.* (5), although recognizing Flagellariaceae and Joinvilleaceae as separate families, present cladograms with these families as sister taxa, a close

relationship consistent with older taxonomic treatments of *Flagellaria* and *Joinvillea* as a single family, Flagellariaceae. However, chloroplast genomes of these genera differ by the two inversions shared between *Joinvillea* and Poaceae, suggesting that Flagellariaceae plus Joinvilleaceae is not a monophyletic group.

Of the taxa studied here, *Joinvillea* has a chloroplast genome that appears most closely related to those of grasses. This result is not unexpected, as a close affinity between these taxa—even a sister-group relationship—has been suggested previously (e.g., ref. 23). Of the several trees identified by the three recent cladistic analyses of the grasses and their allies (5–7), one of those found by Campbell and Kellogg (6) is consistent with our data (Fig. 1) in placing the Joinvilleaceae as sister group to the Poaceae (here indicated by the shared presence of the 6-kb inversion) and including these families in a larger clade (supported here by the presence of the 28-kb inversion) that includes Restionaceae and Ecdeiocoleaceae, but not Flagellariaceae. However, our results are inconsistent with this morphological analysis in excluding Anarthriaceae from this clade. Complete resolution of phylogenetic relationships among these taxa must await further sampling, particularly of the taxonomically complex Restionaceae (24–26).

These results affirm the phylogenetic affinity of grasses with a group of families having generalized eastern Gondwanan geographic distributions (i.e., principally Australasia to India and Africa) and possibly representing an Upper Cretaceous diversification (27). This phylogenetic structure is consistent with independent origins of floral and inflorescence characters related to wind pollination in the grass and sedge (Cyperaceae) families (3–5).

The 28-kb inversion was thought to have been produced either by repeat-mediated intramolecular recombination (21) or by intermolecular recombination involving tRNA genes (11); according to the latter hypothesis, the chimeric *trnfM/G* pseudogene was produced as a result of the recombination event. Our results suggest that the pseudogene is present in all chloroplast genomes having the 28-kb inversion, but whether both were produced simultaneously remains unclear. As Palmer (28) has pointed out, an understanding of the mechanisms likely to have given rise to inversions will be facilitated by study of the unrearranged chloroplast genomes most closely related to those of taxa having rearrangements. Establishing a phylogenetic hypothesis for grasses and their allies makes that approach feasible.

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