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A MOLECULAR PHYLOGENY OF THE GRASS SUBFAMILY PANICOIDEAE (POACEAE) SHOWS MULTIPLE ORIGINS OF C_4 PHOTOSYNTHESIS[1]

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DNA sequence data from the chloroplast gene *ndhF* were analyzed to estimate the phylogeny of the subfamily Panicoideae, with emphasis on the tribe Paniceae. Our data suggest that the subfamily is divided into three strongly supported clades, corresponding to groups with largely identical base chromosome numbers. Relationships among the three clades are unclear. In unweighted parsimony analyses, the two major clades with $x = 10$ (Andropogoneae and $x = 10$ Paniceae) are weakly supported as sister taxa. The third large clade corresponds to $x = 9$ Paniceae. In analyses under implied weight, the two clades of Paniceae are sisters, making the tribe monophyletic. Neither resolution is strongly supported.

Our molecular phylogenies are not congruent with previous classifications of tribes or subtribes. Based on this sample of species, we infer that C_4 photosynthesis has evolved independently several times, although a single origin with multiple reversals and several reacquisitions is only slightly less parsimonious. The phosphoenol pyruvate carboxykinase (PCK) subtype of C_4 photosynthesis has evolved only once, as has the NAD-malic enzyme (ME) subtype; all other origins are NADP-ME. Inflorescence bristles are apparently homologous in the genera *Setaria* and *Pennisetum*, contrary to opinions of most previous authors. Some genera, such as *Digitaria*, *Echinochloa*, and *Homolepis* are supported as monophyletic. The large genus *Paspalum* is shown to be paraphyletic, with *Thrasya* derived from within it. As expected, *Panicum* is polyphyletic, with lineages derived from multiple ancestors across the tree. *Panicum* subg. *Panicum* is monophyletic. *Panicum* subg. *Dichanthelium*, subg. *Agrostoides*, and subg. *Phanopyrum* are unrelated to each other, and none is monophyletic. Only *Panicum* subg. *Dichanthelium* sect. *Dichanthelium*, represented by *P. sabulorum* and *P. koolauense*, is monophyletic. *Panicum* subg. *Megathyrsus*, a monotypic subgenus including only the species *P. maximum*, is better placed in *Urochloa*, as suggested by other authors.

Key words: C_4 photosynthesis; *ndhF*; Panicoideae; Poaceae.

The grass subfamily Panicoideae includes ~3300 species in 206 genera (following the Grass Phylogeny Working Group [GPWG], 2001) and is larger than most angiosperm families. The subfamily is distributed on all continents except Antarctica, and its members are dominant in tropical and warm temperate regions. In addition, it includes some of the world's most important crop plants, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugar cane (*Saccharum officinarum*), common millet (*Panicum miliaceum*), pearl millet (*Pennisetum glaucum*), foxtail millet (*Setaria italica*), and Shama millet (*Echinochloa colona*).

Panicoideae form a monophyletic group, based on their paired florets, the lower of which is staminate or sterile (Brown, 1810, 1814), and on distinctive simple starch grains (Tateoka, 1962; Kellogg and Campbell, 1987; GPWG, 2001). Staminate flower development is apparently uniform throughout the subfamily and unique among the grasses. In staminate flowers, gynoecial development ceases after a clear ridge forms around the nucellus (LeRoux and Kellogg, 1999). This correlates with loss of cytoplasm and nuclei in a small set of subepidermal cells, apparently the result of controlled cell death. This pattern matches that found in maize and *Tripsacum*, both panicoid grasses, in which the gynoecial cell death is known to be under the control of the product of the gene *Tasselseed2* (*TS2*)(DeLong, Calderón-Urrea, and Dellaporta, 1993). The cell death pathway is not active in *Zizania aquatica*, an oryzoid grass that has independently evolved male flowers (Zaitchik, LeRoux, and Kellogg, 2000). Thus, *TS2*-induced cell death is apparently restricted to Panicoideae and may be the genetic basis of the subfamilial synapomorphy.

The monophyly of the subfamily (sensu GPWG, 2001) has been supported by every molecular phylogenetic study to date, including phylogenies of both chloroplast and nuclear genes (reviewed in Kellogg, 1998; Soreng and Davis, 1998; support was relatively weak in Cummings, King, and Kellogg, 1994). In addition, maps of the nuclear genomes of maize, sugar cane, sorghum, pearl millet, and foxtail millet show that they share a common genome arrangement, with linkage group 10 (corresponding to rice chromosome 10) inserted into group 3 and with linkage group 9 inserted into group 7 (Gale and Devos, 1998; Kellogg, 1998; Devos and Gale, 2000). Recent molecular data place the Panicoideae as sister to *Gynerium* (Arundinoideae), with the panicoid/*Gynerium* clade sister to a clade consisting of *Chasmanthium*, *Zeugites* (both Centothecoideae),

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and *Thysanolaena* (Arundinoideae) (GPWG, 2000, 2001). The genera *Eriachne* (tribe Eriachneae, now *incertae sedis*) and *Danthoniopsis* (formerly Arundinelleae, now Centothecoideae) are formally excluded from the subfamily (GPWG, 2001; S. Aliscioni, Darwinion Institute, unpublished data).

Bentham (1881) divided the Panicoideae into six tribes, although more divisions were proposed during the 20th century with the addition of new anatomical and cytological characters (Pilger, 1954; Hsu, 1965; Butzin, 1970). At least seven tribes are commonly recognized, of which by far the largest are Paniceae (101 genera; cf. Clayton and Renvoize, 1986) and Andropogoneae (85 genera). Other tribes include Arundinelleae (12 genera), Hubbardieae (1), Neurachneae (3), Isachneae (5), and Steyermarkochloeae (1); the last tribe is placed in Arundinoideae by Watson and Dallwitz (1992).

The tribe Paniceae, with almost half the genera and 60% of the species of panicoid grasses, is the central phylogenetic problem of the subfamily. There is no evidence that the tribe is monophyletic. It is morphologically diverse and lacks an obvious unifying morphological character. The lemmas are generally, but not always, indurated, and the glumes are often membranous. The inflorescences are commonly described as simple or compound, paniculate or racemose (Clayton and Renvoize, 1986), although preliminary developmental data suggest that these standard descriptors are inaccurate and insufficient to describe the morphological diversity indicated by comparison of adult morphologies (L. G. LeRoux, A. N. Doust, and E. A. Kellogg, unpublished data). Chromosome number may be based on 9 or 10 or more rarely on 8 (Clayton and Renvoize, 1986).

Over the years, the systematics of the Paniceae has involved several criteria. For instance, Brown (1977) divided the Paniceae into four subtribes based on the different photosynthetic pathways among species (see below) and suggested that species with Kranz anatomy have evolved from non-Kranz taxa. Likewise, with the use of morphological features, Clayton and Renvoize (1986) distinguished seven subtribes primarily differentiated by spikelet characters, such as presence of bristles and texture of the upper lemma.

Panicum, as circumscribed by Zuloaga (1987), is the largest genus in the Paniceae; it is highly variable and almost certainly polyphyletic. Zuloaga, Morrone, and Giussani (2000) subdivided *Panicum* into groups with apparent synapomorphies and used these groups as terminal taxa in a morphological phylogenetic analysis of the tribe Paniceae; they concluded that *Panicum* is polyphyletic. In fact, the diversity of this genus encompasses almost all the variation in the tribe. Were it to be treated as a single terminal taxon for a morphological phylogenetic analysis, virtually all phylogenetically informative characters would be polymorphic. Clayton and Renvoize (1986) depicted *Panicum* as a large amorphous blob, out of which most other genera in the tribe have arisen. It appears that for any phylogenetic study, the genus must be divided into putatively monophyletic subgenera or sections.

Based on available molecular sequence data, Andropogoneae, the other large tribe within subfamily Panicoideae, forms a monophyletic assemblage (Mason-Gamer, Weil, and Kellogg, 1998; Spangler et al., 1999; S. Mathews and E. A. Kellogg, unpublished data). However, its position within the subfamily is not resolved. These same investigations showed that the tribe Arundinelleae is polyphyletic (Spangler et al., 1999; Kellogg, 2000).

Diversification of photosynthesis is evident across the grass family, in which C_3, C_4, and C_3/C_4 intermediates occur. The C_4 species differ anatomically and biochemically, but some combinations of anatomy and biochemistry recur frequently. This led Hattersley and Watson (1992) to describe ten different structural-biochemical types within grasses, with the three most common combinations of characters being ''classical NADP-ME,'' ''classical NAD-ME,'' and ''classical PCK'' types. Although many panicoid species use the conventional C_3 photosynthetic pathway, a large number exhibit the C_4 photosynthetic pathway. The tribe Andropogoneae is entirely C_4 ''classical NADP-ME,'' whereas Paniceae includes eight different C_4 types, as well as intermediate C_3/C_4 species (Hattersley and Watson, 1992).

In C_4 plants, enzymes associated with the C_3 pathway are produced only in the bundle sheath, whereas enzymes such as phosophoenol pyruvate carboxylase (PEPC) are strongly upregulated in mesophyll cells (Kanai and Edwards, 1999; Leegood and Walker, 1999); all are produced by nuclear-encoded genes. PEPC catalyzes the production of a four-carbon compound, oxaloacetate, from phosphoenol pyruvate (PEP) plus carbon dioxide (in the form of bicarbonate). The four carbon compound is then modified to malate or aspartate and shunted to the bundle sheath, where the CO_2 is removed. The CO_2 is then supplied directly to Rubisco, which accumulates only in the bundle sheath. The remaining three-carbon compound is transported back to the mesophyll, where it is phosphorylated to regenerate PEP. All C_4 plants are alike in replacing Rubisco with PEPC in the mesophyll. They also have closely spaced leaf veins, with every bundle sheath cell in contact with a mesophyll cell. This is clearly necessary for the continual shuttling of substrates between the two different cell types. Species that translocate malate and decarboxylate with malic enzyme using NADP as a cofactor (NADP-ME) have only a single sheath around the vascular bundles. The single sheath is of procambial origin, has a suberized outer wall, and is positionally and structurally similar to the mestome sheath of C_3 grasses. This combination of anatomy and biochemistry has been called ''classical NADP-ME'' by Hattersley and Watson (1992), in their typology of C_4 variation. Conversely, species that translocate aspartate decarboxylate with either a malic enzyme or PEP carboxykinase (PCK) using NAD as a co-factor (NAD-ME) have a double bundle sheath, the inner part of which is a conventional mestome sheath, while the outer part is parenchymatous and thin walled. Rubisco is expressed in the outer sheath. In NAD-ME species, the outer walls of the bundle sheath cells generally form a regular outline, whereas in PCK species, the outer sheath cells are much less regular in size and shape. These constitute the ''classical NAD-ME'' and ''classical PCK'' types of Hattersley and Watson (1992). In a few members of Panicoideae, intermediate veins are reduced to lines of isolated bundle sheath cells, called ''distinctive cells'' by mystified earlier researchers. These were thought to characterize the Arundinelleae and were interpreted by some as intermediates on the way to full C_4 anatomy (''Arundinelleae'' type sensu Hattersley and Watson, 1992). It is possible, however, that distinctive cells are actually a loss of intermediate veins, rather than a gain.

Diversity of photosynthetic pathways is as great within the genus *Panicum* (sensu Zuloaga, 1987) as within the entire tribe. Some subgenera are photosynthetically homogeneous. For example, both subgen. *Dichanthelium* and subgen. *Phanopyrum* are entirely C_3. *Panicum* subgen. *Panicum* is, anatomically and biochemically, a C_4 ''classical NAD-ME'' type. Al-

though some species within subgen. *Panicum* are anatomically similar to PCK type, biochemically they use the enzyme NAD-ME. Consequently, these species are included in the NAD-ME ''classical PCK'' type (Hattersley and Watson, 1992). Subgenus *Megathyrsus* is a C_4 ''classical PCK'' type (Zuloaga, 1987), while *Panicum* subg. *Agrostoides* is C_4 ''classical NADP-ME'' type. This photosynthetic type is found not only in all Andropogoneae but also in many genera of Paniceae, including *Setaria, Pennisetum,* and *Echinochloa,* to name only a few. Also, *P. prionitis* and *P. petersonii,* both in section *Prionitia,* are biochemically NADP-ME although the outer sheath is still present, which places them in the ''Neurachneae type'' (Hattersley and Watson, 1992).

In this study, we have used sequences from the chloroplast gene *ndh*F to address whether the tribe Paniceae forms a monophyletic assemblage within the panicoid grasses. We also investigated the monophyly and phylogenetic relationships of major genera such as *Panicum* and *Paspalum.* We selected *ndhF* because of its relatively high rate of molecular evolution in the grasses (Clark, Zhang, and Wendel, 1995). Virtually all the proteins involved in C_4 photosynthesis are encoded by nuclear genes (Kanai and Edwards, 1999; Leegood and Walker, 1999); a chloroplast gene should thus provide an independent history from the genes selected for C_4 photosynthesis. Our study provides new insight into the evolution of photosynthetic pathways and relationships of the major phylogenetic lineages of Panicoideae.

MATERIALS AND METHODS

Plant material, DNA extraction, and sequencing—Plants were either grown in the greenhouses of the University of Missouri-St. Louis and Harvard University or were field collected and dried in silica gel. Voucher information is provided as supplementary information at http://ajbsupp.botany.org/. DNA extractions were conducted using modifications of the protocols in Doyle and Doyle (1987), Murray and Thompson (1980), and Saghai-Maroof et al. (1984). Plant tissue was ground in liquid nitrogen and the protocols were scaled down for use with small amounts of either fresh and/or silica gel-dried plant material. In some cases, total DNA was purified with Geneclean III kit (BIO 101, Vista, California, USA). The *ndhF* gene was amplified via the polymerase chain reaction (PCR) using a *Taq*-mediated protocol (Promega, Madison, Wisconsin, USA) in several overlapping fragments: 5F/972R, 5F/1318R, and 972F/2110R. For difficult taxa the gene was amplified in smaller fragments, i.e., using primer pairs 5F/536R, 536F/1318R, 972F/1821R, and 1318F/2110R. In total, we used a battery of 10–14 sequencing primers described by Olmstead and Sweere (1994) with the exception of primer 1821, which was designed by Clark, Zhang, and Wendel (1995). The PCR products were cleaned with the QIAquick PCR purification kit (QIAGEN, Valencia, California, USA), quantified by comparison to a low mass DNA ladder (pGEM 25 and 50 ng; Applied Biosystems, Foster City, California, USA) and then labelled with fluorescent dye terminators (Applied Biosystems) during cycle sequencing (10 μL reactions). Both forward and reverse strands were sequenced with a minimum overlap of 90% for every taxon on an ABI 377 automated sequencer using Long Ranger acrylamide gels (FMC Bioproducts, Rockland, Maine, USA). Assembly and editing of sequences used the software program Sequencher, version 3.1 (Gene Codes, Ann Arbor, Michigan, USA). We used the *ndhF* sequence of *Oryza sativa* (rice) as a reference for aligning our data. The rice gene is 2205 bp long, occupying coordinate numbers 103 637 (5′ end) to 101 433 (3′ end), (Hiratsuka et al., 1989, corrected by Clark, Zhang, and Wendel, 1995). Sequences were translated to check for stop codons and then manually aligned, preserving the reading frame. Gaps corresponding to indels were mapped onto the final trees to determine whether they were synapomorphies or homoplasies. Other gaps were added when contiguous blocks of sequence (contigs) could not be assembled after several attempts at amplification; these were treated as missing data. *Panicum euprepes* is incomplete between nucleotide positions 102 643 and 102 318; *Chaetium bromoides* between 102 294 and 102 203; and the following taxa were sequenced only between nucleotide position 103 580 and the position shown in parentheses: *Tatianyx arnacites* (102 295), *Panicum pedersenii* (102 197), *Panicum piauiense* (101 793), *Panicum ovuliferum* (101 704), *Pennisetum alopecuroides* (101 674), and *Chasmanthium latifolium* (101 643). The aligned data matrix has been submitted to TreeBASE (http://www.herbaria.harvard.edu/treebase) and has also been submitted as supplemental data to the *American Journal of Botany* website (http://ajbsupp.botany.org/).

Taxonomic sampling—In this study, subfamily Panicoideae was considered the ingroup, including sequences of the tribes Paniceae, Andropogoneae, and Arundinelleae. Delimitation of the subfamily follows GPWG (2001), and outgroup selection was based on the GPWG (2001) phylogeny as well as the grass phylogeny proposed by Clark, Zhang, and Wendel (1995). Outgroups included members of the tribes Thysanolaeneae and Centotheceae (Centothecoideae) plus the formerly panicoid genus *Danthoniopsis.* Tribal classification follows the treatments proposed by Clayton and Renvoize (1986), Watson and Dallwitz (1992), and Zuloaga, Morrone, and Giussani (2000).

In all, 78 sequences of the chloroplast gene *ndhF* were generated, 76 of 78 within the tribe Paniceae. The remaining two sequences, for *Danthoniopsis dinteri* (Arundinelleae) and *Chasmanthium latifolium* (Centotheceae), were generated to verify sequences available in GenBank because we were concerned about possible misplacement of the species in preliminary trees. We used our own sequences in the analyses presented here (supplemental material, http://ajbsupp.botany.org/). Additional sequences from the ingroups, Paniceae (*Panicum virgatum* and *Setaria viridis*), Andropogoneae (22 species), and Arundinelleae (two species), and from the outgroup Centothecoideae (three species) were obtained from GenBank; accession numbers are also specified in the supplemental material (http://ajbsupp.botany.org)/.

Effort was made to encompass most of the morphological diversity of the Paniceae (represented by 35 genera), the tribe Andropogoneae (22 genera), and the tribe Arundinelleae (2 genera). Our sample included 19 species of *Panicum,* representing 14 sections in 5 subgenera; throughout this paper we follow the classification of Zuloaga (1987) in discussing *Panicum.* We also included 9 species of *Paspalum,* representing 7 informal taxonomic groups; and 7 species of the large genus *Setaria.* One of the *Panicum* species, listed by Zuloaga (1987) as *P. maxima* in subgenus *Megathyrsus,* is listed in Table 1 as *Urochloa maxima,* following Webster (1987).

Phylogenetic analysis—A maximum parsimony analysis was performed using NONA version 2.0 (Goloboff, 1997a) with all characters equally weighted and gaps scored as missing data. Overall, 3.5% of the data matrix cells were scored as gaps. Separate analyses using implied weights (Goloboff, 1993) were run in Pee-Wee version 3.0 (Goloboff, 1997b) using the same search strategies as in NONA. This reduces the number of trees by reducing the influence of homoplastic characters, which are downweighted in proportion to their number of extra steps (homoplasy). The weighting is based on a concave function, with six different concavities available in the program; 6 is the mildest and 1 the strongest weighting function (Goloboff, 1997b). Searches were done using K = 1, K = 3, and K = 6. In weighted and unweighted analyses, uninformative characters were discarded using the ''pack'' command. All informative characters were considered unordered, and both the ''amb-'' (resolve clades only if they have unambiguous support) command, and ''poly='' (polytomies allowed) command were used. Searches were performed using ''mult*3000,'' which randomizes the order of taxa, creates a Wagner tree, and submits it to branch-swapping by tree-bisection reconnection (TBR). It stores up to 20 most-parsimonious trees in memory and repeats the process 3000 times. The shortest trees retained from the subsearches were then TBR swapped to completion with the ''max*'' command. To estimate the relative stability of individual clades and overall topology of the cladograms, strict consensus trees were generated from the most-parsimonious trees obtained from the NONA and Pee-Wee analyses. Data with equally weighted characters were also analyzed in PAUP*4.01b (Swofford, 1998) with 1000 random addition sequences and no branch swapping; these found 208 islands of equally parsimonious trees. These trees were then used

TABLE 1. Base chromosome number (*x*), anatomical and physiological characters, as mapped on to the cladograms in Fig. 2. Missing or ambiguous data are coded as—; unknown = ?. Primary carboxylating compound produced in the mesophyll may be either a C_3 compound (3-phosphoglycerate) or C_4 compound (oxalacetate). This character is correlated with the number of mesophyll cells between adjacent vascular bundles: more than 4 cells, all C_3 species; 2–4 cells, all C_4 species. Principal enzyme acting on decarboxylation processes within bundle sheaths: na = not applicable, C_3 species; NADP-me = NADP-malic enzyme; NAD-ME = NAD-malic enzyme; PCK = PEP carboxykinase. Position of chloroplasts in the bundle sheaths: abs = absent or peripheral to slightly centrifugal; -fugal = centrifugal; -petal = centripetal. Chloroplast structure: granal or agranal. Number of bundle sheaths may be one or two.

Taxon	(*x*)	1° carboxylating compound	Decarboxylating enzyme	Chloroplast position	Chloroplast structure	Number of bundle sheaths	Reference[a]
Tribe Paniceae (Panicoideae)							
Acroceras zizanioides	9	C_3	na	abs	granal	two	(a and b) HQV; WV; WD (b) B; E; HW, 1992 (c) B; ZMS
Altoparadisium chapadense	?	C_4	— Arundinella anatomy	-fugal	agranal	one	(b) FDZM
Anthaenantiopsis rojasiana	10	C_4	NADP-ME Neurachne type	-fugal	agranal	one	(a and b) MFZD; WD (b) B; HW, 1992
Arthropogon lanceolatus	?	C_3	—	abs	granal	two	(b) FDZM; F, 1982
Arthropogon villosus	?	C_4	NADP-ME	-fugal	agranal	one	(b) FDZM
Axonopus anceps	10	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD (a) MHZE (b) GEB; B; PHS; HW, 1992
Axonopus fissifolius	10	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD (a) NQK (b) GEB; B; PHS; HW, 1992
Cenchrus ciliaris	9, 12	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD (a) BS, 1985; SBS (b) GGE; B; E; PHS; HW, 1992
Chaetium bromoides	13	C_4	PCK	-fugal	granal	two	(b) B; HW, 1992; MZAPA
Digitaria	9, 15, 17						(a and b) WD (a) HZME (b) HK, 1974b; B; E; EKB; UKE; PHS; HW, 1992 (c) He
Digitaria ciliaris	9	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; SB
Digitaria radicosa	9	C_4	NADP-ME	-fugal	agranal	one	
Digitaria setigera	9	C_4	NADP-ME	-fugal	agranal	one	(a) BS, 1986; AVA
Echinochloa	9						(a and b) WD (b) GGE; GEB; GE; E; PHS; HW, 1992 (c) Y
Echinochloa colona	9	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; BS 1986
Echinochloa frumentacea	9	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; Hilu 1994
Echinolaena inflexa	10	C_3	na	abs	granal	two	(a and b) WD; WV (a) GS (b) B; HW, 1992 (c) F, 1994.
Eriochloa punctata	9	C_4	PCK	-fugal	granal	two	(a and b) WD (a) HZME; Q (b) GEB; B; E; PHS; HW, 1992
Homolepis glutinosa	10	C_3	na	abs	granal	two	(a) DP; Sh (a and b) WD; ZSo; WV (b) B; HW, 1992
Homolepis isocalycina	10	C_3	na	abs	granal	two	(a) GS (a and b) WD; ZSo; WV (b) B; HW, 1992
Hymenachne donacifolia	10	C_3	na	abs	granal	two	(a and b) HQV; WD; CR (b) B; HW, 1992
Ichnanthus pallens	10	C_3	na	abs	granal	two	(a and b) WD; WV (a) HQV; HZME (b) B; HW, 1992 (c) St
Lasiacis sorghoidea	9	C_3	na	abs	granal	two	(a and b) WD; D (a) NQK (b) B; HW, 1992
Leptocoryphium lanatum	10	C_4	NADP-ME	-fugal	agranal	one	(a) WD (b) B
Melinis repens	9	C_4	PS-PCK				(a and b) WD; MZ, 1995 (b) E; PHS; PHS; HW, 1992

TABLE 1. Continued.

Taxon	(*x*)	1° carboxylating compound	Decarboxylating enzyme	Chloroplast position	Chloroplast structure	Number of bundle sheaths	Reference[a]
Mesosetum chaseae Luces	8	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD; F, 1990 (b) B; HW, 1992 (c) F, 1990
Ophiochloa hydrolithica	?	C_4	—	-fugal	agranal	one	(b) WD; FDZ, 1993
Oplismenus hirtellus	9, 10, 11	C_3	na	abs	granal	two	(a and b) WD; Scholz, 1981 (a) HQV; HZME (b) B; E; HW, 1992
Otachyrium versicolor	10	C_3	na	abs	granal	two	(a and b) WD (a) DP, 1978 (b) B; HW, 1992 (c) SS
Panicum bulbosum	9	C_4	—	-fugal	agranal	one	(a and b) Z, 1987; ZDM (a) HHMR (b) Do; B; HS; DDH; PHS
Panicum obtusum	10	C_4	NADP-ME	-fugal	agranal	one	(a and b) Z, 1987; GEB; B
Panicum prionitis	10	C_4	NADP-ME Neurachne type	-fugal	agranal	two	(a and b) Z, 1987, 1989 (a) BBBC 1981 (b) B
Panicum ovuliferum	9	C_3	na	abs	granal	two	(a, b and c) ZSM; Z, 1987 (b) B
Panicum koolauense	9	C_3	na	abs	granal	two	(a, b and c) WD; Z, 1987; ZEM, 1993 (a) HZME (b) B; HW, 1992
Panicum sabulorum	9	C_3	na	abs	granal	two	
Panicum aquaticum	9	C_4	—	-petal	granal	two	(a, b and c) Z, 1987; 1989 (b) Do; GGE; B; OM; OMC; HB; ZM 1996
Panicum elephantipes	9	C_4	PCK-like NAD-ME anatomy	-fugal	granal	two	(a, b and c) Z, 1987; 1989 (b) Do; GGE; B; OM; OMC: HB; ZM 1996
Panicum fauriei	9	C_4	NAD-ME	-petal	granal	two	(a and b) Z, 1987 (b) B; ZM, 1996
Panicum nephelophilum	9	C_4	NAD-ME	-petal	granal	two	(a and b) Z, 1987 (b) B; ZM, 1996
Panicum rudgei	9	C_4	—	-petal	granal	two	(a, b and c) Z, 1987; ZDM (b) B
Panicum pedersenii	9	C_4	—	-petal	granal	two	(a and b) Z, 1987; ZDM
Panicum repens	9	C_4	- (PCK-like NAD-ME)	-fugal	granal	two	(a) AVA; BS 1986; SBS (b) M; HW, 1976; B (c) ZDM
Panicum virgatum	9	C_4	- (PCK-like NAD-ME)	-fugal	granal	two	(a) HHMR (b) B; SK; HB; H; PHS; E, 1988; HW, 1992
Panicum laxum	10	C_3	na	abs	granal	two	(a, b and c) Z, 1987; ZEM, 1992 (a) BBBC; NQK (b) B
Panicum euprepes	10	C_3	na	abs	granal	two	(a, b and c) RZ; Z, 1987 (a) MHZE
Panicum millegrana	9	C_3	na	abs	granal	two	(a and b) Z, 1987; 1989 (b) B
Panicum piauiense	10	C_3	na	abs	granal	two	(a and b) Z, 1987; ZS (b) B
Paspalidium geminatum	9	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD; MZ, 1995 (a) AVA (b) B; E, 1977; PHS; HW, 1992
Paspalum	10	C_4					(a and b) WD (a) MHZE; HZME (b) M; HW, 1976, 1992; GGE, 1976; B; E, 1977; UKE; PHS (c) C; MZC
Paspalum malacophyllum	10	C_4	NADP-ME	-fugal	agranal	one	(a) HHQV; HZME; K; NQK
Paspalum conjugatum	10	C_4	NADP-ME	-fugal	agranal	one	(a) DC, 1977; 1983; 1988; HQV; MC; SCSD

TABLE 1. Continued.

Taxon	(*x*)	1° carboxylating compound	Decarboxylating enzyme	Chloroplast position	Chloroplast structure	Number of bundle sheaths	Reference[a]
Paspalum vaginatum	10	C_4	NADP-ME	-fugal	agranal	one	(a) L; O; QB; RM
Paspalum remotum		C_4	NADP-ME	-fugal	agranal	one	(a) HZME
Paspalum paniculatum	10	C_4	NADP-ME	-fugal	agranal	one	(a) DC, 1977; 1983; DP, 1972; Q
Paspalum arundinellum	10	C_4	NADP-ME	-fugal	agranal	one	(a) HQV
Paspalum haumanii		C_4	NADP-ME	-fugal	agranal	one	(a) Burs, 1975; NQB; Sau
Paspalum conspersum	10	C_4	NADP-ME	-fugal	agranal	one	(a) Burs, 1978; DC, 1983; MFBBSS; Q
Paspalum wettsteinii Hack.	10	C_4	NADP-ME	-fugal	agranal	one	(a) DC, 1983, 1988
Pennisetum Rich.	9	C_4					(a and b) WD; MZ, 1995 (b) BG; GGE; HKC; B; E, 1977; LBC; UKE; PHS; HW, 1992
Pennisetum alopecuroides	11	C_4	NADP-ME	-fugal	agranal	one	(a) SBS; XWZ
Pennisetum setaceum	9	C_4	NADP-ME	-fugal	agranal	one	(a) PT; SMRM
Plagiantha tenella	10	C_3	na	abs	granal	two	(a) MHZE (b) WD; ZMVG; HW, 1992 (c) ZMVG
Pseudechinolaena polystachya	9	C_3	na	abs	granal	two	(a and b) WD (b) B; E, 1977; HW, 1992
Sacciolepis indica	9	C_3	na	abs	granal	two	(a and b) WD; WV (b) B; E, 1977; J; HW, 1992
Setaria	9, 10						(a, b and c) WD; P (b) HKE; GGE; E, 1977; UKE; PHS; HW, 1992
Setaria geniculata	9	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; BS, 1985; 1986 (b) GGE; HW, 1976
Setaria lachnea	9	C_4	NADP-ME	-fugal	agranal	one	(a) CM
Setaria macrostachya	9	C_4	NADP-ME	-fugal	agranal	one	(a) B, 1950; Em; GuS
Setaria palmifolia	9	C_4	NADP-ME	-fugal	agranal	one	(a) MS; T
Setaria parviflora	9	C_4	NADP-ME	-fugal	agranal	one	(a) NQK
Setaria sphacelata	9	C_4	NADP-ME	-fugal	agranal	one	(a) S
Setaria viridis	9	C_4	NADP-ME	-fugal	agranal	one	(a) KG; XWZ (b) M, GGE; HW, 1976, B
Steinchisma hians	10	C_3	Intermediate C_3/C_4	-petal	granal	two	(b, c) WD; ZMVG (b) B; HW, 1992
Stenotaphrum secundatum	9	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD; MZ, 1995 (b) B; E, 1977; PHS; HW, 1992
Streptostachys							(b) WD; B (c) MZ, 1991
Streptostachys asperifolia	10	C_3	na	abs	granal	two	(a) MHZE
Streptostachys ramosa	9	C_4	—	-fugal	agranal	one	(a) DP, 1978; MZ, 1991
Tatianyx arnacites	10	C_4	—	-fugal	agranal	one	(a) MHZE (b) WD (c) ZSo.
Thrasya glaziovii	10	C_4	NADP-ME	-fugal	agranal	one	(a and b) WV (b) B; WD; HW, 1992 (c) Burm
Thrasya petrosa	10	C_4	NADP-ME	-fugal	agranal	one	(a and b) WV (a) NQK (b) B; WD; HW, 1992 (c) Burm
Urochloa	7, 9						(a, b and c) WD; MZ, 1992, 1993, 1995, W; Z, 1987; 1989 (a) HZME; MHZE (b) M; GGE; GEB; HW, 1976; B; E, 1977; PHS; MZ, 1992, 1993
Urochloa acuminata	13 + II	C_4	PCK	-fugal	granal	two	(a) MHZE
Urochloa plantaginea	9	C_4	PCK	-fugal	granal	two	(a) PD, 971; R
Urochloa maxima	8, 9	C_4	PCK	-fugal	granal	two	
Urochloa mutica	9	C_4	PCK	-fugal	granal	two	
Tribe Andropogoneae (Panicoideae)							(a and b) WD (b) E, 1977; HW, 1992
Andropogon gerardii	5, 10	C_4	NADP-ME	-fugal	agranal	one	(b) GGE
Apluda mutica	10	C_4	NADP-ME	-fugal	agranal	one	(a) SB
Bothriochloa bladhii	10	C_4	NADP-ME	-fugal	agranal	one	
Capillipedium parviflorum	10	C_4	NADP-ME	-fugal	agranal	one	(a) AVA
Chionachne koenigii	10	C_4	NADP-ME	-fugal	agranal	one	

TABLE 1. Continued.

Taxon	(*x*)	1° carboxylating compound	Decarboxylating enzyme	Chloroplast position	Chloroplast structure	Number of bundle sheaths	Reference[a]
Chrysopogon fulvus	5, 10	C_4	NADP-ME	-fugal	agranal	one	(a) SB; SBS
Cleistachne sorghoides	9	C_4	NADP-ME	-fugal	agranal	one	
Coelorachis selloana	9	C_4	NADP-ME	-fugal	agranal	one	(a) HQV
Coix aquatica	5, 10	C_4	NADP-ME	-fugal	agranal	one	(a) CMP; RN
Cymbopogon flexuosus	5, 10	C_4	NADP-ME	-fugal	agranal	one	(a) La
Dichanthium aristatum	10	C_4	NADP-ME	-fugal	agranal	one	(a) SBS
Elionurus muticus	5, 10	C_4	NADP-ME	-fugal	agranal	one	(a) NQK
Heteropogon contortus	10, 11	C_4	NADP-ME	-fugal	agranal	one	(a) BS, 1985
Hyparrhenia hirta	10, 15	C_4	NADP-ME	-fugal	agranal	one	(a) HD
Ischaemum afrum	10	C_4	NADP-ME	-fugal	agranal	one	(a) HD; SMPS
Microstegium nudum	10	C_4	NADP-ME	-fugal	agranal	one	(b) GEB; UKE
Miscanthus japonicus	19	C_4	NADP-ME	-fugal	agranal	one	(a) A
Phacelurus digitatus	10	C_4	NADP-ME	-fugal	agranal	one	
Schizachyrium scoparium	5, 10	C_4	NADP-ME	-fugal	agranal	one	(b) EKB; GGE
Sorghum bicolor	5, 10	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; MO (b) GGE
Tripsacum dactyloides	9, 10	C_4	NADP-ME	-fugal	agranal	one	(a) DBWH
Zea mays	10	C_4	NADP-ME	-fugal	agranal	one	(a) AVA; AP (b) Do; GGE; HK, 1974a; HKE; PHS; SK; UKE
Tribe Arundinelleae (Panicoideae)							
Danthoniopsis petiolata	9	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD (b) HW, 1992
Danthoniopsis dinteri	9	C_4	NADP-ME	-fugal	agranal	one	(a and b) WD (a) LLP (b) HW, 1992
Arundinella hirta	7, 10, 12, 14	C_4	NADP-ME Arundinella type	-fugal	agranal	one	(a and b) WD (b) SK
Tribe Centotheceae (Centothecoideae)							
Chasmanthium laxum subsp. *sessiliflorum*	6	C_3	na	abs	granal	two	(a and b) WD
Chasmanthium latifolium	6	C_3	na	abs	granal	two	(a) De; Yat (b) SK
Zeugites pittieri	$2n = 46$	C_3	na	abs	granal	two	(a and b) WD
Tribe Thysanolaeneae (Arundinoideae)							
Thysanolaena maxima	11	C_3	na	abs	granal	two	(a and b) WD

[a] References for tabulated information: (a) references documenting chromosome number; (b) references documenting photosynthetic pathway; (c) general references. Abbreviations: A = Adati, 1958; AP = De Aguiar Perecin, 1985; AVA = Ahsan, Vahidy, and Ali, 1994; B = Brown, 1950, 1977; BBBC = Bouton et al., 1981; BG = Brown and Gracen, 1972; BS = Bir and Sahni, 1985, 1986; Burm = Burman, 1987; Burs = Burson, 1975, 1978; C = Chase, 1929; CM = Cáceres and Mazzucato, 1995; CMP = Christopher, Mini, and Pillai, 1989; CR = Clayton and Renvoize, 1986; D = Davidse, 1978; DBWH = Dewald et al., 1987; DC = Dandin and Chennaveeraiah, 1977, 1983, 1988; DDH = Dengler, Dengler, and Hattersley, 1986; De = Delay, 1974; Do = Downton, 1970; DP = Davidse and Pohl, 1972, 1978; E = Ellis, 1977, 1978; EKB = Edwards, Kanai, and Black, 1971; Em = Emery, 1957; F = Filgueiras, 1982, 1990, 1999; FDZ = Filgueiras, Davidse, and Zuloaga, 1993; FDZM = Filgueiras et al., in press; GE = Gutiérrez and Edwards, unpublished in Brown, 1977; GEB = Gutiérrez, Edwards, and Brown, 1976; GGE = Gutiérrez, Gracen, and Edwards, 1974; GS = Gould and Soderstrom, 1967; GuS = Gupta and Singh, 1977; H = Hattersley 1984; HB = Hattersley and Browning, 1981; HD = Hoshino and Davidse, 1988; He = Henrard, 1950; HHMR = Hamoud et al., 1994; HK = Hatch and Kagawa, 1974a, b, 1976; HKC = Hatch, Kagawa, and Craig, 1975; HKE = Huber, Kanai, and Edwards, 1973; HQV = Hofni, Quarín, and Valls 1991[1990]; HS = Hattersley and Stone, 1986; HW = Hattersley and Watson, 1976, 1992; HZME = Hunziker et al., 1998; J = Judziewicz, 1990; K = Killeen, 1990; KG = Koul and Gohil, 1988; L = Llauradó, 1984; La = Lavania, 1987; LBC = Lavergne, Bismuth, and Champigny, 1979; LLP = Li, Lubke, and Phipps, 1966; M = Metcalfe, 1960; MC = Mehra and Chaudhary, 1981; MFBBSS = De Moraes Fernandes et al., 1974; MFZD = Morrone et al., 1993; MHZE = Morrone et al., 1995; MO = Morakinyo and Olorode 1988; MS = Mehra and Shana, 1975; MZ = Morrone and Zuloaga, 1991, 1992, 1993, 1995; MZAPA = Morrone et al., 1998; MZC = Morrone, Zuloaga, and Carbonó, 1995; NQB = Norrmann, Quarín, and Burson, 1989; NQK = Norrmann, Quarín, and Killeen, 1994; O = Okoli, 1982; OM = Ohsugi and Murata 1980; OMC = Ohsugi, Murata, and Chonan, 1982; P = Pensiero, 1999; PD = Pohl and Davidse, 1971, 1974; PHS = Prendergast, Hattersley, and Stone, 1987; PT = Parihar and Tripathi, 1989; Q = Quarín, 1977; QB = Quarín and Burson, 1983; R = Reeder, 1967, 1971; RM = Rao and Mwasumbi, 1981; RN = Rao and Nirmala, 1990; RZ = Renvoize and Zuloaga, 1984; S = Sahni, 1989; Sau = Saura, 1941; SB = Sahni and Bir, 1985; SBS = Sinha, Bhardwaj, and Singh, 1990; SCSD = Sarkar et al., 1976; Sh = Shibata, 1962; SK = Sinha and Kellogg, 1996; SMPS = Spies et al., 1991; SMRM = Sujatha et al., 1989; SS = Sendulsky and Soderstrom, 1984; St = Stieber, 1987; T = Takeoka, 1962; UKE = Usuda, Ku, and Edwards, 1984; W = Webster, 1987; WD = Watson and Dallwitz, 1992; WV = Webster and Valdés-Reyna, 1988; XWZ = Xu, Weng, and Zhang 1992; Y = Yabuno, 1966; Yat = Yates, 1966; Z = Zuloaga, 1987, 1989; ZDM = Zuloaga, Dubcovsky, and Morrone, 1993; ZEM = Zuloaga, Ellis, and Morrone, 1992, 1993; ZM = Zuloaga and Morrone, 1996; ZMS = Zuloaga, Morrone, and Saenz, 1987; ZMVG = Zuloaga et al., 1998; ZS = Zuloaga and Sendulsky, 1988; ZSM = Zuloaga, Saenz, and Morrone, 1986; ZSo = Zuloaga and Soderstrom, 1985.

TABLE 2. Insertions and deletions in *ndhF* for the panicoid grasses. Position of indels refers to coordinates of the *ndhF* of *Oryza sativa* (Hiratsuka et al., 1989). Indels are mapped on the phylogeny in Fig. 1.

Insertion/ deletion	Coordinate number	Code letter[a]	Size (bp)	Indel identity	Taxa
Insertion	102 703	a	6	Autapomorphy	*Setaria sphacelata*
Insertion	102 114	b	6	Homoplasy	*Chaetium bromoides, Danthoniopsis petiolata, D. dinteri*
Deletion	102 114	c	18	Autapomorphy	*Digitaria ciliaris, D. radicosa, D. setigera*
Deletion	102 092	d	9	Homoplasy	*Chaetium bromoides, Danthoniopsis petiolata,*[b] *D. dinteri*
Insertion	102 065	e	6	Synapomorphy	*Axonopus anceps, A. fissifolius, Ophiochloa hydrolithica*
Deletion	101 951	f	15	Homoplasy	*Oryza sativa. Acroceras zizanioides, Panicum sabulorum, P. euprepes*
Deletion	101 739	g	9	Synapomorphy	*Hymenachne donacifolia, Otachyrium versicolor, Panicum laxum, Plagiantha tenella,* and *Steinchisma hians*
Insertion	101 735	h	6	Synapomorphy	$x = 10$ Paniceae clade (aaaaat / aaaaag / aaaact)
		h′		Autapomorphy	*Thysanolaena* (actttt)
Insertion	101 704	i	6	Autapomorphy	*Cleistachne*

[a] Code refers to designations on Fig. 1.
[b] Position differs from that shown by Clark, Zhang, and Wendel (1995).

as starting trees for a heuristic search with TBR branch swapping; the memory limit was reached at 19 500 trees. The strict consensus of these 19 500 trees was then used as a negative constraint tree.

To assess the relative support for clades found in each analysis, bootstrap analyses (Felsenstein, 1985) were performed with PAUP* version 4.01b for UNIX or for Macintosh Power PC (Swofford, 1998) with 1000 replicates in a heuristic search using random taxon entry followed by TBR branch swapping (MULTREES). Constrained analyses were performed with NONA to calculate the number of additional steps it would take to make a monophyletic group. To perform these analyses we used a tree with a fixed monophyletic group as starting point (using the ''force'' command) and carried out a branch-swapping search on the initial tree (''max/'' command) to look for trees with highest fit. To test for significant differences between constrained and unconstrained trees, we did Templeton tests (Templeton, 1983), as implemented in PAUP*4.01b.

Haploid chromosome numbers, leaf structure, and physiological characters related to photosynthetic pathways were obtained from the literature (Table 1) at the generic and specific levels. Although these characters were not included in the analyses, they were added to the matrix and were unambiguously optimized on one of the most-parsimonious trees using Winclada Beta, version 0.9.9 (Nixon, 1999) after the analyses. Optimization of these characters allowed us to look for evolutionary patterns and degree of relationships among the different lineages.

RESULTS

The 107 sequences were visually aligned; of these, 103 species represented subfamily Panicoideae, and four sequences represented outgroups. After excluding amplification primer regions, the data set had a total of 2028 nucleotides between coordinate numbers 103 579 and 101 553 of the rice *ndhF* gene. The alignment required the addition of five indels 6 base pairs (bp) long, one indel 15 bp long, and one indel 18 bp long that lengthened the data set by a total of 45 nucleotide positions, resulting in 2073 columns or characters. Table 2 shows the indels identified in the data matrix after the alignment.

In the aligned data matrix, 770 (38%) characters were variable and 435 (21.4%) of those were phylogenetically informative. There are 269 characters out of 435 that vary between two bases (i.e., two-state characters), 134 between three bases, and 32 with the four nucleotides represented. The sequences were A-T rich: adenine: 26.8%, thymine: 37.3%, guanine: 16.6%, and cytosine: 15.8% (3.5% of the data matrix is shown as gaps).

The phylogenetic analysis with equally weighted characters (NONA) found 27 128 equally parsimonious trees of length (L) = 1472, consistency index (CI) = 0.43, and retention index (RI) = 0.77, excluding uninformative sites; the analysis reached completion after 35 h when running max*, mult*3000. One most parsimonious tree from the equally weighted (NONA) analysis, with branch lengths and bootstrap values, is shown in Fig. 1.

For analyses under implied weights (Pee-Wee), we tried different concavities and chose among different topologies and highest fit. Trees from $K = 6$ are very close to the Nona results, particularly at the deepest branches, and result in higher fit (3517.7). Trees from $K = 1$ had the worst fit (2363.4) and topology is similar to trees from $K = 3$, although with less resolution in minor clades. Here we report only the results from the medium concave function $K = 3$ to show the results most dissimilar to the unweighted analyses. The $K = 3$ analysis ran to completion and found 3600 equally parsimonious trees of length = 1468, consistency index = 0.42, retention index = 0.77, fit = 3111.3, and rescaled index = 0.48. Figure 2 shows a comparison between consensus trees from unweighted and weighted analyses.

The subfamily Panicoideae is strongly supported as monophyletic in all our analyses (99% bootstrap value and eight informative molecular synapomorphies; Fig. 1), with the exception of *Danthoniopsis,* which consistently falls among the outgroups. We included data from two species of *Danthoniopsis,* resequenced *D. dinteri* for verification of the published sequence, and conclude that this result is not artifactual.

Panicoideae is divided into three well-resolved and strongly supported clades, corresponding largely to groups having the same basic chromosome number (Andropogoneae [$x = 10$], Paniceae [$x = 10$], and Paniceae [$x = 9$]). The relationship among these clades is uncertain. Trees with equally weighted characters place Andropogoneae sister to $x = 10$ Paniceae (Fig. 1), although there is no bootstrap support for this relationship. Constraining the trees from equally weighted characters to make Paniceae monophyletic added only one step; a Templeton test indicated that the difference was not significant ($P < 0.706$). Trees with characters under implied weight indicate that the Paniceae is monophyletic, with the $x = 10$ clade sister to the $x = 9$ clade (Fig. 2B). Thus, we cannot be certain whether the Paniceae is monophyletic or not.

The tribe Andropogoneae is strongly supported in all analyses with 17 nucleotide substitutions and 100% bootstrap value (Fig.

1). Analyses with equally weighted characters place *Arundinella hirta* ($x = 7, 10, 12$) (formerly Arundinelleae) as sister to the tribe, as found by previous authors (Mason-Gamer, Weil, and Kellogg, 1998; Spangler et al., 1999; Kellogg, 2000), although not well supported by bootstrap analysis (57%). Analyses with implied weights, however, place *A. hirta* in the Andropogoneae and the clade *Tripsacum-Elionurus-Zea* appears as sister group to the tribe (Fig. 2B). Most members of Andropogoneae are $x = 10$, but some species, i.e., *Coix* and *Sorghum* are $x = 5$; *Cleistachne* and *Coelorachis* are $x = 9$.

The $x = 9$ Paniceae are clearly monophyletic (98% bootstrap, seven nucleotide substitutions; Fig. 1). Although most members of this clade have a chromosome number of $x = 9$, there are a few exceptions, such as *Chaetium* ($x = 13$), a few species of *Setaria* ($x = 10$), and a few species of *Urochloa* ($x = 8$) (Table 1). Within the $x = 9$ Clade, *Digitaria* is represented by three species and forms a robust group (100% bootstrap, branch length 29) (Fig. 1). The species investigated also have a unique 18 bp deletion (coordinate number 102 114, Fig. 1; Table 2). The position of the *Digitaria* clade is not stable; it is basal within the $x = 9$ clade in unweighted analyses, but results from implied weight analysis place *Digitaria* as sister taxa of the ''*Setaria*/*Urochloa*/*Panicum* clade'' (see below for description of this clade). This affects inferences about character evolution (see below).

Acroceras, Echinochloa, Lasiacis, Oplismenus, Panicum ovuliferum (subg. *Dichanthelium,* sect. *Cordovensia*), and *Pseudechinolaena* form a moderately well-supported clade (82% bootstrap, four nucleotide substitutions). Because all the taxa normally have lanceolate leaf blades and are associated with forest shade environment (Davidse, 1978; Clayton and Renvoize, 1986; Zuloaga, Morrone, and Saenz, 1987), we call this the ''Forest Shade Clade.'' Within this clade, *Echinochloa,* represented here by *E. colona* and *E. frumentacea,* forms a robust monophyletic unit (100% bootstrap, 25 mutations; Fig. 1). *Pseudechinolaena, Lasiacis,* and *Oplismenus* form a well-supported and constant group within the Forest Shade Clade (93%). The first two species are also linked in all trees, with 94% bootstrap value.

Panicum millegrana (subg. *Phanopyrum,* sect. *Monticola*) and *Sacciolepis indica* are sister taxa (84% bootstrap, four molecular synapomorphies; Fig. 1). *Panicum koolauense* and *P. sabulorum* (subg. *Dichanthelium,* sect. *Dichanthelium*) are also strongly supported as sisters (100% bootstrap, 18 molecular synapomorphies).

The remaining species of the $x = 9$ Clade fall into a single well-supported group (88% bootstrap, with 6 bp substitutions), here called the *Setaria*/*Urochloa*/*Panicum* clade. All species in this clade exhibit C_4 photosynthesis. The clade is divided into three strongly supported subgroups, each of which represents a single C_4 subtype (Fig. 2). All members of the clade containing *Chaetium, Eriochloa, Panicum maximum* (= *Urochloa maxima*), *Melinis,* and *Urochloa* (98% bootstrap, eight mutations) use PEP carboxykinase as a decarboxylating enzyme, whereas all members of *Panicum* subgenus *Panicum* (98% bootstrap, nine mutations) use NAD-malic enzyme. The clade, including species of *Cenchrus, Panicum bulbosum* (subg. *Agrostoides,* sect. *Bulbosa*), *Paspalidium, Pennisetum, Setaria,* and *Stenotaphrum* (100% bootstrap, and nine changes) uses the NADP-malic enzyme.

Several major genera (*Urochloa, Setaria,* and *Pennisetum*) are paraphyletic. Five species of *Setaria* (*S. macrostachya, S. parviflora, S. palmifolia, S. sphacelata,* and *S. geniculata*) form a monophyletic subclade (95% bootstrap; Fig. 1), but the remainder are placed together with *Paspalidium, Stenotaphrum,* and *Panicum bulbosum,* or are basal to the *Setaria* clade. *Pennisetum* is paraphyletic but forms a monophyletic assemblage with *Cenchrus ciliaris.* The latter species is treated as *Pennisetum ciliare* by Pohl (1980) and Hitchcock (1951) among others.

The $x = 9$ Clade includes members of five subgenera of *Panicum* (subg. *Panicum* [C_4], *Dichanthelium* [C_3], *Agrostoides* [C_4], *Phanopyrum* [C_3], and subg. *Megathyrsus* [C_4], sensu Zuloaga [1987]). Our results show that these subgenera are unrelated to each other. Subgenus *Panicum* is strongly supported as monophyletic, but subgenus *Dichanthelium* is polyphyletic. Subgenus *Dichanthelium* sect. *Dichanthelium* (represented by *P. sabulorum* and *P. koolauense*) is monophyletic and is unrelated to subgenus *Dichanthelium* sect. *Cordovensia* (represented by *P. ovuliferum*). Section *Dichanthelium* is sister to the large clade of C_4 species, the *Setaria*/*Urochloa*/*Panicum* clade, or basal to the $x = 9$ Clade, depending on whether the data are unweighted or weighted, respectively. *Panicum ovuliferum,* on the other hand, appears more closely related to *Echinochloa* (Fig. 1). Subgenus *Megathyrsus* includes only *Panicum* (= *Urochloa*) *maximum*; this species falls in the *Urochloa* clade and is unrelated to other species of *Panicum.* Subgenus *Agrostoides* is represented by *P. bulbosum,* which falls in the *Setaria* clade, and subgenus *Phanopyrum* (represented by *P. millegrana*) is sister to *Sacciolepis.*

The $x = 10$ Paniceae form a robust clade (100% bootstrap, 12 nucleotide substitutions) that includes taxa with a base chromosome number of ten (Fig. 1). The only known exception is *Streptostachys ramosa* with $x = 9$. The $x = 10$ Paniceae share a 6-bp insertion (coordinate 101 735) that represents a synapomorphy of the clade. *Thysanolaena* contains a 6-bp insertion at the same position but the inserted nucleotides are completely different (Table 2).

Phylogenetic relationships among the terminal taxa within the $x = 10$ Paniceae are not fully resolved, but many small groups are well supported. A modest 72% bootstrap value and three base substitutions support the inclusion of *Altoparadisium, Arthropogon, Homolepis, Mesosetum, Panicum euprepes* (subg. *Phanopyrum,* sect. *Lorea*), *P. prionitis* (subg. *Agrostoides,* sect. *Prionitia*), *Streptostachys ramosa,* and *Tatianyx* in a single clade. Members of the clade have little in common in terms of morphology or ecology, so we refer to them as the ''Ambiguous Clade.'' Several species pairs are strongly supported as sisters, but relationships among the pairs are unclear. In all cases, *Homolepis* (C_3) is monophyletic, with two species represented (*H. glutinosa* and *H. isocalycia*) (99% bootstrap and nine common mutations). *Arthropogon* is paraphyletic; *A. villosus* and *Altoparadisium* are in a highly supported monophyletic clade (100% bootstrap, 18 nucleotide substitutions), while *Arthropogon lanceolatus* is together with *Panicum euprepes* and *P. prionitis* in a well-supported clade (87% bootstrap, 4 bp substitutions). *Mesosetum* and *Tatianyx* are sister taxa with ten mutations and 99% bootstrap support.

Panicum laxum (subg. *Phanopyrum,* sect. *Laxa*), *Steinchisma hians, Plagiantha, Hymenachne, Otachyrium* (all C_3), and *Leptocoryphium* (C_4), form a second well-supported clade (89% bootstrap, five mutations; Fig. 1) within the $x = 10$ Paniceae. Internal branches in this clade are strongly supported in bootstrap analyses, and *Leptocoryphium* is basal. Species of the *Hymenachne* to *Steinchisma* clade share a 9-bp deletion


Thysanolaena maxima 11 / 3
Danthoniopsis dinteri (13) 9 / 4
Danthoniopsis petiolata 9 / 4
Zeugites pittieri 2n = 46 / 3
Chasmanthium laxum subsp. sessiliflorum 6 / 3
Chasmanthium latifolium 6 / 3
Arundinella hirta 7, 10, 12, 14 / 4
Elionurus muticus 5, 10 / 4
Zea mays 10 / 4
Tripsacum dactyloides 9, 10 / 4
Chionachne koenigii 10 / 4
Ischaemum afrum 10 / 4
Chrysopogon fulvus 5, 10 / 4
Phacelurus digitatus 10 / 4
Coelorachis selloana 9 / 4
Microstegium nudum 10 / 4
Miscanthus japonicus 19 / 4
Cleistachne sorghoides (1) 9 / 4
Sorghum bicolor (2) 5, 10 / 4
Apluda mutica 10 / 4
Coix aquatica 5, 10 / 4
Cymbopogon flexuosus (2) 5, 10 / 4
Heteropogon contortus (8) 10, 11 / 4
Capillipedium parviflorum 10 / 4
Bothriochloa bladhii 10 / 4
Dichanthium aristatum 10 / 4
Schizachyrium scoparium 5, 10 / 4
Andropogon gerardii 5, 10 / 4
Hyparrhenia hirta 10, 15 / 4
Streptostachys ramosa 9 / 4
Panicum euprepes 10 / 3
Arthropogon lanceolatus ? / 3
Panicum prionitis 10 / 4
Homolepis isocalycia 10 / 3
Homolepis glutinosa 10 / 3
Mesosetum chaseae 8 / 4
Tatianyx arnacites 10 / 4
Altoparadisium chapadense (15) ? / 4
Arthropogon villosus ? / 4
Leptocoryphium lanatum 10 / 4
Hymenachne donacifolia 10 / 3
Plagiantha tenella 10 / 3
Otachyrium versicolor 10 / 3
Panicum laxum 10 / 3
Steinchisma hians 10 / 3
Ichnanthus pallens 10 / 3
Echinolaena inflexa 10 / 3
Panicum piauiense 10 / 3
Streptostachys asperifolia 10 / 3
Ophiochloa hydrolithica (7) ? / 4
Axonopus anceps 10 / 4
Axonopus fissifolius 10 / 4
Anthaenantiopsis rojasiana 10 / 4
Panicum obtusum 10 / 4
Paspalum vaginatum 10 / 4
Paspalum malacophyllum 10 / 4
Paspalum remotum (1) 10 / 4
Paspalum conspersum 10 / 4
Paspalum paniculatum 10 / 4
Thrasya glaziovii (10) 10 / 4
Thrasya petrosa 10 / 4
Paspalum conjugatum 10 / 4
Paspalum haumanii 10 / 4
Paspalum arundinellum 10 / 4
Paspalum wettsteinii 10 / 4
Digitaria radicosa 9 / 4
Digitaria setigera 9 / 4
Digitaria ciliaris 9 / 4
Panicum ovuliferum 9 / 3
Echinochloa colona (7) 9 / 4
Echinochloa frumentacea 9 / 4
Acroceras zizanioides 9 / 3
Oplismenus hirtellus 9, 10, 11 / 3
Lasiacis sorghoidea 9 / 3
Pseudechinolaena polystachya 9 / 3
Panicum millegrana 9 / 3
Sacciolepis indica 9 / 3
Panicum koolauense (2) 9 / 3
Panicum sabulorum 9 / 3
Panicum elephantipes 9 / 4
Panicum nephelophilum 9 / 4
Panicum fauriei 9 / 4
Panicum virgatum 9 / 4
Panicum rudgei 9 / 4
Panicum repens 9 / 4
Panicum pedersenii 9 / 4
Panicum aquaticum 9 / 4
Urochloa acuminata n = 13 / 4
Melinis repens 9 / 4
Urochloa plantaginea 9 / 4
Urochloa mutica 9 / 4
Urochloa maxima 8, 9 / 4
Chaetium bromoides 13 / 4
Eriochloa punctata 9 / 4
Pennisetum alopecuroides 11 / 4
Cenchrus ciliaris 9, 12 / 4
Pennisetum setaceum 9 / 4
Paspalidium geminatum 9 / 4
Stenotaphrum secundatum 9 / 4
Panicum bulbosum 9 / 4
Setaria viridis 9 / 4
Setaria lachnea 9 / 4
Setaria macrostachya (4) 9 / 4
Setaria sphacelata 9 / 4
Setaria palmifolia 9 / 4
Setaria geniculata 9 / 4
Setaria parviflora 9 / 4

(coordinate 101 739), which removes 4 bp of the $x = 10$ inserted sequence plus an additional 5 bp.

The third clade within the $x = 10$ Paniceae clade includes *Axonopus* (C_4) and *Paspalum* (C_4), in addition to *Anthaenantiopsis* (C_4), *Echinolaena* (C_3), *Ichnanthus* (C_3), *Ophiochloa* (C_4), *Panicum obtusum* (C_4) (subgenus *Agrostoides*, section *Obtusa*), *P. piauiense* (C_3) (subg. *Phanopyrum*, sect. *Stolonifera*), *Thrasya* (C_4), and *Streptostachys asperifolia* (C_3) (80% bootstrap, four substitutions; Fig. 1). *Ichnanthus* is the sister of this clade in one of the two possible topologies. There are three major lineages common to all topologies: (1) *Echinolaena* and *Panicum piauiense* (89% bootstrap, four mutations, C_3); (2) *Axonopus*, *Ophiochloa*, and *Streptostachys* (95% bootstrap, seven synapomorphies, C_3 and C_4). Within this clade, the two representatives of *Axonopus*, *A. anceps* and *A. fissifolius*, are placed with the monotypic genus *Ophiochloa* in a very strong clade (100% bootstrap, 18 mutations, C_4); these three taxa share a 6-bp insertion (coordinate number 102 065, Fig. 1, Table 2). (3) *Panicum obtusum*, *Anthaenantiopsis*, plus all species of *Paspalum* and *Thrasya* (100% bootstrap, 12 mutations, C_4). *Paspalum* forms a large paraphyletic group in which *Thrasya* is embedded (93% bootstrap, four mutations); however, *Thrasya*, represented by *T. petrosa* and *T. glaziovii*, is monophyletic (85% bootstrap, three mutations).

The *Panicum* species investigated fall in both the $x = 9$ (*Panicum* subg. *Panicum*, *Panicum* sect. *Dichanthelium*, *P. millegrana*, *P. bulbosum*, and *P. ovuliferum*) and $x = 10$ clades (*P. euprepes*, *P. laxum*, *P. obtusum*, *P. prionitis*, and *P. piauiense*). Forcing *Panicum* to be monophyletic in the traditional sense, including all the species under study, costs 116 extra steps relative to the most-parsimonious trees and 101 steps excluding *Panicum* (= *Urochloa*) *maximum*; both constraints are significantly different from the most-parsimonious topology ($P < 0.0001$).

Correlation with photosynthetic types—Optimization of photosynthetic pathway differs between the trees with equal weights and those with implied weights (Fig. 2A, B); the differences between the trees indicate our uncertainty about some aspects of photosynthetic evolution. Using the implied weights topology, the common ancestor of the panicoid clade appears as C_3 (Fig. 2B); in this case there are eight or nine origins of the C_4 pathway, with the exact number depending on the resolution within the Ambiguous Clade. Using the topology retrieved from the equally weighted analysis, the ancestral state is ambiguous (Fig. 2A). Multiple independent origins of the C_4 pathway are possible, but it is equally parsimonious to postulate a single origin of the C_4 pathway in the common ancestor of the subfamily. In the latter case, the single origin would be followed by multiple losses (reversals to C_3) and then several reversals of the reversals to arrive at the current C_4 condition. We prefer the hypothesis of multiple independent origins because it seems simpler than a hypothesis involving gain-loss-gain of C_4.

When all C_4 species of the subfamily Panicoideae were forced to be monophyletic, the shortest trees were 85 steps longer than the unconstrained trees with C_3 species basal on the tree; if *Danthoniopsis* was excluded from the subfamily, and the remaining C_4 species were constrained to form a clade, the trees were 73 steps longer. In both cases, the Templeton test (1983) indicated that the difference was highly significant ($P < 0.0001$). Within the C_4 constraint clade, species were grouped principally by basic chromosome number and several minor clades were still recognized. When the tree was constrained to match the photosynthetic classification of the Paniceae proposed by Brown (1977) (i.e., C_3, C_4 NADP-me, C_4 NAD-ME, and C_4 PCK species in four different clades), 75 extra steps were necessary to keep this hypothesis, and 72 steps if Andropogoneae and *Arundinella hirta* were included within the C_4 NADP-ME group, Subtribe 1. The molecular evidence thus argues strongly that the C_4 pathway is highly homoplasious.

Assuming that the Panicoideae are ancestrally C_3, C_4 photosynthesis has originated once at the base of the Andropogoneae. There are at least four origins in the $x = 10$ Paniceae clade (Fig. 2): (1) the clade including *Anthaenantiopsis*, *Panicum obtusum*, *Paspalum*, and *Thrasya*; (2) the *Axonopus-Ophiochloa* clade; (3) *Leptocoryphium*; and (4) the Ambiguous Clade. Optimization of C_3/C_4 characters on the basal branch is ambiguous. In the consensus tree from the equally weighted analysis, well-supported groups in the Ambiguous Clade collapse on the ancestral branch (Fig. 2A). Optimization over the two possible resolutions for this clade shows three independent origins for the C_4 pathway. In analyses under implied weights, the ancestral state of this clade is also ambiguous, and two or three origins are possible (Fig. 2B). In addition, *Steinchisma hians* is a C_3/C_4 intermediate (Brown and Brown, 1975; Morgan and Brown, 1979; Morgan, Brown, and Reger, 1980; Brown et al., 1985) and here is clearly derived from C_3 ancestors; there is no evidence that it represents a transition from C_3 to C_4.

Optimization of C_4 in the $x = 9$ Paniceae depends on the position of the *Digitaria* clade, which is basal in the equally weighted analyses (Fig. 2A), and embedded in the clade in analyses under implied weights (Fig. 2B). In the former analyses, there are three independent C_4 origins: (1) *Digitaria*; (2) *Echinochloa*; and (3) the large *Setaria*/*Urochloa*/*Panicum* clade. In analyses with implied weights, C_4 originates only twice in the $x = 9$ species—once in *Echinochloa* and once in the large clade of *Digitaria* plus the *Setaria/Urochloa/Panicum* clade.

Most C_4 Panicoideae use NADP-malic enzyme as a decarboxylating enzyme. Most NADP species have lost their outer bundle sheath and form agranal chloroplasts in the mestome sheath, located over the outer wall in centrifugal position; these characters thus appear in each origin of NADP-ME C_4 (Fig. 2). Two exceptions are *Panicum prionitis*, which has retained an outer bundle sheath similar to that in C_3 species

←

Fig. 1. One of the 27 128 most parsimonious trees ($L = 1472$) obtained with equally weighted characters by NONA, for panicoid grasses based on *ndhF* sequence data. Branch length refers to the number of nucleotide substitutions, shown above branches. Bootstrap values are given below each branch. When no number is provided, bootstrap support is less than <53%. Asterisks show branches that collapse on the consensus tree. Length mutations (indels) characterizing particular clades or taxa are shown according to code letters in Table 2. Squares represent synapomorphies or autapomorphies and circles represent homoplasies. First number following species names indicates base chromosome number (x) and the second number indicates photosynthetic pathway, based on data in Table 1. 3 = C_3, 4 = C_4.

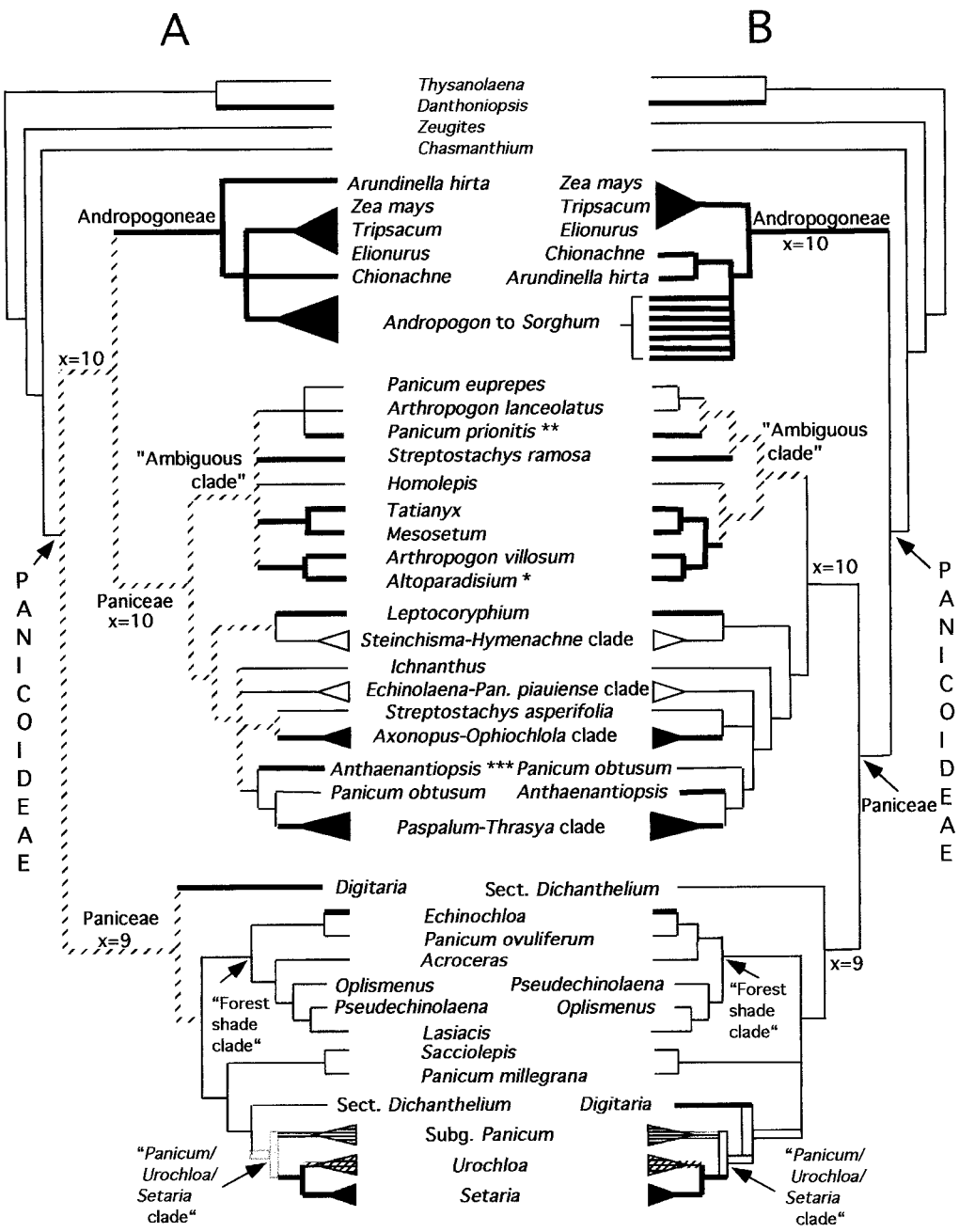

Fig. 2. Comparison of strict consensus trees. (A) Unweighted (NONA) analysis; (B) implied weight (Pee-Wee) analysis. Leaf structural and biochemical characters (Table 1) are optimized on trees. Key to line shadings and groups of taxa: *thin black lines* = C_3, two bundle sheaths, no predominant decarboxylating enzyme, bundle sheath chloroplasts absent, peripheral or slightly centrifugal, granal; *heavy black lines* = C_4, one bundle sheath, predominant enzyme NADP-ME, bundle sheath chloroplasts centrifugal, agranal (''classical NADP-ME type:'' * = isolated bundle sheath cells present; ** = one outer bundle sheath; *** = one or two remnant cells of the outer sheath present); *diagonally hatched lines* = ambiguous, C3 or C_4 NADP-ME; *cross hatching* (*Urochloa* clade) = C_4, two bundle sheaths, predominant enzyme PCK, bundle sheath chloroplasts centrifugal, granal (''classical PCK type''); *horizontal lines* (subgenus *Panicum*) = C_4, two bundle sheaths, predominant enzyme NAD-ME, chloroplast centrifugal or centripetal, granal (''classical NAD-ME type''); *black boxes* = C_4, two bundle sheaths, predominant decarboxylating enzyme ambiguous, bundle sheath chloroplasts centrifugal, granal; *white boxes* = C_4, two bundle sheaths, predominant enzyme NADP-ME, chloroplasts centrifugal, granal.

(Zuloaga, Morrone, and Dubcovsky, 1989), and *Anthaenantiopsis rojasiana*, with remnants of the outer sheath represented by one or two globose cells (Morrone et al., 1993). A few NADP-ME panicoids, including *Arundinella hirta* and *Altoparadisium chapadense* sampled here, have isolated bundle sheath cells in the mesophyll instead of minor veins; this anatomy has thus originated multiple times independently.

The only panicoids in this analysis that use NAD malic enzyme are species of *Panicum* subg. *Panicum*, which is strongly supported as monophyletic. Similarly, all species us-

ing PEP carboxykinase (PCK-type) are in a single well-supported clade. Each of these C_4 types thus originated only once. Both NAD-ME species and PCK species retain their outer bundle sheaths, a plesiomorphic character shared with their C_3 ancestors. Centripetal chloroplasts in the outer bundle sheath is a derived character that appears three times on internal branches of subgenus *Panicum.* The order of evolution of the three C_4 subtypes is uncertain and depends on the type of analysis done. However, if *Digitaria* is basal in the $x = 9$ Clade, then the ancestral decarboxylating enzyme is ambiguous (Fig. 2A). If *Digitaria* is sister to the *Setaria*/*Urochloa*/*Panicum* clade, a clade that includes the three C_4 subtypes, NADP-ME is optimized as ancestral (Fig. 2B). In this case, NAD-ME and PCK are derived from ancestors with the NADP-ME subtype.

DISCUSSION

DNA sequence data from the gene *ndhF* have allowed us to verify the monophyly of the subfamily Panicoideae and to demonstrate its division into three strongly supported clades. Our results thus expand on the work of Gómez-Martínez (1998) and Gómez-Martínez and Culham (2000), based on the chloroplast gene *trnL.* Gómez-Martínez was the first to establish that Paniceae are divided into two clades corresponding to chromosome number; these she correlated with biogeography and called ''the American Paniceae'' ($x = 10$) and ''the Pantropical Paniceae'' (most of the $x = 9$ species). Because the taxonomic sample presented here is biased toward the New World, additional samples from Africa, Asia, and Australia will be necessary to determine whether the distributional hypothesis is supported.

The sample of species presented here is considerably larger than any molecular study to date, but is still lacking several small tribes. We did not have material of tribes Isachneae, Steyermarkochloeae, Hubbardieae (a total of ~112 species, all C_3), nor of Paniceae subtribes Neurachninae (10 species, C_3 and C_4) or Spinificinae (8 species, C_4). Our sample of 59 genera represents slightly more than one-fourth of the total and our sample of species represents ~3% of the total. The results presented here thus reflect whatever limitations are imposed by the set of sequences; data collected since these analyses, however, continue to support the results outlined here (J. Barber, A. Doust, L. Giussani, K. Hiser, and E. Kellogg, University of Missouri-St. Louis, unpublished data).

Zuloaga, Morrone, and Giussani (2000) produced a morphological phylogeny of the Paniceae including >100 genera of the tribe. Their analysis could not test the monophyly of Paniceae because Andropogoneae were not included. The two basic chromosome numbers ($x = 9$ and $x = 10$) have several independent origins on the morphological tree rather than correlating with major clades, as in the molecular tree. (Note that chromosome number was not used in the phylogenetic analysis of Zuloaga, Morrone, and Giussani [2000] due to the lack of information in many genera.) The morphological phylogeny also did not clearly correlate with biogeography. The morphological phylogeny places C_3 species at the base of the tree and the C_4 NADP-ME genera in a derived monophyletic clade (Zuloaga, Morrone, and Giussani, 2000). This contrasts with the *ndhF* phylogeny, in which C_4 NADP-ME appears in multiple independent clades.

Both molecular phylogenies (*ndhF* and *trnL*) and the morphological phylogeny of Zuloaga, Morrone, and Giussani (2000) have several clades in common. All studies find that *Panicum* subg. *Panicum* is monophyletic, as is the group of genera grouped into the *Setaria* clade (*Setaria, Cenchrus, Pennisetum, Panicum bulbosum, Paspalidium,* and *Stenotaphrum,* the latter two genera not included in the *trnL* analysis). In the *ndhF* and the morphological phylogenies *Acroceras* and *Lasiacis* are part of the same clade, but are linked with *Cyrtococcum* and *Microcalamus* in the morphological study; these taxa were not included in the molecular data set. The clade consisting of *Eriochloa, Urochloa* (*Brachiaria* in Gómez-Martínez and Culham, 2000), and *Panicum* (= *Urochloa*) *maximum* appears in the three phylogenies, although the molecular phylogenies also place *Melinis* and *Chaetium* (in the *ndhF* trees) within the group. *Echinolaena* and *Panicum piauiense* (subg. *Phanopyrum* sect. *Stolonifera*) are closely related in the studies, although the genera with which they are associated differ.

Both Zuloaga, Morrone, and Giussani (2000) and the molecular data identify *Panicum* as a polyphyletic genus; within *Panicum,* subg. *Agrostoides* is also polyphyletic. Other genera, such as *Streptostachys* and *Arthropogon,* also appeared as polyphyletic in both studies (Zuloaga, Morrone, and Giussani, 2000).

Our analyses confirm the polyphyly of the tribe Arundinelleae. The taxa included for study (*Arundinella hirta, Danthoniopsis dinteri,* and *D. petiolata*) appear in different and distantly related clades (Fig. 1), with *Danthoniopsis* clearly placed in a basal polytomy with the outgroups. Previous studies (Mason-Gamer, Weil, and Kellogg, 1998; Spangler et al., 1999) have also indicated a polyphyletic origin of the Arundinelleae and the need of further studies to recircumscribe this tribe. Likewise, relationships between the subfamilies Panicoideae and Centothecoideae (the latter including Thysanolaeneae) are not resolved by our analyses and need further investigation.

Morphological correlates of molecularly defined clades—Members of the Forest Shade Clade—*Acroceras, Echinochloa, Lasiacis, Oplismenus, Panicum ovuliferum,* and *Pseudechinolaena*—all have lanceolate leaf blades and are associated with the forest shade environment (Davidse, 1978; Clayton and Renvoize, 1986; Zuloaga, Morrone, and Saenz, 1987). In addition, all taxa in this clade except *Echinochloa* use the C_3 photosynthetic pathway. The ligules of these species are membranaceous, their glumes are herbaceous, and their upper paleas and lemmas are crustaceous, with the margins of the upper lemma tucked into the palea; their primary inflorescence branches are more or less racemose, with the spikelets borne close together on short pedicels. *Acroceras* and *Echinochloa* both have a protuberance on the apex of the palea (perhaps an homologous character for both genera); they are not sisters in our analyses, but the placement of *Acroceras* in the clade is ambiguous. *Echinochloa* is C_4 NADP-ME subtype, with a single bundle sheath.

The *Setaria* clade includes species of *Cenchrus, Paspalidium, Pennisetum, Setaria,* and *Stenotaphrum,* all of which have setae or bristles in the inflorescences, the sole exception being *Panicum bulbosum.* These bristles vary in position and development, with some terminating branches, and others apparently representing modified pedicellate spikelets (Webster, 1988, 1992). Previous authors (e.g., Clayton and Renvoize, 1986) had thought that bristles were not homologous among members of this group, but our molecular data and that of

Gómez-Martinez and Culham (2000) suggest that bristles had a single evolutionary origin.

Setaria is one of the genera with the largest taxonomic sampling, in part because it includes >100 species distributed worldwide. Although the genus is easily recognized because of its bristles that persist on the rachis when the spikelet falls, our study indicates that this genus is paraphyletic. Of the species investigated, five of them (*S. macrostachya, S. parviflora, S. palmifolia, S. sphacelata,* and *S. geniculata*) form a monophyletic subclade. *Setaria viridis* and *S. lachnea* are sisters and together form a clade with *Panicum bulbosum*. The relationship between *Pennisetum* and *Cenchrus* is not surprising in that both have smooth cartilaginaceous to membranous paleas and lemmas, and the dispersal unit is the spikelet-bristle combination (i.e., bristles fall from the rachis along with the spikelets).

All taxa in the *Urochloa* clade (*Chaetium, Eriochloa, Panicum* (= *Urochloa*) *maximum, Melinis* and *Urochloa*) use the PCK subtype of the C_4 photosynthetic pathway, and have the classical morphological correlates of the pathway (Brown, 1977; Watson and Dallwitz, 1992), although there are no other morphological characters that distinguish the clade. Although most species have a base chromosome number of $x = 9$, *Chaetium bromoides* has $x = 13$, an unusual chromosome number within the Paniceae. The paraphyletic *Urochloa* also appears to have undergone an active process of chromosome evolution, with the chromosome number of *Panicum* (= *Urochloa*) *maximum* varying from $x = 9$ to $x = 8$ or 7. *Urochloa acuminata,* which appears basal in the clade, also has $x = 13$ (Morrone et al., 1995).

Panicum subg. *Panicum* is a robust monophyletic unit. (Here and elsewhere in this discussion, the classification of *Panicum* follows Zuloaga, 1987). Physiologically, the group is characterized by the principal activity of NAD-ME enzyme during photosynthesis (see above under ***Correlation with photosynthetic pathways***), and several species have centripetal chloroplasts in the outer part of the parenchymatous sheath. Morphologically, this subgenus includes caespitose plants with membranous-ciliate ligules, linear to linear-lanceolate blades, open and lax inflorescences, spikelets with crustaceous upper lemmas and paleas, and the apex of the palea with simple or compound papillae.

Panicum subg. *Dichanthelium* section *Dichanthelium,* represented by two species (*P. sabulorum* and *P. koolauense*), is sister to the large *Setaria*/*Urochloa*/*Panicum* clade in the equally weighted trees or basal to the $x = 9$ Paniceae clade in the implied weighted trees. The two species are C_3, with foliar dimorphism usually present, and have upper lemmas and paleas covered with simple papillae.

The sister taxon relationship of *Panicum millegrana* (*P.* subg. *Phanopyrum* sect. *Monticola*) and *Sacciolepis indica* is one of several surprises in this study. Both species are C_3, but this is the ancestral condition and does not indicate relationship; they differ by the presence of a spiciform panicle in *Sacciolepis,* with a gibbous upper glume and a smooth upper floret. *Panicum millegrana* bears open and lax panicles, nonswollen upper glumes, and upper florets that are transversely rugose. We have been unable to find any morphological characters shared by the two species.

Digitaria is clearly monophyletic in these analyses, but its position is uncertain, whether sister to all $x = 9$ Paniceae (in equally weighted trees) or sister to the *Setaria*/*Urochloa*/*Panicum* clade (in implied weighted analysis). *Digitaria* species share the C_4 NADP-ME subtype and have only one bundle sheath. *Digitaria* is also characterized by dorsiventrally compressed spikelets with the lower glume reduced, the lower flower absent, the upper lemma and palea cartilaginous.

The clade that includes *Altoparadisium, Arthropogon, Homolepis, Mesosetum, Panicum euprepes, P. prionitis, Streptostachys ramosa,* and *Tatianyx* (the Ambiguous Clade), includes considerable morphological, anatomical, and physiological diversity. These taxa have not been placed together in previous classifications and few obvious morphological features unite them. Some are C_3 with standard C_3 vein spacing and double bundle sheaths, whereas others are C_4 NADP-ME with close vein spacing and variously reduced outer bundle sheaths. *Homolepis* is monophyletic, with the two species investigated having glumes that are equal in length, cartilaginous upper lemmas and paleas covered by silica bodies, and linear hila that extend half the length of the caryopsis (Zuloaga and Soderstrom, 1985). Two of the three species of the recently recircumscribed *Arthropogon* (Filgueiras et al., 2001) are represented here and appear in different clades. *Arthropogon lanceolatus* is grouped with *Panicum euprepes* and *P. prionitis,* while *A. villosus,* a C_4 NADP-ME species, is related to the recently established genus *Altoparadisium* (Filgueiras et al., 2001). *Arthropogon lanceolatus* is a C_3 species with fusoid cells, aristate lower glume, and cartilaginous upper lemma and palea; *P. euprepes* is also C_3 but with stiff and sharp-pointed leaf blades, and open, lax panicles, while *P. prionitis* is C_4 with flat leaves, keeled blades and open panicles.

Species in the clade including *Anthaenantiopsis, Axonopus, Echinolaena, Ichnanthus, Ophiochloa, Panicum piauiense, P. obtusum, Paspalum, Streptostachys asperifolia,* and *Thrasya* share an inflorescence pattern in which the spikelets are arranged in unilateral branches. Most genera included in this large clade are native to America. *Streptostachys asperifolia,* a C_3 non-Kranz species, is sister to the *Axonopus-Ophiochloa* clade. *Axonopus* and the monotypic genus *Ophiochloa* are C_4 ''classical NADP-ME'' type species. In some trees, *Axonopus* appears to be paraphyletic with *Ophiochloa* derived from within it. However, their relationships are not resolved in the consensus trees and *Ophiochloa* differs by nine mutations. *Ophiochloa* differs from *Axonopus* by its hyaline upper floret with the upper palea free at the apex, lower lemma free from the upper glume, and one raceme per inflorescence (Filgueiras, Davidse, and Zuloaga, 1993).

Paspalum is, after *Panicum,* the second largest genus of the Paniceae, with ~320 species. In our study, this genus is represented by nine species belonging to seven informal groups, which include a wide range of morphological features and geographic distribution. These species form a well-supported clade with representatives of *Thrasya* (Fig. 1). *Paspalum* is a paraphyletic assemblage, with two species of *Thrasya* embedded within it. Both genera have unilateral racemes with the lower glume absent or reduced. Additionally, several species of the *Paspalum* group *Decumbentes* are morphologically intermediate between the two genera, with the lower glume always or variably present and varying in length within the same inflorescence, the pedicels partly adnate to the rachis, and the lower lemma coriaceous and sulcate in the middle portion.

From the major clade composed by *Hymenachne, Leptocoryphium, Otachyrium, Panicum laxum, Plagiantha,* and *Steinchisma hians,* the last four genera form a well-supported subclade characterized by an expanded lower palea. Additionally, *Panicum laxum, Plagiantha,* and *Steinchisma* are linked

in another subclade with all three entities having verrucose papillae all over the upper lemma and palea, although this character is not constant in all specimens of *Panicum laxum.*

Implications for classification—Brown (1977) classified the Paniceae based on photosynthetic pathway, dividing it into four major groups. His subtribe 1 included all NADP-ME species, here shown to be polyphyletic. Brown's subtribe 2 included all C_3 species, which he interpreted as basal, and the group from which all other members of the tribe originated. We agree with Brown's point of view, as it is the ancestral condition in the implied weighted trees, and we consider it the most probable state for the ancestor of the panicoid grasses (see below under ***Evolution of C_4 photosynthetic pathways within panicoid grasses***), although the ancestral state is ambiguous in the equally weighted trees. Brown's subtribes 3 and 4 corresponded to the *Urochloa* group (all with the PCK subtype of C_4) and *Panicum* subg. *Panicum* (all with NAD-ME subtype); we have confirmed that these are monophyletic.

Clayton and Renvoize (1986) recognized seven subtribes in Paniceae, based on several exomorphological and anatomical characters. Of the seven, only subtribe Cenchrinae is monophyletic on the molecular tree, although *Cenchrus* and *Pennisetum* were the only genera of the subtribe included. Their intuitive diagram of relationships among genera of the subtribe Setariinae showed the genera organized according to photosynthetic pathway; taxa are grouped largely without regard to chromosome numbers. Nonetheless, their groups of *Otachyrium-Plagiantha-Steinchisma, Setaria-Paspalidium-Stenotaphrum, Paspalum-Thrasya,* and *Urochloa-Eriochloa* correspond to clades in the molecular analyses. The latter group also includes *Brachiaria* s.s.; although we did not include any species of *Brachiaria* s.s. in this analysis, *Urochloa mutica* was treated as *Brachiaria mutica* by Clayton and Renvoize (1986). Clayton and Renvoize (1986) suggested *Panicum* as a possible ancestor from which all Setariinae emerged. Our results, in contrast, show *Panicum* in many places over the tree, with many of these species associated with other genera of Paniceae (see discussion of *Panicum*).

Streptostachys ramosa appears in the $x = 10$ Paniceae clade, although the chromosome count (Davidse and Pohl, 1978) shows that this species is $x = 9$. *Streptostachys ramosa* is separated from the type species of the genus, *S. asperifolia,* a species with a basic chromosome number of $x = 10$ (Morrone et al., 1995), the latter in a well-supported clade together with *Ophiochloa* and *Axonopus.* Morrone and Zuloaga (1991) have pointed out the differences between *S. asperifolia* and the other species of the genus, *S. ramosa* and *S. macrantha,* but they did not make any decision about the taxonomic position of these taxa. Our data suggest that the genus should be split into two, with *S. ramosa* placed in another genus.

Our data show that *Paspalum* is paraphyletic and includes the genus *Thrasya.* To maintain a monophyletic *Paspalum,* therefore, the clade must either be divided into smaller units or *Thrasya* must be merged with *Paspalum.* The latter possibility has already been suggested by morphological studies (Trillo and Rúa, 1999; S. S. Aliscioni, unpublished data).

The *ndhF* data support segregation of *Steinchisma* from *Panicum* (Renvoize, 1988, 1998; Zuloaga et al., 1998) and its close relationship with *Plagiantha* (already established by Zuloaga et al., 1998). The similarity between *Panicum laxum* and *Steinchisma* needs to be tested with addition of more species of subg. *Phanopyrum* sect. *Laxa* of *Panicum.* Species of sect. *Laxa* have been crossed with those of Steinchisma (Brown et al., 1985), suggesting a close relationship between the two groups. It is also remarkable that *Hymenachne* and *Sacciolepis,* two genera usually cited as closely related in the tribe (Pohl and Lersten, 1975), appeared in this analysis in two clearly distinguished clades.

Zuloaga and Soderstrom (1985) removed two species from *Panicum* and included them in the genus *Homolepis,* a conclusion not followed by Clayton and Renvoize (1986) and Renvoize (1998). In this study, one of these two species was included (*H. glutinosa* = *Panicum glutinosum*), and its position as sister with *H. isocalycia* supports its segregation as *Homolepis.*

Brown (1977) placed *Brachiaria, Urochloa,* and *Eriochloa* in a natural group, supported by their PCK physiology. Many of the species of *Brachiaria* included by Brown were later transferred to *Urochloa.* Brown suggested that *Panicum maximum,* the only species of *Panicum* with PCK physiology, should be included within the *Brachiaria* group. Most genera of this C_4 PCK-type group have rough, transversely rugose lemmas and primary inflorescence branches with spikelets on short pedicels, although *U. maxima* and the *Fasciculata* group of *Urochloa* do not fit this general description. Morrone and Zuloaga (1992, 1993), following Webster (1987, 1988) transferred American species of *Panicum* with the PCK syndrome to *Urochloa,* a conclusion strongly supported by the molecular data.

Most genera currently accepted within Paniceae, such as *Acroceras, Brachiaria, Digitaria, Homolepis, Ichnanthus, Otachyrium,* and *Urochloa,* among others, have been gradually segregated from *Panicum,* starting with the pioneering papers of Chase (1906, 1908a, b, 1911), and the relationships of this genus with many other taxa are highlighted in the intuitive evolutionary diagrams of Clayton and Renvoize (1986). In spite of the continuous segregation of genera, *Panicum* is still polyphyletic. *Panicum* subg. *Panicum* and sect. *Dichanthelium* are the only taxa that are monophyletic. *Panicum maximum,* previously classified by Zuloaga (1987) as *P.* subg. *Megathyrsus,* is found within the *Urochloa* clade, and therefore in this paper it is treated as *U. maxima,* as suggested by Webster (1987); this species is characterized by a transversely rugose upper lemma and palea. Species of *Panicum* subg. *Dichanthelium* are distributed in two different clades, with sect. *Dichanthelium* (represented here by *P. sabulorum* and *P. koolauense*) forming an isolated but well-supported clade, unrelated to *P. ovuliferum* (sect. *Cordovensia*), which is close to *Acroceras* and *Echinochloa. Panicum bulbosum,* which belongs to *P.* subg. *Agrostoides* sect. *Bulbosa* (Zuloaga, 1987), is within the *Setaria* clade. The other species of subgenus *Agrostoides, P. prionitis* of sect. *Prionitia,* appears in the $x = 10$ Paniceae clade, related to *P. euprepes* (sect. *Lorea,* subg. *Phanopyrum*) and *Arthropogon lanceolatus.* In turn, *Panicum piauiense* (subg. *Phanopyrum* sect. *Stolonifera*) forms a clade with the genus *Echinolaena. Panicum obtusum* is distantly related to other NADP-ME taxa, such as *Thrasya, Paspalum,* and *Anthaenantiopsis.* Also, *Panicum laxum,* of subg. *Phanopyrum* section *Laxa,* is grouped with *Steinchisma hians* in a strongly supported clade related to *Plagiantha, Hymenachne,* and *Otachyrium.*

These results suggest that the name *Panicum* should be restricted to subgenus *Panicum. Panicum maximum* should be placed in *Urochloa, Dichanthelium* can be raised from the subgeneric to generic level (although the position of section *Cor-*

dovensia is still doubtful), and subgenera *Agrostoides* and *Phanopyrum* split into several small subunits. More species of the latter three subgenera will need to be sampled to reach firm conclusions about the taxonomic position of taxa of this difficult and complex genus.

Evolution of C_4 photosynthetic pathways within panicoid grasses—The number of C_4 origins in Panicoideae is remarkable given the apparent complexity of the pathway. C_4 photosynthesis requires numerous biochemical and anatomical modifications of the plant, apparently involving multiple genetic changes, although, as far as is known, no ''new'' genes or proteins are involved in C_4. The enzymes used are all housekeeping enzymes whose regulation is altered in a tissue-specific manner (Gutiérrez, Gracen, and Edwards, 1974; Prendergast, Hattersley, and Stone, 1987; Sinha and Kellogg, 1996).

Available data on photosynthetic pathway are remarkably good for this group of species. All of the species have been assigned to photosynthetic type based on anatomical criteria. In addition, all outgroup taxa and 63 of the 103 ingroup taxa (61%) have been identified as C_3 or C_4 using either the ratio of ^{13}C to ^{12}C ($\partial^{13}C$), biochemical assays for decarboxylating enzymes, estimates of CO_2 compensation point, immunolocalization of photosynthetic enzymes (see references in Table 1), or some combination of these. These taxa are among those used to establish the strong correlations of photosynthetic pathway with leaf anatomy. Although Brown and Hattersley (1989) postulated that C_4 anatomy might have appeared before C_4 photosynthesis, we see no evidence for this. The one C_3/C_4 intermediate species in our analysis, *Steinchisma hians,* is not sister to a C_4 clade, as we would expect if it were a step along the evolutionary pathway of C_4.

Our data suggest that C_3 photosynthesis is the ancestral condition among panicoid grasses and that C_4 arose at least eight times. The alternative, however, that C_4 arose once, was lost multiple times, and then was regained, is as parsimonious or nearly so. (Note that the exact number of origins could also change slightly if the C_4 subtribes Neurachninae and Spinificinae were included.) In either case, the pathway is highly labile in the subfamily and apparently easy to modify in evolutionary time. The precise number of origins depends on some branches that are poorly supported in this analysis and on the resolution of some polytomies. Despite these caveats, and no matter how ambiguities in our tree are resolved, the character is homoplasious.

Most C_4 panicoids use NADP-ME as a decarboxylating enzyme. These species are similar in having specialized, centrifugally placed chloroplasts in the bundle sheath, without well-developed grana, and having lost the parenchymatous outer sheath around the vascular bundles (''classical NADP-ME type''). However, some NADP-ME species present deviations from this pattern, i.e., *Panicum prionitis* preserves the outer bundle sheath, while *Altoparadisium, Anthaenantiopsis,* and *Arundinella,* among others, have one or two cells that are remnants of the outer sheath. The C_4 ''classical PCK'' and ''classical NAD-ME'' types, and other variants like the ''PCK-like NAD-ME'' type, only appear in the $x = 9$ Paniceae, along with ''classical NADP-ME'' species. Our analyses do not resolve the order of evolution of the three types. In the trees from equally weighted characters, it is not clear which type was first derived from C_3 ancestors. In the trees constructed using implied weights (Fig. 2, right side), PCK and NAD-ME species are derived from an NADP-ME ancestor; this optimization is forced by the position of *Digitaria* sister to the *Setaria*/*Urochloa*/*Panicum* clade. This hypothetical C_4 ancestor would have also had centrifugal chloroplasts with well-developed grana and two bundle sheaths (Fig. 2). Although C_3 species generally have few nonspecialized chloroplasts in the parenchymatous bundle sheath or no chloroplasts at all (Ellis, 1977), the chloroplasts, when present, are slightly disposed towards the outside walls of the cells and the intercellular space (N. Dengler, University of Toronto, personal communication). This attribute could represent an homologous state with that of the inferred C_4 ancestor.

The inferred C_4 ancestral combination of biochemical and structural features has never been found in extant Panicoideae. In the grasses, the characters occur together only in the non-panicoid tribe Eriachneae, in just five species of *Eriachne* and *Pheidochloa gracilis* S. T. Blake (Eriachneae) (Prendergast, Hattersley, and Stone, 1987). Either our findings suggest novel character combinations for the panicoids, or the simple parsimony optimization methods produce results that are not biologically realistic.

The distinctions among NAD-ME, PCK, and NADP-ME biochemistry are not as marked as might appear. The enyzme PCK is only active in species classified as PCK-type, and has almost no detectable activity in ''classical NAD-ME'' and ''classical NADP-ME'' species, and NADP-ME activity is very low in PCK species (Gutiérrez, Gracen, and Edwards, 1974; Prendergast, Hattersley, and Stone, 1987). However, NAD-ME is active not only in species classed as NAD-ME, but also in PCK (Watson and Dallwitz, 1992; Sinha and Kellogg, 1996) and in NADP-ME species (Gutiérrez, Gracen, and Edwards, 1974; Prendergast, Hattersley, and Stone, 1987). In other words, the C_4 subtypes do not actually reflect absolute distinctions in decarboxylating enzyme activity.

Malate is the major C_4 acid formed in the mesophyll of C_4 NADP-ME species, and aspartate is mostly present in PCK and NAD-ME. However, both products are detected in C_3 and all C_4 species, although activity of aspartate and alanine aminotransferases differ significantly among pathways, cells, and organelles (Leegood, 1997).

The ''classical PCK'' species seem to derive, biochemically, from a C_4 NADP-ME ancestor, which corresponds to the optimization in the weighted tree topology (Fig. 2). Such an evolutionary pathway requires a ''switch on'' for the activity of PCK and a simultaneous ''switch off'' for the activity of NADP-ME. *Alloteropsis semialata,* an $x = 9$ species (Watson and Dallwitz, 1992), is the only known panicoid with predominant activity of PCK along with a single mestome sheath (similar to the ''classical NADP-ME'' type; Prendergast, Hattersley, and Stone, 1987). The species might thus represent a transition between NADP-ME and PCK physiology. Similarly, the genus *Chaetium* contains three species, one PCK and the other two NADP-ME. *Chaetium bromoides,* the PCK species, is placed in the PCK clade (the *Urochloa* clade) by our data. The NADP-ME species, *C. cubanum* (Wright) Hitchc. and *C. festucoides* Nees, have distinctive cells similar to *Neurachne* (Brown, 1977; Renvoize, 1987; Morrone et al., 1998) and could represent a link with the supposed NADP-ME ancestor. However, until the positions of *Alloteropsis semialata* and the NADP-ME species of *Chaetium* are determined, these hypotheses remain uncertain.

As shown by Monson (1999), C_4 genes are related to and likely derived from C_3 housekeeping genes. Duplication may be one common mechanism that could generate a new meta-

bolic function for genes. Additional nuclear and organelle gene sequencing and optimization of physiological and biochemical characters on the phylogeny will help to deepen understanding of the evolution of the C_4 pathways and its subtypes among the panicoid grasses. It will be useful to include more representatives of the tribe Paniceae, particularly taxa with deviation from the classical types (i.e., *Neurachneae*), with different pathways among species of the same genus (i.e., *Alloteropsis*), or different C_4 subtypes (i.e., *Chaetium*), as well as the classical representatives of the C_4 subtypes (i.e., *Arthragrostis, Yakirra*) and C_3 genera (i.e., *Entolasia, Ichnanthus*).

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