



Complex Transitions between C3 and C4 Photosynthesis during the Evolution of Paniceae: A Phylogenetic Case Study Emphasizing the Position of *Steinchisma hians* (Poaceae), a C3-C4 Intermediate

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COMPLEX TRANSITIONS BETWEEN C₃ AND C₄ PHOTOSYNTHESIS DURING THE EVOLUTION OF PANICEAE: A PHYLOGENETIC CASE STUDY EMPHASIZING THE POSITION OF *STEINCHISMA HIANS* (POACEAE), A C₃-C₄ INTERMEDIATE

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A two-tiered, nested molecular phylogenetic study of panicoid grasses to explore character state transitions between the C₃ and C₄ adaptive syndromes is presented. A broad survey of 92 panicoid species was sampled for the grass-specific insert sequence in the chloroplast RNA polymerase locus (*rpoC2*), combining published and unpublished sequences. This portion of the study also included an intensive phylogenetic investigation of one clade of seven species that included *Steinchisma hians*, which is notable for exhibiting intermediacy between the C₃ and C₄ photosynthetic types. Both *rpoC2* data and previously published sequences of the F subunit of an NADH-dependent dehydrogenase were analyzed together for this small group. A rigorous phylogenetic investigation of *S. hians* and 13 other species of Panicoideae included in the broad survey was then performed with sequences of both *rpoC2* and the externally transcribed spacer region of the nuclear ribosomal repeat. These 14 species were selected to maximize representation among photosynthetic subtypes. Combined analysis resolved single origins of two photosynthetic subtypes. A reversion of C₄ to C₃ photosynthesis during the evolution of the lineage that includes *S. hians* is identified. These and other recent results indicate that repeated reversions from C₄ to C₃ have occurred. The C₃ species *Panicum laxum* has a strongly supported sister group relationship to *S. hians* (C₃-C₄). The most parsimonious interpretation is that *S. hians* represents an incipient reversal from C₃ to C₄ photosynthesis, beginning with the capacity to compartmentalize photorespiratory metabolism in the bundle sheath tissue.

Keywords: C₃-C₄ intermediacy, carbon metabolism, ETS, external transcribed spacer, NAD-ME, panic grass, Paniceae, PCK, photosynthesis, *rpoC2*.

Introduction

The origin and evolution of complex adaptations, such as C₄ photosynthesis, are of special interest because natural selection must simultaneously operate on apparently unrelated characters for the complex to arise. In the case of C₄ photosynthesis, there is a synchrony of anatomical organization, physiological specialization, and tissue-specific regulation of gene expression. The complexity of the C₄ photosynthetic syndrome argues against multiple independent origins, yet our best phylogenetic efforts to date indicate that it has arisen at least 31 separate times among 18 diverse families of angiosperms and at least four times within the grasses (summarized in Sinha and Kellogg 1996; Kellogg 1999).

The photosynthetic physiological type is invariant within virtually all taxonomic families of angiosperms as well as within individual subfamilies of grasses *sensu* GPWG (2001) (Kellogg 1999). In this regard, the group commonly known as the “panic grasses,” which are classified in the tribe Paniceae, is exceptional because much of the photosynthetic diversity within Poaceae is concentrated in this tribe. Paniceae is uniquely diverse with both C₃ and C₄ species and eight sub-

types of the latter, each documented for multiple taxa (Hattersley 1987; Hattersley and Watson 1992).

Plants that show intermediacy between the C₃ and C₄ photosynthetic conditions are of interest because they contribute to our understanding of the evolution of the C₄ pathway (Kopriva et al. 1996; Monson 1999). These species may represent either nascent independent origins of C₄ photosynthesis or reversals to the C₃ condition from C₄ ancestors. *Steinchisma hians* (Elliot) Nash (synonyms: *Panicum hians* Elliott and *Panicum milioides* Nees ex Trin.) exhibits integrated ultrastructural and biochemical adaptations, resulting in apparent photorespiration rates that are lower than those of closely related C₃ plants, such as *Panicum laxum* Swartz (Brown and Hattersley 1989; Rawsthorne and Bauwe 1997; Monson 1999). More specifically, *S. hians* is an example of a C₃-C₄ intermediate species with strategic arrangements of chloroplasts and mitochondria in the bundle sheath and compartmentalization of glycine decarboxylase in this tissue. These adaptations allow efficient internal recycling of photorespiratory CO₂ through ribulose biphosphate carboxylase/oxygenase (Holbrook et al. 1985; Hylton et al. 1988). The confinement of photorespiratory glycine decarboxylation to the bundle sheath has previously been postulated as a primary event in the evolution of plants toward adopting the C₄ pathway (Rawsthorne 1992; Monson 1999).

We conducted a molecular phylogenetic investigation of the evolutionary position of the C₃-C₄ intermediate *S. hians* among

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Table 1

**Species Included in the Broad Phylogenetic Survey of *rpoC2* in Panicoideae
Together with Voucher and GenBank Accession Numbers**

Species	Voucher (herbarium code) ^a	GenBank accession number
<i>Altoparisidium chapadense</i> ^b		AF372160
<i>Anthaenantiopsis rojasiana</i>	FZ 6747 (SI)	AY174472 ^c
<i>Arthropogon lanceolatus</i> ^b		AF372161
<i>Arthropogon villosus</i>	TF s.n. (SI)	AY174473 ^c
<i>Arundinella hirta</i>	PI 246756	AY174474 ^c
<i>Axonopus fissifolius</i>	RGM 961	AY254806 ^c
<i>Brachiaria mutica</i> ^b		AF372162
<i>Brachiaria serrata</i> ^b		AF000019
<i>Cenchrus agrimonioides</i> ^b		AF372165
<i>Cenchrus ciliaris</i> ^b		AF372164
<i>Cenchrus echinatus</i> ^b		AF372163
<i>Chaetium bromoides</i> ^b		AF372166
<i>Chasmanthium latifolium</i> ^b		U94334
<i>Chionachne koenigii</i>	RS 97–18 (GH)	AY174475 ^c
<i>Coix lachryma-jobi</i>	BK s.n. (GH)	AY174476 ^c
<i>Cymbopogon citratus</i>	KB s.n. (GH)	AY174477 ^c
<i>Danthoniopsis dinteri</i> ^b		AF372167
<i>Digitaria ciliaris</i> ^b		AF372170
<i>Digitaria insularis</i> ^b		AF372173
<i>Digitaria radicata</i> ^b		AF372168
<i>Digitaria setigera</i> ^b		AF372171
<i>Digitaria violascens</i> ^b		AF372172
<i>Echinochloa colona</i> ^b		AF372175
<i>Echinochloa crus-galli</i> ^b		AF372174
<i>Echinolaena inflexa</i> ^b		AF372176
<i>Eriachne mucronata</i>	SJ 9719 (NSW)	AY174478 ^c
<i>Eriochloa punctata</i> ^b		AF372177
<i>Homolepis glutinosa</i>	FZ 6799 (SI)	AY174479 ^c
<i>Homolepis isocalycia</i>	FZ 6938 (SI)	AY174480 ^c
<i>Hymenachne donacifolia</i>	OM s.n. (SI)	AY174481 ^c
<i>Hyparrhenia hirta</i> ^b		U96320
<i>Ichnanthus pallens</i>	FZ 6869 (SI)	AY174482 ^c
<i>Isachne distichophylla</i>	CM 1227 (HAW)	AY174483 ^c
<i>Lasiacis sorghoidea</i>	FZ 6762 (SI)	AY174484 ^c
<i>Leptocoryphium lanatum</i> ^b		AF372178
<i>Melinis minutiflora</i> ^b		AF372179
<i>Melinis</i> (= <i>Rhynchelytrum</i>) <i>repens</i> ^b		AF000020
<i>Mesosetum chaseae</i> ^b		AF372178
<i>Ophiochloa hydrolithica</i> ^b		AF372181
<i>Oplismenus hirtellus</i>	LC s.n. (ISC)	AY174485 ^c
<i>Otachyrium versicolor</i> ^b		AF372182
<i>Panicum aquaticum</i>	FZ 6967 (SI)	AY174486 ^c
<i>Panicum koolauense</i> ^b		AF372169
<i>Panicum elephantipes</i> ^b		AF372193
<i>Panicum euprepes</i>	FZ s.n. (SI)	AY174487 ^c
<i>Panicum fauriei</i> ^b		AF372188
<i>Panicum laxum</i> ^b		AF372184
<i>Panicum miliaceum</i>	MD s.n. (DEK)	AY174488 ^c
<i>Panicum millegrana</i> ^b		AF372191
<i>Panicum mystasipum</i> ^b		AF372192
<i>Panicum nephelophilum</i> ^b		AF372189
<i>Panicum ovuliferum</i> ^b		AF372195
<i>Panicum pedersenii</i> ^b		AF372194
<i>Panicum prionitis</i> ^b		AF372196
<i>Panicum repens</i> ^b		AF372185
<i>Panicum rudgei</i>	FZ 6985 (SI)	AY174489 ^c
<i>Panicum stolonifera</i>	FZ s.n. (SI)	AY174490 ^c

Table 1
(Continued)

Species	Voucher (herbarium code) ^a	GenBank accession number
<i>Panicum tenuifolium</i> ^b		AF372199
<i>Paspalidium geminatum</i> ^b		AF372190
<i>Paspalum conjugatum</i>	OM 3339 (SI)	AY174491 ^c
<i>Paspalum consperum</i>	FZ 6748 (SI)	AY174492 ^c
<i>Paspalum fimbriatum</i> ^b		AF372187
<i>Paspalum malacophyllum</i>	OM 3400 (SI)	AY174493 ^c
<i>Paspalum scrobiculatum</i> ^b		AF372186
<i>Paspalum quadrifarium</i> ^b		AF372197
<i>Pennisetum clandestinum</i> ^b		AF372201
<i>Pennisetum purpureum</i> ^b		AF372200
<i>Pennisetum setaceum</i> ^b		AF372198
<i>Pennisetum</i> sp. ^b		L25383
<i>Pheidochloa gracilis</i>	JB 0158 (GH)	AY174494 ^c
<i>Plagiantha tenella</i> ^b		AF372183
<i>Pseudechinolaena polystachya</i> ^b		AF372202
<i>Saccharum officinarum</i> ^b		U96321
<i>Sacciolepis indica</i> ^b		AF372207
<i>Setaria gracilis</i> ^b		AF372204
<i>Setaria macrostachya</i>	PI 216573	AY174495 ^c
<i>Setaria palmifolia</i> ^b		AF3721
<i>Setaria parviflora</i>	PI 316422	AY174496 ^c
<i>Setaria sphacelata</i>	PI 268145	AY174497 ^c
<i>Sorghum halapense</i> ^b		AY174498 ^c
<i>Steinchisma hians</i> ^b		AF372205
<i>Stenotaphrum secundatum</i> ^b		AF372203
<i>Streptostachys asperifolia</i>	FZ 6941 (SI)	AY174499 ^c
<i>Streptostachys ramosum</i> ^b		AF372206
<i>Tatianix arnaces</i> ^b		AF372209
<i>Thrasya glaziovii</i> ^b		AF372210
<i>Thrasya petrosa</i> ^b		AF372211
<i>Tristachya leucothrix</i>	HB 5018 (DEK)	AY174500 ^c
<i>Urochloa acuminata</i> ^b		AF372212
<i>Urochloa maxima</i> (= <i>Panicum maximum</i>) ^b		AF000021
<i>Urochloa plantaginea</i>	FZ 6767 (SI)	AY174501 ^c
<i>Urochloa reptans</i> ^b		AF372213

^a Abbreviations for plant voucher numbers: BK = B. Kent; FZ = F. Zuloaga; HB = H. Beck; JB = J. Barber; KB = K. Bluemel; OM = O. Morrone; PI = USDA Plant Introduction Station (Pullman, Wash.); RGM = R. Gómez-Martínez; RS = R. Spangler; SJ = S. Jacobs; TF = T. Filgueiras. Herbaria are indicated parenthetically by standard acronyms.

^b From Duvall et al. (2001).

^c First published in this article.

panic grasses of known photosynthetic types and, in most cases, known subtypes. As one marker, we used a grass-specific insert in the chloroplast locus that encodes subunit C2 of an RNA polymerase (*rpoC2*). This insert is found near the center of the *rpoC2* coding regions of grasses, is ca. 450 bp in length, and increases the length of the *rpoC2* coding region by ca. 10% (Cummings et al. 1994). Although the overall chloroplast genome is generally considered to be evolutionarily conservative, this extra coding region in the *rpoC2* locus of grasses is exceptionally labile. The insert region demonstrates higher rates of nucleotide substitution and accumulated insertion/deletion events than the surrounding regions. The molecular evolution of this locus has included numerous slipped-strand mispairing mutations of 21-bp repeat units (Cummings et al. 1994). This knowledge expedites the tasks of nucleotide alignment and phylogenetic analysis of *rpoC2* insert sequences. The

rpoC2 insert was chosen because of its demonstrated use for grass phylogenetics (Barker et al. 1999; Duvall et al. 2001; GPWG 2001). To increase the phylogenetic information in a critical clade, we also combined the *rpoC2* data with sequences of a second chloroplast marker, the F subunit of an NADH-dependent dehydrogenase (*ndhF*), which were previously published (Giussani et al. 2001).

Phylogenies based on molecular markers from different cellular compartments reflect evolutionary history more accurately (Miyamoto and Cracraft 1991). Thus, we also employed sequences of the conserved 3' portion of the external transcribed spacer (ETS) region of nuclear ribosomal DNA. Prior phylogenetic studies of ETS have been largely confined to Asteraceae (Baldwin and Markos 1998; Clevinger and Panero 2000; Linder et al. 2000; Chan et al. 2001; Markos and Baldwin 2001, 2002; Bayer et al. 2002; Lee et al. 2002; Kelch and

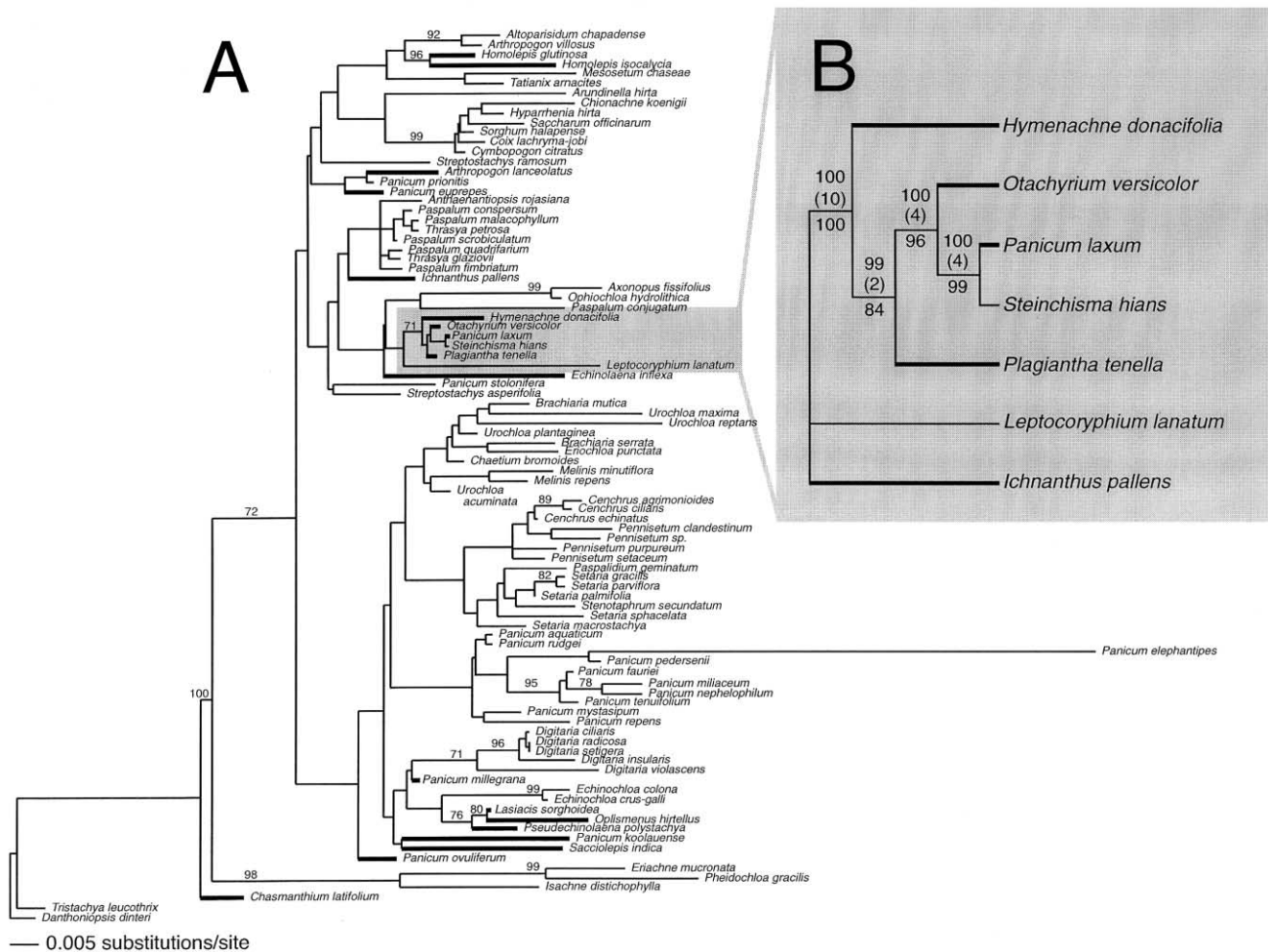


Fig. 1 Phylogenetic survey across Panicoideae using sequences of plastid loci. C₃ lineages in both A and B are indicated by heavier branches. A, Neighbor-joining tree of 92 *rpoC2* sequences. Branch lengths are proportional to the number of steps along a branch. Numbers along the branches are bootstrap values. Note the multiple locations of C₃ lineages. B, Bayesian inference tree of the *Steinchisma hians* lineage. Numbers along the branches are support statistics; clade credibility values (top), Bremer values (middle, parenthesized), and bootstrap values (below the branches).

Baldwin 2003; Saar et al. 2003) and other dicots (e.g., Bena et al. 1998; Chandler et al. 2001). We report here what we believe to be the first use of the ETS locus in grass phylogenetics.

Material and Methods

Sources of DNAs

DNA samples were obtained from the Native Hawaiian Plant DNA Library (Morden et al. 1996) and from the Kellogg DNA Library (J. H. Cota-Sánchez, University of Saskatchewan, Saskatoon, and E. Kellogg, University of Missouri, St. Louis). In addition, seeds of *Panicum miliaceum* were obtained from Gurney's Seed and Nursery Company, Yankton, South Dakota (Duvall s.n., DEK). *Tristachya leucothrix* seeds and vouchers were collected by H. Beck from Blyde River Canyon Nature Reserve, Mpumalanga Province, South Africa (HB5018, DEK). DNA was extracted from seedlings of these two species using standard methods (Doyle and Doyle 1987). The complete list of species

and plant vouchers is provided (table 1). Sampling maximized representation of the photosynthetic variation across panicoid grasses with special attention to the inclusion of putative relatives of *Steinchisma hians*. Photosynthetic condition was determined from the literature (Watson and Dallwitz 1992; Zuloaga et al. 1998), including a recent compilation (Giussani et al. 2001).

rpoC2 Amplification, Sequencing, and Alignment

DNA fragments containing either the grass-specific *rpoC2* insert or the complete ETS region were amplified from the same DNA extracts of each species with *Taq* Polymerase (Promega, Madison, Wis., for *rpoC2*; Failsafe PCR System H from Epicentre Technologies, Madison, Wis., for ETS). Protocols for determining *rpoC2* insert sequences are described in Duvall et al. (2001). Nucleotide sequences of *rpoC2* were translated and aligned as amino acid sequences following the rule-governed alignment method of Barker et al. (1999), as modified

Table 2
Species Included in the Phylogenetic Analysis of
Plastid Loci in the *Steinchisma hians* Lineage
with GenBank Accession Numbers for *ndhF*

Species	GenBank accession number
<i>Hymenachne donacifolia</i>	AY029635
<i>Ichnanthus pallens</i>	AY029638
<i>Leptocoryphium lanatum</i>	AY029640
<i>Otachyrium versicolor</i>	AY029643
<i>Panicum laxum</i>	AY029655
<i>Plagiantha tenella</i>	AY029674
<i>Steinchisma hians</i>	AY029685

Note. Sequences published in Giussani et al. (2001). See table 1 for sources of corresponding *rpoC2* sequences for these species.

by Duvall et al. (2001). These rules were devised to preserve the repeats commonly found in the *rpoC2* insert. After the amino acid sequences were fully aligned, the corresponding nucleotide sequences were aligned in parallel preserving the same gaps. Gaps were coded as binary characters that were appended to the aligned sequences for subsequent maximum parsimony (MP) analysis.

ETS Amplification, Sequencing, and Alignment

Primer sequences flanking the ETS region and used for amplification of ETS were ETS-For (CCTTGCTGCCACGATCCACTGAGAT) and ETS-Rev (GACTACTGGCAGGATCAACC). Two or more products were typically obtained when amplifying with the ETS primers, probably because of multiple priming across the subrepeat structure of the ETS region (Linder et al. 2000). The fragment with a length corresponding to that of the entire ETS region in grasses (1.8–2.2 kb) was gel

purified with the QiaQuick gel extraction kit (Qiagen, Santa Clarita, Calif.) and ligated into a plasmid vector (pGEM-T Easy, Promega). Ligations were used to transform competent *E. coli* cells (strain DH5 α). Crude extracts of *E. coli* clones were used as templates for a PCR reamplification with the universal primers T7 and SP6, or minipreps of the ETS-containing plasmids were performed with Cyclo-Prep Spin Columns (AMRESCO, Solon, Ohio) before sequencing. Clone homogeneity was verified by sequencing multiple clones of two, randomly selected species, *Cenchrus echinatus* (three clones) and *Eriochloa punctata* (five clones). Sequencing was performed on an Applied Biosystems 373 automated instrument. Nucleotide similarity and inferred homology of these sequences to ETS was verified by comparisons against published sequences of ETS from *P. miliaceum* (Klarholz and Hildebrandt, direct GenBank submission AF147502) and *Zea mays* (McMullen et al. 1986; Toloczyki and Feix 1986).

Gapped nucleotide positions in both *rpoC2* and ETS found in half or more of the species were excluded from subsequent analyses, an approach that minimized the detrimental effect of excessive numbers of missing values on phylogenetic analysis while still preserving the character state data found for the majority of the taxa. ETS sequences were aligned with ClustalW as implemented in Gene Inspector 1.5 (Textco, West Lebanon, N.H.), with some further manual adjustments (alignment available on request).

Phylogenetic Analysis

Phylogenetic analyses were performed on three different sets of data.

1. A broad survey of 92 *rpoC2* sequences was performed. Alternative evolutionary models were compared using ModelTest 3.06 (Posada and Crandall 1998). The optimal model was found to be the general time-reversible (GTR) nucleotide substitution model with γ -distributed, among-site rate

Table 3
Species Included in the Broad Phylogenetic Survey of *rpoC2* in Panicoideae
Together with Voucher and GenBank Accession Numbers

Species	Voucher (herbarium code) ^a	GenBank accession number or other source
<i>Altoparadisium chapadense</i>	TF-TYPE (SI)	AY254794 ^b
<i>Cenchrus echinatus</i>	CM 1168 (HAW)	AY254804 ^b
<i>Eriochloa punctata</i>	CM 1273 (HAW)	AY254798 ^b
<i>Oplismenus hirtellus</i>	CM 1007 (HAW)	AY254805 ^b
<i>Panicum laxum</i>	FZ 6756 (SI)	AY254801 ^b
<i>Panicum miliaceum</i>	Previously published	AF147502
<i>Panicum pedersenii</i>	FZ 6926 (SI)	AY254795 ^b
<i>Panicum tenuifolium</i>	CM 1333 (HAW)	AY254796 ^b
<i>Paspalum scrobiculatum</i>	CM 1175 (HAW)	AY254802 ^b
<i>Pennisetum setaceum</i>	CM 1101 (HAW)	AY254797 ^b
<i>Steinchisma hians</i>	FZ 6773 (SI)	AY254800 ^b
<i>Tatianix arnaces</i>	FZ 6965 (SI)	AY254803 ^b
<i>Urochloa maxima</i>	CM 1106 (HAW)	AY254799 ^b
<i>Zea mays</i>	Previously published	McMullen et al. 1986; Toloczyki and Feix 1986

^a Abbreviations for plant voucher numbers: CM = Clifford Morden (see Randell and Morden 1999); FZ = F. Zuloaga; TF = T. Filgueiras. Herbaria are indicated parenthetically by standard acronyms.

^b First published in this article.

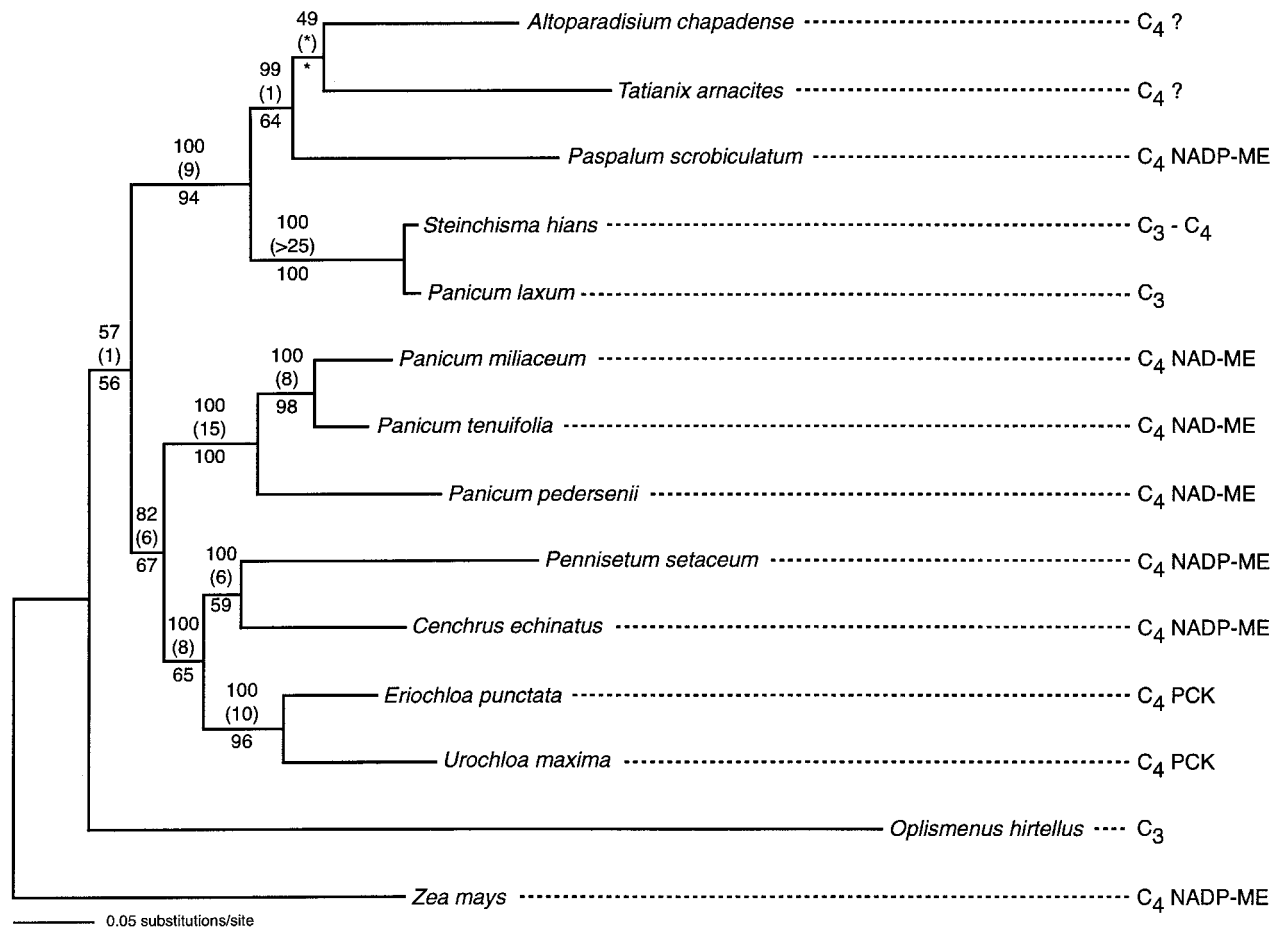


Fig. 2 Bayesian inference tree over combined sequences of *rpoC2* and ETS. Support statistics are indicated as in fig. 1B. Photosynthetic subtype for each taxon is indicated. In two cases, a question mark indicates an unknown subtype. The node indicated with an asterisk was not supported in the MP tree.

variation. DNA distances were calculated under this model with the neighbor-joining (NJ) method (Saitou and Nei 1987) implemented in PAUP* version 4.0b10 (Swofford 2000). Defaults were used for all other parameters. *Danthoniopsis dinteri* was specified as the outgroup (Giussani et al. 2001; GPWG 2001). An NJ bootstrap analysis was also performed with 1000 replicates. Other types of phylogenetic analyses, e.g., Bayesian inference (BI), were not performed because of the large number of gapped positions inserted in the *rpoC2* alignment to accommodate indels relative to the number of terminal taxa.

2. The broad survey identified a clade of six species that clustered with *S. hians*. These species and *Ichnanthus pallens* as the local outgroup were analyzed with both the MP (implemented in PAUP* 4.0b10) and BI (implemented in MrBayes 2.01; Huelsenbeck 2000) methods. The *rpoC2* data from this study were combined with previously published *ndhF* data for the same species to increase the phylogenetic signal over this small subset. A partition homogeneity test was performed in PAUP* 4.0b10 with 1000 random repartitions in branch and bound searches (Farris et al. 1995). Branch and bound MP analyses were conducted to find the most parsimonious tree

and to estimate bootstrap (1000 replicates; Felsenstein 1985) and Bremer values (Bremer 1988). BI analysis was performed under the GTR model (again as indicated by ModelTest analysis) with partitioning by locus and with estimated base frequencies. The analysis was performed for 1 million generations, with trees sampled every 80 generations. The first 2501 trees were discarded by which point the algorithm was observed to have converged on the optimum log-likelihood value. BI analysis produces clade credibility values that approximate posterior probabilities of the tree given the sequence data.

3. The *rpoC2* and ETS sequences were analyzed from 13 species plus the outgroup *Z. mays*. A partition homogeneity test was performed as above. ModelTest analysis again indicated the optimal model to be GTR + Γ + I model. Combined data were analyzed with BI and MP as above. Support of the sequence data for the tree topologies was assessed with the bootstrap and Bremer support methods as above. In addition, each species was scored for photosynthetic type in a three-state, unordered character (0 = C₃, 1 = C₄, 2 = C₃-C₄). These character state transformations were plotted on the combined tree topology using MacClade version 3.0 (Maddison and Maddison 1992).

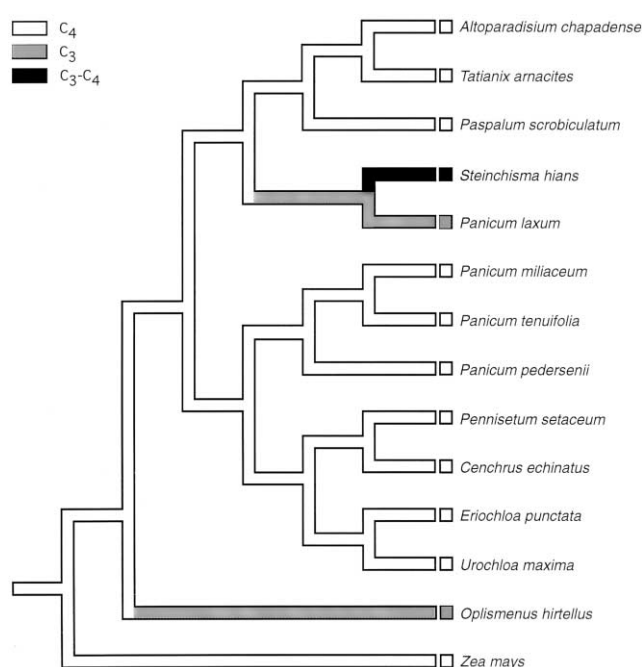


Fig. 3 The tree of fig. 2 is represented here with character state transformations between C₃ and C₄ produced from the computer software MacClade (Maddison and Maddison 1992). Transformations are optimized in the context of the more intensively sampled phylogeny of Paniceae (fig. 1). Note the reversion in *Steinchisma hians* from its ancestral C₃ condition to one of C₃-C₄ intermediacy.

Results

Nucleotide sequences of *rpoC2* were determined for 31 species (table 1). These were aligned together with previously published sequences from 61 other species for a total alignment length of 939 bases, 637 of which were included in the phylogenetic analysis. Analysis of these sequences produced the neighbor-joining tree of figure 1A. *Steinchisma hians* is found here in a derived position in a clade with four other species (bootstrap = 71), all of which are C₃ taxa: *Panicum laxum*, *Otachyrium versicolor*, *Plagiantha tenella*, and *Hymenachne donacifolia*. Basal to this clade was the C₄ species *Leptocoryphium lanatum*. This clade of six species will be hence designated the “*S. hians* lineage.” Also note that C₃ lineages are found at multiple points, suggesting six reversals at minimum from C₄ photosynthesis, across the *rpoC2* phylogeny (fig. 1A).

Previously published sequences of *ndhF* (table 2) were aligned for the *S. hians* lineage together with *Ichnanthus pallescens*, a nearby outgroup on a relatively short branch with a correlated conservative mutation rate. The aligned length of the *ndhF* sequences was 2048 bases, 2039 of which were analyzed (excluding a 9-bp deletion found in the five terminal of these seven species). A partition homogeneity test of these *rpoC2* and *ndhF* sequences did not find incongruence ($P = 1.0$). BI analysis of these combined sequences produced the tree of figure 1B, which is shown with three measures of support: clade credibility (CC), Bremer values (BV), and bootstrap support (BS). Parsimony analysis recovered one fully resolved branch and bound tree of length 162 with the identical to-

polo. The Consistency Index (CI) of the MP tree, excluding uninformative characters, is 0.8235 and the Retention Index (RI) is 0.8286. The topology of this *S. hians* lineage ingroup is identical to the topology of the same species in the broad survey (fig. 1A). The integrity of the *S. hians* lineage has strong support (CC = 100; BV = 10; BS = 100). The derived position of *S. hians* in this lineage is also strongly supported (CC = 100; BV = 4; BS = 99).

Nucleotide sequences of ETS from 12 species were determined (table 3) and combined with two previously published sequences for a total of 14 species. Sequences determined from multiple ETS clones that were obtained from the same species were identical. The total aligned sequence lengths for the *rpoC2* insert and the conserved region of ETS for this subset of species were 578 and 649 bp, respectively. The *rpoC2* sequences were not found to be incongruent with ETS sequences by the partition homogeneity test ($P = 0.539$). Eighteen gaps in the *rpoC2* sequences were encoded as additional binary characters for MP analysis. Gapped positions in either locus that were found in >50% of the taxa were excluded from subsequent analyses, which reduced the number of characters to 491 (*rpoC2*) and 512 (ETS). Sequences from each locus were analyzed separately but failed to produce single most parsimonious trees over these 14 species (results not shown). In both cases, deep branches in the tree were unresolved, although the position of *S. hians* was identical to that in the combined analysis.

BI analysis, partitioned by locus, produced the tree of figure 2. The combined MP analysis produced one fully resolved, most parsimonious, branch and bound tree with a CI, excluding uninformative characters, of 0.5556 and an RI of 0.5060. Note that this tree differed from the BI tree only in that the weakly supported positions of *Tatanix arnatices* and *Paspalum scrobiculatum* (CC = 49) were reversed.

Correlations between positions in the phylogenetic tree and biochemical C₄ subtypes, defined by the principal decarboxylating enzyme, were observed in two instances. The three NAD-specific malic enzyme (NAD-ME) species were in a strongly supported cluster (CC = 100; BV = 15; BS = 100), as were two PEP-carboxykinase (PCK) species (CC = 100; BV = 10; BS = 96). As in all prior analyses, *S. hians* clustered with the C₃ species *P. laxum*, with strong support (CC = 100; BV ≥ 25; BS = 100). Exceptionally, NADP-ME species were found in three different locations in the tree.

Discussion

In this phylogenetic study, chloroplast and nuclear sequences were combined for selected species of Paniceae with the intent of elucidating evolutionary transformations between photosynthetic types. The ETS locus has been little used to date in molecular phylogenetic research on monocots and used not at all among grasses. ETS resembles the much more widely used internally transcribed spacer (ITS) (including ITS1, 5.8S rDNA, and ITS2; Baldwin 1992, 1993) in several respects. Both ETS and ITS (excluding 5.8S rDNA) are transcribed spacer regions under similarly relaxed selective constraints. Both are part of the ribosomal repeat unit and share the same patterns of nuclear genetic inheritance and mutational events. ETS and ITS both demonstrate some degree of concerted evo-

lution in which mutations are rapidly propagated among repeats after they arise (Arnheim et al. 1980; Hillis et al. 1991; Baldwin and Markos 1998).

Despite the similarities of ETS to ITS, the former offers several advantages for intergeneric phylogenetic research. ETS has more than twice the number of variable sites as ITS. We have both the 3' variable region of ETS and ITS sequences from the same 11 panic grass species (M. R. Duvall and D. E. Saar, unpublished data). The average pairwise genetic distance between our partial ETS sequences (0.3398) is more than twice the average ITS distance (0.1495). ETS shows marked substitutional variation along its length. The 5' variable portion of ETS can be used to compare closely related populations, whereas the 3' conserved portion is useful for infrafamilial intergeneric comparisons.

The broad phylogenetic survey of Panicoideae (fig. 1A) is largely congruent with results reported elsewhere (Gómez-Martínez and Culham 2000; Duvall et al. 2001; Giussani et al. 2001). Note that the African species of Arundinelleae, *Tristachya leucothrix*, clusters near the danthonioid outgroup, *Danthoniopsis dinteri*, rather than with *Arundinella hirta*, indicating that the tribal classification of this species is erroneous. Also note that Eriachneae (represented by *Eriachne mucronata* and *Pheidochloa gracilis*) and Isachneae (*Isachne distichophylla*) cluster in a clade that is phylogenetically basal to other Panicoideae, congruent to results obtained with other plastid loci (Duvall et al. 2003). In this phylogenetic survey, the single, unambiguous origins of the NAD-ME (10 representative species) and PCK (also 10 species) subtypes of C₄ photosynthesis are confirmed (first suggested in Gómez-Martínez and Culham 2000 and substantiated in Giussani et al. 2001).

Our results here overwhelmingly support a sister group status of the C₃-C₄ intermediate *Steinchisma hians* to the C₃ species *Panicum laxum* (figs. 1B, 2, 3). (Note that in a recent study of *Panicum* [Aliscioni et al. 2003], *Steinchisma* [three species] is weakly supported [bootstrap = 64%; decay value = 1] as being paraphyletic with *P. laxum*). Moreover, these two species are strongly supported as being terminally derived in a clade with three other C₃ taxa, *Hymenachne donacifolia*, *Otachyrium versicolor*, and *Plagiantha tenella*. Within this lineage, four C₃ species and *S. hians* are supported by a synapomorphic 9-bp deletion in *ndbF* (Giussani et al. 2001; Aliscioni et al. 2003). This *S. hians* lineage is embedded in a largely C₄ clade (fig. 1; Duvall et al. 2001; Giussani et al. 2001), suggesting that the ancestor of the entire *S. hians* lineage underwent a reversal from the C₄ to the C₃ condition. Additional sampling of congeners in this clade would further strengthen this result.

All six species of *Steinchisma* show a similar anatomical pattern of C₃-C₄ intermediacy (Zuloaga et al. 1998). In addition, low CO₂ compensation points have been measured for *Steinchisma decipiens* as well as *S. hians* (Morgan and Brown 1979), confirming that at least two of these species exhibit operational C₃-C₄ intermediate physiology. Thus, it is plausible that the progenitor of this genus experienced a relatively recent reversal toward the C₄ state. These events are depicted in figure 3, using the topology of the tree of figure 2.

The anatomical and physiological adaptations resulting in reduced photorespiratory rates in *S. hians* can be viewed as

initial changes toward a suite of characteristics necessary for the full operation of C₄ photosynthesis (Rawsthorne 1992; Monson 1999). The relatively large numbers of chloroplasts and mitochondria in bundle sheath cells of *S. hians* compared with the surrounding mesophyll tissue (Brown and Hattersley 1989) are necessary for decarboxylation of photorespiratory glycine in the bundle sheath tissue and the subsequent refixation of CO₂. It is of interest that analysis of leaf anatomy in *P. laxum* (C₃) showed a close association of mitochondria and chloroplasts in bundle sheath cells that is similar to the C₃-C₄ sister group species, *S. hians* (Brown et al. 1983; Brown and Hattersley 1989). However, despite the elevated number of these organelles and enclosure of some mitochondria by chloroplasts, *P. laxum* does not exhibit the same capacity as *S. hians* to refix photorespired CO₂ (Holbrook et al. 1985). This observation can be partly explained by the exclusive compartmentation of glycine decarboxylase into the bundle sheath cell mitochondria in *S. hians* compared with the presence of this enzyme in both mesophyll and bundle sheath mitochondria in *P. laxum* (Hylton et al. 1988). The nascent phylogenetic distinction between *S. hians* and *P. laxum* therefore includes a complete division of photorespiratory metabolism between the bundle sheath and the mesophyll in the former species. This adaptation increases overall rates of CO₂ assimilation and photosynthetic nitrogen use and water use efficiency without significant increases in the fixation of carbon into C₄ acids typical of fully evolved C₄ plants (Ku and Edwards 1978; Monson 1999).

Clayton and Renvoize (1986) report that panic grasses have tended to radiate into mesic habitats where selection pressures favoring C₄ metabolism are minimized. The driving selective force for this radiation is not known. However, in such habitats, selection against the energetically more expensive C₄ pathway would be predicted. This trend may explain the repeated reversals from the C₄ condition among panic grasses indicated by recent phylogenetic research (fig. 1A; Duvall et al. 2001). The species of *Steinchisma*, which are distributed from the southern United States to Argentina, are commonly adventives in damp grasslands (Watson and Dallwitz 1992). Competition with native species may have selected for greater photosynthetic efficiency under higher light intensities and higher growth temperatures resulting in the adoption of the C₃-C₄ condition. Whatever the cause, our study suggests an incipient return toward C₄ photosynthesis in *Steinchisma*, a derived genus in an otherwise C₃ lineage, a lineage that itself arose as a reversal from more distant C₄ ancestors.

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