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The Phytochrome Gene Family in Grasses (Poaceae): A Phylogeny and Evidence that Grasses Have a Subset of the Loci Found in Dicot Angiosperms

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The phytochrome nuclear gene family encodes photoreceptor proteins that mediate developmental responses to red and far red light throughout the life of the plant. From studies of the dicot flowering plant ***Arubidopsis, the*** family has been modeled as comprising five loci, ***PHYA-PHYE.*** However, it has been shown recently that the ***Arabidopsis*** model may not completely represent some flowering plant groups because additional ***PHY*** loci related to ***PHYA*** and ***PHYB*** of ***Arubidopsis*** apparently have evolved independently several times in dicots, and monocot flowering plants may lack orthologs of ***PHYD*** and ***PHYE*** of ***Arabidopsis.*** Nonetheless, the phytochrome nucleotide data were informative in a study of organismal evolution because the loci occur as single copy sequences and appear to be evolving independently. We have continued our investigation of the phytochrome gene family in flowering plants by sampling extensively in the grass family. The phytochrome nuclear DNA data were cladistically analyzed to address the following questions: (1) Are the data consistent with a pattern of differential distribution of phytochrome genes among monocots and higher dicots, with homologs of ***PHYA, B, C, D,*** and ***E*** present in higher dicots, but of just ***PHYA, B,*** and C in monocots, and (2) what phylogenetic pattern within Poaceae do they reveal? Results of these analyses, and of Southern blot experiments, are consistent with the observation that the phytochrome gene family in grasses comprises the same subset of loci detected in other monocots. Furthermore, for studies of organismal phylogeny in the grass family, the data are shown to provide significant support for relationships that are just weakly resolved by other data sets.

Introduction

Grasslands occupy about a third of the earth's surface, hosting the progenitors of cultivated grains, and the grass family (Poaceae) has the fifth highest number of species (about 10,000) of any flowering plant family (Clayton and Renvoize 1986). Thus, grasses are ecologically, economically, and evolutionarily significant, and have figured prominently among investigations of plant phylogeny and classification. From these investigations have emerged a number of conclusions that we summarize below.

Certain classifications (e.g., Brown 1814; Roshevits 1937; Tzvelev 1989) have placed all grass genera into two major taxa. More commonly, five or six major subfamilies are recognized, Bambusoideae, Centothecoideae, Pooideae, Chloridoideae, Panicoideae, and Arundinoideae (e.g., Watson et al. 1985; Clayton and Renvoize 1986; Watson and Dallwitz 1992), based on anatomical, chemical, cytological, morphological, and physiological data. Three subfamilies, Chloridoideae, Panicoideae, and Pooideae, are monophyletic (Kellogg and Campbell 1987; Davis and Soreng 1993; Soreng and Davis 1994; Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995; Kellogg and Linder 1995). Furthermore, three sets of data from the chloroplast genome unite Panicoideae, Chloridoideae, Centothecoideae, and Arundinoideae in a monophyletic group (Davis and Soreng 1993; Soreng and Davis 1994; Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995; fig. 1) referred to as the PACC clade (Davis and Soreng 1993). Within the PACC clade placement of arundinoid taxa is ambiguous. Moreover, relationships at the base of the tree are not clearly resolved (fig. 1). However, the *ndh*F data do suggest that a major evolutionary divergence in the family led to the PACC clade on the one hand and a clade comprising bambusoid, oryzoid, and pooid genera (BOP clade; Clark, Zhang, and Wendel 1995) on the other, and are further notable in providing evidence of the ancestral nature of the bambusoid elements Streptochaeteae, Anomochloeae, and Phareae among all grasses.

Lack of strongly supported resolution within and among phylogenies of grasses has been attributed to rapid speciation events that were not accompanied by significant molecular and morphological divergence (Kellogg and Watson 1993; Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995), or to extensive parallel evolution (Kellogg and 'Watson 1993). These may be valid explanations of morphological evolutionary events. However, lack of strong phylogenetic signal in molecular data sets (e.g., low evidential or bootstrap support of phylogenetic hypotheses) also could result from the choice of the molecular tool (e.g., more slowly evolving sequences from chloroplast or ribosomal DNA molecules).

We have investigated the relevance to these questions of DNA sequence data from the phytochrome nuclear gene family. Additionally, we have continued our survey of the phylogenetic distribution of phytochrome genes in flowering plants. Fifteen genera of Poaceae and three genera of the closely related Flagellariaceae, Joinvilleaceae, and Restionaceae were sampled for phytochrome gene and nucleotide diversity. We have shown previously that nucleotide data from phytochrome genes

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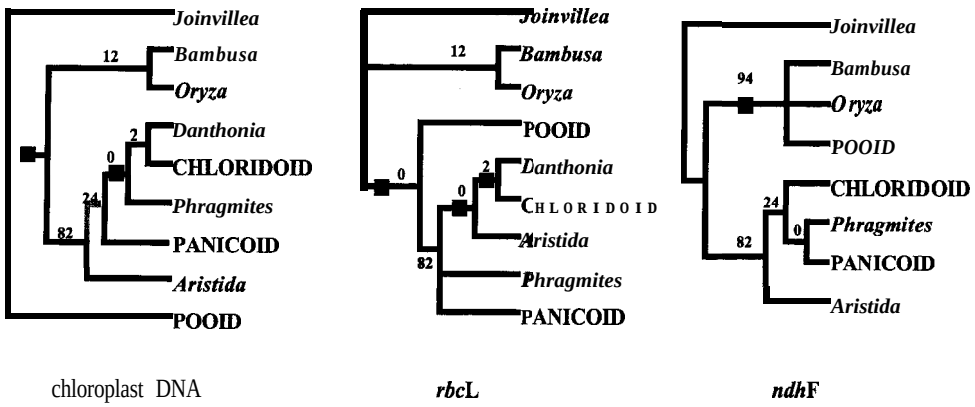

FIG. 1.—Phylogenies of Poaceae inferred from other dam sets (Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995; Davis and Soreng 1993) simplified to represent taxa that were sampled in the phytochrome study. Taxa that were sampled for chloroplast data did not strictly correspond to taxa sampled for phytochrome data; thus, in some cases, taxon labels in the cpDNA and *rbc*L trees are substitutes for closely related sister taxa. Bootstrap values (from 100 replicate resamples of the phytochrome nucleotide data) are on the nodes. The gray shaded branch in the cpDNA phylogeny is inferred from Soreng and Davis (1994). Filled boxes are on branches that collapse because they are not well supported by authors' criteria.

is informative, especially for subfamilial phylogenetic studies (Mathews, Lavin, and Sharrock 1995). Phytochrome genes occur in all land plants and green algae; in flowering plants a family of five related genes has been fully characterized from *Arubidopsis* (Sharrock and Quail 1989; Clack, Mathews, and Sharrock 1994). However, we observed that monocots apparently have a subset of the five phytochrome loci found in *Arubidopsis* and other dicots with triaperaturate pollen, indicating that the presence or absence of some gene family members potentially provides evidence of relationships among major angiosperm lineages (Mathews, Lavin, and Sharrock 1995). From Poaceae, full-length coding sequences of one to two phytochrome gene family members from oat (Hershey et al. 1985), corn (Christensen and Quail 1989), and rice (Kay et al. 1989) are known, and several sets of oligonucleotide primers for amplification of phytochrome genes via the polymerase chain reaction (PCR) are available (e.g., Kolukisaoglu et al. 1995; Mathews, Lavin, and Sharrock 1995; M. Lavin, personal communication; K. Marshall, personal communication), facilitating the use of phytochrome data for phylogenetic studies in Poaceae.

The phytochrome nuclear DNA data were cladistically analyzed to address the following questions: (1) What phylogenetic pattern within Poaceae do they reveal, (2) are they useful for placing anomalous genera, and (3) are the data consistent with a pattern of differential distribution of phytochrome genes among monocots and eudicots, with homologs of *PHYA, B, C, D,* and *E* present in eudicots, but of just *PHYA, B,* and C in monocots?

Table 1
Sources of Phytochrome Sequences Determined in this Study

Flagellariaceae	
Flagellaria indica L.	***R. J. Soreng*** 77 394 BH
Joinvilleaceae	
Joinvillea ascendens Gaudicyh. ex Brongn. & Gris	***H. Moore 10438 NY***
Restionaceae	
Thamnochortus argenteus Kunth.	***Julie Schaller 83284 NY***
Poaceae	
ARUNDINOIDEAE	
Aristida purpurea Nutt.	***Lavin s.n.*** MONT
Danthonia unispicata (Thurber) Munro ex Macoun	***Lavin s.n.*** MONT
Phragmites australis (Cav.) Trin. ex Steudel	***Lavin s.n.*** MONT
BAMBUSOIDEAE	
Bambusa Schreb..	***E. A. Kellogg V6*** A
Oryza sativa L.	***Lavin s.n.*** MONT
CHLORIDOIDEAE	
Bouteloua gracilis (Willd. ex H.B.K.) Lag. ex Griffiths	***Lavin s.n.*** MONT
Calamovilfa longifolia (Hook.) Scribn.	***Lavin s.n.*** MONT
Eragrostis cilianensis (All.) Mosher	***Lavin s.n.*** MONT
PANICOIDEAE	
Panicum capillare L.	***Lavin s.n.*** MONT
Pennisetum setaceum (Forsk.) Chiov.	***Lavin s.n.*** MONT
Zea mays L.	***Lavin s.n.*** MONT
POIIDEAE	
Bromus inermis Leyss.	***Lavin s.n.*** MONT
Poa pratensis L.	***Lavin s.n.*** MONT
Stipa viridula Trin.	***Lavin s.n.*** MONT

NOTE.—Taxonomic arrangement follows Clayton and Renvoize (1986).

Materials and Methods

DNA Isolation

Total DNA was isolated from fresh or dried herbarium material of taxa listed in table 1 by standard methods (Doyle and Doyle 1987). Aliquots were extracted once with phenol : chloroform-isoamyl alcohol (1: 1 volume), and the aqueous portions were purified over sepharose CL-6B columns (Sigma). To assess phytochrome gene and nucleotide diversity in Poaceae, homologous fragments of phytochrome DNA (fig. 2) were sampled from genera of Arundinoideae, Bambusoideae, Chloridoideae, Panicoideae, and Pooideae (table 1) using PCR and DNA cloning and sequencing protocols described by Mathews, Lavin, and Sharrock (1995). We

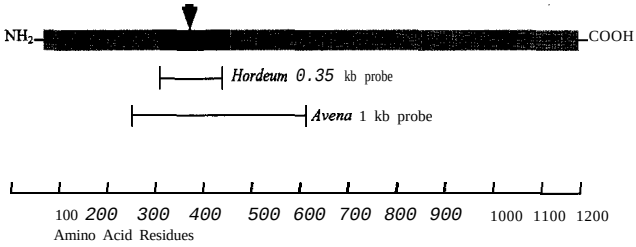

FIG. 2.--Schematic diagram of a phytochrome protein. The black filled rectangle is the region sampled in this study. The triangle denotes the approximate site of chromophore attachment. The coding regions covered by the nucleic acid probes used in Southern blot analysis are indicated.

generated a total of 41 unique homologous phytochrome sequence fragments for comparison, 8 of which were from single clones. We did not purposefully determine both orientations of every sequence, but found that in most cloning experiments, each was detected. Sequences detected in this study are deposited in GenBank under accession numbers U08149, U08166, U08169–U08170, U08177, and U61185–U61220. Additional accessions used in phylogenetic analyses include P19862 and X03242.

DNA Sequence Analysis

Peptide sequences inferred from DNA sequences detected in this study, together with published sequences, were multiply aligned by eye; peptide alignments were the basis of multiple nucleotide sequence alignments. A region of insertion and deletion among paralogous phytochrome sequences occurs within the amplified sequences and downstream from the site of chromophore attachment (Sharrock and Quail 1989; Clack, Mathews, and Sharrock 1994; Mathews, Lavin, and Sharrock 1995). We found this region to be variable among phytochrome sequences from grasses as well, requiring the insertion of gaps. Alignment gaps that were inferred to be homologous were retained in the data matrices and coded as single characters.

Sequences were compared using maximum-parsimony algorithms available in Hennig86 (Farris 1988) and PAUP 3.1 (Swofford 1993), and the maximum-likelihood algorithm in PHYLIP 3.5c (Kishino and Hasegawa 1989; Felsenstein 1993). Minimal-length trees resulted from heuristic search options available in either Hennig86 (mh* and bb* with no upper limit set, or ie*), or in PAUP (CLOSEST or RANDOM data addition sequence, HOLD option set for five trees when applicable, STEEPEST DESCENT, MULPARS, and TBR branch-swapping options activated, with branch swapping on nonminimal trees, and MAXTREES set at 10,000). Support for monophyly of putative clades was evaluated in bootstrap resampling analysis (Felsenstein 1985) and decay analysis (Bremer 1988) using PAUP. For cladistic analyses, trees were rooted by designating ***PHY*** sequences from ***Flagellaria indica, Joinvillea ascendens,*** and ***Thamnochortus*** sp. as outgroups; these taxa represent families inferred from morphological (Campbell and Kellogg 1987; Linder and Rudall 1993) and molecular data (Doyle et al. 1992) to be among the closest relatives of Poaceae. Cladograms rooted at ***Muscari*** (GenBank accession U08171, U08172, U08179) allow detection of phylogenetic structure within outgroup species, but do not differ in other details from those rooted at ***Joinvillea.*** The ***PHY*** sequence from ***Selaginella*** (GenBank accession X61458) was designated as the outgroup for the cladogram of all ***PHY*** sequences from grass genera because strong support for the placement of this sequence in a single ***PHY*** lineage is lacking (Mathews, Lavin, and Sharrock 1995) However, cladograms rooted at a sequence from a monocot or a dicot differ only in their placement of ***PHY*** lineages relative to one another (not shown).

The phytochrome sequence data detected in this investigation were variously combined or partitioned for cladistic analyses. Initially, we treated each sequence as a single operational taxonomic unit (OTU) with 324 characters (nucleotide sites); results from cladistically analyzing this matrix were used to infer homology of the sequences with phytochrome loci of ***Arabidopsis.*** Subsequently, we combined data of each locus sampled, resulting in a matrix of 972 nucleotide characters for 20 taxa, with characters coded as missing data if not all loci were sampled from a given taxon (e.g., from ***Avena*** because the sequence data came from the literature rather than from sampling in this study). We did not detect mutational saturation (e.g., Mindell, Schultz, and Ewald 1995) at third codon positions that might serve as an a priori rationale for differentially weighting codon positions for cladistic analyses (e.g., assigning third codon positions a weight of zero). And, assigning third codon positions a weight of zero did not significantly increase the consistency index of the cladogram of combined data (see ***Results).***

Congruence Analyses

Individual phytochrome gene trees differ in their degree of resolution but do not conflict with one another (see ***Results).*** Thus, we combined the data from the different genes to infer an organismal phylogeny (e.g., Bull et al. 1993), and evaluated its congruence with trees from three molecular data sets from the chloroplast genome. The chloroplast trees differ from one another in their placement of arundinoids, bamboo, rice, and the pooid and PACC clades (fig. 1). However, when branches that are not well supported by the authors' criteria (Davis and Soreng 1993; Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995) are collapsed, bamboo and rice and Pooideae are unresolved relative to the PACC clade (fig. 1). We compared the individual chloroplast trees with the phytochrome tree to detect whether the phytochrome data favored one of the plastid topologies. We did not include the tree inferred from morphological data (Kellogg and Campbell 1987) because subsequent analysis of those data with more powerful algorithms resulted in numerous minimal-length trees whose consensus was largely unresolved (E. Kellogg, personal communication). We measured taxonomic congruence (sensu Swofford 1991, p. 296) in three ways. First, we generated strict consensus trees to identify clades that were common to all phylogenies (Sokal

and Rohlf 1981). Second, in order to estimate the degree of actual conflict among alternate phylogenetic hypotheses, we noted the number of times that each clade on a tree inferred from the chloroplast data sets occurred in bootstrap replicates of the phytochrome data. This analysis assumes that conflict among topologies is sometimes more apparent than real and that a data set might support individual components of a tree while resulting in an overall optimum topology that conflicts with the hypothesis of interest (Mason-Gamer and Kellogg 1996). Third, because the raw data were not available, each of the chloroplast gene trees (fig. 1; *Results*) was treated as a single multistate character from which a matrix of binary characters was derived. In the resulting matrix, each node of the original gene tree becomes a single binary character; thus, the matrix comprises a reduced set of highly consistent characters that support relationships inferred from the original data. The new character matrices can then be combined and cladistically analyzed (Baum 1992; Doyle 1992). We used the approach to infer a tree from the chloroplast gene trees data combined with the phytochrome nucleotide data (i.e., sensu Baum 1992). This approach is not directly comparable to consensus methods that seek to identify identical or compatible components in phylogenetic trees (e.g., Sokal and Rohlf 1981; Bremer 1990; Page 1990). For example, the matrices of combined binary data retain information about the frequency of specific clades among the gene trees; thus, conflicting placement of taxa among gene trees does not necessarily result in an unresolved tree when the binary data are cladistically analyzed (see *Results*). In contrast, specific clades receive a weight of one in most consensus analyses, no matter how many times they occur in rival trees; the result is that conflicting placement of a single taxon in a single tree may produce an unresolved consensus. Only majority-rule consensus trees incorporate information about frequency by retaining clades that occur in some proportion of the rival trees (Margush and McMorris 1981), but the choice of which proportion to specify is arbitrary. A disadvantage of comparing gene trees by recoding them as additive binary data is that it requires that the original trees be taken at face value, i.e., with 100% confidence in each node resolved on the tree. Thus, we stress that results must be interpreted with caution; they cannot be taken as a substitute for results from analysis of a matrix combining the actual sets of nucleotide data.

Analysis of Evolutionary Rates

The proportions of Jukes-Cantor corrected nonsynonymous differences within and among gene lineages were estimated using the program MEGA (Kumar, Tamura, and Nei 1993) and relative evolutionary rates were calculated by the method of Wu and Li (1985).

Southern Blot Analysis

We estimated the number of phytochrome loci in the genome of an exemplar grass by Southern hybridization. Total DNA that was isolated from dark-grown *Oryza sativa* cv. taipei (rice) by standard methods

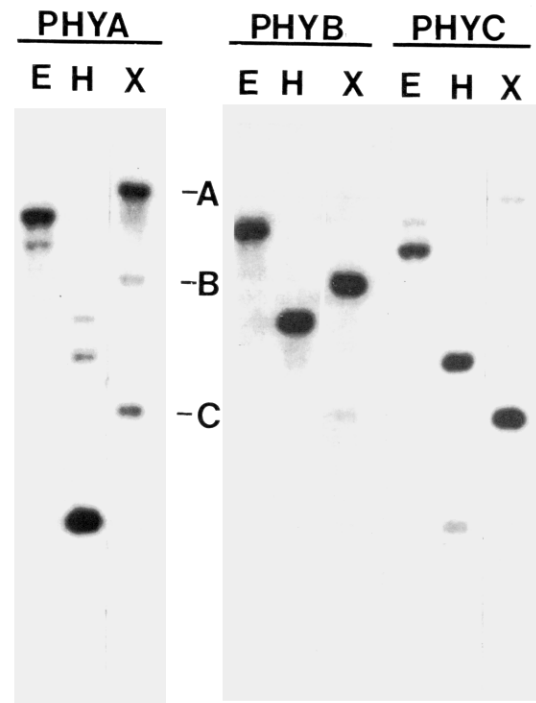

FIG. 3.—Southern blot of rice genomic DNA hybridized with probes specific for *PHYA*, *PHYB*, and *PHYC*. E = *Eco*RI, H = *Hin*dIII, X = *Xho* I; scale of λ*Hin*dIII markers is on the right.

(Doyle and Doyle 1987) was digested in 5-μg aliquots with restriction enzymes, separated on 0.8% agarose gels, and transferred to GeneScreenPlus (DuPont) hybridization membranes according to recommendation of the manufacturers. Three gene-specific heterologous probes (fig. 2), an *Avena sativa PHYA Hin*dIII 1.0-kb insert from cDNA clone pAP3.2 (Hershey et al. 1985), and the *Hordeum vulgare PHYB* and *PHYC Eco*RI/*Hin*dIII inserts from PCR clones mHV238 and mHV228 (Mathews, Lavin, and Sharrock 1995) were ^{32}P-labeled by random priming (Sambrook, Fritsch, and Maniatis 1989). Blots were prehybridized for 12 h at 42°C in 30% formamide, 5 × SSC, 5 × Denhardt's solution, 40 mM $NaPO_4$ (pH 6.8), 0.5% BSA, 1% SDS, and 100 μg/ml denatured salmon sperm DNA and subsequently hybridized to individual heterologous probes for 16 h at 42°C in 30% formamide, 5 × SSC, 40 mM $NaPO_4$ (pH 6.8), 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. Membranes were washed twice for 20 min at room temperature in 2 × wash solution (2 × SSC, 5 mM EDTA, 1.5 mM sodium pyrophosphate, 0.5% SDS) and once for 1 h at 60°C in 2 × wash solution prior to final autoradiography.

Results

Southern Blot Analysis

The blot of total genomic rice DNA reveals multiple bands of hybridization to the 1.0-kb coding-region probe from *PHYA* of *Avena sativa* (fig. 2), one very strongly hybridizing band and one or two additional faintly hybridizing bands in each lane (fig. 3). When the blot is subsequently hybridized with the 0.35-kb probes

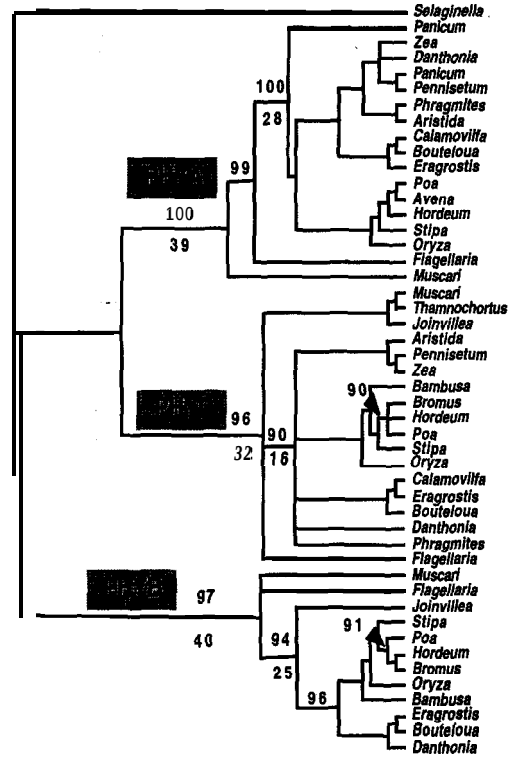

FIG. 4.—Strict consensus of the 24 most parsimonious trees from analysis of phytochrome DNA sequences from grasses, which comprised 203 informative sites. Length = 1,272; consistency index = 0.33; retention index = 0.72. Bootstrap values (from 100 replications) are included on the best supported clades. The numbers of nucleotide mutations supporting ***PHY*** gene lineages are below branches. The names of the homologous ***Arabidopsis PHY*** loci are above branches.

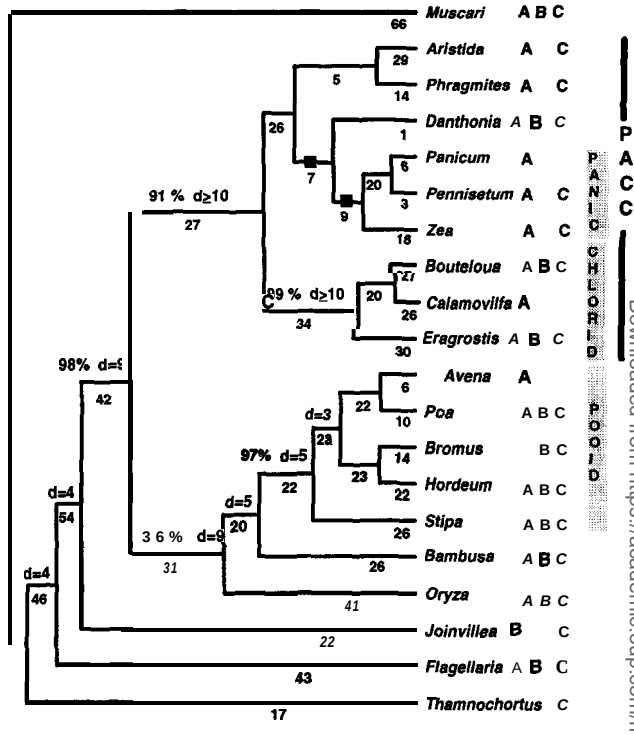

FIG. 5.—Single most parsimonious tree from analysis of combined phytochrome nucleotide data from grasses, which comprised 299 informative sites. Length = 893; consistency index = 0.55; rentention index = 0.53. Bootstrap values from 100 replications and Bremer support (*d*) values are above branches. The numbers of nucleotide mutations supporting clades are below branches. Single uppercase letters represent the ***PHY*** loci that were sampled from each genus; * ***Bambusa PHYA*** is a fragment. Filled boxes are on branches that collapse when just first and second codon positions are searched (108 informative sites).

from ***PHYB*** and ***PHYC of Hordeum,*** in each case one of the weaker bands seen on the *PHYA* blot is strongly detected by the probe. This indicates that, in each lane, the three hybridizing bands very likely correspond to ***PHYA-, PHYB-,*** and PHYC-like sequences. No strong evidence for additional ***PHY*** sequences in rice DNA was seen.

Phylogenetic Analyses: Homology of the Phytochrome Loci

Cladistic analysis of the phytochrome nucleotide data from grasses results in the resolution of three monophyletic gene lineages (fig. 4) that were determined in analyses including sequences from ***Arabidopsis*** {not shown) to comprise homologs of ***PHYA, PHYB,*** and ***PHYC.*** In the phylogeny of phytochrome sequences from grasses the clades are resolved with bootstrap support of 100% ***(PHYA), 97% (PHYB),*** and 96% (***PHYC***), and are supported by 39, 40, and 32 base substitutions, respectively. Two sequences that are most closely related to ***PHYA*** were detected in ***Panicum,*** one of which is very divergent from all ***PHYA***s from grasses and never occurs as the sister group to ***Panicum PHYA (fig. 4).*** Closely related duplicate loci were not detected in any other grass taxon, nor were homologs of ***PHYD*** or ***PHYE,*** consistent with their absence or lack of detection in other monocots (Mathews, Lavin, and Sharrock 1995).

Phylogenetic Analyses: Organismal Phylogeny

In the gene tree of phytochrome sequences from grasses (fig. 4), the ***PHYA*** portion of the tree resolves a clade comprising genes from panicoid, chloridoid, and arundinoid genera as the sister group of a clade comprising sequences from pooid and bambusoid genera, a degree of resolution not observed in the ***PHYC*** clade. The ***PHYB*** clade is resolved similarly to the ***PHYA*** clade, but there are no sequences representing panicoid genera, and the sequence from ***Dunthonia (an*** arundinoid) is the sister group of the chloridoid sequences.

The combined data (fig. 5) resolve two major lineages that are well supported, one comprising genes from bambusoids that are paraphyletic to the genes from pooid taxa, and another that resolves a highly supported PACC clade, similarly to the ***PHYA*** gene phylogeny. Thus, apparent discordance among individual ***PHY*** gene trees simply may result from a lack of resolution in the individual data sets that is overcome when the data are joined; for example, the ***PHYA, PHYB,*** and ***PHYC*** data sets have 51, 69, and 54 informative sites, respectively (for the same set of taxa). A maximum-likelihood analysis of the data results in identical topology (tree not shown), and its major features are maintained in the consensus of two most-parsimonious trees that result when

third codon position nucleotides are excluded from the data set in parsimony analysis (fig. 5).

Congruence Analyses

We compared the organismal phylogeny inferred from the phytochrome data (fig. 5) with the chloroplast trees (fig. 1) because the raw plastid data were not available for combination. Furthermore, we did not summarize the chloroplast topologies in a consensus tree for comparison because we wished to detect whether phylogenetic signal in the phytochrome data favored one of the chloroplast topologies. The strict consensus of all four trees retains just the PACC clade, suggesting that the chloroplast topologies are in conflict with one another and with the phytochrome tree. We did not use methods designed to determine the significance of conflict (e.g., Farris et al. 1994; Huelsenbeck and Bull 1996) because their implementation requires the raw data. Alternatively, we estimated the significance of conflict by determining the number of times clades in the chloroplast trees occur in bootstrap replicates of the phytochrome data. At least one study shows that similar results are obtained from using the bootstrap in this manner and using tests designed to more precisely quantify the conflict (Mason-Gamer and Kellogg 1996). We discovered that relationships of bamboo and rice and of the arundinoid taxa as depicted in the chloroplast trees are infrequently recovered in bootstrap resampling of the phytochrome data set (fig. 1). However, the *ndh*F and phytochrome phylogenies differ only in their depiction of bamboo and rice as paraphyletic or unresolved relative to Pooideae, and in their placement of ***Aristidu (figs.*** 1 and 5). Thus, it is striking that cladistic analysis of the phytochrome data combined with the additive binary coding matrices derived from the chloroplast trees depicted in figure 1 resulted in increased support for most of the clades of the phytochrome phylogeny, while favoring the basal placement of ***Aristida*** in the PACC clade (fig. 6). Notably, topologies that conflict are not well supported (figs. 1 and 5). In the phytochrome phylogeny, low support may result from the lack of ***PHYB*** sequences from both panicoid and arundinoid genera, except ***Danthonia,*** because ***PHYB*** sequences contribute over half of the informative sites to the combined phytochrome data set. Conversely, ***PHYB*** sequences commonly were detected in the chloridoid, bambusoid, and pooid genera, perhaps resulting in the well-supported placement of bambusoid genera. We do not think it likely that ***PHYB*** is absent from most panicoid and arundinoid genera, and are using an alternate oligonucleotide primer pair in PCR to investigate whether the absence of ***PHYB se***quences in our data set might result from nucleotide divergence in the primer sites that we used (see ***Discussion).***

Analysis of Relative Evolutionary Rates

In 25 relative rate tests (Wu and Li 1985) used to evaluate the hypothesis that divergence rates within and among the ***PHY*** loci are clocklike, nine rate differences were significantly different ($P < 0.05$ or 0.01) given a model of rate constancy. All of these differences were among, rather than within, ***PHY*** lineages, except for in the comparison of the two sequences from *Panicum* that are members of the ***PHYA*** clade (table 2). For example, ***PHYC*** lineages are evolving at a significantly slower rate relative to ***PHYA*** and ***PHYB*** sequences, except in *Oryza,* where the full-length ***PHYB*** sequence (GenBank accession X57563) is significantly less divergent compared with the full-length ***PHYA*** sequence (GenBank accession X14172). The sequence from ***Panicum*** that is basal in the ***PHYA*** clade is significantly divergent relative to all other ***PHYA***s from grasses, exemplified by its comparison with ***Poa PHYA*** (table 2).

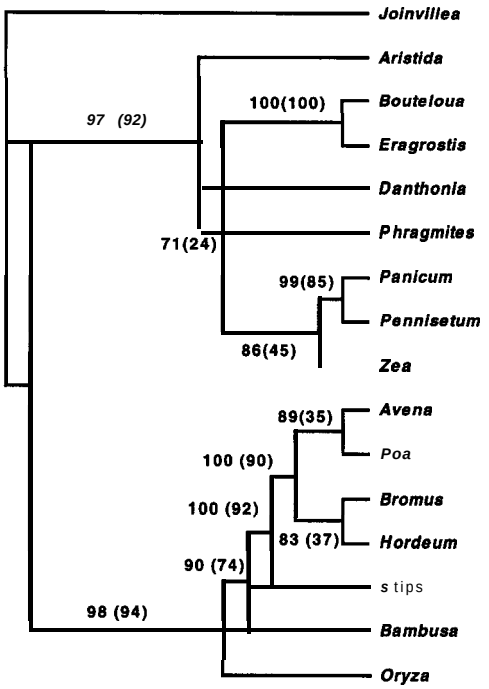

FIG. 6.-Majority-rule consensus from 100 bootstrap replicates of the combined phytochrome and additive binary coding matrices derived from the cpDNA, *rbc*L, and *ndh*F trees. Bootstrap values are above the branches; those from analysis of just the phytochrome data are in parentheses.

Discussion

The Phytochrome Gene Family in Grasses

Results from phylogenetic analyses of phytochrome sequence fragments detected in this study provide further evidence that monocot ***PHY*** loci comprise a subset of the ***PHY*** genes found in nearly all dicot groups surveyed thus far (i.e., in all major subclasses but Magnoliidae, which have not been extensively investigated; Cordonnier-Pratt et al. 1994; Mathews, Lavin, and Sharrock 1995). We detected the same subset of phytochrome loci in grass taxa that we have detected in the monocot subclasses Alismatidae, Arecidae, Zingiberidae, and Liliidae (Mathews, Lavin, and Sharrock 1995), homologs of ***PHYA, PHYB,*** and ***PHYC*** of ***Arabidopsis.***

It is very possible that sequence divergence at the primer sites precludes the amplification of all phytochrome loci present in a genome, or that certain members of the gene family are more readily amplified (see,

Table 2
Relative Rate Tests (Wu and Li 1985) to Detect Rate Asymmetry

Species 1	Species 2	Species 3 (Reference)	$d_{13} - d_{23}$ + S E
Chromophore Region Only (330-594 BP) Compared			
Within lineage tests			
PoaA	PennisetumA	Flagellaria	0.0520 ± 0.0276
BoutelouaB	BambusaB	Flagellaria	0.0286 ± 0.0387
BambusaC	AristidaC	Flagellaria	0.0465 ± 0.0103
OryzaC	PoaC	Flagellaria	0.0024 ± 0.0103
OryzaC	BambusaC	Flagellaria	-0.0232 ± 0.0315
ZeaC	AristidaC	Flagellaria	0.0413 ± 0.0289
ZeaC	BambusaC	Flagellaria	-0.0052 ± 0.3302
ZeaC	OryzaC	Flagellaria	0.0180 ± 0.0310
ZeaC	PhragmitesC	Flagellaria	0.0343 ± 0.0300
Among lineage tests			
OryzaA	OryzaB	Selaginella	0.0639 ± 0.0387
OryzaA	OryzaC	Selaginella	0.0852 ± 0.0547
OryzaB	OryzaC	Selaginella	0.0890 ± 0.0554
BambusaB	BambusaC	Selaginella	0.0919 ± 0.0549
PhragmitesA	PhragmitesC	Selaginella	0.1179 ± 0.0540*
DanthoniaA	DanthoniaB	Selaginella	0.0012 ± 0.0634
DanthoniaA	DanthoniaC	Selaginella	0.1238 ± 0.0548*
DanthoniaA	DanthoniaC	Selaginella	0.1226 ± 0.0552*
AristidaA	AristidaC	Selaginella	0.1350 ± 0.0530*
ZeaA	ZeaC	Selaginella	0.1176 ± 0.0566*
HordeumA	HordeumB	Selaginella	0.0590 ± 0.0676
HordeumA	HordeumC	Selaginella	0.1735 ± 0.0597**
HordeumB	HordeumC	Selaginella	0.1145 ± 0.0560*
PanicumU	PennisetumA	Flagellaria	0.1350 ± 0.0366**
PanicumU	PoaA	Flagellaria	0.0830 ± 0.0409*
Full-Length Coding Sequence (3384 bp) Compared			
Among lineage tests			
AvenaA	ZeaA	Selaginella	-0.0081 ± 0.0161
OryzaA	OryzaB	Selaginella	0.0617 ± 0.0194**

NOTE.—d_{13} and d_{23} are the numbers of nonsynonymous substitutions per site between species 1 and 3, and species 2 and 3, respectively; under the null hypothesis $d_{13} = d_{23}$. SE is standard error.

* $P < 0.05$.

** $P < 0.01$.

e.g., Wagner et al. 1994). However, results from Southern blot analyses of total genomic DNA corroborate the pattern observed by our PCR sampling (see also discussion of variation in sampling strategies in Mathews, Lavin, and Sharrock 1995). Furthermore, the phylogenetic distribution of phytochrome loci apparent in our sampling is consistent with the early divergence of monocots from dicots (Martin, Gierl, and Saedler 1989; Wolfe et al. 1989; Brandl, Mann, and Sprinzl 1992; Manhart 1994; Laroche, Li, and Bosquet 1995), which likely occurred prior to much of the diversification noted in the *PHYB/D/E* gene lineage within dicots. We currently are investigating whether the monocot–dicot split might have preceded the divergence of *PHYB/D* from *PHYE.*

Results of these analyses are pertinent to the use of experimental systems such as *Arubidopsis* to develop a model of phytochrome action in flowering plants. We know that *PHYA* and *PHYB* in *Arabidopsis* have distinct photosensory roles (Reed et al. 1994), and that *PHYD* in *Arubidopsis* does not compensate for the loss of PHYB function in *phyB* null mutants (Reed et al. 1993; Wester et al. 1994); thus, the additional genes in the *PHYB/D/E* subfamily are not necessarily functionally redundant. Further, *PHYC* and *PHYE are* widely distributed and are divergent to a degree that predicts that they also function discretely in photomorphogenesis (Mathews, Lavin, and Sharrock 1995). Thus, although *Arabidopsis* and perhaps other dicots have partitioned red-light-mediated development among five different photoreceptors, monocots, and perhaps certain other flowering plants, potentially function with just three photoreceptors. This phenomenon could result from dicots having additional growth responses to red-light that are mediated by the additional loci, or from monocots accomplishing the same complexity of responses with fewer photoreceptors.

Results from relative rate tests indicate that evolutionary rates among *PHY* lineages in grasses are not clocklike, as evidenced by the slower evolution of *PHYC.* We performed these tests on just fragments of phytochrome genes; further rate heterogeneity was detected in our one comparison involving full-length sequences. Thus, our estimate of rate variability (based on a highly conserved portion of exon I; e.g., Sharrock and Quail 1989) is likely a conservative one. The single anomalous sequence detected in our survey of phytochromes in grasses, the duplicate *PHYA* locus from *Panicum,* is evolving rapidly relative to all other phytochrome sequences from grasses (table 2) and, most significantly, never occurs as a sister group to the *PHYA* homolog in *Panicum,* or to any other *PHYA (fig. 4).* This sequence could be a member of a duplicated *PHYA* lineage in grasses that is under strong selection to diverge, although no additional members of this lineage were detected in other grass taxa. Alternatively, while evidence of the functional status of the sequence is lacking (e.g., no stop codons were detected and the site of chromophore attachment is preserved), its relatively rapid evolution is consistent with the hypothesis that it is from a pseudogene. A pseudogene from *Pisum* (Sato 1990), however, is characterized by a number of deletions and is just 1% divergent from *Pisum PHYA* (M. Lavin, personal communication). Phytochrome fragments cloned from *Zea* and inferred to be pseudogenes have not been characterized (Christensen and Quail 1989).

Utility of Phytochrome Nucleotide Data for Evolutionary Studies

The phytochrome data we generated provide greater resolution to phylogenetic questions in the grass family than molecular data from the chloroplast genome. For example, the divergence of the family into two major lineages observed in both the phytochrome and *ndh*F trees is highly supported by phytochrome characters, but less so with *ndh*F characters, despite the higher number of characters in the *ndh*F data set (2,124 versus 972 phytochrome sites, including gaps in both data sets). The clade comprising bambusoid, oryzoid, and pooid taxa (BOP clade of Clark, Zhang, and Wendel 1995) is supported by 32 phytochrome characters but just 6 *ndh*F characters, while the PACC clade is supported similarly by both data sets (fig. 5; Clark, Zhang, and Wendel

1995). Furthermore, the unambiguous placement of bambusoid genera with Pooideae (bootstrap support of 97%) is retained in all trees that are up to 10 steps longer than the minimal-length tree, and is not altered when third codon position sites are dropped from analyses (fig. 5). Comparative support for pooid and PACC clades also is weaker in the *rbc*L phylogeny than in the phytochrome tree (as evidenced by bootstrap values of 69% vs. 97% and 84% vs. 91%, respectively). In the phytochrome tree, placement of the arundinoid taxa is weakly supported, as it is in trees from all of the other data sets; yet this may result merely from the lack of the more phylogenetically informative ***PHYB*** sequences from arundinoid and panicoid taxa in the data set. Furthermore, deletion of a homologous amino acid in ***PHYB*** shared by chloridoid genera potentially provides a tool to more definitively circumscribe Chloridoideae and, thus, further define structure in the PACC clade. Evidence from analyses of relative evolutionary rates among phytochrome lineages in grasses is consistent with the prediction that additional data from ***PHYB*** loci will help to resolve relationships within the PACC clade and to place genera of uncertain affinity because they are evolving significantly faster than the more commonly sampled ***PHYC*** genes. Thus, the phytochrome data apparently contradict the suggestion of Clark, Zhang, and Wendel(1995, p. 452) that "robust resolution of the branch order among the bambusoid, oryzoid, pooid, and PACC clades will probably not be forthcoming from any reasonable amount of DNA sequencing" because of a "relatively rapid divergence among these ancient lineages."

The discordance that we observed among individual phylogenies of Poaceae is restricted to structure within the PACC clade and to placement of bamboo and rice (figs. 1 and 5), and likely results from lack of resolution in individual data sets (e.g., Olmstead and Sweere 1994). This explanation is supported by results from congruence analyses. For example, among the chloroplast data sets, signal for placement of bamboo and rice is weak (fig. 1). but their placement with Pooideae is enhanced when phtochrome characters are added. Likewise, ***Aristidu*** is basal in the PACC clade in the ***PHYA*** gene tree (not shown) but not in the phylogeny from combined ***PHY*** data, a signal in the data that perhaps is enhanced by addition of chloroplast chraracters (fig. 6).

We suggest that analysis of combined sets of nucleotide data, including from informative loci such as phytochrome loci, likely will provide very robust phylogenetic hypotheses for Poaceae. While a survey of both morphological and molecular characters suggests that a scenario of rapid diversification of grass lineages is plausible (e.g., Kellogg and Watson 1993; Barker, Linder, and Harley 1995; Clark, Zhang, and Wendel 1995), the phytochrome data suggest that it is premature to attribute the lack of resolution among lineages of the grass family to this phenomenon; the hypothesis of rapid radiation can reliably be inferred only in the context of data from additional molecules.

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