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A study of genetic variation and evolution of *Phyllostachys* (Bambusoideae: Poaceae) using nuclear restriction fragment length polymorphisms

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Abstract Phylogenetic and taxonomic difficulties are common within the woody bamboos, due to their unique life cycle, which severely limits the availability of floral characters. To addresss some of these problems, 20 species of woody bamboos in the genus *Phyllostachys* were analyzed using nuclear restriction fragment length polymorphisms (RFLPs). The RFLP data were used to generate genetic distances between all pairs of taxa and to examine the degree of genetic variation within and among bamboo species. The genetic distances were also used to create dendrograms of accessions and species. These trees supported the current division of the genus into two sections and provided some information on the thorny taxonomic problems in this group. We show that RFLPs can be used for species identification and the delineation of species limits.

Key words Bamboo · *Phyllostachys* · RFLP · Variation · Evolution

Introduction

The bamboo subfamily, the Bambusoideae, is one of the five currently recognized subfamilies of the grasses, the Poaceae (Soderstrom and Ellis 1986). The Bambusoideae consists of 850 species of the woody bamboos in one supertribe, the Bambusodae, while the other supertribe, the Olyrodae, contains about 150 species of herbaceous bamboos, or bamboo allies (Clark 1990; Soderstrom 1981). The taxonomy and systematics of the woody bamboos are incomplete in comparison with the other major groups of grasses. Though some species are relatively well defined, the delimitations of genera and even tribes is sometimes tenuous. Very little work has been done on infrageneric relationships. The need to assess the genetic variability and population structure of these species is pressing, however. These plants are vital to many Asian economies and are used on a daily basis by 2.5 billion people worldwide (Manokaran 1990), but a basic knowledge of their biology and genetics which would allow them to be exploited more efficiently is severely lacking.

This degree of uncertainty is a direct result of the unusual life cycle of the woody bamboos. These plants reproduce asexually via complex underground rhizome systems for a number of years. At the end of a characteristic period of vegetative growth, members of a given species will flower synchronously, even if widely separated geographically. For instance, in the late 1960s, accessions of *Phyllostachys bambusoides* flowered in China, the United States, Europe and North Africa, all within a 5- to 10-year period (Soderstrom 1976). However, it is currently unclear whether all clones of a given species flower simultaneously, or just a subset. The vegetative growth phase varies from 1 year to as long as 120 years among bamboo species (Janzen 1976). In fact, some species have never been known to flower. After flowering, the aboveground culms die, and often the rhizome system also dies (Blatter 1930).

With this kind of life cycle, floral characters, the basis of a great deal of plant taxonomy and systematics, are available very infrequently. Therefore, bamboo systematics has to be primarily based on vegetative morphology. Genera are delimited on the basis of rhizome characters and branching patterns, but species determination requires characters such as culm sheath, ligule and auricle morphology (Ohrnberger and Goerrings 1986). This presents a number of problems. First, these vegetative characters are often environmentally influenced, and so are less constant than one could wish for systematic purposes (Wu 1962). Second, characters which delimit species are likely to be subtle (e.g. degree of culm sheath spotting or hairiness). Third, it is difficult to say how 'natural' these types of markers are, i.e. how much they reflect the true evolutionary history of the organisms. Without floral characters, it is unclear

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whether a systematic grouping based on vegetative characters alone reflects the evolution of the species concerned. From a practical standpoint, the variability inherent in the characters makes the identification of individual plants and circumscription of species very difficult, even for experts. For example, *Sasa palmata* has received 25 different names since it was first described in 1913 (Ohrnberger and Goerrings 1986).

To overcome some of these problems, we decided to apply modern molecular techniques to the species of one genus of woody bamboos. The genus *Phyllostachys* was recognized in 1843 by Siebold and Zuccharini and is one of the larger genera of temperate bamboos. *Phyllostachys* is well defined, based on a sulcate internode on the branch-bearing side of the culm and usually two branches per node (McClure 1957). However, the species and infraspecific taxa are not as clearly defined. Two sections, *Phyllostachys* and *Heteroclada* have been defined by Wang et al. (1980a, b), but these definitions are considered to be fairly tentative. In a previous study (Friar and Kochert 1991), we assessed the restriction fragment length polymorphism (RFLP) variability present in this group and determined that nuclear RFLPs would be appropriate markers to infer systematic relationships within bamboo genera. Here we present the results of a comprehensive study on 36 accessions of *Phyllostachys* and one outgroup. We are maintaining this information as a database for the identification of bamboo species. In order to easily add more taxa to the database and phylogeny at a later date, we separately analyzed data from a subset of the probes used in this study to determine the most useful probes for this purpose.

Materials and methods

Leaf specimens representing two genera and 21 species were collected from 37 bamboo accessions at the USDA Byron, Georgia Fruit and Tree Nut Research Station (Table 1). These samples were stored at − 80 °C until DNA was extracted using the SDS procedure previously described for rice (McCouch et al. 1988).

Extracted DNA was digested separately with three restriction enzymes (*Hin*dIII, *Eco*RI or *Eco*RV) for at least 6 h before agarose (2.0%) electrophoresis overnight. The size-fractionated DNA samples were then transferred to nylon membranes by the method of Southern (1975).

Library construction and probing procedure were as in Friar and Kochert (1991). Twelve bamboo genomic probes and 4 rice cDNA probes were used. Ten of the probes were evaluated over all three restriction enzymes, 5 were evaluated over two enzymes and 1 probe was used with only one enzyme (Table 2).

Presence versus absence of 384 restriction fragment bands was scored. Genetic distances were calculated using the Jaccard algorithm (Sneath and Sokol 1973) in the computer program NTSYS-pc (Rohlf 1992). Distances were used to create dendrograms using the neighbor joining program of the PHYLIP package, version 3.4 (Felsenstein 1993). Ten random taxon input orders were used. Restriction fragment presence/absence data were also analyzed using PAUP (phylogenetic analysis using parsimony), version 3.1.1 (Swofford 1993). Heuristic analyses were performed using TBR branch swapping with 100 orders of taxon entry. Uninformative characters were ignored. Confidence limits were placed on groupings with a bootstrap procedure with 100 replications (Felsenstein 1985). For these analyses, *Shibataea kumasaka* was designated as the outgroup, based on the systematic analysis of this group by Ohrnberger and Goerrings (1986). Voucher specimens have been placed in the University of Georgia herbarium (UGA).

For the identification of unknown bamboo accessions from the USDA Plant Introduction facility in Savannah, DNA isolation, Southern blotting and probing procedures were the same as above. Only 2 genomic probes and two restriction enzymes were used. Genetic distances and dendrograms were also generated as above.

Table 1 Bamboo DNA samples. The names are as identified at the USDA Byron, Georgia Fruit and Tree Nut Research Station. An asterisk denotes those samples that were later shown to have been misidentified

Species	Identifier
Phyllostachys angusta A McClure	PI 23237
Phyllostachys angusta B	PI 23237
Phyllostachys arcana McClure	PI 77007
Phyllostachys atrovaginata C.S. Chou & H.Y. Zou	PI 80149
Phyllostachys aurea AA. & C. Riv.	PI 75153
* *Phyllostachys aurea* B	PI 38919
* *Phyllostachys aureosulcata* Yellowgroove A McClure	PI 55713
Phyllostachys aureosulcata Yellowgroove B	BL 1
Phyllostachys aureosulcata C	S 2765
Phyllostachys bambusoides Giant Timber A Sieb. & Zucc.	PI 128787
Phyllostachys bambusoides Slender Crookstem B	PI 146420
Phyllostachys bambusoides White Crookstem C	PI 66785
Phyllostachys decora A McClure	PI 128789
Phyllostachys decora B	PI 128789
Phyllostachys dulcis McClure	PI 73452
Phyllostachys elegans McClure	PI 128778
Phyllostachys glauca McClure	PI 77011
Phyllostachys heterocycla forma *pubescens* (Houzeau Leh.) Muroi	PI 80034
Phyllostachys makinoi Hayata	PI 195284
Phyllostachys meyeri McClure	PI 116768
Phyllostachys nidularia A Munro	PI 63757
Phyllostachys nidularia B	PI 128769
* *Phyllostachys nidularia* C	S 2767
Phyllostachys nidularia Smooth Sheath D	PI 128776
Phyllostachys nigra Black A (Lodd.) Munro	PI 77259
Phyllostachys nigra Bory B	PI 77258
Phyllostachys nigra Henon C	PI 24761
Phyllostachys nuda McClure	PI 103938
Phyllostachys purpurata A McClure	PI 128771
Phyllostachys purpurata Straight Stem B	PI 77001
Phyllostachys purpurata Straight Stem C	PI 128792
Phyllostachys rubromarginata A McClure	PI 66902
Phyllostachys rubromarginata B	PI 67398
Phyllostachys viridiglaucescens (Carriere) A. & C. Riviere	PI 123432
Phyllostachys viridis A (Young) McClure	PI 77257
Phyllostachys viridis Robert Young B	S-2764
Shibataea kumasaka (Zollinger ex Steudel) Makino	PI 89716

Results and discussion

To analyze the phylogenetic relationships within this genus, 12 random genomic bamboo clones were used. These probes had previously been shown to be low- to

Table 2 Probes and enzymes used to detect RFLPs

Probe	Type	Enzymes used	Number of bands/enzyme
BL004	Bamboo genomic	*Hind*III, *Eco*RI, *Eco*RV	10.3
BL007	Bamboo genomic	*Hind*III, *Eco*RI, *Eco*RV	7.6
BL009	Bamboo genomic	*Hind*III, *Eco*RI, *Eco*RV	8.6
BL011	Bamboo genomic	*Eco*RI, *Eco*RV	4.5
BL012	Bamboo genomic	*Hind* III, *Eco*RV	7.0
BL013	Bamboo genomic	*Hind* III, *Eco*RI, *Eco*RV	14.3
BL014	Bamboo genomic	*Hind* III, *Eco*RI	9.5
BL019	Bamboo genomic	*Hind* III, *Eco*RI, *Eco*RV	10.6
BL021	Bamboo genomic	*Hind* III, *Eco*RI, *Eco*RV	12.3
BL022	Bamboo genomic	*Hind* III, *Eco*RI	5.5
BL023	Bamboo genomic	*Hind* III, *Eco*RI, *Eco*RV	6.6
BL046	Bamboo genomic	*Hind* III, *Eco*RI, *Eco*RV	11.0
CDO079	Rice cDNA	*Hind* III, *Eco*RI	6.5
CDO251	Rice cDNA	*Hind*III	8.0
CDO281	Rice cDNA	*Hind* III, *Eco*RI, *EcoRV*	10.6
CDO345	Rice cDNA	*Hind* III, *Eco*RI, *Eco*RV	13.3

moderate-copy number and nuclear in origin (Friar and Kochert 1991). Four rice cDNA probes were also used (Table 2). A total of 384 bands were scored over 43 probe-enzyme combinations, for an average of 8.9 bands per probe-enzyme combination. Numbers of bands ranged from 3 to 23 bands per probe-enzyme combination. Of the 384 bands, 4 (1.0%) were common to all accessions, 34 (8.9%) were unique to a single accession and 346 (90.1%) were informative. Nei and Li's (1979) K distance was computed for several pairs of species in the genus *Phyllostachys*. The greatest value was computed to be 0.024, well within the suggested limit of 0.05 for reliable use of restriction fragment data.

Variation within species

As was previously determined (Friar and Kochert 1991), there was a large amount of variability between *Phyllostachys* species, but little variability within any given species. Genetic distances were calculated between all pairs of taxa in this study. The average genetic distance between accessions of the same species was 0.138, while the average genetic distance between species was 0.508. Most species showed very low levels of genetic distance between individual accessions. For instance, the 2 accessions of *P. angusta* had only a genetic distance of 0.04 between them. Likewise, all 3 accessions of *P. nigra* had an average genetic distance of 0.02 between them. Some accessions of a single species were identical (*P. nigra* B and C, *P. viridis* A and B), leading one to believe that the 2 plants were offshoots of the same rhizome. However, some single accessions of a given species were significantly different from the other representatives of that species. *Phyllostachys bambusoides* A, for instance, had an average genetic distance of 0.48 to the other 2 *P. bambusoides* accessions.

A dendrogram drawn using genetic distances between all of the accessions based on the neighbor joining algorithm is shown in Fig. 1. Most of the accessions form groups consistent with their species identifications. However, several inexplicable groupings were found. For instance, *P. aurea* B consistently grouped with the members of *P. nidularia*, rather than with *P. aurea* A. *Phyllostachys aureosulcata* A grouped with *P. aurea* A rather than with the other members of *P. aureosulcata*,

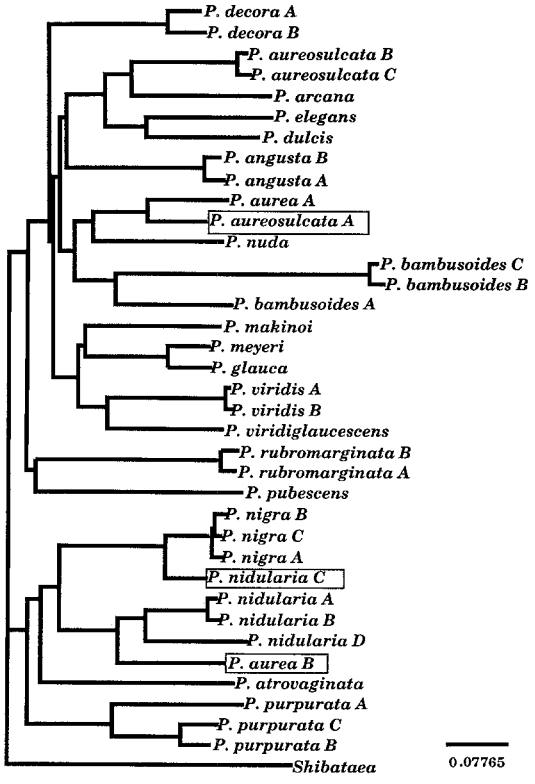


Fig. 1 A dendrogram showing relationships between accessions of bamboo. The taxa in *boxes* were later shown to be misidentified. Branch lengths are proportional to genetic distance (see scale at bottom of figure)

and *P. nidularia* C clustered with the members of *P. nigra* instead of the other members of its species. These anomalous groupings were consistent. We therefore asked for confirmation of the identity of the bamboos from which these samples had been taken, which caused the Byron Introduction Station to update all of their bamboo identifications. All of the plot identifications were confirmed except for the plots from which the anomalous *P. aurea* B and *P. aureosulcata* A samples had been taken. These were shown to have been previously misidentified. The accession labelled *P. aureosulcata* A in this analysis was determined to be a representative of *P. aurea*, which is indeed where it groups in the cluster analysis. The plot from which *P. nidularia* C was taken was discovered to be a mixed plot, containing not only culms of *P. nidularia* but also those of *P. nigra*. This demonstrates the usefulness of this type of analysis in identifying plants of uncertain species.

We have tested this procedure by analyzing several unidentified bamboo clones collected from the Coastal Area Experiment Station in Savannah, Georgia. A number of identifications could be made based on the clustering of the unknown species with known standards (Fig. 2). For example, the plant labelled *P. nigra* A? had been tentatively identified as being a member of that species. This analysis groups it easily with the known members of *P. nigra*, so that identification is supported. On the other hand, the plant labelled *P. viridis* A? does not group with the rest of the members of *P. viridis*, but rather with *P. meyeri*. The tentative identification was therefore likely to have been incorrect.

Variation between species

The amount of genetic distance between species was much larger than the average distance within species. We constructed a dendrogram of species by pooling the distances from accessions to get average pairwise distances for species (Miller and Tanksley 1990), excluding those accessions that were shown to be misidentified. These species relationships are shown in Fig. 3. For the most part, this clustering supports the same relationships between species as the accession tree. The two differences involve *P. makinoi* and *P. angusta*. In the accession tree, *P. angusta* forms a group with *P. aureosulcata*, *P. arcana*, *P. elegans* and *P. dulcis*. However, in the species tree, *P. angusta* groups with *P. glauca* and *P. meyeri*. Likewise, *P. makinoi* moves from being closely grouped with *P. glauca* and *P. meyeri* in the accession tree to appearing related to *P. viridis* in the species tree. These differences may have been the result of pooling the data sets.

This analysis shows two distinct groupings of *Phyllostachys* species that correlate highly with the two infrageneric sections as defined by Wang et al. (1980a, b). Both sections are shown on the species tree. The only difference between the sections Wang defined and the groupings shown on this tree is in the placement of *P. nigra*. Wang et al. (1980a, b) placed *P. nigra* in section *Phyllostachys*. However, in these analyses *P. nigra* groups internal to section *Heteroclada*.

Some of the previous difficulties regarding the classification of this genus can be resolved using this data.

Fig. 2 A dendrogram showing the identification unknown bamboo clones. Taxa shown with *question marks* had been tentatively identified. Branch lengths are proportional to genetic distance (see scale at bottom of figure)

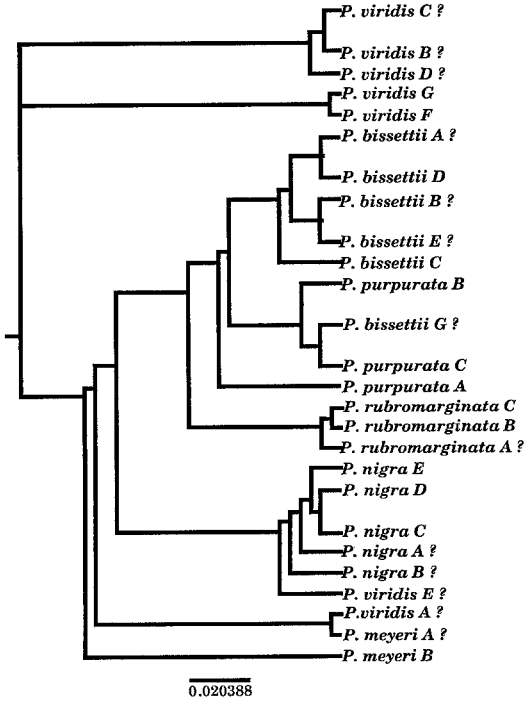
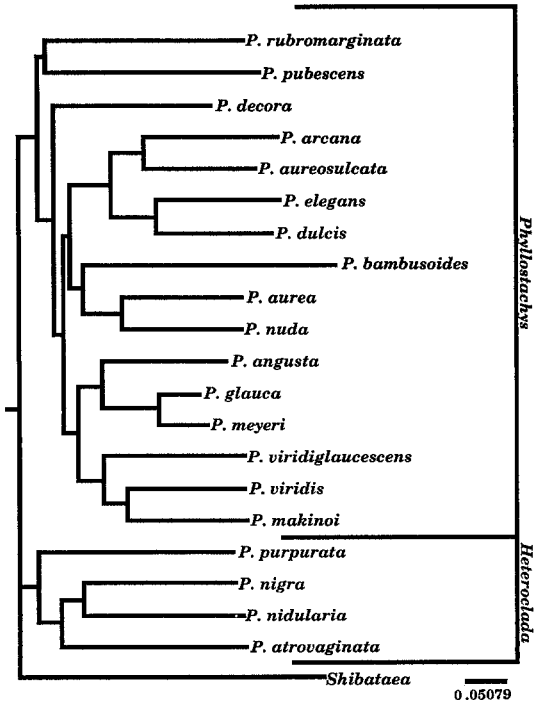


Fig. 3 A dendrogram showing the relationships between bamboo species. The two sections as identified by Wang et al. (1980a, b) are shown on the figure. Branch lengths are proportional to genetic distance (see scale at bottom figure)



Phyllostachys rubromarginata was tentatively assigned to section *Phyllostachys* by Wang et al. (1980a, b). This placement is strongly supported by this analysis. Also, it has been suggested that *P. elegans* was not a 'good' biological species, but rather a form of *P. viridiglaucescens* (Chou and Chu 1980). This analysis supports retaining these species as separate entities. In fact, these 2 species are widely separated within the genus in both the accession and species trees.

It has often been suggested that restriction fragments are unsuitable as characters for use in cladistic studies. To test this premise for this data set, we used the restriction fragment presence/absence data to generate a phylogeny by means of the parsimony algorithm in the computer program PAUP (Swofford 1993). The resulting majority-rule bootstrap consensus tree is shown in Fig. 4. The pattern of associations between accessions within a species are identical in the parsimony and distance trees. A number of the relationships between species are similar between the two analyses also. For instance, *P. nidularia* and *P. nigra* are closely associated in both. The parsimony tree also very clearly shows the delineation of the two tribes as defined by Wang et al. (1980a, b). However, within sections, several of the species relationships are rearranged in the parsimony tree.

Comparisons between this study and previous studies of the infrageneric phylogeny of the genus *Phyllostachys* are difficult to make, as the other studies have used smaller, different subsets of the species than were used here. Wu (1962) analyzed 5 species of this genus using leaf anatomy as a phylogenetic tool. Even after accounting for three nomenclatural changes, the grouping she suggested for these 5 species do not at all agree with those suggested here. For instance, she grouped *P. nigra* with *P. pubescens* and *P. aurea* with *P. makinoi*. Both of these pairs of species are widely separated in our analysis. Chou et al. (1984) used several biochemical markers, including chromatogram patterns of phenolics and flavenoids and isozyme patterns of peroxidases, to analyze 7 species of *Phyllostachys*. The three analyses and an analysis of the combined data all gave very different groupings. However, none of these four clustering patterns are very similar to those presented here. In their study *P. nigra* grouped very closely with the other members of section *Phyllostachys*, unlike its placement in the current study. However, since all of the species used by Chou et al. (1984) were in this section, it is difficult to say how much weight to put on this finding.

In conclusion, RFLPs have shown great utility in determining the amount of genetic variation present within and between bamboo species, which could be of further use in inferring the relationships between species. A large number of new species have recently been named (Wang et al. 1980a, b), but not all of these have been

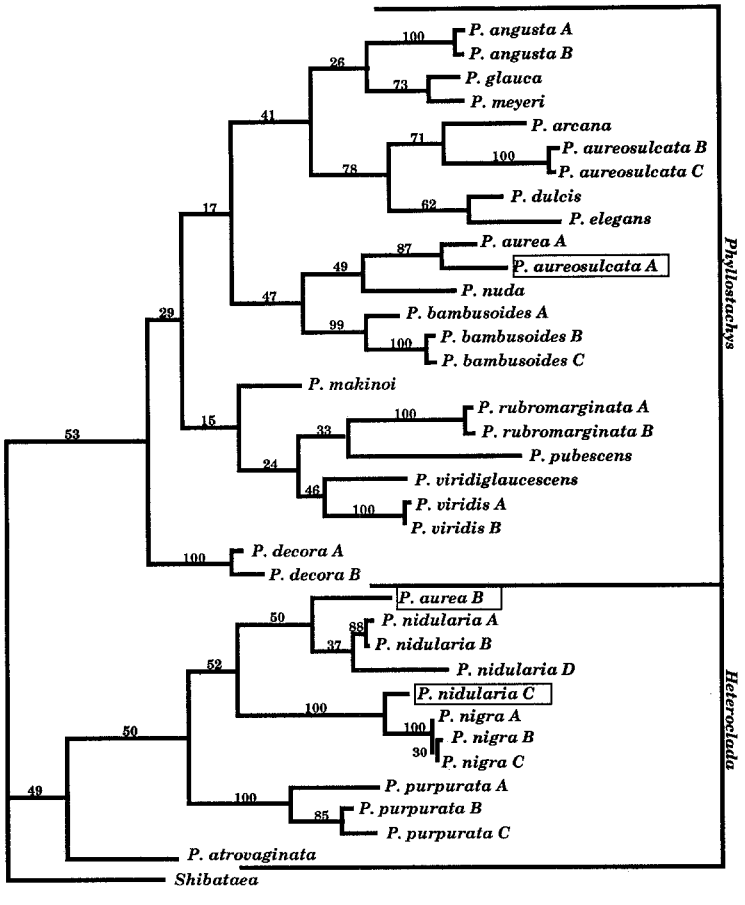


Fig. 4 A majority-rule bootstrap consensus tree from the computer program PAUP; tree length = 915, Consistency Index (CI, excluding uninformative characters) = 0.262, Retention Index (RI) = 0.636. *Numbers above the nodes* represent the number of times, out of 100, that a particular grouping was supported during bootstrapping. As in Fig. 1, taxa which had been misidentified are shown in *boxes*. Sections as defined by Wang et al. (1980a, b) are also shown on this tree

widely accepted as many of them have been considered to be merely forms of acknowledged species. By looking at the degree of genetic distance between these taxa and pre-existing species, one may be able to resolve this confusion. If these new species are actually genetically distinct from any previously described, one may wish to maintain them in cultivation. Most of the native habitats of the bamboos are being destroyed at a rapid rate, so the evaluation of bamboo germ plasm for conservation purposes is urgently needed. Finally, it has been suggested that some genera of bamboo have resulted from hybridization (Clark et al. 1989; Ohrnberger and Goerrings 1986), a hypothesis which could easily be investigated using RFLP technology.

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