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Bamboo germplasm screening with nuclear restriction fragment length polymorphisms

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Summary. Bamboo species are difficult to identify because flowering material is seldom available and taxonomy is of necessity based on vegetative characters. To evaluate the utility of restriction fragment length polymorphism (RFLP) analysis in bamboo systematics and germplasm screening, a library of random genomic probes from a *Phyllostachys nigra PstI* library was constructed. Probes from the library were used to screen bamboo germplasm consisting mostly of temperate bamboos of the genus *Phyllostachys*. RFLP variation was abundant, and species-specific patterns were readily obtained. Chloroplast DNA showed little variation among the bamboo accessions analyzed.

Key words: RFLP – Bamboo – *Phyllostachys* – Chloroplast DNA – Germplasm screening

Introduction

Bamboos are a fascinating group of organisms that play a vital role in the economy and life style of many Asian, African, and American countries (Farrelly 1984; McClure 1956, 1966). The term "bamboo" has traditionally been used to refer to a group of perennial, woody grasses, some of which grow to impressive sizes. The woody bamboos have always been recognized by grass systematists as a monophyletic group (Soderstrom and Ellis 1986), and they are placed in their own tribe, the Bambuseae. These bamboos primarily reproduce asexually by the production of underground rhizomes. Flowering is rare in most species; some accessions have never been known to flower, and some flower at intervals of as long as 120 years. In some species, flowering of given clones appears to occur at about the same time over wide geographic areas, and the flowering shoots die soon after flowering (McClure 1966; Jantzen 1976). Thus, many U.S. plantings of *Phyllostachys bambusoides* flowered and died back to the rhizome system in the late 1960's (Adamson et al. 1978). With this sort of a life cycle, it would seem that the simultaneous flowering of different clones or species would be rare and that there would be little opportunity for genetic exchange. Woody bamboos might thus be expected to evolve slowly, and this idea is supported by the large number of "primitive" flower characters retained in the bamboos. Indeed, bamboos are postulated to be "guardians of ancient flowering systems long since lost in other grasses" (Soderstrom 1981). Despite their inherent interest, however, researchers know very little about breeding systems, population structure, or genetic variability in bamboos, and modern techniques of population genetics and molecular systematics have not been applied to this group. Collections of bamboo exist at several localities world-wide, but there has been little attempt to quantify the genetic variability present.

As opposed to most plant groups, the classification of bamboos must be done primarily using vegetative characters, since flowering is so infrequent and unpredictable. In the absence of floral characters, current bamboo taxonomic descriptions at the species level rely heavily on morphological characters of the culm sheath, the ligule, or the auricle – and distinguishing these requires considerable experience on the part of the observer. In addition, culm sheaths are only available at certain times of the year in some species, and even experts have difficulty in identifying bamboo species. One plant had for "a hundred frustrating, flowerless years, twice been proclaimed a new genus, twice been classified as an existing genus,

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Table 1. Bamboo accessions used for RFLP analysis[a]

1. *Arundinaria amabilis* McClure	PI 110509
2. *Arundinaria funghomii* McClure	TRS 2501
3. *Arundinaria graminea* (Bean) Makino	PI 75147
4. *Arundinaria* sp.	TRS 2523
5. *Arundinaria* sp.	TRS 2524
6. *Arundinaria* sp.	TRS 2527
7. *Bambusa glaucescens* (Willd.) Holttum	PT A-5
8. *Bambusa glaucescens* 'Alphonse Karr'	PI 63959
9. *Bambusa glaucescens* 'Chinese Goddess'	PI 77014
10. *Bambusa glaucescens* 'Silverstrip'	PT A-1
11. *Phyllostachys arcana* McClure	PI 77007
12. *Phyllostachys aurea* A. & C. Riv.	PI 75153
13. *Phyllostachys aureosulcata* McClure	PI 55713
14. *Phyllostachys bambusoides* Sieb. & Zucc.	PI 40842
15. *Phyllostachys bambusoides*	PI 118926
16. *Phyllostachys bambusoides* 'Giant Timber'	PI 128787
17. *Phyllostachys bambusoides* 'Giant Timber'	PI 12180
18. *Phyllostachys bambusoides* 'Giant Timber'	PI 77003
19. *Phyllostachys congesta* Rendle	PI 80149
20. *Phyllostachys decora* McClure	PI 128789
21. *Phyllostachys dulcis* McClure	PI 73452
22. *Phyllostachys elegans* McClure	PI 128778
23. *Phyllostachys glauca* McClure	PI 77011
24. *Phyllostachys makinoi* Hayata	PI 195284
25. *Phyllostachys meyeri* McClure	PI 116768
26. *Phyllostachys nidularia* Munro	PI 128779
27. *Phyllostachys nigra* (Lodd.) Munro 'Black'	PI 49505
28. *Phyllostachys nigra* 'Hale'	TRS 2702
29. *Phyllostachys nigra* 'Henon'	PI 24761
30. *Phyllostachys nigra* 'Henon'	PI 66787
31. *Phyllostachys nigra* 'Henon'	PI 75158
32. *Phyllostachys nuda* McClure	PI 103938
33. *Phyllostachys purpurata* McClure	PI 128771
34. *Phyllostachys purpurata*	PI 128796
35. *Phyllostachys purpurata* 'Straight Stem'	PI 77001
36. *Phyllostachys purpurata* 'Straight Stem'	PI 116711
37. *Phyllostachys rubromarginata* McClure	PI 67398
38. *Phyllostachys viridis* (Young) McClure	PI 77257
39. *Phyllostachys viridis* 'Robert Young'	PI 89718
40. *Pseudosasa japonica* (Sieb. & Zucc.) Makino	PI 75165
41. *Sinobambusa* sp.	PT H-1
42. *Sinocalamus lateriflorus* (Munro) McClure	PT 25835

[a] For some bamboo accessions the Plant Introduction (PI) number is given. For others, a number assigned by Thomas Soderstrom (TRS) is provided. If neither was available, the plot number (PT) where the accession is growing at Byron, Ga. is provided.

and four times received new species names", before flowering revealed its true identity (Soderstrom 1979). The "naturalness" of the morphological characters used to classify this group is also open to debate. The goal of any taxonomic grouping is to reflect evolutionary history, and it is uncertain that the characters traditionally used to classify bamboo fulfill this requirement.

Because of the difficulty in utilizing morphological characters, an accurate, usable classification of these organisms is of importance, as this one group can supply humans with food, shelter, boats, paper, and numerous other goods. This study assesses the usefulness of DNA molecular markers for studying bamboo systematics and evolution and for germplasm screening. Restriction Fragment Length Polymorphism (RFLP) analysis of low-copy-number nuclear DNA has proven very successful for this type of analysis in other plants. As an initial test, we have used a selection of temperate bamboos, largely collected from China, which has been maintained in the United States, first at the Barbour Lathrop Plant Introduction Garden in Savannah, Georgia and later at the USDA Southeastern Fruit and Tree Nut Laboratory at Byron, Georgia.

Materials and methods

The leaves of 61 accessions of bamboo were collected from the USDA Southeastern Fruit and Tree Nut Laboratory at Byron, Georgia (Table 1). The leaves were stored at −80 °C, and DNA was subsequently isolated according to the procedure outlined by McCouch et al. (1988) for rice.

A random genomic library in plasmids was constructed from *Phyllostachys nigra* (PI 77259) DNA that had been digested to completion with *Pst*I. The digested DNA was separated by agarose electrophoresis in low melting point agarose, and the region containing 1–2 kb fragments was cut from the gel under UV light. Fragments were isolated from the gel slices by phenol-chloroform extraction (Sambrook et al. 1989), cloned into pUC8, and transformed into *E. coli* DH5α cells. Bacteria containing recombinant clones were selected on plates containing X-Gal and IPTG. Plasmids were isolated by a miniprep procedure (Wilimzig 1985). We then digested the plasmids with *Pst*I, separated the plasmids from the inserts by electophoresis on agarose gels, and performed Southern (1975) blots onto nylon membranes.

This library was screened by probing the filters first with cotton chloroplast DNA (gift of G. Galau) and then with *P. nigra* (PI 77259) total DNA. Both probes were labelled with ^{32}P d-CTP by nick translation (Rigby et al. 1977). Clones which did not hybridize to either probe were selected as putative low-copy-number, non-chloroplast clones and used for RFLP analysis.

Bamboo genomic DNA filters were prepared by overnight digestion with restriction enzymes (*Eco*RV and *Hin*dIII) followed by agarose (0.8%) electrophoresis and Southern (1975) blotting onto nylon filters. Ten micrograms of DNA was used per gel lane. Isolated inserts (50–100 ng) were labelled by the random-hexamer technique with ^{32}P-dCTP (Feinberg and Vogelstein 1984). Hybridization was carried out at 65 °C overnight with gentle shaking as described in McCouch et al. (1988). Filters were washed sequentially at 65 °C for 20 min in 2 × SSC, 1 × SSC, and 1 × SSC before being exposed to X-ray film with intensifying screens at −80 °C for 1–3 days.

Results

To characterize the bamboo genomic library, 61 randomly chosen clones were digested with *Pst*I, run in 1.2% agarose gels to separate the inserts from the vector, blotted onto nylon membranes, and probed with chloroplast DNA and with total bamboo DNA. This analysis showed that the library of 61 fragments consisted of 26%

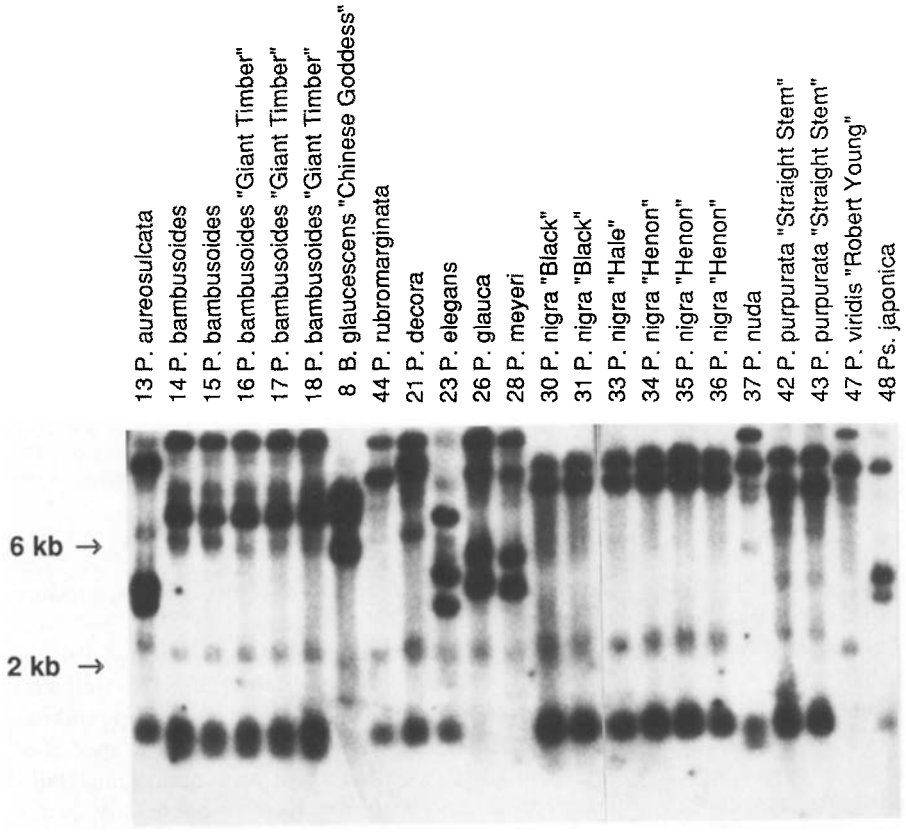

Fig. 1. RFLP patterns from an *Eco*RV digest of bamboo accessions. The probe was random genomic clone BL029

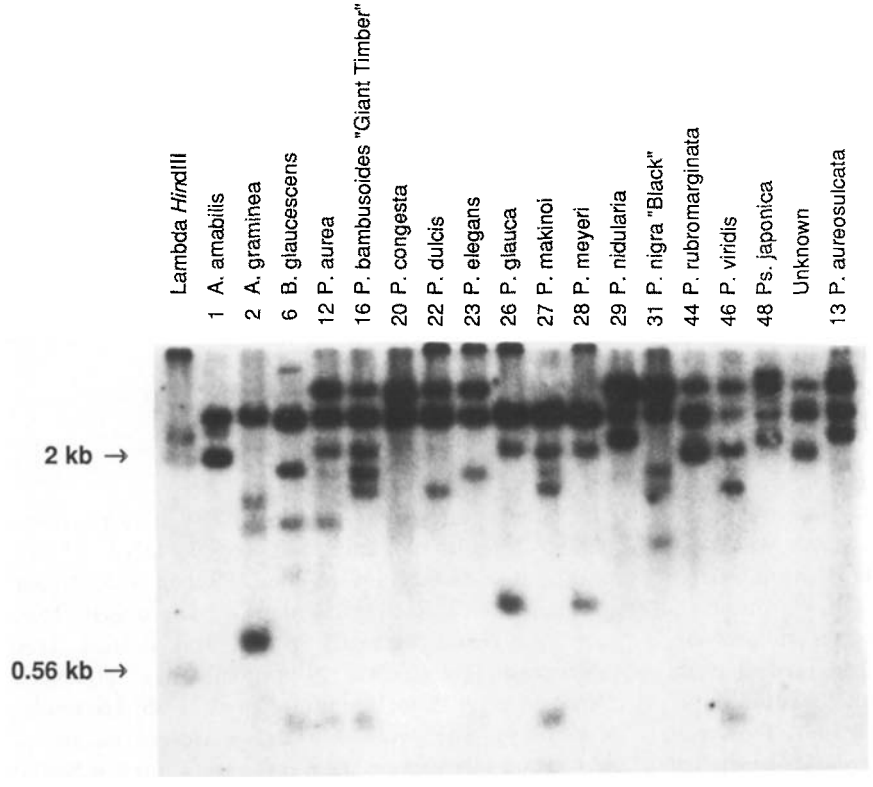

Fig. 2 RFLP patterns from a *Hpa*II digest of 19 bamboo species. The probe was random genomic clone BL017

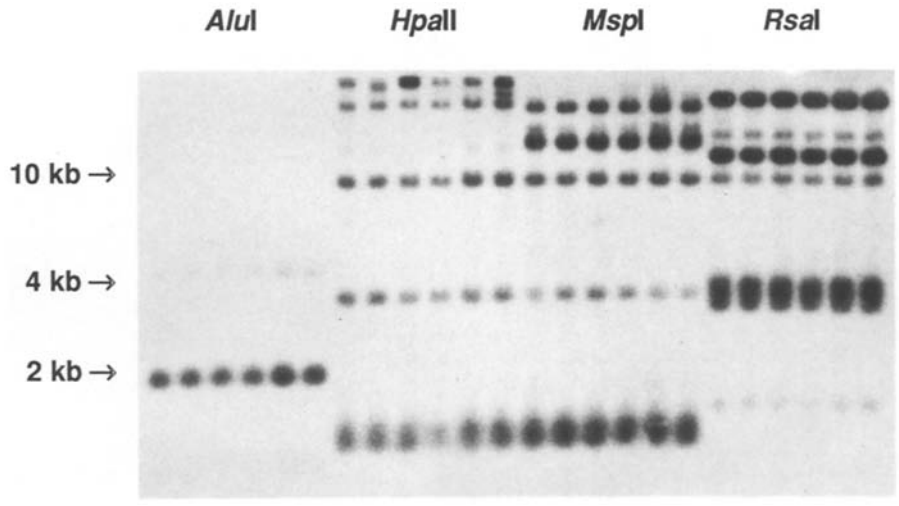

Fig. 3. RFLP patterns from genomic DNA from six accessions of *Phyllostachys nigra* (30, 31, 33, 34, 35, 36 in Table 1) digested with four different restriction enzymes. The probe was random genomic clone BL023

chloroplast sequences, 31% repeated sequences, and 43% low-copy-number nuclear sequences (data not shown). Apparent low-copy-number clones were selected for RFLP analysis.

The bamboo germplasm collection at Byron, Georgia consists largely of different accessions of the genus *Phyllostachys,* although a few accessions of *Arundinaria, Sasa,* and *Bambusa* are also maintained. We selected for analysis one accession of each of the species of *Phyllostachys* in the collection. We also selected three *Phyllostachys* species which were represented by multiple accessions to test whether our probes detected variability within species. Ten randomly selected probes from the random *Pst*I library were tested against bamboo genomic DNA digested with one of three restriction enzymes. All the probes we tested gave multiple bands on bamboo genomic DNAs (Figs. 1, 2, 3). Typically, each probe would detect three to six restriction fragments in genomic digests with any of the restriction enzymes used.

A large amount of genetic variability was present among the bamboo accessions we tested. Different genera could be readily distinguished with any probe/enzyme combination. Almost every probe we tried also uniquely distinguished every species of *Phyllostachys* in the germplasm collection, except *P. glauca* and *P. meyeri,* which were indistinguishable (Figs. 1, 2; Table 3). The restriction fragment haplotypes generated with each probe thus produced a DNA fingerprint highly characteristic at the species level. When multiple accessions of a single species were tested, no variation was seen (Fig. 1). Figure 3 shows RFLP patterns produced by digesting the DNA from six accessions of *Phyllostachys nigra* with four different restriction enzymes and probing with clone BL023. Identical patterns are present in each accession. This figure also shows that methylation polymorphisms may also be present in bamboo DNA, because the enzymes *Hpa*II, which is methylation sensitive, and *Msp*I, which is insensitive to methylation, produce slightly different RFLP patterns.

From visual inspection of our data it seems obvious that the RFLP haplotypes we detect correlate well with the conventional assignment of species in the bamboos we studied. Different accessions of the same species of *Phyllostachys* gave patterns that were quite similar, different species gave patterns which were generally species specific, and the other genera we used as outgroups gave patterns which were very different from the *Phyllostachys* species. Tables 2 and 3 show the number of dissimilar RFLPs for the bamboo species tested using six and eight probe-enzyme complexes, respectively. Since different accessions of the same species had identical banding patterns, only one set of data is shown in Table 3 for those species where we analyzed multiple accessions.

To compare the degree of RFLP variability detected by random genomic probes to that which could be detected by chloroplast probes, total chloroplast DNA from cotton was labelled with ^{32}P-dCTP by nick translation and used to probe the same filter used to produce Fig. 2. RFLP patterns produced in response to the chloroplast DNA probe were monomorphic for these accessions (data not shown).

Discussion

RFLP analysis has proven useful for systematic purposes in many groups of plants. Chloroplast DNA RFLPs have been extensively investigated (Palmer 1985; Palmer and Stein 1986). Restriction maps of chloroplast DNA have been made for many species, and at least three chloroplast DNAs have been completely sequenced (Ohyama et al. 1986; Shinozaki et al. 1986; Hiratsuka et al. 1989). The small size and conserved nature of chloroplast DNA has made it most useful for systematic

Table 2. Number of dissimilar RFLPs between bamboo accessions

	3	4	5	6	7	10	11	12	19	21	24	26	35	36	37	38	42
A. graminae (3[a])	–	0[b]	0[b]	0[b]	12	12	7	9	10	8	7	8	9	9	8	9	8
A. sp. (4)		–	0[b]	0[b]	12	12	7	9	10	8	7	8	9	9	8	9	8
A. sp. (5)			–	0[b]	12	12	7	9	10	8	7	8	9	9	8	9	8
A. sp. (6)				–	12	12	7	9	10	8	7	8	9	9	8	9	8
B. glaucescens (7)					–	0	12	12	11	11	11	12	11	11	13	12	12
B. glaucescens (10)						–	12	12	11	11	11	12	11	11	13	12	12
P. arcana (11)							–	4	7	1	3	6	7	7	5	4	8
P. aurea (12)								–	7	5	1	8	9	9	5	2	10
P. congesta (19)									–	8	8	3	4	4	10	9	10
P. dulcis (21)										–	4	6	8	8	6	5	9
P. makinoi (24)											–	7	8	8	4	1	9
P. nidularia (26)												–	3	3	8	8	10
P. purpurata (35)													–	0	10	9	11
P. purpurata (36)														–	10	9	11
P. rubromarginata (37)															–	5	11
P. viridis (38)																–	10
S. lateriflorus (42)																	–

[a] Taxon numbers from Table 1
[b] 21 fragments analyzed. All other comparisons are based on 24 fragments

Table 3. Number of dissimilar RFLPs between bamboo accessions

	1	2	8	13	14–18	20	22	23	25	27–31	32	33–34	37	39	40	41
A. amabilis (1[a])	–	2/31[b]	7/31	6/31	7/31	7/31	9/31	10/31	10/31	8/31	5/31	7/31	7/31	7/31	7/31	4/17
A. funghomii (2)		–	7/31	14/52	14/52	15/52	17/52	17/52	19/52	14/52	7/41	14/52	13/52	11/52	9/41	6/38
B. glaucescens (8–9)			–	22/52	19/52	19/52	21/52	21/52	21/52	18/52	12/41	18/52	17/52	15/52	12/41	10/38
P. aureosulcata (13)				–	21/52	12/52	17/52	14/52	15/52	16/52	10/41	15/52	19/52	16/52	14/41	13/38
P. bambusoides (14–18)					–	12/52	10/52	12/52	12/52	13/52	6/41	11/52	10/52	6/52	10/41	11/38
P. decora (20)						–	18/52	13/52	13/52	11/52	8/41	9/52	8/52	10/52	12/41	11/38
P. elegans (22)							–	16/52	16/52	12/52	11/41	19/52	16/52	14/52	13/41	13/38
P. glauca (23)								–	0	12/52	11/41	19/52	16/52	14/52	13/41	13/38
P. meyeri (25)									–	19/52	4/41	13/52	10/52	7/52	12/41	13/38
P. nigra (27–31)										–	10/41	14/52	11/52	13/52	10/41	10/38
P. nuda (32)											–	8/41	12/41	2/41	8/41	7/27
P. purpurata (33–34)												–	10/52	9/52	12/41	9/38
P. rubromarginata (37)													–	6/52	12/41	10/38
P. viridis (39)														–	8/41	8/38
Ps. japonica (40)															–	3/37
S. sp. (41)																–

[a] Taxon numbers from Table 1
[b] 2 dissimilar fragments of 31 analyzed

studies at taxonomic levels above the species. Nuclear DNA, however, has been shown to undergo a higher rate of base substitution than chloroplast DNA (Palmer 1990). Therefore it could be more suitable for analysis at the lower taxonomic levels. In humans, where RFLPs of nuclear DNA have been extensively studied, individual-specific DNA fragment patterns have been demonstrated (Jeffreys et al. 1985; Nakamura et al. 1987). In plants, human nuclear DNA probes or probes derived from the bacteriophage M13 can be used to differentiate cultivars of rice (Dallas 1988) and individual plants of other species (Rogstad et al. 1988; Nybom et al. 1990). Libraries of homologous nuclear DNA probes have also been useful in the elucidation of systematic relationships in higher plants (Song et al. 1988a, b, 1990).

All the low-copy-number probes we used detected several fragments in digests of bamboo DNA. *Phyllostachys* species are tetraploids (2n = 4x = 48), so multiple bands might be expected. However, little is known about breeding systems in bamboos or the degree of heterozygosity, and further research is clearly indicated. Our data demonstrate a high degree of RFLP variability in bamboo nuclear DNA as assayed by homologous probes; many random genomic DNA probes were found to be species specific. In contrast, there was much less variation using chloroplast DNA. With the enzymes we

tested, the species of *Phyllostachys* included in our experiments were monomorphic. Doubtless, if we had used more enzymes we might have detected some variation in chloroplast DNA RFLPs, but it is clear that nuclear DNA is much more variable in these organisms. The molecular basis of the RFLP variation that we see is not known. It could represent genomic rearrangement or base sequence change. Bamboo systematics is largely based on vegetative characters, and identification at the species level is often problematic. Our results demonstrate that DNA probes can be used to identify individual species of bamboo. With most probes, different accessions of the same species produced identical RFLP patterns. Therefore, random genomic probes seem to have just the right amount of variability for use at the species level; just two probes and six enzymes were sufficient to distinguish each of the taxa in our study. Data from such a small number of probe/enzyme combinations is not very meaningful in terms of evolutionary relationships, and our data merely demonstrate that such probes can be used for identification of bamboos to the species level. By using additional probe/enzyme combinations, we expect to find probes that will enable us to distinguish different accessions of the same species and to associate DNA fragment patterns with most accessions of the genus *Phyllostachys*. With a database of such information, unknown clones could be conclusively identified, origins of accessions whose history has been lost could be traced, and a valuable additional tool would be available for the study of bamboo systematics, evolution, and population biology, and for germplasm screening. Since bamboos seldom flower, it has not been possible to improve them by conventional plant breeding methods utilizing sexual hybridization. However, a recent report (Nadgauda et al. 1990) has indicated that some bamboos may be induced to flower by tissue culture methods and that conventional plant breeding may become more feasible. RFLP probes of the type we are developing could be used in plant breeding and would be of immediate applicability in any bamboo breeding program that may develop.

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