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Full Length Research Paper

Bamboo species relations revealed by random amplified polymorphism chloroplast DNA

H. Y. Zhang[1], Y. M. Yang[2] and X. Z. Liu[1]*

[1]Key Laboratory of Biodiversity Conservation in Southwest China, State Forest Administration, Southwest Forestry University, White Dragon Temple, Kunming, Yunnan Province-650224, Peoples Republic of China. [2]Bamboo and Rattan Research Institute, Southwest Forestry College, White Dragon Temple, Kunming, Yunnan Province-650224, Peoples Republic of China.

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The polymorphism, similarities and relationships among bamboo species were assessed with random amplified polymorphism chloroplast DNA (RAPD) analysis. The results showed distinct chloroplast DNA differences, present among the species. 175 bands were detected of which 94 were polymorphic (53.1%).3 to 15 bands generated by a single primer of variable lengths were detected, with an average of 7.2 polymorphic bands. The genetic similarity coefficients ranged from 0.543 to 0.954, and most of them were <0.75. Meantime, species relationships were estimated through Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis based on RAPD data. It is a useful method for analysis of the relationship in bamboo species.

Key words: Bamboo species, chloroplast DNA, relationships, random amplified polymorphism chloroplast DNA (RAPD), unweighted pair group method with arithmetic mean (UPGMA).

INTRODUCTION

Bamboos are vital to many Asian economies, having important uses ranging from domestic items to rural housing and raw materials for industry (Dransfield and Widjaja, 1995). Among bamboo species, the vegetative growth phase varies from 1 year to 120 years, moreover some species have never been known to flower (Janzen, 1976). So it is very difficult to get genetic information from the traditional means. Up to now, the basic knowledge of genetics of bamboo is severely lacking, therefore relationships among a lot of bamboo species remain controversial and unclear. Nowadays, greater attention is needed in the classification and identification of bamboos (Hui and Yang, 1998).

In the last few years, in spite of controversies, the use of molecular techniques has contributed to the resolution of many systematic and phylogenetic problems (Zhang et al., 2006). Several techniques such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), Inter -simple sequence repeats (ISSRs), arbitrary fragment length polymorphism (AFLP), and DNA sequencing, have been used with success to clarify relationships at different phylogenetic levels (Thormann et al., 1994; Zhang et al., 2006, 2008b, 2009).

Since RAPD amplification is directed with a single, arbitrary and short oligonucleotide primer, DNA from virtually all sources is amenable to amplification (Liu et al., 2009). However, due to the nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification (Zhang et al., 2005). RAPD markers are dominant markers and have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant breeding (Zhang et al., 2005, 2008a, b). This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of

*Corresponding author. E-mail: hanyaoz@163.com. Tel: (+) 86 871 386 3022. Fax: (+86) 871 3862178.

markers in a short period compared with previous methods (Zhang et al., 2008a). Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. Despite problems such as poor reproducibility, faint or fuzzy products, and difficulty in scoring bands, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labor.

RAPD is now considered as an efficient tool for species identification (Hillis et al., 1996). Despite controversies, it has been applied successfully in the evaluation of interspecies relationships of many different organisms (Wang et al., 1998). Chloroplast DNA is regarded to be more suitable than genomic DNA to study genetic relations among species and genera in RAPD analysis (Wang et al., 1998). Since the chance of co migrating bands being homologous becomes less as populations diverge, it was suggested that RAPD analysis gives more accurate estimates between closely related populations and less accurate estimates for distantly related populations using genomic DNA (Williams et al., 1993; Smith and Williams, 1994). In this report the RAPD procedure was used to assess the level of polymorphism of chloroplast DNA and relationships among bamboo species.

MATERIALS AND METHODS

Plant materials

Samples from fully expanded leaves of bamboo plants were collected from the South West Forest College Bamboo Gardens. The name and number of 22 Bamboo species used in RACDP analysis were: 1.*Bambusa multiplex* cv. *Alphonse-Karr*; 2. *B. multiplex* var. *riviereorum*; 3. *B. multiplex*; 4. *B. ventricosa*; 5. *B. vulgaris* cv. *Wamin*; 6. *B. vulgaris* cv. *Vittata*; 7. *B. pervariabiulis*; 8. *B. intermedia*; 9. *Qiongzhuca tumidinoda*; 10. *Phyllostachys bamsoides flacrima-deae*; 11. *P. heterocycla*; 12. *P. nigra*; 13. *P. nigra* var. *henonis*; 14. *P. aurea*; 15. *Yushania* sp.; 16. *Chimonobambusa yunnanensis*; 17. *Chimonobambusa* spp.; 18. *Dendrocalamus latiflorus*; 19. *D. asper*; 20. *D. sinicus*; 21. *D. semiscandens*; 22. *Neosinocalamus affini*. At least, three independent leaf samples were collected for each species, in order to account for any artificial amplification. The leaves were surface sterilized using the procedure which was essential to remove fungi, which are closely associated with bamboos. The leaves were washed twice with 1% solution of tween 20 for 10 min each. They were then disinfected in 3% solution of sodium hypochlorite for 20 min and rinsed 5 times with sterile distilled water.

Chloroplast DNA extraction and purification

Chloroplast DNA was extracted from leaves as described by Zhou et al. (1998). Additional procedure was used for DNA purification. After complete dissolving, the DNA was heated briefly with TEN buffer and SDS, then potassium acetate was added. The mixture was shaken for a few min (until the precipitate is dissolved), then it was frozen for 30 min. After centrifugation, the supernatant was collected carefully to avoid transfer of pellet particles. The supernatant was poured into ammonium acetate and isopropanol, mixed and incubated at −20°C for 30 min. The precipitated DNA was then pelleted for 10 min in a microfuge (12,000 rpm), washed with ethanol (70 and 99%), dried at room temperature, and re-dissolved in TE-buffer.

RAPD analysis

Amplification was performed in volumes of 0.02 cm^3 containing 0.002 cm^3 of the 10x buffer, and 100 mM each of dNTPs, 0.4 mM primer, 25 ng genomic DNA, and 1 unit of polymerase (Sangon, China). The reaction mixture was overlaid with 0.04 cm^3 mineral oil. Amplifications were carried out using a 2400 Perkin-Elmer Thermal; cycles were programmed for 40 cycles as follows: 30 s at 94°C, 30 s at 36 °C, 1.5 min at 72 °C, with an initial melting of 6 min at 94 °C, and a final extension of 6 min at 72 °C. Amplification products were analyzed by electrophoresis in a 1.5 % agarose gel with 1× TAE buffer (0.004 M Tris-acetate and 0.002 M EDTA).

Data analysis

Only the repeatable bands in the profiles of the DNA electrophoresis were recorded. Individual RAPD fragments for each primer-genotype combination were scored as 1 (presence) or 0 (absence), and a note of their sizes was made. Distance matrices were generated using pairwise similarities, and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was then used to produce a dendrogram. Bands present in all accessions were not used in calculation. The above procedures were performed using STATICA statistical package (STATICA for Windows, StatSoft).

RESULTS AND DISCUSSION

Of the 200 primers used in this study, 61 (30.5%) produced amplification products that were too faint to score or could not be consistently reproduced and 118 (59%) produced monomorphic banding patterns. Thus, only 21(10.5%) out of 200 primers were scored. A total of 175 bands were scored from the comparison of amplifications with 21 primers of DNAs from 22 bamboo species, with an average of 8.3 bands scored per primer (Table 1). 4 to 15 bands generated by a single primer of variable lengths were detected, portion of gel was shown in Figure 1. The polymorphic bands were 94 (53.7%). The average number of bands per primer detected among all the 200 primers tested was 0.465 polymorphic and total 0.875 (invariant plus polymorphic).

Based on the RAPD data of the 22 bamboo species, the pairwise similarities were calculated (Table 2). The result showed that the pairwise similarity between *B. pervariabiulis* and *B. intermedia* was the biggest 0.954 (Table 2), and the pairwise similarity between *N. affini* and *Yushania* sp. was the smallest 0.543 (Table 2). And a dendrogram was produced (Figure 2), the relationships among bamboo species were shown. It was worth noting that not all the species belonged to one genus clustered together in a separate branch, for example, *P. aurea* was not clustering with the other species of *Phyllostachys* species. Some pair similarities of different species of the same genus were smaller than those of the different

Table 1. Chloroplast DNA fragments produced by different primers in the 22 bamboo species.

Primers	Sequence	Total bands	Polymorphic bands
S7	GGTGACGCAG	7	3
S31	CAATCGCCGT	8	5
S38	AGGTGACCGT	4	2
S45	TGAGCGGACA	6	5
S55	CATCCGTGCT	7	5
S71	AAAGCTGCGG	11	4
S73	AAGCCTCGTC	9	3
S77	TTCCCCCCAG	11	6
S93	CTCTCCGCCA	7	5
S103	AGACGTCCAC	8	4
S108	GAAACACCCC	6	5
S114	ACCAGGTTGG	7	3
S116	TCTCAGCTGG	8	4
S119	CTGACCAGCC	8	4
S125	CCGAATTCCC	6	3
S132	ACGGTACCAG	8	3
S154	TGCGGCTGAG	10	6
S160	AACGGTGACC	9	5
S175	TCATCCGAGG	15	10
S180	AAAGTGCGGC	9	4
S183	CAGAGGTCCC	11	5
	Total	175	94

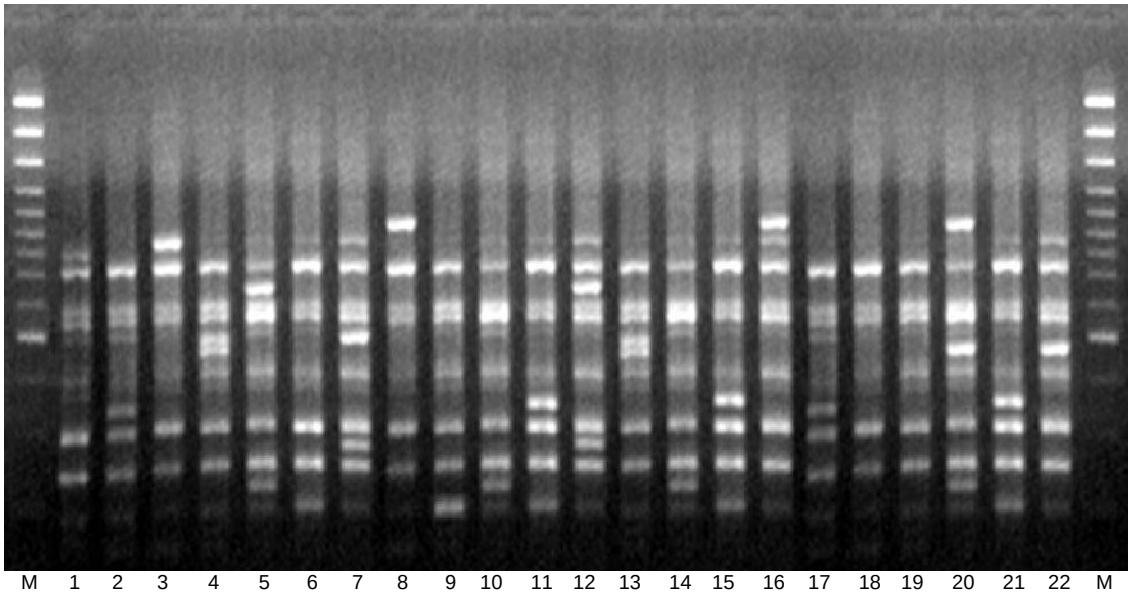

Figure 1. RACDP fingerprints of 22 bamboo species (the primer was S175).M was 100 bp markers.

genera, for example the pair similarity between *P. heterocycla* and *P. aurea* was 0.749, and the pair similarity between *P. aurea* and *Q. tumidinoda* was 0.794 (data was shown in table 2). Morphologic marker has the advantages of simplicity and intuition, but the disadvantage is heavy depending on experience, and traditional taxonomy was depended on very few morphologic markers too much. This might be the reason of that RAPD analysis results were different to the traditional taxonomy.

Table 2. Pairwise similarities (%) of the 22 bamboo species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	100																					
2	90.3	100																				
3	94.3	90.3	100																			
4	91.4	89.7	92.6	100																		
5	89.8	89.8	92	90.3	100																	
6	92.6	89.8	92.6	92	90.3	100																
7	92.6	90.9	92.6	92	90.3	93.2	100															
8	91.4	92	93.7	93.7	92.6	93.2	95.4	100														
9	76.6	76	80	78.3	78.9	78.3	81.7	81.7	100													
10	80	79.4	81.1	78.3	80	81.7	81.7	82.9	89.7	100												
11	80	80.6	82.3	79.4	81.7	81.7	84	84	92	92	100											
12	77.7	76	80	76	78.9	77.1	79.3	80.6	88.6	94.3	92	100										
13	77.1	76.6	80.6	78.9	78.3	78.9	80	81.1	93.7	91.4	91.4	92.6	100									
14	60.6	61.1	64	62.3	62.9	63.4	63.4	63.4	79.4	74.9	74.9	76	82.3	100								
15	73.1	73.7	74.3	0.24	75.4	71.4	76	77.1	71.4	68	68	71.4	70.9	60	100							
16	61.1	61.7	62.3	70.6	61.1	62.9	62.9	62.9	67.4	75.4	68.6	69.7	68	62.9	57.1	100						
17	69.1	69.7	70.3	68.6	69.1	70.9	70.9	70.9	76.6	84.6	77.7	78.9	77.1	68.6	61.7	88.6	100					
18	72.6	70.9	72.6	72	72.6	76.6	74.3	75.4	69.7	68.6	69.7	76.4	69.1	65.1	69.7	56.6	63.4	100				
19	74.3	72.6	73.1	72.6	70.9	77.1	74.9	73.7	68	68	69.1	68	68.6	63.4	69.7	57.1	64	93.7	100			
20	75.4	74.9	74.3	73.7	72	78.3	0.76	74.9	66.9	66.9	0.32	66.9	67.4	62.3	70.3	54.9	61.7	85.7	92	100		
21	72.6	70.9	71.4	70.9	69.1	75.4	74.3	72	65.1	65.1	66.3	65.1	65.7	61.7	67.4	56.6	62.3	86.3	92.6	91.4	100	
22	65.1	66.9	67.4	66.9	62.9	69.1	66.9	66.9	61.1	63.4	65.7	62.3	62.9	54.3	54.3	56	61.7	65.1	71.4	69.1	67.4	100

The number representing species code was shown in the plant materials section.

Although RAPD is regarded as one of the simplest and fastest of DNA-based techniques in genetic studies, and has been used in various genera and species (Thormann et al., 1994; Wang et al., 1998; Zhang et al., 2005), RAPD markers have been discouraged for use in interspecific genetic studies as co-migrating bands from different species that do not necessarily have sequence homology (Staub et al., 1996; Thormann et al., 1994). In this study, genomic DNA was substituted by chloroplast DNA, and the problem was solved. Results suggest that bamboo species, after many years of independent evolution, are indeed genetically distinguishable at the chloroplast DNA level. Friar and Kocher (1991, 1994) also found that the variations of chloroplast DNA were useful in study genetic variation and evolution of *Phyllostachys.* The RAPD technique in general can reveal useful information about the genetic relationship in bamboo species, which morphological markers cannot detect. Therefore, it should be used as a good tool for the assessment of genetic relationship. Our investigations, together with information from other studies, will help to establish simple protocols for molecular analysis with RAPDs in bamboo species under strictly controlled experimental conditions. In conclusion, RAPD has been shown to be useful in studying the genetic similarity among bamboo species and genera.

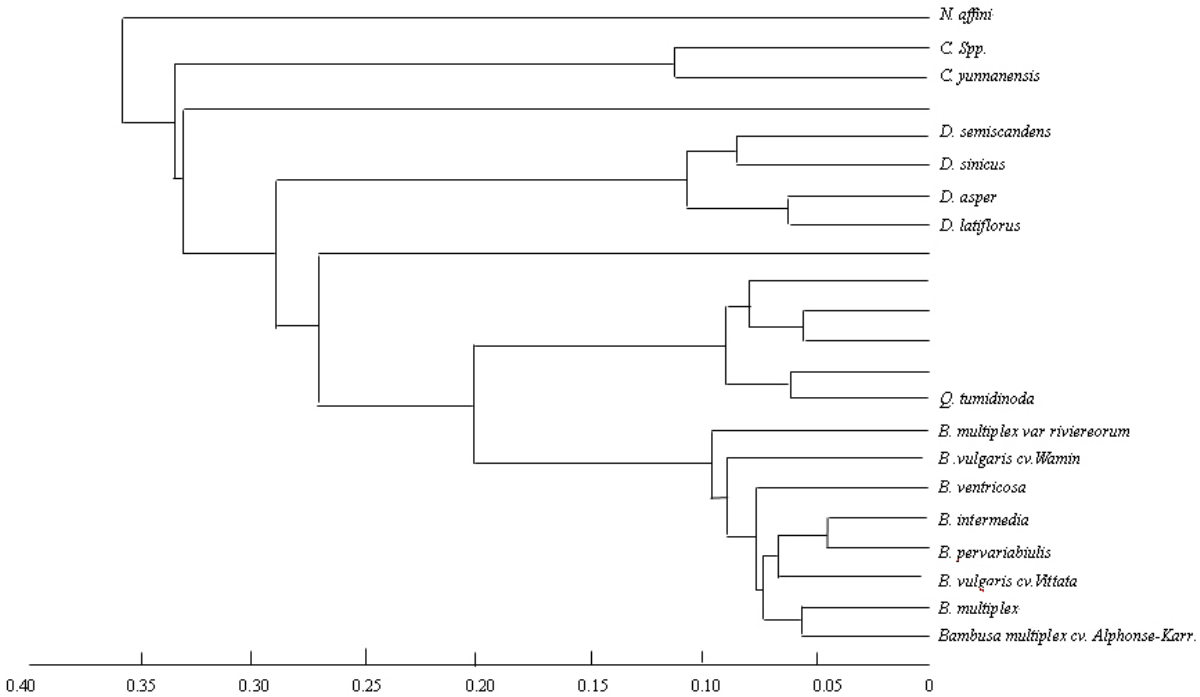

Figure 2. Dendrogram of the 22 bamboo species based on cluster analysis (UPGMA) of the genetic distances calculated from 94 polymorphic RAPD fragments.

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