GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIPS IN THE *BAMBUSA* GENUS AS REVEALED BY RAPD MARKERS

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Abstract. Understanding the genetic diversity and divergence within *Bambusa* has important implications for conservation and sustainable use. In this study, genetic diversity and phylogenetic relationships between 28 species/varieties of *Bambusa* were evaluated based on 16 randomly amplified polymorphic DNA (RAPD) primers screened from 96 primers. A total of 218 bands were amplified using these 16 primers, yielding DNA fragments of 290–3000 bp. The number of bands and percentage of polymorphism were 211 and 96.79%, respectively, indicating high inter and intraspecific genetic diversity within *Bambusa*. In clustering analysis, genetic distances ranged from 0.2139 to 0.7647. The 28 bamboo species/varieties were classified into six groups at a genetic distance of 0.6138, consistent with traditional classification results. Our results indicated a high degree of polymorphism at RAPD loci, suggesting that the RAPD markers are effective for the analysis of genetic diversity and phylogenetic relationships in the genus. The results of this study provide a theoretical basis for germplasm conservation, classification, and evolutionary studies of *Bambusa* species/varieties.
Keywords: *molecular diversity, genetic relationships, RAPD, bamboos*

Introduction

The genus *Bambusa*, which belongs to the Bambusoideae subfamily, is highly abundant in China and has a wide range of applications. More than a hundred *Bambusa* species have been described and classified into three subgenera, i.e., *Subgen Bambusa*, *Subgen Lingnania*, and *Subgen Leleba*, with *Bambusa chungii*, *Bambusa multiplex* cv. *Fernleaf*, and *Bambusa arundinacea* as the respective type species. China, in particular, boasts approximately 60 *Bambusa* species, which are mainly distributed in the eastern, southern, and southwestern regions (Editorial Committee of Flora of China, Chinese Academy of Sciences, 1996; Zhu et al., 2017). *Bambusa* is characterized by sympodial rhizomes and has relatively high economic, aesthetic, and ecological value. Therefore, in-depth research on the genus is beneficial for the conservation of biodiversity within bamboo resources in China (Ma et al., 2007).

Although *Bambusa* species propagate by asexual reproduction, adaptations to different habitats and long-term evolution have resulted in genetic variation. The high genetic diversity in *Bambusa* poses certain challenges for the accurate differentiation and identification of *Bambusa* species (Lou et al., 2011). In addition, as bamboo species have low flowering and fruiting rates, the determination of interspecific relationships

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within *Bambusa* based on morphological markers, such as the morphological characteristics of flowers and fruits, or other markers, such as isozymes, is extremely difficult (Lin et al., 2008). Therefore, the quest for effective markers has become a key topic in phylogenetic studies of *Bambusa*.

Advances in molecular biology have substantially improved DNA marker techniques, enabling the direct comparison of genetic material without the influence of external environmental factors (Liu et al., 2015). Such techniques are increasingly used for analyses of genetic diversity and phylogenetic relationships within *Bambusa*. Randomly amplified polymorphic DNA (RAPD) marker detection, using electrophoresis and PCR (Mutharaian et al., 2018), has several advantages, such as its high sensitivity, low cost, and ease, enabling the acquisition of large quantities of information (Xia et al., 2001); accordingly, this approach is widely used for studies of genetic diversity (He et al., 2019; Tanzeem et al., 2019; Leandro et al.2019; Subramanyam et al., 2010), phylogenetic relationships (Amom et al., 2020; Liu et al., 2016), variety identification (Odunayo et al., 2019; Archana et al., 2013), and DNA fingerprinting (Mei et al., 2014; Afshari et al., 2016). However, few studies have utilized RAPD to evaluate *Bambusa*. In this study, 28 *Bambusa* species/varieties were used as test materials for a RAPD-based analysis of genetic diversity and phylogenetic relationships to provide a theoretical basis for germplasm conservation, classification, and evolutionary studies of *Bambusa* species/varieties.

Materials and methods

Test materials

The test materials were obtained from 28 *Bambusa* species/varieties from the Bamboo Cultivation Base of the Fujian Agriculture and Forestry University, Fuzhou, China in August 2018. *Table 1* provides basic information for the test materials. Fresh uninfested leaves (3–6 g) were harvested from each plant and stored in a -80 °C ultra-low temperature freezer until genomic DNA extraction.

Methods

DNA extraction and detection

Genomic DNA was extracted using the CTAB method (Zhang et al., 2014), and the concentration was measured using a NANODROP 2000 spectrophotometer. Electrophoresis was performed using a 1.0% agarose gel; briefly, 9 μL of template DNA was mixed with 1.5 μL of 6× loading buffer and subjected to 120 V (5 $V \cdot cm^{-2}$) for 30 min, using 1× TBE as the electrophoresis buffer. The electrophoresis results were analyzed using a gel imaging system. The size of the DNA ladder marker (No. B500347, Sangon Biotech) is 100-3000 bp. Total DNA that satisfied the study requirements was diluted to a 20 $ng \cdot \mu L^{-1}$ and stored in a -20 °C freezer before further use.

Primer screening

A total of 96 random oligonucleotide primers of 10 bp were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and used for the preamplification of the total DNA of certain *Bambusa* species/varieties. Then, 16 primers that produced bands with high

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clarity, stability, degree of polymorphism, and reproducibility were selected for the RAPD analysis with the total DNA of all 28 bamboo species/varieties. *Table 2* shows a list of selected primers and sequences.

Table 1. *Summary of test materials*

No.	Species/variety name
1	*Bambusa sinospinosa*
2	*B. rutila*
3	*B. subaequalis*
4	*B. gibba*
5	*B. ventricosa*
6	*B. remotiflora*
7	*B. cerosisssima*
8	*B. textilis* cv. *Maculata*
9	*B. textilis* var. *gracilis*
10	*B. tulda*
11	*B. eutuldoides McClure* var. *viridi-vittata*
12	*B. pervariabilis*
13	*B. longispiculata*
14	*B. tuloides*
15	*B. subtruncata*
16	*B. boniopsis*
17	*B. vulgaris*
18	*B. vulgaris* cv. *Vittata*
19	*B. vulgaris* cv. *Wamin*
20	*B. gibboides*
21	*B. albo-lineata*
22	*B. lenta*
23	*B. contracta*
24	*B. multiplex*
25	*B. multiplex* cv. *Alphonse-Karr*
26	*B. multiplex* cv. *Silverstripe*
27	*B. multiplex* cv. *Fernleaf*
28	*B. multiplex* var. *riviereorum*

Table 2. *List of primers and sequences*

Primer	Sequence 5′–3′	Primer	Sequence 5′–3′
S4	GGACTGGAGT	S69	CTCACCGTCC
S5	TGCGCCCTTC	S431	TCGCCGCAAA
S13	TTCCCCCGCT	S1219	CTGATCGCGG
S26	GGTCCCTGAC	S2093	TCGGTGAGTC
S32	TCGGCGATAG	S1408	GTTACGGACC
S36	AGCCAGCGAA	S1412	CCTGTACCGA
S45	TGAGCGGACA	S1420	CTTCTCGGAC
S67	GTCCCGACGA	S1421	TCCCAGCAGA

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RAPD-PCR and the detection of amplification products

PCR was performed using a LabCycler PCR System (SensoQuest International Ltd). The reaction system for RAPD-PCR consisted of the following: 2.0 μL of 10× buffer, 3.5 mmol·L^{-1} Mg^{2+}, 0.4 mmol·L^{-1} dNTPs, 0.6 μmol·L^{-1} primer, 3.0 U of *Taq* polymerase, 40 ng of template DNA, and sterile ddH_2O for a total volume of 20 μL.

The RAPD-PCR protocol was as follows: initial denaturation at 94 °C for 2 min, denaturation at 94°C for 30 s, annealing at 34 °C for 30 s, and extension at 70 °C for 90 s for 38 cycles; final extension at 72 °C for 7 min. The PCR products were stored in a 4 °C refrigerator, and detection was subsequently performed by electrophoresis on a 1.0% agarose gel.

Statistical analysis

POPGENE version 3.2 was used to determine the total number of bands and the number of polymorphic bands amplified by each primer (Yeh et al., 1999). The bands produced at the same site by different primers were counted for each sample, assigning a value of 1 if a band was present and 0 if a band was absent. DPS 18.10 was used to analyze genetic diversity, perform a clustering analysis, and construct a phylogenetic tree of the tested *Bambusa* species/varieties (Tang et al., 2013).

Results and analysis

Analysis of RAPD marker polymorphism

Among 96 random RAPD primers used for preamplification, 16 primers produced bands with high clarity, degree of polymorphism, and stability and were selected for marker amplification from all 28 *Bambusa* species/varieties. *Figures 1* and *2* show the representative gel electrophoresis results obtained with primers S32 and S431. In total, 218 clear bands were obtained with the 16 primers, and 211 of these bands were polymorphic. On average, 13.63 bands and 13.19 polymorphic bands were obtained for each primer. The percentages of polymorphism for the primers ranged from 77.78% to 100% and the average percentage was 96.79% (*Table 3*). Each primer produced 6–21 bands and 5–21 polymorphic bands, with fragment lengths of 190–3000 bp. These results indicate that genetic diversity is high within *Bambusa*. This high diversity suggests that the genus possesses a strong ability to adapt to various environments. Furthermore, interspecific genetic differences within *Bambusa* can be effectively elucidated by RAPD markers.

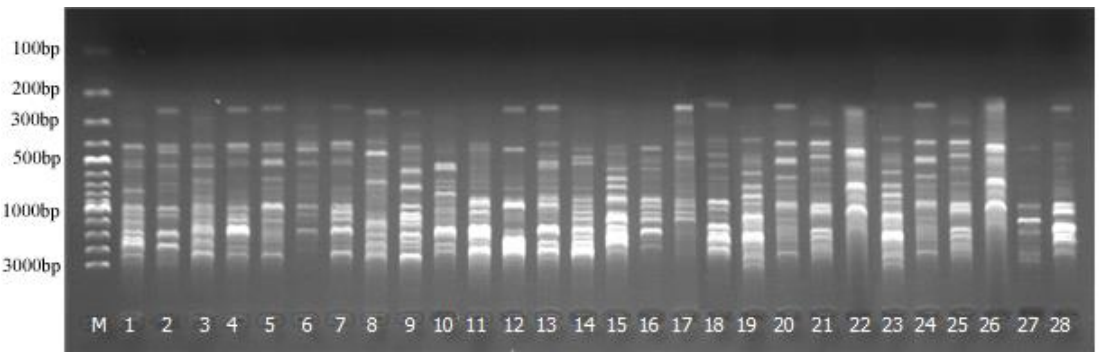

Figure 1. *Electrophoresis results for 28 Bambusa samples amplified using RAPD S32*

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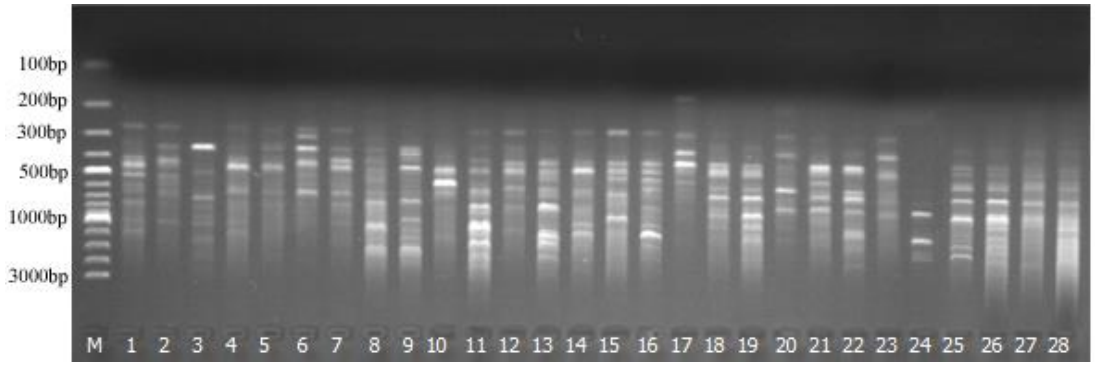

Figure 2*. Electrophoresis results for 28 Bambusa samples amplified using RAPD S431*

Table 3. *RAPD primers and percentages of polymorphism*

Primer	Total no. of bands	No. of polymorphic bands	Percentage of polymorphism (%)
S4	14	14	100
S5	12	12	100
S13	17	17	100
S26	10	9	90.00
S32	21	21	100
S36	9	7	77.78
S45	6	5	83.33
S67	13	12	92.31
S69	13	13	100
S431	15	15	100
S1219	12	11	91.67
S2093	13	13	100
S1408	14	13	92.86
S1412	15	15	100
S1420	17	17	100
S1421	17	17	100
Total	218	211	96.79
Average	13.63	13.19	96.79

Analysis of genetic distances and genetic diversity among the Bambusa species/varieties

For the 16 primers, a data matrix was generated from the genomic fingerprint, and pairwise genetic distances among the various *Bambusa* species/varieties were calculated using DPS 18.10 based on Jaccard's formula (Yhang et al., 2010) (*Table 4*). The average genetic distance was 0.5597 and the range was 0.2139–0.7647, indicating a relatively high average and a high degree of variation. As shown in *Table 4*, the genetic distances within the genus *Bambusa* were mostly around 0.5, indicating good genetic stability. The genetic distances between variants or cultivars of the same species were lower than those for comparisons between different species. For instance, the genetic distances among *B. multiplex* cv. *Alphonse-Karr*, *B. multiplex* cv. *Silverstripe*, *B. multiplex* cv. *Fernleaf,* and *B. multiplex* var. *riviereorum*, belonging to *Bambusa*

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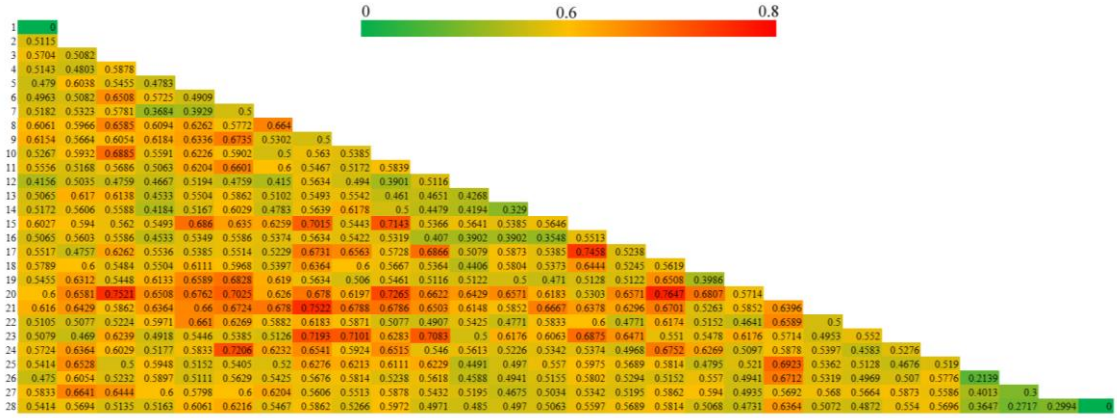
subgen. *Leleba*, were relatively low, while the genetic distance between *B. subaequalis* (belonging to *Bambusa* subgen. *Bambusa*) and *B. subtruncata* (belonging to *Bambusa* subgen. *Leleba*) was high. These results demonstrate that the phylogenetic relationships among *Bambusa* species/varieties are consistent with the morphological classification and that RAPD markers are effective indicators of genetic diversity in *Bambusa* species/varieties.

Table 4. *The genetic distance matrix for 28 Bambusa species/varieties*



1	0																										
2	0.5115	0																									
3	0.5704	0.5082	0																								
4	0.5143	0.4803	0.5878	0																							
5	0.479	0.6038	0.5455	0.4783	0																						
6	0.4963	0.5082	0.6508	0.5725	0.4909	0																					
7	0.5182	0.5323	0.5781	0.3684	0.3929	0.5	0																				
8	0.6061	0.5966	0.6585	0.6094	0.6262	0.5772	0.664	0																			
9	0.6154	0.5664	0.6054	0.6184	0.6336	0.6735	0.5302	0.5	0																		
10	0.5267	0.5932	0.6885	0.5591	0.6226	0.5902	0.5	0.563	0.5385	0																	
11	0.5556	0.5168	0.5686	0.5063	0.6204	0.6601	0.6	0.5467	0.5172	0.5839	0																
12	0.4156	0.5035	0.4759	0.4667	0.5194	0.4759	0.415	0.5634	0.494	0.3901	0.5116	0															
13	0.5065	0.617	0.6138	0.4533	0.5504	0.5862	0.5102	0.5493	0.5542	0.461	0.4651	0.4268	0														
14	0.5172	0.5606	0.5588	0.4184	0.5167	0.6029	0.4783	0.5639	0.6178	0.5	0.4479	0.4194	0.329	0													
15	0.6027	0.594	0.562	0.5493	0.686	0.635	0.6259	0.7015	0.5443	0.7143	0.5366	0.5641	0.5385	0.5646	0												
16	0.5065	0.5603	0.5586	0.4533	0.5349	0.5586	0.5374	0.5634	0.5422	0.5319	0.407	0.3902	0.3902	0.3548	0.5513	0											
17	0.5517	0.4757	0.6262	0.5536	0.5385	0.5514	0.5229	0.6731	0.6563	0.5728	0.6866	0.5079	0.5873	0.5385	0.7458	0.5238	0										
18	0.5789	0.6	0.5484	0.5504	0.6111	0.5968	0.5397	0.6364	0.6	0.5667	0.5364	0.4406	0.5804	0.5373	0.6444	0.5245	0.5619	0									
19	0.5455	0.6312	0.5448	0.6133	0.6589	0.6828	0.619	0.5634	0.506	0.5461	0.5116	0.5122	0.5	0.471	0.5128	0.5122	0.6508	0.3986	0								
20	0.6	0.6581	0.7521	0.6508	0.6762	0.7025	0.626	0.678	0.6197	0.7265	0.6622	0.6429	0.6571	0.6183	0.5303	0.6571	0.7647	0.6807	0.5714	0							
21	0.616	0.6429	0.5862	0.6364	0.66	0.6724	0.678	0.7322	0.6788	0.6786	0.6503	0.6148	0.5852	0.6667	0.6378	0.6296	0.6701	0.5263	0.5852	0.6396	0						
22	0.5105	0.5077	0.5224	0.5971	0.661	0.6269	0.5882	0.6183	0.5871	0.5077	0.4907	0.5425	0.4771	0.5833	0.6	0.4771	0.6174	0.5152	0.4641	0.6589	0.5	0					
23	0.5079	0.469	0.6239	0.4918	0.5446	0.5385	0.5126	0.7193	0.7101	0.6283	0.7083	0.5	0.6176	0.6063	0.6875	0.6471	0.551	0.5478	0.6176	0.5714	0.4953	0.552	0				
24	0.5724	0.6364	0.6029	0.5177	0.5833	0.7206	0.6232	0.6541	0.5924	0.6515	0.546	0.5613	0.5226	0.5342	0.5374	0.4968	0.6752	0.6269	0.5097	0.5878	0.5397	0.4583	0.5276	0			
25	0.5414	0.6528	0.5	0.5948	0.5152	0.5405	0.52	0.6276	0.6213	0.6111	0.6229	0.4491	0.497	0.557	0.5975	0.5689	0.5814	0.4795	0.521	0.6923	0.5362	0.5128	0.4676	0.519	0		
26	0.475	0.6054	0.5232	0.5897	0.5111	0.5629	0.5425	0.5676	0.5814	0.5238	0.5618	0.4588	0.4941	0.5155	0.5802	0.5294	0.5152	0.557	0.4941	0.6712	0.5319	0.4969	0.507	0.5776	0.2139	0	
27	0.5833	0.6641	0.6444	0.6	0.5798	0.6	0.6204	0.5606	0.5513	0.5878	0.5432	0.5195	0.4675	0.5034	0.5342	0.5195	0.5862	0.594	0.4935	0.5692	0.568	0.5664	0.5873	0.5586	0.4013	0.3	0
28	0.5414	0.5694	0.5135	0.5163	0.6061	0.6216	0.5467	0.5862	0.5266	0.5972	0.4971	0.485	0.497	0.5063	0.5597	0.5689	0.5814	0.5068	0.4731	0.6364	0.5072	0.4872	0.554	0.5696	0.3647	0.2717	0.2994	0

Cluster analysis of Bambusa species/varieties

Based on the RAPD-PCR amplification results, phylogenetic relationships within *Bambusa* were analyzed with the raw genotypic data matrix using DPS 18.10 (Tang et al., 2013). A cluster analysis was performed based on genetic distances using the Nei–Li maximum distance method (Zaya et al., 2017), and a dendrogram was constructed (*Fig. 3*; *Table* 5). The clustering results indicate that the 28 *Bambusa* species/varieties could be classified into six groups at a genetic distance of 0.57 (L1). (1) Group 1 (genetic distance = 0.6038) included 8 species/varieties (*B. sinospinosa*, *B. remotiflora*, *B. rutila*, *B. contracta*, *B. vulgaris*, *B. gibba*, *B. cerosisssima*, and *B. ventricosa*); Group 2 (genetic distance = 0.5634) included 4 species/varieties (*B. textilis* cv. *Maculata*, *B. textilis* var. *gracilis*, *B. tulda*, and *B. pervariabilis*); (3) Group 3 (genetic distance = 0.5484) included 3 species/varieties (*B. subaequalis*, *B. vulgaris* cv. *Vittata*, and *B. vulgaris* cv. *Wamin*); (4) Group 4 (genetic distance = 0.4651) included 4 species/varieties (*B. eutuldoides McClure* var. *viridi-vittata*, *B. longispiculata*, *B. tuloides*, and *B. boniopsis*); (5) Group 5 (genetic distance = 0.5776) included 7 species/varieties (*B. albo-lineata*, *B. lenta*, *B. multiplex*, *B. multiplex* cv. *Alphonse-Karr*, *B. multiplex* cv. *Silverstripe*, *B. multiplex* cv. *Fernleaf*, *B. multiplex* var. *riviereorum*); (6) Group 6 (genetic distance = 0.5303) included 2 species/varieties (*B. subtruncata* and *B. gibboides*).

Groups 1 and 2 exhibited a genetic distance of 0.7193, with *B. sinospinosa*, *B. rutila*, *B. gibba*, and *B. ventricosa* (all belonging to *Bambusa* subgen. *Bambusa*) forming a distinct cluster, and *B. remotiflora*, *B. cerosisssima*, *B. textilis* cv. *Maculata*, and *B. textilis* var. *gracilis* (all belonging to *Bambusa* subgen. *Lingnania*) clustering together. However, *B. subaequalis*, which belongs to *Bambusa* subgen. *Bambusa*, did not cluster with species in the same subgenus; instead, it formed a group with the majority of members of *Bambusa* subgen. *Leleba*, as evidenced by the shorter genetic distances. In

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Group 3, *B. vulgaris* cv. *Vittata* and *B. vulgaris* cv. *Wamin*, which are both members of *Bambusa* subgen. *Leleba* and variants of *B. vulgaris*, did not cluster together with *B. vulgaris*; instead, they formed a cluster with *B. subaequalis* of *Bambusa* subgen. *Bambusa* (genetic distance, 0.5484). In Group 5, *B. multiplex* was distinctly clustered with its cultivated variants *B. multiplex* cv. *Alphonse-Karr*, *B. multiplex* cv. *Silverstripe*, *B. multiplex* cv. *Fernleaf*, and *B. multiplex* var. *riviereorum*, consistent with traditional classification results. However, *B. multiplex* cv. *Alphonse-Karr*, *B. multiplex* cv. *Silverstripe*, *B. multiplex* cv. *Fernleaf*, and *B. multiplex* var. *riviereorum* did not clustered together with *B. multiplex*; instead, they clustered together with *B. multiplex*, *B. lenta*, and *B. albo-lineata* (genetic distance, 0.5776). This indicates that divergence in *B. multiplex* resulted in greater genetic similarity with *B. lenta* and *B. albo-lineata*.

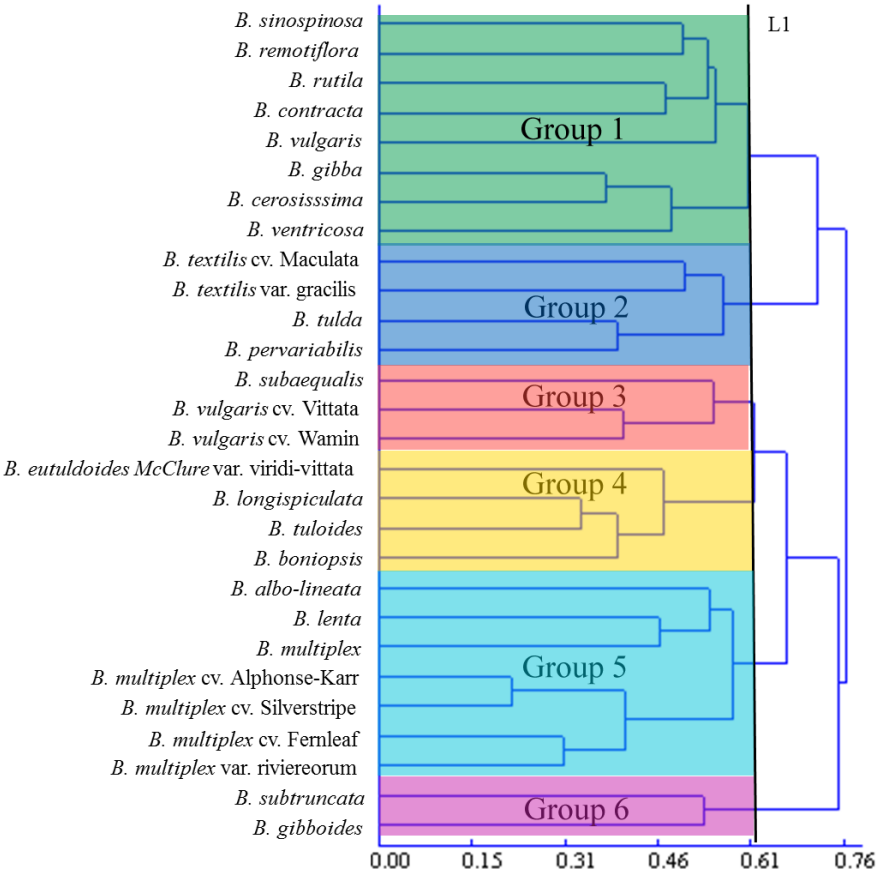

Figure 3. *Dendrogram of the 28 Bambusa species/varieties based on RAPD markers*

Discussion

Previous research has demonstrated that the main determinants of the stability and reproducibility of RAPD analyses are temperature conditions, reagent concentrations, and the duration of various PCR steps (Bi et al., 2011). The application of the RAPD method for the investigation of interspecific relationships (including subspecific classes) within *Bambusa* is rare, with a literature search yielding a single study by Nayak et al (2003) involving only a few *Bambusa* species. In this study, we adopted the RAPD technique for the analysis of genetic differences and phylogenetic relationships among 28 *Bambusa* species/varieties. The amplification patterns of certain primers consisted of

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a variety of bands with significant differences. Amplification using 16 primers produced 218 clear bands, with 96.79% (211 bands) polymorphism. Therefore, a high level of polymorphism within *Bambusa* was detected with RAPD molecular markers, demonstrating that the approach is practical and effective for genetic analyses of the genus.

Table 5. *Clustering and genetic distances of the 28 Bambusa species/varieties*

T Number	I Linked level	J Indexing of clustering order	Distance
1	26	25	0.2139
2	28	27	0.2994
3	14	13	0.3290
4	7	4	0.3684
5	12	10	0.3901
6	16	13	0.3902
7	19	18	0.3986
8	27	25	0.4013
9	24	22	0.4583
10	13	11	0.4651
11	23	2	0.4690
12	5	4	0.4783
13	6	1	0.4963
14	9	8	0.5000
15	20	15	0.5303
16	2	1	0.5385
17	22	21	0.5397
18	18	3	0.5484
19	17	1	0.5517
20	10	8	0.5634
21	25	21	0.5776
22	4	1	0.6038
23	11	3	0.6138
24	21	3	0.6667
25	8	1	0.7193
26	15	3	0.7521
27	3	1	0.7647

The average genetic distance range of values were high. These results show that *Bambusa* possesses high genetic diversity and relatively complex interspecific genetic relationships (Zhan et al., 2015). The genus is highly influenced by various factors, such as geographical location, climate fluctuations, and various evolutionary processes (Lou et al., 2011), leading to the establishment of a diverse gene pool.

A clustering analysis based on RAPD markers showed that the 28 *Bambusa* species/varieties could be clearly distinguished. The species/varieties were classified into 6 groups, consistent with results based on morphological properties. Compared with Nayak et al. (2003), a larger number of primers and samples were used in this study. However, the results of the two studies were largely consistent and in agreement

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with Loh et al. (2000), who adopted amplified fragment length polymorphism (AFLP) markers for the analysis of genetic diversity and relationships. In Group 3, *B. vulgaris* cv. *Vittata* and *B. vulgaris* cv. *Wamin*, both members of *Bambusa* subgen. *Leleba* and variants of *B. vulgaris*, were not initially clustered with *B. vulgaris*; instead, they were clustered with *B. subaequalis* of *Bambusa* subgen. *Bambusa* and only formed a cluster with *B. vulgaris* at a genetic distance of 0.7647. This can be explained by variation in *B. vulgaris* cv. *Vittata* and *B. vulgaris* cv. *Wamin* or anthropomorphic disturbance; further research is required to determine the exact cause. In Group 5, except for *B. albo-lineata*, the genetic distances among other species/varieties were less than 0.5, indicating that genetic traits within the group were relatively stable. All species/varieties within this group belong to *Bambusa* subgen. *Leleba* according to the traditional classification described in the Flora of China. In particular, *B. multiplex* cv. *Alphonse-Karr* and *B. multiplex* cv. *Silverstripe*, which are both cultivated varieties of *B. multiplex*, exhibited high genetic similarity. Sun et al. (Sun et al., 2005) utilized ribosomal DNA ITS sequences in a study of *B. subaequalis* and *B. multiplex* cv. *Fernleaf*, and suggested that the two species are sister species. Based on the results of the present study, *B. subaequalis* and *B. multiplex* cv. *Fernleaf* are closely related, further supporting the sister-group relationship.

Conclusions

Genetic diversity and phylogenetic relationships within *Bambusa* can be reliable and reproducibly evaluated based on RAPD markers. The 28 tested *Bambusa* species/varieties possess high genetic diversity and complex phylogenetic relationships. When combined with morphological features, the results of this study can provide a theoretical basis for germplasm conservation, classification, and evolutionary analyses of *Bambusa* species/varieties. It is obligatory to fortify the collection, identification, and excavation of the germplasm resources of *Bambusa* to augment the genetic diversity of breeding materials. Continuous development of biotechnology, in bamboo research will be required to address more problems. Bamboo genome research, gene mechanism, transgenic technology, and cloning technology can be a new direction in bamboo research.

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