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Molecular identification of bamboo genera and species based on RAPD-RFLP markers

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Highlights

- We investigated the potential of RAPD-RFLP (Random Amplified Polymorphic DNA, Restriction Fragment Length Polymorphism) in detecting genetic relationships among bamboo genera and species.
- RFLP profiles resolved in acrylamide gels revealed high number of markers, which accurately differentiated species and genera, based on cophenetic correlation coefficients.
- We recommend RAPD-RFLP for analyses of genetic diversity and divergence among bamboo genera, species and varieties.

Abstract

Bamboo species have a very significant ecological and economic impact. Determining morphological and genetic differences among bamboo genera and species are crucial to explore desirable traits for breeding purposes. Several advances have been made in the taxonomy of bamboos by using molecular fingerprinting tools and next generation sequencing technologies. Nevertheless, classical molecular markers such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and ISSR (Inter Simple Sequence Repeats) also provide an accurate discrimination among genera and species. Moreover, the RAPD-RFLP (Random Amplified Polymorphic DNA, Restriction Fragment Length Polymorphism) method, in which amplification products from RAPD are digested with restriction enzymes, is a reliable, fast and cost-effective method for fingerprinting. RAPD-RFLP has been scarcely used in the literature and no report regarding bamboo taxonomy is available with this method. Here we explored the molecular (RAPD, RAPD-RFLP) variation among genera (*Bambusa*, *Dendrocalamus*, *Guadua* and *Phyllostachys*) and species of bamboo cultivated in Brazil. Both molecular markers allowed clear distinction among the genera studied. Moreover, high cophenetic correlation values in UPGMA clusters indicated their potential for discriminating bamboo species. The digestion of RAPD products (RFLP) resulted in high number of polymorphic bands and produced very characteristic profiles for each genus with three enzyme combinations (*HindIII/HaeIII*, *HinfI/RsaI*, and single digestion with *MspI*). We recommend RAPD-RFLP as a reproducible and informative method for screening differences among genera, species and varieties of bamboos. Providing a cost-effective and accurate method for species identification and characterization is straightforward for bamboo conservation, management and breeding.

Keywords molecular screening; molecular markers; restriction enzymes; genetic divergence; *Dendrocalamus*; *Bambusa*

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Abbreviations

RAPD – Random Amplified Polymorphic DNA
AFLP – Amplified Fragment Length Polymorphism
ISSR – Inter Simple Sequence Repeats
RFLP – Restriction Fragment Length Polymorphism
UPGMA – Unweighted Pair-Group Methods with Arithmetic Mean

1 Introduction

Bamboos (sub-family Bambusoideae, family Poaceae) comprise approximately 1290 species, which are naturally distributed all over the world (Hamzah et al. 2016). Most of the species richness is found in Pacific Asia and South America (Bystriakova et al. 2003; Das et al. 2008). Bamboos are used for a wide range of applications such as for umbrella handles, fishing rods, mud huts, mats, baskets (Bystriakova et al. 2003), concrete reinforcement (Ghavami 2005), building material for supporting structures (Van der Lugt et al. 2006), charcoal preparation (Xiong et al. 2014; Xia et al. 2016), cellulose extraction (Li et al. 2015), hybrid microfibers fabrication (Yu et al. 2014), and forages for maintaining environmental conditions (Panizzo et al. 2016). Due to their fast growth rate (5–7 years to mature) and some potential fuel characteristics, e.g., low ash content, alkali index or heating value, they might also be a valuable resource for energy (Bystriakova et al. 2003). Research on their pharmaceutical applications are of increasing interest (Ho et al. 2013). The combination of fast growth, natural vegetative propagation and their adequacy for several products makes bamboos suitable for replacing other perennial and woody species for various industrial applications (Bonilla et al. 2010; Hamzah et al. 2016).

In a scenery of increased disturbance to the environment, bamboos arise as promising alternatives for sustainable use of plant genetic resources (Vogtlander et al. 2010), reforestation, restoration of degraded areas and carbon sequestration (Bonilla et al. 2010). The knowledge of botanical and ecological aspects of bamboos, along with their genetic resources is essential for their conservation and discovery of new potential applications. Moreover, considering the multiple species and their morphological similarities in several instances, it is important to distinguishing such species at the taxonomical level for proper establishment of strategies aimed at their conservation. A well-designed conservation project relies on proper constituted germplasm banks or repositories, in which species and accessions are described by their morphological and genetic features (Generoso et al. 2016).

Taxonomy of bamboos has been traditionally set based on morphological traits for different parts of the plants such as rhizomes, branches, leaves, inflorescences and fruits. Several classification systems have been proposed and phylogenetic analyses have provided genetic relationships based on morphological and molecular analyses (Das et al. 2008). Particular characteristics of bamboo species have also been determined by scanning electron microscopy, analyzing specific cellular or

subcellular structures (Gomes and Neves 2009). However, the molecular identity of each species has been more acknowledged in recent years, as various studies have provided high-confidence results for the differentiation at the genus, species and accession levels (Yeasmin et al. 2015).

Molecular studies have provided significant advances in the comprehension of various research questions with bamboos. First, for molecular identification and phylogenetic relationships, RAPD markers were used for distinguishing species of the genera *Bambusa*, *Dendrocalamus*, *Dinocloa* and *Cephalostachyum* (Nayak et al. 2003). Two specific RAPD markers from *Bambusa balcooa* Roxb. and *B. tulda* Roxb. were converted to SCAR (Sequence Characterized Arbitrary Regions) in order to obtain proper markers for the molecular identification of the species (Das et al. 2005). Another class of markers, the AFLP (Amplified Fragment Length Polymorphism), were screened for detecting genetic variability and defining phylogenetic relationships among bamboo species from a collection of Singapore (Loh et al. 2000) and India (Waikhom et al. 2012). As alternative dominant genetic markers, ISSR (Inter Simple Sequence Repeats) markers have been used to distinguishing bamboo species (Lin et al. 2010; Mukcherjee et al. 2010). The genetic diversity has been another research question addressed to the within-species level, such as a group of *Ochlandra travancorica* Benth accessions from India, combining RAPD and AFLP markers (Nag et al. 2013). The association between morphological traits and molecular markers such as RAPD in bamboo collections has also been a research problem of concern (Shalini et al. 2013). As bamboos have evolved with natural proclivity for vegetative propagation, the clonal structure of populations has been the motive for investigation in a community of *Phyllostachys pubescens* (Mazel) Ohwi (Isagi et al. 2004).

To specifically address phylogenetic and taxonomic questions, however, it has been usual the analysis of plastid markers due their higher sequence conservation. One major study with that purpose on bamboos was performed with five plastid markers that were screened to analyze the phylogenetic relationships among 33 bamboo taxa. It enabled the categorization of bamboos according to the tribes they belonged, as well as their major distribution areas throughout the world (Kelchner et al. 2013). To better acknowledge the molecular screening of bamboo species, a recent review has compiled the various studies conducted so far (Yeasmin et al. 2015).

Therefore, the use of molecular markers has revolutionized the extent of resolution to what phylogenetic and population genetic studies could achieve. The development of Polymerase Chain Reaction (PCR) pulsed the numerous derivative techniques for genetic analyses aforementioned, such as RAPD (Williams et al. 1990). Genuine concern, however, has been described about RAPD markers for their low reproducibility and, therefore, lack of resolution. However, some line of evidence has shown that this issue might be circumvented if the PCR conditions are optimized, especially the annealing temperature and the concentration of reaction components (Scoric et al. 2012; Lencina et al. 2016). An alternative and simple method for genotyping bamboos is PCR-RFLP (Polymerase Chain Reaction, Restriction Fragment Length Polymorphism). It is based on the digestion of PCR products with different combinations of restriction enzymes. After digestion, the products are separated by gel electrophoresis (agarose or acrylamide). PCR-RFLP has been frequently conducted with the digestion of PCR products of ITS (Internal Transcribed Spacer) regions in plants (Biswas et al. 2013) and organelle genes and genomes (Schlogl et al. 2007). Little work has also been shown with the digestion of RAPD products with restriction enzymes in tree species, such as *Pinus resinosa* Aiton (DeVerno and Mosseler 1997). RAPD-RFLP profiles have revealed great potential for distinguishing plant species and accessions (DeVerno and Mosseler 1997). Nonetheless, no reports on the application of such method for bamboos have been provided so far.

As bamboos have been of increasing interest in a wide range of applications and more attention has been dedicated to its identification and conservation, we fingerprinted a set of bamboo species integrating a collection of the Bamboo Active Germplasm Bank (AGB) from Brazil, using

RAPD and RAPD-RFLP markers. This AGB encompasses various bamboo species of ecological and economic value. However, we performed molecular analyses with samples of the most important species cultivated for the market and industries (economic aspect) in Brazil and other countries, such as those from genera *Bambusa*, *Dendrocalamus* and *Phyllostachys*. Moreover, we aimed at representing the Neotropical genus *Guadua*, with species also native to Brazil, with potential for ornamental and economic usage, but of special conservation concern, as they are native to the Amazon.

Therefore, this study was aimed at validating RAPD-RFLP as a method for estimating the genetic relationships among bamboo taxa (genera and species), providing a simple and cost-effective strategy for their molecular categorization with reliable results. To achieve such goal, we examined a few species representatives within each of the following genera, considering their economic and ecological importance: *Bambusa*, *Dendrocalamus*, *Phyllostachys* and *Guadua*. This method might be employed to further examine wider sets of species and populations of bamboos from the AGB or that are yet to be collected, distinguished and incorporated to germplasm banks for conservation and breeding.

2 Material and methods

2.1 Plant materials

We sampled 13 taxa from the Bamboo Active Germplasm Bank (AGB) at the Development and Research Station (UPD/DDD/APTA/SAA) (23°21'S, 47°51'W), Tatuí, São Paulo State, Brazil. The following taxa were analyzed: *Bambusa vulgaris* Schrad. ex J.C. Wendl., *B. vulgaris* var. *vittata* Rivière & C. Rivière, *B. beecheyana* Munro, *Dendrocalamus giganteus* Wallich ex Munro, *D. asper* (Sult. & Sult. F.) Backer ex k. Heyne, *Phyllostachys edulis* (Carrière) J. Houz., *P. heterocyclus* (Carrière) Mitford., *Guadua amplexifolia* J. Presl and *G. superba* Huber. Four samples of *Guadua* unidentified at the species level were also collected from a National Forest Reserve from Acre, North Brazil, and fingerprinted to assess their relationships with the other taxa. Culms of each species were collected on their locations, sectioned and placed on pots with soil in a greenhouse. Three leaf samples were collected for each species and DNA extractions were performed for each one separately.

2.2 DNA extraction

DNA extraction was carried out according to Doyle (1990), with modifications. Approximately 50 mg of leaf powder was homogenized in 700 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 1% PVP) and maintained in microtubes at 60 °C for 30 min. Then, 650 µL of chloroform: isoamyl alcohol (24:1) was added to each sample. After centrifugation at 12 000 g, the supernatant of each sample was transferred to a new microtube and the same volume of chloroform: isoamyl alcohol (24:1) was added. This step was once more repeated. The DNA contained in the supernatant was precipitated in one volume of isopropanol (1:1) and washed in ethanol 70% and 95% for salt removal. Pellets were air-dried and suspended in Tris-RNase (Tris HCl pH 8.0 and RNase 10 µg mL⁻¹) solution and incubated 30 min at 37 °C. DNA concentration was estimated with the spectrophotometer Nanodrop 2000 (Thermo Scientific™) and sample quality was evaluated with 1% agarose gel stained with ethidium bromide.

Table 1. RAPD primers used for fingerprinting bamboo species from a Brazilian collection.

Identification	Sequence
OPC-20	5'-ACTTCGCCAC-3'
OPV-17	5'-ACCGGCTTGT-3'
OPG-17	5'-ACGACCGACA-3'
OPAN-03	5'-AGCCAGGCTG-3'
OPB-10	5'-CTGCTGGGAC-3'
OPAW-20	5'-TGTCTAGCC-3'
OPAW-08	5'-CTGTCTGTGG-3'
OPJ-01	5'-CCCGGCATAA-3'
OPJ-15	5'-TGTAGCAGGG-3'
OPJ-04	5'-CCGAACACGG-3'
OPI-15	5'-TCATCCGAGG-3'
OPL-04	5'-GACTGCACAC-3'

2.3 RAPD

RAPD reactions were prepared with 20 ng of template, 1 X PCR reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.6 mM MgCl₂, 0.12 mM dNTP, 0.16 μM of each primer (Table 1), 1 U of Taq DNA polymerase (Invitrogen™) and were completed to 25 μL with sterilized pure water (Milli-Q). PCR was performed as follows: initial denaturation at 94 °C, 40 cycles of 94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min; and final extension at 72 °C for 7 min. RAPD products were separated on agarose gel 1.5% stained with ethidium bromide (10 mg mL⁻¹) and visualized in UV Transilluminator (Molecular Imaging, Locus Biotechnology). RAPD fragments were scored as presence/absence of bands. A binary matrix (1 and 0) was generated for further genetic analyses. Only bands with strong intensity in the gel were considered for further analyses.

2.4 RAPD-RFLP

The RAPD products from the primer OPV17, which presented considerable number of bands with clear resolution, were used for the analysis. The RAPD products were used to perform digestions with different restriction enzyme combinations (double digestion with *HindIII/HaeIII* and *HinfI/RsaI*; and single digestion with *MspI*). Digestions were performed with 5 μL of PCR product, 5 U of each enzyme and 1 μL of 10x Thermo Scientific Tango Buffer (33 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg mL⁻¹ BSA).

The digestions were performed for two hours at 37 °C. For enzyme deactivation, temperatures were raised to 70 °C for 15 min. Digestion products were separated on 6% polyacrylamide gel and visualized by silver staining, following similar procedure as Creste et al. (2001). The derived RFLP fragments were scored as presence/absence of fragments and a binary matrix was generated for the statistical analysis. We performed manual scoring of the bands, considering only those with clear visual identification and with strong intensity.

2.5 Statistics

From the binary matrices, we determined the total number of bands per primer and the number of unique bands (band appearing in only one species) per species and per primer. The polymorphic information content (PIC) of each marker was calculated using the formula $PIC = 1 - \sum p_i^2$, in

which p_i is the frequency of the band i in a total of k bands observed for each primer (Weiler et al. 2010). The genetic relationships among the taxa were assessed based on Jaccard's similarity indexes and dendrograms were generated using the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA). Clustering consistency was analyzed with a bootstrap test with 1000 replications. Cophenetic correlations were estimated based on the comparison of dissimilarity and cophenetic matrices (Sokal and Rohlf 1961). The genetic relationships of the bamboo taxa were also addressed by a principal coordinate analysis (PCoA), using the two first coordinates to explain the relationships. The analyses were performed for RAPD and RAPD-RFLP separately, as well as for both matrices combined. Softwares DendroUPGMA (Garcia-Vallve et al. 2009) and PAST (Hammer et al. 2001) were used to compute the statistics.

3 Results

3.1 Levels of polymorphism with RAPD markers

The twelve primers used in this work were effective in detecting genetic differences among genera and species. We only analyzed the bands with strong intensity and that could be clearly distinguished. In total, 83 bands were detected from the RAPD analyses (Table 2). Fig. 1 shows the electrophoretic profile of OPV-17. One primer, however, provided a single fragment in all taxa (OPC-20), showing no variation among them. The number of bands detected per marker ranged from 1 (OPC-20) to 12 (OPI-15). The average number of bands per marker obtained (6.91) was reasonably high, considering the RAPD method. Furthermore, the average polymorphic information content per marker showed considerably high levels (PIC=0.74) (Table 2), reinforcing the potential of such technique in detecting variation at the genus and species levels.

Table 2. Total number of bands (N), species with unique bands (UB) and number of unique bands detected with RAPD and RAPD-RFLP markers in bamboo species.

Marker	N	UB	PIC	Unique bands per species												
				BV	BVV	BB	DG	DA	PE	PH	GA	GS	G1	G2	G3	G4
OPC-20	1	0	0.00													
OPV-17	5	1	0.74				1									
OPG-17	7	2	0.85			1			1							
OPAN-03	7	1	0.71			1										
OPB-10	10	2	0.78			1				1						
OPAW-20	9	4	0.91	1	1		1			1						
OPAW-08	5	1	0.88		1											
OPJ-01	5	3	0.94							1	2					
OPJ-15	6	1	0.56			1										
OPJ-04	7	1	0.84										1			
OPI-15	12	2	0.89							1	1					
OPL-04	9	1	0.81							1						
Total	83	19	0.74	1	2	4	2	0	1	5	3	0	1	0	0	0
RAPD-RFLP																
<i>MspI</i>	33	9	0.86		2	4		1		2						
<i>HindIII/HaeIII</i>	26	14	0.94			5		1	2		4			1	1	
<i>HinfI/RsaI</i>	35	11	0.93			4			3				3			1
Total	94	34	0.91	0	2	13	0	2	5	2	4	0	3	1	1	1

BV – *Bambusa vulgaris*. BVV – *Bambusa vulgaris vittata*. BB – *Bambusa beecheyana*. DG – *Dendrocalamus giganteus*. DA – *Dendrocalamus asper*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocycla*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp.

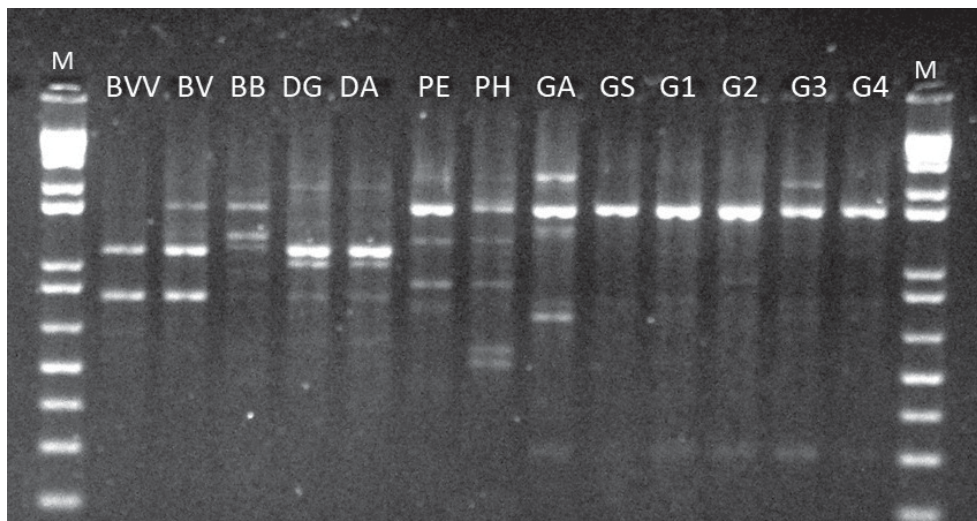


Fig. 1. RAPD profile of OPV-17 for 13 bamboo taxa. BVV: *Bambusa vulgaris vittata*. BV: *Bambusa vulgaris*. BB: *Bambusa beecheyana*. PE: *Phyllostachys edulis*. PH: *Phyllostachys heterocyclus*. DA: *Dendrocalamus asper*. DG: *Dendrocalamus giganteus*. GA: *Guadua amplexifolia*. GS: *Guadua superba*. G1, G2, G3 and G4: *Guadua* spp. M – 1 Kb Plus Ladder DNA.

The RAPD screening also provided unique bands among species. With the exception of OPC-20, all other markers exhibited unique bands across the whole analysis panel. A single unique band has been detected at OPV-17 and OPL-04, respectively for *D. giganteus* and *P. heterocyclus*. From five bands detected at OPJ-01, three bands were uniquely encountered in *P. heterocyclus* and *G. amplexifolia*. *B. beecheyana* was differentiated from the other species based on four unique bands at four distinct loci (amplified from OPG-17, OPAN-03, OPB-10 and OPJ-15). *P. heterocyclus* was distinguished by five markers, each at a different locus (OPB-10, OPAW-20, OPJ-01, OPI-15, OPL-04). In total, 19 unique bands (22.8% from the total) were distinguished.

3.2 Levels of polymorphism with RAPD-RFLP

The digestion of OPV-17 with the three enzyme combinations (*MspI*, *HindIII/HaeIII* and *HinfI/RsaI*) resulted in a high number of bands, as revealed by acrylamide gels (Fig. 2). In total, 94 bands were distinguished from the gels and used in the statistical analysis (Table 2). Digestion with *MspI* revealed 33 distinguishable bands, while *HindIII/HaeIII* and *HinfI/RsaI* digestions allowed scoring 26 and 35 distinct bands, respectively. The polymorphism revealed by only these three markers (PIC=0.91) with the digestion of a single primer was higher than the average polymorphism obtained with the RAPD markers (PIC=0.74) (Table 2).

Moreover, bands were detected from the RAPD-RFLP profiles. In total, 34 unique bands were identified as unique among the taxa with the three enzyme combinations, approximately 79% higher than with the 12 RAPD primers. In average, around 11 unique bands were detected per enzyme combination. Interestingly, *B. beecheyana* was distinguished from the other taxa with 13 unique bands from the total of the three digestions, representing more than 38% of the total number of unique bands.

With the increased representation provided by RFLP, unique bands were also detected within the genus *Guadua*. While with the whole set of RAPD markers it was possible to identify four unique bands, it raised to 10 bands with RFLP markers, showing higher discriminatory power of this method.

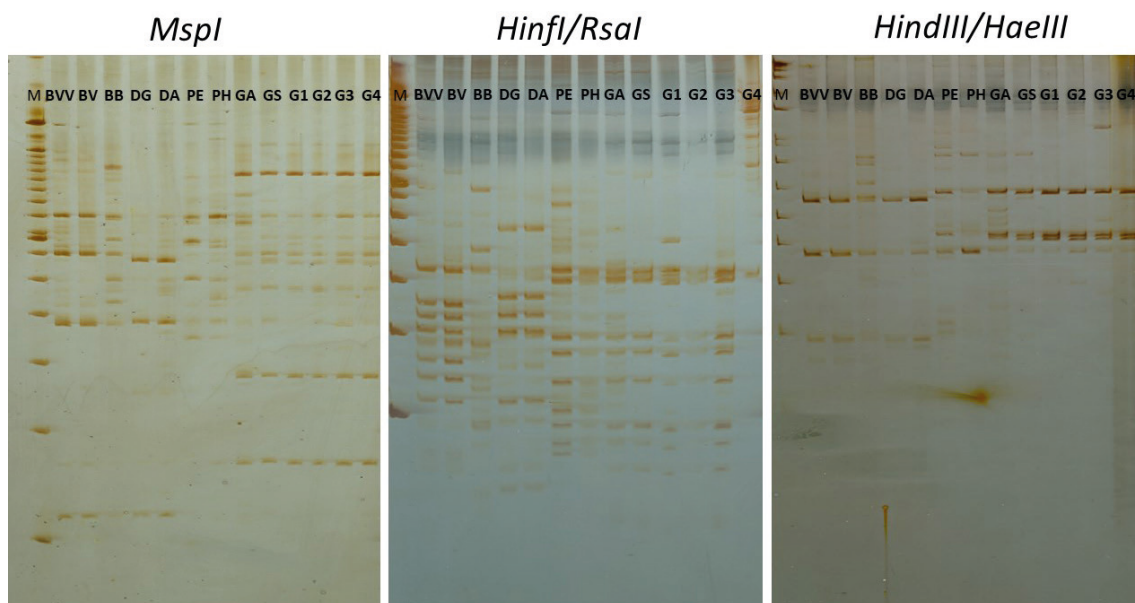


Fig. 2. RAPD-RFLP profile from the digestion of RAPD products from primer OPV17 with three restriction enzyme combinations. BVV – *Bambusa vulgaris vittata*. BV – *Bambusa vulgaris*. BB – *Bambusa beecheyana*. DG – *Dendrocalamus giganteus*. DA – *Dendrocalamus asper*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocycla*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp. M – 100 bp marker (Ladder).

3.3 Genetic relationships among the bamboo taxa

The RAPD screening was able to differentiate each genus in different groups, based on the clustering analysis with Jaccard's similarity index through the UPGMA approach (Fig. 3a). The bootstrap resampling analysis strengthened the clustering reliability. The *Guadua* genus was separated as a group with a bootstrap value of 89%. *Phyllostachys*, *Dendrocalamus* and *Bambusa* were grouped with a low bootstrap (42%), but when each genus was considered as a separate group, the bootstrap values were of at least 83% for *Bambusa*, and then 92% for *Phyllostachys* and 100% for *Dendrocalamus*. Each genus was then considered as a separate group. As a high cophenetic correlation was detected from the clustering analysis ($r=0.972$), each species could actually be considered as a separate group, demonstrating that RAPD markers were able to reliably distinguish the bamboo taxa.

Similar results were obtained with RAPD-RFLP, except for certain distinctions between the dendrograms. In case of the RAPD-RFLP clustering, *Phyllostachys* was no longer included within the same group that *Bambusa* and *Dendrocalamus* (Fig. 3b). Conversely, a considerably high bootstrap value (71%) matched *Phyllostachys* and *Guadua* within the same subgroup (Fig. 3b). As the cophenetic correlation coefficient was similarly high ($r=0.961$) as it was for RAPD, each species could be fitted in separate subgroups. In comparison to RAPD, higher bootstrap values matched three genera subgroups, ranging from 90% (*Guadua*) to 100% (*Dendrocalamus*). However, *B. beecheyana* was matched with a low bootstrap value (53%) to the other members of *Bambusa*. A joint analysis of RAPD and RFLP data, in general, revealed similar clustering to the one provided by only RAPD-RFLP (Figs. 3b–c).

The four *Guadua* taxa with no identification to the species level revealed different clustering results from the two techniques. From the RAPD analysis, the most similar taxa were two of *Guadua* unidentified at the species level, exhibiting 97% of similarity (Table 3, Fig. 3a). Taxa 1 and 2 were defined in a different subgroup, according to the cophenetic correlation, and with similarity of 83%. Alternatively, with the RFLP data, taxa 1, 2 and 4 were more similar (Table 4,

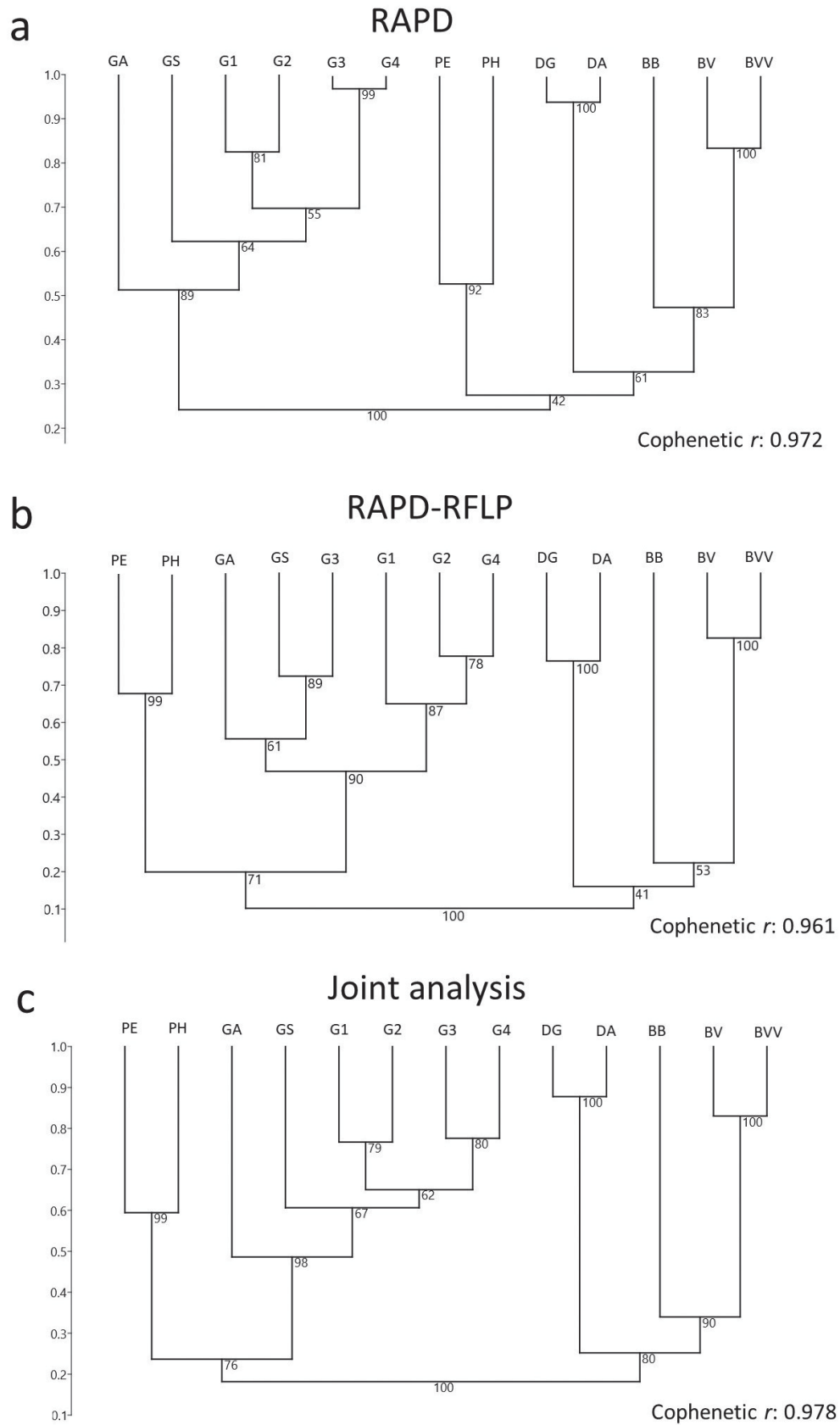


Fig. 3. Dendrogram of 13 bamboo taxa based on (a) RAPD and (b) RAPD-RFLP markers, and the (c) joint analysis. BVV – *Bambusa vulgaris vittata*. BV – *Bambusa vulgaris*. BB – *Bambusa beecheyana*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocycla*. DA – *Dendrocalamus asper*. DG – *Dendrocalamus giganteus*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp.

Table 3. Jaccard's similarity coefficient among 13 bamboo taxa, based on 83 RAPD markers.

	BV	BVV	BB	DG	DA	PE	PH	GA	GS	G1	G2	G3	G4
BV		0.833	0.486	0.304	0.318	0.325	0.271	0.212	0.204	0.245	0.235	0.18	0.184
BVV			0.459	0.283	0.295	0.3	0.25	0.216	0.184	0.226	0.216	0.184	0.188
BB				0.372	0.39	0.3	0.304	0.265	0.234	0.226	0.216	0.208	0.213
DG					0.938	0.213	0.275	0.264	0.286	0.296	0.264	0.212	0.216
DA						0.222	0.286	0.275	0.298	0.308	0.275	0.22	0.224
PE							0.526	0.333	0.244	0.189	0.2	0.217	0.222
PH								0.447	0.28	0.268	0.259	0.255	0.26
GA									0.61	0.521	0.489	0.467	0.477
GS										0.683	0.692	0.55	0.564
G1											0.825	0.683	0.659
G2												0.737	0.711
G3													0.968

BV – *Bambusa vulgaris*. BVV – *Bambusa vulgaris vittata*. BB – *Bambusa beecheyana*. DG – *Dendrocalamus giganteus*. DA – *Dendrocalamus asper*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocyclus*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp.

Fig. 3b), while taxon 3 fitted the same group as *G. amplexifolia* and *G. superba*. With the joint analysis of RAPD and RFLP data, a similar grouping than the one provided by the RAPD data was showed (Fig. 3c).

Another approach used to address the genetic relationships among the 13 bamboo taxa was a principal coordinate analysis (PCoA), which is based on the genetic distances among each species (Figs. 4a–c). In general, RAPD, RAPD-RFLP and the joint analysis revealed similar dispersion of the taxa across the graphs. The first two coordinates were able to explain more than 50% of the genetic variation among the bamboo species. One important highlight, however, was that the genus *Guadua* was more distinguished from the other genera from the PCoA graphs than with the dendrograms. The distinction among the taxa within the *Guadua* subgroup, however, presented variable profiles, as also revealed from the UPGMA clustering.

Table 4. Jaccard's similarity coefficient among 13 bamboo taxa, based on 94 RAPD-RFLP markers.

	BBV	BV	BB	DG	DA	PE	PH	GA	GS	G1	G2	G3	G4
BBV		0.826	0.209	0.172	0.188	0.114	0.154	0.106	0.098	0.086	0.121	0.093	0.121
BV			0.238	0.172	0.188	0.14	0.154	0.13	0.098	0.086	0.121	0.119	0.121
BB				0.1	0.143	0.157	0.146	0.192	0.196	0.116	0.146	0.213	0.146
DG					0.765	0.025	0.028	0.023	0.057	0	0.036	0.054	0.036
DA						0.047	0.051	0.067	0.108	0.062	0.1	0.103	0.1
PE							0.677	0.311	0.3	0.098	0.128	0.227	0.1
PH								0.341	0.333	0.108	0.111	0.22	0.111
GA									0.571	0.371	0.382	0.541	0.382
GS										0.519	0.538	0.724	0.538
G1											0.65	0.433	0.65
G2												0.5	0.778
G3													0.556G

BBV – *Bambusa vulgaris vittata*. BV – *Bambusa vulgaris*. BB – *Bambusa beecheyana*. DG – *Dendrocalamus giganteus*. DA – *Dendrocalamus asper*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocyclus*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp.

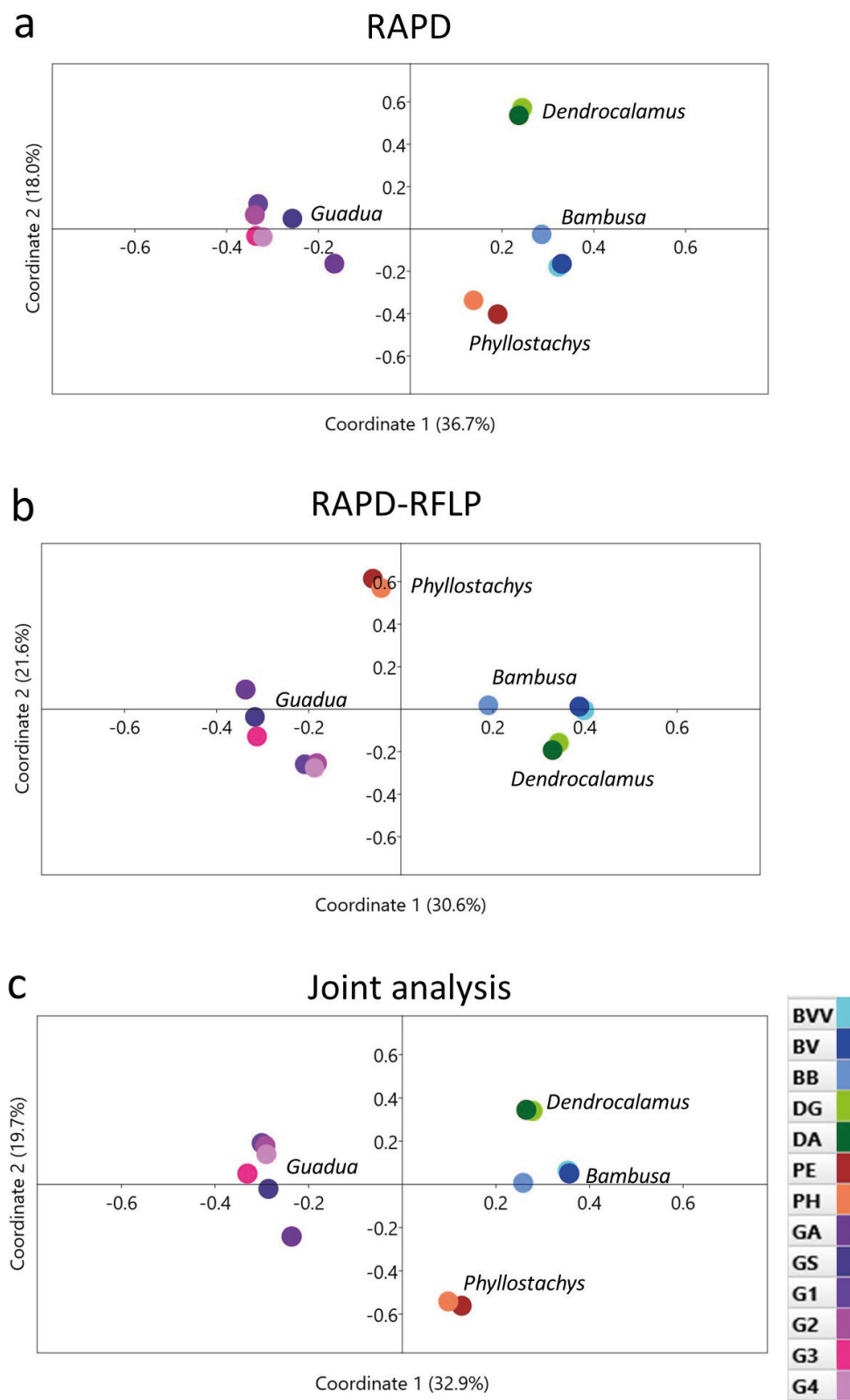


Fig. 4. Principal coordinate analysis of 13 bamboo taxa based on (a) RAPD, (b) RAPD-RFLP and (c) the joint analysis. BVV – *Bambusa vulgaris vittata*. BV – *Bambusa vulgaris*. BB – *Bambusa beecheyana*. DG – *Dendrocalamus giganteus*. DA – *Dendrocalamus asper*. PE – *Phyllostachys edulis*. PH – *Phyllostachys heterocycla*. GA – *Guadua amplexifolia*. GS – *Guadua superba*. G1, G2, G3 and G4 – *Guadua* spp.

4 Discussion

We were able to discriminate 13 bamboo taxa based on RAPD and RAPD-RFLP methods. Both methods allowed the distinction among genera and species, with similar resolution. However, our results revealed higher number of markers with the digestion of the fragments generated from the amplification of a single RAPD primer with the three enzyme combinations, which has the potential of increasing the discriminatory power among genera and species. One inherent difference of the screening of RAPD and RAPD-RFLP markers, however, was that the RAPD-RFLP markers were run on an acrylamide gel, which enables the detection of higher number of loci, usually with potential of detecting bands with even less than 100 bp with sufficient resolution for distinction.

RAPD markers have been used in studies aimed at the analysis of genetic diversity and divergence among bamboos (Nayak et al. 2003; Lalhruaitluanga and Prasad 2009; Shalini et al. 2013). Despite these markers have been argued about their limitations, such as their dominance, uncertain locus homology and sensitivity to the conditions of the PCR reactions (Yeasmin et al. 2015). In this case, the reproducibility problem might be overcome if the conditions or the reactions are optimized (Scoric et al. 2012). Our reactions were carefully prepared and maintained at homogeneous conditions to perform the RAPD screening. Moreover, the digestion of the RAPD products with the restriction enzymes produced a high number of bands, allowing a reliable estimation of the phylogenetic relationships among the taxa examined. Our results are the first report on the application of RAPD-RFLP markers on the genetic characterization of bamboo species. Previous studies have used classical RFLP markers only, which involved the design of probes, Southern hybridization and autoradiography for revealing molecular markers. In bamboos, an example was the study of Friar and Kockert (1994), dedicated to the genetic diversity and evolutionary analysis of the genus *Phyllostachys*.

Both methods used in our work revealed similar clustering patterns by the UPGMA method (Fig. 3), as well as by the principal coordinate analysis (PCoA) (Fig. 4). The high cophenetic correlation encountered for RAPD clusters ($r=0.972$) and RAPD-RFLP clusters ($r=0.961$) reinforced that each taxon might be considered independently, showing the high discriminatory power of the markers. Moreover, two components of the PCoA explained more the half of the genetic divergence observed, and a clear separation among the genera (*Bambusa*, *Dendrocalamus*, *Phyllostachys* and *Guadua*) is detected from this multivariate approach.

Our work was not aimed at a taxonomic categorization of the species and genera, as a limited set of species and genera were studied. However, some inferences might be described. Considering the clustering among genera (with no regards to the cophenetic correlations), *Bambusa* and *Dendrocalamus* were grouped together by both RAPD and RAPD-RFLP methods, as well as the combined analysis of all markers. Species of *Bambusa* and *Dendrocalamus* are naturally distributed in Asia. Earlier studies have determined the preferential distribution of bamboos according to climatic zones in India, the second richest country in bamboo species after China (Bystriakova et al. 2003; Yeasmin et al. 2015). Taxonomically, *Bambusa* and *Dendrocalamus* both belong to the tribe Bambuseae. These genera are predominant in the dry tropical regions of India, but also occur on temperate and subtropical regions of the country (Ahmed 1996). *B. vulgaris* occurs in the tropics of Asia. *D. asper* is distributed in India and southeastern Asia, whereas *D. giganteus* occurs in southern Myanmar and northern Thailand (Benton 2015). Considering that we sampled only one individual of each species and each genus integrating various species, the groupment between *Bambusa* and *Dendrocalamus* was coherent.

The genus *Phyllostachys* belongs to a different tribe, the Arundinarieae. In general, species of *Phyllostachys* are native to China and Japan (Benton 2015). In our analysis, the clustering between

Phyllostachys and the other genera was different between RAPD and RAPD-RFLP markers. With the former class of markers, *Phyllostachys* was grouped with *Bambusa* and *Dendrocalamus* (Fig. 3a), whereas with the latter *Phyllostachys* was clustered with *Guadua*. The genus *Guadua* is predominantly Neotropical. Therefore, *Guadua* species occur mainly in Central and South America (Yeasmin et al. 2015). The separation between *Guadua* and the other genera, considering their natural distribution is then reasonably expected.

To confirm aforementioned clustering results and, more importantly, to further understand the conflicting results of the position of *Phyllostachys* in the groups from each marker (Fig. 3), our results might be compared with a wider investigation of the phylogenetic relationships among bamboos based on five plastid markers performed by Kelchner et al. (2013). In the study, 33 bamboo species were phylogenetically resolved from a total of 6.7 kb sequence-data of coding and non-coding regions of chloroplast genomes, enabling the categorization of bamboos in four major lineages: Paleotropical Woody, Neotropical Woody, Temperate Woody and Herbaceous. Among those, the genus *Guadua* was placed within Neotropical Woody bamboos, while *Bambusa* was within the Paleotropical Woody clade. By extension of this interpretation, *Dendrocalamus*, which was not analyzed by Kelchner et al. (2013), belongs to the same tribe as *Bambusa*, which supports the results obtained in this study, showing higher similarity between *Dendrocalamus* and *Bambusa* than with the other genera (Fig. 3). Moreover, the *Phyllostachys* member analyzed by Kelchner et al. (2013) along with other species of the tribe Arundinaceae were placed within the Temperate Woody clade, which was phylogenetically more distant than between Paleotropical Woody and Neotropical Woody clades. It might explain the lack of consistency of the positioning of *Phyllostachys* in our study, as with only RAPD markers (Fig. 3a) it was placed within the same group of *Dendrocalamus* and *Bambusa*. Conversely, with RAPD-RFLP (Fig. 3b) and the joint analysis (Fig. 3c), the *Phyllostachys* group was placed alongside with the *Guadua* group. Probably, the inclusion of more taxa would provide better resolution for this clustering result, which is beyond the main purpose of our work.

As the first analysis involving RAPD-RFLP analysis in bamboos, we were able to demonstrate its applicability for studies aimed at the genetic characterization of genera and species of bamboo with such as low-cost and fast-screening method. We recommend the use of RAPD-RFLP method for further investigations of genetic diversity within species collections, such as accessions of different locations. Genetic analyses that have no requirement of sequencing technologies might be efficiently conducted with the employment of RAPD-RFLP markers or other PCR-RFLP strategies. The wide diversity among and within species has to be better understood to aid information for breeding and conservation programs of bamboos.

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