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Genetic analysis of *Apuleia leiocarpa* as revealed by random amplified polymorphic DNA markers: prospects for population genetic studies

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ABSTRACT. *Apuleia leiocarpa* (Vogel) J.F. MacBride is a hardwood species native to South America, which is at serious risk of extinction. Therefore, it is of prime importance to examine the genetic diversity of this species, information required for developing conservation, sustainable management, and breeding strategies. Although scarcely used in recent years, random amplified polymorphic DNA markers are useful resources for the analysis of genetic diversity and structure of tree species. This study represents the first genetic analysis based on

DNA markers in *A. leiocarpa* that aimed to investigate the levels of polymorphism and to select markers for the precise characterization of its genetic structure. We adapted the original DNA extraction protocol based on cetyltrimethyl ammonium bromide, and describe a simple procedure that can be used to obtain high-quality samples from leaf tissues of this tree. Eighteen primers were selected, revealing 92 bands, from which 75 were polymorphic and 61 were sufficient to represent the overall genetic structure of the population without compromising the precision of the analysis. Some fragments were conserved among individuals, which can be sequenced and used to analyze nucleotide diversity parameters through a wider set of *A. leiocarpa* individuals and populations. The individuals were separated into 11 distinct groups with variable levels of genetic diversity, which is important for selecting desirable genotypes and for the development of a conservation and sustainable management program. Our results are of prime importance for further investigations concerning the genetic characterization of this important, but vulnerable species.

Key words: *Apuleia leiocarpa*; RAPD markers; Genetic diversity; DNA; Molecular markers

INTRODUCTION

Apuleia leiocarpa (Vogel) J.F. MacBride (Fabaceae) is a tree species native to the tropical forests of South America, popularly referred to as the 'Queen' of trees from the southern states of Brazil and commonly known as grapia. *A. leiocarpa* is a diploid ($2n = 2x = 26$) species with the karyotype 20 m + 4 sm + 2 t (Auler and Battistin, 1999), a monoecious tree and probably alogamous. *A. leiocarpa* is naturally distributed from Peru to Uruguay. In Brazil, it occurs from the South to the North and Northeast (Carvalho, 2003). Its wood has a high value and is of great interest to the timber industry, since it is suitable for use in external structures in civil construction, woodwork, energy, and cellulose, among other applications (Carvalho, 2003). Furthermore, *A. leiocarpa* has medicinal potential, since a seed protein (ApulSL) with antimicrobial activity was recently isolated and characterized (Carvalho et al., 2015). Its fruit extract was tested as a biocide, and was found to exhibit limited *in vitro* cytotoxicity and have high potential as a source of prototypes in the development of drugs with antibiofilm activity (Silva et al., 2015). Because of its high wood quality and industrial application, *A. leiocarpa* has to be considered a priority for conservation (de Medeiros et al., 2011). The tree is highly endangered and threatened with extinction, being categorized as vulnerable, according to Brazilian records (CNCFLORA, 2012). It is also described as critically endangered according to a decree of the Rio Grande do Sul State (Decree no 52.109, from December 1, 2014, https://www.mprs.mp.br/ambiente/legislacao/id9025.htm?impressao=1).

Knowledge on the ecology and genetic structure of natural populations of tropical tree species remains scarce, but is essential for defining strategies toward their conservation, breeding, and sustainable management (Konzen, 2014). Genetic studies are even more necessary for species at risk of extinction, with limited distribution, and rare frequency in native forests (Juchum et al., 2007), as they help to identify areas with higher genetic variability, which can

be prospected for species conservation (Santos et al., 2007; Martins-Corder et al., 2009).

Several molecular techniques are currently available for the analysis and detection of genetic variability at the DNA level. Molecular markers have been developed and employed in plants (Rossetto and Rymer, 2013). Such methods have been fundamental for genetic analyses, including fingerprinting, discovery of diversity within and among populations, genetic structure and paternity detection, and gene mapping (Konzen, 2014). Random amplified polymorphic DNA (RAPD) is a molecular marker that allows considerable sampling of an individual genome and is able to detect variation at relatively low costs in non-coding and coding regions. RAPD markers represent different-sized DNA fragments obtained by amplification using primers constituted by arbitrary nucleotides (generally 10 bp) (Williams et al., 1990).

RAPD markers are especially appropriate for use in species where no prior information about the genetic diversity status and genetic structure are available. The procedure is simple, fast, and associated with relatively low cost when compared to other techniques. Conversely, some common drawbacks of this technique are its low reproducibility, low number of markers detected per primer, and the low information content per locus due to its dominant inheritance. Nevertheless, some studies have noted that the issue of reproducibility might be circumvented if PCR conditions are optimized, especially the annealing temperature and the concentrations of reaction components (Scoric et al., 2012). Moreover, this issue might be related to marker selection and marker sampling, and may therefore represent more than just a technical issue (Ramos et al., 2008). Several forest tree species have recently been characterized using RAPD markers, including *Caesalpinia pulcherrima* (L.) Sw. (Rodrigues et al., 2012), *Poincianella pyramidalis* (Mendes et al., 2014), *Calophyllum brasiliense* (Schühli et al., 2013; Mendonça et al., 2014), *Hancornia speciosa* Gomes (da Silva et al., 2012), and *Nectandra megapotamica* (Costa et al., 2015). Some species at serious risk of extinction have also been analyzed using RAPD markers, such as *Dalbergia nigra* (Fabaceae) (Juchum et al., 2007), *Amburana cearensis* (Fr. Allem) A. C. Smith, *Myracrodruon urundeuva* M. Allem., and *Schinopsis brasiliensis* Eng. (Santos et al., 2007).

Considering that *A. leiocarpa* is a priority for conservation due to its environmental and economic importance, knowledge on its genetic variability and structure are of fundamental value. To date, the genetic knowledge of the species is restricted to a few sequences deposited in NCBI/GenBank (http://www.ncbi.nlm.nih.gov/genbank/), annotated as genes coding for chloroplast and ribosomal proteins, which are used as phylogenetic markers (Doyle et al., 1997; Bruneau et al., 2008). Therefore, in the present study, we describe the genetic analysis of an *A. leiocarpa* population from the southern State of Rio Grande do Sul, in Brazil, using RAPD markers. Furthermore, we present the first evidence of genetic variability in *A. leiocarpa* based on DNA markers and select suitable RAPD primers to genetically characterize this species. An analysis of the number of markers needed to reliably estimate the genetic diversity of the species is also presented. Finally, we outline bands that are potentially conserved in this species, which are suitable for sequencing and for the detection of polymorphisms at the nucleotide level.

MATERIAL AND METHODS

Adaptation of the DNA extraction protocol for *A. leiocarpa*

Seeds were harvested from three adult individuals of a natural population of *A. leiocarpa* located in Santa Maria, RS. The progenies of these trees have been used for several clonal propagation techniques aimed at maintaining genetic resources of the species

at Universidade Federal de Santa Maria. Leaf tissues were collected from eighty 3-year-old plants derived from three adult plants. Moreover, leaf tissues were also collected from eight adult plants of the natural population, in addition to those previously described. Therefore, in total, 88 samples were used for the genetic analyses. After collecting, leaf tissues were lyophilized (Christ®) for 3 days until completely dehydrated, and stored at room temperature in glass flasks containing silica gel until DNA extraction was performed.

The extraction method was adapted from that described by Doyle and Doyle (1990). Leaf tissues of all 88 individuals were frozen in liquid nitrogen and ground to a fine powder. Approximately 50 mg leaf powder from each plant was transferred to individual Eppendorf tubes and vigorously shaken. CTAB extraction buffer (700 µL) was added to each sample. The samples were kept in a hot water bath at 65°C for 25 min, and slightly shaken every 10 min. Afterwards, 650 µL chloroform/isoamyl alcohol (24:1) was added to the extract, which was then centrifuged at 14,000 rpm for 7 min. From the supernatant, 500 µL was pipetted and transferred to a new tube containing isopropanol (1:1) for DNA precipitation. After vigorous shaking, samples were centrifuged at 14,000 rpm for 7 min. The supernatant was entirely discarded and the pellet containing the nucleic acids was washed twice with 70% ethanol to remove salt. The pellets were air-dried and diluted with 30 µL Tris-HCl buffer containing RNAse (10 µg/mL). DNA concentration was estimated using a Nanodrop 2000 (Thermo Scientific™) and sample quality was evaluated on a 1% agarose gel stained with ethidium bromide.

RAPD reactions

DNA samples were diluted to 20 ng/µL and RAPD reactions were adapted from the original protocols described by Williams et al. (1990). PCR was performed in a final volume of 25 µL, containing 20 ng DNA, 1X Reaction Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.6 mM $MgCl_2$, 0.12 mM dNTP mix, 0.16 µM primers (Table 1), 1 U Taq DNA polymerase (Platinum, Invitrogen™), and sterile ultrapure water. Amplification conditions consisted of initial denaturation at 94°C, followed by 40 cycles at 94°C, 37°C (primer annealing temperature) and 72°C (extension by Taq) for 1 min each, and a final extension at 72°C for 7 min.

Amplification products were run on 1.2% agarose gel stained with ethidium bromide in an electrophoresis cube with TBE (1X) buffer at 60 V for approximately 2 h. The gels were visualized with an UV transilluminator and photographed with an EDAS 290 (Kodak) system. Twenty-six RAPD primers obtained from Operon Technologies (Eurofins) were initially tested. Based upon their band quality, 18 RAPD primers were used in the genetic analysis (Table 1).

RAPD scoring and polymorphism analysis

In this study, only very clear fragments in the gel, with medium-to-strong intensity were analyzed. From the fingerprints, we generated a binary matrix of data, where RAPD markers were scored based on the presence (1) or absence (0) of a band. Bands were considered polymorphic when their frequency was lower than 99%. The relative contributions to the polymorphism and the total polymorphism were calculated as the ratio between the number of polymorphic bands and the total number of bands per primer. The polymorphic information content (PIC) was calculated using the formula $PIC = 1 - \Sigma p_i^2$, in which p_i is the frequency of band i in a total of k bands observed for each primer (Weiler et al., 2010).

Table 1. Identification and sequences of RAPD primers used for the analysis of *Apuleia leiocarpa* individuals, number of amplified bands, number of polymorphic bands, and percentage of polymorphism.

Primer		Sequence	Number of fragments		Polymorphism (%)
			Amplified	Polymorphic	
1	OPC-20	5'-ACTTCGCCAC-3'	4	4	4.34
2	OPV-17	5'-ACCGGCTTGT-3'	2	1	1.08
3	OPG-17	5'-ACGACCGACA-3'	6	5	5.43
4	OPL4-525	5'-GACTGCACAC-3'	7	6	6.52
5	OPAN-03	5'-AGCCAGGCTG-3'	3	2	2.17
6	OPB-10	5'-CTGCTGGGAC-3'	3	2	2.17
7	OSAW-08	5'-CTGTCTGTCG-3'	8	8	8.69
8	OPJ-01	5'-CCCGGCATAA-3'	9	9	9.78
9	OPJ-04	5'-CCGAACACGG-3'	3	3	3.26
10	OPI-15	5'-TCATCCGAGG-3'	3	3	3.26
11	OPAN-01	5'-ACTCCACGTC-3'	8	7	7.60
12	OPAW-14	5'-GGTTCTGCTC-3'	8	6	6.52
13	OPAS-02	5'-GTCCTCGTGT-3'	6	6	6.52
14	OPA-13	5'-CAGCACCCAC-3'	8	3	3.26
15	OPAS-08	5'-GGCTGCCAGT-3'	4	4	4.34
16	OPAS-14	5'-TCGCAGCGTT-3'	3	2	2.17
17	OPJ-05	5'-CTCCATGGGG-3'	2	1	1.08
18	OPAN-19	5'-ACCACGCCTT-3'	5	3	3.26
Total			92	75	81.45

Detection of the most adequate number of markers for genetic analysis

Optimization was performed using the GENES software (Cruz, 2006) to verify if the number of bands obtained through the RAPD technique was sufficient to obtain precise estimates of genetic structure. Through this software, we obtained estimates of the correlations between the original dissimilarity matrix with other matrices produced when different numbers of bands were randomly sampled from the total. These data allowed the deviation sum squares (SQ_D) and the stress (E) value to be estimated, which indicate the adjustment between the original and sampled matrices. The ideal number of bands was determined when $E < 0.05$, as indicated by Kruskal (1964). This approach, along with the PIC and the relative contribution of each primer to the variability, based on multivariate approach on the Euclidian distances, allowed the selection of the most suitable primers and an adequate number of markers. In this step, we used the Action version 2.5 software.

Genetic distances and clustering

We analyzed the genetic structure among the individuals of *A. leiocarpa*. Two matrices of genetic distances were generated, one considering the entire set of markers and the second only with the selected bands from the optimization analysis. The matrices were compared through Mantel's test with 1000 permutations (Mantel, 1967) and the significance was evaluated through a *t*-test. A dendrogram was generated with the unweighted pair group method with arithmetic mean (UPGMA) approach considering only the selected markers. Clustering consistency was analyzed with cophenetic correlations between dissimilarity and cophenetic matrices (Sokal and Rohlf, 1962). The cutoff was defined according to Mojena (1977), using the formula $Pc = m + k\ Sd$, where, m is the mean of the values of distance, corresponding to different stages, k is a constant (1.25), and Sd is the standard deviation. These analyses were performed with the GENES software (Cruz, 2006).

RESULTS

DNA extraction protocol and RAPD primer test

Overall, the DNA extraction protocol used in this study yielded high-quality DNA samples for most individuals. DNA quality was assessed through the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}), and read using a spectrophotometer, with an average of 1.92 detected. In general, pure DNA has an A_{260}/A_{280} ratio close to 1.8. If RNA is present, it might be greater than 1.8. In this study, all samples were treated with RNAse (10 μg/mL) for 30 min and no RNA signal was detected at the bottom of the agarose gels (data not shown), indicating that most of the RNA was degraded. Overall, six samples were discarded from the analysis, as their A_{260}/A_{280} ratios were considerably higher than 2.0, and their A_{260}/A_{230} ratios were higher than 2.5. Normally, A_{260}/A_{230} ratios should be at a maximum of 2.2.

Thereafter, we standardized the amplification conditions and obtained high-intensity fragments on the gel, which enabled data to be scored with higher precision. Initially, we evaluated 26 RAPD primers, from which 18 were selected, revealing polymorphic markers with good resolution for visual scoring (Figure 1). The other RAPD primers (OPAN-04, OPAN-02 OPAW-20, OPJ-15, OPAW-13, OPAM-17, OPJ-03, and OPAN-02) were discarded from the analysis, since no suitable resolution for interpretation was obtained. From the 88 individuals initially fingerprinted, 82 were considered for the analysis because of their resolution and band-pattern in the gel. The individuals corresponded to the six samples with ratios A_{260}/A_{230} out of the range of good quality.

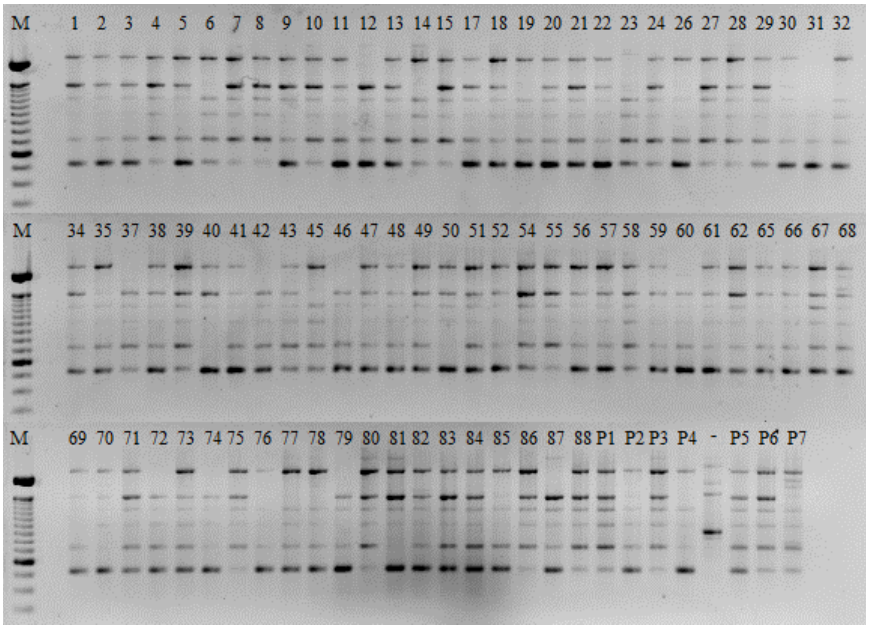

Figure 1. Random amplified polymorphic DNA (RAPD) profile of 88 *Apuleia leiocarpa* individuals amplified using OPAS-02. M: 100-bp ladder (Invitrogen™). P: Adult trees. Numbers represent all young individuals grown from seeds and sampled in this study. The lanes marked with a dash represent samples that were not included to the analysis of this marker.

Levels of polymorphism

The 18 RAPD primers analyzed provided 92 bands, from which 75 were polymorphic. An average of four bands per primer was detected (Table 1). The high number of polymorphic loci (P = 81.7%) encountered in this study indicates that this method has potential for use in the genetic characterization of *A. leiocarpa*.

The highest number of polymorphic bands was detected with primers OSAW-08 and OPJ-01, with eight and nine bands, respectively (Table 1). Furthermore, all bands from these primers were polymorphic. These two primers, along with OPL4-525, OPAN-01, OPAW-14, and OPAS-02, explained almost half (45.6%) of the total polymorphism among the individuals. Conversely, OPV-17 and OPJ-05 revealed the lowest number of bands (2), with only one polymorphism per primer. OPA-13 revealed eight bands, with only three polymorphic, presenting the lowest percentage of polymorphic bands.

PIC values varied from 0.09 to 0.67 for the primers. OSAW-08 and OPJ-01 were the most informative markers, as the highest number of polymorphic bands was found using these markers (Table 2 and Figure 2). The PIC estimates the discriminatory power of a particular primer. In the case of RAPD, the PIC is calculated according to the number of bands revealed for each primer, which is associated with the genetic variability and the number of individuals being analyzed (Malone et al., 2007). Therefore, primer OPJ-01 exhibited both high polymorphism and a high number of amplified fragments (Tables 1 and 2). Nevertheless, higher numbers of bands per primer do not have direct implications for the information content. The OPA-13 primer, for example, was able to amplify eight fragments. However, lower levels of polymorphism were detected, implying an overall lower PIC value. From the eight bands of OPA-13, five were monomorphic (Table 1), representing the lowest percentage (0.38) of polymorphic bands in this study.

Table 2. Frequency of RAPD bands and polymorphism information content (PIC) for 18 RAPD primers used for the genetic analysis of *Apuleia leiocarpa*.

Primer		Number of bands and their frequency									PIC*
		1	2	3	4	5	6	7	8	9	
1	OPC-20	0.80	0.32	0.85	0.48						0.62
2	OPV-17	0.91	1.00								0.09
3	OPG-17	0.26	1.00	0.90	0.22	0.53	0.99				0.58
4	OPL4-525	0.63	0.54	0.98	0.75	0.79	0.62	0.86			0.45
5	OPAN-03	0.77	0.84	1.00							0.24
6	OPB-10	1.00	0.54	0.72							0.43
7	OSAW-08	0.91	0.69	0.08	0.45	0.94	0.23	0.90	0.40		0.67
8	OPJ-01	0.70	0.49	0.78	0.60	0.68	0.34	0.95	0.36	0.51	0.64
9	OPJ-04	0.54	0.60	0.90							0.54
10	OPI-15	0.83	0.75	0.95							0.29
11	OPAN-01	0.74	0.05	0.99	0.98	0.38	0.37	0.98	0.98		0.54
12	OPAW-14	0.34	0.23	0.46	0.75	1.00	1.00	0.43	0.97		0.58
13	OPAS-02	0.86	0.79	0.79	0.61	0.92	0.89				0.34
14	OPA-13	1.00	0.22	0.75	1.00	1.00	0.37	1.00	1.00		0.37
15	OPAS-08	0.99	0.99	0.44	0.55						0.45
16	OPAS-14	1.00	0.71	0.17							0.61
17	OPJ-05	0.34	1.00								0.55
18	OPAN-19	0.85	0.79	1.00	1.00	0.70					0.24

*PIC = 1 - Σpi^2, where pi is the frequency of the fragment i from a total of k fragments considered for each primer (Weiler et al., 2010).

Primers with PIC values higher than 0.5 have high information content. Values ranging from 0.25 to 0.49 are intermediate and those less than 0.25 have weak discriminatory power

among individuals (Botstein et al., 1980). By those standards, OSAW-08, OPJ-01, OPC-20, OPAS-14, OPG-17, OPAW-14, OPJ-05, OPAN-01, and OPJ-04 were highly informative, with PIC values higher than 0.5.

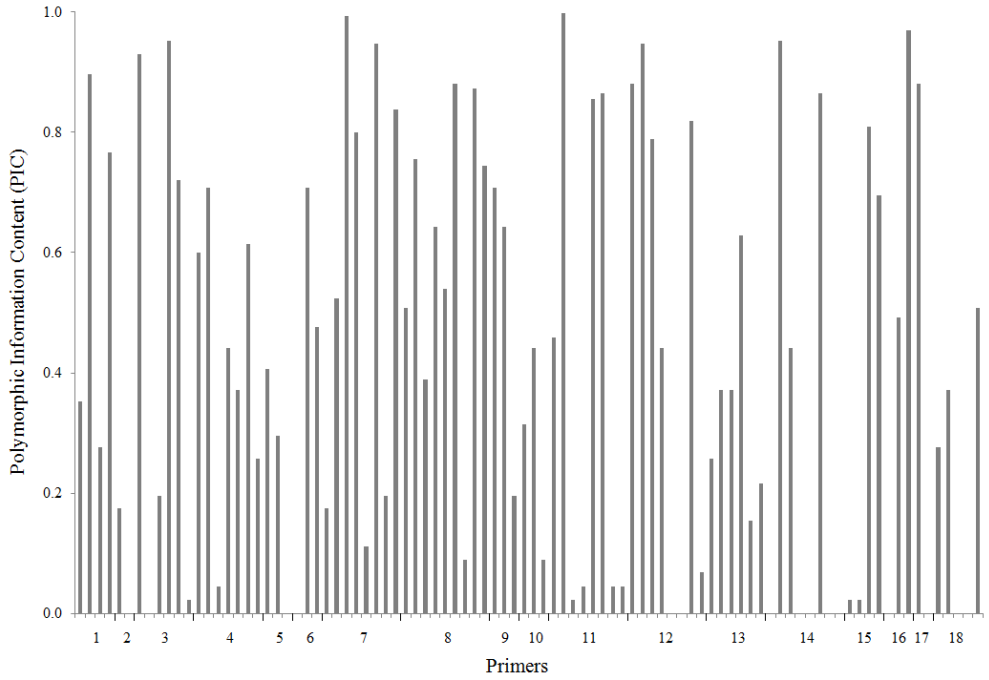

Figure 2. Average polymorphic information content (PIC) of 18 primers used for the genetic analysis of *Apuleia leiocarpa*.

Determination of an adequate number of markers for diversity analyses

Considering the high number of polymorphic bands in this study, we further investigated the number of markers that would be sufficient for reliably estimating the genetic variability of *A. leiocarpa*. Analyses on the association between polymorphic bands and the magnitude of the correlation of similarity values with different numbers of bands indicating the precision of the analysis (Figure 3). We found a high correlation estimate (r = 0.942), with low values of the square sum of deviations (SQ_D = 2.83) and stress value (E = 0.048) with 61 bands, indicating a consistent adjustment between the original similarity matrix and the new matrix with the 61 markers. Stress values below 0.05 indicate there is enough precision, according to the model of this test (Kruskal, 1964). Therefore, the 75 polymorphic bands used in this study were sufficient to reliably characterize the genetic variability of the species.

The 61 markers detected by the analysis were defined by 11 RAPD primers: OPC-20, OPG-17, OPL4-525, OSAW-08, OPJ-01, OPJ-04, OPAN-01, OPAW-14, OPAS-02, OPA-13, and OPAS-08. OPJ-01 revealed fragments with a frequency below 0.05, which could represent rare polymorphisms within the species.

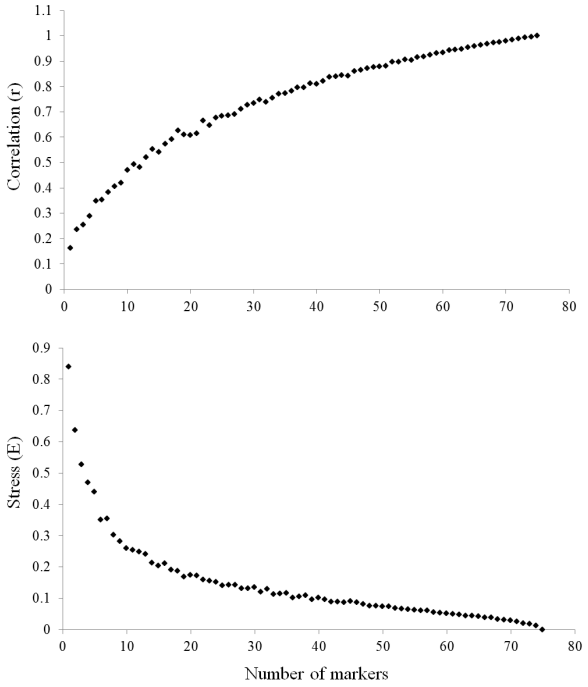

Figure 3. Correlation (*r*) and stress (*E*) values among similarity matrices obtained for different numbers of polymorphic fragments used for the genetic analysis of *Apuleia leiocarpa.*

Genetic distances among the individuals

The genetic distances among *A. leiocarpa* individuals was analyzed considering two sets of markers: all the markers detected (92 bands), and only the markers of the 11 primers selected when the adequate number of markers (61 bands) was determined. Considering all the markers used in this analysis, the maximum distance was observed between individuals 1 and 32 (D = 0.435), while the lowest distance was detected between individuals 54 and 62 (D = 0.116). Similar results were obtained when only the 11 primers recommended in this study were considered. In this case, the genetic distances were slightly increased between individuals 1 and 32 (D = 0.456) and 54 and 62 (D = 0.118). Mantel's correlation, considering the distance matrices with all bands and with the bands revealed by the selected primers, was high (0.92) and highly significant ($P < 0.01$), indicating that the selected primers were able to represent the dissimilarity coefficients among the individuals. Thereafter, we performed a clustering analysis based on the genetic distances with only the 61 polymorphic bands identified by the 11 primers selected in this study.

The individuals were grouped according to the hierarchical method UPGMA, based on Jaccard's genetic distances (Figure 4). The analysis allowed 11 groups to be distinguished, based on a cut-off limit of 0.3401, as determined by the method described by Mojena (1977). These results revealed variable levels of genetic diversity among groups. This clustering was considered reliable, as the cophenetic value was significant at 0.1% ($r = 0.676$ and $P = 0.001$). Given that r was slightly lower than 0.7, some distortions might have occurred during the conversion of the matrix data to the dendrogram.

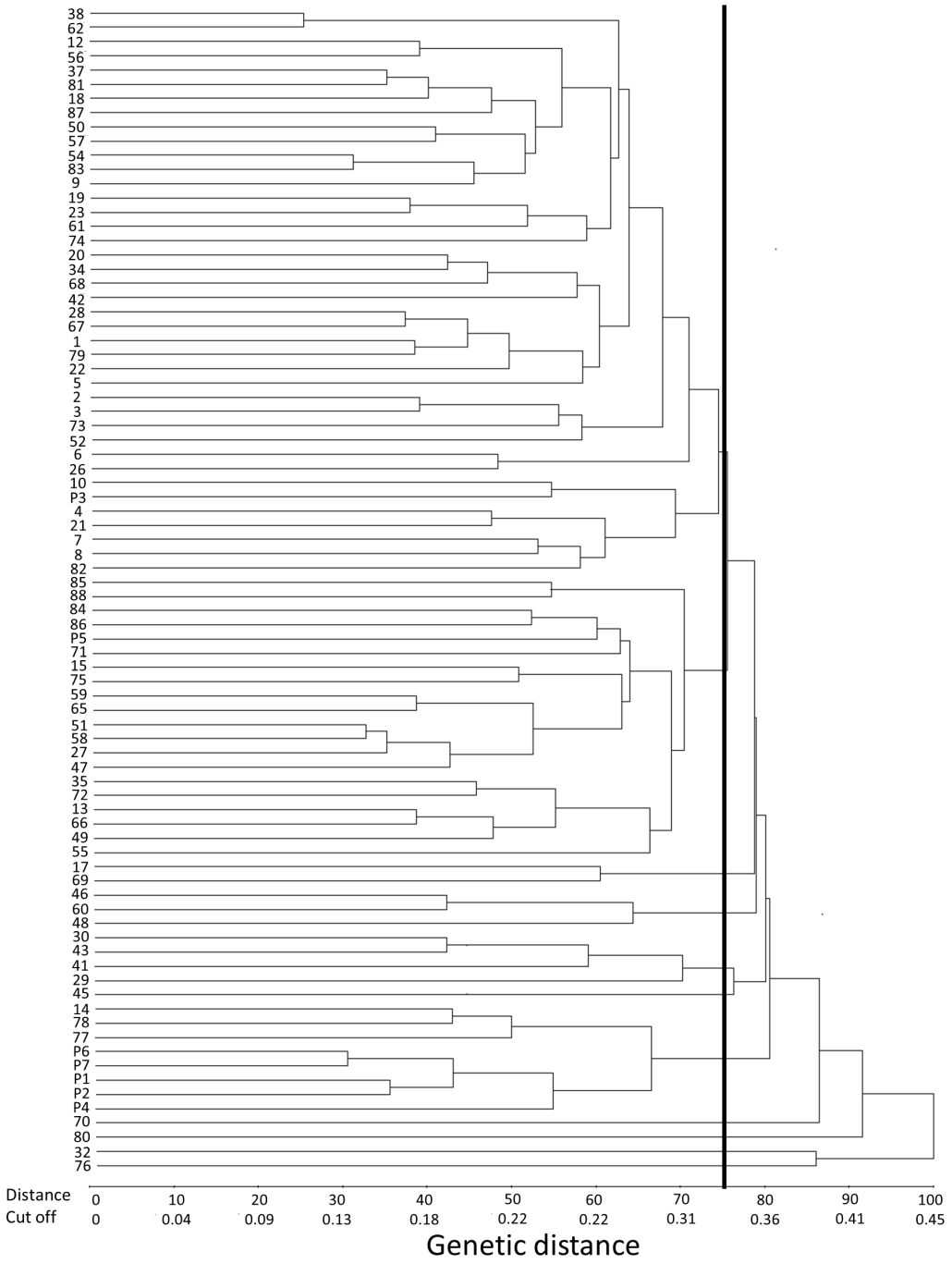

Figure 4. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Jaccard's index, representing the genetic similarity among *Apuleia leiocarpa* genotypes. P: Adult trees. Numbers represent all young individuals obtained from seeds of *A. leiocarpa*.

DISCUSSION

Adaptation of the DNA extraction protocol

This is the first report of a DNA-based analysis of genetic variation in *A. leiocarpa*. In general, the DNA extraction protocol adapted for *A. leiocarpa* provided high-quality samples, as confirmed by spectrophotometric measurements (A_{260}/A_{280} = 1.92), and by the high-quality electrophoretic profiles observed following amplifications (Figure 1). The PCR results obtained confirmed the quality of the DNA used as a template in the subsequent analysis (Kidwell and Osborn, 1992). Furthermore, we detected no apparent differences between the DNA obtained from adult leaf samples and that obtained from leaves of 3-year-old leaf samples, which might contain different amounts of phenolic compounds and polysaccharides, which could negatively affect DNA extraction. The irreversible interaction among these compounds could lead to the inability of different enzymes to manipulate DNA (Manoj et al., 2007).

Levels of polymorphism

The level of polymorphism detected in this study (81.45%) was higher than that reported previously with forest species. *D. nigra*, a species with a similar level of vulnerability to *A. leiocarpa*, presented only 39% polymorphism (Juchum et al., 2007). Similarly, only 38% of bands observed for *Dalbergia oliveri* were polymorphic (Phong et al., 2011). However, a study utilizing a *N. megapotamica* population revealed polymorphism levels of 99% (Costa et al., 2015).

The PIC values obtained for *A. leiocarpa* were also higher than those reported in previous studies (0.09 to 0.67, based on 18 primers), which further validates the usefulness of RAPD markers for this species. In *D. oliveri*, PIC values varied from 0 to 0.544 among 29 tested primers (Phong et al., 2011).

Polymorphism levels may be interpreted in different ways. First, the primers used in the studies of Juchum et al. (2007) and Phong et al. (2011) might have flanked regions with less polymorphism, even though statistical analyses revealed such markers would be sufficient to reliably estimate diversity. Furthermore, the present study was performed with the progeny of three individuals and six other adult individuals from a natural population. The studies of Juchum et al. (2007) and Phong et al. (2011) were conducted with only adult individuals, which might be related to different levels of diversity. Ultimately, from an ecological perspective, the levels of perturbation of highly endangered species such as *D. nigra* (Juchum et al., 2007) and *D. oliveri* (Phong et al., 2011) could have already reduced their genetic diversity. The intensive exploitation and fragmentation of *A. leiocarpa*, however, might not yet have had a significant impact on the species. Such effects might manifest in future generations, as fewer individuals are available for outcrossing, which may raise inbreeding levels and ultimately reduce the diversity and fitness of *A. leiocarpa* populations (Konzen, 2014). Conservation of the remnant populations and the continuous evaluation of the genetic diversity of the populations over time are necessary to prevent such events. Moreover, screening populations with higher resolution molecular markers is necessary, such as microsatellites.

Presence of monomorphic fragments

Of 92 bands obtained, 17 were monomorphic (present) among all individuals. Although

monomorphic bands contribute significantly to lowering values of genetic diversity, these could be useful for another kind of analysis. As the availability of sequences for the species is scarce in GenBank, we could isolate these monomorphic fragments in order to delineate nucleotide polymorphisms among *A. leiocarpa* individuals following sequencing. Monomorphic bands detected on agarose gel revealed nucleotide polymorphisms, thus showing their usefulness as informative markers (Holla et al., 2014). Nucleotide diversity analyses also provide important information on how polymorphisms are shaped in populations based on a few nucleotide variations from a sequence, those referred to as single nucleotide polymorphisms (Konzen, 2014). As nucleotide polymorphisms exhibit inherently low mutation rates (Nielsen, 2000), monomorphic fragments from *A. leiocarpa* could be sequenced and analyzed for use in phylogeographic studies in order to identify the spatial origin of the species. Furthermore, it is possible to obtain possible markers associated with coding sequences, which could be identified as specific genes from *A. leiocarpa*. In our study, the primers OPB-10, OPA-13, OPAS-14, and OPJ-15 exhibited monomorphic fragments, which have potential use for sequencing purposes and nucleotide diversity analyses across populations with the presence of those fragments (Figure 3). Polymorphic fragments could also be sequenced, with the aim of identifying potential genes that are variable throughout populations of the species. Sequenced fragments could be converted to co-dominant markers, such as sequenced characterized amplified regions (SCAR), with high specificity (Nietsche et al., 2000).

Determining an adequate number of RAPD markers for genetic analysis and genetic clustering

In this study, we detected a high number of polymorphic bands. Therefore, we further investigated the number of bands that would be sufficient to characterize the genetic variability of *A. leiocarpa*, resulting in 61 bands (represented by the markers OPC-20, OPG-17, OPL4-525, OSAW-08, OPJ-01, OPJ-04, OPAN-01, OPAW-14, OPAS-02, OPA-13, and OPAS-08). Increasing the number of markers beyond that would not significantly change the levels of variability or the precision of the similarity matrices among individuals. This number of bands varies according to the species (Souza et al., 2014), and might represent a limiting factor for RAPD markers. In fact, analysis of only 61 bands was sufficient to sustain the precision of the analysis. Analysis of 61 bands would reduce the number of reactions necessary to characterize the genetic diversity and structure of *A. leiocarpa*.

Studies on other tree species identified different numbers of bands for use in their genetic characterization using RAPD markers. In *Psidium guajava*, 117 bands were used to analyze the genetic diversity (Gomes-Filho et al., 2010). In *Erythrina velutina*, 106 bands were detected (Souza et al., 2014). Analysis of *Pothomorphe umbellata* was based on only 25 bands (Valle et al., 2013). Although no data from sufficiency analyses were available, those studies revealed high rates if polymorphism, at 87.9, 85.7, and 96.1%, respectively.

The 61 markers defined by the method of Kruskal (1964) accurately represented the genetic distances among individuals, and therefore the clustering analysis. From the UPGMA analyses and the cophenetic coefficient, 11 groups were formed by the defined cut off on the dendrogram. This indicates the usefulness of the identified RAPD markers for representing the differences among individuals, which might be employed in further studies aimed at characterizing more populations and defining the genetic structure of the species across its natural distribution.

Significance and implications of the results

Our study makes an important contribution to an initial understanding of the genetic diversity of *A. leiocarpa*. The existence of large genetic distances among individuals implies a source of high genetic diversity, which can be used to select superior genotypes. This also has implications for programs aimed at the conservation and sustainable management of the species, since it is highly vulnerable to extinction.

RAPD markers are able to provide a precise estimate of the genetic structure of the species; however, such markers lack precision for other estimates. Furthermore, such markers have been largely replaced by others with higher genomic coverage and precision, such as inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers (Konzen, 2014). For more in-depth analyses, such as heterozygosity and inbreeding levels, and outcrossing rates, microsatellite markers are more precise. However, at present, no microsatellite library is available for *A. leiocarpa*.

We recommend performing further investigations on the genetic diversity status of *A. leiocarpa* for a better assessment of diversity patterns across a wider range of natural populations. Continuous monitoring of species diversity must also be encouraged, as proposed elsewhere (Martins-Corder et al., 2009). The use of the RAPD markers provided in this study, as well as the development of microsatellite markers and the analysis of nucleotide polymorphisms, is highly recommended for exploring the genetic diversity and for engaging on conservation and breeding strategies for *A. leiocarpa*.

In conclusion, this study provides the first evidence of DNA-level variation for *A. leiocarpa*. We provide a simple and effective adaptation of a previously described method for extracting high-quality DNA from leaf tissues. Importantly, we show the adequacy and potential of using RAPD markers for the analysis of genetic variability and structure of populations of this species. A high number of polymorphic bands were detected, along with consistently high genetic distances among individuals. The individuals were separated in 11 distinct groups with variable levels of genetic diversity, which is important for selecting desirable genotypes and developing conservation and sustainable management programs. We also found conserved fragments among the individuals, which can be sequenced and used to analyze nucleotide diversity parameters through a wider set of individuals and populations of *A. leiocarpa*. Our results are of prime importance for further investigations concerning the genetic characterization of such an important, but vulnerable species.

Conflicts of interest

The authors declare no conflict of interest.

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