Crop Breeding and Applied Biotechnology 10: 266-270, 2010
Brazilian Society of Plant Breeding. Printed in Brazil

Variability in *Macadamia integrifolia* by RAPD markers

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Received 27 October 2009

Accepted 22 March 2010

ABSTRACT - *Macadamia is one of the most valuable commercial nuts and its culture is a good alternative for small farmers or for large properties with a low capacity for the mechanization of its areas. Macadamia clones, due to their morphological proximity exhibited some difficulties in field identification that could difficult the right plant distribution. The objective of this work was to genetically characterize, by RAPD markers, different macadamia clones under cultivation. Leaves from 'HAES-741', 'HAES-660', 'IAC-920BX', 'IAC-920', 'HAES-344', 'IAC-420', 'HAES-816', 'IAC-1.21', 'Shimada', 'IAC - Campinas B', 'IAC - Campinas A' and 'HAES-Aloha' were donated by Queen Nut Macadâmia. DNA was extracted and amplified with 15 primers and the binary data (presence/absence of bands) collected was used and dendrograms constructed. Clone identification, via RAPD was possible using primers Op G18, C19 and C7. Most of the genetic variation is inside the Brazilian or Hawaiian groups rather than between the groups and could allow inbreeding or croxssing between distant clones.*

Key words: Nut, clone identification, DNA.

INTRODUCTION

The macadamia tree belongs to Proteaceae, the same botanical family as *Grevillea*. Originating in Australia, it was introduced to Hawaii in 1878 and to Brazil in 1931. Actually there were 10 known species, but just three were used as food (*Macadamia integrifolia*, *Macadamia tetraphyla* and *Macadamia ternifolia*). Culture expansion occurred from the 80's, precisely to the beginning of the 90's. It found good growing conditions in South America, from Bahia (NE, Brazil) to Uruguay. It is one of the most lucrative cultures in value per area. As a recent culture in Brazil, the information about the culture and the recommended technologies has initially been based on the research results of Australia and Hawaii (Sacramento 2003). Since 1940 IAC (Instituto Agronômico de Campinas) started a process of selection and culture conduction in the local conditions (Sobierjaski et al. 2006).

The need for genetic resources in agriculture implies that there is an increase of modern methods and bio techniques to reach success in sustainable agriculture, and from these processes molecular markers have been used (Vilela-Morales and Valois 2000). Morphological markers have a narrow availability for some cultures for which the number of molecular markers is higher as for example in corn, peas and tomato (Ferreira and Grattapaglia 1998).

Many molecular biology techniques are available today for variability detection at DNA level, e.g., for genetic polymorphism evaluation (Lima 2003). These techniques allow, virtually, an unlimited number of molecular markers that can search the whole genome of the organism in study (Borba 2007). PCR technology was developed in the 80's

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causing a revolution in the basic biological research that allowed the understanding of areas of breeding, as well as very high number of specific DNA fragments which are visible in a electrophoresis gel with specific dyes (Ferreira and Grattapaglia 1998).

Molecular markers could define precisely the fingerprint of any organism, and in the case of plants it is important to plant protection and to follow the offspring, which allows acceleration and facilitates the processes of selection and the recombination of superior individuals (Salla 2002).

Among the markers available today, the detection of RAPD (Random Amplified Polymorphic DNA) markers is distinct for its simplicity, fast execution, low cost, the small quantity of DNA necessary, and the possibility of the study of species for which molecular information is scarce (Williams et al. 1990). RAPD has been used for different numbers of species, independent of the strategy of reproduction with success, like in *Citrus* (allogamous, Oliveira et al. 2000, Oliveira et al. 2001), *Passiflora* (allogamous, Crochemore et al. 2003), *Coffea* (autogamous, Diniz et al. 2005; allogamous, Ferrão et al. 2008), *Aspidosperma* (allogamous, Torezan et al. 2005) and *Zea mays* (self-fertilized, Souza et al. 2008). The aim of this work was to study the genetic similarity among germplasm accessions of twelve clones of *Macadamia* from two selection groups, Hawaiian and Brazilian, to check the proximity of them with the germplasm analysed, through the use of RAPD markers.

MATERIAL AND METHODS

The experiment was conducted at UNOESTE's laboratory of Molecular Genetics and Cytogenetics in Presidente Prudente, SP. For DNA extraction, the method described by Doyle and Doyle (1987) was used, with modifications. The leaf tissue of twelve clones of *Macadamia integrifolia* 'HAES-741', 'HAES-660', 'HAES-344', 'HAES-816' and '*HAES-Aloha*' constituting the Hawaiian group and 'IAC-420', 'IAC-920 BX', 'IAC-920', 'IAC-1.21', 'Shimada', 'IAC-Campinas B', 'IAC-Campinas A' as the Brazilian group were used.

Tissue was ground in a mortar with a pestle under liquid nitrogen. Buffer composed of Tris-EDTA pH 8.0, 500 mM CTAB and 2% ß-mercaptoethanol at 65 ºC at a 1:10 ratio (w:v) was added to the powder, and the mix was incubated at that temperature for 40 min. After that, chloroform:isoamyl alcohol (24:1) was added and mixed carefully for 10 min. The mixture was centrifuged at 12,000 Xg for 15 min. The aqueous phase was collected and cooled in an ice bath. 7.5M ammonium acetate and isopropyl alcohol were added to a final concentration of 6% and 54%, respectively. The mixture was placed at -20 ºC, 24 h. A new cycle of centrifugation was done and the liquid phase discarded. Pellets were washed twice with 70% ethanol at 65 ºC, one minute each and air-dried in an air flow cabinet. DNA was dissolved in a TE buffer, pH 8.0, and quantified by a spectrophotometer at 260/280 nm and adjusted to 10 $\mu g.\mu L^{-1}$.

Amplifications were done as in Williams et al. (1990), with modifications. Genomic DNA was amplified in a reaction volume of 25 µL containing 10% of Tris KCl buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 0.4 µM of primer, 0.2 µM of each dNTP, one Taq unit and template DNA (25 and 50 ng). RAPD amplifications were performed in a thermocycler with the following steps: 94 ºC, three minutes for initial denaturation, followed by 43 cycles of one min at 94 ºC, one min at 37 °C for primer annealing and 30 s at 72 ºC for chain elongation. One extra step of five minutes at 72 ºC was done at the end for a final elongation. Only bands present in amplifications of both 25 and 50 ng of template DNA were considered. One hundred twenty Operon primers, series A, B, D, G, X and Y, were evaluated. Amplification products were separated by gel electrophoresis in a 1.5% agarose gel with ½X TBE buffer. The gel was stained with ethidium bromide and pictures were captured by a CCD machine (Alpha-Inmotech) and by ChemiImager software.[2]

Bands were used to construct a similarity matrix based on the Jaccard coefficient, coding 1 as present and 0 as absent. The grouping analysis was done using the UPGMA algorithm (Unweighted Pair-Group Method Using an Arithmetic Average). This analysis was performed with the software Gene (Cruz 2006). Molecular variance analysis (AMOVA) was calculated by total decomposition of its components among and between accessions using the square distances (Excoffier et al. 1992) with the software Arlequim (Excoffier et al. 2006).

RESULTS AND DISCUSSION

The 12 samples of the *Macadamia integrifolia* were amplified with the 15 selected primers, generating 130 bands with an average of 8.7 bands per primer, all of them polymorphic. The fragment size ranged from 300 to 1550 bp (Table 1). The number of bands according to Telles et al. (2001) is more important than the primer number, an idea

Table 1. Nucleotide sequences, number of bands and number of polymorphic bands of each primer used for RAPD-PCR analysis of *Macadamia integrifolia*

Primers	Nucleotide sequence (5'→3')	Number of bands	Polymorphic bands	Polymorphism (%)	Fragment size (pb)
C7	GTCCCGACGA	12.0	11.0	91.7	320-1520
C9	CTCACCGTCC	8.0	4.0	50.0	300-1260
C19	GTTGCCAGCC	11.0	10.0	90.9	350-1340
D8	GTGTGCCCCA	6.0	4.0	66.7	530-1420
G5	CTGAGACGGA	12.0	11.0	91.7	310-1460
G7	GAACCTGCGG	8.0	8.0	100.0	500-1350
G11	TGCCCGTCGT	7.0	6.0	85.7	340-1180
G17	ACGACCGACA	7.0	7.0	100.0	360-1110
G18	GGCTCATGTG	13.0	13.0	100.0	310-1480
X8	CAGGGGTGGA	7.0	5.0	71.4	350-1550
X11	GGAGCCTCAG	6.0	5.0	83.3	540-1190
X20	CCCAGCTAGA	7.0	7.0	100.0	340-1120
Y6	AAGGCTCACC	6.0	5.0	83.3	310-1210
Y9	AGCAGCGCAC	8.0	8.0	100.0	360-1530
Y14	AGCCGTGGAA	12.0	12.0	100.0	300-1370
Average		8.7	7.7	87.6	
Total		130.0	116.0		

that is in accordance with several authors regarding genetic estimates by the RAPD technique. Dias et al. (2004) argued that the better results for RAPD use may be done, according the literature, with at least 160 bands. In this case, 130 bands were found (Table 1) with good polymorphism (89%) and with bands clearly visible and with high reproducibility, because they were amplified by two different DNA concentrations (25 and 50 ng).

Allogamous species, by definition, are much more variable than autogamous (Yanaka et al. 2005). *Macadamia*, as an allogamous, in the majority of the species, is cross pollinated, in this case by animals (hummingbirds, bees and butterflies). In some allogamous species such as *Aspidosperma* (Torezan et al. 2005), *Medicago sativa* (Crochemore et al. 1996), *Bromus inermis* and *B. riparius* (Ferdinandez and Coulman 2002) variability among populations is higher than within population. In *Lolium multiflorum*, these values can reach 98% within populations (Vieira et al. 2004), which consequently reduce the possibilities of genetic drift and endogamy contributing to the maintenance of high diversity inside the species.

In this study, *Macadamia* clones from different plant breeding programs were grouped together in the branches of the tree (Figure 1). Since this species is distributed in a large area with complex environments, its gene-flow is rich within the species. Even in the case of high genetic similarity between two clones ('HAES-344' and 'IAC-420').

F_{ST} estimates the gene flow between two populations or species, where low values indicate introgression between populations and consequently high genetic variability. On the other hand, a low gene flow leads to high F_{ST} values

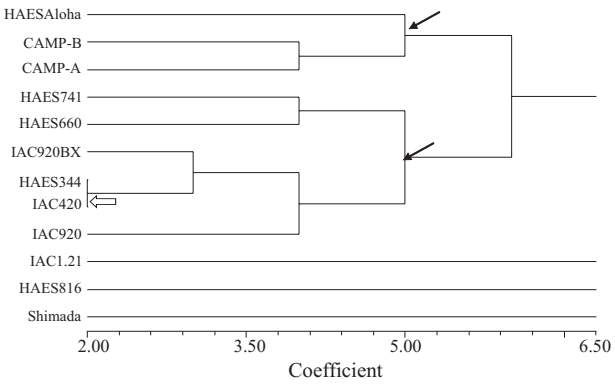

Figure 1. Similarity among accesses of macadamia estimated by 15 RAPD primers.

indicating that populations became endogamic and consequently, for alogamous species, more vulnerable to gene erosion. In this case, the F_{ST} values are low indicating a very high variability in the suited clones (Table 2). For plants grouped as Brazilian, this value is lower than the average or than the Hawaiian group. It means that there were variabilities to be exploited especially if clones as 'IAC-Campinas A or B' would be crossed with 'Shimada' or 'IAC-1.21', which could maintain a high variability and improve some qualities in the offspring. The crosses between 'IAC-Campinas A' x 'IAC-Campinas B', 'IAC-920' x 'IAC-420', 'IAC-420' x 'IAC-920BX'or 'IAC-920' x 'IAC-920BX' could also be done with the expectation of good genetic gain, by fixing some characters. In our case, F_{ST} for all samples (0.0328) and its sub samples (Hawaiian 0.0439 and Brazillian 0.0249) shows that there was a high gene flow among all samples, which could be used in favor of plant breeding programs (Table 2).

Table 2. Wright's measurement of population differentiation (F_{ST}) using RAPD markers between groups in *Macadamia integrifolia*

Group	F_{ST}	Numbers of samples	Polymorphic band in the group
Hawaiian	0.0439	5	75
Brazilian	0.0249	7	94
Average	0.0328	12	

It is well known that domestication strongly reduces sequence diversity in genes controlling traits of interest (Allard 1988, Wang et al. 1999, Salamini et al. 2002). In populations submitted to constant selection, where just the superior individuals were promoted for reproduction, the alleles controlling characters of interest had their frequency increased; leading to diversity loss in crop plants (Salamini et al. 2002). It is also well documented that although

most domestication traits are quantitatively controlled, the dramatic morphological changes that accompanied domestication may be due to relatively few genes (Koinange et al. 1996). The decrease in genetic variability that is detected in populations due to driven selection (Wang et al. 1999) was not found in this work.

The OP G18 primer (Figure 2) isolated the clones 'HAES-741', 'IAC-920BX', 'IAC-920', 'HAES-816', 'HAES-660' and 'IAC-420'. The primer Op C19 (Figure 2) isolated the clone 'HAES-344' and put it into four pairs and the others into one trio, and the primer Op C7 (Figure 2) did not isolate any of the 12 clones. However, using the combination of two primers together the clones 'HAES-Aloha', 'Shimada', 'IAC-1.21', 'Campinas B' and 'Campinas A' were isolated. The clone 'Shimada' could be identified using the primer G18 and C7. In the tree generated by the C7, this clone is combined with 'HAES-660', which, by its turn, is isolated in the G18. For 'Campinas B' and 'IAC-1.21' the primer combination is G18 and C19. In the G18 'Campinas B' is combined with 'IAC-1.21' and in C19 with the clone 'HAES-816'. However, 'HAES-816' is alone in the tree of the G18 primer. 'IAC-1.21' is combined with 'HAES-741' in the primer C19, but this one is alone in the G18. The identification of 'Campinas A' could be done by combining C19 and C7 primers. In the C7 this clone is grouped with 'HAES-344', however in C19 this one is isolated. 'HAES-Aloha' could be identified by two combinations either with C7 and C19 or with C7 and G18, but the first is the easiest. In primer C19 this clone is combined with 'Campinas A', but in the C7 primer this clone comes with 'IAC-1.21'. These three primers allowed the distinction of the twelve clones of *Macadamia integrifolia*, easily, by the isolation of seven cultivars and by combination of two primers that exhibited the difference between the last five (Figure 2). Variability in *Macadamia integrifolia* is high enough to permit inbreeding and selection among clones. Three of the fifteen primers used allowed the identification of the twelve *Macadamia* clones.

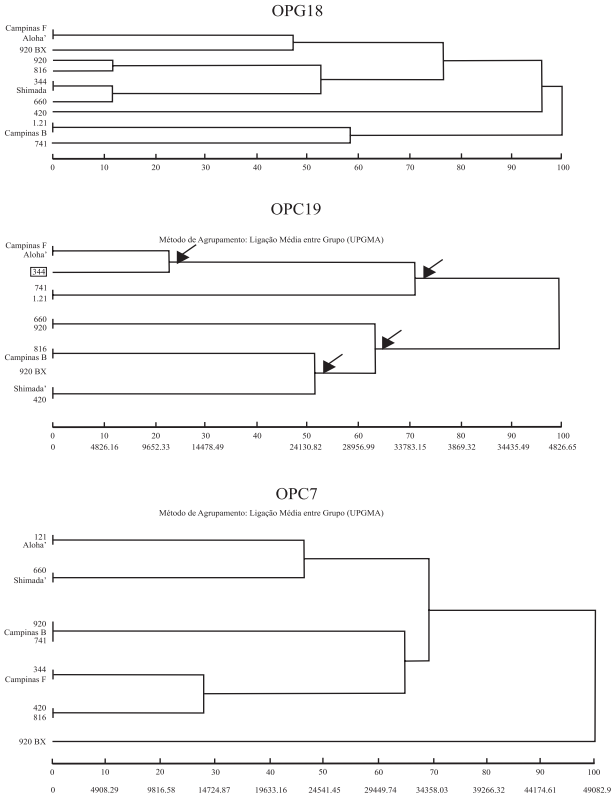

Figure 2. UPGMA tree based on primer of *Macadamia integrifolia* accessions.

Variabilidade em *Macadamia integrifolia* por marcadores RAPD

RESUMO - *A Macadamia é uma das mais valiosas nozes e sua cultura é uma boa alternativa para pequenos agricultores ou grandes propriedades com pouca capacidade de mecanização. Devido à semelhança morfológica, existem algumas dificuldades de identificação, o que poderia atrapalhar a correta distribuição destes. O objetivo deste trabalho foi caracterizar geneticamente, por meio de marcadores RAPD, diferentes clones. Folhas dos clones 'HAES-741', 'HAES-660', 'IAC-920BX', 'IAC-920', 'HAES-344', 'IAC-420', 'HAES-816', 'IAC-1.21', 'Shimada', 'IAC - Campinas B', 'IAC - Campinas A' e 'HAES-Aloha' foram cedidas pela Queen Nut Macadâmia. O DNA foi extraído e amplificado com 15 primers e os dados binários (presença/ausência de bandas) obtidos foram analisados e os dendrogramas construídos. Identificação Clonal, por meio de RAPD, foi possível utilizando-se os primers Op G18, C19 e C7. A maior parte da variação genética está dentro dos grupos Brasileiro ou Havaiano do que entre grupos, permitindo tanto endogamia como cruzamento entre clones distintos.*

Palavras-chave: Nozes, identificação clonal, DNA.

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