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ORIGINAL ARTICLE

Genetic diversity of sacha inchi accessions detected by AFLP molecular markers

Diversidade genética em acessos de sacha inchi utilizando marcadores moleculares AFLP

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ABSTRACT: Sacha inchi (*Plukenetia volubilis* L.) is a species native to the Amazon region for which studies of genetic diversity are required to ensure the success in programs to breed cultivars for agriculture. The purpose of this study was to assess the genetic diversity among sacha inchi accessions using amplified fragment length polymorphism (AFLP) markers. Thirty-seven accessions of the sacha inchi germplasm bank of Embrapa Amazônia Ocidental were analyzed. The markers were identified with four primer combinations. The percentage of polymorphic loci was estimated and the similarity of accessions analyzed by calculating the arithmetic complement of the Jaccard coefficient and cluster analysis. The primers revealed 191 polymorphic loci. The parameters of the similarity values in class intervals were on average 0.739 and variance 0.01. The estimates of the number and percentage of the genetic distance values per class interval were highest in the range of 0.7 to 0.79, (248 and 37.24%, respectively); the highest percentage, 69.22%, corresponded to values of genetic distance above 0.7, showing mostly high genetic diversity among the accessions. The results indicated a geographic structure among accessions in relation to their origin, generating consistent and valuable data for breeding programs and species conservation.

RESUMO: *Sacha inchi (Plukenetia volubilis L.) é uma espécie nativa da Amazônia. Mostram-se necessários estudos de sua diversidade genética para possibilitar avanços em programas de melhoramento, visando a estabelecer cultivares para a agricultura. O objetivo deste trabalho foi estudar a diversidade genética entre acessos de sacha inchi com o uso de marcadores moleculares AFLP. Foram analisados 37 acessos de sacha inchi, que pertencem ao Banco de Germoplasma da Embrapa Amazônia Ocidental. Para obtenção dos marcadores, foram utilizadas quatro combinações de primers. Foi estimada a porcentagem de locos polimórficos e realizada a análise de dissimilaridade entre os acessos, por meio do cálculo do complemento aritmético do coeficiente de Jaccard, além de construção de dendograma. Os primers revelaram 191 locos polimórficos. Os parâmetros dos valores de dissimilaridade em intervalos de classe foram uma média igual 0,739 e variância 0,01. As estimativas do número e da porcentagem dos valores de distância genética por intervalos de classe foram maiores no intervalo de 0,7 a 0,79, com 248 e 37,24%, respectivamente; note-se que a maior porcentagem, 69,22%, corresponde a valores de distância genética acima de 0,7, mostrando predominância de alta diversidade genética entre os acessos. Os resultados obtidos mostraram a existência de estruturação geográfica entre os acessos em função de sua origem, gerando informações consistentes e de alto valor para programas de melhoramento genético e de conservação da espécie.*

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1 Introduction

The genetic variability is high in the Amazon region and has been exploited by man long before the time of the discovery of the Americas. However, a large portion of the species found in this biome is to date partially or totally unknown to the scientific society, requiring studies to better understand their populations and possible uses. Sacha inchi (*Plukenetia volubilis* L.), of the Euphorbiaceae family, is a woody vine native to the Amazon region, whose oil extracted from the seeds has very interesting qualities, with great potential for pharmaceutical, nutritional and aesthetic uses.

Sacha inchi nut oil has a high unsaturated fatty acid content (approximately 93% of the total) (HAMAKER et al., 1992) of which about 45.2% consists of α-linolenic acid (omega 3) and 36.8% of linolenic acid (omega-6) (FOLLEGATTI-ROMERO et al., 2009), which are essential fatty acids that are not synthesized by the human body, making their intake necessary. These fatty acids are precursors of prostaglandins, thromboxanes and prostacyclins, which is a group of substances involved in regulating the blood pressure, heart rate, vascular dilation, blood clotting, lipolysis, integrity of cell membranes, immune response, central nervous system, and inhibition of platelet aggregation (NOVELLO; FRANCESCHINI; QUINTILIANO, 2010).

In Manaus, Amazonas, the Embrapa Amazônia Ocidental maintains a sacha inchi germplasm bank with 37 accessions collected in the Brazilian Amazon. Despite the known benefits of consumption of sacha inchi oil, so far no studies or descriptions have addressed the genetic diversity among the accessions of this collection, to deepen the knowledge and make information available for breeding programs.

The development of tools for genetic analysis at the molecular level has made a more detailed analysis of the evolutionary origin of plant genomes possible, as well as the evaluation of the degree of genetic variability in plant groups (GANGA et al., 2004).

Molecular markers that detect the polymorphism in DNA sequences have been widely used in studies of genetic diversity. Among these markers, the technique of amplified fragment-length polymorphism (AFLP) markers can detect a large number of polymorphic loci and is reproducible, fast and reliable (KARDOLUS; ECK; BERG, 1998). This technique can be used for any plant species and has been applied in studies, e.g., of yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) by Ganga et al. (2004), mango (*Mangifera indica* L.) by Santos (2008) and in buriti (*Mauritia flexuosa* L.), by Gomes et al. (2011).

Our objective was to estimate the genetic diversity among accessions of the sacha inchi germplasm bank of Embrapa Amazônia Ocidental-CPAA using AFLP markers.

2 Materials and Methods

The 37 studied accessions were taken from the sacha inchi germplasm bank of Embrapa Amazônia Ocidental, located in Manaus-AM (3° 8' S, 59° 52' W). Of the accessions, 25 were collected in the State of Amazonas in 1992 and 12 on the farm "Nova Jerusalém" in Careiro Castanho -AM in 2012, S 3° 31' 45.0"; W 59° 49' 07.9"). Young leaves of accessions were collected at Embrapa, placed in plastic bags with silica and stored at –20 °C in the Plant Breeding Laboratory of the Federal University of Amazonas (UFAM).

The DNA was extracted using the cationic detergent CTAB 2% (Cationic *Hexadecyltrimethyl Ammonium Bromide*) (DOYLE; DOYLE, 1987), with proteinase K, according to the protocol modified by Ferreira and Grattapaglia (1998). The DNA was quantified by the comparative method in 1% agarose gel using markers with known molecular weight (50-100 ng). The agarose gel was ethidium bromide-stained (FERREIRA; GRATTAPAGLIA, 1998) and visualized under UV transillumination.

For the DNA digestion reactions, the combination of restriction enzymes *Eco*RI/*Mse*I was used, with 200 ng genomic DNA; 5.0 µL One Phor All buffer; 10X (OPA; Amersham); 0.5 µL BSA solution (Bovine Serum Albumin) (10 µg μL^{-1}); 0.5 µL *Mse*I enzyme (10 units/µL, New England Biolabs), and 0.4 µL *Eco*RI enzyme (12 units/µL, Gibco), in a final volume of 50 µL. The reactions were performed for 3 h at 37 °C and then the enzymes were inactivated at 70 °C for 15 min.

In the pre-amplification reaction, primers complementary to sequences of restriction enzymes sites were used with a selective nucleotide, using the primer combination *EcoRI*-A/ *MseI*-C. The pre-amplification products were diluted by adding 40 µL of ultrapure water for amplification.

For selective amplification, 11 primer combinations were used in a random sample of 10 plants of the collection to select the best combinations: *EcoRI-AAC/ MseI-CAC*, *EcoRI-AAC/ MseI-CGC*, *EcoRI-AAC/ MseI-CTC, EcoRI-ACA/ MseI-CCA*, *EcoRI-ACA/ MseI-CGC*, *EcoRI-ACA/ MseI-CAT*, *EcoRI-AGC/ MseI-CAC*, *EcoRI-AGC/ MseI-CAT*, *EcoRI-ATC/ MseI-CCA, EcoRI-ATC/ MseI-CTC,* and *EcoRI-AGT/ MseI-CGC*. For the reactions of selective amplification via PCR (Polymerase Chain Reaction), 2.5 µL of the pre- amplification product was diluted in a thermocycler (Techine TC-512).

Prior to electrophoresis of the samples, a pre-run was conducted at constant 50 W for 1 h for gel heating and cleaning. After sample application, electrophoresis was performed at the same voltage in a ~4-h run.

The bands were developed by silver nitrate staining, according to a protocol proposed by Creste, Tulmann and Figueira (2001). The polymorphic loci were analyzed for presence/absence of the amplified fragment. In the description of AFLP markers, a code was adopted in which the first character corresponds to the digestion enzymes used (*Eco*RI = E and *Mse*I = M), and the second and third character to the numbers that identified the combinations according to the variation in the primer extensions.

After this identification the size of the amplified fragment of the locus was added to the number of bases. From the resulting fragments, a binary matrix was constructed, adopting the numbers one and zero to indicate the presence and absence of fragments, respectively.

The data of the binary matrix, obtained from molecular markers were used to construct a dissimilarity matrix by the arithmetic complement of the Jaccard coefficient (JACCARD, 1908), based on the expression :

$$D_j = 1 - \left(\frac{a}{a+b+c} \right)$$

where *a*, *b* and *c* stand for the presence of a band in both accessions, presence of a band in the first and absence in the second accession, and absence in the first and presence in the second, respectively.

To obtain the dendrogram, the similarity matrix was used in the unweighted pair group method with arithmetic average (UPGMA). The distance between the groups was determined by averaging the distances between pairs of individuals from different groups by the expression:

$$d_{(ij)k} = \frac{n_i}{n_i + n_j} d_{ik} + \frac{n_i}{n_i + n_j} d_{jk}$$

Where $d_{(ij)k}$ is the distance between group (*ij*), with inner size n_i and n_j, respectively, characterizing *i*, *j* and *k* as individuals or groups (CRUZ; FERREIRA; PESSONI, 2011).

Data were processed in the Bioinformatics Laboratory of the Federal University of Viçosa, using software GENES (CRUZ, 2013).

3 Results and Discussion

The four primer combinations *EcoRI-ACA/ MseI-CGC, EcoRI-AAC/ MseI-CAC, EcoRI-AAC/ MseI-CGC,* and *EcoRI-ATC/ MseI-CCA* showed a higher number of polymorphic loci, better quality of amplification and were selected for analysis in all plants of the collection. A total of 191 polymorphic markers were obtained for the four primers analyzed in the accessions. The genetic diversity detected between most accessions is high (Table 1). The dissimilarity of accession A2U20 to 11 accessions from the germplasm bank (A1M14, A2M01, A2M02, A2M03, A2M04, A2M05, A2M06, A2M09, A2M10, and A2U15) was approximately 0.9, and the lowest dissimilarity values were observed between the accessions A2M03 and A2M04 (0.338), A1M3 and A1M4 (0.368), and A1M12 and A1M15 (0.373).

The parameters of dissimilarity values in class intervals (mean 0.739, variance 0.01 and symmetry -0.649) (Table 2) showed that the distribution tail is tilted to the right, because the values were above average. The flatness (kurtosis) was estimated at 3.47, indicating that the distribution in question is higher (tapered) and more concentrated than normal distribution (Lilliefors $\alpha = 5\%$). It was found that 37.24% of the values are in the class interval ranging from 0.7 to 0.79 and 69.22% have distance values above 0.7, showing a mostly high genetic diversity among the accessions.

Cluster analysis based on genetic distances showed a relationship between the similarity degree and the origin of accessions, cutting at the fusion level of 0.75*, estimated by the Mojema index (Figure 1). It was observed that group A consisted of 22 accessions, of which only one had been collected from the farm "Nova Jerusalém", group B had three accessions collected from "Nova Jerusalém", and group C five accessions from "Nova Jerusalém" and one from the interior of the State, group D containing two accessions from Embrapa and group E one accession from "Nova Jerusalém" and one from Embrapa, while the accessions A1U28 and A2U20 formed the groups F and G, respectively.

The dendrogram showed geographic structuring within the germplasm bank, where accessions from the interior of the state were more similar to each other compared to the estimated similarity among accessions collected on the farm "Nova Jerusalém", since most of the accessions from the state interior were concentrated in group A and those from "Nova Jerusalém" formed separate groups. This shows that accessions from the interior have a narrower genetic base than the others (Figure 1). On the other hand, the presence of accession A2U19 within group A and the presence of accessions from the interior in the groups formed by accessions from "Nova Jerusalém" shows some degree of genetic similarity, regardless of their origin. When evaluating cassava accessions using RADP markers, Costa, Cardoso and Ohaze (2003) stated the efficiency of this marker in the study of genetic diversity, although they detected no geographic structure. Colombo, Second and Charrier (2000) investigated the polymorphism generated by RAPD markers in 126 cassava genotypes and concluded a weak genetic structure in cassava, which can cause overlapping of genotypes from different locations.

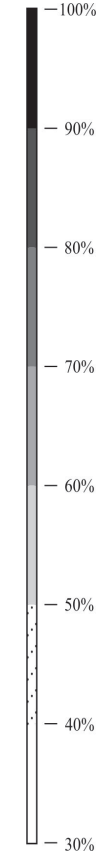
To generate a segregating population from divergent parents, crosses are recommended in the accession combinations: A2U20-A1M14, A2U20-A2M06, A2U20-A1M11, A2U20-A2U15, A2M08-A1U29, A2U20-A2M10, A2U01-A1U28, A2U20-A2M05, A2U20-A1U29, and A2U10-A1U28. These combinations showed the greatest distance between pairs estimated by the arithmetic complement of the Jaccard coefficient, respectively. Accession A2U20 was most dissimilar and was not clustered in the estimated dendrogram at any fusion level, making a potential parent for breeding programs using this germplasm bank accessions (Figure 1).

Many breeding studies have addressed the identification of parents with high genetic divergence (GANGA et al., 2004; COSTA et al., 2006; NICK et al., 2010), with a view to hybridizations that would produce segregation in the progenies, to increase the probability of superior genotypes. Thus, the description of germplasm is essential for breeding programs, ensuring the availability of information about the accessions of a bank and facilitating the work of selecting potential gene donors. These donors can also be used to eliminate duplicates and prevent the loss of genetic resources, which is fundamental for the success of a breeding program and agricultural production of the crop.

The results provided useful information for breeding programs and for the sacha inchi germplasm bank of Embrapa Amazônia Ocidental, so far no papers in the literature quantified the genetic variability of accessions of this species using AFLP markers, which generate a great amount of markers and ensure high reliability in data generation. In the test study of ISSR markers randomly selected from natural sacha inchi populations from neighboring communities in the city of Pucallpa in Peru, an average of 16 polymorphic bands for the primers used was obtained. The authors concluded that the rate of polymorphic bands in the studied population is high and that these markers are informative to estimate genetic parameters of this species in future studies (KRIVANKOVA et al., 2012).

Table 1. The genetic dissimilarity detected in 37 *Plukenetia volubilis* accessions with AFLP markers, where the upper-right diagonal shows the dissimilarity values calculated by the arithmetic complement of the Jaccard coefficient and the lower-left diagonal shows the graphical analysis obtained from the dissimilarity matrix, where each color represents a class corresponding to the dissimilarity percentage between the accessions, and each class is represented in the legend.

Acesso	A1M01	A1M02	A1M03	A1M04	A1M05	A1M06	A1M07	A1M08	A1M09	A1M10	A1M11	A1M12	A1M13	A1M14	A1M15	A2M01	A2M02	A2M03	A2M04	A2M05	A2M06	A2M07	A2M08	A2M09	A2M10	A2U01	A2U02	A2U03	A2U15	A2U16	A2U19	A2U20	A2U22	A2U24	A2U27	A1U28	A1U29
A1M01		0.773	0.672	0.687	0.527	0.690	0.630	0.720	0.635	0.543	0.737	0.597	0.773	0.797	0.583	0.618	0.813	0.766	0.759	0.821	0.823	0.732	0.750	0.730	0.745	0.724	0.645	0.600	0.786	0.690	0.744	0.750	0.833	0.745	0.793	0.804	0.717
A1M02			0.552	0.630	0.726	0.778	0.771	0.650	0.744	0.761	0.800	0.697	0.684	0.652	0.659	0.809	0.557	0.784	0.721	0.621	0.692	0.709	0.671	0.711	0.809	0.838	0.829	0.813	0.806	0.859	0.737	0.892	0.737	0.754	0.719	0.821	0.764
A1M03				0.368	0.590	0.694	0.746	0.616	0.738	0.646	0.758	0.667	0.671	0.698	0.592	0.763	0.627	0.734	0.728	0.708	0.781	0.716	0.676	0.717	0.806	0.785	0.729	0.758	0.841	0.833	0.771	0.774	0.729	0.702	0.661	0.800	0.758
A1M04					0.541	0.645	0.794	0.514	0.702	0.642	0.806	0.681	0.649	0.712	0.608	0.725	0.667	0.645	0.658	0.721	0.809	0.727	0.726	0.699	0.797	0.738	0.647	0.672	0.831	0.722	0.753	0.807	0.757	0.649	0.700	0.828	0.712
A1M05						0.738	0.611	0.724	0.640	0.589	0.648	0.581	0.743	0.781	0.589	0.603	0.672	0.703	0.731	0.788	0.806	0.787	0.754	0.774	0.772	0.790	0.731	0.700	0.850	0.747	0.774	0.804	0.750	0.727	0.754	0.807	0.678
A1M06							0.571	0.722	0.788	0.727	0.828	0.797	0.827	0.910	0.727	0.784	0.839	0.787	0.810	0.828	0.905	0.754	0.694	0.802	0.860	0.727	0.689	0.623	0.792	0.764	0.775	0.727	0.824	0.673	0.755	0.895	0.823
A1M07								0.831	0.782	0.737	0.653	0.734	0.897	0.855	0.685	0.725	0.814	0.901	0.918	0.859	0.845	0.849	0.705	0.862	0.833	0.885	0.818	0.776	0.830	0.870	0.862	0.851	0.836	0.833	0.857	0.870	0.754
A1M08									0.556	0.674	0.761	0.608	0.479	0.690	0.644	0.493	0.686	0.500	0.480	0.699	0.813	0.658	0.798	0.613	0.819	0.696	0.750	0.760	0.800	0.620	0.628	0.882	0.795	0.731	0.716	0.863	0.760
A1M09										0.602	0.708	0.579	0.551	0.805	0.618	0.506	0.707	0.619	0.583	0.734	0.779	0.646	0.795	0.633	0.750	0.662	0.720	0.744	0.797	0.610	0.633	0.890	0.750	0.800	0.787	0.842	0.727
A1M10											0.618	0.547	0.659	0.778	0.430	0.619	0.693	0.640	0.588	0.705	0.797	0.698	0.741	0.608	0.753	0.737	0.658	0.606	0.784	0.631	0.726	0.812	0.767	0.753	0.757	0.797	0.714
A1M11												0.589	0.800	0.768	0.657	0.656	0.741	0.824	0.831	0.776	0.839	0.829	0.884	0.833	0.729	0.862	0.866	0.850	0.800	0.836	0.859	0.960	0.754	0.849	0.808	0.771	0.647
A1M12													0.586	0.667	0.373	0.470	0.683	0.675	0.636	0.697	0.788	0.689	0.773	0.710	0.813	0.734	0.848	0.817	0.828	0.795	0.710	0.845	0.753	0.754	0.797	0.724	0.667
A1M13														0.631	0.674	0.568	0.687	0.514	0.472	0.642	0.731	0.543	0.740	0.547	0.859	0.677	0.785	0.781	0.873	0.671	0.596	0.892	0.785	0.754	0.812	0.821	0.829
A1M14															0.697	0.718	0.696	0.757	0.733	0.712	0.750	0.808	0.812	0.791	0.862	0.891	0.890	0.879	0.796	0.902	0.791	0.964	0.791	0.800	0.804	0.877	0.825
A1M15																0.550	0.676	0.685	0.652	0.738	0.827	0.727	0.695	0.689	0.818	0.815	0.707	0.747	0.831	0.777	0.714	0.812	0.738	0.770	0.757	0.764	0.680
A2M01																	0.767	0.547	0.600	0.808	0.840	0.716	0.849	0.634	0.814	0.706	0.775	0.803	0.845	0.663	0.649	0.912	0.819	0.779	0.800	0.826	0.700
A2M02																		0.621	0.597	0.500	0.647	0.656	0.579	0.702	0.818	0.833	0.788	0.763	0.815	0.844	0.815	0.902	0.710	0.725	0.731	0.895	0.859
A2M03																			0.338	0.638	0.743	0.636	0.718	0.529	0.849	0.729	0.714	0.740	0.847	0.597	0.653	0.913	0.793	0.765	0.768	0.877	0.835
A2M04																				0.594	0.736	0.541	0.662	0.459	0.855	0.704	0.709	0.733	0.822	0.558	0.606	0.901	0.741	0.775	0.743	0.867	0.827
A2M05																					0.588	0.565	0.576	0.600	0.867	0.823	0.831	0.794	0.759	0.821	0.769	0.927	0.723	0.764	0.768	0.842	0.848
A2M06																						0.617	0.655	0.691	0.808	0.825	0.868	0.814	0.780	0.838	0.809	0.961	0.717	0.830	0.833	0.776	0.833
A2M07																							0.603	0.423	0.785	0.645	0.694	0.701	0.762	0.679	0.648	0.871	0.747	0.803	0.788	0.815	0.855
A2M08																								0.659	0.912	0.871	0.829	0.812	0.894	0.860	0.745	0.818	0.729	0.806	0.864	0.857	0.935
A2M09																									0.725	0.592	0.655	0.642	0.722	0.482	0.596	0.906	0.768	0.800	0.774	0.851	0.830
A2M10																										0.500	0.623	0.625	0.538	0.610	0.841	0.935	0.783	0.840	0.796	0.923	0.706
A2U01																											0.632	0.551	0.558	0.525	0.784	0.826	0.870	0.765	0.815	0.930	0.776
A2U02																												0.442	0.691	0.552	0.768	0.815	0.811	0.763	0.787	0.908	0.809
A2U03																													0.587	0.500	0.778	0.787	0.809	0.680	0.736	0.897	0.806
A2U15																														0.672	0.865	0.957	0.800	0.837	0.816	0.922	0.796
A2U16																															0.674	0.894	0.817	0.812	0.779	0.919	0.784
A2U19																																0.838	0.640	0.741	0.679	0.795	0.778
A2U20																																	0.877	0.744	0.810	0.907	0.925
A2U22																																		0.741	0.679	0.817	0.791
A2U24																																			0.595	0.857	0.821
A2U27																																				0.813	0.712
A1U28																																					0.792
A1U29																																					



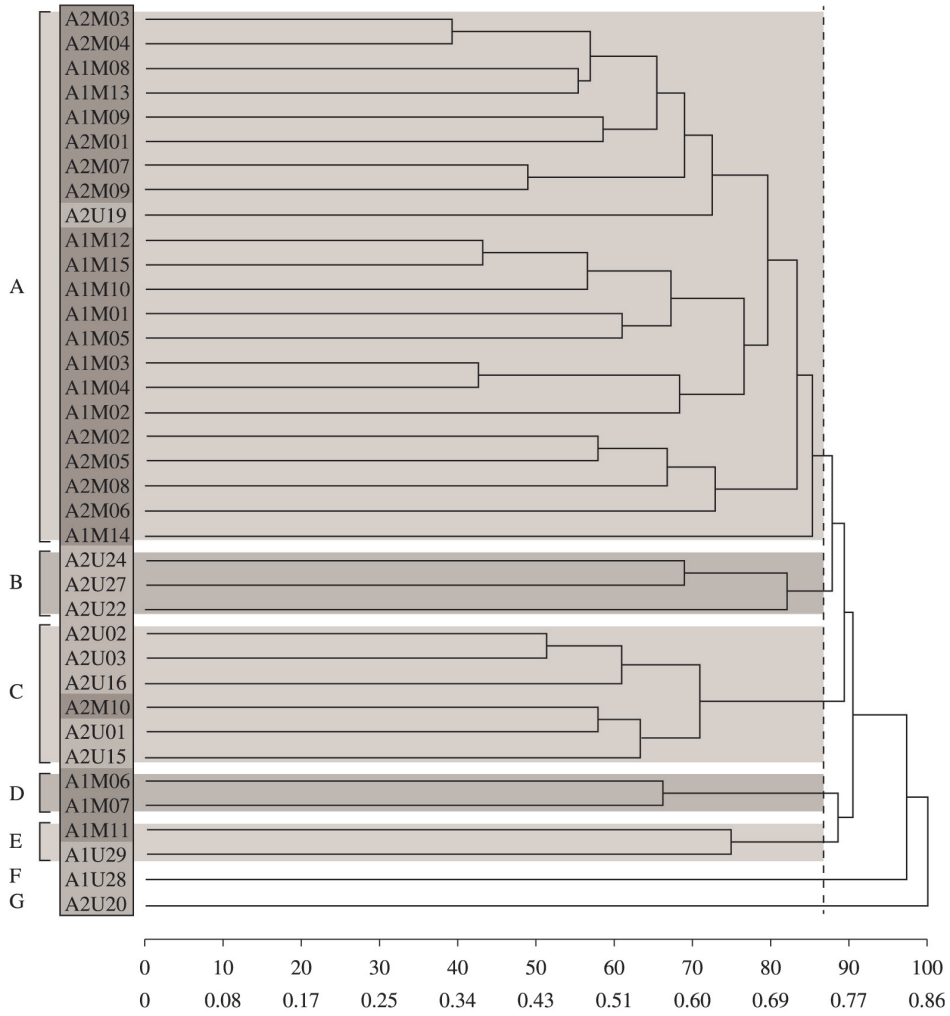


Figure 1. Dendrogram of 37 *Plukenetia volubilis* accessions, constructed by the unweighted pair-group method based on arithmetic averages (UPGMA); accessions from the state interior are marked in dark gray and those from the farm "Nova Jerusalém" in light gray; the vertical dotted line represents the estimated cut by the method of Mojema (0.75*) and A, B, C, D, E, F, and G are the groups formed at this fusion level.

Table 2. Distribution of estimates of the number and percentage of genetic distances by class intervals and the associated parameters mean, variance, symmetry and flatness (kurtosis).

Interval estimate	Number f distances in the interval	%
0	0	0
0.1-0.19	0	0
0.2-0.29	0	0
0.3-0.39	3	0.45
0.4-0.49	10	1.51
0.5-0.59	53	7.95
0.6-0.69	139	20.87
0.7-0.79	248	37.24
0.8-0.89	188	28.23
0.9-0.99	25	3.75
1	0	0
Mean	0.739	-
Variance	0.01	-
Symmetry	–0.649	-
Flatness (kurtosis)	3.47	-

The AFLP markers were effective for the assessment of genetic variability by detecting and quantifying the genetic diversity in 37 accessions that compose the sacha inchi germplasm bank of Embrapa Amazônia Ocidental, a dendogram showing the geographic structuring of the accessions, according to their origin. The results serve as input for future breeding programs of this species, assisting in the selection of potential parents for crosses.

4 Conclusions

The sacha inchi accessions in the germplasm bank of Embrapa Amazônia Ocidental contain genetic variability that can be exploited in breeding programs of the species.

Diversity was observed among the accessions of the sacha inchi germplasm bank as well as a geographic structure, according to the location of collection.

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