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SHORT NOTE [NOTA CORTA]

MORINGA GENETIC DIVERSITY FROM GERMPLASM BANK USING RAPD MARKERS

[DIVERSIDAD GENÉTICA DE GERMOPLASMA DE MORINGA UTILIZANDO MARCADORES RAPD]

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SUMMARY

The Moringa (*Moringa oleifera* Lam.) is a tree species with the known value of food, medicine and water treatment, and is promising for bioenergy use. Our objective was to evaluate, using RAPD markers, the genetic diversity of sixteen accessions from Germplasm Bank (BAG) of Embrapa Coastal Tablelands, Sergipe, Brazil. We estimate the diversity indices, and genetic similarity between accessions. The Shannon index and the index of genetic diversity (H) were 0.33 and 0.22, respectively. We observed by Jaccard similarity that, the accessions MO1 and MO12 are the most similar (0.27), and MO13 and MO16 are the most divergent (0.69). By UPGMA and PCoA groupings, we identified that MO1, MO2, MO12 and MO13 are genetically isolated. The results are important in designing strategies for conservation, but because of low diversity detected in this investigation, new activities of collection should be realized, with integration and characterization of new accessions, to ensure the ever increasing diversity of the collection.

Key words: *Moringa oleifera* Lam.; DNA; variability; genetic resources.

RESUMEN

Moringa (*Moringa oleifera* Lam.) es una especie arbórea con valor alimenticio, medicinal, en el tratamiento de agua, y también puede ser utilizada para producción de bioenergía. Nuestro objetivo fue evaluar, mediante marcadores RAPD, la diversidad genética del Banco de Germoplasma (BAG) de Moringa perteneciente a la Embrapa Mesetas costeras Costeiros, Sergipe, Brasil. Estimamos los índices de diversidad, y la similitud genética entre las 16 accesiones. El índice de Shannon y el índice de diversidad genética (H) fueron 0.33 y 0.22, respectivamente. Hemos observado que por la similitud de Jaccard, las accesiones MO1 y MO12 son los más similares (0,27), y MO13 y MO16 son los más divergentes (0.69). Por las agrupaciones UPGMA y PCoA, identificamos que dos pares de accesiones (MO1, MO2, MO12 y MO13) están genéticamente aislados. Los resultados son importantes para estrategias de conservación. Debido a baja diversidad detectada, nuevas actividades de recolección deben realizarse, con la integración y la caracterización de las nuevas accesiones, para garantizar el aumento de la diversidad en la colección.

Palabras clave: *Moringa oleifera* Lam.; DNA; variabilidad; recursos genéticos.

INTRODUCTION

Moringa (*Moringa oleifera* Lam., Moringaceae) is a perennial species, native of India. It is widely cultivated in tropical and subtropical countries, and it was introduced in Brazil during the 1950s. The species presents food, medicinal and cosmetic value. In addition, the seeds can be used to oil extraction and water treatment (Bezerra *et al.*, 2004).

The State of Sergipe has a potential area for Moringa production. However, more information about the species is needed, mainly concerning the characterization of genotypes of interest (Dos Santos *et al.*, 2011b). Given the importance of species and their need for conservation, the Embrapa Coastal Tablelands created in 2009, the Moringa Germplasm Bank (BAG) located in municipality of Nossa Senhora das Dores city.

The genotypes with immediate potential or future should be kept in Germplasm banks (BG's). The primary importance of BG's are the ability to provide to genetic variability for improvement programs, besides, help efforts to reconcile the conservation of agricultural biodiversity and sustainable development (Nass, 2007).

Studying the variability of such collections allows to identify duplicates, to estimate genetic linkage among accessions, to quantify genetic variability in the collection, to propose collections based on observed genetic diversity and to identify suitable breeding genotypes to improvement (Ferreira and Grattapaglia, 1995).

Molecular markers are used to detect genetic variation of genotypes of interest at the DNA level. The RAPD (Random Amplified Polymorphic DNA) is one of the tools used, especially in the characterization of genetic resources for rapid quantification of diversity (Silveira *et al.*, 2009). With the help of statistical methods, the RAPD technique has been effective in detecting the diversity of population in various types of organisms. Besides its low cost and speed, this technique has the advantage, even without prior knowledge of the genome, of requiring little amount of DNA for analysis (Goulão *et al.*, 2001).

RAPDs markers were used by Souza *et al.* (2001) successfully to evaluate the hypothesis of the existence of three races in peach palm; estimate the diversity and separation of cultivars in acerola (Salla *et al.*, 2002), peach and nectarine (Lima *et al.*, 2003), plum (Bianchi *et al.*, 2003), banana (Padmesh *et al.,* 2009), mangaba (Costa *et al.*, 2011) and moringa from Africa (Mgendi *et al.*, 2010). Muluvi *et al.* (1999) using AFLP found high level of genetic diversity in Moringa from Kenya and India, indicating the genetic potential of the species.

The aim of the study was the molecular characterization and genetic diversity of the moringa Germplasm Bank belonging to Embrapa Tabuleiros Costeiros, using RAPD markers.

MATERIAL AND METHODS

We used 16 accessions of Moringa Germplasm Bank belonging to Embrapa Tabuleiros Costeiros. These accessions are from exchanged germplasm of University of Florida, USA. It were introduced in the Campo Experimental Jorge do Prado Sobral belonging to Embrapa Tabuleiros Costeiros, located in the municipality of Nossa Senhora das Dores (Sergipe State).

The DNA extraction was based on Doyle and Doyle (1991) methodology. 300 mg young leaves were macerated in liquid nitrogen, adding 800 mL of extraction CTAB buffer (2% of CTAB, EDTA – 0.5 M pH 8.0, Tris-Cl – 1 M pH 8.0, NaCl 5M, 2% de PVP).

Each RAPD reaction was performed in 25μL volume containing 50 ng genomic DNA, 1X PCR buffer (GIBCO-BRL), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 U *Taq* DNA polymerase (GIBCO), 30 ng/μL primer , 20 μL ultrapure water.

The PCR amplifications were performed using a PTC-100 thermocycler (Programmable Thermal Controller - MJ Research, Inc.) and subjected to a cycle of 96°C for 5 minutes for initial denaturation, followed by 35 cycles of denaturation at 96°C for 45 seconds, 36°C for 45 seconds for primer annealing, 72°C for 45 seconds for extension, and finally one cycle of 72°C for 5 minutes for final extension (Silva and Martins, 2006). We tested 17 primers (A3, A4, A8, A12, A15, A16, A18, IDT02, IDT3, IDT15, S01, S18, W02, W13, W19, B02, B18) with 14 primers Operon (Operon Technologies, USA) and three IDT primers (Integrated DNA Technologies, Germany) (Table 1).

For electrophoresis, 10 μL of PCR products were mixed to 1.5 μL of blue juice (0.01% bromophenol blue, 40% glycerol). We used 1.5% agarose gel (1X TEB - 89 mM TRIS, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system Sunrise (Gibco BRL), carried out at a constant voltage of 100 V for 90 minutes. Gel was stained with ethidium bromide solution (5 mg/mL) for 15 minutes, and the amplification products visualized under ultraviolet light using a Gel Doc L-Pix image system (Loccus Biotecnologia, Brazil).

The electrophoretic profile of each gel was transformed into a binary matrix of presence (1) and absence (0) and which was subsequently used for all analysis. Bootstrap procedure was applied to calculate variance of the genetic distance obtained from markers, and was obtained from 5,000 bootstrap random draws using the DBOOT software (Coelho, 2000). Polymorphic information content (PIC) is a parameter that provides an estimate of the discriminatory power of molecular marker per primer and was calculated according to Ghislain *et al.*, (1999). The marker index (MI) was determined as a product of PIC and the number of polymorphic bands per assay unit as described in Zhao *et al.*, (2007). To measure the genetic diversity, we used Genalex v.6.3 (www.anu.edu.au/BoZo/GenAlEx/) and calculated the Shannon Index (I) (Brown and Weir 1983) as well as genetic diversity (H) as described by Lynch and Milligan (1994) and Maguire *et al.*, (2002) for dominant markers.

The data matrix of the RAPD scores was generated and similarity coefficients were calculated using Jaccard's arithmetic complement index. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster algorithm. In order to determine the robustness of the dendrogram, the data was bootstrapped with 5,000 replications using FreeTree software (http://web.natur.cuni.cz/flegr/programs/freetree.htm). For visualization of the cluster we used the TreeView package (http://web.natur.cuni.cz/flegr/programs/freetree/TreeView.exe). Principal Coordinates Analysis (PCoA) was performed using the software XLSTAT (http://www.xlstat.com/) based on similarity matrix of Jaccard.

RESULTS AND DISCUSSION

The 17 primers generated a total of 95 fragments, 59 polymorphic (62%). Primers with the highest number of polymorphic fragments was IDT15 and S01 (seven). Primers IDT3 and B18 did not show polymorphic fragments (Table 1). The primers used resulted in a banding pattern that was distinct, as seen in Figure 1 which shows the RAPD profiles of the primer IDT 02.

The polymorphism found can be considered high (62%), and similar values were found in other studies. In a study in cultivated and non-cultivated Moringa from Tanzania (Africa), a total of 98 fragments were scored and 86 of them (89.6%) were polymorphic using 12 RAPD primers (Mgendi *et al.*, 2010). The selection stage of these markers is critical to the efficiency of the work of characterization and can be done through literature review or tests in the laboratory (Silva *et al.*, 2008).

There is a directly proportional relationship between the number of fragments analyzed and the coefficient of variation (CV) (Figure 2). The results indicate a clear of decreasing CV with increasing number of fragments. According to Moura *et al.* (2005), there is a point where the enlargement in the number of fragments does not show a significant increase in experimental accuracy and not justifying the extra effort in labor. From 55 fragments there is a stabilization of the CV, with value less than 10%, suggesting that the results obtained by the fragments used in this study (59) can be used for analysis of diversity.

To access the Moringa, the PIC value ranged from 0.00 to 0.41 and MI from 0.00 to 2.07. For the variables of diversity, the Shannon index (I) with average value of 0.33, and the genetic diversity index (H) showed a mean value of 0.22 (Table 1).

Table 1. Primers, totals (TF) and polymorphic fragments (PF%), polymorphic information content (PIC), marker index (MI), Shannon index (I) and genetic diversity (H) between 16 accessions belonging Moringa (*Moringa oleifera* Lam.) Germplasm Bank from Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe State, Brazil).

Primers	Sequence 5' – 3'	TF	PF%	PIC	MI	I	H
A3	AGT CAG CCA C	3	100	0.36	1.07	0.52	0.36
A4	AAT CGG GCT G	4	50	0.25	0.50	0.34	0.25
A8	GTG ACG TAG G	3	67	0.26	0.52	0.38	0.26
A12	TCG GCG ATA G	3	67	0.13	0.27	0.23	0.13
A15	TTC CGA ACC C	4	75	0.17	0.52	0.29	0.17
A16	AGC CAG CGA A	4	100	0.39	1.55	0.57	0.39
A18	AGG TGA CCG T	6	100	0.23	1.35	0.37	0.23
IDT02	TGA TCC CTG G	8	62	0.14	0.69	0.21	0.14
IDT3	TGC CGA GCT G	2	0	0.00	0.00	0.00	0.00
IDT15	GGT CGG AGA A	9	78	0.30	2.07	0.44	0.30
S01	CTA CTG GCG T	8	87	0.26	1.82	0.40	0.26
S18	CTG GCG AAC T	9	62	0.16	0.79	0.24	0.16
W02	ACC CCG CCA A	7	29	0.12	0.24	0.17	0.12
W13	CAC AGC GAC A	4	75	0.34	1.02	0.48	0.34
W19	CAA AGC GCT C	9	67	0.17	1.02	0.27	0.17
B02	AGT CAG CCA C	4	100	0.41	1.65	0.60	0.41
B18	AAT CGG GCT G	8	0	0.00	0.00	0.00	0.00
Average		6	62	0.22	0.89	0.33	0.22

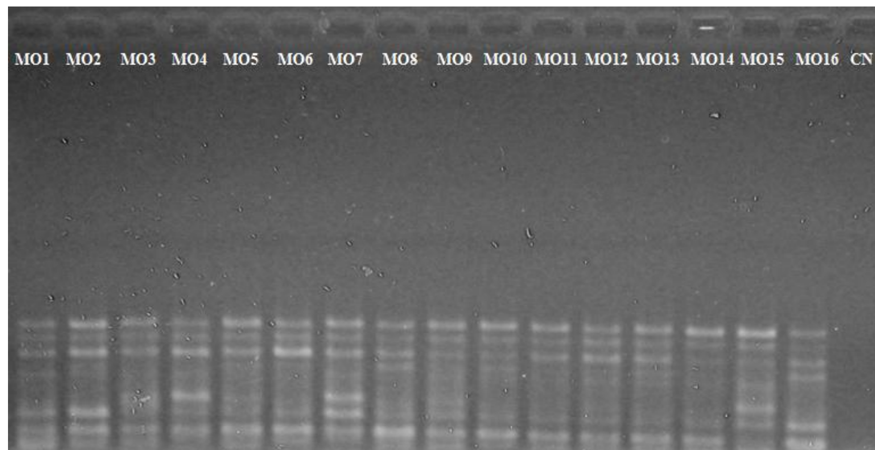

Figure 1. RAPD profiles generated by IDT 02 primer and negative control (CN) between 16 accessions belonging Moringa (*M. oleifera* Lam.) Germplasm Bank from Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe State, Brazil).

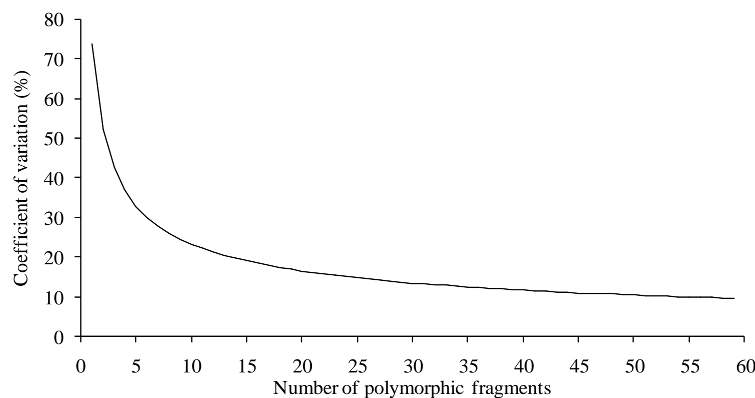

Figure 2. Coefficient of variation for the number of polymorphic fragments using RAPD markers between 16 accessions belonging Moringa (*M. oleifera* Lam.) Germplasm Bank from Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe).

Genetic diversity indicated by I (0.00 - 0.52) and H (from 0.00 to 0.41) in BAG Moringa can be considered low since the values were equal to or less than 0.55, or is in the BAG for a low diversity, being necessary to insert a new accessions to promote increased diversity and more likely to use these resources. The values found are similar to those obtained for other tree species *Populus tremuloides* (0.58 to 0.69, Yeh *et al*, 1995), *Fitzroya cupressoides* (0.42 to 0.56; Allnut *et al.*, 1999) and *Swietenia macrophylla* (0.41 to 0.27, Gillies *et al.*, 1999). The average genetic diversity (H) obtained is in agreement with those found for other species. To *Trichilia pallida* Swartz, ranged from 0.27 to 0.33 (Zimback *et al.*, 2004) and *Aspidosperma polyneuron*, diversity averaged 0.28 (Torezan *et al.*, 2005).

The average similarity (Table 2) between accessions was 0.48, ranging from 0.27 (MO1 and MO12) to 0.69 (MO13 and MO16). This variability expressed should be used in breeding programs and studies on conservation and multiplication of top accessions. RAPD primers were used to investigate the genetic diversity between and within cultivated and non-cultivated provenances of Moringa from Costal regions of Tanzania, and the cluster analysis showed five clusters with similarity ranging from 54% to 96% (Mgendi *et al.*, 2010).

According to UPGMA clustering, we indentified three distinct groups (Figure 3). The first group (G1) was formed by ten accessions, second (G2) by four and third (G3) that only two accessions. Among the groups, G3 stood alone, indicating that these accessions are the most genetically distant.

Table 2. Genetic similarity of 16 accessions from Moringa (*Moringa oleifera* Lam.) Germplasm Bank belonging to Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe state).

	MO1	MO2	MO3	MO4	MO5	MO6	MO7	MO8	MO9	MO10	MO11	MO12	MO13	MO14	MO15	MO16
MO1	-	0.45	0.30	0.33	0.35	0.42	0.41	0.46	0.49	0.45	0.47	***0.27***	0.35	0.45	0.54	0.46
MO2	0.45	-	0.33	0.38	0.46	0.41	0.40	0.42	0.38	0.45	0.42	0.29	0.38	0.44	0.50	0.46
MO3	0.30	0.33	-	0.64	0.57	0.50	0.45	0.38	0.40	0.36	0.44	0.34	0.29	0.43	0.44	0.35
MO4	0.33	0.38	0.64	-	0.65	0.55	0.50	0.45	0.47	0.41	0.46	0.36	0.34	0.47	0.51	0.45
MO5	0.35	0.46	0.57	0.65	-	0.68	0.57	0.47	0.49	0.50	0.47	0.47	0.47	0.53	0.50	0.47
MO6	0.42	0.41	0.50	0.55	0.68	-	0.62	0.50	0.45	0.55	0.61	0.43	0.51	0.53	0.54	0.46
MO7	0.41	0.40	0.45	0.50	0.57	0.62	-	0.56	0.55	0.49	0.64	0.50	0.54	0.51	0.53	0.45
MO8	0.46	0.42	0.38	0.45	0.47	0.50	0.56	-	0.63	0.50	0.44	0.47	0.44	0.60	0.61	0.57
MO9	0.49	0.38	0.40	0.47	0.49	0.45	0.55	0.63	-	0.45	0.50	0.36	0.43	0.59	0.60	0.56
MO10	0.45	0.45	0.36	0.41	0.50	0.55	0.49	0.50	0.45	-	0.56	0.38	0.52	0.45	0.54	0.58
MO11	0.47	0.42	0.44	0.46	0.47	0.61	0.64	0.44	0.50	0.56	-	0.44	0.58	0.58	0.64	0.51
MO12	***0.27***	0.29	0.34	0.36	0.47	0.43	0.50	0.47	0.36	0.38	0.44	-	0.53	0.54	0.56	0.51
MO13	0.35	0.38	0.29	0.34	0.47	0.51	0.54	0.44	0.43	0.52	0.58	0.53	-	0.58	0.55	***0.69***
MO14	0.45	0.44	0.43	0.47	0.53	0.53	0.51	0.60	0.59	0.45	0.58	0.54	0.58	-	0.64	0.64
MO15	0.54	0.50	0.44	0.51	0.50	0.54	0.53	0.61	0.60	0.54	0.64	0.56	0.55	0.64	-	0.65
MO16	0.46	0.46	0.35	0.45	0.47	0.46	0.45	0.57	0.56	0.58	0.51	0.51	***0.69***	0.64	0.65	-

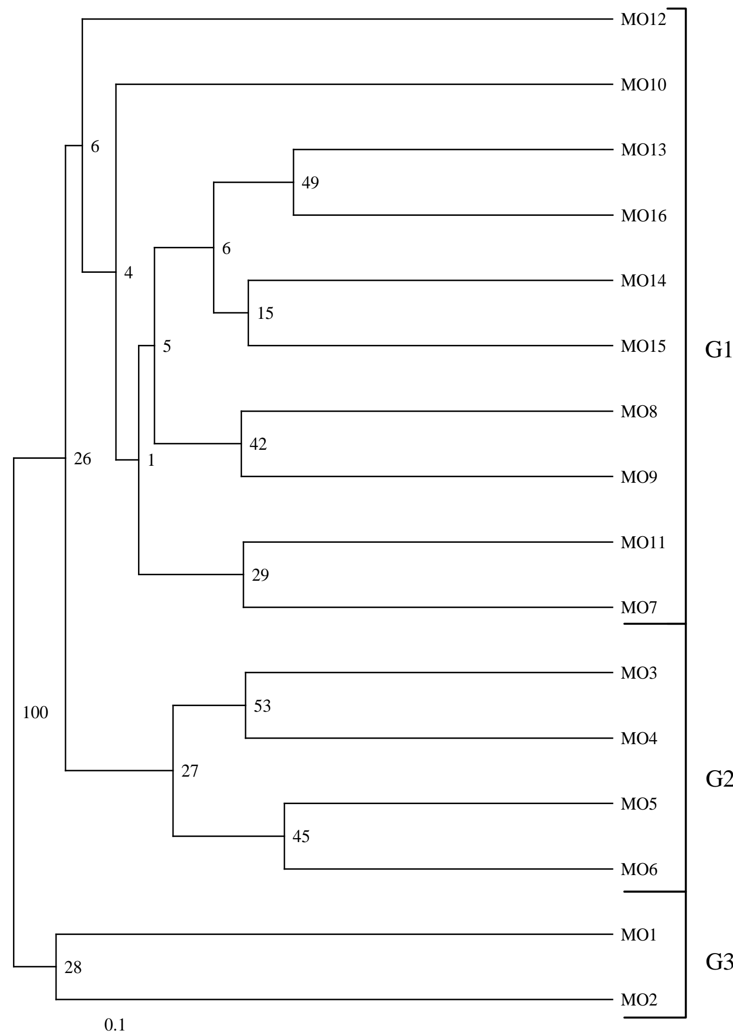

Figure 3. Cluster generated from the Jaccard similarity coefficient and UPGMA clustering using RAPD markers between 16 accessions from Moringa (*Moringa oleifera* Lam.) Germplasm Bank belonging to Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe state).

According by PCoA (Figure 4), the first two axes represent 38.29% of the variation. We indentified four groups, the first one (I) formed by two accessions, second (II) for seven third one (III) by two and fourth (IV) for five. The groups (I) and (III) were isolated, suggesting that these are the most different groups.

Some differences were found in contrasting two clustering methods. According to UPGMA analysis accesses MO1 and MO2 were more related, and to PCoA were MO1 and MO2, and MO12 and MO13. In addition, the group G1 of UPGMA clustering is equivalent II and III to the PCoA, indicating that the groups are subgroups.

The regrouping has been used by some authors (Costa *et al.*, 2011; Dos Santos *et al.*, 2011a) as an alternative to make the results more revealing. The use of more than one method of grouping prevents erroneous inferences because of differences in classification, optimization and ordering of groups. Thus, this option is adopted in the allocation of materials within a particular subset of genotypes (Arriel *et al.*, 2006).

Information on the genetic diversity can assist in a breeding program avoiding duplication or mixtures of genotypes in studies and germplasm conservation programs (Pinheiro *et al.*, 2003). Molecular characterization of genetic diversity from Germplasm Bank can provide useful data to assist the breeder in the identification and selection of clones or parents to establish the basic breeding program.

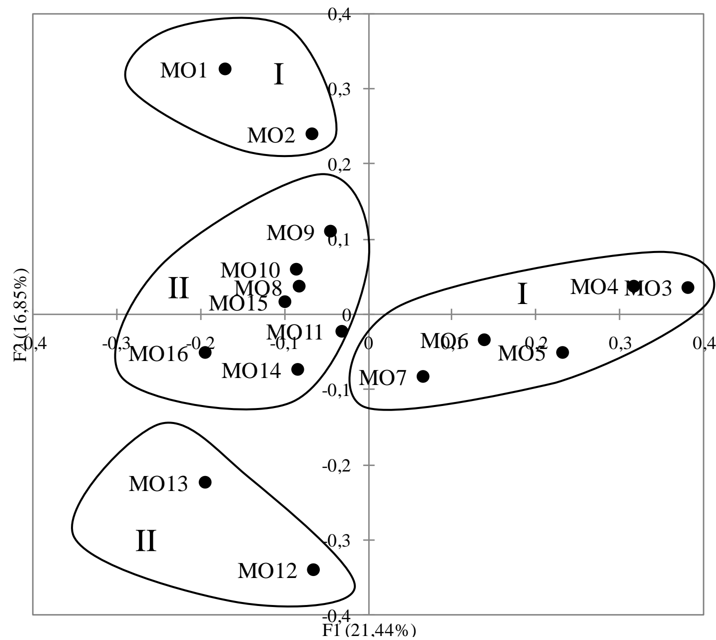

Figure 4. Principal Coordinates Analysis (PCoA) obtained from the Jaccard similarity coefficient using RAPD markers between 16 accessions from Moringa (*Moringa oleifera* Lam.) Germplasm Bank belonging to Embrapa Coastal Tablelands (Nossa Senhora das Dores, Sergipe state).

CONCLUSIONS

The accessions MO1, MO2, MO12 and MO13 are genetically isolated, and the information generated can be used to design strategies and conservation of germplasm in future breeding programs of the species. Because of low diversity detected in this investigation, to Moringa Germplasm Bank is fundamental to continue inclusion of new accessions.

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