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The genetic variability using sequencing of the ribosomal internal transcribed spacer (ITS) region in cultivars of the cowpea [*Vigna unguiculata* L. (Walp).]

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Cowpea is a predominant crop in the small farms of the Brazilian semi-arid region, where several varieties of cowpea with high genetic variability are planted. Due to their genetic diversity, good adaptation to marginal environments and growth in low-input systems, these varieties, often called "landraces," are of great interest for use in breeding and biodiversity conservation programs. The present study describes twelve varieties and three cultivars of cowpea, chosen on the basis of their high frequency of planting by farmers in the Paraíba and Pernambuco States. Total DNA was extracted from the plant seeds. In order to observe the variability of the studied material, four Intersimple sequence repeat (ISSR) markers, the amplified ribosomal DNA restriction analysis (ARDRA) technique using various endonucleases (*Alu*I, *Hinf*I, *Hpa*II, *Rsa*I and *Nru*I), and amplification and sequencing of the ribosomal internal transcribed spacer (ITS1) and ITS2 regions were employed. The results show that two assays, the fingerprinting and the sequencing of the ribosomal ITS1 and ITS2 regions, were sufficient to detect the variability of the cowpea germplasm used by small farmers in the Brazilian semi-arid region. Although the varieties often received the same designation, high diversity may have occurred within a variety, according to the origin of the cowpea seed. This work represents efforts to guide preservation of cowpea biodiversity in semi-arid regions.

Key words: *Vigna unguiculata* L. (Walp.), coat seeds, intersimple sequence repeat (ISSR), amplified ribosomal DNA restriction analysis (ARDRA), diversity.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important grain legume cultivated in all tropical and subtropical regions of the world, as well as in South-East Europe and in the United States. In small Brazilian semi-arid farms, cowpea is one of the predominant crops. In general, local varieties of cowpea are preferentially cultivated, as the government-supplied cultivars do not satisfactorily meet the needs of the producers and consumers due to either type of maturation and plant cycle or to the color, shape and size of the grains, despite the high productive potential and resistance to virus diseases of the cultivars developed by government agencies. Often, varieties with quite distinct phenotypic characteristics (such as color and size of seed or growth habit) have the same name in

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distinct micro-regions.

Local varieties are important in several regions and countries where cowpea is cultivated, particularly in Africa (Adjei-Nsiah et al., 2008; Ghalmi et al., 2010). Due to their genetic diversity, good adaptation to marginal farming environments and ability to grow in systems of low inputs, these varieties, often called "landraces", are of great interest for use in breeding programs and fordevelopment of new cropping systems.

Accordingly, various efforts to understand the genetic diversity of the preferred cultivars and to preserve this diversity have been undertaken by researchers from different countries (Ghalmi et al., 2010). These studies show that a complex interaction of factors is responsible for the observed diversity patterns in cowpea.

Molecular markers can be used as tools for studying the genetic diversity between individuals within a population and between populations, or between related species, and for determining the identity of a cultivar variety (Arnao et al., 2008). The Intersimple sequence repeat (ISSR) analysis has many advantages in assessing genetic diversity (Gupta et al., 1994) because it is based on the amplification of microsatellite sequences between adjacent regions of DNA via polymerase chain reaction (PCR). ISSR technology uses highly polymorphic targets, it is reproducible, does not require prior knowledge of the genome and is relatively inexpensive (Vieira et al., 2009).

Recently, analyses of the ribosomal DNA (rDNA) spacers and comparative studies of the nucleotide sequences of the rDNA genes using the Amplified ribosomal DNA restriction analysis (ARDRA) technique, have been applied to the study of the phylogeny and taxonomy of plants, fungi and bacteria (Yadav et al., 2011). While the regions of ribosomal genes are highly conserved within species, regions of the internal transcribed spacer (ITS) evolve faster; therefore, evolution may vary interspecifically on the sequence of bases and in length (Gerbi, 1985), being frequently used for taxonomy of species and genera (Menezes et al., 2010).

To date, no studies have been published that have used the ISSR markers, the ARDRA technique and sequencing of the 18S rDNA to examine cowpea cultivars in use by small family farmers in the Brazilian semi-arid region states. The objective of this study was to use molecular markers (ISSR and ARDRA) and sequencing the ITS region of rDNA to estimate the genetic diversity and investigate the genetic relationships between local varieties of cultivated cowpea in the States of Pernambuco and Paraíba.

MATERIALS AND METHODS

Thirteen local cowpea varieties were used, including four from Paraiba (Corujinha-PB, Sedinha-PB, Canapú-PB and Azul-PB) and eight from Pernambuco (Sempre Verde-PE, Sedinha-PE, Maratuã-PE, Canapú-PE, São Sebastião-PE, Costela de Vaca Branca-PE, Corujinha-PE and Costela de Vaca Marrom-PE). In addition to these, the cultivars that were recommended for use were the BRS Pujante, the IPA206 and the IPA207. Some of the varieties in use have the same denomination in two states, but it has been observed that they are sufficiently divergent in color, format and size, suggesting possible genetic differences; for this reason, these varieties were included in the study. For easy identification of the studied materials, each variety was given the initials of its state of origin after the variety name. Total DNA was extracted from seeds. The Invisorb Spin Plant Mini Kit from Invitek was used and followed the manufacturer's suggestions with slight modifications in the preparation of samples. As the seed coat has high levels of compounds such as tannic acid, phenol and cyanide which causes interference in the amplification of DNA were removed from seeds were separated from the pistil. After this procedure, liquid nitrogen was added at a rate of 60 mg to the vegetable material to obtain a fine powder, and DNA extraction was performed. The quantification was performed in an agarose gel containing 0.8% agarose in 0.5 × Tris/Borate/EDTA (TBE) buffer using the bromothymol blue buffer and the SybrGold (Invitrogen) stain. Electrophoresis was run on 100 V, and the product was viewed under ultraviolet (UV) light using a Gel Doc L-Pix image-Loccus software system.

For genotypic characterization of cowpea varieties and cultivars by amplification of the ITS1 and ITS 2 regions, two primers were used: ITS1 (5'-TTC CGT AGG TGA ACC TGC GG-3') and ITS2 (5'-TCC TCC GCT TAT TGA TAT GC- 3') (White et al., 1990). For amplification by PCR, a 15 µl final volume was used under the following conditions: 2 µl of DNA (20 to 40 ng), 0.4 mM of each primer, 10 × Taq polymerase buffer, 10% DMSO, 1 U Taq polymerase, 200 mM of mix dNTP´s, and 2.5 mM $MgCl_2$. The amplification cycles used included an initial denaturation (5 min at 94°C) and further 30 cycles of: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and one last extension step of 5 min at 72°C. The amplified fragments were separated by agarose gel electrophoresis on a gel containing 0.8% agarose in 0.5 × TBE buffer, in bromothymol blue buffer and stained with SybrGold (Invitrogen). The resultant fragments were viewed under UV light and photographed under UV light using a Gel Doc L-Pix image-Loccus software system. After purification with 7.5M ammonium acetate, the PCR products were treated separately with endonucleases: *Alu*I, *Hinf*I, *Hpa*II, *Nru*I and *Rsa*I. Digestion was performed in a final volume of 20 µl (9 µl of water, 8 µl of amplified purified product, 2 µL of the specified buffer for each enzyme and 1 µl of endocuclease). The enzyme digestion temperature varied depending on the enzyme used, and all reactions were incubated overnight. Then, the restriction fragments were separated by agarose gel electrophoresis at 80 V for 3 h on a gel containing 2.5% agarose in 0.5 × TBE buffer. The running buffer was supplemented with bromothymol blue, and SybrGold was used for staining. Gels were then viewed under ultraviolet light and photographed in under UV light using a Gel Doc L-Pix image-Loccus software system. The PCR products of the ITS1 and ITS2 regions were purified after the reaction in a final volume of 100 µl.

This volume was subdivided: one part was used for the endonuclease digestions, and the other part was used for sequencing. The purification consisted of adding 8 µl of 7.5 M ammonium acetate and 208 µl of 100% ethanol to samples in 1.5 ml microtubes and centrifuging those at 13,000 rpm for 20 min at room temperature. The supernatant was removed and 150 µl of ice cold 70% ethanol was then added to before centrifugation for 5 min at 13,000 rpm. The microtube was poured onto a paper towel to allow the pellet to dry overnight. DNA-containing pellets were then re-suspended in 30 µl of ultrapure water and kept at -20°C until sequencing was performed. Total DNAs were used at a dilution of 1:500. The ISSR analyses used the following primers: UBC01-5´-AC^8-3´, UBC808 - 5´- AG^8C- 3´, UBC809 - 5´- AG^8G - 3´; UBC810 - 5´-GA^8T-3´. For amplification by PCR, a final volume of 15 µl was used and contained 20 to 40 ng of genomic DNA (1 µl), 0.4 µM

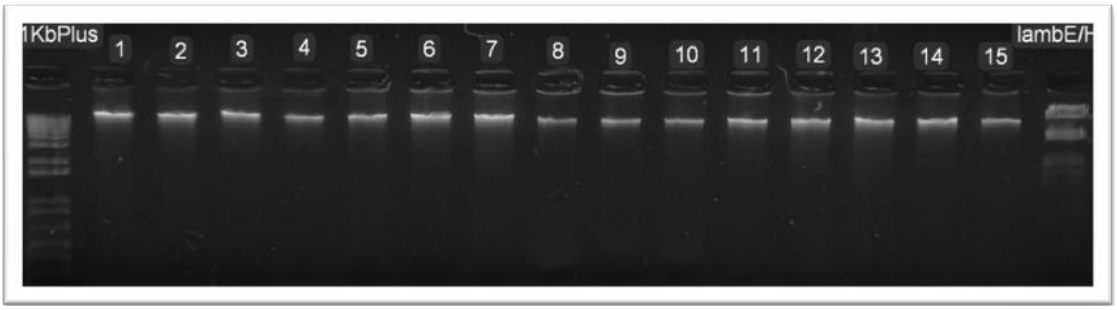


Figure 1. Extraction of genomic DNA from cowpea seeds. Lane 1. Corujinha-PB (CRJPB), Lane 2. Sedinha-PB (SDHPB), Lane 3. IPA207, Lane 4. Canapú-PB (CNPPB), Lane 5. Azul-PB, Lane 6. Sempre Verde-PE (SVPE), Lane 7. Sedinha-PE (SDHPE), Lane 8. Maratauã-PE (MRTPE), Lane 9. Canapú-PE (CNPPE), Lane 10. São Sebastião-PE (SSPE), Lane 11. BRS Pujante-PE (BRSPPE), Lane 12. Costela de Vaca Branca-PE (CVBPE), Lane 13. IPA206, Lane 14. Corujinha-PE (CRJPE), Lane 15. Costela de Vaca Marrom-PE (CVMPE). DNA ladder 1 Kb Plus (Invitrogen) and Lambda E/H.

primer, 10% Taq polymerase buffer (Invitrogen), 10% DMSO, 1U Taq polymerase enzyme (Invitrogen), 200 mM of each dNTP, and 2.5 mM of $MgCl_2$. The amplifications were conducted in a PCT-100 (MJ-Research-Peltier) thermal cycler. The amplification cycles used included an initial denaturation (5 min at 94°C) and further 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension step of 5 min at 72°C. To eliminate the possibility of contamination, a negative control was always used. The amplified products were separated by agarose gel electrophoresis on a gel containing 1.2% agarose in 0.5 × TBE buffer and were then stainedwith SybrGold and viewed under UV light. The repeated bands were punctuated and denoted as either present (1) or absent (0) in ISSR markers and ARDRA, and each feature was treated independently. For each primer, the number of different frequency bands and the bands were designated as polymorphics. A genetic similarity calculation was measured using the Simple Matching (SM) coefficient where N_{ij} is the number of bands present in both genotypes *i* and *j*, N_i is the number of bands present in genotype *i*, and N_j is the number of bands present in genotype *j*. The genetic similarity was converted to genetic dissimilarity. The dissimilarity matrix produce was used to generate a cophenetic matrix (Figure 7), the adjustment between the dissimilarity matrix and the dendrogram being estimated from the cophenetic correlation coefficient (r). The clustering analysis was performed by calculating the similarity of SM coefficient, and the results generated an array of genetic distance.

This array was visualized as dendrograms constructed using the NTSYSpc program (Numerical Taxonomy and Downloads Analysis System) version 2.1 (Rohlf, 1998) and by applying the SM coefficient of similarity. For the analysis of the genetic distance between variants, Unweighted pair group method with arithmetic mean (UPGMA) clustering was used with the parameters of the Sequential agglomerative hierarchical nested cluster analysis (SAHN) program, and the construction of a phylogenetic tree was completed using the TREE plot (Sokal and Sneath, 1963) program. For sequencing reactions, the oligonucleotides ITS1 and ITS4 were used. Sequencing was performed on the MegaBace 1000 DNA sequence (Amersham Biosciences) employing the DNA sequencing NTBIO platform of EMBRAPA Genetic Resources and Biotechnology - CENARGEN. The electrophoresis parameters used for sequencing were: 40 s injection of sample under 1 Kv voltage and 5 Kv running voltage for 240 min. For construction of the phylogenetic tree, the nucleotide sequences obtained from the different isolates were subjected to alignment by the program BioEdit (Hall, 1999). Nucleotide sequences were aligned with the ClustalW program, and phylogenetic analysis was conducted using neighbor-joining (with 1000 bootstrap replications) and pairwise deletion of nucleotides according to the Tamura-Nei model made by MEGA program, version 4 (Tamura et al., 2004).

RESULTS

Extracted DNA from all seeds resulted in a high-quality material as can be viewed in Figure 1.The four primers used for ISSR showed to be polymorphic and the few tracks of amplified products that were observed are shown in Figure 2. These primers are characterized by their ability to generate profiles with strong banding; the amplified products range from 6 to 11 bands with polymorphic DNA bands ranging from 300 to 2000 oligonucleotides in length. The dendrogram of similarity (Figure 3) of the four ISSR markers showed the formation of three ISSRs clusters (Clusters 1, 2, and 3), with genetic distances of 67, 66 and 58%, respectively.

The first cluster contains two subclusters: 1A and 1B (Figure 3). In subcluster 1A, Corujinha-PB and Canapú-PB varieties showed 81% similarity to one another, while the Azul-PB showed 73% similarity in relation to the other two varieties. Along another branch, Maratauã-PE and Canapú-PB varieties showed 88% similarity, the greatest similarity presented using the SM coefficient, while São Sebastião-PE showed a less than 77% similarity to the other two varieties. In subcluster 1B, the highest level of similarity (85%) was observed between Corujinha-PE and Costela de Vaca Marrom-PE. These varieties showed 81% similarity with respect to the IPA206 and IPA207 cultivars used in this work as control *V. unguiculata* species, and 73% in relation to the Costela de Vaca Branca-PE variety. In this cluster, the two varieties, Costela de Vaca Branca and Costela de Vaca Marrom, both from Pernambuco, showed distinct genotypes.

In Cluster 2, Sedinha-PB variety and the cultivar IPA207 showed 82% similarity, and Costela de Vaca Branca and Costela de Vaca Marrom varieties showed

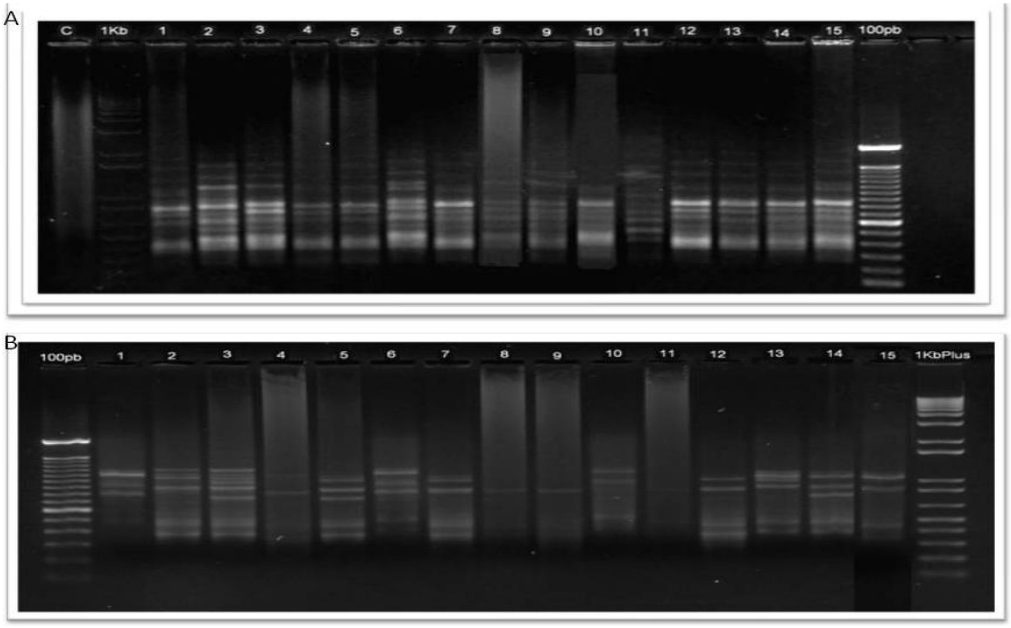


Figure 2. ISSR patterns of cowpea varieties and cultivars generated by primers: UBC 808 (A) and UBC 810 (B). Lane 1. Corujinha-PB (CRJPB), Lane 2. Sedinha-PB (SDHPB), Lane 3. IPA207, Lane 4. Canapú-PB (CNPPB), Lane 5. Azul-PB, Lane 6. Sempre Verde-PE (SVPE), Lane 7. Sedinha-PE (SDHPE), Lane 8. Maratauã-PE (MRTPE), Lane 9. Canapú-PE (CNPPE), Lane 10. São Sebastião-PE (SSPE), Lane 11. BRS Pujante-PE (BRSPPE), Lane 12. Costela de Vaca Branca-PE (CVBPE), Lane 13. IPA206, Lane 14. Corujinha-PE (CRJPE), Lane 15. Costela de Vaca Marrom-PE (CVMPE). DNA Ladder 1 Kb Plus and 100 pb (Invitrogen).

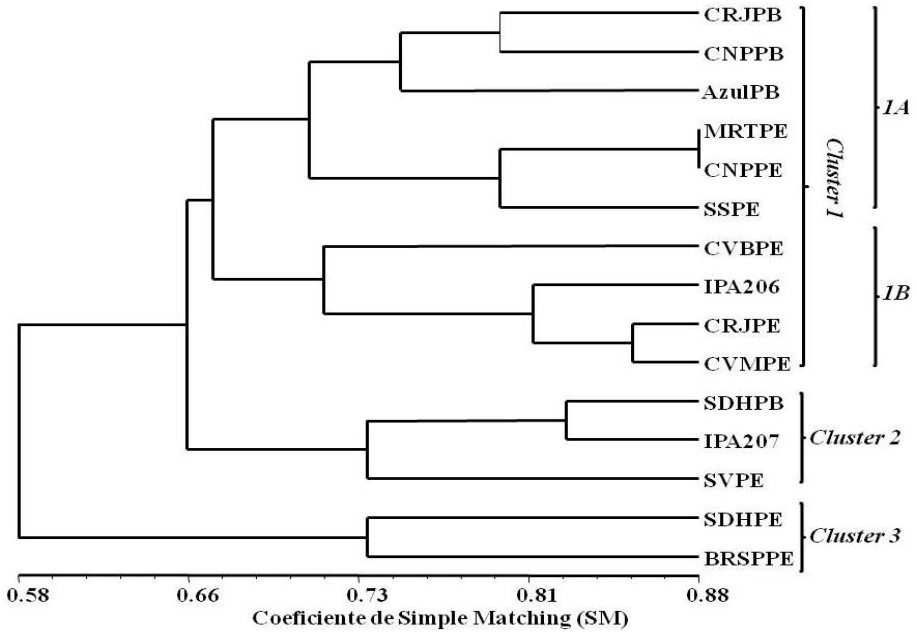


Figura 3. UPGMA dendrogram showing the relationship among cowpea varieties and cultivars using ISSR Markers (UBC 01, UBC 808 , UBC 809; UBC 810. 1. Corujinha-PB (CRJPB), 2. Sedinha-PB (SDHPB), 3. IPA207, 4. Canapú-PB (CNPPB), 5. Azul-PB, 6. Sempre Verde-PE (SVPE), 7. Sedinha-PE (SDHPE), 8. Maratauã-PE (MRTPE), 9. Canapú-PE (CNPPE), 10. São Sebastião-PE (SSPE), 11. BRS Pujante-PE (BRSPPE), 12. Costela de Vaca Branca-PE (CVBPE), 13. IPA206, 14. Corujinha-PE (CRJPE), 15. Costela de Vaca Marrom-PE (CVMPE).

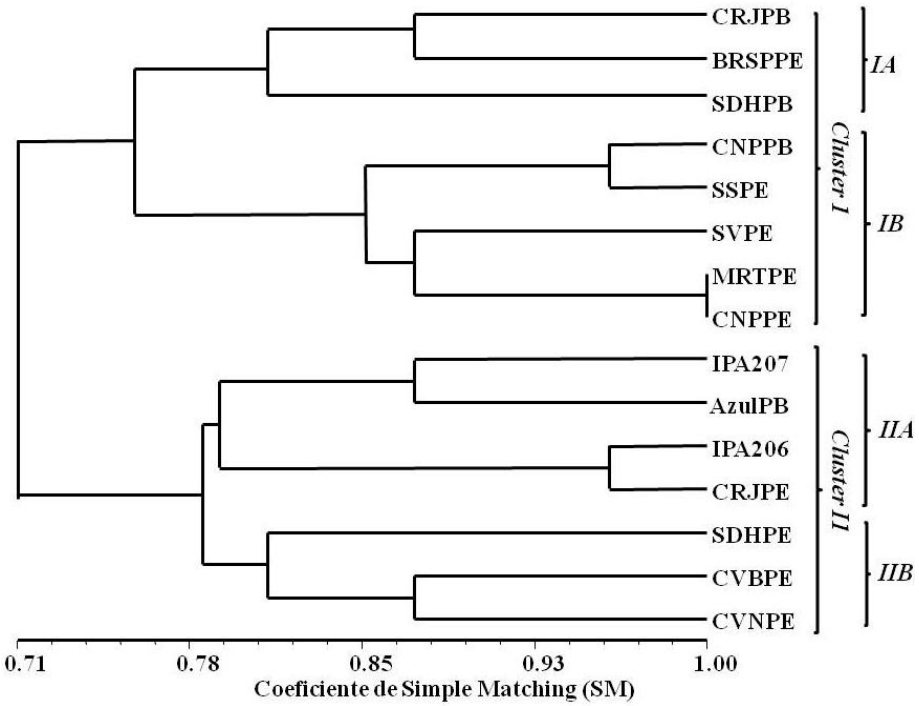


Figure 4. UPGMA dendrogram showing the relationship among cowpea varieties and cultivars using ARDRA techniques with six 06 different endonucleases (*Alu*I, *Hinf*I, *Hpa*II, *Nru*I e *Rsa*I. 1. Corujinha-PB (CRJPB), 2. Sedinha-PB (SDHPB), 3. IPA207, 4. Canapú-PB (CNPPB), 5. Azul-PB, 6. Sempre Verde-PE (SVPE), 7. Sedinha-PE (SDHPE), 8. Maratauã-PE (MRTPE), 9. Canapú-PE (CNPPE), 10. São Sebastião-PE (SSPE), 11. BRS Pujante-PE (BRSPPE), 12. Costela de Vaca Branca-PE (CVBPE), 13. IPA206, 14. Corujinha-PE (CRJPE), 15. Costela de Vaca Marrom-PE (CVMPE).

73% similarity in relation to the other two. The third cluster is formed only by Sedinha-PE and BRS Pujante-PE varieties, and these shares 73% similarity (Figure 3).

In this paper, we show that cowpea varieties with the same title present different genetic traits. For example, in the case of varieties denominated Canapú (Canapú-PB and Canapú-PE), which, even positioned in the same Cluster 1, actually show a genetic distance of 73% (Figure 3).Using the ARDRA technique, a dendrogram with 2 clusters, each with 2 subclusters, was generated (Figure 4). Varieties with the same name, but with different origins (Pernambuco and Paraíba) such as Sedinha, Canapú and Corujinha, showed a large genetic distance. Sedinha and Corujinha from different states were grouped into different subclusters (IA and IIB, and IA and IIA respectively). However, varieties denominated as Canapú, but originated from two different states, showed less genetic distance and both clustered into subcluster IB. Once again, Maratauã-PE and Canapú-PB varieties showed maximal similarity. Canapú-PB and São Sebastião-PE, as well as Corujinha- PE and IPA206 varieties, showed high similarities to one another (greater than 95%). The other varieties, and cultivar IPA206, have higher genetic variability when analyzed by ARDRA techniques. These finding have not been previously reported in the literature using these techniques, for cowpeas.

Although the dendrogram formed by the compilation of the two techniques (Figure 5) also comprises three clusters, also it revealed an increase of the variability for those varieties and cultivars with genetic distances between 60 and 85% and generated three new subcluters (2A, 2B and 2C). Once again, both Canapu (PE and PB) varieties were grouped in the same cluster and Sedinha-PE and BRS Punjante-PE varieties formed the out group.

Data analysis using the dominant program NTSYS-pc showed that the method is to explore and to visualize similarities or dissimilarities of data was constructed for principal coordinate analysis of dominant data (Figure 7), and observed associations between fifteen cowpea cultivars obtained by analysis coordinate primary similarity coefficients of SM calculated from the 720 bands generated by two combinations of five endonucleases in ARDRA analyzes and four combinations of primers ISSR where cultivars CRJPB, SDHPB and IPA207 showed a distance of 1.0.

The sequencing of the ITS1 and ITS2 regions of the cowpea varieties and cultivars resulted in a phylogenetic tree (Figure 6), with two main Clusters (1 and 2). Along

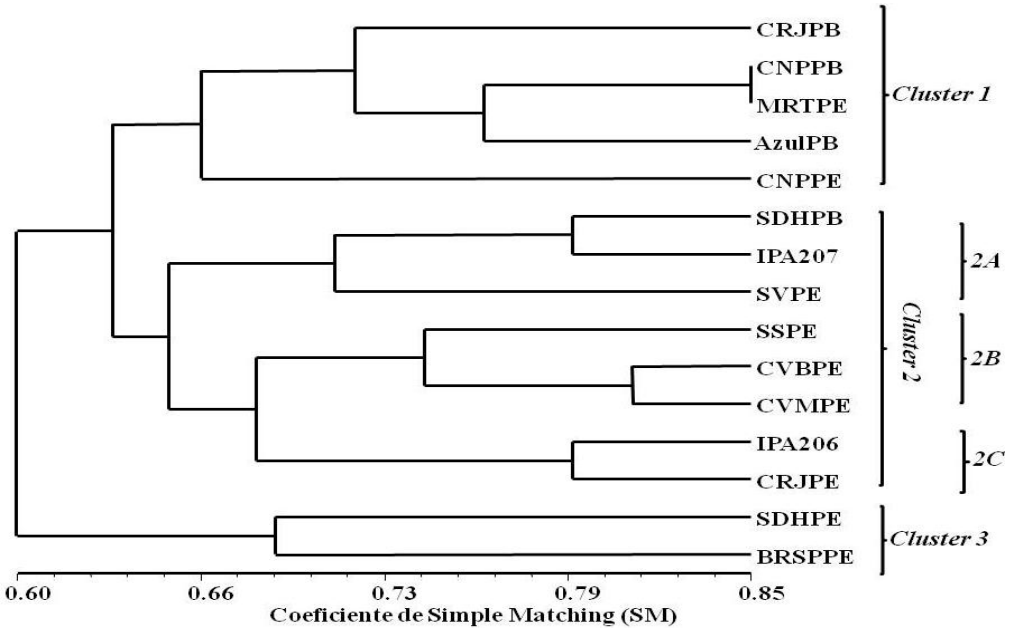


Figure 5. UPGMA dendrogram showing the relationship among cowpea varieties and cultivars using ISSR markers and ARDRA techniques. 1. Corujinha-PB (CRJPB), 2. Sedinha-PB (SDHPB), 3. IPA207, 4. Canapú-PB (CNPPB), 5. Azul-PB, 6. Sempre Verde-PE (SVPE), 7. Sedinha-PE (SDHPE), 8. Maratauã-PE (MRTPE), 9. Canapú-PE (CNPPE), 10. São Sebastião-PE (SSPE), 11. BRS Pujante-PE (BRSPPE), 12. Costela de Vaca Branca-PE (CVBPE), 13. IPA206, 14. Corujinha-PE (CRJPE), 15. Costela de Vaca Marrom-PE (CVMPE).

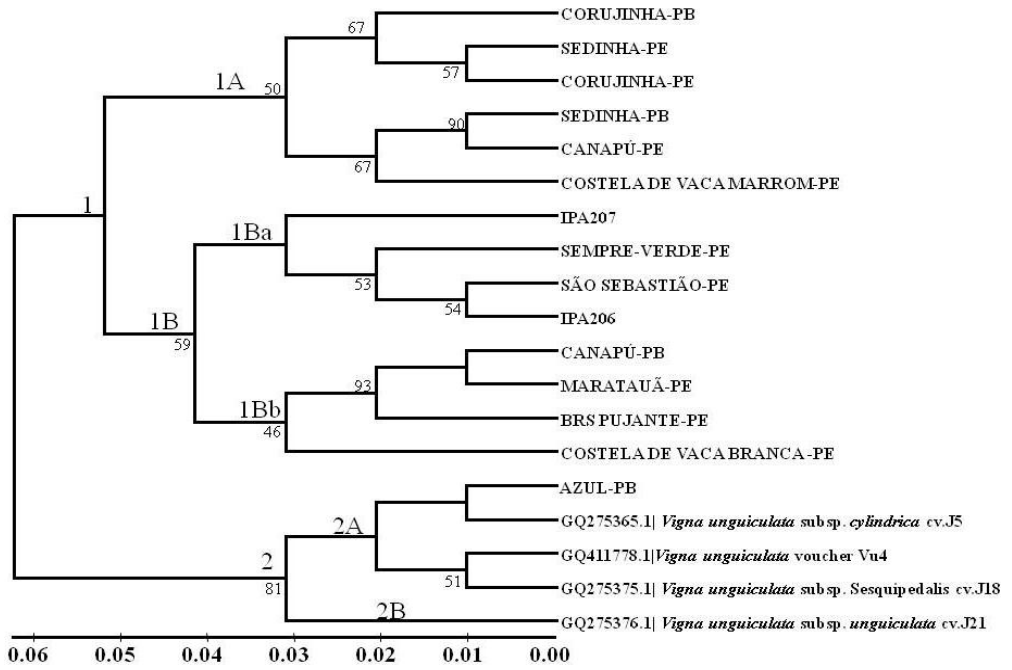
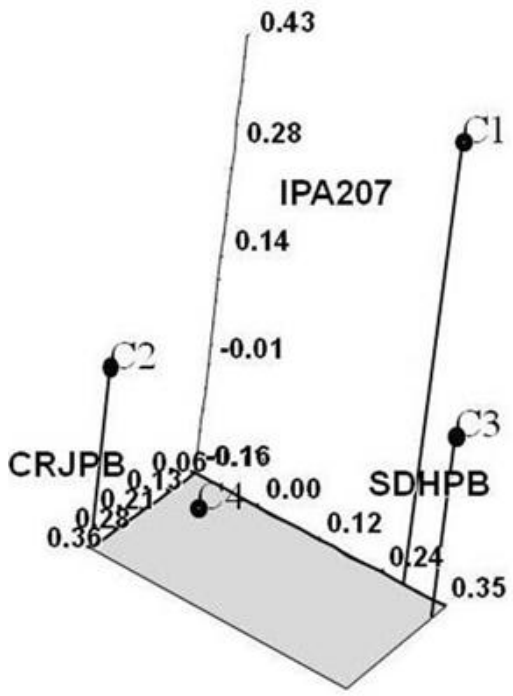


Figura 6. Phylogenetic tree of cowpea varieties and cultivars based in the sequencing of the ITS1 and ITS2 regions using BioEdit and ClustalW programs and Tamura-Nei model computed by the MEGA v.4 program. 1. Corujinha-PB (CRJPB), 2. Sedinha-PB (SDHPB), 3. IPA207, 4. Canapú-PB (CNPPB), 5. Azul-PB, 6. Sempre Verde-PE (SVPE), 7. Sedinha-PE (SDHPE), 8. Maratauã-PE (MRTPE), 9. Canapú-PE (CNPPE), 10. São Sebastião-PE (SSPE), 11. BRS Pujante-PE (BRSPPE), 12. Costela de Vaca Branca-PE (CVBPE), 13. IPA206, 14. Corujinha-PE (CRJPE), 15. Costela de Vaca Marrom-PE (CVMPE).



Cultivars	OTUi	OTUj	Dist
CRJPB	1,0000		
SDHPB	3,1864	1,0000	
IPA207	3,1864	6,2962	1,0000
CNPPB	3,6137	3,1864	3,1864
AZULPB	3,6137	3,1864	3,1864
SVPE	3,1864	4,9936	4,9936
SDHPE	3,0052	3,0052	3,0052
MRTPE	3,6137	3,1864	3,1864
CNPPE	3,0251	3,0251	3,0251
SSPE	3,1864	4,0989	4,0989
BRSPPE	1,3894	1,3894	1,3894
CVBPE	3,1864	4,0989	4,0989
IPA206	3,1864	4,0989	4,0989
CRJPE	3,1864	4,0989	4,0989
CVMPE	3,1964	4,0989	4,0989

Graphic Matrix Cophenetics

Matrix Cophenetics

Figure 7. Matrix cophenetics obtained of UltrametricDis and Graphics Mod3D plot among cowpea varieties and cultivars using ISSR markers and ARDRA techniques. 1. Corujinha-PB (CRJPB), 2. Sedinha-PB (SDHPB), 3. IPA207, 4. Canapú-PB (CNPPB), 5. Azul-PB, 6. Sempre Verde-PE (SVPE), 7. Sedinha-PE (SDHPE), 8. Marataúã-PE (MRTPE), 9. Canapú-PE (CNPPE), 10. São Sebastião-PE (SSPE), 11. BRS Pujante-PE (BRSPPE), 12. Costela de Vaca Branca-PE (CVBPE), 13. IPA206, 14. Corujinha-PE (CRJPE), 15. Costela de Vaca Marrom-PE (CVMPE).

with the studied varieties, four sequences of the *V. unguiculata* species, reiterated from GenBank at National Center for Biotechnology Information (NCBI) were also used. With the exception of the Azul-PE variety, all varieties and both studied cultivars are grouped in Cluster 1, composed by subclusters 1A (with Corujinha-PB, Sedinha-PE, Corujinha-PE, Sedinha-PB, Canapú-PE and Costela de Vaca Marrom-PE) and 1B (which consists of all other cultivars and varieties). The IPA206 cultivar showed 70% similarity to the IPA207 cultivar, and 80 and 90% similarity with Sempre Verde-PE and São Sebastião-PE varieties, respectively (Figure 6). In branch 1B, behaved as monophyletic crude to cultivar BRS Pujante-PE (80%) and the variety Costela de Vaca Branca-PE (70%). The Costela de Vaca Marrom-PE variety was highly genetically different than Costela de Vaca branca, and these cultivars appeared in different groups (1A), confirming that their genetic differences are related to their phenotypic differences. The Canapú-PB and Marataúã-PE varieties showed 90% similarity to each other.

The four subspecies of *V. unguiculata* (*cylindrica*, *voucher*, *sesquipedalis* and *unguiculata*), which were accessed through GenBank, formed the Cluster 2, along with the Azul-PB variety. *V. unguiculata unguiculata* cvJ21 demonstrated the largest genetic distance in relation to the other subspecies (70%). The subspecies *V. unguiculata* voucher Vu4 and the subspecies *sesquipedalis* cvJ18, demonstrated a similarity of 90% among themselves and 80% similarity to both the subspecies *cylindrica* cvJ5 and to Azul-PB variety.

DISCUSSION

The discriminatory potential of the ISSR markers depends on the variety and frequency of microsatellites, which change with the species; this factor explains why simple sequence repeats are the target of the present study. The sequences of nucleotide repeats were anchored to allow the analysis of multiple loci on a single reaction, that is, multiplexed (Ajibade et al., 2000). The reproducibility of fragments generated by ISSR markers exceeds those of analyses of arbitrary sequences of

primers (RAPD). However, Xavier et al. (2005) observed in studies of genetic variability with 45 cowpea allows to discriminate the accessions from different countries, and groups of genotypic Brazilian landraces were grouped into a single group, suggesting a limitation of the genetic basis and there can be a tendency to group themselves according to their origins.

The study of genetic relationships of several species of the genus *Vigna* using the ISSR markers (Ajibade et al., 2000), showed that cultivated varieties of cowpea cluster closely with the subspecies *V. unguiculata*, and that, although clearly separated, these species were also very close to *V. triphylla* and *V. reticulata*. On a sub-generic level, these authors observed that the clustering of taxa differs from the currently accepted classification. Similar data studied with microsatellites genomic shows a powerful tool to evaluate local varieties not yet explored, what comes tbc the data found in this work (Badiane et al., 2012).

There is a growing concern about conservation of the genetic characteristics of cowpea varieties used by the small-scale farmers in the Brazilian semi-arid. In this sense, this work intends to contribute in this field. According to Yang et al. (1996), Culley and Wolfe (2001) and Arnao et al. (2008), the markers used in this work have great potential and population - level polymorphic the polyphasic studies could bring greater clarification of the genetic diversity of these varieties (Zhanou et al., 2008).

A very important factor in the genetic diversity of cowpea according to Nagalakshmi et al. (2010), which is the main contribution to a good rating in the genotypes and the environment that influences a particular character. The character that less influences these characteristics may reveal that it was the least affected in evolution. The results obtained from the sequencing of ITS1 and ITS2 regions suggest that the *V. unguiculata* studied varieties do not have genetic purity. While studying the cowpea lineages of the Sahara regions (likely the center of origin for the species) Pasquet (2000) observed a low variation within and between accessions of cowpea, probably due to the extreme isolation in the Oasis.

Asare et al. (2010) studied the genetic diversity in cowpea in Ghana using SSR markers observed results similar to those found in our study that there is a need for future studies that will conserve and gestionar the cowpea germplasm in this country having as starting point the selection of parental lines for breeding program. Studied data from phenotypic traits in cowpea through analysis of diversity using hybridization protocol and observed that the improvement of culture reveals the potential for reproduction and genetic improvement program in Nigeria (Adewale et al., 2011). In the Brazilian semi-arid, cowpea is grown from seeds obtained from the same field or purchased at fairs, without regard to the genetic purity of the material. The fingerprinting markers and the ITS1 and ITS2 regions of the rDNA sequencing were sufficient to detect the variability of the cowpea germplasm used by small farmers of the Brazilian semi-arid region. Although cowpea varieties often receive the same denomination, a particular variety can contain high diversity, according to the source of the seed. A clear understanding of genetic variation and differences between populations may be helpful for the conservation of cowpea, and therefore efforts to preserve this biodiversity in the Brazilian semi-arid can be a great contribution to this endeavor.

Conclusions

The use of ISSR markers revealed greater genetic variability in the varieties and cultivars investigated and suggested that varieties with the same titles may have different genetic traits. The compilation of the ISSR and ARDRA techniques increased the variability among the studied varieties and cultivars, whose genetic distances varied between 60 and 85%, indicating the need for additional molecular markers. The sequencing of the ITS1 and ITS2 regions from cowpea varieties and cultivars showed that these do not have genetic purity in the Brazilian semi-arid region because the cowpea is grown from seeds obtained from the same cultivated field or acquired in trade fairs.

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