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RESEARCH ARTICLE

ENZYMATIC AND MICROSATELLITE MARKERS FOR THE CHARACTERIZATION OF MILLET CULTIVARS

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ABSTRACT

In this study the objective was to characterize millet cultivars through enzymes and microsatellite markers and select microsatellite primers and enzymatic systems to identify cultivars. Twelve millet cultivars, which were tested in seven enzymatic systems (ADH, CAT, EST, GOT, MDH, IDH and ACP) and 123 pairs of microsatellite primers were evaluated. By the enzymes ACP and CAT was not observed sufficient polymorphism to cultivars differentiation. Sixty pairs of microsatellite primers were selected. About the enzymatic systems evaluated, EST presented more polymorphic to distinguish cultivars. By the primers PSMP2008, PSMP2045 and PSMP2056 is possible to distinguish all cultivars used in this study. It is possible to identify millet cultivars by means of enzymes and microsatellite markers.

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INTRODUCTION

Due increasing the no-tillage area in the country, and land cover potential offered for no-tillage practice, the demand for millet seeds have grown substantially. This increased need for seeds causes companies to invest in breeding programs to develop new cultivars, which creates a lot of work, time and investment. Millet (*Pennisetum glaucum* (L.) R. Br.) is a species that has good grain production, large mass production, fast growth, adaptability to low soil fertility and drought due to these characteristics has been grown direct sowing system in the Brazilian savannah region. To ensure the right of the breeder on the cultivar developed, in the country was approved the Cultivars protection Law. However, for the law effective implementation, the production control and illegal seeds sale has been a major challenge, which has compromised the seeds quality available to farmers, with direct effects on productivity. Thus, certification of genetic purity cultivars, safely, it becomes an indispensable practice in quality control programs for companies to ensure the genetic characteristics of the cultivars developed by the breeder, in addition to providing allowances for the cultivars protection (GRATAPAGLIA and FERREIRA, 1996). For the cultivars protection it is required by the breeder the DHE test to meet the homogeneity, cultivar distinctiveness and descriptors stability criteria. Morphological descriptors have been used to characterize the cultivars and ensure their protection. However, these markers have disadvantages such as need of a large descriptors number, mostly in adult or whole plant. In addition to the spent time and the physical space need to genotypes assessment, some morphological markers can be influenced by the environment. Thus, enzymes' molecular markers and DNA have particularities that make them extremely useful for the cultivars protection and certification of genetic purity in seed lots. Microsatellite markers have characteristics that make them safe markers for cultivars characterization. Typically, few locus ensure complete differentiation of the interest genotypes (Schuster *et al*., 2006). The enzymes markers are codominant and depending on the enzymatic system used can be considered useful for the cultivars characterization. In this study the objective was to characterize millet cultivars through enzymes and microsatellite markers and select microsatellite primers and enzymatic systems to identify cultivars.

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MATERIAL AND METHODS

The work was conducted at the Seeds Laboratory Center of the Agriculture Departament - Federal University of Lavras. Twelve millet cultivars (ADR 300, ADR 500, ADR 7010, ADR 7020, ADR 8010, ANSB MC, ANM 17, ANM 30, IPA BULK 1BF, BN-1, BN-2 and BRS 1501) were used. For the enzymes extraction and DNA were sampled 200 millet seeds of each cultivar according to the methodology described by Mendonça Neto (2013). Enzymatic systems used were: alcohol dehydrogenase (ADH - EC 1.1.1.1), catalase (CAT - EC 1.11.1.6), esterase (EST - 3.1.1.1), glutamate oxaloacetate transaminase (GOT - EC 2.6.1.1), malate dehydrogenase (MDH - EC 1.1.1.37), isocitrate dehydrogenase (IDH - EC 1.1.1.42), acid phosphatase (ACP - EC 3.1.3.2), according to the methodology described by Alfenas (2006). For the microsatellite markers (SSR) polymerase chain reactions were performed (PCR) in a medium with a final volume 25µl containing 3µl of genomic DNA solution (10ng/µL), 0.75µl of each primer (10µM) with a compound mix: 15.55µl ultrapure water; 2.5µl buffer 10X; 1.0µl MgCl2 25mM; 1.25µl DNTP 10 mM; 0.2µl Platinum® Taq DNA Polymerase 5U/µl. The amplification program consisted 94 °C starting temperature for five minutes, five amplification cycles at 94 °C for 30 seconds, 57 °C for 30 seconds. In this stage was adapted a Toch Down, decreasing 0.5 °C every cycle, 72 °C for 30 seconds. Followed by another 25 cycles started at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds. The extension was performed at 72 °C for five minutes. One hundred twenty three pairs of specific primers for the species described in works (BUDAK *et al*., 2003; MARIAC *et al*., 2006; YADAV *et al*., 2007; KAPILA *et al*., 2008; ALLOUIS *et al*., 2001; QI *et al*., 2001; QI *et al*., 2004) were tested. The gels analyzes of SSR following the methodology described by Mendonça Neto (2013). The primer and the enzymes results were used in the preparation of a matrix to calculate the estimates of genetic similarity using the Jaccard coefficient. It was performed the genotypes grouping using the average method unweighted averages of similarities (UPGMA), calculations were made through the XLSTAT program (ADDINSOFT®, Version 2015.4.1).

RESULTS AND DISCUSSION

In this work, it was not considered relative differences to the bands intensities formed in the gels. So, for easy results viewing were also built zymograms as schemas in which each band was represented by a dash, after the polymorphism analysis. Are presented below, the relative electrophoretic patterns for the seven enzymatic systems adopted for the identification of the twelve millet cultivars evaluated in this work. In Figure 1 it can be seen that by the ADH system was possible to distinguish the cultivars into two groups. The first consists of the cultivars: ADR 300; ADR 7010; ADR 7020; ADR 8010 and BRS 1501, and the second by the cultivars: ADR 500; ANM 17; ANM 30; ANSB MC; IPA BULK 1BF; BN-2 and BN-1. It was possible by this system to differentiate cultivars from the first group those from the second group by the presence of a third isoform with lower molecular weight. In the first group isoenzyme is dimer formed from two peptides and in the second monomer group consisting of a peptide. Several authors use the ADH system in surveys to identify cultivars with different results. Bonow *et al*. (2009), working with rice seeds; Ferreira *et al*. (2009) with the species Gladíolo and Salgado *et al*. (2006) working with maize hybrids, they could not separate cultivars by this system. As to Mendonça Neto *et al*. (2013) observed polymorphism among the millet cultivars when using ADH enzyme as a marker. By the criteria adopted in this study, the electrophoretic pattern of the CAT enzyme was not polymorphic enough to be used to help distinguish the cultivars.

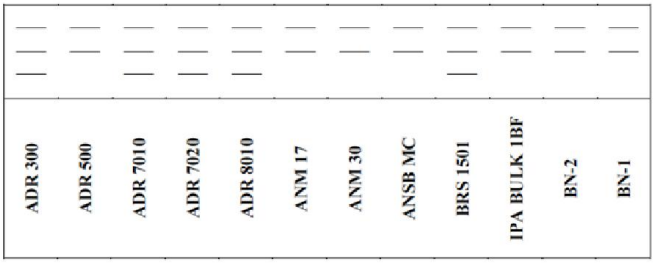

Figure 1. Zymogram of alcohol dehydrogenase isoenzyme (ADH) in seeds of millet cultivars

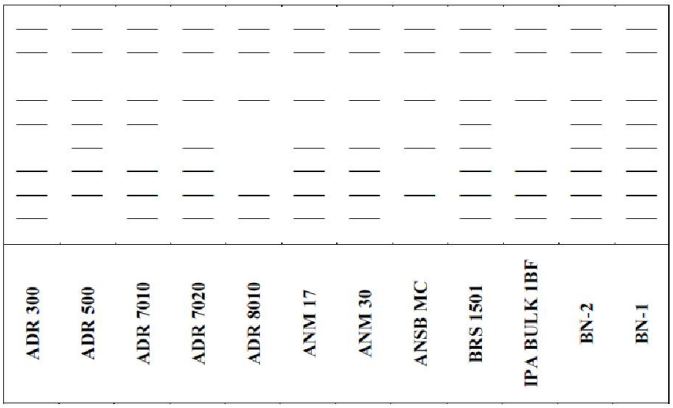

Figure 2. Zymogram of esterase isoenzyme (EST) on seeds of millet cultivars

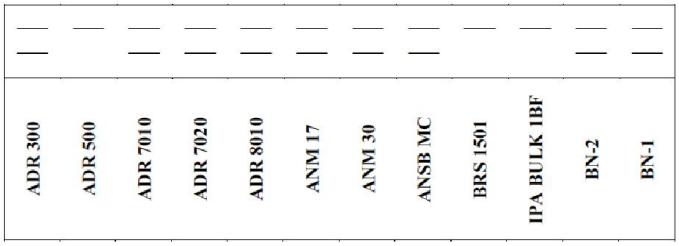

Figure 3. Zymogram of glutamate oxaloacetate transaminase isoenzyme (GOT) in seeds of millet cultivars

In the zymogram of esterase enzyme, Figure 2, it was observed polymorphism, whereby it was possible to distinguish the cultivars into seven groups. The first consists of the cultivars ADR 300, ADR 7010, the second constituted by the ADR 500; the third by the cultivars ADR 7020; ANM 17; ANM 30; the fourth by the ADR 8010; the fifth by the ANSB MC; the sixth by BRS 1501; BN-2 and BN-1 and seventh by the IPA BULK 1BF. It is noticed that by this enzyme system the cultivars; ADR 500; ADR 8010; ANSB MC and IPA BULK 1BF, were

distinguished from each other. The esterase has been considered important markers for the cultivars characterization to be easy detecting and frequent expression in seeds. Bonow *et al*. (2009) observed polymorphism among rice cultivars when using this system. It was noted that even in rice seeds infected with pathogens and damaged this marker showed as an important resource for identifying cultivars. Mendonça Neto *et al*. (2013) also observed polymorphism when using esterase enzyme for the characterization of millet cultivars BN-2, ADR 300 and ADR 500. This characterization was also observed in this study because the cultivars BN-2; ADR 300 and ADR 500, were placed in different groups. Salgado *et al*. (2006) found that by this system, there was Mendelian segregation for the maize hybrid UFLA 8/3 which made safer genetic purity certification. Ferreira *et al.* (2009) extracting enzyme of leaves gladioli were able to separate all cultivars when using this system.

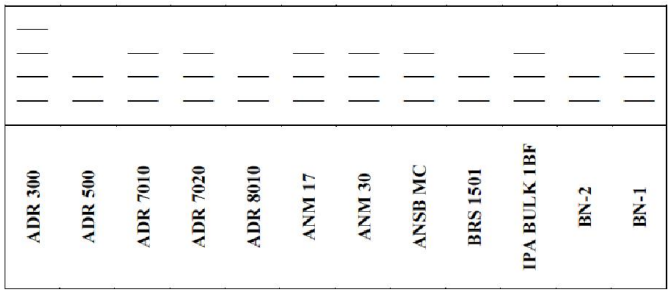

Figure 4. Zymograms of malate dehydrogenase isoenzyme (MDH) in seeds of millet cultivars

The enzyme GOT electrophoretic pattern, Figure 3, only two groups were formed, the first by the cultivars; ADR 300; ADR 7010; ADR 7020; ADR 8010; ANM 17; ANM 30; ANSB MC; BN-2; BN-1, and the second by the ADR 500; BRS 1501 and IPA BULK 1BF. Mendonça Neto *et al*. (2013) using this system in millet were able to distinguish the cultivars ADR300; ADR500; BN-2; IPABULK 1BF; BRS 1501 and ADR7010.

In this work the cultivars ADR500; BRS1501 and IPABULK 1BF were not distinct from one another, but were separated of the cultivars ADR300, ADR7010 and BN-2, which were not distinct from each other. By the MDH system, Figure 4, it was possible to separate the cultivars into three groups being the first compound by the ADR 300; the second by the cultivars ADR 500; ADR 8010; BRS 1501 and BN-2 and the third by the ADR 7010; ADR 7020; ANM 17; ANM 30; ANSB MC; IPABULK 1BF and BN-1. For this system it was possible to distinguish the cultivar ADR 300 from all other cultivars evaluated. Menezes *et al.* (2008) separated cotton cultivars regardless of the seeds physiological quality from which the enzyme was extracted. It is observed by the Figure 5 that the IDH system was possible to distinguish the cultivars BRS 1501 and IPA BULK 1BF from each other and all of them that composing a single group. With this system Salgado *et al.* (2006) were able to separate maize hybrid UFLA 8/3 and UFLA 7/4 of their respective progenitors. Vieira *et al*. (2009) were able to differentiate soybean cultivars UFV16 and Garantia with this system and Bonow *et al.* (2009) working with rice was not successful in separating cultivars. By the ACP system was not observed polymorphism to distinguish cultivars. Freitas *et al.* (2000) working with seven cultivars of elephant grass and its hybrids with millet, concluded that while not useful for identifying cultivars this system contributed to analyze the degree of genetic similarity.

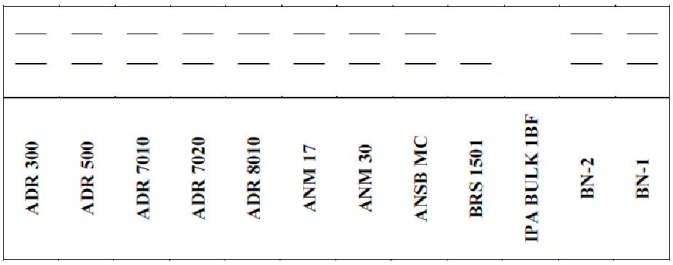

Figure 5. Zymogram of isocitrate dehydrogenase isoenzyme (IDH) in seeds of millet cultivars

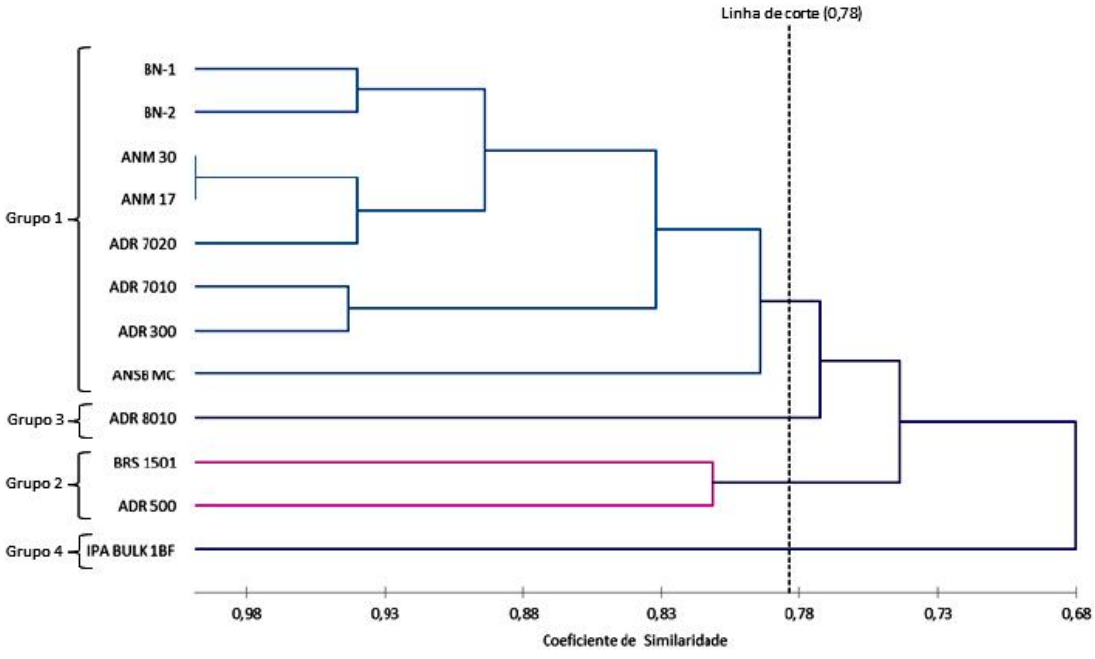

Figure 6. Dendrogram of similarity of the twelve millet cultivars, obtained by cluster analysis UPGMA, estimated by Jaccard coefficient, based on isoenzyme analysis in seeds

Regarding the genetic similarity study considering the cut line, Figure 6, were formed four cultivars groups constituted as follows: Group 1: ADR 300; ADR 7010; ADR 7020; ANM 17; ANM 30; ANSB MC, BN-2; BN-1; Group 2: ADR 500; BRS 1501 Group 3: ADR 8010; Group 4: IPA BULK 1BF. By the Figure 6, it is observed less genetic similarity between the cultivar IPA BULK 1B and other cultivars. This can be explained by the fact that cultivar IPA BULK 1BF have been developed from a selection of 400 different progenies (TABOSA *et al*., 1999). For cultivars BRS 1501 and ADR 500 was observed close similarity 0.81 forming a group that has close similarity 0.74 in relation to the groups formed by other cultivars. It can be seen the higher similarity value 1.0 between cultivars ANM 17 and ANM 30 having similarity 0.94 with the cultivar ADR 7020. Between the cultivars BN-1 and BN-2 were observed similarity 0.94 these cultivars have been developed from phenotypic mass selection with intention to improve features cultivars from the *Goiás* state (BRAZ *et al*., 2004). Between the cultivars ADR 7010 and ADR 300 were observed similarity 0.94. The cultivar ADR 7010 is a hybrid and probably the cultivar ADR 300 has been used in the cultivar development process. Cultivar ANSB MC has similarity 0.80 with the other cultivars of the group 1.

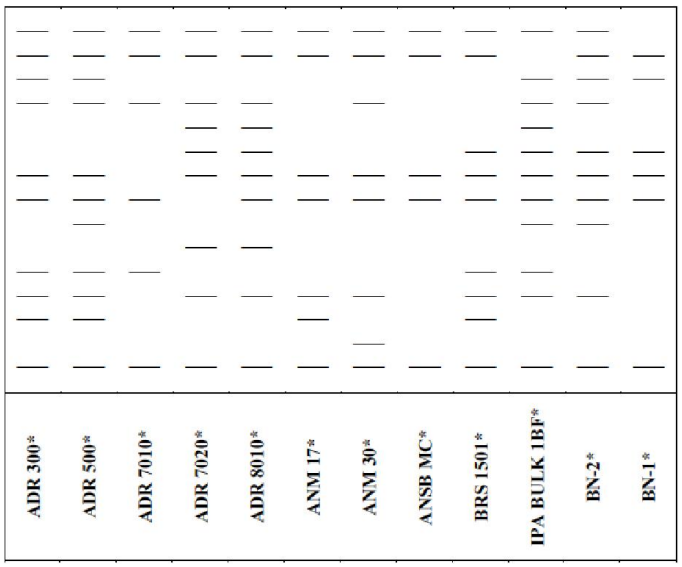

Figure 7. Zymogram from the primer amplification PSMP2008 in seeds of millet cultivar. Note: * Cultivars identified

In general, it is observed higher similarity values between the materials of the same breeder company. Thus, the cultivars from the company *Adriana Sementes* (ADR) were grouped in group 1 with exception of ADR 500 which is variety of a population introduced of Africa and India and ADR 8010 which is a hybrid derived from different parents of the other cultivars developed by the company. In the same group 1 are inserted all cultivars from the company *Agronorte Pesquisa e Sementes* (AN). Group 2 contains the cultivars ADR 500 and BRS 1501 of *Embrapa* Maize and Sorghum, in group 3 is the cultivar ADR 8010 and in group 4 the cultivar from the *Instituto Agronômico de Pernambuco* - IPA (IPA BULK 1BF). Mendonça Neto *et al*. (2013) working with identifying millet cultivars observed similarities ranging from 0.50 to 1.00 among cultivars witnesses and marketed lots. In the same study, it was observed similarity 0.5 from the cultivar BN-2 in relation to the other cultivars studied, the cultivars ADR 500, ADR 7010 and BRS 1501 showed similarity 0.80 with each other, the cultivar IPA BULK 1BF presents similarity 0.87 with the cultivar ADR 300. Among the 123 primers tested, 60 presented polymorphism, were selected for presentation in this work the primers PSMP2008 (Figure 7), PSMP2045 (Figure 8) and PSMP2056 (Figure 9) where it was possible to distinguish all cultivars used. In these primers the polymorphic pattern of each cultivar was unique, being with this possible the separation of each cultivar. Several authors used microsatellites in researches to identify cultivars with different results. Mendonça Neto *et al*. (2013) working with millet seeds were able to distinguish the cultivars ADR 300, ADR 500, ADR 7010, BN-2, IPA BULK 1BF and BRS 1501 with four primers. Also working with millet Kapila *et al*. (2008) used 25 primers to assess the genetic diversity of 421 millet accesses of the Nigeria and found low number of polymorphic fragments. Bonow *et al*. (2009), working with rice, emphasized that only five primers were enough for distinguishing all genotypes studied and the microsatellite analysis allowed the characterization and individualization of all cultivars studied as the subspecies. Vieira *et al*. (2009) working with 53 soybean cultivars and 283 microsatellite markers, they concluded that 53 microsatellites evaluated presented higher informativeness and it is possible to detect differences in the evaluated germplasm. Salgado *et al*. (2006) working with maize found that microsatellite markers were able to distinguish the hybrids studied from their parental lines securely.

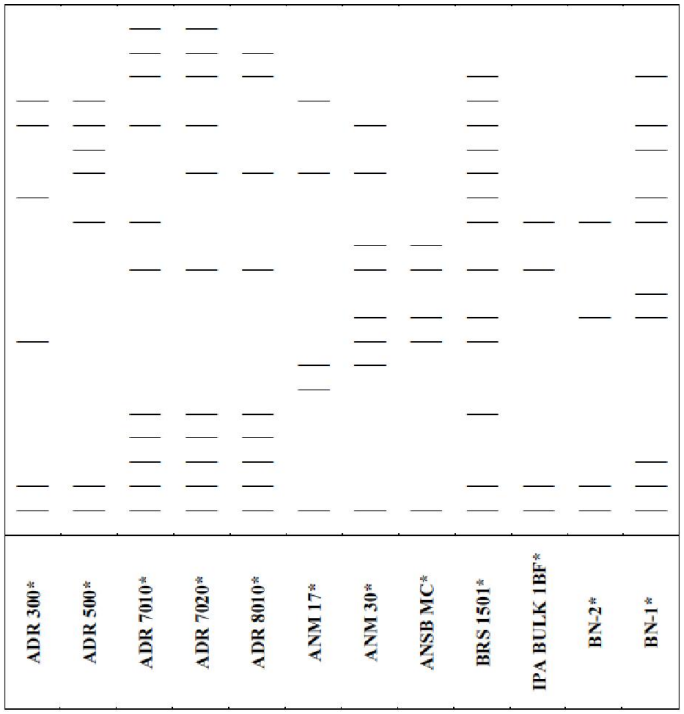

Figure 8. Zymogram from the primer amplification PSMP2045 in seeds of millet cultivar. Note: * Cultivars identified

Several authors working with soybean and microsatellite markers in cultivar identification studies obtained different results. Oliveira *et al*. (2010) evaluated 32 soybean cultivars with 48 microsatellite markers and distinguished all cultivars,

including cultivars with high similarity rate. Priolli *et al*. (2002) identified 184 of 186 soybean cultivars evaluated. Garcia *et al*. (2007) evaluated 69 microsatellites and selected 10 to be routinely used in the soybean cultivars characterization. Ribeiro *et al*. (2013) identified six primers with high genetic diversity values, which were able to differentiate all 48 cultivars analyzed. From unique patterns observed for the cultivars it is possible to use marker to certify the genetic purity of a particular cultivar in relation to others.

In the specific millet case there are not many registered cultivars for the marketing of seed and protected, which makes feasible this type of marker for the certification of genetic purity and certification of seeds origin.

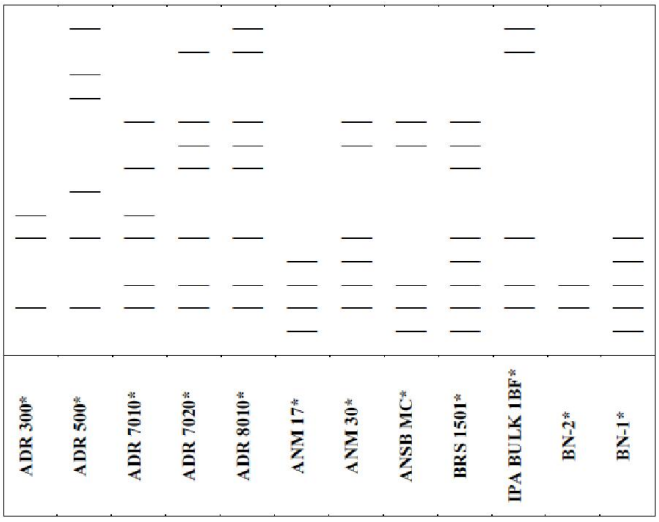

Figure 9. Zymogram from the primer amplification PSMP2056 in seeds of millet cultivar. Note: * Cultivars identified

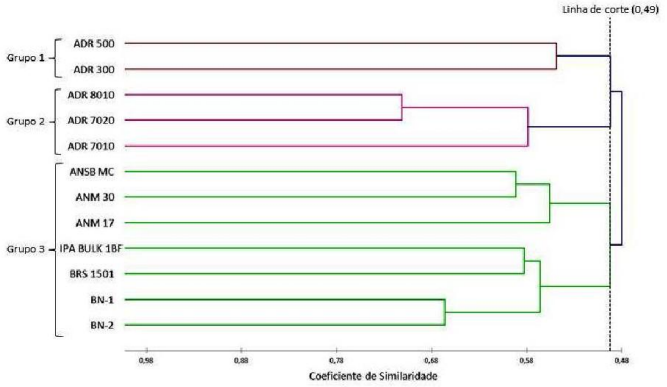

Figure 10. Dendrogram of similarity of the twelve millet cultivars, obtained by cluster analysis UPGMA, estimated by Jaccard coefficient, based on microsatellite analysis

Regarding the genetic similarity study considering the cut line, Figure 10, were formed three cultivars groups constituted as follows: Group 1: ADR 300 and ADR 500; Group 2: ADR 7010, ADR 7020 and ADR 8010 and Group 3: ANM 17, ANM 30, ANSB MC, BRS 1501, IPA BULK 1BF, BN-2 and BN-1. For the similarity calculation, Figure 10, the zymograms of 60 polymorphic primers were used. The results obtained by microsatellite markers can be observed that the higher similarity value (0.71) was found between the cultivars ADR 8010 and ADR 7020 can infer that populations in common may have been used in their development. These two cultivars presented similarity index of approximately 0.58 regarding to cultivate ADR 7010. As observed for protein markers the cultivars BN-1 and BN-2 were grouped with 0.66 similarity and showed similarity 0.56 with the cultivars IPA BULK 1BF and BRS 1501 these with 0.58 similarity. In this same group can be verified that the cultivar ANSB MC and ANM 30 have similarity 0.59 and that are similar to ANM 17 in 0.55. The similarity between the cultivars of this group is 0.49. The cultivar ADR 300 has similarity 0.55 in relation to cultivate ADR 500, these cultivars were selected within populations introduced of Africa and India. The number of SSR markers used was higher than that used for the enzymes. As the regions of the genome evaluated using these markers are distinct, it expects different indexes of similarity when compared to each other. To identify cultivars aiming to the certification of genetic purity, when higher the similarity, higher the difficulty to find a marker that can be used for the certification of genetic purity in seed lots, mainly aimed to the marketing of legal seeds.

Conclusions

It is possible to identify millet cultivars by means of enzymes and microsatellite markers. From the enzymatic systems evaluated the esterase appears more polymorphic for the distinguish cultivars. By the primers PSMP2008, PSMP2045 and PSMP2056, it is possible to distinguish all the millet cultivars used in this study.

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