

Molecular marker-based genetic diversity analysis of scantily studied Brazilian accessions of a medicinal plant, *Morinda citrifolia* L. (noni)

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ABSTRACT. *Morinda citrifolia* L., commonly known as noni, has been used for the treatment of various diseases for over two centuries. It was introduced and widely disseminated in Brazil because of its high market value and ease of adaptation to the soil and climatic conditions of the country. The aim of this study was to estimate the genetic variability of noni accessions from the collection of Embrapa Agroindústria Tropical in Brazil. We evaluated 36 plants of the 13 accessions of noni from the germplasm collection of *M. citrifolia*. Several methods of DNA extraction were tested. After definition of the method, the DNA of each sample was subjected to polymerase chain reactions using 20 random amplified polymorphic DNA primers. The band patterns on agarose gel were converted into a binary data matrix, which was used to estimate the genetic distances between the plants and to perform the cluster analyses. Of the total number of markers used in this study, 125 (81.1%) were polymorphic. The genetic distances between the genotypes ranged from 0.04 to 0.49. Regardless of the high number of polymorphic bands, the genetic variability of the noni plants evaluated was low since most of the genotypes belonged to the same

cluster as shown by the dendrogram and Tocher's cluster analysis. The low genetic diversity among the studied noni individuals indicates that additional variability should be introduced in the germplasm collection of noni by gathering new individuals and/or by hybridizing contrasting individuals.

Key words: *Morinda citrifolia*; Genetic variability; RAPD; Polymorphic markers; Germplasm collection

INTRODUCTION

The species *Morinda citrifolia* L., popularly known as noni, originates from southeast Asia and has been used by the inhabitants of Polynesia for more than 2000 years for the treatment of several diseases (León and Poveda, 2000), such as high blood pressure, constipation, malaria, and even cancer (Rodríguez and Pinedo, 2004). Owing to its widespread dissemination, it is now found in various parts of the world, such as the tropical regions of Africa, the Caribbean, Australia, China, Malaysia, Indonesia, India, and some South American countries, such as Ecuador, Surinam, and Brazil (McClatchey, 2002; Correia et al., 2011). It is a species that adapts very well to coastal regions, from sea level up to 400 m altitude (Lübeck and Hannes, 2001) and has tolerance to saline soils and water deficit. These environmental characteristics favor Brazil as a potential producer of this species. Cultivation of noni is quite recent in Brazil, and is carried out in an empirical manner. The demand for noni is high mainly because of its high market value and the ease of obtaining seeds and seedlings, which are traded online (Sousa et al., 2010). It is grown in various states of Brazil, including Pará (Otelo, 2006), Maranhão (Silva, 2010), Sergipe, and Ceará, among others (Correia et al., 2011).

Owing to the medicinal properties attributed to it, noni has awakened the interest of producers and the pharmaceutical industry. However, there is still a lack of basic information, such as the cultivation practices, time of harvest, nutritional requirements, and genetic characterization of noni. This lack of information can compromise the quality of the final product due to the inadequate management practices. For example, the lack of information regarding the choice of the best genotype for cultivation, might limit the exploitation of genetic potential for the purpose of maximizing important characteristics of this plant.

Random amplified polymorphic DNA (RAPD) markers have been used until now to analyze the diversity, genetic relationships, and germplasm management of various species. Other markers, such as simple sequence repeat (SSR) markers, have also been used for the same purpose, but this becomes non-viable in the absence of genetic information. Other examples of the use of RAPD markers for studying genetic diversity can be cited, such as for artichoke (Sharaf-Eldin et al., 2015), bamboo (Desai et al., 2015), grass (Ghariani et al., 2015), and lemongrass (Bishoyi et al., 2016), among others. These markers have already been successfully used for studying genetic variation among the three species of noni (Singh et al., 2011) and for determining the germplasm diversity of *M. citrifolia* for plant breeding and germplasm management in India (Singh et al., 2012). However, in Brazil, no studies involving molecular markers in noni have been conducted thus far. Thus, the aim of this research was to study the magnitude of genetic divergence through RAPD markers among the accessions of noni that constitute the germplasm collection of Embrapa Agroindústria Tropical in Brazil.

MATERIAL AND METHODS

We evaluated 36 plants from the 13 accessions of noni grown by seed from the *M. citrifolia* germplasm collection (Table 1) of Embrapa Agroindústria Tropical established in the experimental field in Pacajus, CE, Brazil. Leaves were collected from two or three plants of each accession of noni, and were immediately put in 15 mL tubes, and then placed in liquid nitrogen for transport to the molecular biology laboratory, where the DNA of each plant was extracted, following the protocol described by Diniz et al. (2005). The NanoDrop 2000 system (Thermo Scientific, Waltham, MA, USA) was used to quantify and evaluate the purity of the DNA.

Table 1. Plant codes and passport data of 13 accessions of noni (*Morinda citrifolia* L.) of the noni germplasm collection of Embrapa Agroindústria Tropical, Brazil.

Plant genotype	Accession	Collection point	Place of origin
NN01-01, NN01-02, NN01-03	CNPAT 1	Aracaju-SE	Paramaribo, Suriname
NN02-01, NN02-02, NN02-03	CNPAT 2	Aracaju-SE	Austrália
NN03-01, NN03-02, NN03-03	CNPAT 3	Tomé-Açu-PA	Japão
NN04-01, NN04-02, NN04-03	CNPAT 4	Campinas-SP	Fiji
NN05-01, NN05-02, NN05-03	CNPAT 5	Aracaju-SE	Paramaribo, Suriname
NN06-01, NN06-02, NN06-03	CNPAT 6	Tomé-Açu-PA	Japão
NN07-01, NN07-02, NN07-03	CNPAT 7	Guaiúba-CE	Unknown
NN08-01, NN08-02	CNPAT 8	Fortaleza-CE	Unknown
NN09-01, NN09-02, NN09-03	CNPAT 9	Uruoca-CE	Unknown
NN10-01, NN10-02, NN10-03	CNPAT 10	Santa Quitéria-CE	Teresina - PI
NN11-01, NN11-02	CNPAT 11	Crato-CE	Maranhão
NN12-01, NN12-02	CNPAT 12	Euzébio-CE	Unknown
NN13-01, NN13-02, NN13-03	CNPAT 13	Caicó-RN	Unknown

Polymerase chain reactions (PCR) were carried out using 20 RAPD primers (OPL01, OPA08, OPAA04, OPAA06, OPO02, OPN01, OPAB05, OPW10, OPR05, OPAB10, OPN17, OPW08, OPR06, OPW07, OPN02, OPAA03, OPAA02, OPL02, OPR07, and OPN14; Operon Technologies, Alameda, CA, USA). The PCRs were carried out in 25- μ L reaction volume containing 1X PCR buffer, 6 mM MgCl₂, 2.17 μ M each primer, 200 μ M dNTP, 35 ng DNA, 1 U Taq DNA polymerase, and ultra-pure water. Reactions were carried out in the thermocycler Techne TC-512 (Techne Ltd., Cambridge, UK) adjusted for initial denaturation at 96°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 35°C for 1 min, and extension at 72°C for 1:30 min, concluding with a final extension at 72°C for 7 min. The amplified fragments were separated on 1.8% agarose gel in 1X TBE (Tris-borate-EDTA) buffer and stained with ethidium bromide, and the images were captured in an imaging system under ultraviolet light.

The patterns of the presence/absence of markers (bands) of each RAPD initiator were codified in a binary matrix in which '0' and '1' referred to the absence and presence of the marker, respectively. The polymorphism of RAPD markers was estimated by calculating the percentage of polymorphic markers with regard to the total number of fragments amplified.

The genetic distance matrix was constructed using the arithmetic complement of the Jaccard Coefficient. To determine the optimum number of amplified markers necessary for studying genetic diversity, the correlation estimates (r) and the stress value (E) were obtained from the dissimilarity matrix. The number was considered satisfactory when the correlation coefficient was ≥ 0.95 , and the stress was less than 0.05 (Kruskal, 1964). The dendrogram was constructed via the unweighted pair group method using arithmetic averages (UPGMA). The

bootstrap analysis was also carried out to verify the consistency of the bifurcations of the dendrogram. The mean value of the genetic distance matrix was used for the separation of the clusters. Tocher's clustering method was used to analyze genetic divergence (Rao, 1952). Statistical analyses were carried out using the GENES software (Cruz, 2016).

RESULTS AND DISCUSSION

All 20 RAPD primers used in this study led to the amplification of the target DNA and exhibited polymorphism. In all, 154 bands were produced, with an average of 7.7 bands per primer. Of these, 125 bands were polymorphic (81.1%). The number of polymorphic bands per primer ranged from 4 (OPAA2, OPAB5, and OPW8) to 11 (OPAA4).

The high number of polymorphic bands might be due to the fact that the samples were obtained from distinct plants, even when dealing with the same accession. Furthermore, although noni has been described as a preferentially self-pollinating plant (Nelson, 2003), it also has pistillate florets (Waki et al., 2008), suggesting the possibility of cross-pollination, which might be associated with high number of polymorphic bands.

Thus, in spite of the high number of polymorphic bands, the genetic variability of these noni plants was low, showing the need to gather other individuals to increase the variability in this noni germplasm collection. The reason for this was that five clusters were formed, and most of the genotypes belonged to the same cluster of the dendrogram (Figure 1). In this cluster, the magnitude of the distances between the genotypes was not sufficient to separate them into distinct clusters, showing that the high number of polymorphic bands did not result in high genetic differentiation.

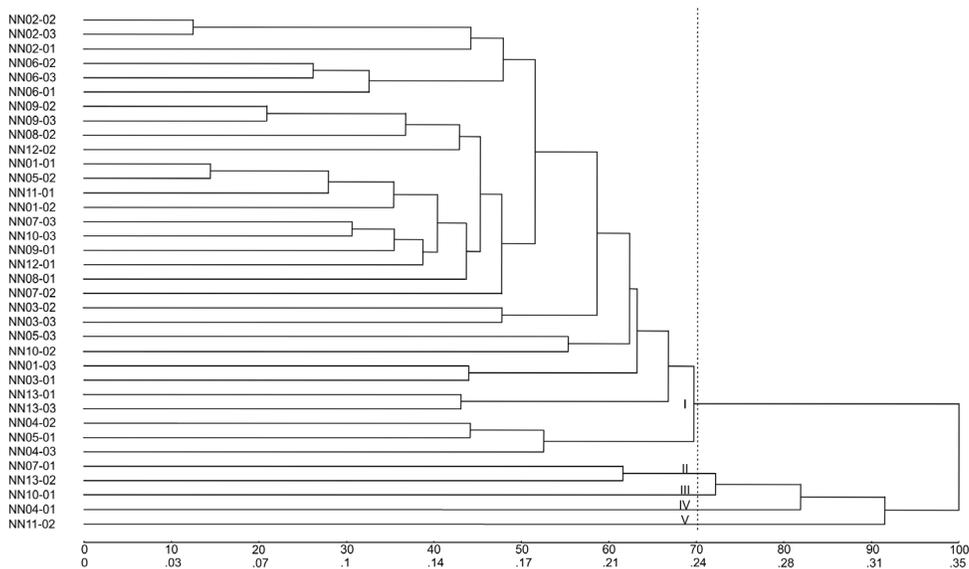


Figure 1. Clustering pattern of 36 noni genotypes of noni (*Morinda citrifolia* L.) from the germplasm collection at Embrapa Agroindústria Tropical, Brazil. The dendrogram was constructed by using the unweighted pair group method using arithmetic averages (UPGMA) based on Jaccard's dissimilarity coefficient. The mean value of the genetic distance matrix was used for separation of the clusters.

Analysis of the optimum number of markers indicated that it was possible to represent the same level of genetic diversity with 96 polymorphic bands ($r = 0.976$; $E = 0.049$) as was defined by 129 polymorphic bands generated by the 20 RAPD primers (Figure 2). As the number of polymorphic bands (129) generated in this study was greater than the optimum number of markers (96), it can be inferred that our results were consistent.

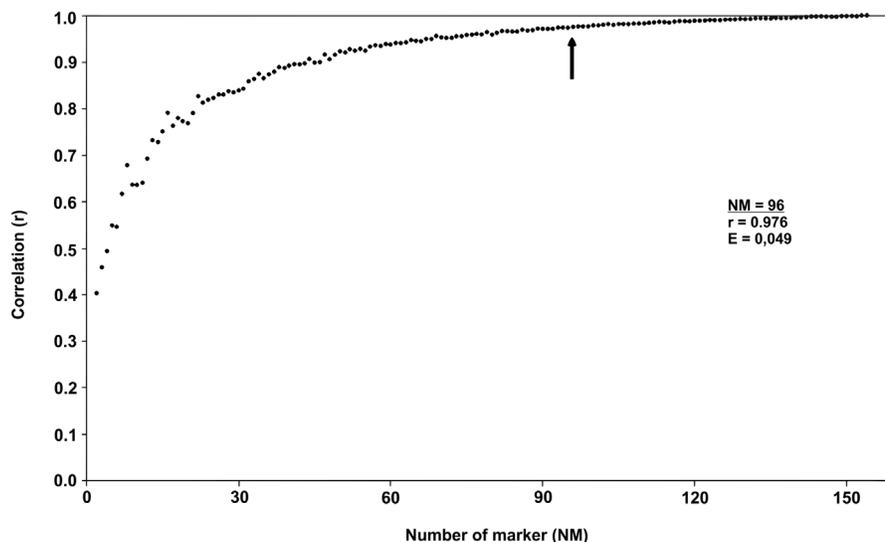


Figure 2. Analysis of the optimum number of markers based on the relationship between correlation coefficient (r) and number of fragments obtained for noni (*Morinda citrifolia* L.) genotypes from the germplasm collection at Embrapa Agroindústria Tropical, Brazil.

According to Tocher's cluster analysis and optimization based on genetic distances, the genotypes were arranged into four clusters of dissimilarity. Of the 36 genotypes, 31 were present in the first cluster. The genotypes NN07-01, NN13-02, and NN10-01 formed the second cluster. The third and fourth clusters were formed by the genotypes NN04-01 and NN11-02, respectively (Table 2).

Table 2. Clustering of 36 noni (*Morinda citrifolia* L.) genotypes from the germplasm collection at Embrapa Tropical Agroindustry based on Tocher's clustering method. The analysis were run based on Jaccard's dissimilarity matrix.

Cluster	Genotypes
I	NN02-02, NN02-03, NN05-02, NN01-01, NN11-01, NN06-03, NN06-02, NN06-01, NN10-03, NN01-02, NN09-03, NN08-02, NN09-01, NN12-01, NN07-03, NN08-01, NN12-02, NN07-02, NN02-01, NN09-02, NN03-02, NN03-01, NN03-03, NN13-01, NN10-02, NN05-03, NN04-03, NN01-03, NN04-02, NN13-03, and NN05-01
II	NN07-01, NN13-02, and NN10-01
III	NN04-01
IV	NN11-02

In the dendrogram generated by the UPGMA method, the genotypes belonging to the accession CNPAT 2 (NN02-01, NN02-02, and NN02-03) remained close, in the same branch of the cluster analysis. The same can be observed for the accession CNPAT 6 (NN06-01, NN06-02, and NN06-03) (Figure 1). This indicates the genetic proximity of these plants coming from the same accession, even though they were derived from distinct seeds; thus,

they were probably half-sibs. The other clusters showed little relationship with the origin of the seeds. It should be noted that an accession is a sample of live material, which represents an individual or various individuals of a population (Walter and Cavalcanti, 2005).

The distribution of the genotypes in the dendrogram (Figure 1) was similar to that observed in the Tocher's cluster analysis (Table 2), except for the genotype NN10-01. Thus, cluster analysis by the UPGMA method and the Tocher's method were mostly in agreement, even with different criteria for classification of the genotypes. This shows the robustness of the cluster analyses formed. The bootstrap analysis also confirmed the consistency of the dendrogram. This information allows the choice of parents, aiming to implement plant breeding studies based on the evaluated germplasm.

Among the hierarchical methods, the cluster analysis by the UPGMA is the most used method in this type of study in which the separation of the clusters might vary depending on an arbitrary cut-off point. In this study, this cut-off point was defined by the mean value of the distances between all pairs of genotypes. On the other hand, the cluster analysis performed by the Tocher's method follows the optimization method, in which the clusters are mutually exclusive and independent because their calculation takes into consideration the fact that a greater genetic distance exists between the individuals of different clusters than between the individuals of the same cluster (Amaral Júnior and Thiébaud, 1999).

The genetic distance between the genotypes ranged from 0.04 (between the genotypes NN02-02 and NN02-03) to 0.49 (between the genotypes NN13-02 and NN13-03). Considering that the genotypes NN02-02 and NN02-03 belong to the same accession, the low genetic distance (0.04) between them suggests that these individuals have a high degree of kinship. In contrast, the high genetic distance (0.49) between the genotypes NN13-02 and NN13-03 suggests that these genotypes came from distinct plants, even though they belonged to the same accession. The usefulness of RAPD markers in the detection of the absence of correlation between the degree of genetic similarity of the accessions and their respective origins was also reported for accessions of pitahaya, the fruit of *Hylocereus undatus* (Junqueira et al., 2010).

RAPD molecular markers have been successfully used in studying the degree of genetic diversity between the noni plants on the Asian continent. The studies of Singh et al. (2011), Singh et al. (2012), and Patel et al. (2014) have revealed high genetic diversity in *M. citrifolia* plants in different regions of India. This was probably due to the fact of that region being considered the center of origin of this species.

CONCLUSION

To our knowledge, this is the first report of the genetic variability of the accessions of *M. citrifolia* (noni) in Brazil. In this study, the evaluation of the genetic diversity of noni led to the identification of its contrasting genotypes, which can be used as parents in future gene combinations in breeding programs seeking to increase the variability in segregating populations. The genotypes NN02-02 and NN11-02 were found to be the most divergent. In addition, the evaluation of genetic diversity allowed the basis for new sampling, aiming to expand the genetic variability of the noni germplasm collection of Embrapa Agroindústria Tropical in Brazil.

Conflicts of interest

The authors declare no conflict of interest.

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