ISSN 2349-0837
Volume 7 Number 2
Journal Of Advance In Agriculture

POLYMORPHISM IN *LIPPIA ALBA* CLONES FROM THE METROPOLITAN REGION OF RIO DE JANEIRO

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ABSTRACT

In Brazil, the natural occurrence of the species *Lippia alba* (Mill) N. E. Brown is broad and diffuse, which justifies a high phenotypic variation and the occurrence of several chemotypes. Furthermore, the molecular methods help to determine the genetic variability with greater precision, in relation to the morphological methods. Thus, the objective of this work was to evaluate the genetic variability of different genotypes of *L. alba* from the Metropolitan region of the State of Rio de Janeiro using ISSR markers. The experiment was conducted at the Institute of Agronomy of the UFRRJ, from twenty genotypes of *L. alba*. Thirteen ISSR primers were used, obtaining 97 amplified bands and 74 polymorphic bands, equivalent to 77.89% of the amplified bands. The dendrogram (Neighbor Joining) formed five groups and just the genotypes UFRRJ LA01 and UFRRJ LA02 were grouped by the same geographic region and chemotype. The lowest similarity was observed in the grouping that joined the accessions UFRRJ LA14 and UFRRJ LA06. These results confirm the genetic variation between the genotypes, even when originating from the same region. *L. alba* genotypes from the Metropolitan Region of Rio de Janeiro show genetic and phytochemical variability.

Indexing terms/Keywords

Genetic variability, Polymorphism, Essential oil.

Academic Discipline And Sub-Disciplines

Genetic; Breeding Plants; Medicinal Plants

SUBJECT CLASSIFICATION

Cell Biology; Biotechnology

TYPE (METHOD/APPROACH)

Research article

INTRODUCTION

The species *Lippia alba* (Mill) N. E. Brown, popularly recognized as lemongrass or brazilian lemongrass is a native species that occurs in all regions of Brazil, being widely used as a medicinal and aromatic plant. Besides its classic use by folk medicine in the treatment of gastric diseases, fever, asthma and as a tranquilizer, it also has promising economic potential for the pharmaceutical, aroma and perfumery industries (Val et al., 2009; Jannuzzi et al., 2011; Bottignon et al., 2011).

Since South America is the largest center of genetic diversity of this species (Rufino et al., 2012) and the Atlantic Forest is one of the naturally occurring biomes, studies which aim at the botanical identification, the phytochemical characterization and molecular structure of this plant in natureare urgently needed (Zucchi et al., 2013). The use of molecular markers helps to identify plant species, to evaluate the present genetic variability, and it can also assistin the conservation of genetic variability of species in active germplasm banks (Blank et al., 2015). Among the *L. alba* chemotypes, the most frequently mentioned in Brazil are citral, citral-myrcene, citral-limonene, carvone-limonene, β-caryophyllene and linalool (Matos, 1996; Silva et al., 2006; Jannuzzi et al., 2011).

Among the many types of existing molecular markers, the ISSRs (Inter-Simple Sequence Replication) offer a microsatellite-based method, which does not require prior knowledge of the genome (Goulão and Oliveira, 2001). Considering the high reproducibility and the large number of polymorphic loci at a lower cost than other markers, the use of ISSR has been increasingly frequent in breeding programs for genotype selection, as well as in genetic diversity studies, “fingerprinting”and assisted selection (Costa, 2010).

Therefore, the present study was developed to evaluate the genetic variability of the genotypes of *L. alba* (Mill) N. E. Brown from the Metropolitan Region of the State of Rio de Janeiro, using the ISSR technique.

https://cirworld.com
DOI : 10.24297/jaa.v7i2.6221

1 MATERIAL E AND METHODS

1.1 Experimental Site Description

Genetic material from 20 *L. alba* genotypes which are kept in the collection of the Department of Plant Science of the Federal Rural University of Rio de Janeiro (UFRRJ) was used in this study. The plants were from three locations in the Metropolitan Region of Rio de Janeiro (Campo Grandeneighborhood, Queimados-RJ and Seropédica-RJ). The experimental area is located at the geographic coordinates 22 ° 45 'S, 43 ° 41' W and 25 m altitude. The climate of the region is Aw, according to Köppen's classification. In Table 1, the accessions are identified by site of origin and chemotype.

Table 1: Accessions of *L. alba* and corresponding chemotypes.

Accessions	Origin	Chemotype*
UFRRJ LA1	Campo Grande - RJ	Citral (neral-geranial)
UFRRJ LA2	Campo Grande - RJ	Citral (neral-geranial)
UFRRJ LA3	Campo Grande - RJ	Carvone-limonene
UFRRJ LA4	Campo Grande - RJ	Linalool
UFRRJ LA5	Seropédica – RJ	Citral (neral-geranial)
UFRRJ LA6	Seropédica – RJ	Citral-myrcene
UFRRJ LA7	Queimados – RJ	Carvone-limonene
UFRRJ LA8	Queimados – RJ	Carvone-limonene
UFRRJ LA9	Queimados – RJ	Citral-myrcene
UFRRJ LA10	Queimados – RJ	β-caryophyllene-β-citral
UFRRJ LA11	Queimados – RJ	Citral (neral-geranial)
UFRRJ LA12	Queimados – RJ	Citral (neral-geranial)
UFRRJ LA13	Queimados – RJ	β- caryophyllene -β-myrcene
UFRRJ LA14	Queimados – RJ	Citral-myrcene
UFRRJ LA15	Queimados – RJ	Citral (neral-geranial)
UFRRJ LA16	Queimados – RJ	Citral-myrcene
UFRRJ LA17	Queimados – RJ	Citral-myrcene
UFRRJ LA18	Queimados – RJ	Citral (neral-geranial)
UFRRJ LA19	Queimados – RJ	Citral (neral-geranial)
UFRRJ LA20	Queimados – RJ	Citral (neral-geranial)

*Chemical characterization was performed by gas chromatography coupled to mass spectrometry (GC-MS) at the analytical center of the Postgraduate Program in Chemistry of the Institute of Exact Sciences of UFRRJ.

1.2 Establishment of Experiment and Data Collection

Samples of adult plant tissue of *L. alba*, previously immersed in liquid nitrogen, were brought to the laboratory of Molecular Biology of the Department of Phytotechnology of UFRRJ, for the extraction of genomic DNA. In this step, the methodology suggested by Doely and Doely (1990), with some adaptations, was followed.

For each genotype, 200 to 300 mg of vegetable tissue were homogenized in liquid nitrogen with the aid of gral and pistil. Then, part of the material was transferred to amicrotube and 850 µL of extraction buffer solution (CTAB) was added. The mixture was pre-homogenized and incubated in a water bath at 65 °C for 40 minutes. During incubation, the tubes were shaken every 10 minutes in order to homogenize the suspension.

Two partitions were made with organic solvent. In the first one, 800 µL of solution containing chloroform and isoamyl alcohol (24:1) were added and the mixture was homogenized by shaking during 10 minutes. Next, the emulsion was centrifuged at 16000 rpm for 5 minutes, and thereafter the supernatant was transferred to new tubes. The tubes received 650 µL of solution containing phenol, chloroform and isoamyl alcohol (24:24:1) and centrifuged again. After centrifugation, the upper phase of the tube was transferred to a new tube, where 450 µl of cold (-20 °C) isopropanol was added. This

mixture was gently homogenized and then packaged in the freezer at -20 °C for two and a half hours to precipitate the nucleic acids.

After this period, a new centrifugation was carried out at 1400 rpm for 10 minutes, and the supernatant was discarded at the end. The tubes were washed twice, one with 70% alcohol and one with 95% alcohol. The tubes were then placed to dry for 15 minutes. Following this period, the precipitate was resuspended in 200 µL of TE buffer containing RNAse at the concentration of 4 µg mL^{-1} and incubated at 37 °C for 30 minutes for RNA degradation.

After the treatment with RNAse, 2µL of the DNA of each genotypes were extracted for quantification and evaluation of the purity in spectrophotometer (of the Nanodroptype). Finally, an aliquot was used according to the concentration determined for dilution in 1x TE to obtain the working solution in the concentration of 12.5 ng μL^{-1}.

The DNA was subjected to the polymerase chain reaction (PCR) using 13 single repeated sequence internal tags (ISSR). Amplification products were analyzed by 1.2% agarose gel electrophoresis, subjected to a continuous current of 75 V for approximately three and a half hours, stained with ethidium bromide and photodocumented in the Eagle Eye® II system (Stratagene). As a result 96 bands were obtained, of which, 84 were polymorphic. Each polymorphic band was characterized as present (1) or absent (0) for all genotypes, resulting in an array of binary data.

1.3 Analysis

The dendrogram, representing the genetic similarity between each pair of genotypes, was made using the Jaccard coefficient as a measure of distance using the Darwin software version 6.5 (Perrier et al., 2006) by the Neighbor-Joining grouping method (Saitou et al., 1987).

2. RESULTS

With the 13 markers used, 21 monomorphic bands and 74 polymorphic bands were observed, totaling 95 amplified bands. The number of bands per primer varied from 9 ((GA) 8C) to 3 ((GA) 8YT), with a mean of 6.78 bands per primer. Of the 95 amplified bands, 77.89% were polymorphic bands (Table 2).

Table 2. ISSR markers used in the DNA amplification of 20 accessions of Brazilian lemongrass (*Lippia alba*), with their respective sequences, annealing temperature (AT), total number of bands (TNB), number of polymorphic bands (PB), Number of monomorphic bands (MB) and percentage of polymorphism (PP). Seropédica-RJ, 2015.

Primer	Sequência*	AT (°C)	TNB	PB	MB	PP
ISSR 1	$(AC)_8CT$	47	6	3	3	50
ISSR 2	$(GAA)_6AA$	50	9	9	0	100
ISSR 3	$(GA)_8C$	47	9	6	3	66
ISSR 4	$C\ (GA)_7$	50	7	3	4	42
ISSR 5	$T\ (GA)_8$	50	7	5	2	71
ISSR 6	$(GA)_8C$	50	7	5	2	71
ISSR 7	$(GA)_8YT$	47	3	2	1	66
ISSR 8	$(GT)_8YC$	47	9	8	1	88
ISSR 9	$TA\ (CAG)_4$	50	9	8	1	88
ISSR 10	$(CA)_6AC$	40	6	6	0	100
ISSR 11	$(CA)_6AG$	40	9	7	2	77
ISSR 12	$(GAG)_3GC$	40	8	6	2	75
ISSR 13	$(GATA)_4$	45	6	6	0	100
Total			95	74	21	71

* Y = C or T; e R = A or G.

In the dendrogram (Figure 1), the formation of five groups can be observed.

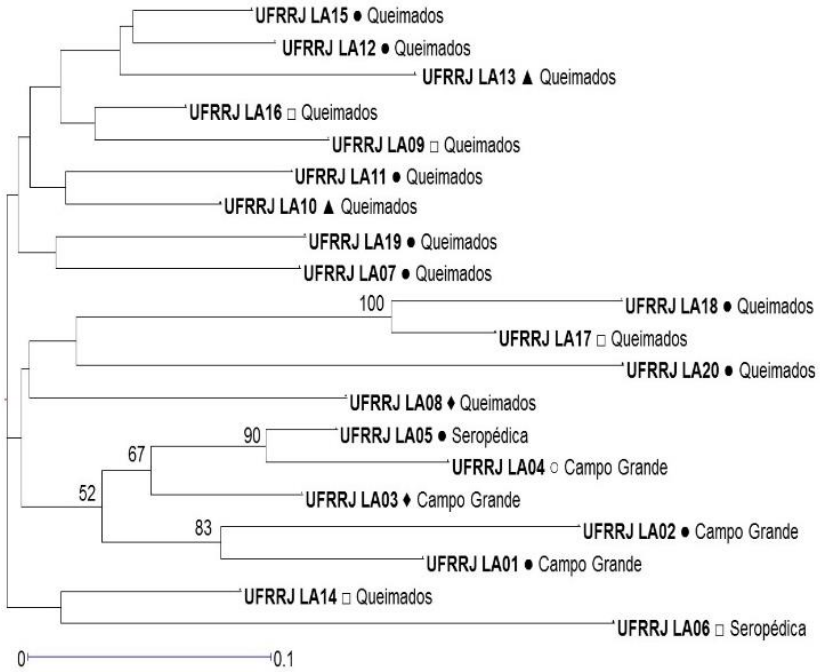

Fig 1: Dendrogram obtained by the Neighbon Joining method using the Jaccard's coefficient of similarity for the samples of twenty genotypes of *L. alba*.

Chemotypes: ● citral; □ citral-myrcene; ♦ carvone-limonene; ▲ ß-caryophyllene; ○ linalool.

The group 1 is composed by the accessions (UFRRJ LA17 and LA18), the group 2 by the accessions (UFRRJ LA04 and 05), the group 3 by the accessions (UFRRJ LA01 and LA02), the group 4 with three accessions (UFRRJ LA03, LA04 and LA05) and the group 5 with fiveaccessions(UFRRJ LA01, LA02, LA03, LA04 and LA05).

In the first group, it was verified that the UFRRJ LA 17 and LA18 accessions presented the highest genetic similarity, both of which are from the same region and have similar chemotypes, citral and citral-myrcene, respectively. The group 2 (UFRRJ LA04 and 05) collected two accessions of different chemotypes and regions. Although Group 3 presented (UFRRJ LA01 and LA02), the same chemotype of group 1, it formed an isolated grouping. The groups 4 and 5 were formed by the predominance of accessions from the same region (Campo Grande), and by three distinct chemotypes (citral, carvone-limonene and linalool).

Even though some accessionshave been grouped from the same geographic region, subgroups were formed varying the chemotype accession. According to these results, it is evident that the use of these markers was not efficient in separating plants by their chemotypes. Because of this, it is necessary to increase the number of individuals analyzed and to expand the collection to other municipalities of the state of Rio de Janeiro and to use a wider set of primers, by selecting those associated with specific chemotypes.

3. DISCUSSION

The ISSR markers used in the twenty accessions of *L. alba* resulted in a percentage of polymorphism equal to 71%, suggesting genetic variability between the accessions. According to Kernodle et al. (1993), the variation in the number of bands amplified by primers is influenced by several factors, such as the primer structure, the annealing temperature and the lesser number of annealing in the genome.

The number of polymorphic fragments used in the analyses play is quite variable (Estopa et al., 2006). A study developed with *L. alba* genotypes in the southern region of Brazil (Manica-Cattani et al., 2009), using six ISSR primers and four RAPD primers, detected 120 amplified fragments and 55 polymorphic fragments with ISSR markers representing 82.08% polymorphism. When selecting superior genotypes of Aroeira (*Schinus terebinthifolius* Raddi), Souza et al. (2014) used nineteen ISSR primers and obtained 104 tags and 79.92% polymorphic bands; whereas Shinwari et al. (2010) found values of polymorphic bands of 47.2% and 27.4% for the species *Mentha spicata* and *Mentha royleana*, respectively.

When assessing the genetic variability among populations of Carqueja and Auler (2004) showed that 96.7% of the existing variability is distributed within the populations, against only 3.3% among populations. On the other hand, Santos and Corrêa (2006), when evaluating 16 individuals of Santa-Maria grass, observed low polymorphism, allowing them to infer that the species has spread in the region from a few specimens or may reflect the little polymorphic nature of the group.

4. CONCLUSION

Molecular data indicate genetic variation among the accessions of the *L. alba* germplasm collection of the Federal Rural University of Rio de Janeiro.

The ISSR markers used were not efficient to separate the *L. alba* accesses evaluated in this study by chemotype.

5. ACKNOWLEDGMENTS

This work was supported with financial resources and scholarships by the Foundation for Research Support of the State of Rio de Janeiro (FAPERJ), National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES).

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DOI : 10.24297/jaa.v7i2.6221