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Characterization of the genetic variability among *Caesalpinia pulcherrima* (L.) Sw. (Fabaceae) plants using RAPD molecular markers

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ABSTRACT. The species *Caesalpinia pulcherrima*, which belongs to the Fabaceae family, is frequently used as a hedge or street tree in urban landscapes, parks and gardens. It flowers profusely, and the flowers, depending on the variety, may be pink, orange or yellow. As flower characteristics are essential for determining the commercial value of ornamental species, the main objective of this work was to identify polymorphisms in *C. pulcherrima* plants that produce flowers of different colors using RAPD molecular markers. For this study, 30 adult plants were randomly chosen on streets located in Jaboticabal city, São Paulo State, Brazil, and leaf samples were collected for DNA extraction. Among these trees, 20 have orange flowers, eight have yellow flowers and only two produce pink flowers. From the 140 tested RAPD primers, 94 primers amplified defined fragments that resulted in 246 bands, which were 100% monomorphic. Thus, a polymorphism was not detected by any of the primers tested. It was concluded that the RAPD technique is not an efficient method for detecting polymorphisms and that more specific molecular markers should be tested. Additionally, the morphological characteristic "flower color" may be controlled by several genes or by the association of them.

Keywords: peacock flower, flower color, monomorphism.

Caracterização da variabilidade genética entre árvores de flamboyanzinho [*Caesalpinia pulcherrima* (L.) Sw., Fabaceae] por marcadores moleculares RAPD

RESUMO. O flamboyanzinho (*Caesalpinia pulcherrima*, Fabaceae) é muito utilizado como cerca-viva e na arborização de ruas, parques e jardins. De florescimento exuberante, suas flores podem apresentar coloração rosa, laranja ou amarela, conforme a variedade. Como características florais são essenciais para a definição do valor comercial de espécies ornamentais, o objetivo principal do trabalho foi verificar a existência de polimorfismo entre plantas de *C. pulcherrima*, que produzem flores de diferentes colorações, por marcadores moleculares RAPD. Foram escolhidas aleatoriamente 30 plantas adultas, cultivadas no município de Jaboticabal, Estado de São Paulo, para a retirada de amostras foliares para a extração de DNA. Dentre essas matrizes, 20 possuem flores alaranjadas, oito, flores amarelas, e apenas duas produzem flores de coloração rosa. Dos 140 *primers* de RAPD avaliados, 94 foram capazes de ampliar fragmentos definidos, gerando 246 bandas, das quais se observou 100% de bandas monomórficas, indicando que nenhum dos *primers* utilizados detectou polimorfismo entre os tratamentos. Concluiu-se que a técnica para detecção de polimorfismo por marcadores RAPD não foi eficiente, e que existe a necessidade de se testarem marcadores moleculares mais específicos. Além disso, a característica morfológica "cor de flor" é, possivelmente, controlada por vários genes ou pela combinação deles.

Palavras-chave: flamboyanzinho, cor de flor, monomorfismo.

Introduction

The *Caesalpinia pulcherrima* (L.) Sw. species, which belongs to the Fabaceae family, is a perennial shrub or small tree that is frequently used as a hedge or street tree in urban landscapes, parks and gardens. Its trunk is woody, branched and thorny, and it can reach a height of 4 m. Its leaves are compound and bipinnate. It flowers profusely throughout the year, but mainly during spring and summer. Its racemose inflorescences are composed, depending on the variety, by pink, orange or yellow flowers with long stamens. The fruit is legume-like, and plants are easily propagated by seeds (LORENZI; SOUZA, 2008).

Flower characteristics, especially color and size, are used to determine the commercial value of ornamental species. Therefore, it is necessary to define the relationship between these characteristics and their genes and to identify these genes with the help of molecular markers (YE et al., 2008). The use of molecular markers may also contribute to studies on the genetic divergence of flowers from the same species in which color is a distinct morphological characteristic (REZENDE et al., 2009; SILVA et al., 2005). Molecular markers have been successfully used in the genetic analysis of plants and in characterizing the existing variability among individuals (BECKMANN et al., 2006). In addition to identifying the genetic parentage among species (DE BENEDETTI et al., 2001), they can also be used as a basis for new crossings and for defining basic clones, new hybrids and mutants (DA MATA et al., 2009; PINHEIRO et al., 2003).

Some of the most commonly used DNA molecular markers include the following: restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites (SSRs) (FERREIRA; GRATTAPAGLIA, 1998; KARP et al., 1996; MELO et al., 2001). The RAPD technique is a variation of the polymerase chain reaction (PCR) protocol. Unlike PCR, it uses a single oligonucleotide primer, which usually contains 10 bases, instead of a pair of primers. The sequence of the primer is randomly built, which results in the amplification of an unknown target sequence. For this reason they are called random or arbitrary primers (GUERRERO et al., 1997).

In addition to facilitating studies on traditional species (e.g., maize, tomato and rice), the RAPD technique can also be used to investigate species on which there is little data. Applications for this technique include the following: attaining the genetic fingerprints of individuals, varieties and populations; analyzing the genetic structure and diversity in natural populations, breeding populations and gene banks; establishing phylogenetic relationships among different species; building genetic maps of high genomic coverage; and locating genes of economic interest (FERREIRA; GRATTAPAGLIA, 1998).

The objectives of this work were to identify polymorphisms among *C. pulcherrima* plants that produce flowers of different colors using RAPD molecular markers and to determine the efficiency of these markers in achieving the first objective.

Material and methods

The study was carried out in the Laboratory of Bacteria Genetics and Biotechnology of the Department of Applied Biology on Agriculture and Livestock at the State University of São Paulo (FCAV/UNESP) located in Jaboticabal city, São Paulo State, Brazil.

The plant material was collected from *C. pulcherrima* plants growing in Jaboticabal city (21°15’ S, 48°18’ W, 590 m of altitude). Thirty trees were chosen according to their flower production, flower color and phytosanitary conditions. Twenty of these trees have orange flowers, eight have yellow flowers and only two produce pink flowers (Table 1). One leaf sample was collected for DNA extraction from each of the chosen plants.

Table 1. Identification of the 30 samples of *Caesalpinia pulcherrima* (L.) Sw. (Fabaceae) cultivated in Jaboticabal city and analyzed with RAPD primers. Jaboticabal, São Paulo State, Brazil (2009).

Sample	Description	Code[1]	Origin
01	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
02	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
03	*C. pulcherrima* “pink”	PF	Jaboticabal, São Paulo State
04	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
05	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
06	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
07	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
08	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
09	*C. pulcherrima* “pink”	PF	Jaboticabal, São Paulo State
10	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
11	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
12	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
13	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
14	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
15	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
16	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
17	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
18	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
19	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
20	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
21	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
22	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
23	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
24	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
25	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
26	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
27	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
28	*C. pulcherrima* “yellow”	YF	Jaboticabal, São Paulo State
29	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State
30	*C. pulcherrima* “orange”	OF	Jaboticabal, São Paulo State

[1]YF = yellow flowers; OF = orange flowers; PF = pink flowers.

DNA was extracted for genotyping from young leaves, without obvious injuries, according to the technique described by Lodhi et al. (1994). The DNA was quantified and analyzed for quality by a spectrophotometer and by electrophoresis in an agarose gel. In the spectrophotometer, the ratio between the absorbances 260 and 280 nm (nucleic acids/proteins) was determined and used to verify the integrity of the DNA samples using 0.8% agarose gels. For the electrophoresis analysis in an agarose gel, the DNA was analyzed by the intensity of the ethidium bromide fluorescence dye under an UV light.

The amplification reactions were performed in a thermocycler (PTC-100 Programade Thermal Controller – MJ Research, Inc.) using the following protocol: 94°C for two minutes; 94°C for one minute, 36°C for one minute and 72°C for one minute (38 cycles); 72°C for seven minutes; and finally, a cycle of 4°C for 15 minutes to cool the samples.

Each amplified reaction was in a total volume of 20 μL containing 8.5 μL of H_2O with 3 μL of primer, 0.4 μL of dNTP, 0.5 μL of $MgCl_2$, 2 μL of TEB 1X buffer, 0.5 μL of Taq DNA Polymerase and 5 μL of plant genomic DNA (20 ng 200 μL^{-1}) and was dyed with ethidium bromide (0.5 μL mL^{-1}); each amplified reaction was analyzed by electrophoresis in a 1.5% agarose gel. For primer synthesis, oligos were purified by a Tris-EDTA elution, and aliquots of 10 μL were dry air lyophilized and resuspended in 200 μL of TE according to the manufacturer's recommendations. Gels were analyzed in an UV transilluminator and registered through a photodocumentation system.

A total of 140 RAPD primers were tested, which were obtained from Operon Technologies kits C and F and from the UBC RAPD Primer Synthesis Project Oligonucleotide Set 100/3 kit acquired from the Nucleic Acid/Protein Service Unit at the University of British Columbia (UBC) located in Vancouver, Canada.

Results and discussion

Among the 140 primers tested, 94 primers were able to amplify defined fragments resulting in 246 bands that were 100% monomorphic. These results suggest that these primers are not able to detect polymorphisms among individuals (Figure 1).

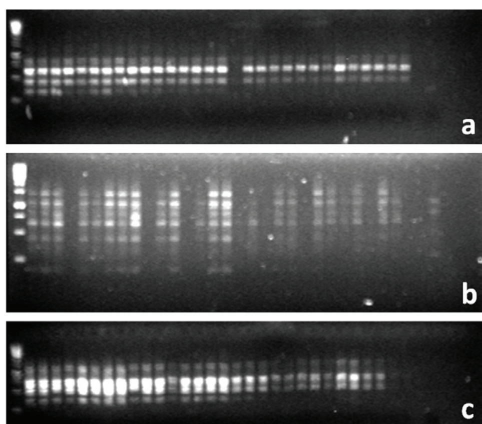

Figure 1. Amplification products from RAPD markers: F7 (a), F12 (b) and F13 (c) from leaf samples of *Caesalpinia pulcherrima* (L.) Sw. (Fabaceae) obtained from a 1.5% agarose gel. Jaboticabal, São Paulo State, Brazil (2009).

The generation of monomorphic bands suggests that the specimens analyzed may have been derived from the same plant (DE BENEDETTI et al., 2001). These results also suggest that related individuals have been bred to each other, especially when the seeds are harvested for the local landscape. These seeds may then be fertilized by related plants, which would prevent the formation of a diverse population in the municipality. If this is the case, then different genotypes should be brought into the region to improve, through new crossings, the agronomic characteristics of the descendent specimens. This recommendation has also been suggested by Pinheiro et al. (2003) for saffron accesses.

An analysis of carnation plants, which descended from a single specimen, produced similar results. Some of these plants had morphological characteristics that were distinct from the mother plant, such as flowers with different shapes and colors, but these differences could not be detected by the RAPD technique. In that study, it was concluded that the gene, or genes, that control these characteristics may manifest themselves only through associations with each other (NIMURA et al., 2003).

Additionally, Chung et al. (2001), when analyzing the characteristics of gerbera cultivars, such as flower color, diameter and stem, found polymorphisms among the samples using the RAPD technique. They could not, however, detect a genetic relationship among the morphological characteristics studied. They found that there was a highly inconsistent connection between the molecular markers and the morphological characteristics and that many of the genes that may be responsible for the characteristics studied were located on different chromosomes. Furthermore, molecular markers show similarities among individuals by site sampling, while morphological characteristics measure how equal the individuals are according to variables whose expression levels depend on the number of potentially epistatic genes (GRIVET; NOYER, 2003).

Sekhon and Gupta (1995) concluded that a precise correlation between the distances obtained from phenotypic and molecular data cannot be expected once each data group only represents one part of the genome. Additionally, Moser and Lee (1994) emphasized that the number of markers as well as the size and variability of the analyzed population are factors that may affect the distance obtained from molecular data. Furthermore, due to the complex nature of the relationship between the genotypic and phenotypic variations, phenotypically different genotypes may show variations in a few loci, while phenotypically similar genotypes may be completely different.

Additionally, Wiering and De Vlaming (1984) verified that in petunia plants, at least 35 genes are responsible for determining flower color. Therefore, the *C. pulcherrima* flower color could be controlled by several genes, which would explain the monomorphism among the genotypes even if they show different phenotypes. Rezende et al. (2009), working on the genetic divergence among gerbera cultivars through RAPD markers, also concluded that flower color may be controlled by several genes. Interestingly, they detected a polymorphism among their samples.

In some cases, depending on the species, very few genes may be responsible for a single morphological characteristic, such as flower color. However, the association of several other genes is still required for pigment synthesis (ABE et al., 2002), and these genes may not be among those amplified by the tested primers (HOSOKI et al., 1997).

In regards to the association among plant groups that show genetic proximity to their phenotypic characteristics, it was not possible to establish a link among individuals that possessed similarities of genotype and phenotype characteristics in some plant species, such as gerbera (DA MATA et al., 2009), tree peony (HOSOKI et al., 1997) and *Zinnia elegans* (YE et al., 2008). Therefore, it was concluded that morphological characteristics, such as the length of the flower stem, flower color and inflorescence shape, may be controlled by a small number of genes, which may not be detectable by RAPD molecular markers. These markers detected a lower genetic diversity than the diversity found by morphological analysis (DA MATA et al., 2009).

It has also not been possible to make a genetic distinction among some rose varieties using the RAPD technique, even if they are clearly distinguished by their flower color (DE BENEDETTI et al., 2001). Additionally, in chrysanthemum cultivars, the RAPD technique has not been able to identify polymorphisms in same species of plants possessing different morphological characteristics. Therefore, it appears that high morphological variability is also common in other species (WOLFF; RIJN, 1993).

Conclusion

The RAPD technique is not effective at detecting polymorphisms among *C. pulcherrima* plants; therefore, more specific molecular markers should be tested.

The morphological characteristic "flower color" may be controlled by several genes or by the association of them.

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