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Genetic divergence among *Dimorphandra* spp. accessions using RAPD markers

Divergência genética entre acessos de *Dimorphandra* spp. usando marcadores RAPD

Cláudia Pombo Sudré[I*] Rosana Rodrigues[I] Leandro Simões Azeredo Gonçalves[I] Ernane Ronie Martins[II] Messias Gonzaga Pereira[I] Marilene Hilma dos Santos[I]

ABSTRACT

*The genus Dimorphandra has distinguish relevance considering either medicinal or biodiversity aspects because it includes two species that are economically important flavonoids sources for pharmachemical industry (**D. mollis** Benth. and **D. gardneriana** Tul.), and species endemic to Brazil, such as **D. jorgei** Silva and **D. wilsonii** Rizz., threatened by extinction. In order to evaluate variability among accessions of **D. mollis** (fava-d'anta), **D. gardneriana** and **D. wilsonii**, it was collected fruit from individual plants from three Brazilian states in a total of 57 accessions, which were analyzed with RAPD markers. It was used 20 seeds per progeny; the DNA was extracted from fully-formed young leaves, which were collected in bulk. The data were analyzed using a binary matrix, in which the score one represented presence of a band and zero, absence. The similarity matrix was developed by using the arithmetic complement of the Jaccard index, later grouped based on the Neighbor Joining algorithm. It was found considerable intra and inter specific variability in **Dimorphandra** spp., which were separated into four groups. Though genetic variability was found, the collecting trips showed that most of these areas are subject to loss of genetic resources of fava-d'anta due to the following factors: continuous anthropic activity, propensity for natural fires, and loss of natural seed dispersers (large fruit-eating mammals). Therefore, protections of these areas and* ex situ *conservation are essentials for the maintenance of genetic variability of these species.*

Key words: *fava-d'ant, molecular characterization, plant genetic resource, germoplasm.*

RESUMO

*O gênero Dimorphandra tem grande relevância, sobretudo nos aspectos medicinais e de biodiversidade, por incluir duas espécies que são importantes economicamente como fontes de flavonoides para indústria farmacoquímica (**D. mollis** Benth. e **D. gardneriana** Tull.), e espécies endêmicas do Brasil, como a **D. jorgei** Silva e **D. wilsonii** Rizz., sendo esta ameaçada de extinção. Objetivando avaliar a variabilidade entre acessos de **D. mollis**, **D. gardneriana** e **D. wilsonii**, foram realizadas coletas de frutos separados por planta em três estados brasileiros em um total de 57 acessos que foram analisados por meio da técnica RAPD. Utilizaram-se vinte sementes por progênie e o DNA, extraído de folhas definitivas, jovens, e coletadas em bulk. Os dados foram analisados utilizando-se uma matriz de dados binários. Para formação da matriz de dissimilaridade, foi utilizado o complemento aritmético do Índice de Jaccard e posteriormente o agrupamento pelo algoritmo de Neighbor Joining. O estudo revelou que existe variabilidade entre e dentro das espécies de **Dimorphandra** spp., o qual formou quatro grupos. Apesar da variabilidade genética constatada, as expedições de coleta demonstraram que a maioria das áreas amostradas está sujeita a perdas de recursos genéticos de fava-d'anta devido aos seguintes fatores: ocorrência de ação antrópica contínua; propensão a incêndios naturais; e diminuição dos dispersores naturais de sementes (grandes mamíferos frugíveros). Assim, a proteção destas áreas e a conservação* ex situ, *são primordiais para manutenção da variabilidade genética dessas espécies.*

Palavras-chave: *fava-d'anta, caracterização molecular, recursos genéticos vegetais, germoplasma.*

INTRODUCTION

The market for medicinal plants increases about 10% each year and only for phytoterapics is expected an annual movement of US$ 20 billions

[I]Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), 28013-602, Campos dos Goytacazes, RJ, Brasil. E-mail: cpombo@uenf.br. *Autor para correspondência.
[II]Universidade Federal de Minas Gerais (UFMG), Montes Claros, MG, Brasil.

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worldwide. In Brazil, the value of this market is estimated in about US$ 400 millions with potential to reach US$ 2 trillion only using Brazilian native plants (RAMOS, 2010). Demand for native medicinal plants in Brazil tends to increase with the discovery of substances that still cannot be produced synthetically. This is the case for fava-d'anta (***Dimorphandra*** spp.), which fruits have considerable concentrations of flavonoids, especially rutin and quercetin. According to HUBINGER et al. (2009), content of flavonoids can reach about 10.25% in dried ground fruit. These are among the most commonly exported pharmacological products produced in Brazil, accounting for 50% of world production. In 2008, the Brazilian exports of rutin and quercetin summed US$ 6.2 million and US$ 2.6 million respectively (abiquifi.org.br).

This genus occurs mainly in *cerrado* (savannah-like), ecosystem from several Brazilan states, such as Amazonas, Bahia, São Paulo, Maranhão, Pará, Piauí, Ceará, Tocantins, Pernambuco, Minas Gerais, Goiás, Mato Grosso and Mato Grosso do Sul. The number of known species varies from 11 to 43 in the genus *Dimorphandra* (GONÇALVES, 2007; INPI, 2009). Among these, the ***D. mollis*** Benth. and ***D. gardneriana*** Tul. are considered the most important because they are most frequently found in nature, being the most collected and used in chemical and pharmaceutical industry (GONÇALVES et al., 2010; CUNHA et al., 2009). Nevertheless, ***D. wilsonii*** Rizz. is also considered potentially important because the fruit size as much when compared with the other two species, producing at least three times more rutin (FERNANDES et al., 2007).

Due to the threat of extinction, there is concern about surviving and maintaining the species ***D. mollis*** and ***D. gardneriana***, since they are used commercially only through extractivism. An analysis to study the genetic erosion risk was made on 32 populations of ***D. mollis*** Benth. in northern Minas Gerais state indicated that 41% of the populations had risk of genetic erosion of 40% or above. The main risk factors that contributed most were extractive harvesting, lack of protection of local habitats and forest fires (SOUZA & MARTINS, 2004).

D. wilsonii, an endemic species of Minas Gerais state, is on the Red List of Threatened Plants of the IUCN (International Union for Conservation of Nature and Natural Resources) in the category "critically endangered" and currently fewer than 50 mature plants are found in nature, located on private properties in the cities of Paraopeba, Pequi, Lagoa Santa and Sete Lagoas, all in the state of Minas Gerais (SOUZA et al., 2009).

In order to take proper measures to help the ***Dimorphandra*** spp. germplasm conservation, it is necessary to have information on the genetic structure of the populations, as well as variability between populations. One of the tools used for this purpose is molecular markers, which allow inferences on genetic diversity among and within populations (SCHLÖTTERER, 2004; SCHULMAN, 2007; HUANG et al., 2009).

In studies of plant conservation, the Random Amplified Polymorphic DNA (RAPD) is one of the techniques indicated for species for which there is little genetic information and that are rare or threatened by extinction, since it uses short random sequence primers, requires little material for analysis and is relatively fast (LYNCH & MILLIGAN, 1994; LACERDA et al., 2002). Genetic variability was examined among accessions of ***D. mollis***, ***D. gardneriana*** and ***D. wilsonii***, from several Brazilian localities using RAPD markers.

MATERIAL AND METHODS

Obtaining vegetal material

Fruits of the species ***D. mollis*** Benth. (fava-d'anta), ***D. gardneriana*** Tul. and ***D. wilsonii*** Rizz. were collected in 57 accessions from 19 localities of three Brazilian states: Minas Gerais, Maranhão and Tocantins (Table 1). Areas with elevated anthropic influence were included, mainly due to extractive harvesting of fava-d'anta fruit, along with areas within a conservation unit, named Lajeado State Park, Tocantins state.

Fruit from one to ten plants of each accession were collected, generally from five plants, depending on the availability at the time they were harvested. Twenty seeds were collected from each plant to produce seedlings. The seeds were removed, mechanically scarified with the help of a carborundum stone and planted in containers filled with organic substrate, maintained in a greenhouse. As a divergent standard, ***Cassia grandis*** L.f. that belongs to the same subfamily as the genus *Dimorphandra*, was included for the analysis.

Young leaves were collected from 57 accessions and from the ***C. grandis*** L.f. accession. After harvesting, the leaves were taken to the laboratory and macerated. Part of the macerated material was frozen in liquid N_2 and placed in 15mL tubes, capped, labeled, and maintained at -86ºC. Another part of the material was used to extract DNA.

Isolation of genomic DNA

Genomic DNA was extracted using the CTAB method (DOYLE & DOYLE, 1990), modified by DAHER et al. (2002). DNA was quantified in 0.8% agarose gel using the High DNA Mass Ladder (Invitrogen®, USA). Samples were stained with a mixture

Table 1 - Geographic references of the ***Dimorphandra*** spp accessions collected in Brazilian States.

City	Accession number	Region/State	Latitude	Longitude	Altitude (m)	Species
Bocaiúva	01	North/MG[1/]	S17°10'	W43°43'	799	***D. mollis*** Benth.
Brasília de Minas	08	North/MG	S16°10'	W44°29'	810	***D. mollis*** Benth.
Montes Claros	05	North/MG	S16°25'	W44°02'	845	***D. mollis*** Benth.
Coração de Jesus	05	North/MG	S16°51'	W44°09'	738	***D. mollis*** Benth.
Barra do Corda	05	Central/MA	S05° 30'	W45°14'	83	***D. gardneriana*** Tul.
Barra do Corda	05	Central/MA	S05° 30'	W45°14'	83	***D. gardneriana*** Tul.
Paraopeba	02	Metropolitana/MG	S19º 16'	W44º24'	733	***D. wilsonii*** Rizz.
Japonvar	02	North/MG	S16°02'	W44°14'	798	***D. mollis*** Benth.
Jequitaí	02	North/MG	S17°13'	W44°29'	559	***D. mollis*** Benth.
Chapadinha	01	Northeast/MA	S03° 44'	W43° 21'	105	***D. gardneriana*** Tul.
Mirabela	02	North/MG	S16°16'	W44°09'	792	***D. mollis*** Benth.
Olhos d'Água	03	North/MG	S17°26'	W43°37'	780	***D. mollis*** Benth.
Patrocínio	01	Alto do Parnaíba/MG	S18° 56'	W46°59'	965	***D. mollis*** Benth.
Pirapora	01	North/MG	S17° 20'	W44°56'	489	***D. mollis*** Benth.
Claro dos Poções	07	North/MG	S 16°57'	W44°16'	730	***D. mollis*** Benth.
Palmas	02	TO	S10° 00'	W48°15'	625	***Dimorphandra* sp.**
Palmas	03	TO	S10° 00'	W48°15'	625	***Dimorphandra* sp.**
Palmas	01	TO	S10° 00'	W48°15'	625	***Dimorphandra* sp.**
Uberlândia	01	Triângulo Mineiro/MG	S18° 55'	W48° 16'	863	***D. gardneriana*** Tul.

[1/]MG = Minas Gerais State; MA = Maranhão State; TO = Tocantins State.

of Blue Juice with Gel Red (1:1) to visualize the bands in the gel and the image recorded using MiniBis Pro.

RAPD analysis

Sixteen RAPD primers randomly chosen were tested, because there was no information available about polymorphic primers for ***Dimorphandra*** spp. considering this a molecular marker (Table 2). The amplification reactions were run in a Mastercycler gradient (Eppendorf) thermocycler in a final volume of 15μL, containing 10ng of DNA, 10mmol L^{-1} Tris HCl (pH 8.3), 50mmol L^{-1} KCl, 2.0mmol L^{-1} $MgCl_2$, 100mM of dNTP, 0.4mM of primer, and 0.75U of Taq DNA polymerase. The reaction mixture was submitted to 45 amplification cycles after an initial denaturation step at 95°C for 1min. Each cycle consisted of 1min at 94°C, 1min at 36°C and 2min at 72°C. There was a final extension step at 72°C for 7min. The amplification products were submitted to electrophoresis in 1.4% agarose gels. The bands were visualized by staining with a mixture of Blue Juice with Gel Red (1:1) and the images recorded with MiniBis Pro.

Data analysis

The data were obtained by visual evaluation of the strongest and most consistent bands. A binary matrix was prepared, with 1 and 0 corresponding to presence and absence of the band, respectively. A similarity matrix was constructed using the arithmetic complement of the Jaccard index, and then clustered by Neighbor Joining (SAITOU & NEI, 1987). All of the analyses were done using the program DARwin 5 (PERRIER et al., 2003).

RESULTS AND DISCUSSION

According to the RAPD analysis, each primer produced easily detected bands of variable intensity, and nonspecific bands, which were discarded. The 16 primers used produced 100 bands (Table 2). Out of these, 96 were polymorphic and four monomorphic. The number of polymorphic bands per primer varied from one to 11, with a mean of six bands per primer. The degree of polymorphism for each primer varied from 60 to 100% (Table 2), with a mean of 96%.

The dendrogram obtained using Neighbor Joining algorithm allowed us to separate the accessions into five distinct groups (Figure 1). Accessions 58 and 35, 40 and 47 were the most distant from the others; accession 58 was of the species ***C. grandis*** L.f. Although the accessions 35, 40 and 47 had formed a separate cluster, seeds from these accessions were collected in Tocantins state and they were originally identified as ***Dimorphandra*** sp. based on phenotypic

Table 2 - Number of polymorphic and monomorphic bands obtained with RAPD markers in 57 accessions from 19 Brazilian localities of ***Dimorphandra*** spp. using 16 primers.

Primer	Sequence 5'→ 3'	Polymorphic bands	Monomorphic bands	% of polymorphism
OPAA11	ACCCGACCTG	8	1	89
OPA12	TCGGCGATAG	8	0	100
OPAA16	GGAACCCACA	6	0	100
OPAA18	TGGTCCAGCC	4	0	100
OPAA19	TGAGGCGTGT	9	0	100
OPAE06	GGGGAAGACA	6	0	100
OPAE07	GTGTCAGTGG	11	0	100
OPAE09	TGCCACGAGG	1	0	100
OPAE08	CTGGCTCAGA	3	0	100
OPAE15	TGCCTGGACC	4	0	100
OPAE18	CTGGTGCTGA	3	2	60
OPAE20	TTGACCCCAG	7	1	87.5
OPAW09	ACTGGGTCGG	6	0	100
OPBO7	GGTGACGCAG	10	0	100
OPB19	ACCCCCGAAG	6	0	100
OPD19	CTGGGGACTT	4	0	100
Total	16	96	4	

traits. However, it was not possible to cluster these three accessions together with other Dimorphandra representatives only using RAPD markers. It can be seen that these accessions are distant from accessions of ***D. mollis*** Benth., ***D. gardneriana*** Tul. and ***D. wilsonii*** Rizz., leading to suppose that they are of a different species. The International Plant Names Index has 43 species of this genus registered, so that confusion in species recognition is possible.

The accessions of ***D. mollis*** had a mean intragroup distance, based on the Jaccard coefficient, of 0.2304 (±0.08); which were allocated to the same group based on the Neighbor Joining algorithm. The same was found for accessions of ***D. gardneriana*** Tul., though with a mean intragroup distance of 0.3738 (±0.09). The species ***D. mollis*** was collected from 13 localities in Minas Gerais, Brazil, covering three mesoregions in this state, with a maximum distance of 600km between collection sites. On the other hand, ***D. gardneriana*** was collected from fewer localities of Maranhão and Minas Gerais states. Also, two accessions of ***D. gardneriana*** collected in Barra do Corda, Maranhão, came from different management regimes, one in a cultivated area and the other in a region where it occurs naturally. Accession 29 (Uberlândia, Minas Gerais) presented the greatest genetic distance from other ***D. gardneriana*** accessions; geographically, it is 2000km from the collection localities in Maranhão. There are few records of ***D. gardneriana*** in Minas Gerais and then, investigations in this state could be useful to prospect genetic variation for breeding programs.

Two accessions of ***D. wilsonii***, from Paraopeba, Minas Gerais, had a genetic distance of 0.3594; they were in the same cluster based on Neighbor Joining. In 2007, when these two accessions were collected, only 11 plants were recorded, all of them outside protected areas, in locations impacted by anthropization (FERNANDES et al., 2007). Nevertheless, it can be seen that there is variability between these genotypes.

The results obtained demonstrate the serviceable of RAPD markers for studying genetic diversity among ***Dimorphandra*** spp. accessions. The usefulness of this type of marker in allogamous plants was demonstrated by PAMIDIAMARRI et al. (2009), who evaluated interspecific genetic diversity with RAPD and AFLP markers, among *Jatropha* accessions and concluded that both types of markers were concordant and that they can be used to identify interspecific hybrids, for marker-assisted selection and for managing genetic resources. LEAL et al. (2010), comparing RAPD and microsatellites markers for studying popcorn lines, an allogamous plant, as is fava-d'anta, concluded that both techniques provide consistent information and can be used to study genetic diversity in popcorn.

Data obtained by OLIVEIRA et al. (2008), using RAPD markers in a study of 44 genotypes of ***D. mollis*** Benth. from six localities in northern Minas

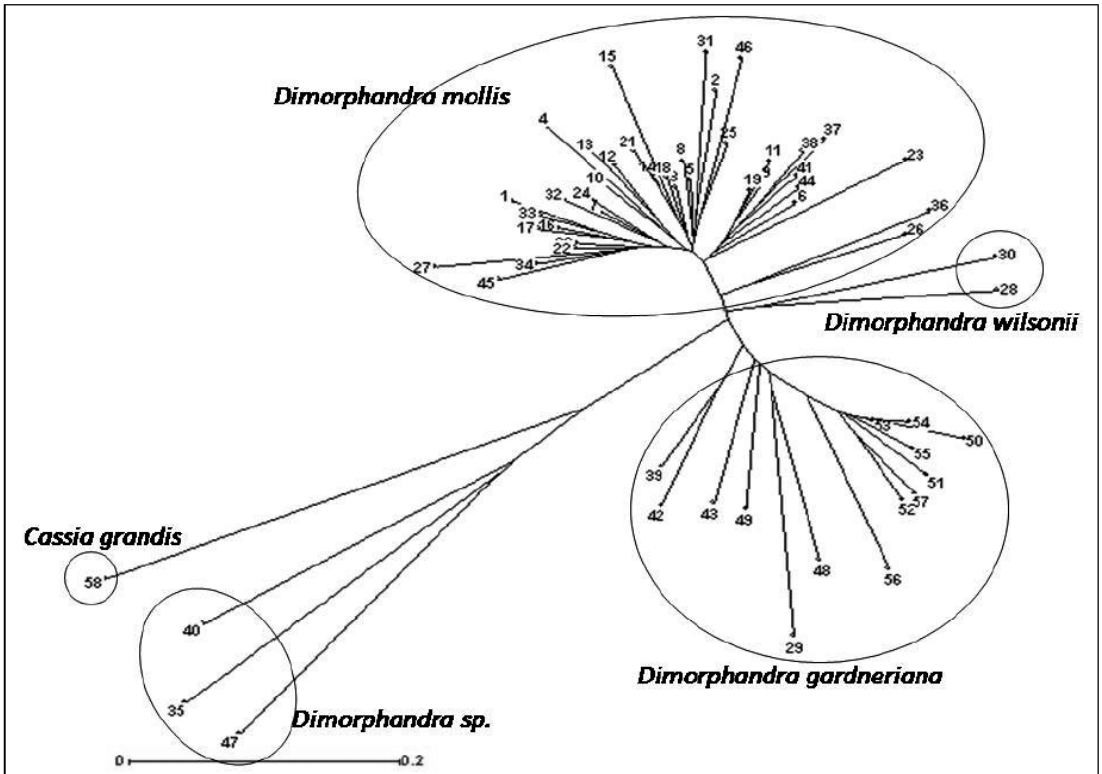

Figure 1 - Dendrogram of genetic divergence among 57 accessions from 19 Brazilian localities of ***Dimorphandra*** spp. and ***Cassia grandis*** L.f., using the Jaccard coefficient based on the Neighbor Joining algorithm from RAPD data. [***D. mollis*** (1-25-27; 31-34; 36-38; 41; 44-46); ***D. gardneriana*** (29; 39; 43; 48-57); ***D. wilsonii*** (28; 30); ***Dimorphandra*** sp. (35; 40; 47); and ***Cassia grandis*** (58)].

Gerais, demonstrated that the populations were constituted by closely-related genotypes, since they found a low degree of polymorphism. The maximum distance between the collection locations was 270km.

The genotypes that were analyzed in this study came from areas up to 2000km apart, allowing us to find diverse genotypes with potential for use in breeding programs. Consequently, though most of the areas from which collections were made, are under continuous anthropic influence, besides being prone to fires and reduced seed distribution, the last due to a decrease in natural seed dispersers (large fruit eating mammals), there is still considerable genetic diversity between and within species of ***Dimorphandra*** spp. Therefore, prospection in new areas, protection in areas which already have been studied and *ex situ* protection is primordial for the maintenance of genetic variability in these species.

CONCLUSION

There is genetic variability among and within species ***Dimorphandra*** spp. using RAPD markers. Three accessions (35, 40 and 47) need further investigations to cluster them in the genus *Dimorphandra*.

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