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Genetic diversity among air yam (*Dioscorea bulbifera*) varieties based on single sequence repeat markers

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ABSTRACT. *Dioscorea* is the largest genus in the Dioscoreaceae family, and includes a number of economically important species including the air yam, *D. bulbifera* L. This study aimed to develop new single sequence repeat primers and characterize the genetic diversity of local varieties that originated in several municipalities of Brazil. We developed an enriched genomic library for *D. bulbifera* resulting in seven primers, six of which were polymorphic, and added four polymorphic loci developed for other *Dioscorea* species. This resulted in 10 polymorphic primers to evaluate 42 air yam accessions. Thirty-three alleles (bands) were found, with an average of 3.3 alleles per locus. The discrimination power ranged from 0.113 to 0.834, with an

average of 0.595. Both principal coordinate and cluster analyses (using the Jaccard Index) failed to clearly separate the accessions according to their origins. However, the 13 accessions from Conceição dos Ouros, Minas Gerais State were clustered above zero on the principal coordinate 2 axis, and were also clustered into one subgroup in the cluster analysis. Accessions from Ubatuba, São Paulo State were clustered below zero on the same principal coordinate 2 axis, except for one accession, although they were scattered in several subgroups in the cluster analysis. Therefore, we found little spatial structure in the accessions, although those from Conceição dos Ouros and Ubatuba exhibited some spatial structure, and that there is a considerable level of genetic diversity in *D. bulbifera* maintained by traditional farmers in Brazil.

Key words: *Dioscorea bulbifera*; Genetic structure; Germplasm; Microsatellite; Microsatellite development; Traditional agriculture

INTRODUCTION

Dioscorea bulbifera L. belongs to the Dioscoreaceae family, section Opsophyton, and is one of the most important cultivated species within the genus (Lebot, 2009), although it is also considered an invasive weed in North America (Croxton et al., 2011). It has high therapeutic potential, and is considered a potential species to replace *D. deltoidea* Wall. ex Griseb., as the main source of diosgenin. Although only traces of diosgenin have been detected in *D. bulbifera*, it is widely used in traditional medicine (Correa, 1978; Narula et al., 2003; Sougata et al., 2012).

D. bulbifera is the only species of the genus that originated in both Asia and Africa, and is still found in the wild. Asian varieties have less angular, more spherical, and less toxic tubers than African varieties (Lebot, 2009). According to Martin and Ortiz (1963), *D. bulbifera* with N = 9 has been found in Africa and the Americas, while individuals with N = 10 have been found in Asia. Using flow cytometry, Obidiegwu et al. (2009a) found diploid (2X), triploid (3X), tetraploid (4X), hexaploid (6X), and octoploid (8X) ploidy levels in *Dioscorea* species, while the three *D. bulbifera* accessions studied were hexaploids (6X). *D. bulbifera* is believed to have been introduced into Brazil by Dutch settlers (Correa, 1978). It is known globally as the air yam, aerial yam, or air potato, and in Brazil it has several vernacular names that have all been inspired by the shape of its bulbils, such as cará do ar (air yam), cará moela (gizzard yam), cara fígado (liver yam), cará paquera (flert yam), cará de árvore (tree yam), cará de cipó (liana yam), and cará preto (black yam) (Bressan et al., 2005; Veasey et al., 2010).

Microsatellites, or simple sequence repeats (SSRs), are widely used molecular markers because of their codominant and highly polymorphic nature, their random distribution throughout the genome, and their high reproducibility (Sosinski et al., 2000). Several SSR primers have been developed and used to assess the genetic diversity and structure of yam species, such as cultivated *D. alata* (Tostain et al., 2006; Sartie et al., 2012; Siqueira et al., 2011, 2014), *D. trifida* (Hochu et al., 2006; Nascimento et al., 2013), and *D. cayenensis/D. rotundata* (Tostain et al., 2007; Obidiegwu et al., 2009b; Sartie et al., 2012; Scarcelli et al., 2005, 2013; Mengesha et al., 2013; Silva et al., 2014). Recently, 14 SSR loci were developed for *D. bulbifera* accessions from China using the dual-suppression polymerase chain reaction (PCR) technique (Yan et al., 2014).

The first study of *D. bulbifera* using molecular markers was by restriction fragment length polymorphism (RFLP) of cpDNA (Terauchi et al., 1991), and compared 15 accessions from Africa and Asia and their differentiation. The genetic diversity of *D. bulbifera* accessions from Africa, Asia, and Polynesia was subsequently assessed using random amplified polymorphic DNA (RAPD) (Ramser et al., 1996). This marker was also used to assess *in vitro D. bulbifera* regenerants by Dixit et al. (2003) and Narula et al. (2003). Amplified fragment length polymorphism (AFLP) markers were used to investigate the genetic diversity of 48 yam accessions from Ethiopia, including five *D. bulbifera* accessions (Tamiru et al., 2007). This study concluded that *D. bulbifera* was closer to *D. alata* than to *D. cayenensis*, *D. rotundata*, and other Ethiopian accessions (Tamiru et al., 2007). This marker was also used by Malapa et al. (2005) to investigate relationships between *D. bulbifera* and other *Dioscorea* spp. Croxton et al. (2011) compared accessions from Florida, USA with those from Africa and China using cpDNA sequences, and showed that the Florida accessions were more closely related to those introduced from China than to those from Africa. *D. bulbifera* was also evaluated using cpDNA sequences by Scarcelli et al. (2011), although the emphasis of this study was on African accessions of *D. rotundata*, *D. abyssinica*, and *D. prehensilis.* In Brazil, isozyme markers were used to study genetic diversity among local varieties of *D. bulbifera* from the Vale do Ribeira, São Paulo State (Veasey et al., 2014). The only studies that have used SSR markers in air yams are Yan et al. (2014), as mentioned above, and Croxton et al. (2011), who only used two SSR primers in addition to the cpDNA sequences.

With the objective of obtaining further information on the genetics of *D. bulbifera*, the present study aimed to develop new SSR primer pairs and assess the genetic diversity and structure of accessions from different states and municipalities of Brazil.

MATERIAL AND METHODS

Forty-two *D. bulbifera* accessions were evaluated in this study, with 25 collected in the state of São Paulo, 13 collected in the state of Minas Gerais, and one each collected in the states of Mato Grosso, Goiás, Pernambuco, and Piauí (Table 1; Figure 1). Some of the accessions were obtained in local markets, but most of them represent local varieties obtained from local farmers.

Enriched genomic library development and SSR genetic characterization

Genomic DNA was extracted from previously lyophilized leaves using a DNeasy® Plant Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK), with the addition of polyvinylpyrrolidone in one of the stages of the process to eliminate phenolic compounds and polysaccharides. DNA was quantified on 1% agarose gels, stained with Blue Green (LGC Biotecnologia, São Paulo, SP, Brazil), and photodocumented.

An enriched genomic library was constructed using the methodology developed by Billotte et al. (1999). After sequencing, the sequences obtained were analyzed and edited in the SeqMan™ II program (DNASTAR Inc.). This program forms contigs from two or more reads, and directly deletes data of poor quality from several automatic sequencers. Microsat software (CIRAD, France) was used to exclude clone sequences and adapters. After this procedure, we identified regions containing microsatellites using the SSRIT program (Temnykh et al., 2001), which is available at http://www.gramene.org/db/searches/ssrtool. Primers were then designed using the PrimerSelect program (DNASTAR Inc.) under the following conditions: fragment

size of 100-300 bp, GC content of between 40 and 60%, annealing temperature between 45° and 60°C, primer size between 18 and 22 bp, and an absence of hairpins or dimers.

Table 1. Characteristics of *Dioscorea bulbifera* accessions collected in Brazil.

No.	Accession	Municipality	Community	Folk name
1	DGC-25	Iguape-SP	Momuna	Cará moela
2	DGC-30	Iguape-SP	Peropava	Cará moela
3	DGC-317	Iguape-SP	Peropava	Cará moela
4	DGC-54	Cananéia-SP	Agrossolar	Cará paquera
5	DGC-58	Cananéia-SP	Aroeira	Cará moela
6	DGC-63	Cananéia-SP	Porto Cubatão	Cará moela
7	DGC-65	Cananéia-SP	Prainha	Cará moela
8	DGC-66	Cananéia-SP	Prainha	Cará moela
9	DGC-102	Iporanga-SP	Betarí	Cará paquera
10	DGC-118*	Iporanga-SP	Amapá	-
11	DGC-122	Anhembi-SP	-	-
12	DGC-35	Campinas-SP	(IAC)	Áspero normal
13	DGC-218	Ubatuba-SP	Fazenda da Caixa	Cará moela
14	DGC-219	Ubatuba-SP	Sertão Ubatumirim	Cará moela
15	DGC-220	Ubatuba-SP	Quilombo Camburi	Cará moela
16	DGC-221	Ubatuba-SP	Sertão do Ingá	Cará moela
17	DGC-222	Ubatuba-SP	Sertão das Cutias	Cará moela
18	DGC-223	Ubatuba-SP	Sertão do Ingá	Cará moela
19	DGC-224	Ubatuba-SP	Sertão do Ingá	Cará moela
20	DGC-225	Ubatuba-SP	Sertão do Ingá	Cará moela
21	DGC-227	Ubatuba-SP	Rio Escuro	Cará moela
22	DGC-230	Ubatuba-SP	Sertão do Quina	Cará moela
23	DGC-244	São José dos Campos-SP	-	Cará moela
24	DGC-383	São José dos Campos-SP	-	-
25	DGC-387	São Luiz do Paraitinga-SP	-	-
26	DGC-274	Conceição dos Ouros-MG	Cesários	Cará de árvore
27	DGC-275	Conceição dos Ouros-MG	Sertãozinho	Cará de cipó
28	DGC-276	Conceição dos Ouros-MG	Sertãozinho	Cará de árvore
29	DGC-277	Conceição dos Ouros-MG	Pereiras	Cará de árvore
30	DGC-280	Conceição dos Ouros-MG	Três Cruzes	Cará figado
31	DGC-373	Conceição dos Ouros-MG	Barbosas	Cará moela
32	DGC-375	Conceição dos Ouros-MG	Bernardino	Cará moela
33	DGC-376	Conceição dos Ouros-MG	Maias	Cará moela
34	DGC-379	Conceição dos Ouros-MG	Barro Branco	Cará moela
35	DGC-380	Conceição dos Ouros-MG	Campo do Meio	Cará de árvore
36	DGC-381	Conceição dos Ouros-MG	Campo do Meio	Cará cipó
37	DGC-384	Conceição dos Ouros-MG	Ribeirão Pequeno	Cará
38	DGC-385	Conceição dos Ouros-MG	Ribeirão Pequeno	Cará de árvore
39	DGC-245*	Pirenópolis-GO	-	Cará moela
40	DGC-134	Tangará da Serra-MT	-	-
41	DGC-169	Regeneração-PI	Chapada Gugel	Inhame figo
42	DGC-273	Camocim de São Félix-PE	Santa Lusia	Cará moela

Asterisks indicate accessions obtained in local markets.

PCRs for the primers developed in this study were conducted in a 10-µL volume containing 10 ng genomic DNA template, 2.5 U *Taq* DNA polymerase (LGC Biotecnologia), 10X amplification buffer (Mg^{2+} free), 5% bovine serum albumin, 3.6 mM $MgCl_2$, 0.4 µM of each primer, and 2.5 mM of each dNTP using an automated thermal cycler (Bioer Technology, Hangzhou, China). For the primers developed by Tostain et al. (2006), we conducted a PCR in a 10-µL volume that contained 10 ng genomic DNA template, 0.5 U *Taq* DNA polymerase (LGC Biotecnologia), 10X amplification buffer (Mg^{2+} free), 2.0 mM $MgCl_2$, 0.6 µM of each primer, and 2.0 mM of each dNTP.

Figure 1. *Dioscorea bulbifera* accession sampling locations in Brazil. 1, Iguape-SP; 2, Cananéia-SP; 3, Iporanga-SP; 4, Anhembi-SP; 5, Campinas-SP; 6, São José dos Campos-SP; 7, Ubatuba-SP; 8, São Luiz do Paraitinga-SP; 9, Conceição dos Ouros-MG; 10, Pirenópolis-GO; 11, Tangará da Serra-MT; 12, Camocim de São Félix-PE; 13, Regeneração-PI.

The PCRs were conducted using a MyCycler™ Thermal Cycler (Bio-Rad Laboratórios Brasil Ltd., São Paulo, SP, Brazil) with the following steps: an initial denaturing step for 5 min at 94°C, followed by 10 preamplification cycles (30 s at 95°C, 30 s at the initial annealing temperature of 60°C, decreasing by 1°C at each cycle, and 50 s at 72°C), followed by 30 cycles of denaturing (30 s at 95°C, 30 s at 50°C, and 50 s at 72°C), and a final extension step for 5 min at 72°C. The amplified products were separated on denaturing 7% polyacrylamide gels (C.B.S. Scientific, USA), and the products were visualized by silver staining according to the methods of Creste et al. (2001).

Statistical analysis

Because *D. bulbifera* is a polyploid species, microsatellite bands were assessed as the presence/absence of bands as dominant markers. The number of bands (alleles) per locus and the percentage of polymorphism were obtained for all analyzed accessions. To compare the efficiency of the markers in the identification of genotypes, parameter D (Tessier et al., 1999) was estimated, which confers the power of discrimination of each primer. This parameter was calculated according to the formula:

$$D_j = 1 - C_j = 1 - \sum_{i=1}^{1} pi \frac{(N_{pi} - 1)}{N - 1} \qquad \text{(Equation 1)}$$

where D is the probability that two randomly selected individuals have different band patterns, C is the probability that two randomly selected individuals have a similar band pattern, and N is the number of individuals analyzed.

Using the Jaccard similarity coefficient, cluster (unweighted pair group method with arithmetic mean) and principal coordinate analyses were conducted using PAST software (Hammer et al., 2001). The accuracy of the groupings was estimated from simulations with resampling, using 10,000 bootstraps. We also conducted a Bayesian analysis using the Structure program (Pritchard et al., 2000), which does not rely on prior information on possible groups based on the origin of the accessions. This analysis was run using the admixture model with correlated allele frequencies and repeated eight times for each K (number of assumed clusters), with a burn-in of 250,000 interactions that was followed by 500,000 Markov Chain Monte Carlo interactions. The most probable number of clusters was chosen using the ΔK method (Evanno et al., 2005).

RESULTS

The genomic library was obtained and sequenced from 86 colonies (clones) containing the insert. Twenty-one colonies had microsatellite sequences, representing an enrichment of 24.4% of the library according to the protocol described by Billotte et al. (1999), who consider enrichment above 20% as high. Most of the repeat motifs (six of the seven primer pairs selected) were classified as dinucleotides, and all of them were classified as perfect. In total, we found 26 regions containing microsatellites. Among the 26 microsatellites obtained, seven SSR markers were selected (Table 2). Designing the remaining primer sequences was not possible because of their low quality. All of the SSR markers had synthesized amplicons, and only one (Db7) exhibited a monomorphic pattern.

Table 2. Characteristics of microsatellite primers developed for *Dioscorea bulbifera*.

Name	Forward and reverse sequences	GenBank No.	Ta (°C)	Size (pb)	Motif
Db2	F: CACGACCTCCTGGAAGACAACT R: ATATAGCACGGGAGGCACAAAC	KC110758	53	214	$(GAC)_4$
Db3	F: TTTTACCCAGGATTTAGAAGAA R: GGACTGGAGCCACAAGATT	KC110759	50	279	$(CA)_8$
Db4	F: TCTCGCTGTTCTCGTGTTCTTC R: GTCCGATTTGATGGTGCTTCTC	KC110760	55	194	$(GA)_{17}$
Db5	F: TGTCTATTATATTGCTCTTTCT R: CGTTTCTAATTTCTGGGTAT	KC110761	50	284	$(GT)_4$
Db6	F: AAGCCGGTATCATTCAACAAAA R: CCCTCGCCAACATCAAGTAA	KC110762	53	170	$(AC)_8$
Db7	F: CCGCAAGGCTCAAAAAGTTAGG R: TCGTGGATGAAGATGGGTGGAC	KC110763	53	104	$(GA)_4$
Db8	F: TCCCAAGAAATCCAGAATA R: ATGCATGCCAAAACAAATA	KC110764	50	139	$(TG)_8$

Ta = annealing temperature.

Thirty-three alleles (bands) were found for the 10 polymorphic loci analyzed, including the six primers developed in this study and four other loci obtained from the literature (Tostain et al., 2006), ranging from two to four alleles per locus, with an average of 3.3 alleles per locus and 100% polymorphism. The discrimination power (D) parameter ranged from 0.113 to 0.834, with an average of 0.595.

Neither principal coordinate nor cluster analysis could separate the accessions

according to their origins (Figures 2 and 3). In addition, Bayesian analysis did not detect any groups (data not shown), confirming that the *D. bulbifera* accessions did not exhibit any spatial structure. However, 13 accessions from Conceição dos Ouros, Minas Gerais were clustered above zero on the principal coordinate 2 axis, which explained 12.1% of the total variation (Figure 3), and all of the accessions from Ubatuba, São Paulo (except for one accession, number 17) were clustered below zero on principal coordinate 2 axis, indicating a slight separation between these two locations. Other accessions from São Paulo and the other states were scatted without any clear pattern.

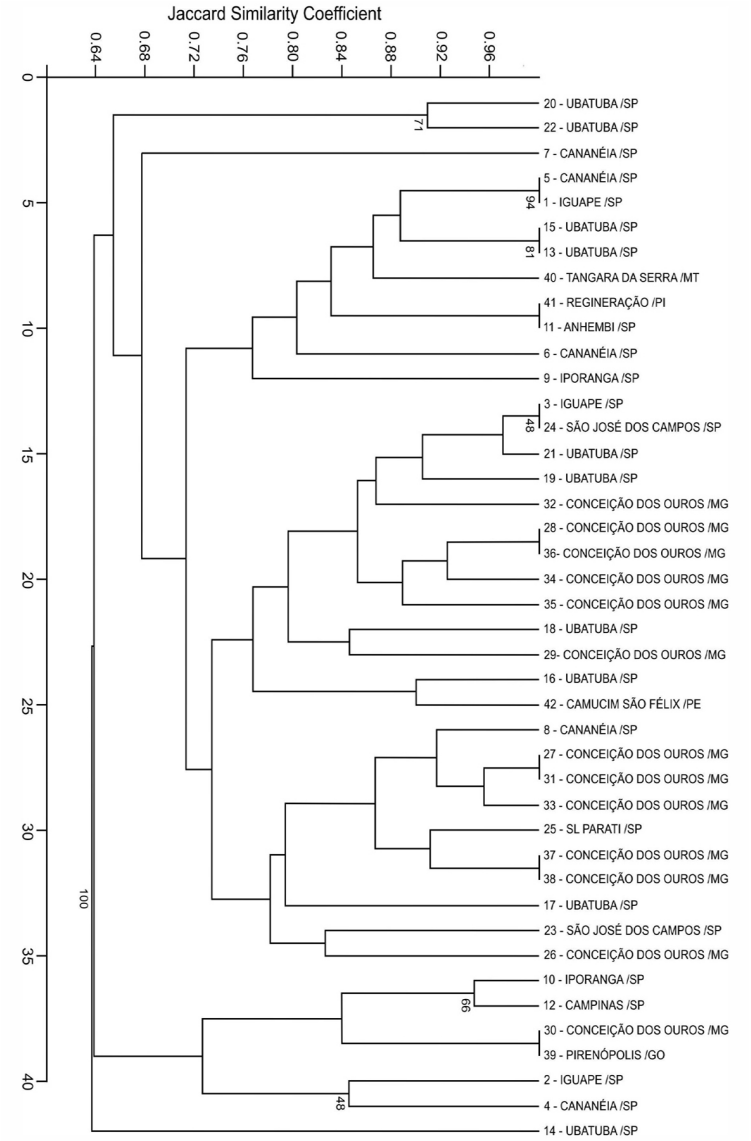

Figure 2. Cluster analysis of 42 *Dioscorea bulbifera* accessions from Brazil based on 10 simple sequence repeat loci.

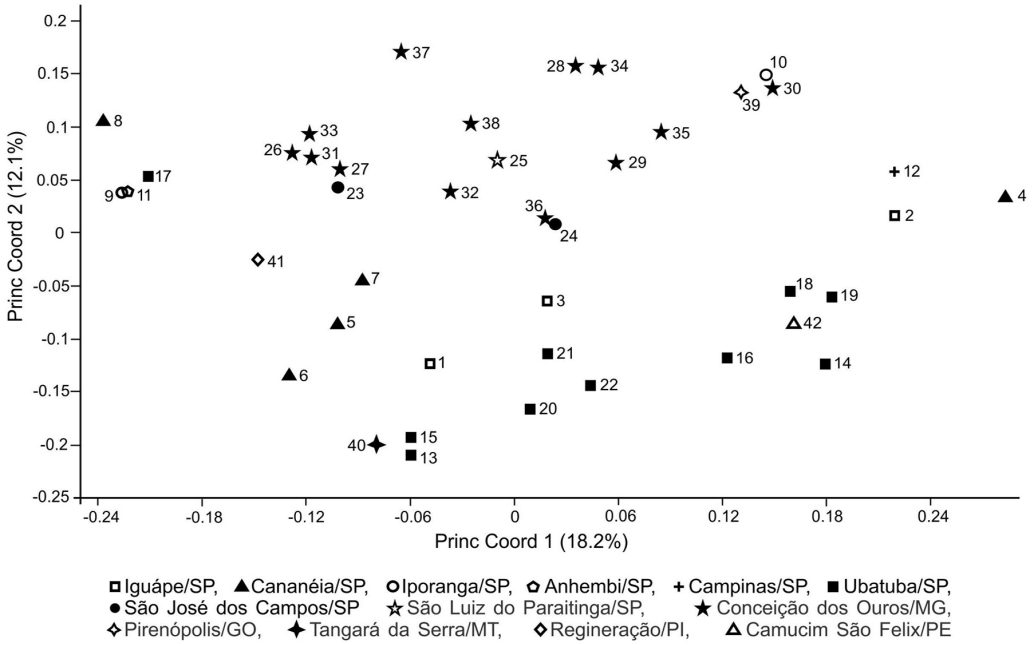

Figure 3. Principal coordinate analysis corresponding to 30.3% of the total variation in 42 *Dioscorea bulbifera* accessions from Brazil based on 10 simple sequence repeat loci.

The cluster analysis classified the accessions into two groups, with one group containing one accession from Ubatuba (number 14) and the other group the remainder of the accessions; therefore, no clear pattern was found in terms of accession origin in the two groups (Figure 2). Although not supported by the bootstrap analysis, three subgroups were identified in the group with most of the accessions, and one of these contained all of the Minas Gerais accessions. However, the 25 accessions from São Paulo were scattered into both groups and subgroups, both in the cluster analysis and in the principal coordinate analysis. Even accessions from the same municipality, such as Ubatuba or Iguape, were scattered into different groups and subgroups. Eight duplicates were found among the accessions; three of them included two accessions each from Conceição dos Ouros, Minas Gerais and a fourth with one accession from Conceição dos Ouros, Minas Gerais and one from Pirenópolis, Goiás. The other duplicates included accessions from different municipalities of São Paulo, and one included an accession each from Anhembi, São Paulo and Regeneração, Piauí. These two accessions had very distant origins and were clustered together.

DISCUSSION

The most frequent class of microsatellite found in this study was dinucleotide, corresponding to six of the seven primer pairs selected. The same dinucleotide predominance has been found for *D. cayenensis* (Silva et al., 2014) and *D. alata* (Tostain et al., 2006; Siqueira et al., 2011). Microsatellite loci of the perfect dinucleotide type are ideal for obtaining highly informative markers of high polymorphism levels, because mutation rates are higher in dinucleotides than in

tri- or tetranucleotides and are therefore widely used in genetic studies (Ellegren, 2004).

Locus amplification yielded 33 alleles (bands), with an average of 3.3 alleles per locus. Croxton et al. (2011) analyzed two SSR loci among *D. bulbifera* accessions with different origins (USA, Africa, and China) and found two and four alleles for each loci, respectively, with an average of 3.0 alleles, which is similar to our study. Siqueira et al. (2014) obtained an average of 5.1 alleles per locus among 89 *D. alata* accessions using 12 SSR loci. The average number of alleles per locus we found in *D. bulbifera* was also lower than the averages found by Hochu et al. (2006), Tostain et al. (2006), and Obidiegwu et al. (2009b), who found 7.6, 7.3, and 8.4 alleles per locus, respectively, in several species of *Dioscorea*. However, Sartie et al. (2012) found an average of 3.7 alleles per locus in *D. alata*, *D. cayenensis*, *D. dumetorum*, and *D. rotundata*.

The discrimination power values were calculated in order to compare the efficiency of the SSR markers for *D. bulbifera*. This parameter indicates a high level of polymorphism when its value is close to one. In our study, an average of 0.595 was found for this parameter, which may be considered a moderate to high value. Therefore, these primers had adequate discriminatory power, allowing them to detect high polymorphism among the genotypes. Siqueira et al. (2014) found an average of 0.92 for *D. alata* accessions, while Nascimento et al. (2013) found a lower average value (0.79) for *D. trifida* accessions, but still higher than the values found in this study.

Although a slight spatial structure was observed in both the principal coordinate and cluster analyses with the clustering of the Minas Gerais accessions, the other accessions were not classified into groups according to their origins, suggesting that high rates of gene flow have occurred between the accessions. Because the first two principal coordinates explained only 30.3% of the total variation and only one group was detected among the accessions in the Bayesian analysis, we conclude that the *D. bulbifera* accessions analyzed in this study have no spatial structure, although a slight spatial structure was detected in the principal coordinate analysis. An absence of genetic structure has been observed among *D. alata* accessions (Siqueira et al., 2014), suggesting that this result may be due to the behavior of farmers, who exchange tubers among their neighbors and friends resulting in a mixture of genotypes.

Only a few studies have been conducted on the genetic diversity of *D. bulbifera*. Ramser et al. (1996) assessed plants originating from Africa, Asia, and Polynesia, and found that the accessions were grouped according to their geographical origin. Terauchi et al. (1991) examined *D. bulbifera* using RFLP from cpDNA, and found a clear geographical distinction between accessions. Veasey et al. (2014) studied the genetic diversity of 17 *D. bulbifera* accessions from Vale do Ribeira using isozymes, and found high genetic diversity and non-spatial structuring among the accessions, agreeing with the results of our study.

Yam diversity may have been maintained by social and economic dynamics between local communities and traditional and ethnic groups that manage this diversity in different ways, depending on their history and the context in which they exist. Yam tubers can travel great distances from their places of origin thanks to migratory flows, because farmers can move to another state or municipality, or even use the tubers as gifts between friends and family, which is common in traditional societies (Siqueira, 2011).

Malapa et al. (2005) found no geographical differences between *D. alata*, *D. nummularia*, and *D. transverse* accessions (of Asian, African, and Melanesian origins, respectively) using AFLP markers, which confirmed the results of Lebot et al. (1998) who used isozyme markers for *D. alata*, and found that clones of these plants were widely distributed. For *D. cayenensis/D. rotundata*, Obidiegwu et al. (2009b) found no relationship between the

accessions and their geographical areas of collection, possibly because the cultivars travelled large distances by human migration as clones. Yam cultivation is part of a dynamic, ongoing process, as observed by Scarcelli et al. (2013), as new genotypes of *D. rotundata* are regularly introduced and become widely used varieties. According to these authors, it may be possible to introduce new varieties that are better adapted to a novel biotic or abiotic environment (Scarcelli et al., 2013). However, *D. bulbifera* is not consumed in Brazil as much as *D. rotundata* is in Africa, and some questions remain to be answered, such as how traditional systems have created such genetic diversity, and for how long traditional agriculture will preserve these accessions in the field.

Conflicts of interest

The authors declare no conflict of interest.

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