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Spatially structured genetic diversity of the Amerindian yam (Dioscorea trifida L.) assessed by SSR and ISSR markers in Southern Brazil

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Abstract	*Dioscorea trifida* L. (Dioscoreaceae) is among the economically most important cultivated Amerindian yam species, whose origin and domestication are still unresolved issues. In order to estimate the genetic diversity maintained by traditional farmers in Brazil, 53 accessions of *D. trifida* from 11 municipalities in the states of São Paulo, Santa Catarina, Mato Grosso and Amazonas were characterized based on eight Simple Sequence Repeats (SSR) and 16 Inter Simple Sequence Repeats (ISSR) markers. The level of polymorphism among the accessions was high, 95 % for SSR and 75.8 % for ISSR. The SSR marker showed higher discrimination power among accessions compared to ISSR, with *D* parameter values of 0.79 and 0.44, respectively. Although SSR and ISSR markers led to dendrograms with different topologies, both separated the accessions into three main groups: I—Ubatuba-SP; II—Iguape-SP and Santa Catarina; and III—Mato Grosso. The accessions from Amazonas State were classified in group II with SSR and in a separate group with ISSR. Bayesian and principal coordinate analyzes conducted with both molecular markers corroborated the classification into three main groups. Higher variation was found within groups in the AMOVA analysis for both markers (66.5 and 60.6 % for ISSR and SSR, respectively), and higher Shannon diversity index was found for group II with SSR. Significant but low correlations were found between genetic and geographic distances ($r = 0.08$; $p = 0.0007$ for SSR and $r = 0.16$; $p = 0.0002$ for ISSR). Therefore, results from both markers showed a slight spatially structured genetic diversity in *D. trifida* accessions maintained by small traditional farmers in Brazil.

Keywords (separated by '-')	*Dioscorea trifida* - Genetic diversity - Genetic structure - Molecular markers - Traditional agriculture - Yams
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RESEARCH ARTICLE

Spatially structured genetic diversity of the endangered Amerindian yam (*Dioscorea trifida* L.) assessed by SSR and ISSR markers in Southern Brazil

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Abstract *Dioscorea trifida* L. (Dioscoreaceae) is among the economically most important cultivated Amerindian yam species, whose origin and domestication are still unresolved issues. In order to estimate the genetic diversity maintained by traditional farmers in Brazil, 53 accessions of *D. trifida* from 11 municipalities in the states of São Paulo, Santa Catarina, Mato Grosso and Amazonas were characterized based on eight Simple Sequence Repeats (SSR) and 16 Inter Simple Sequence Repeats (ISSR) markers. The level of polymorphism among the accessions was high, 95 % for SSR and 75.8 % for ISSR. The SSR marker showed higher discrimination power among accessions compared to ISSR, with *D* parameter values of 0.79 and 0.44, respectively. Although SSR and ISSR markers led to dendrograms with different topologies, both separated the accessions into three main groups: I—Ubatuba-SP; II—Iguape-SP and Santa Catarina; and III—Mato Grosso. The accessions from Amazonas State were classified in group II with SSR and in a separate group with ISSR. Bayesian and principal coordinate analyzes conducted with both molecular markers corroborated the classification into three main groups. Higher variation was found within groups in the AMOVA analysis for both markers (66.5 and 60.6 % for ISSR and SSR, respectively), and higher Shannon diversity index was found for group II with SSR. Significant but low correlations were found between genetic and geographic distances ($r = 0.08$; $p = 0.0007$ for SSR and $r = 0.16$; $p = 0.0002$ for ISSR). Therefore, results from both markers showed a slight spatially structured genetic diversity in *D. trifida* accessions maintained by small traditional farmers in Brazil.

Keywords *Dioscorea trifida* · Genetic diversity · Genetic structure · Molecular markers · Traditional agriculture · Yams

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Introduction

The genus *Dioscorea*, family Dioscoreaceae, represents an important food source in the humid and subhumid tropics (Ayensu and Coursey 1972). This genus consists of more than 600 species, of which only 10 are used for human consumption (Lebot 2009). *Dioscorea trifida* L., originating in South America, is

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among the economically most important species, including *Dioscorea cayenensis* Lam. and *Dioscorea rotundata* Poir., originating in Africa, and *Dioscorea alata* L., originating in Asia (Coursey 1976).

Dioscorea trifida is an herbaceous, autotetraploid (x = 20 and 2n = 4x = 80), viny, and perennial plant (Bousalem et al. 2006), with quadrangular winged stems without spines but with deeply lobed leaves, usually arranged alternately or rarely opposite (Montaldo 1991). The plants are dioecious, with small unisexual flowers that, when fertilized, produce inedible encapsulated fruits (Stephens 2009). Its reproduction occurs by allogamy or vegetative propagation (Montaldo 1991). The tuber, the edible plant structure, has a high nutritional quality and astringent, antimicrobial and diuretic properties, which allow its use for combating malnutrition and treatment of diseases such as diabetes and high cholesterol levels (Ramos-Escudero et al. 2010). The main limiting factor for growing *D. trifida* is potyviruses (genus *Potyvirus*, family Potiviridae), which causes a variety of symptoms on the leaves of infected plants (Odu et al. 2004). Potyvirus infection can cause significant economic damage and process of genetic erosion of the crop (Bousalem et al. 2010).

The evolutionary history of *D. trifida* is controversial. Although it occurs very frequently in various countries of Latin America, and the Amazon has been reported as a possible center of origin and diversification of this species (Degras 1993), the lack of information about its origin and domestication process is still evident. It is believed that *D. trifida* originated on the border between Brazil, Guyana, French Guyana and Suriname, and was domesticated by indigenous peoples in these regions (Pedralli 1998). Recent studies conducted in French Guiana revealed the presence of wild relatives of *D. trifida*, being the first direct genetic evidence of possible places of origin for this species (Bousalem et al. 2010).

In the Amazon, Clement (1999) observed the existence of several areas with large concentrations of genetic resources related to different crop species. *D. trifida* was present in some of these sites, such as in the Northwestern Amazonian Center, the Central Amazonian Center, the Middle Orinoco Minor Centre and the Guiana Minor Centre, indicating the close relationship of these areas with the evolutionary history of *D. trifida*. In archaeological excavations in Panama, *D. trifida* tubers were found together with cassava (Piperno et al. 2000, Dickau et al. 2007). As cassava was domesticated in southwestern Amazon Basin (Olsen 2004) and quickly spread throughout Tropical America (Piperno et al. 2000, Dickau et al. 2007), *D. trifida* could have been domesticated and propagated by the same tribes involved in the process of cassava domestication (Bousalem et al. 2010), possibly being the first yam species cultivated by indigenous peoples in the Amazon (Degras 1993). The cultivation of *D. trifida* in Brazil has taken place since then, mainly by small rural farmers (Pedralli 1998). In recent surveys, the occurrence of this species was observed in the Central West, South and Southeast Brazil (Bressan et al. 2005; Veasey et al. 2010). It is also an important crop in the Amazon (Velez 1998).

Despite the geo-cultural and socioeconomic importance of *D. trifida*, few studies are conducted to explore its potential and to develop conservation strategies for this crop. In Brazil, there are few institutions currently involved in research related to the yam crop; therefore, new studies are important to add information for breeding programs and conservation strategies. As the cultivation and consumption of yam are very intense in family agricultural production systems practiced by traditional communities, these systems provide a favorable environment for the generation and maintenance of genetic diversity of this crop (Veasey et al. 2010). However, the socioeconomic pressures faced by farmers in recent years have caused the loss of plant genetic resources, specifically *D. trifida*, and biodiversity losses can be severe and irreversible. In this context, there is a need to estimate the genetic diversity of *D. trifida* maintained by traditional farmers to assist in developing strategies to preserve the species and lessen losses caused by various socioeconomic pressures on the yam crop.

Various molecular biological techniques are available to detect genetic variability of natural populations and cultivated plants. Among these techniques, microsatellites or Simple Sequence Repeats (SSR) are very effective because they are codominant, multi-allelic, highly polymorphic and show good reproducibility (Oliveira et al. 2006). In order to study the genetic diversity of *D. trifida*, Hochu et al. (2006) developed eight SSR primers specific for this species, which were used for the analysis of 24 cultivars, showing high polymorphism. These primers were used by Bousalem et al. (2006) to assess the inheritance pattern of *D. trifida*, from the analysis of parental genotypes and

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offspring, where the tetraploid behavior of the species was reported.

Another marker used in genetic diversity studies is Inter Simple Sequence Repeats (ISSR), which were developed to explore microsatellite repeats without the need to use of DNA sequencing (Zietkiewicz et al. 1994; Reddy et al. 2002). ISSR markers are very stable, dominant, multi-allelic, present good reproducibility and generate a large number of polymorphic fragments (Mattioni et al. 2002; Wolfe 2005). Few genetic diversity studies have been performed in the *Dioscorea* genus based on ISSR molecular markers. Among them, Zhou et al. (2008) analyzed the level of genetic diversity among different cultivars of *Dioscorea opposita* Thunb., widely used in traditional Chinese medicine and Wu et al. (2009) evaluated the relationship and genetic variability among accessions of *D. alata.* Both studies found that ISSR provided a good assessment of genetic diversity of yam and valuable information to help in selecting parents for future yam breeding programs.

The aim of this study was to characterize 53 accessions of *D. trifida* originating in traditional communities in the States of Santa Catarina, São Paulo, Mato Grosso and Amazonas, using ISSR and SSR markers, in order to verify the level of genetic diversity maintained by farmers in these regions in Brazil. The study describes the spatially structured genetic variation of *D. trifida* maintained by these farmers and the genetic diversity that is concentrated within the different sampling sites.

Materials and methods

Plant materials

We evaluated 53 accessions of *D. trifida* collected from 11 municipalities in the States of Sao Paulo (SP), Santa Catarina (SC), Mato Grosso (MT) and Amazonas (AM), located between latitudes 14°43′S and 26°15′S and longitudes 44°01′W and 62°05′W (Fig. 1; Table 1). In each visited municipality, a collection was conducted so as to seek greater representation of the genetic variability, taking into account morphological variation and information from farmers. Three accessions (the two accessions from Amazonas and one from Ubatuba, SP) were acquired in local markets. Accessions were collected in the form of tubers, which were grown in pots placed in a greenhouse at the Luiz de Queiroz College of Agriculture, University of Sao Paulo, in Piracicaba, SP, located at 22°43′S latitude and 47°25′W longitude, where young leaves were collected for DNA extraction.

DNA extraction and quantification

Young, newly expanded leaves were collected and stored at 4 °C for 7 days in a CTAB gel, containing 30 mg CTAB, 350 mg NaCl and 70 ml of distilled water (Rogstad 1992). After this period, the gel excess was removed from plant tissues with the aid of a paper towel. The fragments were then macerated in 1 mL STE buffer [0.13 mg saccharose, 45 mL of Tris–HCL (1 M), 150 mL of EDTA (0.5 M), completing with distilled water to a final volume of 1.5 mL] and subjected to DNA extraction by the method of Doyle and Doyle (1990). DNA concentration was estimated in a 1 % agarose gel, using a TBE 10X running buffer, stained in ethidium bromide. A final concentration of 5 ng/μL was obtained for the PCR analysis.

Amplification of SSR and ISSR

For the SSR amplification, 10 primer pairs developed by Hochu et al. (2006) and Tostain et al. (2006) were tested (Table 2). PCR was conducted in a 16 μL reaction volume containing: 5 ng of genomic DNA in a 5× reaction buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 5 pmol of forward primer, 5 pmol of reverse primer, and 5 U/μL *Taq* DNA polymerase (Promega, Madison, USA). The amplification reactions were performed in a MyCycler Thermal Cycler model BioRad thermocycler using the following steps: 1) denaturation at 94 °C for 5 min, followed by 30 cycles [30 s at 94 °C, 30 s at annealing temperature (touchdown of 50–60 °C) and 30 s for 72 °C], and a final stage of extension of 5 min at 72 °C for the PCR reactions with specific primers for *D. trifida* (Hochu et al. 2006); 2) denaturation at 94 °C for 5 min, followed by 35 cycles [30 s at 94 °C, 1 min at a temperature of annealing (touchdown 50–60 °C) and 1 min at 72 °C], and a final extension of 8 min at 72 °C for PCR reactions with heterologous primers (Tostain et al. 2006).

Electrophoresis was performed on denaturing 7 % polyacrylamide gel, with a constant power of 70 W for the time necessary for separating the amplified fragments in each primer, using 10 and 100 bp DNA

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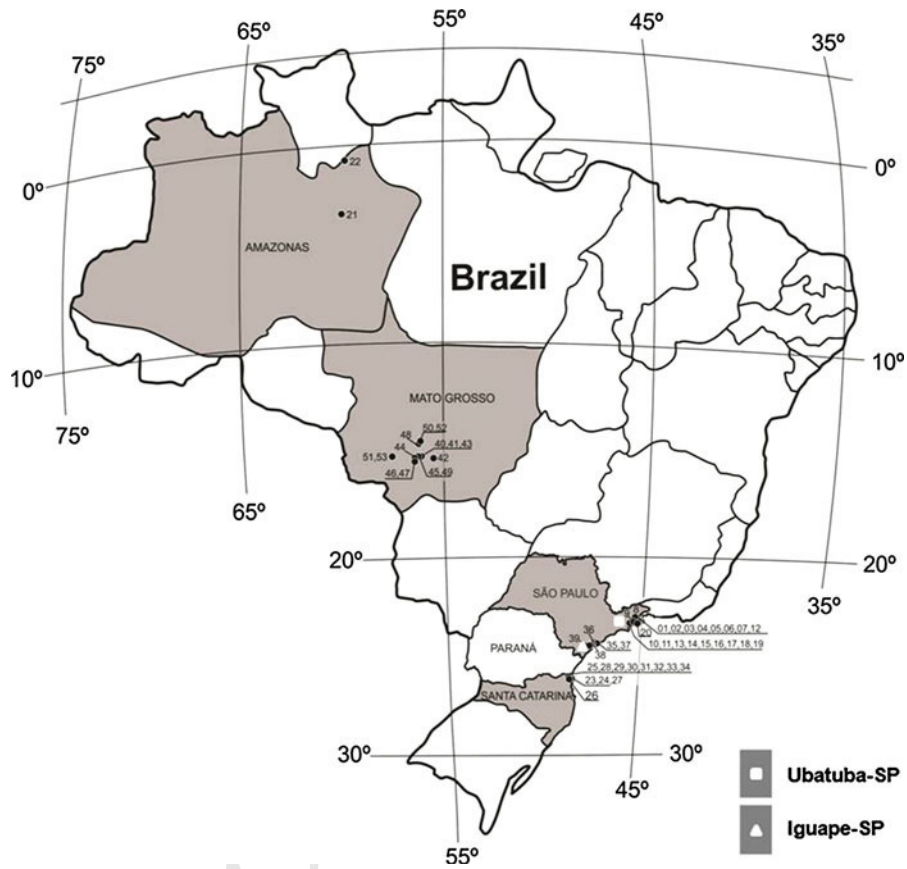

Fig. 1 Collection sites of *D. trifida* in Brazil. Details of each accession are given in Table 1

Ladder (Invitrogen™, São Paulo, Brazil) as markers. The gels were stained using silver nitrate methodology (Creste et al. 2001) for the revelation of microsatellite bands, which were photographed with a digital camera and evaluated in a transiluminator.

For the ISSR analysis 20 primers were tested according to the Wolfe (2000, 2005) protocol (Table 3). PCR reactions were performed in a final volume of 30 µL containing: 5 ng of genomic DNA in a 5× reaction buffer, 2.0 mM $MgCl_2$, 2.33 mM dNTPs, 10 pmol primer and 5 U/µL Taq DNA polymerase (Promega, Madison, USA). The amplification of the DNA template were performed in MultiGene Thermal Cycler thermocycler (Labnet International, Inc.) according to the following amplification conditions: 90 s at 94 °C, 35 cycles at 94 °C for 40 s, followed by 46 cycles (52 °C for 45 s, 72 °C for 90 s, 94 °C for 45 s, 44 °C for 45 s), and a final stage of extension at 72 °C for 5 min (Wolfe 2000).

The products resulting from the amplification reactions were subjected to electrophoresis on a 2 % agarose gel in TBE buffer 10X for 140 min at 90 V and stained with ethidium bromide. A 100 bp DNA Ladder (Invitrogen™, Carlsbad, USA) was used as a marker. Additionally, we used control samples previously amplified with success. The gel was photographed over ultraviolet light source with Syngene photodocumentation system (Synoptics Ltda., Cambridge, United Kingdom). For statistical analysis we considered only robust and unambiguous bands. We discarded the bands that showed low intensity or coalescing with other bands.

Statistical analysis

Due to the tetraploid behavior of *D. trifida*, as described by Bousalem et al. (2006), the band patterns of the SSR and ISSR markers were both interpreted as binary data, presence (1) and absence (0) of bands, generating data matrices that were subjected to the following statistical programs. Genetic diversity analyses were based on POPGENE Software, version 1.3 (Yeh et al. 1997), where we obtained the number of bands observed per primer, number of polymorphic

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Table 1 *Dioscorea trifida* accessions used in this study collected in Brazil, including accession and identification number (ID) in the Germplasm Bank, origin (community, municipality, state), popular name and geographic coordinates

Accession	ID	Geographic localities	Popular name	Lat/Long
01	180	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°15′S/44°01′O
02	181	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará branco	23°17′S/44°05′O
03	182	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°18′S/44°52′O
04	183	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°17′S/44°51′O
05	184	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°18′S/44°51′O
06	185	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°18′S/44°51′O
07	187	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará branco	23°18′S/44°51′O
08	191	Sertão das Cutias, Ubatuba, São Paulo	Cará roxo	23°22′S/44°58′O
09	193	Rio Escuro, Ubatuba, São Paulo	Cará branco	23°28′S/45°08′O
10	195	Sertão do Ingá, Ubatuba, São Paulo	Cará cobrinha	23°31′S/45°13′O
11	196	Sertão do Ingá, Ubatuba, São Paulo	Cará branco	23°31′S/45°13′O
12	197	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°17′S/44°51′O
13	198	Sertão do Ingá, Ubatuba, São Paulo	Cará roxo	23°31′S/45°13′O
14	201	Sertão do Ingá, Ubatuba, São Paulo	Cará roxo	23°31′S/45°14′O
15	203	Sertão do Ingá, Ubatuba, São Paulo	Cará roxo	23°31′S/45°14′O
16	204	Rio Escuro, Ubatuba, São Paulo	Cará roxo	23°28′S/45°08′O
17	208	Araribá, Ubatuba, São Paulo	Cará roxo	23°32′S/45°15′O
18	210	Sertão de Ubatumirim, Ubatuba, São Paulo	Cará roxo	23°29′S/45°10′O
19	216	Fazenda da Caixa, Ubatuba, São Paulo	Cará roxo	23°31′S/45°14′O
20	217	Feira de Ubatuba, Ubatuba, São Paulo	Cará roxo	23°27′S/45°09′O
21	236	Feira de Manaus, Manaus, Amazonas	Cara roxo	03°08′S/60°01′O
22	237	Feira de Barcelos, Barcelos, Amazonas	Cará	0°58′S/62°55′O
23	281	Pirabeiraba, Joinville, Santa Catarina	Cará	26°10′S/48°55′O
24	282	Pirabeiraba, Joinville, Santa Catarina	Cará mimoso	26°09′S/48°56′O
25	283	Pirabeiraba, Joinville, Santa Catarina	Cará	26°09′S/48°58′O
26	285	Acaraí, São Francisco do Sul, Santa Catarina	Cará pão	26°11′S/48°53′O
27	286	Pirabeiraba, Joinville, Santa Catarina	Cará mimoso	26°15′S/48°37′O
28	287	Pirabeiraba, Joinville, Santa Catarina	Carcanhá de nego	26°09′S/48°59′O
29	290	Pirabeiraba, Joinville, Santa Catarina	Cará mimoso	26°09′S/48°59′O
30	292	Pirabeiraba, Joinville, Santa Catarina	Cará	26°09′S/48°59′O
31	297	Pirabeiraba, Joinville, Santa Catarina	Cará	26°09′S/48°59′O
32	298	Rio da Prata, Joinville, Santa Catarina	Cará	26°11′S/48°58′O
33	301	Rio da Prata, Joinville, Santa Catarina	Cará mimoso	26°11′S/48°58′O
34	302	Pirabeiraba, Joinville, Santa Catarina	Cará	26°10′S/48°57′O
35	312	Icapara, Iguape, São Paulo	Cará São João branco	24°40′S/47°27′O
36	313	Cavalcanti, Iguape, São Paulo	Cará-pipa	24°43′S/47°45′O
37	323	Icapara, Iguape, São Paulo	Cará São João roxo	24°40′S/47°27′O
38	328	Momuna, Iguape, São Paulo	Cará São João roxo	24°42′S/47°40′O
39	329	Momuna, Iguape, São Paulo	Cará São João branco	24°42′S/48°40′O
40	335	Carumbé, Acorizal, Mato Grosso	Cará roxo	15°08′S/56°12′O
41	336	Carumbé, Acorizal, Mato Grosso	Cará roxo	15°08′S/56°12′O
42	340	Rio dos Couros, Cuiabá, Mato Grosso	Cará pé de anta	15°36′S/55°48′O
43	343	Carumbé, Acorizal, Mato Grosso	Cará branco	15°08′S/56°12′O

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Table 1 continued

Accession	ID	Geographic localities	Popular name	Lat/Long
44	344	Sela Dourada, Nobres, Mato Grosso	Cará do Joaquim	15°36′S/56°48′O
45	345	Santo Antônio do Barreiro, Jangada, Mato Grosso	Cará roxo	15°08′S/56°17′O
46	350	Sela Dourada, Nobres, Mato Grosso	Cará branco	15°34′S/56°46′O
47	351	Sela Dourada, Nobres, Mato Grosso	Cará mão de anta	15°30′S/56°42′O
48	352	Timbozal, Rosário Oeste, Mato Grosso	Cará mão de anta	14°51′S/56°23′O
49	355	Chapada Vacaria, Acorizal, Mato Grosso	Cará roxo	15°03′S/56°08′O
50	361	Sela Dourada, Nobres, Mato Grosso	Cará roxo	14°43′S/56°15′O
51	364	Barranco Alto, Rosário Oeste, Mato Grosso	Pombinho branco	15°14′S/57°59′O
52	366	Sela Dourada, Nobres, Mato Grosso	Cará roxo cumprido	14°43′S/56°15′O
53	368	Barranco Alto, Rosário Oeste, Mato Grosso	Cará roxo	15°17′S/57°50′O

Table 2 List of SSR primers used to evaluate 53 *Dioscorea trifida* accessions, including primer sequence, annealing temperature (T_A); size range of SSR bands in base pairs (bp), number of bands (N_B), number of polymorphic bands (N_{PB}), percent polymorphism (P) and discriminating power (D)

Primer code	Sequence (5′–3′)	T_A (°C)	Size range (bp)	N_B	N_{PB}	P (%)	D
Da1A01[1]	F: TAT AAT CGG CCA GAG G	51–53	202–205	2	2	100.0	0.97
	R: TGT TGG AAG CAT AGA GAA						
Dab2C05[1]	F: CCC ATG CTT GTA GTT GT	51–52	168–192	5	5	100.0	0.91
	R: TGC TCA CCT CTT TAC TTG						
MTI2[2]	F: TCATCAAGAGCATCAAAAAAC	50–52	121–131	6	6	100.0	0.71
	R: GCCTCGTCTTTGAAGTTGGT						
MTI3[2]	F: TAACAAACAAAAAATGAAAC	55–59	156–205	13	13	100.0	0.85
	R: TAACAGTGATTGAGCTAGGA						
MTI4[2]	F: ACTTGGTGTTGTTGGATTGC	50–58	101–111	8	8	100.0	0.61
	R: TATCACTCCCCAGACCAGA						
MTI10[2]	F: TCGTGTCCATCTTGCTGCGT	55–58	143–198	11	11	100.0	0.61
	R: GAAAAGCGGAGATGAAGAGCA						
MTI11[2]	F: CTCTTTTGCTTCTCATTTCA	55–56	124–137	5	4	80.0	0.72
	R: ATGTAGCCAATCCAAAATAG						
MTI12[2]	F: CTGCCAGCGTTCCGATTC	55–60	100–123	6	5	83.0	0.92
	R: CGTAGGACCTCTCGCATCAG						
Average		–	–	7.0	6.75	95.0	0.79

[1] Tostain et al. (2006); [2] Hochu et al. (2006)

bands, percent polymorphism and estimated the Shannon index according to the following formula: $H' = -\sum_{i=1}^{s} pi \log pi$, where pi is the frequency of each species, for i ranging from 1 to S (richness).

In order to compare the efficiency of the markers in the genotypic identification, the discrimination power (D) (Tessier et al. 1999) was estimated for each primer. This parameter was calculated according to the formula: $D_j = 1 - C_j = 1 - \sum_{i=1}^{I} pi \frac{(Npi-1)}{N-1}$, where D is the probability that two randomly selected individuals have a different and distinct banding pattern from each other; C is the probability that two randomly selected individuals have a similar band pattern, and N is the number of individuals analyzed.

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Table 3 ISSR primers used to evaluate 53 *Dioscorea trifida* accessions, including primer sequence, annealing temperature (T_A), size range of ISSR bands in base pairs (bp), number of bands (N_B), number of polymorphic bands (N_{PB}), percent polymorphism (P), and discriminating power (D)

Primer code	Sequence (5′–3′)	T_A (°C)	Size range(bp)	N_B	N_{PB}	P (%)	D
UBC 7	(CT)8-RG	48	300–1,300	13	11	84.6	0.37
UBC 814	(CT)8-TG	50	500–1,100	8	6	75.0	0.76
UBC 843	(CT)8-RA	48	600–1,200	6	6	100.0	0.82
UBC 844	(CT)8-RC	50	300–1,300	9	2	22.2	–0.35
UBC 898	(CA)6-RY	48	300–1,300	12	6	50.0	–0.48
UBC 899	(CA)6-RG	54	300–1,300	9	7	77.8	0.63
JOHN	(AG)7-YC	54	100–1,200	13	9	69.2	0.81
UBC 901	(GT)6-YR	50	300–1,300	8	8	100.0	0.67
UBC 902	(GT)6-AY	50	500–900	4	4	100.0	0.77
AW3	(GT)6-RG	54	500–800	4	3	75.0	0.75
OMAR	(GAG)4-RC	50	300–900	8	8	100.0	0.54
DAT	(GA)7-RG	54	300–800	9	2	22.2	–0.16
TERRY	(GTG)4-RC	50	300–800	9	5	55.6	0.49
MAO	(CTC)4-RC	50	400–1,300	8	8	100.0	0.36
MANNY	(CAC)4-RC	48	300–1,000	11	9	81.8	0.48
GOOFY	(GT)7-YG	54	300–900	6	6	100.0	0.56
Average	–	–	–	8.56	6.25	75.8	0.44

DARwin software, version 5.0 (Perrier and Jacquemoud-Collet 2006), was used to perform a cluster analysis, based on Jaccard similarity coefficient and the UPGMA method. The stability of the groupings was assessed based on estimates of genetic dissimilarity through the procedure of resampling with 1,000 bootstraps. Values higher than 70 % in the nodes that join the groups indicate homogeneity among accessions. Software NTSYS-pc (Rohlf 1992) was used to conduct a principal coordinate analysis (PCoA) and obtain scatter plots.

To confirm the reliability of the groups obtained in the cluster analysis and PCoA, we conducted a Bayesian analysis using the software Structure (Pritchard et al. 2000; Pritchard and Donnelly 2001; Falush et al. 2007), which does not rely on prior information on possible groups, for example based on the origin of the accessions. The Structure software was run using the admixture model, correlated allele frequencies and repeated ten times for each K (number of assumed clusters) with a burn-in of 500,000 interactions followed by 500,000 interactions MCMC (Markov Chain Monte Carlo). The most likely number of clusters was chosen using the ΔK method (Evanno et al. 2005).

In order to identify the proportion of genetic variation between and within groups obtained using the software Structure, which coincided with the groups of PCoA and cluster analysis, a molecular variance analysis (AMOVA) was carried out with Arlequin software (Schneider et al. 2000). Another parameter analyzed was the correlation between matrices of genetic and geographic distances, as well as between genetic distance matrices for SSR and ISSR markers, through the Pearson correlation (r), whose significance was evaluated by Mantel (1967) test, using NTSYS-pc software (Rohlf 1992).

Results

Eight SSR and 16 ISSR primers were selected based on the presence of well defined and with good resolution bands (Tables 2, 3). We obtained 56 bands or amplification products with sizes ranging from 101 pb to 205 pb for SSR and 137 bands ranging from 100 bp to 1,300 pb for ISSR, in a total of 193 bands, with an average of 7.0 bands/primer for SSR and 8.56 bands/primer for ISSR. The number of polymorphic bands for SSR and ISSR was 54 and 100, with an average of 6.75 and 6.25 polymorphic bands per primer, respectively. The level of polymorphism was high, 95 % for SSR and 75.8 % for ISSR. Parameter D value for SSR was 0.79, while for ISSR was 0.44, demonstrating that although the ISSR marker has generated a greater number of bands, the SSR marker

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showed greater discriminatory power between the accessions.

The Jaccard coefficient among 53 accessions of *D. trifida* ranged from 0.40 to 0.96, with a variation of 56 % similarity for SSR marker and from 0.66 to 0.97, with a variation of 31 % for ISSR. Although the two types of markers are located mostly in neutral regions and related to different sequences of the genome, the correlation between genetic matrices obtained from SSR and ISSR markers was high ($r = 0.57$; $p = 0.0002$), demonstrating similar relationships between data from both marker classes.

Although ISSR and SSR markers generated dendrograms with different topologies (Figs. 2, 3), in general, both dendrograms showed the formation of the same groups, with a few exceptions. Despite the low bootstrap values, below 60 % and thus not shown in the dendrograms, it was possible to identify three well-defined groups: group I (accessions from Ubatuba-SP), group II (accessions from Iguape-SP and Santa Catarina-SC) and group III (accessions from Mato Grosso-MT). The yam varieties collected in Iguape-SP and Santa Catarina showed higher genetic similarity, while the varieties from Ubatuba-SP and Mato Grosso were more divergent and classified into distinct groups. All accessions were grouped according to their collection locations for both markers, except the accessions from Amazonas, which changed their position in the dendrogram according to the molecular marker analyzed. These two accessions were classified into a separate group (group IV) in the ISSR analysis while in the SSR analysis they were classified in group II. Also, within group II, accessions from Santa Catarina were apparently better separated from those from Iguape-SP in the SSR than in the ISSR cluster analysis. Although variations obtained in the PCoA,

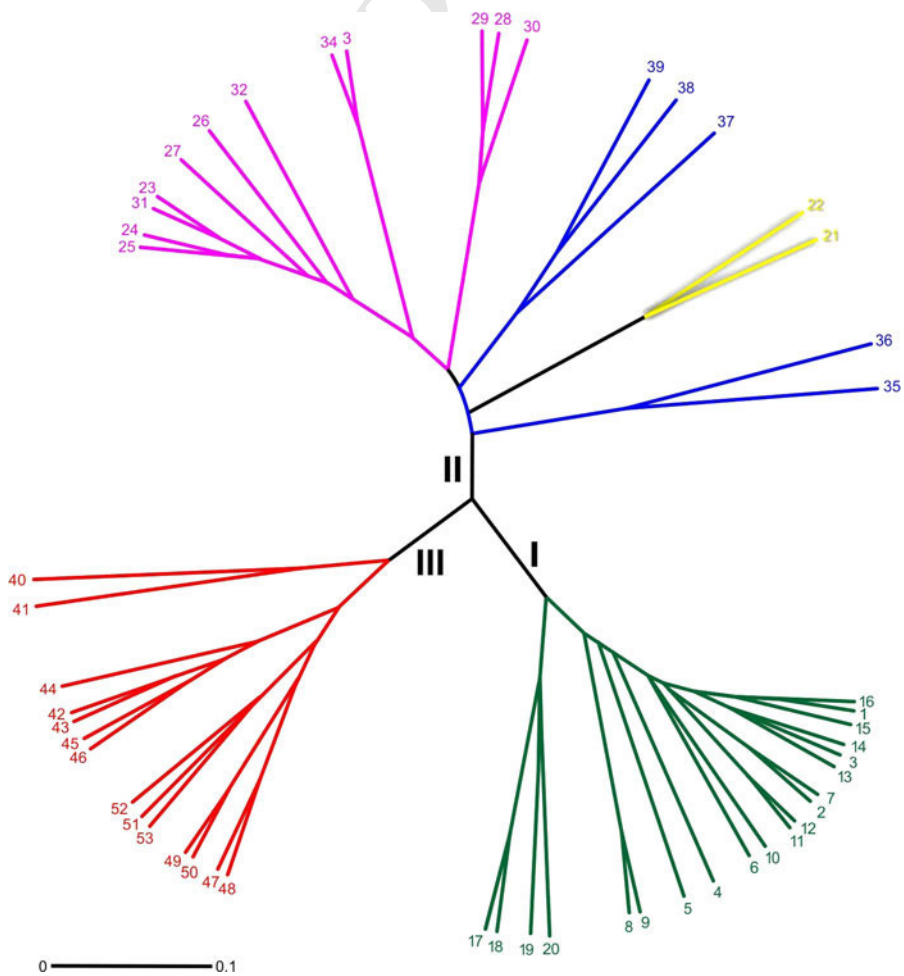

Fig. 2 UPGMA dendrogram based on eight SSR primers showing the genetic relationships among 53 accessions of *D. trifida*: group I [accessions from Ubatuba-SP (*green*)]; group II [accessions from Iguape-SP (*blue*), Santa Catarina (*pink*) and Amazonas (*yellow*)]; and group III [accessions from Mato Grosso (*red*)]

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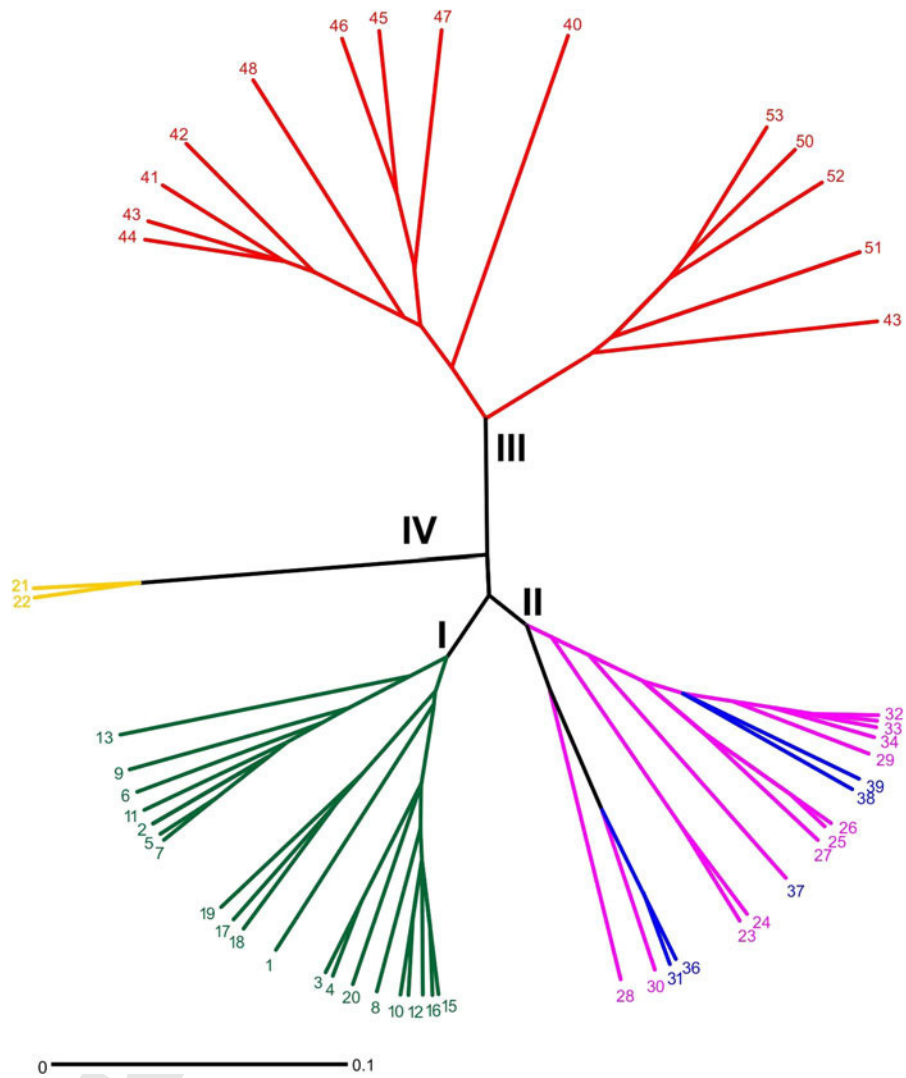

Fig. 3 UPGMA dendrogram based on 16 ISSR primers showing the genetic relationships among 53 accessions of *D. trifida*: group I [accessions from Ubatuba-SP (*green*)]; group II [accessions from Iguape-SP (*blue*) and Santa Catarina (*pink*)]; group III [accessions from Mato Grosso (*red*)] and group IV [accessions from Amazonas (*yellow*)]

whose first two principal coordinates represented 35.7 % of total variation, were not significant, a scatter plot from data obtained with SSR separated the genotypes in the same groups obtained in the scatter plot of the data obtained from ISSR, whose first two principal coordinates represented 31.6 % (not shown data). So, both markers seem to be useful in discriminating the genetic diversity of *D. trifida* accessions.

The Bayesian analysis performed with Structure software for the SSR and ISSR data confirmed the groups obtained in the SSR cluster analysis and the PCoA, since the value of *k* was equal to three, showing that the accessions are genetically structured in three groups (Fig. 4). Based on SSR data, the two Amazonian accessions showed more than 90 % of their genetic constitution similar to accessions from Iguape-SP and Santa Catarina (Fig. 4a), while based on ISSR data, the same accessions showed more than 60 % similarity to those from Mato Grosso and more than 30 % to those from Ubatuba-SP (Fig. 4b). Comparing both analysis, the SSR marker (Fig. 4a) showed some exceptions to the groups formed in the dendrogram for this marker (Fig. 2), such as accessions no. 17 and 18, from Ubatuba-SP, showing more than 50 % similarity to the group from Iguape-SP and Santa Catarina, while accession no. 40, from Mato Grosso, showed more than 80 % similarity to the group from Iguape-SP and Santa Catarina. The ISSR marker (Fig. 4b), on the other hand, showed a group pattern with great similarity to the ISSR dendrogram in Fig. 3.

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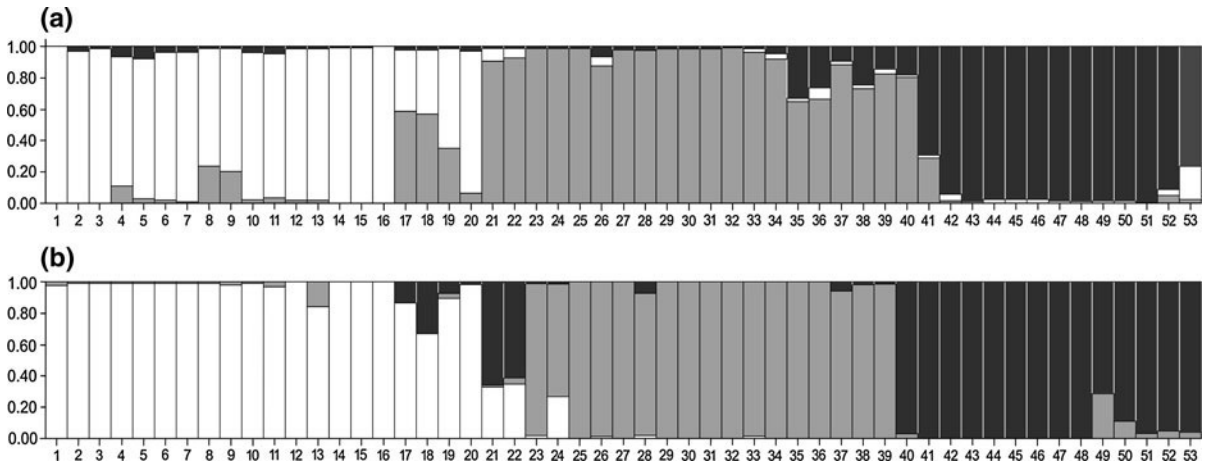

Fig. 4 Structure of the genetic diversity of 53 *D. trifida* accessions based on Bayesian approach for eight SSR markers (**a**) and 16 ISSR markers (**b**). Each accession is represented by a *vertical bar*. Accessions 1–20 originated from Ubatuba-SP; accessions 21–39 originated from Iguape-SP, Santa Catarina and Amazonas; and accessions 40–53 originated from Mato Grosso

From the analysis of variance considering the three groups formed in the Bayesian analysis and two comparison levels, within and between groups, differences were found between the genetic material studied for both the SSR data ($\Phi st = 0.39$; $p = 0.0000$) and for ISSR data ($\Phi st = 0.33$; $p = 0.0000$) (Table 4). Genetic variation was greater within than between groups for both SSR (60.6 %) and ISSR (66.5 %) markers (Table 4). A low positive correlation was identified between genetic and geographical distances for both SSR data ($r = 0.08$; $p = 0.0007$) and ISSR data ($r = 0.16$; $p = 0.0002$), demonstrating a slight spatial structure of genetic material in the geographic area sampled.

The Shannon diversity index obtained from SSR data indicated that the State of São Paulo showed greater genetic diversity (0.40), followed by Mato Grosso (0.31) and Santa Catarina (0.27) (Table 5). Similar results were obtained for ISSR data, but with lower values (0.28, 0.21 and 0.19, respectively). The Amazonas State had the lowest diversity indexes (0.09 for SSR and 0.03 for ISSR), due to the low number of accessions sampled. Considering the groups formed in the Bayesian analysis and in the cluster analysis with SSR, group II (accessions from Iguape-SP, Santa Catarina and Amazonas) had the highest diversity index (0.40), followed by group III (0.31) with accessions from Mato Grosso, and group I (0.30) (accessions from Ubatuba-SP) for SSR, while lower and similar values for the three groups were obtained for ISSR data.

Table 4 Analysis of molecular variance (AMOVA) for 53 accessions of *D. trifida* L. considering three groups, according to the Bayesian analysis on Structure software: group I (accessions from Ubatuba-SP), group II (accessions from Iguape-SP, Santa Catarina and Amazonas), and group III (accessions from Mato Grosso)

Variation source	DF	SSR		ISSR	
		SQ	Total variation (%)	SQ	Total variation (%)
Among groups	3	150.295	39.37	221.218	33.53
Within group	49	288.346	60.63	528.404	66.47
Total	52	438.642			

$\Phi st = 0.3937$ for SSR and $\Phi st = 0.3353$ for ISSR; *DF* degrees of freedom, *SQ* sum of squares

* Value p^1(1,023 permutations) = 0.0000

Discussion

Genetic diversity and comparative analysis of SSR and ISSR markers

The large amount of molecular markers available to estimate genetic diversity allows us to make comparisons in order to determine which technique is best suited for a particular crop (Biswas et al. 2010). Choosing the most appropriate technique depends on the purpose of the research, reproduction mode of the

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Table 5 Shannon diversity index (*H'*) of accessions of *D. trifida* classified according to State, to both municipalities in São Paulo (Ubatuba and Iguape), and to Bayesian analysis on Structure software: group I (accessions from Ubatuba-SP), group II (accessions from Iguape-SP, Santa Catarina and Amazonas), and group III (accessions from Mato Grosso)

	H'	
	SSR	ISSR
States		
São Paulo	0.40	0.28
Mato Grosso	0.31	0.21
Santa Catarina	0.27	0.19
Amazonas	0.09	0.03
Average	0.27	0.18
Groups		
Group I	0.30	0.21
Group II	0.40	0.20
Group III	0.31	0.22
Average	0.34	0.21

species and its genetic structure (Badfar-Chaleshtori et al. 2012), as well as their ability to estimate heterozygosity (Vogel et al. 1996). However, difficulties in relating fragment patterns for specific loci and genotypes in the genomes of polyploid species such as *D. trifida* and other tuberous plants limit the use of heterozygosity estimates to assess different molecular markers in these species (McGregor et al. 2000).

This study showed that it is possible to use both ISSR and SSR techniques for characterizing and discriminating morphologically distinct or similar yam accessions. Also, both ISSR and SSR results highlight the importance of traditional farmers in maintaining high genetic diversity among their local varieties. The eight SSR primer pairs were highly polymorphic and informative among the 53 *D. trifida* accessions analyzed in this study. The heterologous primers Da1A01 and Dab2C05 developed by Tostain et al. (2006) for *D. alata* L., *Dioscorea abyssinica* Hochst. ex Kunth and *Dioscorea praehensilis* Benth, showed 100 % polymorphism, and high discrimination power among accessions, equal to 0.97 and 0.91, respectively. These primers were also suitable for analyzing *D. trifida* accessions in the transferability tests conducted by Tostain et al. (2006). The primers specific for *D. trifida*, developed by Hochu et al. (2006), besides providing good resolution of bands in the gel electrophoresis, showed high polymorphism, with an average of 93.8 %, and a high number of bands per primer (7 bands, on average). We highlight here primers MTI3 and MTI10 that revealed 13 and 11 bands, respectively (Table 2). In contrast, only five bands were revealed by primer MTI11, similar to results obtained by Hochu et al. (2006), which found only three bands for this primer.

Except for primers UBC 844, UBC 898 and DAT, which showed low polymorphism and low discrimination power among the accessions analyzed, all the other ISSR analyzed primers were highly polymorphic with a high number of bands per primer, especially for the UBC 7, UBC 898, JOHN and MANNY primers. These primers revealed more than 10 bands (Table 3), as well as high discrimination power among accessions, with *D* ranging from 0.36 to 0.82. The high percentage of polymorphism (75.8 %, on average) observed for ISSR was also reported by Zhou et al. (2008), analyzing 28 cultivars of *Dioscorea opposita* based on seven ISSR primers, which had a total of 65 fragments with 83 % polymorphism. High levels of polymorphism are common in ISSR, such as the results obtained for potatoes, with 90 % polymorphism (Prevost and Wilkinson 1999) and sweet potato, with 62.2 % (Huang and Sun 2000).

Mantel test results revealed that data obtained with the SSR and ISSR markers are correlated ($r = 0.5$; $p = 0.0002$), although they represent different genomic sequences. Several studies have also noted the existence of high correlation between different techniques of molecular markers in different species (Belaj et al. 2003; Biswas et al. 2010).

Genetic structure and conservation strategies

In Brazil, a center of diversity and domestication of various species, studies of genetic diversity are most often associated with economically important crops (Clement et al. 2010). Roots and tuber crops such as yams have been neglected by breeding and conservation research (Siqueira 2011). In this context, yam is considered an underutilized crop, subject to selection of interesting characters by the traditional communities, where farmers maintain varieties of their preference. Therefore, there is a low level of marketing and exchange of these materials when compared, for example, to other root crops, such as cassava, potato and sweet potato (Siqueira 2011). With this in mind, *D. trifida* can be affected by the process of genetic drift,

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whose effect is intensified because of the presence of dioecy, which requires a male and a female plant in the same site for genetic recombination to occur between individuals (Mignouna et al. 2003). Although this species is allogamous in favorable climatic conditions, it is strongly maintained by vegetative propagation in cultivated fields, and in most cases, the clones of the same individuals are collected and planted for several years. However, the accessions analyzed in this study showed high genetic variability, with a variation in the similarity coefficient of Jaccard equal to 31 and 56 % for SSR and ISSR data, respectively. Veasey et al. (2012), by analyzing accessions of the same species collected in the Vale do Ribeira, São Paulo, based on isozymes, observed a variation in the similarity coefficient of Jaccard equal to 83 %.

In the cluster analysis, based on the Jaccard coefficient and UPGMA method, as well as in the PCoA and the Bayesian analyses, three genetically distinct and consistent groups were identified, with similar or identical membership. One of the three groups mentioned above consisted of accessions from Iguape municipality in the southern coast of the São Paulo State, which were grouped with accessions collected in the north coast of Santa Catarina, in the municipalities of Joinville and São Francisco do Sul. A second group was formed by accessions from the north coast of São Paulo, in Ubatuba municipality, and a third group classified the accessions collected in Mato Grosso, a region well apart from the others. The exception was the Amazonian accessions, purchased in local markets. Their group membership depended on the marker type or on the genomic region sampled.

The interesting fact in this study was the separation of accessions from the south and north coasts of São Paulo. Within Vale do Ribeira, in the south coast of São Paulo, Veasey et al. (2012) had already noticed a spatially structure in genetic variation along a much smaller geographic scale with isozyme markers. *D. trifida* local varieties from Vale do Ribeira were grouped according to their municipalities. Two clusters (with 100 % bootstrap) were obtained in the cluster analysis, one with varieties from Iguape municipality and the other with varieties from Cananeia municipality. The same genetic structure was observed in the present study, but at a higher geographic scale, separating accessions among the north and south coastal areas in São Paulo State. The explanation for this finding is perhaps the introduction of accessions of this species by waves of migrants from different regions or even different ethnic groups. On the other hand, there was a greater similarity of accessions on the south coast of São Paulo, Iguape, with those from Santa Catarina, suggesting that accessions from these two geographically adjacent regions have the same origin.

This dynamics can also be related to indigenous influence on the domestication of *D. trifida*, which has been cultivated by indigenous people from the coastal areas to the Central West region of Brazil (Pedralli 1998). Among the indigenous groups involved in this process, we highlight the Guaraní, who are very itinerant and widely scattered throughout Brazil, including the coast of São Paulo (Ladeira 1992). This ethnic group traveled over long distances carrying with themselves various species of edible plants, among which *D. trifida* (Schmitz and Gazzaneo 1991). This species is, therefore, strongly associated with these indigenous people who, on the other hand, have a strong influence upon the traditional populations in the Vale do Ribeira, São Paulo (Veasey et al. 2012), where the Iguape municipality is located.

The maintenance of genetic variation is a major objective for conservation (Hamrick and Godt 1996) and knowledge of variation within and among populations provides essential information in the formulation of appropriate conservation strategies (Francisco-Ortega et al. 2000). In this study, most of the genetic variability was observed within groups (60.6 and 66.5 % for SSR and ISSR, respectively). In agreement with this result, in a study with wild and cultivated Guinea yams from south and south west Ethiopia, Mengesha et al. (2013) observed that most of the microsatellite diversity was found within rather than among populations. However, our study showed that even among groups the genetic variability was high (39.4 and 33.5 % for SSR and ISSR, respectively). Veasey et al. (2012), comparing two groups of *D. trifida* accessions analyzed with eight SSR primer pairs, one group including seven accessions from Iguape and Cananéia municipalities in São Paulo and one accession from Mato Grosso, and another group with four accessions from the Amazon, observed that most of the variation was between groups (62.9 %) compared with the variation within groups (37.1 %), in contrast to our findings. This result showed that the Amazonian accessions were genetically different from the other accessions.

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In the present study, we observed a large genetic difference among accessions collected from the different locations analyzed, i.e., between groups, since the values of *Φst* was 0.3937 for SSR and 0.3353 for ISSR, which corresponds to a low gene flow among the regions studied. This pattern demonstrates that, unlike other species grown in traditional agriculture, such as cassava (Siqueira et al. 2009) or sweet potato (Veasey et al. 2008), *D. trifida* is a more regionalized crop or less scattered when compared to other yam species, such as *D. alata* (Bressan et al. 2011; Siqueira et al. 2012). This observation can be related to historical and socioeconomic factors, such as the different ways of using these tubers and variation in the preference of varieties over time and space (Veasey et al. 2010). The positive correlation between genetic and geographical distances obtained in the Mantel test, confirms the structure of these materials in the geographic area sampled. Although the value of this correlation was low, the fact that it is significant at 1 % confirms the spatially structure of the genetic variation, which is consistent with the observed *Φst* value. However, the low correlation values could be due to possible recent exchange of materials among these regions, carried out by non-governmental organizations working with indigenous groups, to encourage the exchange of genotypes and inserting new material to prevent genetic erosion of these varieties. Thus, exchange fairs are held, allowing the acquisition and supply of new genotypes. There is little information about these indigenous groups, such as the Guaraní group mentioned above, who have survived various pressures, such as land struggles, beyond sociocultural pressures of modern societies (Arruda 1999). These difficulties are common to both indigenous groups and for farmers that grow various species in a traditional way in the tropics.

Considering the genetic differences among the genotypes grown in the studied locations, the accessions of São Paulo showed higher diversity, followed by Mato Grosso, Santa Catarina and Amazonas. The average level of genetic diversity present in accessions of *D. trifida* was 0.27 and 0.18 among states, for SSR and ISSR data, respectively. Considering the groups formed in the Bayesian analysis, using Structure software, higher diversity was found among accessions in group II for SSRs, which includes accessions from three states (São Paulo, Santa Catarina and Amazonas). However, for the ISSR marker, the highest diversity was found for group III (among Mato Grosso accessions), although the values were very similar among the three groups. In general, the average level of genetic diversity was 0.34 and 0.21, for SSR and ISSR data, respectively. These indices are greater than the values found for perennial herbs ($H' = 0.17$) and to those species with wide geographic distribution ($H' = 0.20$) (Hamrick and Godt 1989). However, studies by Zhou et al. (2008) with varieties of *D. opposita*, widely used in Chinese medicine, showed $H' = 0.32$, a relatively high value and in most cases superior to that obtained for *D. trifida* in this study. It is noteworthy that several factors influence the level of genetic diversity of a species, among them, the geographic distribution, life cycle, reproductive system, dispersal patterns, population size, among others (Hamrick et al. 1991; Gaudeul et al. 2000; Zhou et al. 2008).

The level of genetic diversity observed among accessions is directly related to the fact that *D. trifida* is a polyploid species and reproduces both by outcrossing and vegetative propagation. Thus, individuals are usually highly heterozygous, preserving the allelic diversity at the individual level (Veasey et al. 2008; Siqueira et al. 2009). However, the emergence of variant plants arising from seeds, result of genetic recombination, is unlikely, since the occurrence of flowering and fruiting in this species was not reported and detected by farmers during plant collection.

The genetic diversity level displayed by the accessions collected in the States of São Paulo, Mato Grosso and Santa Catarina was expected because of the wide distribution of the crop in these regions. However, we also found genetically similar varieties, which can be related to the fact that the collection has been restricted to a few farmers and a few communities in Ubatuba, SP, where practically all the collection was made in the community of the Sertão de Ubatumirim, and in the community Pirabeiraba in Santa Catarina. The similarity between accessions corroborates the names given by farmers in the studied regions. In São Paulo, except for accession 195, called *cara cobrinha* (little snake yam), varieties were obtained with only two popular names, *cara roxo* (purple yam) and *cara branco* (white yam), the latter being represented in smaller numbers. In Mato Grosso, most accessions also received the name *cara roxo*. In Santa Catarina, the predominant designations for *D. trifida* were *cara* and *cara mimoso*.

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In conclusion, our results suggest that both markers were useful for evaluation of genetic diversity and assessing differentiation between *D. trifida* populations in Brazil. However, the SSR marker detected higher diversity indices while the ISSR marker seemed more efficient in the clustering of the different genotypes, being able to separate the two Amazonian accessions in the cluster analysis. But both markers detected high levels of genetic diversity for accessions of *D. trifida* maintained by traditional farmers in the states of São Paulo, Mato Grosso, Santa Catarina and Amazonas. The high within-group variation found is quite interesting for the maintenance of the crop over time. Knowledge of the genetic relationships among accessions is an important information for the efficient use and conservation of this species, both *ex situ* in genebanks, or *in situ*, within the aim of conservation in the rural property, known as on-farm conservation. Considering that high genetic diversity was found both within and between groups of accessions from different regions visited, the collection and conservation strategies should consider a large number of individuals from all regions sampled in order to cover all the genetic diversity present in these materials. On-farm conservation, in the case of *D. trifida*, is quite interesting for considering the socio-cultural factors involved in the evolution of the species, considering that agricultural practices, through the cultivation and artificial selection, allows the accumulation over time of morphological traits of agronomic interest, providing an enrichment of genetic variability not only of this species, but of other crops as well.

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