SCIENTIFIC NOTE

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Genetic variability assessment in the genus *Passiflora* by SSR markers

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The genus *Passiflora* encompasses many species that are endemic to the Brazilian territory, including some with economic value. Studies on genetic diversity in this genus are fundamental because they allow understanding genetic variability and distance. The present study aimed to determine the genetic variability and distances among 10 species of the genus *Passiflora* by using microsatellite markers (Simple Sequence Repeat, SSR). Twenty-eight heterologous microsatellite markers were tested, but only 12 were used in the diversity analysis because they amplified in at least 80% of the species. A clear separation was observed among the subgenuses studied, as well as wide variation among the accessions of *Passiflora*. This knowledge enables breeders to explore diversity and transfer favorable alleles found in wild species.

Key words: Breeding, genetic diversity, *Passiflora*.

INTRODUCTION

The genus *Passiflora* groups species are known as passion fruit. It comprises about 530 species, of which approximately 140 have diversified in the Brazilian territory. Eighty-two are endemic to Brazil, which makes the country a center of genetic diversity of the genus (Bernacci et al., 2013). The diversity of wild passion fruit species in Brazil is a potential to be explored and a promising research field in various aspects of plant breeding.

The wild species of *Passiflora* have characteristics of interest to passion fruit culture, including longevity, adaptation to adverse weather conditions, extended flowering period, higher concentration of chemicals of interest to pharmaceutical and cosmetic industries and resistance to diseases (Meletti, 2011). The latter is one of the main goals of passion fruit breeding programs (Junqueira et al., 2006). In spite of the importance of this crop and the huge number of species found in Brazil, there is still little knowledge about diversity in this genus, compared to other species. Genetic variability measurement is a fundamental activity for plant breeding and conservation of many species. Among other alternatives, it can be performed through morphological characterization, in which inheritable traits are observed, measured, and documented (Vicente et al., 2005). However, since the number of descriptors is small and some traits are affected by environmental changes, morphological characterization has been limited to germplasm documentation and registration of cultivars. Therefore, DNA molecular markers have contributed to estimate variability, since they can detect significant differences at DNA level. Molecular markers can perform characterization with greater precision and in large scale (Varshney et al., 2005).

Microsatellites stand out among molecular markers in the analysis of genetic diversity because they are co-dominant, multi-allelic, polymorphic, and reproducible. Thus, highly informative content can be generated (Schlötterer, 2004). Comparative studies on *Passiflora* have been very successful when using Randomly Amplified Polymorphic (RAPD) molecular markers (Viana et al., 2003; Pérez et al., 2007; Viana et al., 2010), inter-simple sequence repeat (ISSR) (Santos et al., 2011), and chloroplast genes (Yockteng and Nadot, 2004). However, there are only a few studies on the use of microsatellite markers for the characterization of *Passiflora* involving several species. Microsatellite markers have been developed for some species of the genus *Passiflora*, and for *P. edulis* Sims (Oliveira et al., 2005), *P. alata* Curtis (Pádua et al., 2005), *P. cincinnata* Mast. (Cerqueira-Silva et al., 2012), and *P. contracta* Vitta (Cazé et al., 2012). However, microsatellites have not been developed yet for most species of this genus. Thus, this study aimed to investigate the cross-amplification of microsatellite markers for 10 species of *Passiflora* and quantify the genetic variability among the genotypes.

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MATERIAL AND METHODS

Germplasm and extraction of genomic DNA

Fifty-five genotypes of *Passiflora* spp. comprising 10 species were evaluated. They were obtained from the germplasm collection of the Universidade Estadual do Norte Fluminense (UENF) and the Empresa Brasileira de Pesquisa Agronômica EMBRAPA (Brazilian Enterprise for Agricultural Research). The plants were germinated and grown in a greenhouse at the UENF research support unit (Table 1).

Young leaves were collected and stored at -80 °C in an ultrafeezer. About 50 mg macerated tissue were transferred to 2 mL tubes and immersed in liquid N_2 for DNA extraction, in accordance with the protocol of Doyle and Doyle (1990), with modifications. The integrity of the extracted DNA was checked on 1% agarose gel and GelRed staining. A spectrophotometer (NanoDrop 2000c UV-Vis spectrophotometer, Thermo Scientific, Wilmington, Delaware, USA) was used for quantification, with absorbance read at 260 nm wavelength.

Optimization of the polymerase chain reaction (PCR)

Primers developed for *P. edulis* (Oliveira et al., 2005) and *P. alata* (Pádua et al., 2005) were used to assess cross-amplification in the species analyzed (Table 2). The primers that amplified in most species were used in the analysis of variability in the genus *Passiflora*. The PCR was performed with 10 ng DNA, 0.5 μM primers and 0.5 U *Taq* DNA Polymerase, 0.02 mM dNTP, and 1.5 mM magnesium chloride and PCR buffer (1X), with a final volume of 13 μL per sample.

The amplifications were conducted in a thermal cycler (Veriti 384-Well Thermal Cycler, Applied Biosystems, Thermo Scientific). The amplification program consisted of the following steps: a cycle of 4 min at 94 °C for initial denaturation, 35 cycles of 1 min at 94 °C, 1 min at a specific temperature for each primer pair and 3 min at 72 °C, and a cycle of 7 min at 72 °C for a final extension. The amplified fragments were then separated on 4% Metaphor agarose gel, stained with GelRed gel and subjected to UV light for visualization of the results (Fotodocumentador MiniBIS Pro, Bio-Imaging Systems, Jerusalem, Israel).

Twenty-eight primers were tested, with optimal annealing temperature for each primer ranging from 56 to 61 °C. Separation of fragments was performed in high resolution Metaphor agarose gel. This strategy is effective for the analysis of simple sequence repeat (SSR) markers, since it is efficient, economical, easy to perform, and enables the separation of 20-800 bp alleles (Asif et al., 2008).

Table 1. Accessions of *Passiflora*, its origin, and the number of plants used in the analysis of genetic diversity.

Accession	Species	Origin	Number of plants
BGP235	*Passiflora alata* Curtis	Brasília	3
BGP016	*Passiflora cincinnata* Mast.	Alagoas	4
BGP268	*P. cincinnata*	Bahia	3
BGP274	*P. cincinnata*	Bahia	4
BGP275	*P. cincinnata*	Bahia	3
BGP290	*P. cincinnata*	Bahia	2
BGP008	*Passiflora gibertii* N.E. Br.	São Paulo	2
BGP198	*P. gibertii*	Indisponível	2
UENF	*P. gibertii*	Rio de Janeiro	3
BGP237	*Passiflora setacea* DC.	Bahia	3
BGP238	*P. setacea*	Bahia	3
BGP272	*P. setacea*	Bahia	1
UENF	*P. setacea*	Rio de Janeiro	4
UENF	*P. setacea*	Bahia	2
UENF	*Passiflora mucronata* Lam.	Bahia	2
São Francisco	*P. mucronata*	Rio de Janeiro	2
UENF	*Passiflora micropetala* Mart. ex Mast	Rio de Janeiro	1
UENF	*Passiflora caerulea* L.	Bahia	1
UENF	*Passiflora suberosa* L.	Rio de Janeiro	4
UENF	*Passiflora coccinea* Aubl.	Rio de Janeiro	2
UENF	*Passiflora edulis* Sims	Rio de Janeiro	4

UENF: Universidade Estadual do Norte Fluminense.

Table 2. Identification of the loci used for genotyping 56 accessions of *Passiflora*.

Locus	Starter "foward"	Starter "reverse"	Reference
PE03	gcagcgagggaagaaaaa	tgagacatcgtgcgtgaa	Oliveira, 2006
PE04	atgcttttggaaatccgttt	tgctcatgcaaagtcactgg	Oliveira, 2006
PE08	ccggatacccacgcatta	tctaatgagcggaggaaagc	Oliveira, 2006
PE11	gcataagttgtcggtcttgg	cctcgaacctctatcatcca	Oliveira, 2006
PE12	cgtaatattgtttgggcact	atcatgggcgaactcattt	Oliveira, 2006
PE13	aagcaccccaatcgttga	ccccctgccacctgagta	Oliveira, 2006
PE18	ccgtgaaccaaccatttctc	ccgtgaaccaaccattttctc	Oliveira, 2006
PE20	aggatcaccatagaaaaccat	gttaggttggcattgctctt	Oliveira, 2006
PE23	caatcccttgacccataga	cgtccatccttctccttt	Oliveira, 2006
PE24	tcaaactgaactcgtaaagg	gtgctgggagactgatgtt	Oliveira, 2006
PE27	ttgctcattgcactcatcct	gcagacatttcctggagca	Oliveira, 2006
PE35	attatgcctaaaaacccaaa	tgatccagaggttgagagg	Oliveira, 2006
PE37	caaaaggataggcctgatgtc	tgcttggtcatccactgaag	Oliveira, 2006
PE38	gatcggtcctcggttagac	agtcacacagcatgagaaatc	Oliveira, 2006
PE41	atcggggttcgcttatttg	cgttcatcctttagtgggcta	Oliveira, 2006
PE42	gtcacttcattcttcctttcc	ttagcccactcaaacacaa	Oliveira, 2006
PE58	gcaatttcaccatcttctgct	gcaatttcaccatcttctgct	Oliveira, 2006
PE66	ccatagtcccaacaagcatc	gctgtggaccctaactcagtc	Oliveira, 2006
PE74	ccctcttatcaatagcgttgg	gcacgagcacgagtatttatt	Oliveira, 2006
PE90	tcaggaagattgcatgttagt	ctgggttttgtttatgttgc	Oliveira, 2006
A07FP1	ggaagtgaaggagaagaaga	ccctctggttgtctacctac	Pádua et al., 2005
A08FP1	cacatttgccgtcactgg	cggcatacgataaatctcctg	Pádua et al., 2005
A06FP1	gggcggaagaaaagagaag	gaaacacacgatgcgaaaa	Pádua et al., 2005
A01FP3	agagtcgtctaaccctcttgc	tcttgcttacgcgtggacta	Pádua et al., 2005
A01BP3	gcgggattctcttgccttac	acaaaacacatcagccacca	Pádua et al., 2005
A08GP1	taaccgacttcgcccaca	gagcaggggaagaaaaga	Pádua et al., 2005
A09DP1	tggcaatttggtggttga	ccttaaccggcgttgga	Pádua et al., 2005
A03AP3	gccttagcttgcaactttcg	ggaggcaacccgagtataaa	Pádua et al., 2005

Statistical analysis

Genetic diversity was estimated by the number of alleles per polymorphic locus, observed heterozygosity (percentage of heterozygous individuals), and gene diversity (expected heterozygosity), which consists of the expected proportion of heterozygous individuals for the allelic frequencies observed and the polymorphic information content (PIC). The genetic distance was obtained by Shared Allele distance, which is based on the sharing of alleles among the genotypes assessed. The Unweighted Pair Group Method using Arithmetic Average (UPGMA) hierarchical method was used to group them. The Principal Coordinates Analysis was developed with the average distances for each species, which allowed the investigation of the distribution of the species of the genus

Passiflora in the two-dimensional Cartesian plane. All values were obtained by using the Powermarker software system (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Transferability

Out of the 28 microsatellite primers used, 12 were transferred to most species. The primers developed for *P. edulis* presented higher transferability rate, *P. cincinnata* was the species with the highest cross-amplification rate (80%) while *P. micropetala* Mart. ex Mast presented the lowest (35%). The low percentage of cross-amplification of these markers in *P. micropetala* may have resulted from the accumulation of molecular differences between *P. edulis* and *P. micropetala*. Out of the eight primers developed for *P. alata*, only two amplified in most species studied (A08FP1, A07FP1) (Table 3). The loci PE13, PE37, PE41, PE66, PE74, PE90, and A08FP1 were transferred to all the analyzed species (Table 4).

Twelve of the primers tested obtained a transferability rate equal to or higher than 80%. Out of these, the primer A08FP1 amplified in all species (Table 4). Aiming to confirm the presence of hybrids in the cross between *P. sublanceolata* and *P. foetida* L., Santos et al. (2011) used the same primer, which was polymorphic for the parents, whose progeny presented bands belonging to both parents. This indicates that the primer in question is highly polymorphic and can be used in studies with species on the genus *Passiflora*. Genetic variability quantification could be performed, since it was possible to achieve the transferability of 12 microsatellites for the species studied.

Table 3. Cross-amplification of 10 species of *Passiflora* using developed primers for *Passiflora edulis* and *Passiflora alata*.

Loci	P.ed	P.al	P.se	P.mu	P.gi	P.ci	P.co	P.ca	P.su	P.mi
PE03	1	-	-	-	-	1	-	-	-	-
PE04	1	-	-	1	-	1	-	-	-	-
PE08	1	1	1	1	1	1	1	-	1	1
PE11	1	-	1	-	-	-	-	-	-	-
PE12	1	-	-	-	1	1	-	-	-	-
PE13	1	1	1	1	1	1	1	1	1	1
PE18	1	1	1	1	1	1	1	1	1	-
PE20	1	1	1	1	1	1	1	1	1	-
PE23	1	1	-	-	-	1	-	-	-	-
PE24	1	-	-	-	-	-	-	-	-	-
PE27	1	-	1	-	-	1	-	-	-	-
PE35	1	-	-	-	-	1	-	-	-	-
PE37	1	1	1	1	1	1	1	1	1	1
PE38	1	1	1	1	1	1	1	1	1	-
PE41	1	1	1	1	1	1	1	1	1	1
PE42	1	-	-	-	-	-	-	-	-	-
PE58	1	-	-	-	-	-	-	-	-	-
PE66	1	1	1	1	1	1	1	1	1	1
PE74	1	1	1	1	1	1	1	1	1	1
PE90	1	1	1	1	1	1	1	1	1	1
T (%)	100	55	60	55	55	80	50	45	50	35
A07FP1	-	1	1	1	1	1	1	1	1	-
A08FP1	1	1	1	1	1	1	1	1	1	1
A06FP1	-	1	-	-	-	-	-	-	-	-
A01FP3	-	-	-	-	-	-	-	-	-	-
A01BP3	-	-	-	-	-	-	-	-	-	-
A08GP1	-	1	1	-	-	-	-	-	-	-
A09DP1	-	1	-	-	-	-	-	-	-	-
A03AP3	-	1	-	-	-	-	-	-	-	-
T, %	12.5	75	37.5	25	25	25	25	25	25	12.5

T: rate of transferability; 1: observed amplification; -: without amplification. P.ed: *Passifora edulis*, P.al: *Passiflora alata*, P.se: *Passiflora setacea*, P.mu: *Passiflora mucronata*, P.gi: *Passiflora gibertii*, P.ci: *Passiflora cincinnata*, P.co: *Passiflora coccinea*, P.ca: *Passiflora caerulea*, P.su: *Passiflora suberosa*, P.mi: *Passiflora micropetala*.

Table 4. Transferability rate observed for microsatellite loci.

Locus	Transferability (%)
PE13, PE37, PE41, PE66, PE74, PE90, A08FP1	100
PE08, PE18, PE20, PE38	90
A07FP1	80
PE04, PE12, PE23, PE27, PE35	30
PE03, PE11, A08GP1	20
PE24, PE42, PE58, A06FP1, A09DP1, A03AP3	10

Genetic diversity

A low number of alleles was observed for all loci, ranging from 2 (PE37 and PE38) to 5 (PE66), and mean of 3.42. A total of 41 alleles were obtained for the 12 loci analyzed. Similar allelic variation was found by Cazé et al. (2012) when they were characterizing SSR loci developed for *P. contracta* in which the number of alleles per locus ranged from 2 to 9, with a mean equal to 5. Cerqueira-Silva et al. (2012) also found few alleles per locus, from 2 to 9 alleles. Oliveira et al. (2005) obtained up to 20 alleles at one locus and an average of 5.3 alleles per locus in a sample of 43 individuals. This shows that a low number of alleles per locus and few polymorphic microsatellite markers have been characteristic of the genus (Cerqueira-Silva et al., 2012). It may suggest that these loci concentrate in preserved regions, with low mutation rate.

The locus PE66 presented a greater number of alleles (5) while four alleles were found in locus PE18. Both were dinucleotide microsatellites with nine replicates. According to Weber (1990), the number of alleles per locus is related to the number of replicates in the microsatellite, which explains the polymorphism found in the locus PE66. The values of expected heterozygosity (He) ranged from 0.33 (PE37) to 0.69 (PE08), with a mean of 0.57 (Table 5). These values reveal the wide genetic variability found in the genus *Passiflora*. Similar values were found by Cerqueira-Silva et al. (2012), who evaluated accessions of *P. cincinnata* and obtained He = 0.51, and Pádua et al. (2005), who obtained He = 0.52, using accessions of *P. alata*. Observed heterozygosity (Ho) ranged from 0.00 (PE37) to 0.981 (PE66), with mean of 0.52. Six of the loci analyzed presented heterozygosity higher than expected. Oliveira et al. (2005) evaluated accessions of *P. edulis* and found similar results for Ho, which ranged from 0.0 to 1.0. Cazé et al. (2012) used seven microsatellite loci to study the genetic structure of *P. contracta* and obtained Ho = 0.50.

It is important to highlight that high values of Ho are expected in studies involving the genus *Passiflora*, since most species of this genus have the self-incompatibility system and are predominantly alogamous, with a large amount of heterozygous loci. However, Reis et al. (2011)

Table 5. Characterization of 12 microsatellite loci used in the molecular characterization of species of *Passiflora*.

Locus	TA(°C)	A	Number of alleles	He	Ho	PIC
PE13	60	0.4909	3.00	0.6094	0.3091	0.5313
PE18	60	0.5943	4.00	0.5829	0.6415	0.5359
PE74	58	0.5096	3.00	0.5497	0.9808	0.4495
PE38	58	0.7245	2.00	0.3992	0.0612	0.3195
PE37	60	0.7857	2.00	0.3367	0.0000	0.2800
PE41	60	0.5192	4.00	0.5627	0.9423	0.4707
PE66	60	0.3889	5.00	0.6799	0.9815	0.6213
PE90	60	0.5568	4.00	0.6010	0.7273	0.5429
A07FP1	54	0.6000	3.00	0.5588	0.4500	0.5071
A08FP1	54	0.4615	3.00	0.6354	0.7115	0.5612
PE08	56	0.4022	4.00	0.6999	0.1522	0.6453
PE20	56	0.4688	4.00	0.6274	0.2917	0.5567
Average		0.5419	3.42	0.5702	0.5208	0.5010

A: Frequency of the allele with greater frequency, He: expected heterozygosity, Ho: observed heterozygosity, PIC: polymorphic information content.

found low heterozygosity values (He = 0.20 and Ho = 0.15) while studying populations of passion fruit from two cycles of recurrent selection. The authors suggest that low molecular variability in this case can be attributed to the loss and fixation of alleles by the selection of agronomically favorable genotypes.

The polymorphic information content (PIC) calculated to estimate how much each primer was informative among the accessions studied ranged from 0.28 (PE 37) to 0.64 (PE08). Botstein et al. (1980) considered a marker highly informative when it presented PIC above 0.5, reasonably informative when its values ranged between 0.25 and 0.50, and mildly informative when values were below 0.24. According to this classification, PE08 and PE66 were considered highly informative, with PIC values of 0.64 and 0.62, respectively, while loci PE37 (0.28) and PE38 (0.31), which presented lower PIC values, were reasonably informative. The lower PIC values observed for these two loci can be attributed to the concentration of gene frequencies, which leads to deviation from the condition of maximum information content of a locus. This occurs when all alleles have similar frequencies.

Regarding the locus PE37, 78.6% of the allele frequencies were concentrated in only one of the alleles. The same occurred in locus PE38, in which 72.4% of the allelic frequency was concentrated in only one allele. Such frequencies explain the lower PIC values and indicate that, in the present work, these loci generate the lowest content of information on the accessions analyzed. The first two main coordinates explained 70% of the variability between the groups (Figure 1). This value indicates that a two-dimensional graphical representation is appropriate to display the relationships among the species studied.

The graphical analysis of the main coordinates revealed dissimilarity between species belonging to the subgenus *Decaloba* (*P. suberosa* L. and *P. micropetala*) and those allocated in the subgenus *Passiflora*. Wide genetic variability was found within the subgenus *Passiflora* and molecular similarity was verified among *P. edulis*, *P. setacea* DC., *P. cincinnata*, *P. gibertii* N.E. Br., and *P. mucronata* Lam. The work of Muschner et

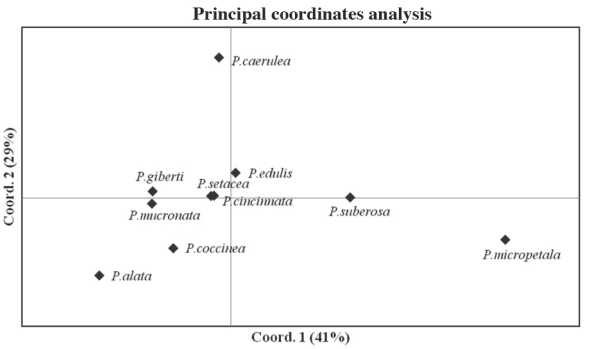

Figure 1. Genetic distance between species of the genus *Passiflora* obtained by the Principal Coordinates Analysis.

al. (2003) used preserved sequences of plastid DNA in order to know the phylogenetic relationships of this genus and corroborated the closeness among these species. The genetic distance between accessions ranged from 0.05 to 0.8, which indicates significant diversity among the accessions analyzed. The smallest distance was observed between ALA235p1 and ALA235p3 (0.05), which belong to the species *P. alata*. The greatest dissimilarity was observed between the species *P. suberosa* and *P. edulis* (0.80). The high divergence between the species *P. edulis* and *P. suberosa* was also found in studies of Crochemore et al. (2003).

The dendrogram favored the formation of three major groups (Figure 2). Group I allocated the species *P. suberosa* and *P. micropetala*, group II consisted of accessions of *P. setacea* and *P. edulis*, and group III gathered *P. cincinnata*, *P. caerulea* L., accessions of *P. setacea*, *P. alata*, *P. coccinea* Aubl., *P. gibertii*, and *P. mucronata*. The grouping provided clear distinction between the subgenuses *Decaloba* and *Passiflora*. The latter is subdivided into two groups. The subgenus

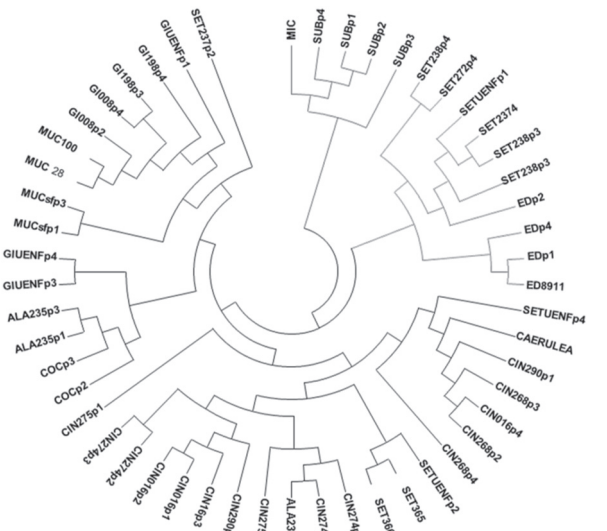

Figure 2. Dendrogram obtained by cluster analysis, using the shared allele genetic distance between accessions of 10 species of *Passiflora* and UPGMA clustering method.

Decaloba had diversified prior to the subgenus *Passiflora* (Muschner et al., 2012). It explains the high molecular dissimilarity between *Passiflora* and *Decaloba*, thus accumulating molecular and morphological differences, including in modes of reproduction, flower size and number of chromosomes. The closeness among the species of the subgenus *Passiflora* is explained by its recent diversification (Muschner et al., 2012). Group II showed closeness between the two species, which indicates the potential use of interspecific crosses. Cerqueira-Silva et al. (2009) quantified the genetic diversity between *P. edulis* and *P. setacea* through physicochemical descriptors of the fruit. These authors observed that the variability between these species can be exploited by means of interspecific crosses aiming to increase the content of soluble solids and pulp production. Furthermore, these crosses may generate hybrids bearing genes resistant to *Cowpea aphid-borne mosaic potyvirus* (CABMV), to which *P. setacea* is resistant (Fonseca et al., 2009).

Similarity among the species allocated in group III was also observed in a study conducted by Muschner et al. (2003), who used preserved sequences to perform the first study on molecular phylogeny of the genus. The sharing of alleles among these species and the use of loci in preserved regions may have contributed to this grouping. *Passiflora setacea* can be found in groups II and III, which reveals intraspecific variability in this species. This study showed the proximity between *P. setacea* and *P. edulis* of *P. cincinnata* was also found in a study on the phylogeny of the genus *Passiflora* (Muschner et al., 2003). Several studies have used molecular markers to assess the genetic variability among *Passiflora* species. Crochemore et al. (2003) used RAPD markers and found a clear separation and a significant diversity among the 11 species. Viana et al. (2010) used both RAPD and morphological markers to quantify the variability among six species of this genus. However, no relationship was observed among the species. Santos et al. (2011) found enormous genetic variability while using ISSR markers to access the variability of *P. edulis* from different sources.

The wide diversity observed is a trait of the genus *Passiflora* resulting from factors such as cross-pollination and self-incompatibility system. Knowledge of genetic diversity is fundamental for the conservation and maintenance of genetic resources in breeding programs (Costa et al., 2012) and helps understanding kinship between genotypes and identifying the best parents to obtain higher genetic gains in segregating populations (Viana et al., 2003; Ganga et al., 2004).

CONCLUSIONS

Heterologous primers amplified in most species analyzed, which enabled them to be used in many molecular studies on *Passiflora*. These markers allowed the estimation of the genetic distance between species and the clear discrimination of the subgenuses *Decaloba* and *Passiflora*. Wide genetic diversity was found among the species studied, which demonstrates potential to be used in plant breeding programs, since the interspecific crosses can be explored aiming to transfer favorable alleles.

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