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Genetic diversity of *Passiflora setacea* in different regions of Bahia, Brazil, through SSR markers

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Abstract

Passiflora setacea DC. is a wild species of passion fruit popularly known as 'maracujá-do-sono' or 'sururuca'. It has been recognized for its potential in passion fruit genetic improvement, due to its resistance to several phytopathogens. The purpose of this study was to evaluate the genetic diversity among and within wild populations of *P. setacea* DC. from different regions of Bahia state, Brazil, revealed by SSR markers. In this regard, 147 samples of plants were collected from 18 localities and six different identity territories in the state. Six pairs of primers were able to amplify polymorphic loci for all individuals. We performed principal coordinates analysis (PCoA) and molecular variance analysis (AMOVA) using Nei genetic distances between individuals of each locations. In addition, we performed two Bayesian analyzes, using Structure and Structurama softwares. High levels of genetic differentiation between populations were observed, as well as the absence of correlation between genetic and geographic distances using the Mantel test. The populations have moderate polymorphism and diverge into two groups: one including populations from the southwestern distribution range, while the other contains all other populations. The existence of two genetic groups was supported by both Bayesian analyses of genetic structure. These results indicate that these populations should be considered for conserving the diversity of *P. setacea* in germplasm banks, as well as the use in genetic improvement programs of passion fruit.

Keywords: Passion fruit, genetic structuring, genetic resource management, microsatellites, variability

Introduction

Brazil is the largest producer of passion fruit, *Passiflora* spp., in the world, with around 593.429t produced in 2019 (IBGE, 2020). Passion fruit tree is grown throughout Brazil and the state of Bahia is the largest national producer (IBGE, 2020). To guarantee Brazilian production and productivity, it is necessary to obtain cultivars resistant to diseases. Diseases such as fusariosis or passion fruit woodiness disease (PWD), caused by cowpea aphid-borne mosaic virus (CABMV), can render large cultivation areas unfeasible or even an entire producing region (Junqueira et al., 2005 ; Barbosa & Santos Filho, 2017). In spite of its economic importance, studies about the characterization of the diversity of *Passiflora* species in Brazil are limited.

In this context, passion fruit breeding programs in Brazil have been working to incorporate wild species in their crosses, as these may contain resistance genes against important disease agents (Faleiro et al., 2019). *Passiflora setacea* DC. (Passifloraceae, Malpighiales), is a wild species of passion fruit popularly known as 'maracujá-do-sono' or 'sururuca'. Also, it is an endemic Brazilian species native to Cerrado, Caatinga and Atlantic Forest from some states of Brazil, including Bahia state (Machado et al., 2017). Within Bahia, its distribution covers central and southern regions, including Chapada Diamantina plateau (Nunes & Queiroz, 2001). Characterization of genetic variation of *Passiflora* species is essential for its effective conservation, management of germplasm and efficient utilization in breeding programs (Oluoch et al., 2018).

Then, genetic improvement programs involving this species are considered strategic for the generation of a resistant hybrid (Junqueira et al., 2005; Oliveira et al., 2013; Faleiro et al., 2019). In fact, interspecific crosses involving *P. setacea* were successful and several hybrids

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resistant to CABMV were identified (Freitas et al., 2015; Sacomann et al., 2018). In this context, knowing the genetic diversity of *P. setacea* populations is an important strategy to understand the species reproductive mechanisms that is necessary for the efficiency of the breeding program. For such purpose, simple sequence repeat (SSR) or microsatellites are efficient molecular markers (Vieira et al., 2016; Wu et al., 2018), that have been successfully used in many studies of genetic diversity of *Passiflora* species in Brazil (Oliveira et al., 2005; Pádua et al., 2005; Cazé et al., 2012; Cerqueira-Silva et al., 2012; Silva et al., 2014), including *P. setacea* (Paiva et al., 2014; Vianna et al., 2019).

Thus, the purpose of this study was to evaluate the genetic diversity among and within wild populations of *Passiflora setacea* collected in different regions of Bahia, Brazil, using SSR markers.

Material and Methods

In this study, 147 young leaf samples of *P. setacea* were collected in 18 locations, from six different identity territories from the state of Bahia (Table 1, Figure 1) (SEPLAN, 2020). One specimen of *P. setacea* from Licínio de Almeida was deposited as a voucher (ALCB 116823) in Herbarium Alexandre Leal Costa (ALCB), at Institute of Biology of Federal University of Bahia (UFBA).

Table 1. List of cities, identity territories of Bahia state were *Passiflora setacea* samples were collected and number of individuals (N).

Pop	Cities	Identity Territories[1]	N
AMA	Amargosa	Vale do Jequiriçá	10
JAC	Jacobina	Piemonte da Diamantina	10
JEQ	Jequié	Médio Rio de Contas	10
CAE	Caetité	Sertão Produtivo	9
CON	Contendas do Sincorá	Sertão Produtivo	10
LIC	Licínio de Almeida	Sudoeste Baiano	10
POC	Poções	Sudoeste Baiano	10
VIT	Vitória da Conquista	Sudoeste Baiano	10
ABA	Abaíra	Chapada Diamantina	8
AND	Andaraí	Chapada Diamantina	10
BON	Bonito	Chapada Diamantina	5
IBI	Ibicoara	Chapada Diamantina	9
LEN	Lençóis	Chapada Diamantina	5
MOR	Morro do Chapéu	Chapada Diamantina	10
PAL	Palmeiras	Chapada Diamantina	5
RIO	Rio de Contas	Chapada Diamantina	6
SEA	Seabra	Chapada Diamantina	5
UTI	Utinga	Chapada Diamantina	5
TOTAL			147

[1]Identity Territories of Bahia state, Brazil (SEPLAN, 2020).

Figure 1. Location of *P. setacea* populations collected in the state of Bahia. Author: C.P. Fazolato.

DNA extraction was performed using fresh leaves, according to Doyle and Doyle (1987). A set of 55 SSR pairs of primers, previously designed for several species of *Passiflora* (Oliveira et al., 2005), was tested in order to amplify DNA from *P. setacea*. However, only six pairs of primers amplified with reproducible and high-quality bands. Polymerase chain reaction (PCR) was performed in a 15 µL final volume mix containing 1X reaction buffer, 9 to 12 ng of DNA template, 0.3 µM of each primer and 1.0 U Taq DNA polymerase (Invitrogen Co., Carlsbad, CA, USA). Sequences of these primers, along with each SSR repeat motif, annealing temperature, dNTP and $MgCl_2$ concentration were optimized as shown in Table 2.

The PCR products were electrophoresed on 6% polyacrylamide gels ran at 1.600 V and 60 W in 1X Tris-Borate-EDTA (TBE) buffer and the size of the fragments was estimated based on a 50 bp ladder. Finally, gels were stained with silver nitrate (Creste et al., 2001).

Table 2. Primer sequences, annealing temperature (TA °C), dNTP and $MgCl_2$ concentrations used for each SSR primer amplified in *Passiflora setacea* samples.

Locus	Primer sequences	TA (°C)	dNTP (mM)	$MgCl_2$ (mM)	Allele range
PE08	F: CCGGATACCCACGCATTA R: TCTAATGAGCGGAGGAAAGC	56	0.15	1.5	274-302
PE09	F: GGAAATCCGAAAACTGGTTG R: GGGCCTTTATCCATGTTTGA	58	0.20	1.5	268-305
PE90	F: TCAGGAAGATTGCATGTTAGT R: CTGGGTTTTGTTTATGTTGC	60	0.20	2.5	245-266
mPe-UNICAMP02	F: TCGAGTGAGATTGGCAGTG R: TTGGCTTCGAGGAGAAGAA	58	0.20	1.5	165-171
mPe-UNICAMP05	F: TCGGTCTTCGTATTCAACTCTG R: GAGGAACTGGCATCGCAT	61	0.15	1.5	194-218
mPe-UNICAMP09	F: GGGCCGTTGTCAAAGTAGT R: GAGGTTAAGGCAAGCACTG	61	0.15	1.5	250-268

The SSR profiles of all individuals were manually transformed into an allele matrix. Then, the variables number of loci (N), number of effective alleles (NE), average proportion of polymorphic loci (P), expected average heterozygosity (He), Nei genetic distances, Shannon's diversity (I), genetic structuring (Gst) and gene flow (Nm) were estimated using GenAlEx 6.5 (Peakall & Smouse, 2012). The Shannon diversity index (I) was estimated using the equation: $I= -1\times\sum[pi\times Ln(pi)$ where p_i is the frequency of an allele within the population. The level of inbreeding was measured as fixation index (F) using equation: (He-Ho)/He = 1 - Ho/He

The gene flow was estimated using the equation: $Nm= [(1/Fst)-1]/4$. Estimates of genetic structuring (Gst) and gene flow (Nm) were obtained from allelic frequencies. Principal coordinates analysis (PCoA) and molecular variance analysis (AMOVA) were performed using Nei genetic distances between individuals of each locations within GenAlEx 6.5. The dendrogram was performed based on the distance matrix by the MEGA X 10.0.5 software (Kumar et al. 2018) by the UPGMA methods (Unweighted Pair-Group Method Using Arithmetic Averages).

Also, a cluster analysis using the Ward algorithm on pairwise Nei genetic distances between the locations was performed within software PAST (Hammer et al., 2001). In this analysis, correlation of geographic and Nei unbiased genetic distances between locations was tested using a Mantel test (Smouse et al., 1986) in the program PAST (Hammer et al., 2001). Geographic distances (in km) between locations based upon GPS coordinates were calculated through the website of National Institute for Space Research (INPE) (http://www.dpi.inpe.br/calcula). In the genetic structuring of populations analysis, a Bayesian approach was used in the software Structure 2.3.4 (Pritchard et al., 2000). Ten runs were done by setting the *K* from 1 to 5, with 100,000 burn-in period and 500,000 Markov Chain Monte Carlo (MCMC) replicates. All analyses were performed using an admixture model with correlated allelic frequencies. The results were analyzed within Structure Harvester (Earl & Holdt, 2012) to calculate the most likely *K* value using the ΔK method (Evanno et al., 2005). A second Bayesian genetic structuring analysis was performed using Structurama (Huelsenbeck et al., 2011). This analysis consisted of 500,000 MCMC replicates following a Dirichlet process prior with parameter a set as a random variable with a gamma probability distribution (with shape and scale parameters set to one).

Results

The number of alleles per locus ranged from two (loci PE08 and mPe-UNICAMP02) to five (loci mPs-UNICAMP09 and PE90). Shannon diversity index of *P. setacea* observed here was 0.493 (Table 2). Mean observed heterozygosity (Ho) and mean expected heterozygosity (He) from the analysed wild populations of *P. setacea* were 0.408 and 0.306, respectively (Table 2). Populations from 'Licínio de Almeida' and 'Caetité' were the most polymorphic ones (P = 100% and 100%; I = 0.699 and 0.67, respectively; Table 2), while 'Lençóis' (P = 33.33%; I = 0.273) was the one showing the lowest polymorphism. The analysis of molecular variance (AMOVA) showed that 45% of the variation observed was among populations and 55% was within populations. Mean genetic divergence between populations (Gst) was 0.221, indicating high levels of genetic differentiation (Yeh, 2000).

As an exploratory method, the PCoA did not identify population groups within *P. setacea*. Populations

from 'Poções' and 'Andaraí' showed the widest dispersion of individuals. The ΔK method selected K = 2 as the most likely cluster arrangement for the Structure Bayesian clustering analyses, suggesting the existence of two different genetic pools within *P. setacea* in Bahia (Figure 2 and 3).

Table 3. Population, number of individuals (N) and mean values of genetic diversity parameters for each *Passiflora setacea* population, considering all SRR loci analysed: Mean number of alleles (Na), Number of effective alleles (Ne), Percentage of polymorphic loci (P), Observed heterozygosity (Ho), Expected heterozygosity (He), Shannon diversity index (I) and Fixation index (F). For populations' acronym, see Table 1.

Pop	N	Na	Ne	P	Ho	He	I	F
AMA	10	1.833	1.534	50.00%	0.317	0.224	0.368	-0.118
JAC	10	1.667	1.412	50.00%	0.300	0.193	0.307	-0.415
JEQ	10	2.000	1.703	66.67%	0.400	0.301	0.481	-0.305
CAE	9	2.667	1.858	100.00%	0.352	0.385	0.670	0.269
CON	10	1.667	1.475	50.00%	0.367	0.217	0.341	-0.575
LIC	10	2.333	1.933	100.00%	0.600	0.451	0.699	-0.282
POC	10	2.500	1.919	83.33%	0.500	0.427	0.694	-0.160
VIT	10	1.500	1.487	50.00%	0.467	0.247	0.343	-0.889
ABA	8	2.333	1.832	66.67%	0.381	0.329	0.568	-0.162
AND	10	2.167	1.734	83.33%	0.350	0.335	0.537	0.058
BON	5	1.667	1.516	50.00%	0.433	0.253	0.377	-0.716
IBI	9	2.500	2.071	66.67%	0.519	0.399	0.678	-0.290
LEN	5	1.500	1.397	33.33%	0.333	0.180	0.273	-0.862
MOR	10	1.833	1.598	50.00%	0.333	0.230	0.383	-0.402
PAL	5	2.167	1.838	83.33%	0.367	0.407	0.621	0.070
RIO	6	2.000	1.717	66.67%	0.500	0.331	0.519	-0.497
SEA	5	2.333	1.737	83.33%	0.500	0.367	0.610	-0.334
UTI	5	2.000	1.457	66.67%	0.333	0.237	0.402	-0.317
Mean		2.037	1.679	66.67%	0.408	0.306	0.493	-0.268

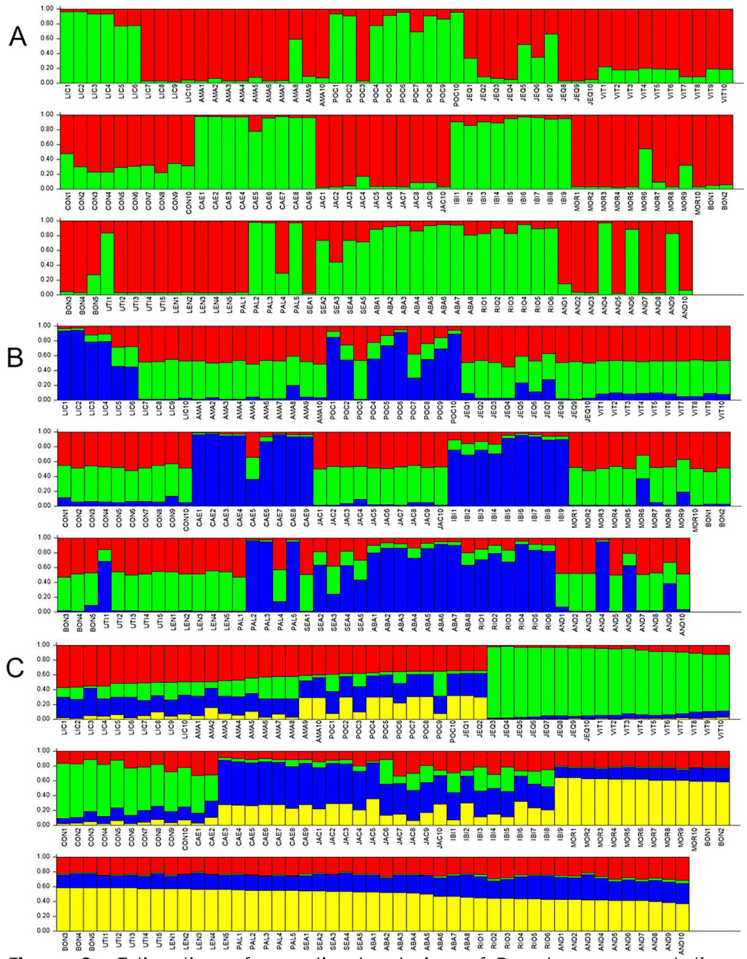

Figure 2. Estimation of genetic structuring of *P. setacea* populations, based on SSR data, in the software Structure. A. K=2. B. K=3. C. K=4. Structure analyses considered K=2 the most probable. For populations' acronym, see Table 1.

However, due to the conflicting results from PCoA and Bayesian inference, and considering the technical limitations of Structure, another Bayesian analysis with the software Structurama was conducted to test genetic clustering. This analysis also supported the existence of two genetic groups within *P. setacea* (57% PP against 42% for K = 3, and 1% for K = 4), confirming the results of Structure. Thus, the results obtained in the Bayesian inference analyses were considered as valid.

Furthermore, almost all analysed populations shared both genetic pools. The clusters were not correspondent to the six different identity territories of the state of Bahia evaluated here (Figure 4). Group I included populations from 'Sudoeste Baiano' (Licínio de Almeida and Poções), 'Sertão Produtivo' (Caetité) and 'Chapada Diamantina' (Ibicoara, Palmeiras, Seabra, Abaíra and Rio de Contas) (Figure 4). Group II included populations of all identity territories evaluated in the state of Bahia: 'Sudoeste Baiano' (Vitória da Conquista), 'Vale do Jequiriçá' (Amargosa), 'Médio Rio de Contas' (Jequié), 'Sertão Produtivo' (Contendas do Sincorá), 'Piemonte da Chapada' (Jacobina), and 'Chapada Diamantina' (Morro do Chapéu, Bonito, Utinga, Lençóis and Andaraí).

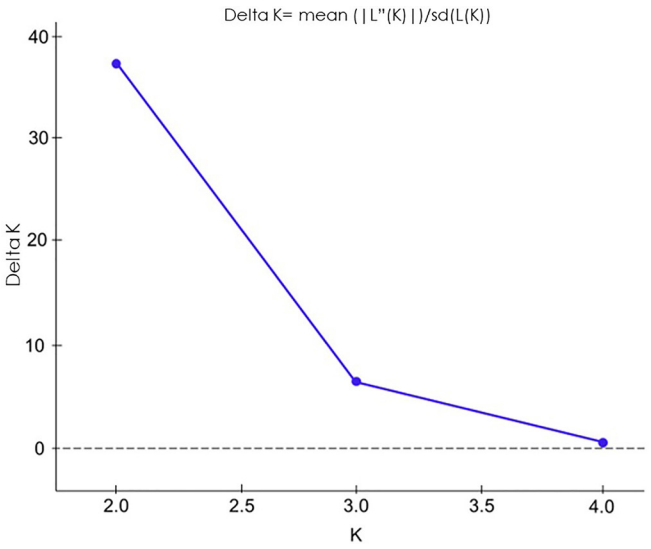

Figure 3. Delta K values compared to the number of groups (K) in the 18 populations of *Passiflora setacea*.

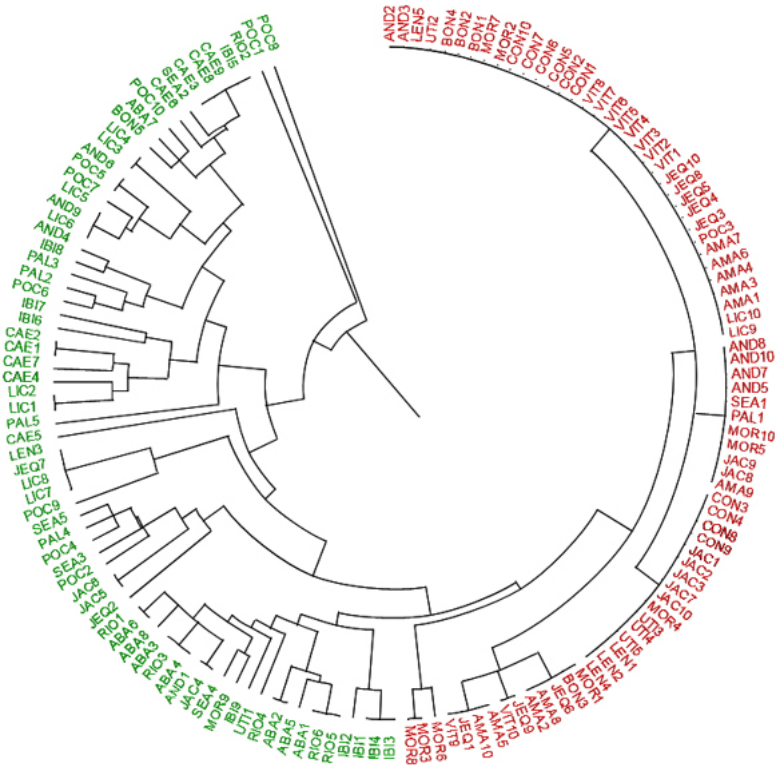

Figure 4. Dendrogram produced by the UPGMA method, obtained from the non-envisaged genetic distance of Nei among individual of *P. setacea*, using data from SSR markers. The population acronym colors indicate the genetic group detected by the Bayesian inference analysis in the Structure program (group 1 = green; group 2 = red). For populations' acronym, see Table 1.

Discussion

The low number of polymorphic SSR loci obtained here has been observed in other *Passiflora* species and has been suggested to be characteristic of the genus (Cerqueira-Silva et al., 2012). Similar values of the number of alleles per locus (up to seven alleles

per SSR locus) have been found in genetic diversity studies (31 SSR markers) of wild populations of *P. alata*, *P. edulis*, *P. cincinnata*, and *P. setacea* (Pádua et al., 2005; Cerqueira-Silva et al., 2014a). Shannon diversity index of *P. setacea* founded, 0.493 (Table 1), indicate a high genetic diversity. Pereira et al. (2015) found similar values with inter simple sequence repeats (ISSR) and resistance genes analogs (RGA) markers (I = 0.51 and 0.34, respectively). This might be an advantage for crop breeding because species with high genetic variability allows the choice of favorable alleles in selective breeding (Bernardes et al., 2020).

Results from other SSR genetic diversity studies of germplasm bank accessions of *P. setacea* found similar mean values of Ho = 0.25-0.34 and He = 0.36-0.41 (Cerqueira et al., 2014a; b). High levels of heterozygosity have been reported in several studies of SSR markers from other species of *Passiflora* (*P. alata* and *P. edulis,* Oliveira et al., 2005; *P. cincinnata,* Cerqueira-Silva et al., 2012), supporting it as a common characteristic of the genus. This behaviour can be related to the occurrence of auto-incompatibility mechanisms present in most species within subgenus *Passiflora* (Pereira et al., 2015). Observed heterozygosity (Ho) values were higher than expected heterozygosity (He) values in most analysed populations, except for 'Caetité' and 'Palmeiras'. This excess of heterozygotes indicate that these populations are not in Hardy-Weinberg equilibrium and reveal the action of evolutionary factors on them, as gene flow, natural selection and geographic range (Oluoch et al., 2018). The fixation index (F) observed was negative for almost all populations and the average value was -0.268. This low level of inbreeding can be explained for the complex method of self incompatibility in passion fruit, characterized by rejecting self-pollen (Madureira et al., 2014).

The lowest polymorphism in 'Lençóis' location observed was probably related to the low number of individuals analysed, considering this population was found in a well-preserved area. All other populations had levels of variation ranging from high (83.3%) to low (50%). The mean level of polymorphism among *P. setacea* populations was high (66.7%) (Bustamante et al., 2016). In this context, anthropic actions are probably influencing the genetic diversity of *P. setacea* since most populations were collected along roadsides and other highly disturbed areas.

Polymorphism values recovered here were higher than detected with ISSR and RGA markers (from 37% to 73%) in other populations of *P. setacea* from Bahia (Pereira et al., 2015). Despite being co-dominant markers that are useful for genetic structuring studies of cultivated plants, RGA markers can present lower polymorphism values than SSR markers (Ren et al., 2013). Thus, low polymorphism values recovered by Pereira et al. (2015) might probably be a result of the selection of markers and population sampling, which was restricted to only two identity territories in the state of Bahia (SEPLAN, 2020).

The results of AMOVA were similar to those observed by Pereira et al. (2015) for ISSR and RGA markers with *P. setacea* and by Martínez et al. (2020) for *Passiflora* spp. with ISSR markers. Furthermore, a similar variation percent (42.56% between populations) was observed in *P. alata* with RAPD markers, suggesting that populations were genetically structured (Loss et al., 2006). High levels of Gst were also detected in other genetic diversity studies using SSR, ISSR and RGA markers (Gst = 0.36, 0.38, 0.42, respectively) on populations of *P. setacea* (Cerqueira-Silva et al., 2014a; Pereira et al. 2015). Despite these high levels of divergence, no population presented exclusive alleles. Estimated gene flow was moderate (1.179, based upon Fst) and congruent with the high population structuring. In the same context, estimated gene flow observed in populations of *P. setacea* from southern Bahia with ISSR and RGA markers were low (0.86 and 0.68, respectively) (Pereira et al., 2015).

Pollen and seed dispersal determine gene flow between populations, playing a fundamental role in population structuring. The self incompatibility in most of the *Passiflora* species (Madureira et al., 2014), makes cross-pollination necessary to produce fruit (Ocampo et al., 2017). Although flowering occurs throughout the year, spontaneous fruit setting rate of *P. setacea* is low (Ataíde et al., 2012). *P. setacea* has nocturnal anthesis and its principal pollinators are bats, together with a low number of pollination events attributed to small invertebrates (Teixeira et al. 2019). Besides transporting large amounts of pollen from multiple genotypes, bats frequently move pollen through long distances between plants. Thus, they promote the increasement of gene flow and the decreasement of genetic differentiation between plant populations (Fleming et al., 2009). Considering bats are this species' main pollinator, high levels of gene flow and low values of population structuring in *P. setacea* were expected. Oliveira and Ruggiero (2005) suggested that seed dispersal in *P. setacea* is zoochoric, due to its tasty fruits. In general, passionflowers are consumed and dispersed by birds and small mammals that favor the occurrence of a more intense gene flow (Shiels & Walker, 2003; Mendiondo & García, 2006). However, the dispersal

agent of *P. setacea* is still unknown.

Based on the results observed here, the accessions of *P. setacea* of 'Caetité' (group I), 'Licínio de Almeida' and 'Andaraí' (group II) (Figure 1) should be considered in the conservation of the variability of this species in germplasm banks. These accessions are equally important for the programs of genetic improvement of passion fruit, as they present high levels of genetic variation, unique alleles, representing the two genetic sets observed in this study. Also, the populations of *P. setacea* presented mean pairwise Nei's genetic distances of 0.15 (data not shown). The phenetic analysis supported the existence of two groups (Figure 1C). These groups were congruent with the results of the Structure and Structurama Bayesian analyses.The Pearson correlation coefficient from the Mantel test was 0.1067 (P = 0.1848), supporting no correlation between genetic and geographic distances of populations within *P. setacea*. Similarly, Pereira et al. (2015) also found no correlation of genetic variation and geographic distance in other populations of *P. setacea* from Bahia using ISSRs and RGAs markers. This can be explained for the self-incompatibility in *Passiflora* genus and its outcrossing pollination (Ocampo et al. 2017).

Conclusions

Our findings reveal the existence of a substantial amount of genetic variability in *P. setacea* from different regions of Bahia state. Their populations have moderate polymorphism and diverge into two groups: one group includes populations from the southwestern distribution range, while the other contains all other populations. The region 'Vitória da Conquista' stands out as a source of genetic resources for this species, since the populations of *P. setacea* collected there presented a private allele.

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