Characterization and selection of ISSR molecular markers in species of *Passiflora* spp.

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ABSTRACT. The objective to be achieved with the search for molecular markers of low cost and good reproducibility for the selection and genetic characterization of passion fruit was to select ISSR (Inter Simple Sequence Repeat) primers for different unimproved varieties and cultivars of *Passiflora*, in order to subsidize molecular genetic studies. To achieve this goal, 23 ISSR starters were tested for eight unimproved varieties and 12 cultivars of *Passiflora* spp. The amplifications were obtained by electrophoresis on 2% agarose gel, photocumented under transilluminator with ultraviolet light. The ISSR primers showed satisfactory results. Among the 23 primers, 16 obtained values above 60% of efficiency when classified as good (presence of two marks or more). As a consequence of the results presented, it can be attested that such primers are suitable for studies of polymorphism, structure and genetic diversity in species of the *Passiflora* genus, in addition to assisting conservation and breeding programs.

Keywords: Diversity, passion fruit, genetic improvement, marker selection.

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INTRODUCTION

The Passifloraceae family is divided into two tribes (*Paropsieae* and *Passiflorieae*) (Cervi, 2005). Representatives of the *Passiflorieae* tribe are found in Brazil and dispersed in four genera (*Ancistrothyrsus; Dilkea; Mitostemma and Passiflora*). Among them, is the genus *Passiflora*, which stands out with the largest number of species (Mondin; Cervi and Moreira, 2011). In Brazil, the genus has an average of 525 species (Cervi; Imig, 2013), of which, many are endemic (Bernacci et al. 2016), because they occur in environments which are inhospitable for the growth and survival of many plant species (Bernacci et al. 2005).

Plants of this genus have high economic value. Its flowers, due to their exuberance, are used as ornamental. The fruits are highly nutritious and appreciated by the food industry for processing pulps. While that, leaves and peels of the fruits are rich in glycosylated flavonoids which are used to produce anxiolytic phytotherapics and sleep inducers (Cunha et al. 2004). In addition to these uses, the mesocarp has shown a biosorbent potential to remove metals from aqueous solutions of industrial origin (Zamberlan, 2007, Costa et al. 2015).

Genetic characterization studies in passion fruit species have been carried out for decades using different molecular markers (Cerqueira-Silva et al. 2014, Cerqueira-Silva et al. 2015). Molecular markers make it possible, in general, to differentiate genetically related individuals (Borém; Miranda, 2005). Providing molecular genetic information with potential use related to the most varied practical developments, ranging from the indication of preferential crosses to the genetic mapping of genes of interest and or understanding of specific metabolic routes are important for the advancement of genetic improvement (Cerqueira-Silva et al. 2014, Faleiro, 2018, Souza et al. 2020).

Even with this initiative, there is still much to be done, as these studies are restricted to some accesses maintained in Active Germplasm Banks (AGB), where most species of wild passion fruit are susceptible to genetic erosion even before their

previous botanical knowledge. Thus, it is necessary to study the morphological and genetic characterization of a greater number of species to guide a conservationist action for the genus.

Many species are conserved *ex situ* in AGB. Such conservation allows scientific research of agronomic and biotechnological nature applied to genetic improvement. To be successful in these studies, it is necessary to know the genetic diversity, including the main morpho-agronomic descriptors that exist between species since together these information make the use of genetic resources effective (Jesus et al. 2013; Ferreira, 2005). Therefore, the uses of molecular markers are essential tools from biotechnology at this stage (Pereira, Pereira and Viana, 2005). Among the most used molecular markers are AFLP (Amplified Fragment Lenght Polymorphism), RGA (Resistance Genes Analogs), ISSR (Inter Simple Sequence Repeats), RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), in addition to SNP (Single Nucleotide Polymorphism) (Cerqueira-Silva et al. 2014; Faleiro, 2007).

The ISSR marker (Zietkiewicz et al. 1994) was developed using RAPD technology and the SSR microsatellite with few *tandem* repeat sequences (Reddy et al. 2002; Zolet et al. 2017). This marker involves the amplification of a region of DNA located between two microsatellite loci using primers with a single binding motif in the initial 3 'or final 5' position by a limited nucleotide (Zietkiewicz et al. 1994). This marker is presented both in the nuclear genome and in organelles, and provides a genotyping system with stability, reliability, speed and dominance achieving high genetic variability and data reproducibility (Moraga-Suazo et al. 2012; Souza et al. 2015). These characteristics are very efficient in the identification of polymorphisms in studies of genetic diversity in wild species as well as in studies of generations (Kumar, et al. 2010). Given the above, the objective was to characterize and select ISSR starters for different unimproved varieties and cultivars of *Passiflora* spp., in order to subsidize molecular genetic studies of *Passiflora* spp.

MATERIAL AND METHODS

Plant Material

Young leaf tissues from 20 representative accessions of eight unimproved varieties and 12 cultivars of passion fruit were used, they were present at the Active Germplasm Banks (AGB) *Flor da Paixão* of the Agricultural Research Center Cerrados (CPAC) of Embrapa Cerrados located in Brasília-DF, under the geographical coordinates (S15.6041265, W47.7119669), available on the platform http://alelobag.cenargen.embrapa.br (Table 1).

Table 1. Accessions of *Passiflora* spp. from the Active Germplasm Bank *Flor da Paixão* of the Embrapa Cerrados (CPAC), used in the study.

Code	Species	Cultivar/Commercial name
PA1	*Passiflora alata* Curtis	Maracujá-doce or Maracujá-açu
PE1	*Passiflora edulis* Sims	Maracujá-amarelo
PS1	*Passiflora setácea* DC.	Maracujá-sururuca, Maracujá-de-boi and Maracujá-do-sono
PQ2	*Passiflora quadrifaria* Vanderpl.	Maracujá-da-Amazônia
PC1	*Passiflora cincinnata* Mast.	Maracujá da Caatinga or Maracujá do mato
PC4	*Passiflora coccinea* Aubl.	Maracujá-poranga
PI1	*Passiflora incarnata* L.	Maracujá-vermelho
PT4	*Passiflora tenuifila* Killip	Maracujá-alho
BRS1	*P. incarnata x P. assiflora edulis*	BRS Céu do Cerrado
BRS2	*P. coccinea x P. setacea*	BRS Estrela do Cerrado
BRS3	*P. edulis x P. edulis*	BRS Gigante Amarelo
BRS4	Selection of *P. edulis*	BRS Minimaracujá Roxo
BRS5	Selection of *Passiflora alata*	BRS Mel do Cerrado
BRS6	Selection of *Passiflora setacea*	BRS Pérola do Cerrado
BRS7	*P. incarnata x (P. quadrifaria x P. setacea)*	BRS Rosea Púrpura
BRS8	*P. setacea x (P. coccinea x P. setacea)*	BRS Roseflora
BRS9	*P. edulis*	BRS Rubi do Cerrado
BRS10	*P. coccinea x (P. coccinea x P. setacea)*	BRS Rubiflora
BRS11	*P. edulis*	BRS Sol do Cerrado
BRS12	Selection of *P. tenuifila*	BRS Vitta Fruit

DNA extraction and quantification

Access DNA was extracted using the CTAB 2% method (*Cationic Hexadecyl Trimethyl Ammonium Bromide* according to Faleiro et al. (2003). Nucleic acids were quantified by spectrophotometry in the absorbance ratios in ng / µL a, A260 / 230 and A260 / 280, using BioDrop® µLITE (Whitehead Scientific). For better attribution of the results they were submitted to a 1% (w / v) agarose gel stained with GelRed® (Biotium) for 90 min at 90 V. They were compared with the molecular weight marker ((DNA Lambda; Invitrogen) at concentrations of 25 and 75 ng / µL. The gels were visualized in UV and recorded in a Kodak photo-documentation system (KODAK MI Software). Subsequently, the extracted DNA samples were diluted in TE buffer, standardized at 50ng.µL^{-1}, with a final volume of 100 µL. In possession of this stage, the samples were conditioned on dry ice and taken to the Laboratory of Applied Molecular Genetics at the State University of Southwest Bahia, *Campus* Itapetinga-BA (LGMA-UESB), where they were stored at -20 ° C until the moment of polymerase chain amplification reaction (PCR).

Amplification tests

For the amplification tests 23 ISSR (Inter-Simple Sequence Repeat) primers were used, as recommended by Embrapa Mandioca and Fruticultura, available in Embrapa's free access to scientific information repository (Alice Repository) (Costa et al. 2010; Costa et al. 2011) (Table 2).

Table 2. ISSR (Inter-Simple Sequence Repeat) primers used in the study and their respective nucleotide sequences.

	Primers	Sequence 5 '- 3'
1	TriGGA3`RC	GGA GGA GGA GGA GGA RC
2	DiGA3`T	GAG AGA GAG AGA GAG AT
3	TriCAG3`RC	CAC CAC CAC CAC CAC RC
4	TriAAG3`RC	AAG AAG AAG AAG AAG RC
5	DiCA3´G	CAC ACA CAC ACA CAC AG
6	DiCA3`RG	CAC ACA CAC ACA CAC ARG
7	DiCA3`YG	CAC ACA CAC ACA CAC AYG
8	DiGA3`C	GAG AGA GAG AGA GAG AC
9	DiGA3` RC	GAG AGA GAG AGA GAG ARC
10	TriCAC3`RC	CAC CAC CAC CAC CAC RC
11	TriCAC3`YC	CAC CAC CAC CAC CAC YC
12	TriCAC5`CY	CAC CAC CAC CAC CAC CY
13	TriGTG3`YC	GTG GTG GTG GTG GTG YC
14	TriTGT3`YC	TGT TGT TGT TGT TGT YC
15	TriAAC3`RC	AAC AAC AAC AAC AAC RC
16	TriACG3`RC	ACG ACG ACG ACG ACG RC
17	TriAGA3`RC	AGA AGA AGA AGA AGA RC
18	TriTGG3`RC	TGG TGG TGG TGG TGG RC
19	TriCGA3`RC	CGA CGA CGA CGA CGA RC
20	TriCGC3`RC	CGC CGC CGC CGC CGC RC
21	TriGAC3`RC	GAC GAC GAC GAC GAC RC
22	TriGCA3`RC	GCA GCA GCA GCA GCA RC
23	TriGCC3`RC	GCC GCC GCC GCC GCC RC

The PCR reactions were performed with a total volume of 16 µL, with 8 µL of DNA at 2 ng, 1 µL of the primer, 0.11 µLTaq DNA Polymerase, 1 µL of the dNTP mix, 1 µL of Magnesium Chloride (MgCl2), 1.7 µL of 10X Buffer, and 3.19 of ultra-pure water (qsp). The programs adopted for the amplification reactions followed temperature recommendations made by *Embrapa Mandioca and Fruticultura* for the same starters tested in *Passiflora edulis* (Costa et al. 2010; Costa et al. 2011). These were composed of: 95 ° C for 5 minutes for initial denaturation; followed by 34 cycles (94 ° C for 50 seconds, 48 ° C for 50 seconds for annealing, 72 ° C for 1 minute for extension); and 5 minutes at 72 ° C for final extension.

The amplification products were subjected to a horizontal electrophoresis system in 2% agarose gel (m/v) and TBE 0.5X buffer (Trisborate-EDTA) with a voltage of 120 for 2 hours. The Red Biotium ® safe Gel (Uniscience) was used as an intercalating and the 1 Kb Plus Ladder standard (Invitrogen, Carlsbad, CA, USA) was used as the molecular weight standard of the polymorphic marks obtained. Subsequently, the gels were exposed to the ultraviolet transilluminator and documented in a Kodak photo-documentation system (KODAK MI Software).

Descriptive analysis and selection of markers

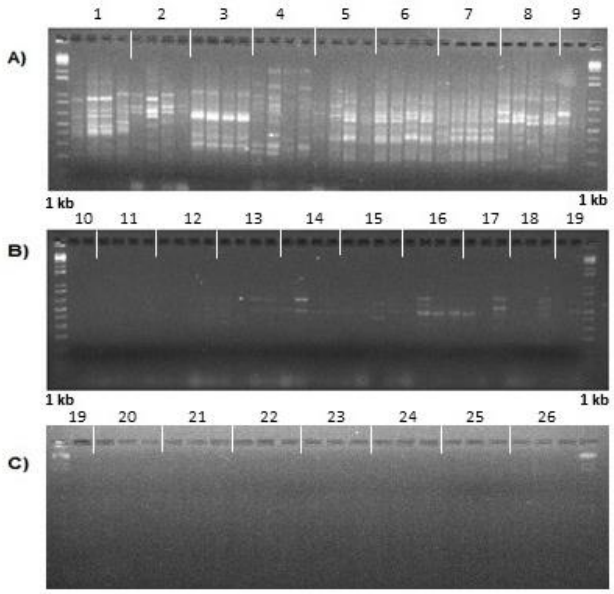

Figure 1. Amplification profile generated by agarose gel electrophoresis (2%), from ISSR markers in accessions and commercial varieties of passion fruit (*Passiflora* spp.). A) Good profile: presence of visible bands; B) reasonable: presence of barely visible bands; C) inadequate: no bands / marks.

Based on the amplification patterns, a presence (+) and absence (-) matrix of the polymorphic bands was made. After characterizing the markers for each access, the percentage of marks for each category was calculated using the formula:

$$quality = \frac{no.species\ amplified}{no.species\ used} \times 100$$

as well as the efficiency of the markers for each access by the formula:

$$efficiency(\%) = \frac{efficient\ starter\ number\ by\ species}{total\ number\ of\ initiators} \times 100.$$

RESULTS AND DISCUSSION

The efficiency of the 23 markers in discriminating the accessions of passion fruit (unimproved varieties and cultivars), (in the columns) as well as the DNA quality of each sample before amplification by ISSR (in the lines) are shown in Table 3.

It was observed that out of the 20 accessions, two (PS1 and PQ2) presented bands from the use of all primers (line). And five (primers 1, 11, 14, 15 and 21) of the 23 ISSR primers showed 100% efficiency with bands in all samples (column) (Table 3). These values were higher than those reported by Souza et al. (2020) in studies with the same specimens from 17 combinations of RGA markers. In this study, the authors observed the presence of bands for all combinations, which ranged from 17.6 for cultivars BRS Roseflora, BRS sol do Cerrado, BRS Vitta at 70.5% for the unimproved variety of *Passiflora edulis*. Only one of the RGA combinations (S1 + As1) achieved 100% efficiency with bands in all samples.

When comparing the results of the present study with the analysis of RGA made by Souza et al. (2020) it was observed that the ISSR was more efficient, in addition to presenting the advantage of a single initiator. While regarding the RGA, although having the same nature as a dominant marker, amplification tests are made possible by the combination of two or more primers, which increases the cost of the analysis.

Meira et al. (2020) by genetically characterizing 36 accessions *of Prosopis juliflora* SW. A.D. through the same 17 pairs of RGA and 23 ISSR, they observed that the two markers were efficient with a similarity coefficient of 0.87 and 0.88. However, by the discriminant analysis of the main component, the ISSR made it possible to genetically distance the accessions in two groups, while the RGA presented a homogeneous distribution.

Santana (2011) in studies of genetic diversity selection of 17 accessions of umbu-cajazeira (*Spondias* sp) with ISSR primers, among which nine were used in this study (DiGA3`T, TriAAG3`RC, DiCA3`YG, DiGA3`C, DiGA3` RC, TriCAC3`RC, TriGTG3`YC, TriAAC3`RC and TriCGA3`RC) (Table 2), observed a total of 249 bands. The average genetic variability between accessions observed by the author ranged from 0.247 to 0.665 and an average polymorphism of 80%. For (Meira et al. 2019) this percentage is considered high, because in a study of genetic diversity of 10 populations of *Lippia rotundifolia* Cham. assessed using the ISSR markers of the UBC series, the authors obtained genetic diversity between 0.132 to 0.204 and 56.57% polymorphism.

Table 3. Descriptive presentation of the classification of ISSR markers considering the occurrence (+) and the absence (-) of amplification in eight unimproved varieties and 12 cultivars of passion fruit (*Passiflora* spp).

Accession	Primers ISSR																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Total	(%)
PA1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	22	95.6
PC1	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	21	91.3
PE1	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	22	95.6
PS1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	23	100
PI1	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	22	95.6
PC4	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	22	95.6
PT4	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	21	91.3
PQ2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	23	100
BRS1	+	+	-	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	15	62.2
BRS2	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	+	-	+	+	-	+	-	-	9	39.1
BRS3	+	+	-	-	-	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	16	69.5
BRS4	-	+	-	-	-	+	-	-	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-	13	56.5
BRS5	-	+	+	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	16	69.5
BRS6	-	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	15	65.2
BRS7	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	-	-	11	47.8
BRS8	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	-	10	43.5
BRS9	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-	10	43.5
BRS10	-	+	-	-	-	+	-	-	-	-	+	-	-	-	+	+	+	+	-	-	+	-	-	8	34.8
BRS11	-	+	+	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	18	78.2
BRS12	-	+	-	-	-	+	-	-	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	13	56.5
Total	8	20	10	7	7	15	8	9	16	16	20	7	19	14	20	20	17	19	15	16	20	15	12		
(%)	40	100	50	35	35	75	40	45	80	80	100	35	95	70	100	100	85	95	75	80	100	75	60		

PA1: *P. alata* Curtis; **PC1***: P. cincinnata* Mast.; **PE1***: P. edulis* Sims; **PS1***: P. setacea* DC*;* **PI1***: P. incarnata* L.; **PC4***: P. coccinea* Aubl.; **PT4***: P. tenuifila* Killip*;* **PQ2***: P. quadrifaria* Vanderpl.; **BRS1**: BRS Céu do Cerrado; **BRS2**: BRS Estrela do Cerrado; **BRS3**: BRS Gigante Amarelo; **BRS4**: BRS Minimaracujá Roxo; **BRS5**: BRS Mel do Cerrado; **BRS6**: BRS Pérola do Cerrado; **BRS7**: BRS Rosa Púrpura; **BRS8**: BRS Roseflora; **BRS9**: BRS Rubi do Cerrado; **BRS10**: BRS Rubiflora; **BRS11**: BRS Sol do Cerrado; **BRS12**: BRS Vitta Fruit.

Although the species reported by the aforementioned authors belong to other families, this information attributes safety to genetic characterization studies based on the present marker. Out of the 23 tested primers, 21 were effective for *Passiflora cincinnata* Mast (PC1). In recent studies with the same species, Almeida (2017) reported 33.33% of polymorphic relevance, in which out of 15 primers tested, 5 presented 100% polymorphism. According to the author, the genetic divergence presented is attributed to the fact that the species is allogamous, which increases the possibility of forming different combinations between alleles with consequent genetic variability (Hartl; Clark, 2010). Applying this study using 31 markers in 25 wild passion fruit species, Sousa et al. (2015) observed that 20 primers had polymorphic loci. In view of the studies with the same primers in different species of the genus, it was observed that these markers, in addition to enabling molecular studies, also inform the divergence between species, whose characteristic makes this marker a potential tool for the selection of taxa in wild species of *Passiflora* L.

The 12 cultivars analyzed showed a lower percentage of amplification than non-improved species. The values of the cultivars varied between 8 to 16 bands, corresponding to 34.8 to 69.5%. Whereas the unimproved plants had an average of 22 bands with a percentage ≥ 95% (Table 3). However, five cultivars showed a percentage above 60% efficiency (BRS1, BRS3, BRS5, BRS6, BRS11). Satisfactory results were also found by Fonseca et al. (2017), in which they sought the molecular characterization of ornamental varieties of passion fruit (BRS Rubiflora, BRS Rosea Púrpura, BRS Roseflora, BRS Céu do Cerrado, BRS Estrela do Cerrado and BRS Pérola do Cerrado), using other ISSR molecular markers, which obtained 125 marks and 91% polymorphism. As a result, the fact that the cultivars had a smaller number of bands than the unimproved varieties does not decrease the efficiency of the markers, since this information is important in the genetic characterization of the passion fruit accessions in the AGB.

According to Melo Júnior et al. (2012), the amplification efficiency as well as the diversity index varies according to the species and the molecular marker. However, the use of the initiator depends on the objective of the study. The ISSR is recommended for the characterization of genetic diversity of unimproved species or poorly studied cultivars. Subsequently in possession of the preliminary screening carried out with the ISSR, other primers may be used. An example of this use would be to meet the demand regarding the detection of pathogen resistance genes, for example, which is the specificity of RGA (Meira et al. 2020; Souza et al. 2020).

However, the present study was a preliminary trial for the characterization and efficiency of ISSR primers, with limited numbers of individuals. For this reason, more detailed studies are recommended regarding intraspecific diversity, since accesses that presented a low percentage of bands for a given initiator, may indicate conserved regions, differentiating those that do not have the same region. Therefore, the intra and interspecific discriminatory analysis is important for the characterization, conservation and identification of species within AGBs (Hartl; Clark, 2010). Nonetheless, the selection of the markers made it possible to know its viability for the genus *Passiflora*, as well as to contribute to future research on the genetic structure of other accessions inserted in the same collection of the AGB from these previously screened primers.

CONCLUSION

ISSR primers are efficient for analysis of unimproved varieties and cultivars of *Passiflora*. Primers 1 (TriGGA3`RC); 11 (TriCAC3`YC); 14 (TriTGT3`YC); 15 (TriAAC3`RC) and 21 (TriGAC3`RC), can be used to support molecular genetic studies of the species of the genus.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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