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AMPLIFICATION AND SELECTION PROFILE OF ISSR MARKERS FOR GENETIC STUDIES IN *Calotropis procera*[1]

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ABSTRACT – Sodom apple is a plant species adapted to various ecosystems and has stood out for its economic and ecological importance. We evaluated the amplification profile of 23 ISSR primers and selected polymorphic loci for genetic studies of a natural population of *Calotropis procera* by collecting and extracting genomic DNA from 33 individuals. Genomic DNA was extracted using the sorbitol protocol and 2% CTAB and the ISSR amplification products were resolved by electrophoresis. Based on the amplification profile, the 23 primers were classified as suitable, moderate, and unsuitable. We described the quality of primers considering the total number of bands, mean bands per primer, percentage of polymorphism, Nei's genetic diversity (expected heterozygosity – He), assuming Hardy-Weinberg equilibrium and the polymorphic information content (PIC). All ISSR primers showed an amplification profile, which generated 173 bands with an average of 7.5 loci per primer. However, only 18 out of the 23 tested primers allowed visible and high-quality amplification, which were classified as suitable and polymorphic. We also observed a mean of 0.30 and 0.24 for PIC and He estimates, respectively. The DiCA3`RG, TriAGA3`RC, and TriCGC3`RC primers were highly transferable to *C. procera* (they presented quality for amplification with good reproducibility), with PIC values higher than 0.40, He higher than 0.30, and polymorphism higher than 86%.

Keywords: Genetic diversity. Apple of Sodom. Molecular markers. Polymorphism.

PERFIL DE AMPLIFICAÇÃO E SELEÇÃO DE MARCADORES ISSR PARA ESTUDOS GENÉTICOS EM *Calotropis procera*

RESUMO – Algodão de seda é uma espécie vegetal adaptada a vários ecossistemas e tem se destacado pela importância econômica e ecológica. Nós avaliamos o perfil de amplificação de 23 iniciadores ISSR e selecionamos loci polimórficos para estudos genéticos de uma população natural de *Calotropis procera* por meio da coleta e extração de DNA genômico de 33 indivíduos. O DNA genômico foi extraído usando o protocolo sorbitol e CTAB 2% e os produtos de amplificação ISSR foram resolvidos por eletroforese. Com base no perfil de amplificação, os 23 iniciadores foram classificados como adequados, razoáveis e inadequados. Descrevemos a qualidade dos iniciadores considerando o número total de bandas, média das bandas por iniciador, porcentagem de polimorfismo, diversidade genética de Nei (heterozigosidade esperada - He), assumindo o equilíbrio de Hardy-Weinberg e conteúdo das informações polimórficas (PIC). Todos os iniciadores ISSR apresentaram perfil de amplificação, os quais geraram 173 marcas com média de 7,5 loci por iniciador. Porém, dos 23 iniciadores testados, apenas 18 permitiram amplificação visível e de alta qualidade, que foram classificados como adequado e polimórfico. Também observamos média de 0,30 e 0,24 para as estimativas de PIC e He, respectivamente. Os iniciadores DiCA3`RG, TriAGA3`RC e TriCGC3`RC foram altamente transferíveis para *C. procera* (apresentaram qualidade para amplificação com boa reprodutibilidade), com valores de PIC superiores a 0,40, He superior a 0,30 e polimorfismo superior a 86%.

Palavras-chave: Diversidade genética. Flor de seda. Marcadores moleculares. Polimorfismo.

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INTRODUCTION

Calotropis procera Ait. R. Br. is an exotic species, belonging to the family Apocynaceae, subfamily Asclepiadoideae, and tribe Asclepiadeae (SENNBLAD; BREMER, 2002). It occurs naturally in Africa and Asia and is widely distributed in arid, semi-arid, and tropical regions of America (SOBRINHO et al., 2013). The species was introduced in Brazil in the 19th century as an ornamental plant and adapted in regions with rainfall of 150 to 1000 mm, ranging from excessively drained, sandy to degraded soils (MOREIRA et al., 2018).

C. procera plays an ecological role as a colonizer of environments due to its good adaptation even in very dry environments, such as phytophysiognomies of the Caatinga and ecotone areas, and its anemochoric character. Its nature as a primary pioneer plant also makes it widely distributed along roadsides, pasture areas, and anthropic environments, whose occupation characterizes it as ruderal, an indicator of degraded areas and maintainer of animal feed in the dry season in the Northeast (FABRICANTE; OLIVEIRA; SIQUEIRA-FILHO, 2013; GARCEZ; CÂMARA; VASCONCELOS, 2014; MOREIRA et al., 2018).

In addition to the good adaptation, studies have reported this plant as hypotensive, analgesic, anti-inflammatory, and antibacterial against strains of *Klebsiella pneumoniae*, *Shigella flexneri*, *Hafnia alvei*, and *Staphylococcus aureus*, in addition to muscarinic anticholinergic activity (GALLEGO-OLEA et al., 2008). The compounds produced from the plant secondary metabolism also give the species biological activities of agricultural importance such as bactericidal, antimicrobial, and larvicidal, which act as allelochemicals (RANI et al., 2017).

Although the species is exotic, widely distributed, and well-adapted, acting as a forage and medicinal support, little is known about its genetic distribution and intrapopulation variability (MOREIRA et al., 2018). The closest contribution in this regard was the research carried out by Almeida et al. (2017), who studied the physiological quality of seeds from natural populations of *C. procera*. Considering the lack of molecular genetic studies regarding the genetic variability of this species, studies focused on populations are necessary to understand the distribution of their subpopulations given the wide geographic occupation. Prior information regarding the selection of molecular markers is required for this type of study (CRUVINEL; MELO; LACORTE, 2017).

Among several molecular markers, the ISSR (Inter Simple Sequence Repeat) marker, which is a dominant marker based on the combination of RAPD and SSR marker technologies, is one of the tools applicable in plants of different families and origins. It is fundamental for the study of the genetic diversity of plants with little known population structure, such as *C. procera*. This marker detects small fragments of DNA repeated in tandem. Although the bands produced by the ISSR marker are not visibly strong such as those obtained by microsatellites, it guarantees good reproducibility, a high degree of polymorphism, and low cost (ZOLET et al., 2017).

In the case of ruderal species whose molecular knowledge is limited, testing markers previously used in commercial species before developing and characterizing species-specific primers are recommended as a means of saving time and resources. In this context, we evaluated the transferability of 23 ISSR markers previously selected for *Passiflora edulis* (SANTOS et al., 2011) for 33 accessions of *C. procera* Ait. R. Br. through the amplification profile characterization.

MATERIAL AND METHODS

The research was conducted at the Laboratory of Applied Molecular Genetics of the State University of Southwest Bahia (LGMA/UESB), Campus Juvino Oliveira, Itapetinga, BA, Brazil, under the coordinates −15.25′19″S and −40.27′75″ W. The plant material consisted of fresh leaves from 33 *C. procera* plants, collected in the municipality of Itororó, micro-region of Southwest Bahia, between the coordinates 15°571′S and 40°2.271′W. The population was georeferenced with a Garmin Oregon GPS (Global Position System) receiver. They were registered at SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) under the registration number A102BCC. After collection, the material was packed in a paper bag, sealed with adhesive tape, identified, placed in a styrofoam box with ice, and sent to LGMA, where it was stored in a refrigerator until the genomic DNA extraction.

Extraction, purification, and quantification of genomic DNA

DNA extraction followed the protocol described by Russell et al. (2010), with modifications. In this step, 1 mL of sorbitol buffer was used in 0.5 g of plant material, which was macerated and centrifuged for 10 min at 5,700 rpm at 15 °C. Subsequently, the supernatant was discarded, and 1 mL of sorbitol buffer was again added to the pellet; this procedure was repeated until the mucilage was completely removed. The second step continued with the addition of 1 mL of 2% CTAB and incubation at 60 °C for 1 h. After incubation, 700 µL of chloroform and isoamyl alcohol in a 24:1 ratio was added and homogenized manually for 20 min. After this process, the material was again centrifuged for 10 min at 13,000 rpm at 15 °C, the supernatant

was transferred to another microtube, and sodium acetate (CH_3COONa) at the proportion 1:10 (v/v) and cold isopropanol at the proportion 2:3 (v/v) were added. After this step, the samples were kept overnight at −20 °C. After incubation, centrifugation was performed for 30 min under the same conditions as above. The pellet was washed twice with 500 µL ice-cold ethanol. The pellet was dried at room temperature for 24 h, and the genomic DNA was resuspended with Milli-Q water q.s. to 60 µL.

Subsequently, the amount and purity of DNA were estimated in a BioDrop µLITE spectrophotometer (Whitehead Scientific) at the ratio of A260/A230 and A260/A280 nm absorbance. The concentration of each sample allowed standardizing the DNA at 50 ng μL^{-1} to be used in the PCR reaction. The material was also subjected to a 1% (w/v) agarose gel electrophoresis at 120 V for 90 minutes. The samples were stained with 0.2% (w/v) Gel Red (Invitrogen) and their concentration was compared to known aliquots of phage lambda (λ) DNA (Invitrogen). After electrophoresis, the gels were visualized in a transilluminator under ultraviolet light and photo-documented in a Kodak camera.

Amplification of genetic material

Twenty-three ISSR (Inter Simple Sequence Repeat) primers were used for amplifying the genetic material of the 33 individuals (Table 1). The reaction was performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems®).

Table 1. ISSR primers 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

R: purine (A or G); Y: pyrimidine (C or T).

The amplification tests were performed as recommended by Embrapa Cassava & Fruits for wild passion fruit species (*Passiflora edulis*) available at Embrapa open access repository to scientific information (Alice Repository). The PCR reaction was prepared from 8 µL of 2 ng DNA, 1.7 µL of 10X PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 1.0 µL of $MgCl_2$, 1.0 µL of

2.5 mM dNTP mix, 0.11 µL of Taq DNA polymerase (LGC Biotechnology), 3.19 µL of Milli-Q water, and 1.0 µL of primer, totaling 16 µL of final volume. This reaction was started at 94 °C for 5 minutes for denaturation, followed by 34 cycles (94 °C for 50 s for denaturation, 48 °C for 60 s for annealing, and 72 °C for 60 s for extension) and a final extension at 72 °C for 5 min. After the PCR reaction, 6 µL aliquots of each amplified product were electrophoresed in a 2% (m/v) agarose gel for 2 h with 0.5x TBE running buffer at 120 V for 120 minutes. The material was stained and visualized as previously described for genomic DNA.

Data analysis

The generated products were classified according to the amplification profile into suitable, moderate, and unsuitable, presenting bands of easy visualization, bands of difficult visualization, and visible absence of bands. Then, a table of binary data was built with the presence and absence of bands, which was submitted to genetic analyses. The total number of bands, the average band for each primer, the percentage of polymorphism (PP), the Nei's genetic diversity index (He), and the polymorphic information content (PIC) were considered in this step. The accuracy of results was verified by extrapolating the binary data in 999 random permutations, Bootstrap, thus assuming that the population is at Hardy-Weinberg equilibrium. The GenAlex and Genes software were used to enable the analyses (PEAKALL; SMOUSE, 2012; CRUZ, 2013).

RESULTS AND DISCUSSION

The 23 ISSR primers allowed the amplification and satisfactory visualization of fragments from the PCR reactions from the genomic DNA of 33 individuals of *C. procera*. Of this total, 18 primers enabled good amplification, being visible and of high quality, and classified as suitable. The other primers showed barely visible marks and were classified with a moderate standard, but as reproducible as those considered suitable. Figure 1 shows examples of amplifications considered suitable (DICA3`RC; DICA3`YG) and reasonable (DICA3`G and DIGA3`C).

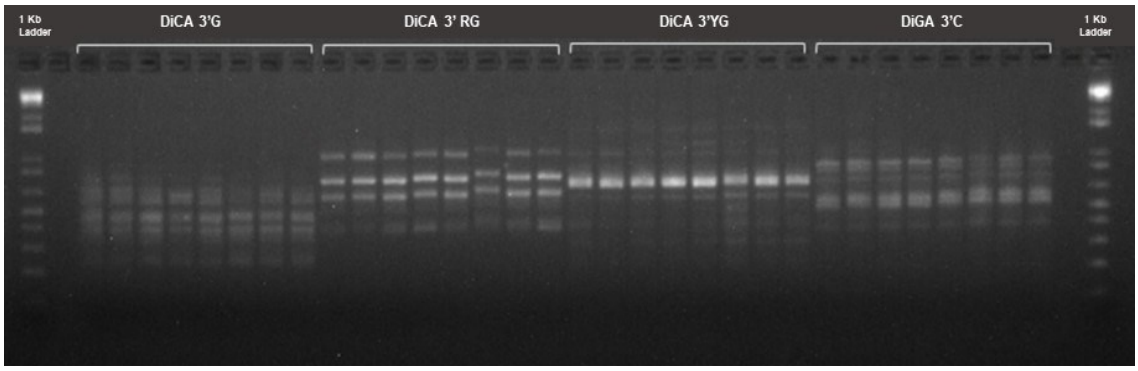

Figure 1. Amplification profile of ISSR primers in *Calotropis procera* Ait. R. Br. accessions.

The primers generated 173 bands with an average of 7.5 bands per primer, in which all were polymorphic, with an average of 6.7 bands. The average percentage of polymorphism (PP%) was 89.60%. The amplification product revealed that 47.8% of the primers (2, 3, 5, 6, 7, 8, 11, 12, 17, 19, and 22) were 100% polymorphic, 43.48% showed polymorphism between 80 and 90% (primers 1, 4, 10, 13, 14, 15, 18, 20, 21, and 23), and only 8.70% (primers 9 and 16) showed a low polymorphism, with 50 and 38%, respectively (Table 2).

Regarding the ISSR response in the PCR reaction, the annealing temperature is known to be a factor that directly contributes to the specificity of the molecular marker. In this context, the ISSR marker has a varied annealing temperature, ranging from 40 to 55 °C (NASCIMENTO et al., 2017), when compared to other dominant markers, such as RAPD (Random Amplified Polymorphic DNA), which anneal at a temperature between 34 and 37 °C (MISHRA et al., 2015; PRIYA; MANIMEKALAI; RAVICHANDRAN, 2015).

This observation is important to contextualize the result presented in this research relative to the annealing temperature. A study with 20 genotypes of *C. procera* using 23 primers of the same ISSR nature submitted to different annealing temperatures presented band averages lower than those obtained in this study (SILVA et al., 2016). The authors reported that only 10 primers with temperatures between 45 and 50 °C showed satisfactory amplification, generating 53 fragments with an average of 5.3 bands per primer. Four of the primers considered satisfactory were used in this study, which corresponded to primers 6, 11, 13, and 14 (Table 2). Primers 6 and 14 showed fragments superior to those

obtained, with 13 and eight bands at 45 °C. Primer 11 had the lowest number, with three bands for all tested temperatures (45 to 50 °C). However, primer 13 remained stable relative to the annealing temperature, which presented five bands both at the temperatures adopted by Silva et al. (2016), between 45 and 50 °C, and at the temperature adopted in the present study, at 48 °C, as recommended by Santos et al. (2011) in *P. edulis* Sims and corroborated by the tests carried out for *Prosopis juliflora* (Sw) DC, *Passiflora setacea* DC., and *Passiflora cincinnata* Mast. at LGMA/UESB. The results indicated it as the best annealing temperature for the primers and, therefore, it was applied in this context for *C. procera* (Table 2).

Table 2. Genomic DNA amplification profile of *Calotropis procera* Ait. R. Br. accessions through ISSR primers.

N	Primer			Number of brands		*PP (%)	*He	*PIC
	*Code	*Sequence (5'-3')	*FA (pb)	Total	Polymorphic			
1	DiCA3'G	$(CA)_8G$	300-1000	8	7	88	0.21	0.26
2	DiCA3'RG	$(CA)_8RG$	650-2000	7	7	100	0.29	0.36
3	DiCA3'YG	$(CA)_8YG$	300-2000	8	8	100	0.24	0.29
4	DiGA3'C	$(GA)_8C$	200-2000	8	7	88	0.27	0.34
5	DiGA3'RC	$(GA)_8RC$	400-1000	9	9	100	0.26	0.32
6	DiGA3'T	$(GA)_8T$	200-1200	5	5	100	0.33	0.43
7	TriCAC3'RC	$(CAC)_5RC$	300-1650	11	11	100	0.19	0.23
8	TriCAC3'YC	$(CAC)_5YC$	400-1200	9	9	100	0.30	0.38
9	TriCAC5'CY	$(CAC)_5CY$	350-1650	6	3	50	0.12	0.15
10	TriCAG3'RC	$(CAG)_5RC$	400-1650	5	4	80	0.20	0.24
11	TriGTG3'YC	$(GTG)_5YC$	200-1200	8	8	100	0.25	0.30
12	TriTGT3'YC	$(TGT)_5YC$	400-1650	10	10	100	0.28	0.35
13	TriAAC3'RC	$(AAC)_5RC$	300-2000	6	5	83	0.18	0.22
14	TriAAG3'RC	$(AAG)_5RC$	200-2000	6	5	83	0.23	0.28
15	TriACG3'RC	$(ACG)_5RC$	300-1000	6	5	83	0.26	0.32
16	TriAGA3'RC	$(AGA)_5RC$	300-2000	8	3	38	0.10	0.11
17	TriTGG3'RC	$(TGG)_5RC$	300-2000	9	9	100	0.32	0.41
18	TriCGA3'RC	$(CGA)_5RC$	300-2000	7	6	86	0.23	0.27
19	TriCGC3'RC	$(CGC)_5RC$	300-2000	9	9	100	0.30	0.37
20	TriGAC3'RC	$(GAC)_5RC$	300-850	7	6	86	0.29	0.38
21	TriGCA3'RC	$(GCA)_5RC$	300-1000	10	9	90	0.28	0.36
22	TriGCC3'RC	$(GCC)_5RC$	300-1000	6	6	100	0.25	0.30
23	TriGGA3'RC	$(GGA)_5RC$	400-1650	5	4	80	0.24	0.30
	Total			173	155	-	-	-
	Mean			7.5	6.7	89.6	0.24	0.30

*R: purine (A or G); Y: pyrimidine (C or T); FA: fragment amplitude; PP: percentage of polymorphism; He: Nei's genetic diversity index (expected heterozygosity); PIC: polymorphic information content.

Other similar studies have been conducted aiming at the conservation of plant species. Costa et al. (2015) tested 19 ISSR primers for 15 individuals of *Hancornia speciosa* Gomes, a species of the same family as *C. procera*, of which only six were different, and found an average of 10.5 per primer. The authors reported their efficiency for the genetic characterization of the population, which showed low variability, with He = 0.18. Jimenez et al. (2015) used six primers in 38 genotypes annealed at 50 °C and obtained an average of nine bands per primer, with 85% polymorphism and He equal to 0.30. Moreover, 24 genotypes tested with 10 ISSR primers from the UBC series, submitted to 53 °C for annealing, resulted in 57 bands, with an average of 5.7 bands, 57.89% polymorphism, and He equal to 0.18 (SOARES et al., 2017). In contrast, 15 ISSR primers of 10 natural populations in the Sergipe State also showed good amplification, with 100% polymorphism. However, these populations showed

a low level of genetic diversity, with He between 0.03 and 0.1 (SOARES et al., 2016).

Chagas et al. (2015) also conducted similar tests for 13 individuals of the species *Elaeis guineenses* Jacq. to test six primers with temperatures ranging from 47 to 53 °C and observed an average number of 11.3 bands. According to the data, the amplification pattern of the studies has a lower quality than that found in *Calotropis procera*. However, the authors considered them to be of sufficient quality for genetic analyses.

Moreover, 77% of the primers for *Catharanthus roseus* L. did not show a suitable amplification profile. The primers showed an average of six bands, of which 60% were polymorphic (IBRAHIM et al., 2013). Finally, nine *Nerium oleander* L. populations were analyzed from the combination of two ISSR submitted to an annealing temperature of 43 °C, which showed an average of 66% of polymorphism and He = 0.19, which was also considered high. This result once again emphasizes the ISSR efficiency for species of the same family. The divergence in the polymorphic profile is explained by plants belonging to different genera (LAZZARO et al., 2017).

Other dominant markers such as RAPD are also used to estimate the genetic diversity of species of the family Apocynaceae. Some authors have reported that the markers have low polymorphism values, with averages between 16 and 44% (LIEDE-SCHUMANN et al., 2012; IBRAHIM et al., 2013; PRIYA; MANIMEKALAI; RAVICHANDRAN, 2015). For instance, RAPD showed a total of 16% polymorphism using 20 markers in diversity analysis of the genus *Calotropis* (PRIYA; MANIMEKALAI; RAVICHANDRAN, 2015). In the same approach, Torezan et al. (2005) and Shahnawaz et al. (2012) found satisfactory percentages of polymorphism of 78.5 and 85%, respectively.

The potential of markers to identify polymorphism among individuals in the population showed results with average values of 0.24 and 0.30 for the He and PIC estimates, respectively. These results corroborate the idea of the efficiency of these markers for the genetic characterization of *C. procera*. However, some markers showed PIC values lower than 0.25, such as primer 16 (TriAGA3'RC), with PIC = 0.11, primer 13, with PIC = 0.22, and primer seven (TriCAC3'RC), with PIC = 0.23 (Table 1). According to Xie et al. (2010), PIC values higher than 0.25 indicate moderate polymorphic information. Costa et al. (2015) reported similar PIC values (0.37) when using ISSR markers on the genetic diversity of mangabeira (*Hancornia speciosa* Gomes) obtained from a natural population.

As previously reported, only one out of the 23 ISSR primers (TriAGA3'RC) was not effective to be used in the genetic diversity of the species due to the low PIC value and the low percentage of polymorphism and heterozygosity (PP = 38% and He = 0.10), the lowest values among the results. The other primers had satisfactory PIC values, which could be transferred to contribute to future molecular genetic assays of naturalized populations of *C. procera*. Nine ISSR primers (6, 8, 11, 12, 15, 17, 19, 20, and 21) were well representative for PIC and He values (Table 2). Three of them showed polymorphism higher than 80% and six were 100% polymorphic (Table 2). Primers 6, 17, and 20, which correspond to the nucleotides DiGA3'T, TriTGG3'RC, and TriGAC3'RC, can be prioritized in the population genetics of the species because they have the best PIC and He values.

Differences in the average number of bands and the percentage of polymorphic loci reported above for species of the same family showed that both primers of the same dominant nature and primers of different nature of ISSR markers respond differently to amplification of genetic material. Thus, the polymorphic response depends on the species and the population, as each sample has its genotypic specificity. However, ISSR primers have shown promise to a greater or lesser extent to be explored in genetic screening of species of the family Apocynaceae. The annealing temperature of 48 °C recommended by Embrapa for *Passiflora edulis* and tested in other species by the LGMA/UESB team was used for the first time in the 23 ISSR primers applied to *C. procera* and was superior to the data reported in the literature, with an average 89.60% polymorphism, expected average heterozygosity of 0.24, and average polymorphic information content of 0.30. Therefore, the high percentage of polymorphism generated for these 23 primers, as well as their profile amenable to analysis, attests to their informative potential for future molecular analyses and transferability of primers to this species without the need to change the amplification process, thus saving time and inputs for the development of new markers.

However, the transfer test of ISSR primers from *Passiflora edulis* to *Calotropis procera* confirm their usefulness as a tool that precedes studies of plant genetics, as well as using them in future analyses of intra-and inter-populational diversity of *C. procera*, thus contributing to conservation studies of plant genetic resources (ARAYA et al., 2017). This study will also allow optimizing the molecular characterization of the species under study, naturalized in Brazil with wide genotypic diversity.

CONCLUSION

The 23 ISSRs for the 33 individuals of *Calotropis procera* obtained from the naturalized population of Itororó, in the southwest of Bahia, showed good transferability and a high percentage of polymorphism. The primers DiGA3'T, TriTGG3'RC, and TriGAC3'RC stood out for their

suitable quality in the amplification of genomic DNA of the species. Therefore, they are recommended for future molecular genetic characterization studies.

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