Isolation of microsatellite markers for Copernicia prunifera (Miller) H. E. Moore (Arecaceae)

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Short Report

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Abstract

Markers based on simple sequence repeats (SSR) or microsatellites have characteristics that make them widely used in population genetics studies: they are abundant and evenly distributed throughout the genome, highly polymorphic and can be transferred between species of the same genus. The present study describes the isolation and characterization of microsatellite markers for *Copernicia prunifera* (carnauba palm). Seventeen primer pairs were developed with base-pair sizes ranging from 113 to 250 bp. Subsequently, the primers were submitted to a polymerase chain reaction (PCR) and all were successfully amplified, with the primers Cop02, Cop07, Cop10 and Cop15 presenting the best amplification patterns. After amplification tests, new reactions were performed, which were adjusted to optimize primer protocols, eliminating unspecific sequences and increasing locus sharpness. The annealing temperatures that presented the best amplification patterns ranged between 52 and 59 ºC. These markers can be used in genetics studies of *C. prunifera* populations and further tested in other *Copernicia* species.

Introduction

The Arecaceae family has predominantly tropical distribution, with 181 genera and approximately 2,600 species (Baker and Dransfield 2016). There are about 42 genera and 208 species in Brazil. The genus *Copernicia* has about 13 species, with Cuba being the center of dispersal. In Brazil, the genus has only two species, *Copernicia prunifera* and *Copernicia alba* (Lorenzi et al. 2004; Costa et al. 2022).

Copernicia prunifera (Miller) H. E. Moore, popularly known as the carnauba palm, is native to the semiarid region of Northeast Brazil. It usually occurs in monodominance and can be found in the states of Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe (Leitman et al. 2015). The species has several uses, such as urban landscaping, fodder for animals and raw material for handicraft products (Lorenzi et al. 2010). However, the main product from carnauba is the leaf-covering wax, used for various purposes such as the production of capsules, food products, car and floor polishes and electronic components (Carvalho Neto et al. 2019; Freitas et al. 2019).

The high commercial value of these products increases of the exploitation of forest resources, causing negative impacts on the vegetation and alteration of the survival, growth and reproduction of trees, modifying the structure and the dynamics of populations (Santos et al. 2021). Thus, there is a need for strategies for the management, conservation and improvement of forest species, considering the mode of reproduction, the cross-breeding system and the genetic structure in these populations (Silva et al. 2017; Santos et al. 2021).

For these purposes, codominant molecular markers have been used in genetic studies of natural populations, mainly to determine reproductive patterns and gene flows (Sebbenn et al. 2011; Ramos et al. 2019). Among these, markers based on simple sequence repeats (SSR) are the closest to ideal for population genetics studies because they are abundant, multi-allelic and evenly distributed throughout

the genome. Moreover, they present the highest informative content per gene locus among all classes of molecular markers (highly polymorphic) (Kalia et al. 2011). Also, they are easily obtained through PCR (polymerase chain reaction) assays, enabling rapid evaluation of many individuals for a large number of loci (Nazareno et al. 2011). They can also be transferred between species of the same genus (Laindorf et al. 2019), and once developed, can be shared between different laboratories.

Small amounts of DNA are sufficient for analyzing these markers, and because they are multi-allelic, they present satisfactory results for reproductive, evolutionary and conservationist purposes (Hedrick 1999). During the process of obtaining primer pairs that amplify repetitive microsatellite regions, steps to characterize the abundance of different repetitive motifs, library constructs, and DNA sequencing are required (Bacon et al 2011; Nazareno et al. 2011). Thus, the present study describes the isolation and characterization of microsatellite markers (SSR) for *Copernicia prunifera*.

Material And Methods

Construction of the genomic library

The development of the library was performed based on the protocol of Billotte et al. (1999). DNA was extracted from leaf tissue samples of *Copernicia prunifera* individuals from natural populations in Rio Grande do Norte, using the method described by Doyle and Doyle (1987).

Genomic DNA was digested using the restriction enzyme RsaI, and fragments obtained were ligated to adapters (Rsa21–5’ CTC TTG CTT ACG CGT GGA CTA 3’ e Rsa25–5’ TAG TCC ACG CGT AAG CAA GAG CAC A 3’). PCR pre-amplification was performed and the obtained fragments were purified using the QIAquick PCR Purification Kit (Qiagen). A biotin-labeled probe was used to hybridize DNA fragments containing microsatellites, and streptavidin-linked magnetic beads were used to capture the selected sequences.

PCR was used to amplify the selected fragments, which were cloned with a pGEM®-T Easy Vector System (Promega) and subjected again to PCR to check the cloning. After binding to the vector, the transformation into competent XL1-Blue cells was performed to amplify the insert. Transformed cells were cultured in Luria-Bertani (LB) medium containing 100 $mg.mL^{-1}$ of ampicillin at 37°C. Then, plasmid DNA from recombinant colonies was isolated and sequenced in an automated sequencer to amplify microsatellite-containing inserts.

The sequences obtained were transformed into FASTA format and subsequently submitted to the WebSat program (http://wsmartins.net/websat/) to identify microsatellite-containing regions (Martins et al. 2009). After identifying the microsatellite regions, the forward (F) and reverse (R) primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000). Criteria were followed, such as absence of redundant bases and presence of SSR distances between 20 and 60 bp. Also, we assured that they consisted of 18 to 22 nucleotides without repetitive sequences and contained percentages of G and C bases of 40 to 60%. Additionally, they started at 5'and ended at 3' with two bases

(G or C, or G and C, if possible). It was also necessary to have an annealing temperature between 45 and 60°C, with a maximum difference of 1°C between the temperatures of the F and R primers, so they could be used in the same reaction and not hybridize to each other or self-hybridize. The stages of construction of the genomic library are illustrated in Fig. 1.

Primer testing

The 17 primer pairs constructed were tested using DNA from three *C. prunifera* individuals from different populations, to verify changes in the annealing region of the primer. The sampled individuals were located in the municipalities of São Miguel do Gostoso, Serrinha and Ipanguaçu in the state of Rio Grande do Norte, Brazil, at coordinates 5º 07' S, 35º 41' W; 6º 14' S, 35º 29' W; and 05º 31' S, 36º 48' W, respectively. The DNA extraction was performed using the CTAB method (Doyle and Doyle 1987). After extraction, the obtained DNA was quantified with an Epoch spectrophotometer.

PC reactions were performed in a 96-well Veriti thermocycler in a final volume of 15 µL containing 1X buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl), 0.7 µM from each primer pair, 250 µM of each dNTPs, 1 U Taq DNA polymerase, 0.25 mg BSA, 1.0 mM $MgCl_2$, and 9 ng DNA. The PCR protocol consisted of initial denaturation at 94°C for 5 min (one cycle), followed by 35 cycles at 94°C for 1 min, annealing for 1 min (ranging from 50 to 62°C) and 72°C for 1 min, then a final extension step at 72°C for 30 min (one cycle).

The amplification product was checked by 3.0% agarose gel electrophoresis and GelRed dye in 1x Tris-Acetate-EDTA buffer (TAE) at 100 V for 2.5 hours using a molecular weight (ladder) of 1 kb. After testing for amplification occurrence, new reactions were performed, which were adjusted to optimize primer protocols, eliminating unspecific sequences and increasing locus sharpness.

Results And Discussion

The 17 primer pairs constructed had base-pair sizes ranging from 113 to 250 bp (Table 1). When subjected to the PCR, all primers were amplified, with primers Cop02, Cop07, Cop10 and Cop15 presenting the best amplification results.

Table 1 Characteristics of 17 microsatellite primers developed for *Copernicia prunifera*. Locus name, GenBank accession number, primer sequence (F: forward, R: reverse), repeated sequence, base pair fragment size (bp) and annealing temperature (Ta)

Locus	GenBank acession n°		Primer sequence (5'-3')	Repeated sequence	Size (bp)	Ta (ºC)
Cop01		F:	AATTTACCCTTCTGGAGCATCA	(TCT)8	152	52
	MT154309	R:	CGCAACCTCATCTCCTTCA			
Cop02		F:	TGAGAAAGACGCACAACCAG	(ATA)4	194	52
	MT154310	R:	AAGCAACTGTGACCCCACTC			
Cop03		F:	GGAGTTTCAGCCAAGTTAGCC	(GA)21	168	55
	MT154311	R:	CATGGGTAGCGTGACCTTC.T			
Cop04		F:	GCAATAACCACCCTCTGCAT	(CT)17... (TG)3(TA)6	156	56
	MT154312	R:	TGTGATGGTGAGGACACTCAA			
Cop05		F:	CCCAGAGGCAGAGCAAATAG	(TA)6	166	54
	MT154313	R:	AGCCATTCCTGGTTACTCATTC			
Cop06		F:	ACACAACTTGCTGCCCAATA	(TC)13... (CA)11	173	52
	MT154314	R:	GACCGAAAGTTGTTGGACTAAAG			
Cop07		F:	GCCTGACGGACAATCAGAAA	(TA)5 (TG)19	158	59
	MT154315	R:	GGCTTGCTCAATCAAGTCCA			
Cop08		F:	CCATATGGTCGACCTGTCA	(CT)14	242	53
	MT499327	R:	GGGGGCTAGTGTGGGTAG			
Cop09		F:	CTCGTTCTAACACTCGTATCTTGGT	(AC)9 (TC)8	169	54
	MT154316	R:	ACACGGGGAGGTAGTGACAA			

Cop10		F:	AAAGTCTCAAAGTTTCCTCTACTCA	(CT)25	167	52
	MT154317	R:	TGTGACATCTTCCATGCTTC			
Cop11		F:	TGACATTTGTCACTTGTCTTACTCT	(CT)19	198	52
	MT154318	R:	AGAGATCGTTCCCACAAAGA			
Cop12		F:	CTTGCCATTGAGTCAGCTTG	(CT)18	155	52
	MT154319	R:	GTGAAGGCTTTCGAGTACGG			
Cop13		F:	AATGAGGAGGAGTTGGGTTT	(GT)16	113	54
	MT154320	R:	ACTTTGCAAACTGCTTTATTCC			
Cop14		F:	GACGTTGTATGGAGCCCACT	(CT)8	184	55
	MT154321	R:	TTGCTAGGGGAATCATGAGG			
Cop15		F:	CAGGCCAGCATCACAAGAT	(ATAA)5	250	55
	MT154322	R:	CCCACGACAAAAACAGTGG			
Cop16		F:	GGGTTTGAGCCTTTCATTCTC	(TTAA)4... (TTAA)4	213	55
	MT154323	R:	AGTGGACTTCCCCTCATGG			
Cop17		F:	ACTCGGACTCTCCACTCTCG	(AGA)9	157	56
	MT154324	R:	TCTTCCCCAAGCCTCATAGA			

Figure 2 shows the agarose gel photographed under ultraviolet light with the amplification profile of one of the constructed primers. The annealing temperatures that presented the best amplification patterns were between 52 and 59 ºC, as reported in Table 1.

Studies conducted with different species of Arecaceae have demonstrated the occurrence of a range of annealing temperature amplitudes and variation of these temperatures in relation to other genera of the same family. Laindorf et al. (2019), studying *Syagrus romanzoffiana* (Arecaceae), reported that the annealing temperature of 51 ºC was efficient for all markers tested. Bacon et al. (2011) built primers for

Pritchardia martii (Gaudich.) H. Wendl. with annealing temperatures ranging from 49 a 58 ºC, while Barbosa and Almeida (2018), working with *Syagrus coronata* (Martius) Beccari, selected temperatures between 58 to 60 ºC. This variation is probably because different genera respond differently to temperature variation. In some genera, higher temperatures allow nonspecific sequence reduction and target sequence optimization, while the opposite is true in others. In the present study, we observed that the lower temperatures favored the amplification of the target sequence and the minimization of nonspecific sequences.

Research has demonstrated the ability of microsatellite transfer between species of the same genus. Neiva et al. (2016), using microsatellites developed for *Acrocomia aculeata* (Jacq.) Lodd. ex Mart., obtained high transferability rates for the species *Acrocomia emensis* (Toledo) Lorenzi. Ramos et al. (2012) found similar results with primers developed for *Astrocaryum aculeatum* Meyer, verifying the transfer efficiency of primers built for other Arecaceae species. Therefore, the primers designed in the present study can be used in different *Copernicia* species, supporting future research. Specifically in the case of *Copernicia prunifera*, the primers can be helpful in population genetics studies, since there is a demand to quantify the genetic diversity of the distribution range of this species. Thus, we recommend further research to create a proposal to identify genotypes for *ex situ* conservation in germplasm banks and to support strategies to develop productive planted forests (Santos et al. 2021). This will be an alternative to preserve the global genetic diversity and evolutionary potential (Costa et al. 2022).

Conclusion

The primers amplified were efficient for the tested DNA, with Cop02, Cop07, Cop10 and Cop15 presenting the best amplification results. These primers can be helpful in further research, enabling the development of management practices and conservation of the species in the areas of occurrence. Besides this, the obtained primers can be used in studies with other species of the genus *Copernicia*.

Declarations

Conflict of interest

The authors declare they have no conflict of interest.

Contributions

LGP, FAV and CGF conceived the study. LGP, KPTC, CGF, FAV and AGN were responsible for data collection. LGP and FAV wrote the original draft. LGP, KPTC and FAV formatted the graphical presentation of the data, reviewed and edited the manuscript. LGP, CGF and FAV were responsible for project administration.

Ethical approval

Not applicable.

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Figures

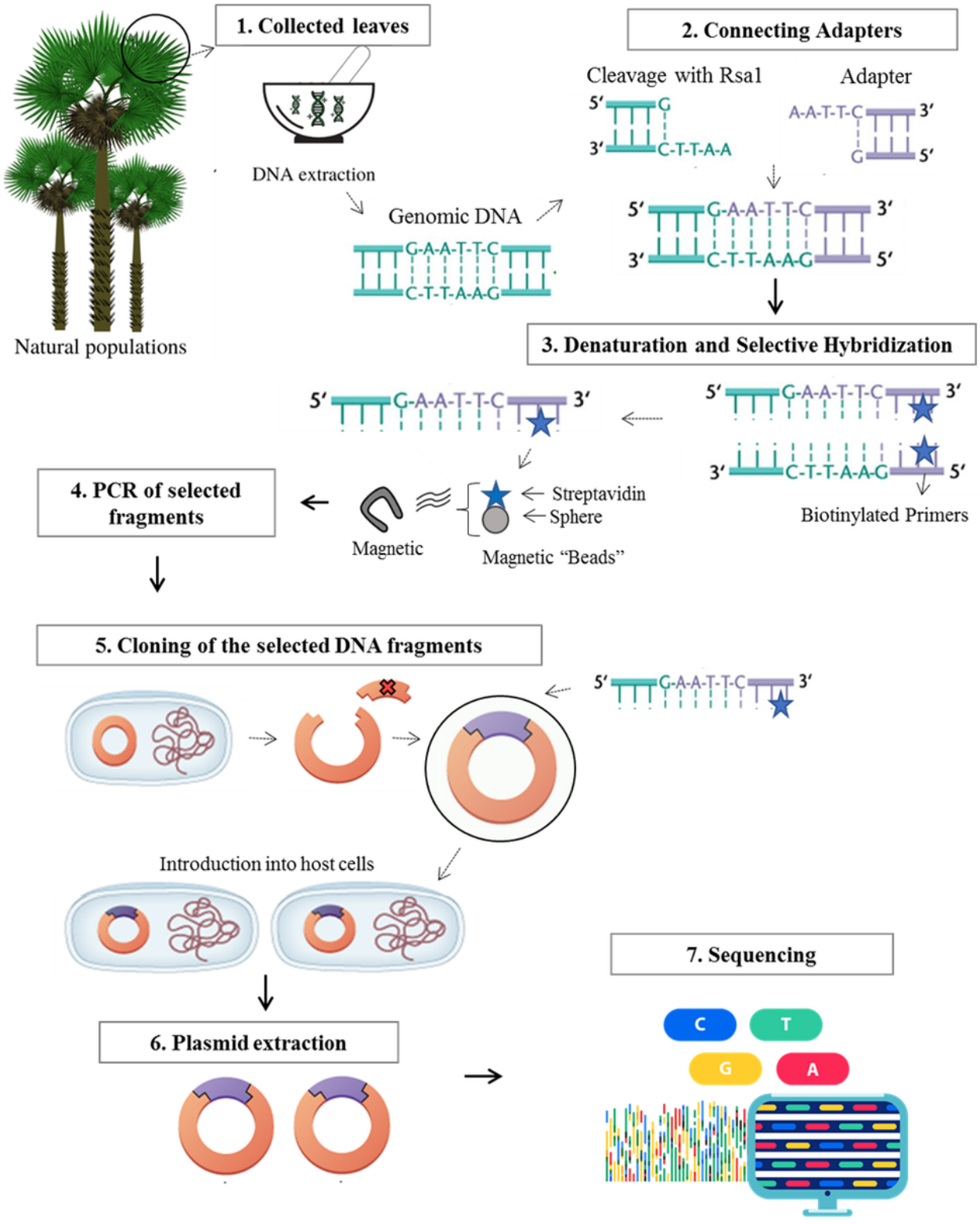

Figure 1

Graphical scheme illustrating the genomic library construction method

Source Klug et al. (2010), modified

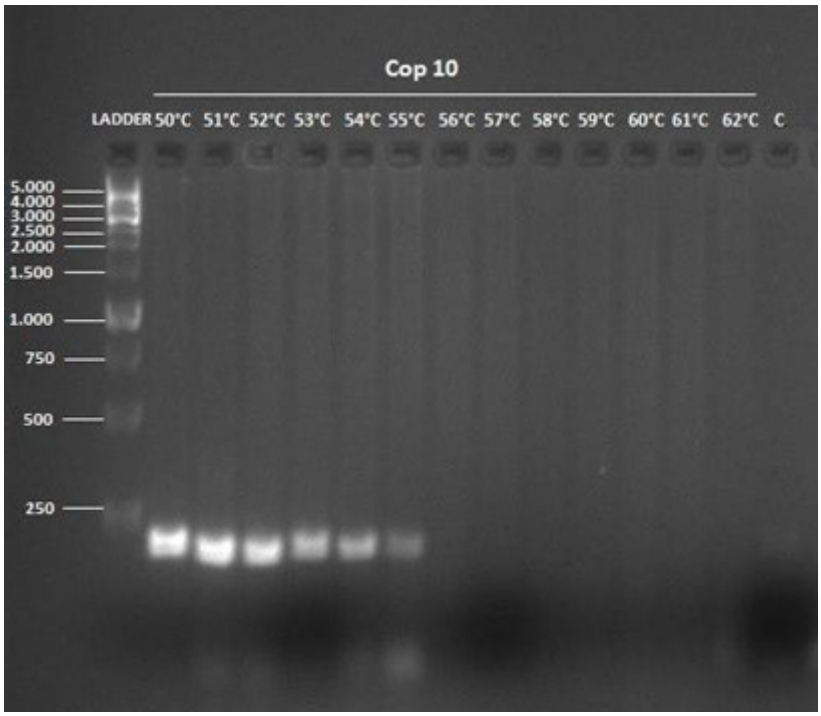

Figure 2

Fragment pattern resulting from amplification of Cop10 primer from *Copernicia prunifera*. The scale on the left indicates the number of base pairs using the 1 kb marker