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ISSR Primer Selection for Genetic Variability Analyses with Jaborandi (*Pilocarpus microphyllus* Stapf ex Wardlew., Rutaceae)

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Abstract

Pilocarpus microphyllus Stapf ex Wardlew. (jaborandi) is native to the North and Northeast regions of Brazil. Intensive exploitation for the extraction of pilocarpine, a valuable alkaloid for the pharmaceutical industry, has killed off or brought about a loss of vigor of naturally occurring plant populations. As a result, the species is officially listed as an endangered species of the Brazilian flora. Genetic diversity is required for the adaptation of populations to environmental changes, and maintaining it is a central objective for biological conservation. ISSRs are dominant markers widely used in genetic diversity studies of endangered species, allowing for the identification of genotypes and cultivars, as well as helping in phylogenetic studies based on DNA fingerprinting. This study presents an ISSR primer selection for genetic structure analyses of natural populations and cultivated collections of *P. microphyllus*.

Keywords: Jaborandi; Pilocarpine; Selection of markers

Introduction

The Rutaceae family has a pantropical distribution and includes approximately 161 genera and 2,070 species [1]. In Brazil, 33 genera and 193 species have been recorded, and among these there are 14 species known of *Pilocarpus Vahl* (jaborandi), of which 11 are endemic to Brazil [2]. *Pilocarpus microphyllus Stapf* ex Wardlew. is native but not endemic, occurring in the north and northeast of Brazil, more specifically in eastern Pará, western and northern Maranhão and northern Piauí. It is a shrub growing in open forests, and frequent in the rocky outcrops known as "canga". In forest interiors, the species can be found in areas with high light incidence [3].

Pilocarpus microphyllus contains a significant amount of secondary metabolites with diverse pharmacological properties, and specially stimulants of the secretive and diuretic systems [4]. Among these, the alkaloid pilocarpine stands out, being known for stimulating salivation and transpiration and for its use in the composition of eye drops [4]. The intensive collection of wild *P. microphyllus* populations has caused widespread loss of populations and mortality or loss of vigor in those that remain, reducing the size of the plants and their leaves [5]. Consequently, the species was officially included in the list of the endangered species of the Brazilian native flora [6].

Successful adaptation of plant populations to environmental changes depends on their genetic diversity, and maintaining this diversity has a central role in biological conservation programs [7]. There are several types of molecular markers that can easily detect genetic diversity levels within natural populations and cultivated materials [8]. ISSR markers, developed by ZIETKIEWICZ et al. [9], are PCR based technique which is easy, quick, simple and economical. The advantages of ISSRS over other markers like AFLPs, RAPDs and more specific SSRs are reproducible due to their better stringency (high annealing temperature), require no gene sequence information and no prior genetic studies are required for these analyses [10,11]. ISSR markers have been successfully used for the assessment of genetic diversity and gene pool origin in Amw plants [12-16].

An important step prior to the analysis of the genetic diversity of a population is the development of appropriate markers in order to avoid those that either fail to amplify or generate only few fragments and low polymorphism levels. It is known that the nucleotide sequence of ISSR primers recognize sequences arbitrarily distributed in the genome and the preliminary stage of primer selection and testing is essential prior to using them to evaluate all the samples of the studied population.

The aim of this study was to select suitable ISSR marker applicable to the evaluation of natural populations and germplasm resources of *P. microphyllus*, in order to estimate the genetic diversity of such an endangered and economically important plant species [17].

Materials and Methods

For marker selection eight individuals of P. microphyllus were used, two from each sampled population in Piauí (Parnaíba and Luzilandia), Maranhão (Mata Roma) and Pará (Parauapebas) (Table 1 and Figure 1). Young leaves were collected from each individual and immediately stored in 2% NaCl-saturated CTAB buffer [18].

Genomic DNA was extracted from leaf fragments in 2% CTAB as described by Doyle and Doyle [17], with some modifications. Approximately 200 µg of young leaves were macerated with extraction buffer [2% CTAB, Tris-HCl 0.1 mM (pH 8.0), 20 mM EDTA (pH

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Population	Collection Site	Voucher	Coordinates
TL	Tabuleiros Litorâneos, Parnaíba, Piauí	Rocha, J. A. 258	3°6'S, 41°47'W
LU	Assentamento Cutias, Luzilândia, Piauí	Rocha, J. A. 255	3°41'S, 42°28'W
MR	Barra dos Macacos, Mata Roma, Maranhão	Silva, F.M.M. 257	3°31'S, 43°6'W
PA	Flona Carajás, Parauapebas, Pará	Silva, F.M.M. 253	6°2'S, 50°12'W

Table 1: Identification and location of sampled populations of *Pilocarpus microphyllus*.

8.0), 1.4 M NaCl and 2% β-mercaptoethanol]. After the maceration, the material was transferred to 1.5 mL polypropylene microtubes and incubated in a water bath at 60°C for 20 min. After cooling the extract, 800 µL of a chloroform-isoamyl alcohol (24:1, v/v) solution were added and the mix was shaken for one hour. The homogenized contents of the tubes were centrifuged for 10 min at 13,000 rpm. The resulting supernatant (~500 µL) was transferred to a new tube, and 300 µL of isopropanol (0.6 volumes) were added, being carefully mixed by inversion and stored overnight in a freezer (−20°C). Then, the tubes were centrifuged at 13,000 rpm for 5 min, concentrating the precipitated DNA. The resulting pellet was washed with 1 mL of 70% ethanol and centrifuged for 5 min at 13,000 rpm (this process was repeated three times) and finally it was left to dry overnight at room temperature. The DNA obtained was re-suspended in 100 µL in TE buffer [10 mM Tris-HC1 (pH 8.0) and 0.1 mM EDTA] for 48 h or until the pellet was homogenized in the solution.

A total of 48 primers [19,20] were tested in eight individuals of P. *microphyllus* ('TL1', 'TL2', MR1', 'MR2', 'PA1', ´PA2', 'LU1', 'LU2'). The PCR procedures were conducted in a final volume of 10 µL containing: enzyme buffer 1×, 1 mM dNTPs, 2.5 mM $MgCl_2$, 50 µM primer, 0.8 U of Taq polymerase enzyme and 25 ng of genomic DNA [19]. The PCR reactions were carried out in a Techne TC-412 thermocycler, with a program consisting of initial denaturing for 4 min at 94°C, followed by 35 cycles of (1) denaturing for 30 s at 94°C, (2) annealing of the primer for 45 s at 50.4-52.0°C (depending on the primer used; Table 2) and extension for 2 min at 72°C; the final extension occurred for 7 min at 72°C. The products of each reaction were separated in a 1.8% agarose gel electrophoresis in 0.5x TBE buffer, stained with ethidium bromide (1.5 µL/100 mL of gel), under 100 V and 159 mA for 3.5h, and then photographed under ultraviolet light.

For the analysis of the amplified fragments, a binary matrix was constructed by the visual criteria of presence (1) and absence (0) of every molecular weight observed with their respective primers. The same weight (1) was given for all accounted fragments, independent of their fluorescence intensity on the gel. Indistinct fragments, with poor visualization, were counted as missing data, remaining neutral in the data analysis.

The discriminating power of each primer was determined by means of three parameters: (1) PIC (polymorphism information content); (2) MI (marker index): and (3) RP (resolving power). The PIC value for each amplified fragment was calculated as PICi = 2 fi (1 – fi), PICi being the individual PIC of marker i, fi the frequency of fragments present in the marker per accession and 1 – fi the frequency of absent fragments [21]. Then, the mean PIC values were obtained for each primer. The MI was obtained using the following formula: MI = PIC × n × np/(np + nm), n being the average number of fragments per primer, np the number of polymorphic fragments and nm the number of monomorphic fragments [22]. The RP of each primer was calculated as follows: RP = Σ Ib, where Ib is the level of information of each fragment. The Ib can be represented as an interval of 0-1 by the following formula: Ib = 1 – (2 × |0.5 – fi|) [23]. Additionally, the correlation between the indices was tested using the Pearson coefficient with aid of the software BioEstat 5.0 [24].

The genetic distance between accessions was estimated using the Dice coefficient [25] using the program DARWIN 5.0.158. Subsequently, the dissimilarity matrices obtained were used to generate a phenogram in the same program, using the neighbor-joining algorithm (30.000 bootstrap replications).

Results and Discussion

Of the 48 primers tested, 34 were informative, generating 336 bands or fragments, ranging from 100-1000 bp in size, of which 226 (67%) were polymorphic (Table 2). The total number of amplified fragments (TNF) in each ISSR reaction ranged from five (UBC 847, UBC 887, UBC 888 and UBC 890) to 19 (UBC 825), with an average of 9.8 bands per primer (Table 2). The polymorphic fragments were categorized according to their respective intervals: unique fragments ($0 < x \leq 0.2$), rare fragments ($0.2 < x \leq 0.4$), frequent fragments ($0.4 < x \leq 0.6$), common fragments ($0.6 < x \leq 0.8$) and shared fragments ($0.8 < x < 1.0$) [20] (Table 2 and Figure 2).

Four genotypes (TL1, TL2, LU1 and LU2) presented unique fragments (data not shown), which were amplified with the primers

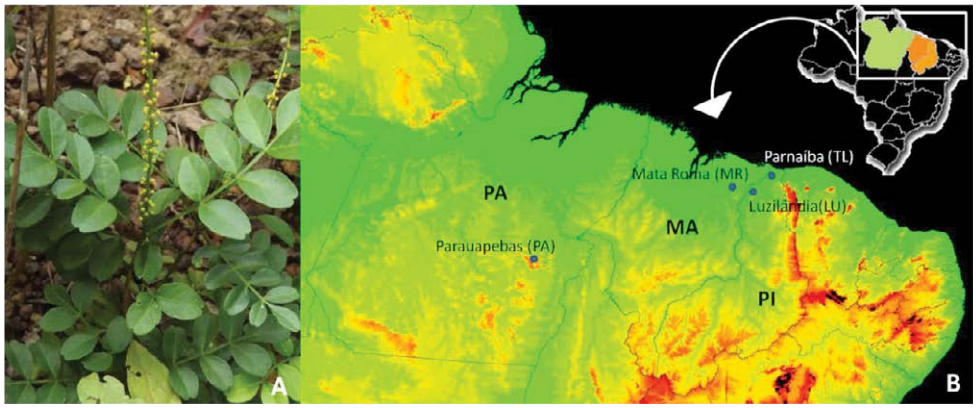

Figure 1: *Pilocarpus microphyllus* in its natural habitat (A). Collection site of the sampled populations of *P. microphyllus* (B) (program DIVA-GIS 7.5; HIJMANS et al. 2001).

Primer UBC [repetitions][a]	Ta(°C)[b]	TNF[c]	NPF[d]	NMF[e]	fp(%)[f]	NUF[g]	NRF[h]	NFF[i]	NCF[j]	NSF[K]	MPICL	MIM	RPN
807 [$(AG)_8$T]	50.4	14	13	1	92	0	4	2	6	1	0.388	3.56	8.78
809 [$(AG)_8$G]	52.8	8	5	3	62	0	0	2	3	0	0.300	1.85	4.25
810 [$(GA)_8$T]	50.4	16	11	5	68	0	1	2	7	1	0.280	1.90	6.57
811 [$(GA)_8$C]	52.8	8	3	5	37	0	1	1	1	0	0.160	0.59	2.00
812 [$(GA)_8$A]	50.4	12	7	5	58	0	0	1	1	5	0.164	0.94	2.75
813 [$(CT)_8$T]	50.4	11	10	1	90	1	1	2	2	4	0.338	3.04	5.66
814 [$(CT)_8$A]	50.4	7	5	2	71	0	1	2	1	1	0.309	2.18	3.66
815 [$(CT)_8$G]	52.0	6	6	0	100	0	2	2	1	1	0.408	4.03	3.71
816 [$(CA)_8$T]	50.4	16	11	5	68	0	1	1	5	4	0.244	1.66	5.42
818 [$(CA)_8$G]	52.8	7	4	3	57	0	1	0	0	3	0.182	1.03	1.66
821 [$(GT)_8$T]	50.4	12	10	2	83	0	5	2	3	0	0.388	3.19	7.75
824 [$(TC)_8$G]	52.0	14	13	1	92	1	2	0	9	1	0.401	3.68	9.42
825 [$(AC)_8$T]	50.4	19	10	9	52	0	1	1	5	3	0.179	0.93	4.75
826 [$(AC)_8$C]	52.8	6	5	1	83	0	0	2	3	0	0.385	3.17	4.00
827 [$(AC)_8$G]	52.8	7	2	5	28	0	0	1	1	0	0.125	0.35	1.50
828 [$(TG)_8$A]	52.8	12	5	7	41	0	1	1	0	3	0.136	0.56	2.28
830 [$(TG)_8$G]	52.8	11	11	0	100	0	0	2	9	0	0.423	4.18	7.25
831 [$(AT)_8$YA]	50.4	9	6	3	66	0	1	0	3	2	0.236	1.55	3.00
843 [$(CT)_8$RA]	52.0	8	8	0	100	1	6	0	1	0	0.425	4.20	5.25
847 [$(CA)_8$RC]	54.0	5	4	1	80	1	2	0	1	0	0.337	2.66	3.00
853 [$(TC)_8$RT]	52.0	14	14	0	100	2	3	3	3	3	0.367	3.63	7.42
855 [$(AC)_8$YT]	52.0	11	1	10	9	0	0	1	0	0	0.045	0.04	1.00
856 [$(AC)_8$YA]	52.0	17	9	8	52	0	1	3	3	2	0.211	1.10	5.42
858 [$(TG)_8$RT]	52.0	9	3	6	33	0	1	1	0	1	0.131	0.43	2.00
860 [$(TG)_8$RA]	52.0	9	2	7	22	0	1	0	0	1	0.065	0.14	0.75
873 [$(GACA)_4$]	52.0	12	11	1	91	0	1	2	8	0	0.380	3.44	7.00
884 [HBH$(AG)_7$]	52.0	8	2	6	25	0	0	1	2	0	0.109	0.27	1.50
885 [BHB$(GA)_7$]	52.0	10	6	4	60	0	1	1	1	3	0.209	1.24	3.25
886 [VDV$(CT)_7$]	52.0	8	8	0	100	0	0	2	6	0	0.428	4.23	5.14
887 [DVD$(TC)_7$]	52.0	5	4	1	80	0	2	1	1	0	0.362	2.86	3.00
888 [BDB$(CA)_7$]	52.0	5	3	2	60	0	0	0	3	0	0.262	1.55	2.00
889 [DBD$(AC)_7$]	52.0	7	3	4	42	0	0	2	1	0	0.174	0.73	2.25
890 [VHV$(GT)_7$]	52.0	5	4	1	80	0	0	1	2	1	0.310	2.45	2.28
891 [HVH$(GT)_7$]	52.0	8	7	1	87	0	0	0	5	2	0.316	2.73	3.42
MEAN	-	9.8	6.6	3.2	67	0.17	1.17	1.23	2.85	1.23	0.270	2.06	4.09
TOTAL	-	336	226	110	67	6	40	42	97	42	-	-	-

Table 2. Primers tested for generating polymorphic ISSR markers among *Pilocarpus microphyllus* genotypes, showing the respective levels of polymorphism and information generated by each primer.
[a]Degenerate bases used: Y (C or T); [b]annealing temperature; [c]total number of fragments; [d]number of polymorphic fragments; [e]number of monomorphic fragments; [f]frequency of polymorphic fragments; [g]number of unique fragments; [h]number of rare fragments; [i]number of frequent fragments; [j] number of common fragments; [k]number of shared fragments; [l] mean PIC; [m]marker index, [n] resolving power.

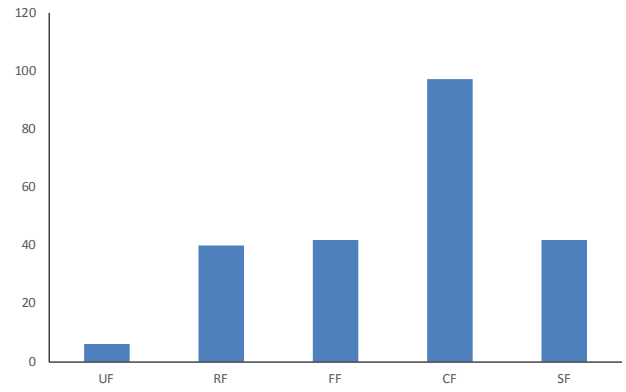

Figure 2: Distribution of polymorphic fragments for the tested ISSR primers in *Pilocarpus microphyllus*. Unique fragments (UF), rare fragments (RF), frequent fragments (FF), common fragments (CF) and shared fragments (SF).

UBC-813, UBC-824, UBC-843, UBC-847 and UBC-853. The primer UBC-853 generated the highest number of unique fragments (2 out of 6; Table 2). A total of 40 rare fragments were amplified, comprising 11.9% of the total number of amplified fragments (TNF) and 17.6% of the polymorphic fragments, with an average of 1.17 per primer. The highest number of rare fragments (six) was observed for the primer UBC-843 (Table 2). The number of frequent fragments was 42 (12.5% of the TNF and 18.5% of the recorded polymorphism), with an average of 1.23 fragments per primer, and a higher value (three) for the primers UBC-853 and UBC-856 (Table 2). Ninety-seven common fragments were amplified, comprising 28.8% of the TNF and 42.9% of the number of polymorphic fragments (NPF), with an average of 2.85 fragments per primer, and the maximum of nine fragments generated by the primers UBC-824 and UBC-830 (Table 2). The shared fragments were visualized 42 times, showing the same values observed for the frequent fragments. In turn, the primer UBC-812 generated the highest number of shared fragments (five; Table 2).

The mean PIC values varied between 0.045 (UBC-855) and 0.428 (UBC-886), with an average of 0.270 (Table 2). The average MI was 2.06, varying between 0.04 (UBC-855) and 4.23 (UBC-886) (Table 2). A strong positive correlation was observed between PIC and MI values ($r=0.979$; $p<0.0001$). The average RP was 4.09 and values ranged from 1.00 (UBC-855) to 9.42 (UBC-824). There was also a strong positive correlation between RP and PIC ($r=0.724$; $p<0.0001$), similar to that observed between MI and RP ($r=0.722$; $p<0.0001$).

Of the 48 primers tested in P. *microphyllus*, 26 were polymorphic. Thus, more than 50% generated polymorphic fragments, eight were completely monomorphic (UBC-811, UBC-827, UBC-828, UBC-855, UBC-858, UBC-860, UBC-884 and UBC-889), and 14 showed no amplification (UBC-820, UBC-829, UBC-832, UBC-833, UBC-837, UBC-838, UBC-839, UBC-846, UBC-849, UBC-852, UBC-859, UBC-878, UBC-882 and UBC-883).

The primers UBC-807, UBC-810, UBC-812, UBC-813, UBC-816, UBC-821, UBC-824, UBC-825, UBC-830, UBC-853, UBC-856, UBC-873, and UBC-885 were the ones that showed the highest number of amplified fragments, with at least ten fragments and a minimum proportion of 50% of polymorphism. The primer UBC-873 was the best one, generating 12 bands, 91% of which were polymorphic, corroborating results obtained by other authors for other plant species [26-29].

The number of fragments by ISSR primer may vary considerably, depending on the species. Some authors [3,30-33], observed a total number of bands per primer lower than 10 (about five) with the primers UBC-807, UBC-810, UBC-812, UBC-813, and UBC-856, which were relatively uninformative in their respective analyses. However, other authors, such as NAN et al. [27,34-37] reported an abundant generation of bands for these same primers, showing an average of 20 fragments per primer. This discussion shows the importance of knowing the profile of bands of a certain primer before the beginning of a study of the genetic variability of a given species.

The PIC value is another criterion that should be taken into account in the selection of primers. Average PIC values similar to the obtained for P. *microphyllus* (0.270) have been described for several other species (e.g. [29,38,39]). The RP and MI can also be used to infer differences between genotypes. Therefore, the average values of these parameters (4.09 and 2.06, respectively) are also indexes of discrimination that have been widely used to measure the information content generated by molecular markers in various plant species, such as sesame (*Sesamum indicum* L.) [25] castor (*Ricinus communis* L.) [20,40], potato (*Solanum tuberosum* L.) [23], physic nut (*Jatropha curcas* L.) [41] and barley (*Hordeum vulgare* L.) [22].

Of the 48 ISSR tested primers, 34 are suitable for studies of genetic diversity in P. microphyllus, especially the following thirteen: UBC-807, UBC-810, UBC-812, UBC-813, UBC-816, UBC-821, UBC-824, UBC-825, UBC-830, UBC-853, UBC-856, UBC-873, and UBC-885. This cited primers evidenced the highest levels of polymorphism among the selected individuals, with an average amplification of 14 fragments per primer, of which 11 were polymorphic, i.e. 78.5% of polymorphism (Figure 3).

Figure 3 shows the phenetic tree dissimilarity for the eight accessions. The high number of replications used (30.000), produced basal branches with high support values (98,90,89,80 and 63). Also observed the formation of three groups being the first (TL-1, LU-1 and LU-2), formed by natural accessions present in the state of Piaui, and TL-1, cultivated individual also present in Piauí. The second group (MR-1 and MR-2) that are present in natural accessions Maranhão,

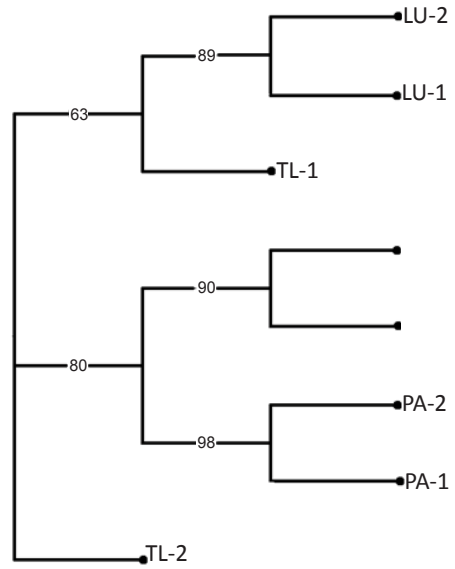

Figure 3: Phenogram Constructed of simple coefficients ISSR data , showing the structure of eight accessions of *P. microphylus*

and the third group (PA-1 and PA-2) individuals collected in Pará. At the same time there is the proximity the second and third groups, tending to form one group. And the TL-2 access, appears as an external individual to groups formed, may be explained by the TL accesses, are grown in other states and not natural populations.

Conclusion

According to the indices analyzed, such as PIC, MI, and RP, the 34 primers indicated herein are informative for genetic diversity analysis in the studied species, and can be used in future studies aimed at the diversity characterization and conservation of natural populations. As there was a significant positive correlation among all the tested indices, any of the three can be taken into account in discriminating more informative polymorphic markers for future studies of genetic diversity among P. *microphyllus* natural populations.

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