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EVALUATION OF MICROSATELLITE ALLELIC PATTERNS TO DNA FINGERPRINTING IN RUBBER TREE CLONES

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Abstract

The rubber tree (*Hevea brasiliensis*) is native to the Amazon region, and it is widely exploited due to natural rubber produced from latex. There are many clonal varieties, without certification tests. In order to determine a genetic certification, 15 clones were genotyped to identify their genetic pattern. Ten microsatellites were used to determine a subset of alleles exclusive for each genetic profile. The genetic estimates obtained were: number of alleles per locus (*N*), expected (H_E) and observed (H_O) heterozygosity, Polymorphic Information Content (*PIC*) and Discriminatory Power (*DP*). The number of alleles (*N*) ranged from five to 14, with an average of 9.2. The H_E mean (0.80) was higher than H_O (0.60), indicating a selection for homozygotes. The locus informativeness was verified with *PIC* (0.77) and *DP* (0.90) means showing high polymorphism. The dendrogram represented the formation of three groups related to geographical origin. Clone MDF 180 presented the highest genetic divergence. Two genic pools represented the genetic composition of genotypes. Based on allelic profiles, a set of two microsatellites (A2365 and A2368) was able to distinguish all examined clones. The genetic certification using microsatellite fingerprinting proved to be an alternative to morphological traits.

Keywords: Genetic Certification. SSR. Variety Identification.

1. Introduction

The rubber tree [*Hevea brasiliensis* (Will. Ex Adr. De Juss.) Muell. Arg.] is a plant native to Amazon (Gonçalves et al. 1989). Natural rubber has unique physico-chemical characteristics, making it a strategic and irreplaceable material for more than 50 thousand industrialized products (Jain et al. 2009; Rippel and Bragança 2009).

The natural rubber of *H. brasiliensis* has made this species the most used for commercial exploration, as this culture has desirable levels of quality and quantity of rubber polymers in the composition of latex (Gonçalves and Fontes 2009). However, phytosanitary problems caused by the fungus *Microcyclus ulei* (Henn.) Arx brought many damages to the crop. In an attempt to overcome productivity problems, studies were carried out to select genotypes that had promising characteristics for genetic improvement programs, such as resistance to climatic stress and diseases. For this purpose, many plants have been removed from their naturally occurring site to compose *ex-situ* collections, serving as sources of genetic resources for breeding programs (Souza et al. 2015).

Ex-situ collections have redundancies due to duplicate accessions (Shan et al. 2007). These collections have operational costs for their maintenance, making it crucial to correctly characterize their accessions,

both to differentiate and exclude duplicates safely and to identify genotypes. Molecular characterization is a tool capable of optimizing the set of accessions in a collection, grouping a sufficient number of genotypes that clearly represent the genetic diversity of the analyzed sample (Souza et al. 2015).

To access genetic diversity within germplasm banks it is necessary to collect, evaluate, characterize, conserve and document to assist breeding programs with the germplasm necessary for the development of new varieties (Gimenes et al. 2000). The evaluation and characterization of germplasm are essential activities to adequately conserve genetic resources. These activities, in addition to providing better knowledge of germplasm available, generate information that helps the collection management, facilitate researchers' access to new gene pools and increase the efficient use of materials.

Molecular markers have been successfully used in rubber tree. Microsatellites have been widely used, both with clonal varieties and for natural rubber tree populations (Saha et al. 2005; Le Guen et al. 2009; Gouvêa et al. 2010; Souza et al. 2015; Souza 2018). They are ideal markers for studies of genetic diversity, as they are highly polymorphic, multi-allelic and codominant (Kalia et al. 2011; Fortes et al. 2016).

Thus, the objective of this work was to perform the molecular identification of rubber tree clones for genetic certification.

2. Material and Methods

Plant material

Fifteen clones of *H. brasiliensis* were collected from Embrapa Acre (Rio Branco/AC) collection (Table 1). Genomic DNA was extracted from young leaflets according to the method described by Doyle and Doyle (1990). The DNA quantification was determined according to Qubit® 4.0 (Thermo Fisher Scientific Inc) fluorometric quantifications system.

Table 1. Accessions of *H. brasiliensis* per geographical and genitor cross origin.

	Genotype	Country Source	Cross origin
1	CD 1174	Guatemala	Avros 1581 x MDF 180
2	FDR 5802	Brazil	Harbel 67 x CD 47
3	CDC 312	Guatemala	Avros 308 x MDX 40
4	CDC 56	Guatemala	MDX 91 x RRIM 614
5	FX 3864	Brazil	PB 86 x FB 38
6	PMB 01	Brazil	Primary clone
7	FDR 5240	Brazil	Harbel 68 x TU 42-525
8	FDR 5597	Brazil	Harbel 68 x TU 42-525
9	FDR 5788	Brazil	Harbel 8 x MDF180
10	MDX 624	Brazil	Avros 1581 x MDF*
11	FDR 5665	Brazil	Harbel 62 x MDX 25
12	MDF 180	Peru	Primary clone
13	FDR 4575	Brazil	Harbel 8 x IAN 3893
14	MDX 607	Brazil	Avros 1581 x MDF*
15	RRIM600	Malaysia	TJIR 1 x PB 86

* = unknown accession.

Analysis of microsatellites

Ten microsatellites developed by Le Guen et al. (2009) (Table 2) were used. Amplification reactions were performed as described by Souza et al. (2015). The amplification products were genotyped in an automatic AB 3500xL sequencer (Applied Biosystems). The samples were multiplexed, organized based on the size of the fragments and the type of fluorophore (Souza 2018). The readings of the samples were performed using the GeneScan-600 LIZ® (Thermo Fisher Scientific Inc) standard in the GeneMarker program version 2.7.4 (SoftGenetics, State College, Pennsylvania, USA). Genotyping failures were repeated following the genotyping protocol on polyacrylamide gels (Creste et al. 2001).

Table 2. Description of the ten microsatellite loci, including the primer sequence, repetitive motif and allelic amplitude.

Loci	Sequence F (5′-3′)	Sequence R (5′-3′)	Repetitive motif	Amplitude allelic (bp)
A2365	CACGACGTTGTAAAACGACGCTATCC ATCAGGCAA	CTCCTTCTTTCTCCCC	CT	190–228
A2368	CTATTCTACATTCTCCATGTC	CACGACGTTGTAAAACGACCTTCTTA TTTTACTGGGCT	CT	229–271
A2389	CTTTCTTTTGGTCTTTCTC	CACGACGTTGTAAAACGACGCAACT CATCCACCAC	CT	188–228
A2406	GTCCACAGAAATAAAACTCA	CACGACGTTGTAAAACGACAGCCAT TTTCTCACCTC	CT	113–171
A2413	ATCCAAACCTGCTCATAC	CACGACGTTGTAAAACGACGACCCC TATCCAAAAGA	CT	286–322
A2684	CACGACGTTGTAAAACGACTATGCG AACAAGGAAAG	ACAGGGATTTACACATACAA	CT	258–300
A2736	CACGACGTTGTAAAACGACGCAACC TGATGAATAAAGA	AAATGAGAAACAAGAAGACC	CT	102–148
BAC55 B02	GATGCCCTTGATTATGTT	CACGACGTTGTAAAACGACACTTAAT GGGCTTTTCC	CT	153–177
TA2163	ATGCAACAGAGTAGGAGA	CACGACGTTGTAAAACGACTCAAAG CAAATGAAGTG	(CT) (CA)	194–242
TAs2558	ACTCAATACAAAGGAAGGT	CACGACGTTGTAAAACGACAGTTCC AAAGGTCGTG	(CT) (CA)	218–256

The parameters to select the loci to fingerprint analysis considered: A – absence of “stutter” bands; B - markers with high discriminatory power and C - minimum number of markers that can discriminate varieties.

Statistical analysis

Microsatellites were characterized in terms of the number of alleles per locus (*N*), expected (H_E) and observed (H_O) heterozygosity, Polymorphic Information Content (*PIC*) for each locus and the average of these estimates for all loci, using the following equations, present in the Tools for Population Genetic Analyzes - TFPGA version 1.3 software (Miller 1997). To compare the efficiency of the markers for identifying the varieties, the Discriminatory Power (*DP*) was estimated for each locus (Tessier et al. 1999).

The genetic distance analyzes were calculated using Rogers' modified distance (Wright 1978), based on the distance matrix generated by the TFPGA program version 1.3 (Miller 1997), using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) for the dendrogram construction in the MEGAX software (Kumar et al. 2018).

The number of groups (K) was obtained using the STRUCTURE version 2.3.4 program (Pritchard et al. 2000), based on Bayesian statistics. These groups are made up of individuals who share the same gene pool. The program was run with K ranging from K = 1 to K = 10. Twenty independent simulations were made for each K, using the “admixture” model, independent allele frequencies, 10,000 burn-in and 100,000 Monte Carlo simulations of Markov Chains (MCMC). The determination of the most likely K number was performed using the Structure Harvester v. 0.6.9 (Earl and Vonholdt 2012), based on ΔK values according to Evanno et al. (2005). The allocation of accessions within the groups was carried out according to the probability of each individual belonging to each of the groups.

3. Results and Discussion

All the ten tested microsatellites were polymorphic (Table 3). The number of alleles per locus (*N*) ranged from five (BAC55-B02) to 14 (TA2163), with an average of 9.2 (Table 3). Other clones presented similar estimates when genotyped also with microsatellites (Silva 2019).

Table 3. Characterization of ten microsatellites in 15 clones of *Hevea brasiliensis* with the genetic parameters: number of alleles per locus (*N*); rare alleles (*A*) expected heterozygosity (H_E); observed heterozygosity (H_O); polymorphic information content (*PIC*) and discriminatory power (*DP*).

Locus	*N*	*A* (<0,05%)	H_E	H_O	*PIC*	*DP*
A2365	9	3	0.83	0.80	0.80	0.96
A2368	9	5	0.73	0.53	0.71	0.84
A2389	8	5	0.59	0.46	0.57	0.78
A2406	9	3	0.88	0.26	0.85	0.93
A2413	9	1	0.87	0.73	0.84	0.96
A2684	7	2	0.62	0.40	0.60	0.83
A2736	9	3	0.83	0.60	0.80	0.92
BAC55-B02	5	0	0.76	0.73	0.74	0.89
TA2163	14	6	0.93	0.60	0.90	0.97
TAs2558	13	8	0.88	0.86	0.86	0.97
Total	92	36	-	-	-	-
Mean	9.2	3.6	0.80	0.60	0.77	0.90

The expected heterozygosity (H_E) ranged from 0.59 (A2389) to 0.93 (TA2163), with an average of 0.80 (Table 3). The values of observed heterozygosity (H_O) varied between 0.26 (A2406) and 0.86 (TAs2558) with an average of 0.60. All loci had H_O values lower than H_E. This result indicates a deficit of heterozygotes. This genetic profile had also been reported in other studies (Le Guen et al. 2009; Souza et al. 2015; Souza 2018). *H. brasiliensis* has a mixed reproductive system and preferentially cross-pollinating with an inbreeding rate above 20% (Paiva 1992). The deficit in heterozygotes may be the result of the pressure for homozygotes exerted by a natural crossing of related plants due to the longevity of a perennial species with a long breeding cycle, as well as the controlled crossing to obtain cultivars (Laurance et al. 2004; Secco 2008). This fact is consistent with the origin of the analyzed genotypes, with only two primary clones (PMB 01 and MDF 180).

The primary clones presented rare alleles, except for PMB 01, which shared an allele with clone RRIM 600 at locus A2736 and with clones FX 3864 and RRIM 600 with locus BAC55-B02. It is important to note that these three individuals were collected close to Brazil.

The values of the polymorphic information content (*PIC*) ranged from 0.57 (A2389) to 0.90 (TA2163), with an average of 0.77 (Table 3). These values indicate that the microsatellites used in the study are highly informative. Botstein et al. (1980), defined those markers with *PIC* values greater than 0.5 are highly informative. In addition, *PIC* can be used to establish relationships between closely related materials, such as paternity or the probability that parents are heterozygous as well as to know the parents from the progeny genotype (Botstein et al. 1980). Perseguini et al. (2012) obtained an average of 0.59 (between 0.23 to 0.86), values slightly lower than the present study. This may be related to the EST-SSR marker used. These loci are more conserved and tend to access less polymorphism. On the other hand, they can be more transferable between species within the genus (Scott et al. 2000; Eujayl et al. 2001).

The set of microsatellites analyzed showed Discriminatory Power (*DP*) between 0.78 (locus A2389) to 0.97 (locus TA2163 and TAs2558), with an average of 0.93 (Table 3). *DP* estimates allow you to select loci that are most likely to differentiate between two individuals. Lower *DP* values (0.07 to 0.90, with an average of 0.40 per locus) were also detected with EST-SSRs (Perseguini et al. 2012).

Microsatellites A2365 and A2368 with *DP* 0.96 and 0.84, respectively, presented a unique allelic profile to identify the accessions. Locus A2365 discriminated nine individuals with distinct profiles and two shared profiles: one for individuals CDC 56, FDR 5597 and FDR 5788 and another for accessions MDF 180 and MDX 607. These genotypes were discriminated by locus A2368, which presented nine exclusive profiles and a common profile among six individuals (CDC 312, FDR 5240, FDR 5597, MDX 624, FDR 5665 and MDX 607) (Table 4).

Table 4. Genotype based on microsatellite fingerprints for locus A2365 e A2368.

Allele+ / Clones	193-196	194-198	194-230	196-196	196-198	196-212	196-230	198-198	198-212	198-213	202-219	248-255	250-250	250-255	250-257	255-255	255-260	255-269	255-274	257-269	267-270
CD 1174					+											+					
FDR 5802								+									+				
CDC 312						+							+								
CDC 56							+					+									
FX 3864									+										+		
PMB 01											+							+			
FDR 5240			+										+								
FDR 5597									+				+								
FDR 5788									+						+						
MDX 624	+												+								
FDR 5665										+			+								
MDF 180				+																	+
FDR 4575										+										+	
MDX 607				+									+								
RRIM 600		+												+							

Besse et al. (1993) identified 73 Wickham clones with minisatellites probes, but this analysis is laborious and evolves manipulation of radioactive phosphorous. The alleles accessed by microsatellites A2365 and A2368 were effective for varietal identification of the 15 clones tested and have methodological advantages for routine use, as safe security without radioactive residue. This microsatellite set can be widely applied in studies with rubber tree clones, allowing a direct, fast, low-cost analysis, capable of guaranteeing the correct discrimination and certification of this set of genotypes.

No redundant accessions were identified (Figure 1). The most divergent pair were MDX 624 vs FDR 5802 clones with a genetic distance of 0.85.

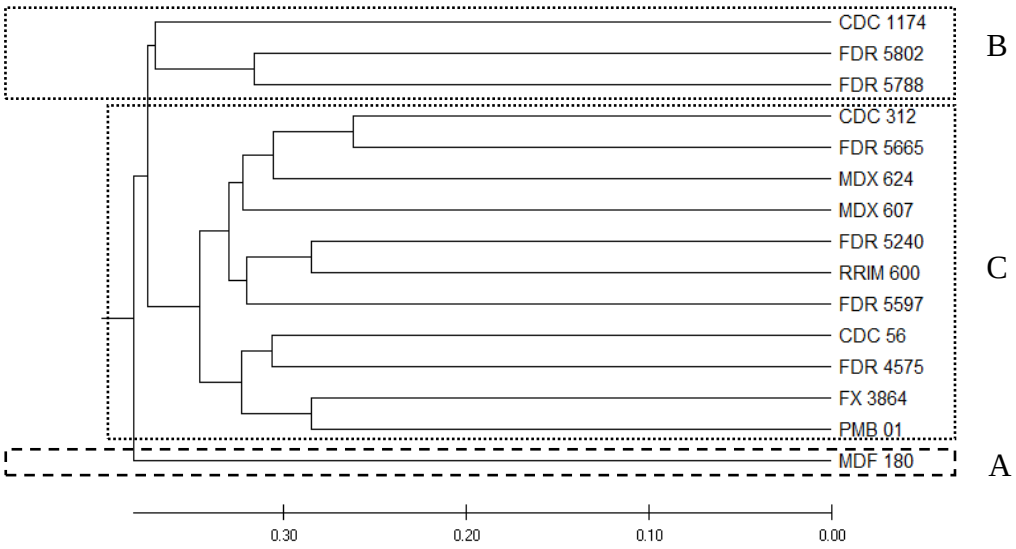

Figure 1. Dendrogram with 15 genotypes of *H. brasiliensis*, according to Rogers modified genetic distance for grouping in the UPGMA method using ten microsatellites. Dotted lines highlight the three formed groups (A, B and C).

The closest individuals were CDC 312 and FDR 5665 clones, which presented 0.52 of genetic distance. Their origins as germplasm are documented as Guatemala and Brazil, respectively. It can be explained by ancestry composition, because both clones have one parent from the same MDX line and consequently share a common genetic base.

Clone clusters were formed relating to the origin and parental (Figure 1). Group A formed only by clone MDF 180, Group B with varieties CDC 1174, FDR 5802 and FDR 5788 and Group C with clones CDC 312, FDR 5665, MDX 624, MDX 607, FDR 5240, RRIM 600, FDR 5597, CDC 56, FDR 4575, FX 3864 and PMB 01. When verifying the source and parental data, relationships were found that explained the groupings. Group A has its origins in Madre de Dios - Peru, one of the species' naturally occurring sites, and its clonal lineage is widely used in controlled crosses to obtain new varieties. Group B varieties originate in Brazil and Guatemala with parents of the Harbel and MDF clonal line, justifying the similarity of these genotypes. Group C individuals have origins in Brazil, Guatemala, and Malaysia, however individuals from Guatemala and Malaysia are products of plant crosses originating from a naturally occurring area, which corroborates their similarity (Table 1).

The similarity of individuals within the group may be related to the restricted genetic base formed by plants collected by Henry Wickham in Pará, which gave rise to several clonal strains such as RRIM and Harbel that participated in crossings of most common commercial clones (Webster and Baulkwill 1989). IAN, MDX and MDF clones are varieties created in the native region of the species, and the crossing of these parents can rescue a diversity similar to the natural individuals of the Amazon region, becoming genetically close (Webster and Baulkwill 1989). The proximity between the varieties within the native region of the species is due to the distribution of its genetic structure through dispersion mechanisms such as the hydrographic network of the Amazon basin that cross countries like Peru, Bolivia, Colombia, and Brazilian states like Pará and Amazonas. The possible differences are related to the natural isolation of plants in key areas (Le Guen et al. 2009).

The Bayesian analysis of the STRUCTURE program and the statistics of Evanno et al. (2005) indicated that the highest delta K value has two distinct groups (clusters) (K = 2). From this data it was possible to obtain a histogram showing the genetic composition of each individual and their respective grouping by similarity to ancestry (Figures 2 and 3).

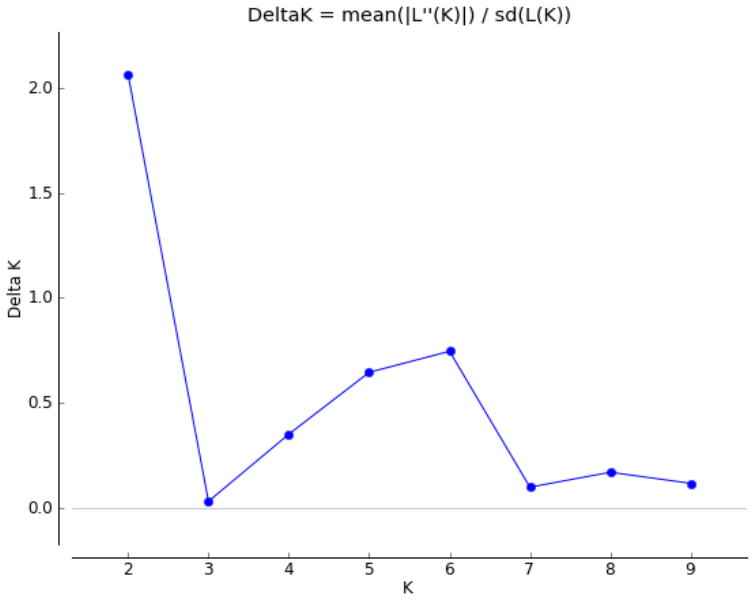

Figure 2. Delta chart (K) with K = 2, obtained by applying the statistical model of Evanno et al. (2005), in the STRUCTURE program, based on the analysis of 15 clones of *H. brasiliensis*.

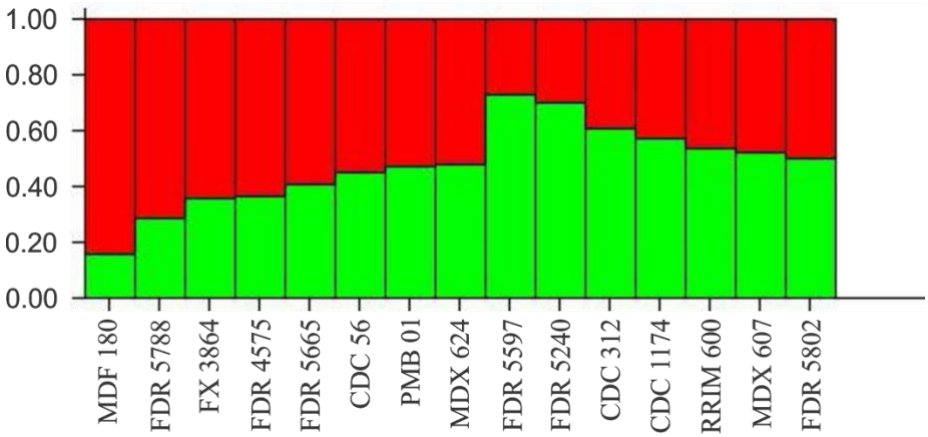

Figure 3. Histogram relating the 15 genotypes of *H. brasiliensis* according to the Bayesian analysis generated by the STRUCTURE program. Columns represent individuals and the colors represent the two gene pools (K = 2).

The histogram (Figure 3) shows the genetic composition and the proportion of each pool per clone. The red pool predominated the composition of individuals CDC 56, FX 3864, PMB 01, FDR 5788, MDX 624, FDR 5665, MDF 180, and FDR 4575. Clones with common parental origin had a similar genetic composition. FDR 5240 and FDR 5597 have the same parents and showed a predominance of the green pool in their genetic composition. Clone FDR 5788 has as its parent the primary clone MDF 180, justifying its similar composition pool. Individuals CDC 1174, MDX 624 and MDX 607 also had parents of the MDF source but were grouped in the green pool. This is due to the second parent of their crosses, belonging to the clonal lineage Avros (Indonesia), which have a restricted genetic base and are closely related to the Amazon region. RRIM 600 from Malaysia was found, which also has a strong genetic relationship with Amazonian genotypes due to ancestry (Gonçalves et al. 1999). This genetic relatedness was also verified in other genetic studies (Souza et al. 2015; Souza 2018).

This analysis represents a robust complementary tool for certifying the clones' authenticity, enabling the tracking of their geographical origin by relating other individuals based on their ancestry. It satisfactorily complements traditional morphological characterization and could be adopted in breeding programs.

4. Conclusions

Microsatellites provided a robust fingerprint analysis. The genotypic profiles allowed to access molecular diversity to characterize, identify, and provided genetic certification in 15 rubber tree clones. It is a powerful tool for breeding programs.

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