Identification *in silico* of SSR markers for genotyping *Hevea* sp. clone gardens in Colombia

Identificación in silico de marcadores SSR para genotipificar jardines clonales de *Hevea* sp. de Colombia

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ABSTRACT

The rubber crops profitability depends largely on genotypes established in plantations, meaning that clone identity must be ascertained. This work was aimed at identifying commercial clones *Hevea* sp. by microsatellites. Primers were designed from sequences reported in Genbank using Primer3, PrimerQuest and OlgoPerfect software for PCR amplification of microsatellites. The primers so obtained were thermodynamically analysed by Oligo Analyzer 3.1 software and experimentally evaluated on 12 *Hevea* sp. clones. The 15 of the 561 microsatellite markers were selected; they had 2- and 3-bp repeat motifs and 11- to 23-bp repeat extension ranges. The most informative ones were microsatellites amplified with SSRH103, SSRH134, SSRH510 and SSRH516 primers with seven alleles and SSRH403 primers with eight alleles. Four microsatellite markers were sufficient for discriminating 10 of the 12 clones. Clustering analysis involved all the markers on the clones evaluated here, showing Brazilian clones' narrow genetic base compared to Asiatic ones. The current work provides new markers and joins work published by other authors for identifying and diversity studies of natural rubber clone.

Key words: polymorphism, molecular markers, *Hevea brasiliensis*, SSR, clone, PCR**,** natural rubber.

RESUMEN

La rentabilidad del cultivo de caucho depende en gran medida de los genotipos establecidos en plantación, por lo tanto es necesario asegurar la identidad de los clones. Este trabajo tuvo como objetivo identificar clones comerciales de *Hevea* sp. mediante microsatélites. Se diseñaron primers a partir de secuencias reportadas en el Genbank con los programas Primer3, PrimerQuest[SM] y OlgoPerfect[SM] para amplificación por PCR de microsatélites. Los primers obtenidos se analizaron termodinámicamente mediante el programa Oligo Analizer 3.1 y se evaluaron experimentalmente sobre 12 clones de *Hevea* sp. Se seleccionaron 15 de 561 marcadores microsatélites con motivos de repetición de 2 y 3 pb y rangos de extensión entre 11 y 23 repeticiones. Los más informativos fueron: con siete alelos los microsatélites amplificados con lo primers SSRH103, SSRH134, SSRH510 y SSRH516, con ocho alelos los primers SSRH403. Cuatro marcadores microsatélites fueron suficientes para discriminar diez de los 12 clones. El análisis de agrupamiento realizado con la totalidad de los marcadores sobre los clones evaluados, evidencia la estrecha base genética de los clones brasileños respecto a los asiáticos. Este trabajo aporta nuevos marcadores y se suma a los publicados por otros autores, para la identificación y estudios de diversidad de clones de caucho natural.

Palabras clave: polimorfismo, marcadores moleculares, *Hevea brasiliensis*, SSR, clon, PCR, caucho natural.

Introduction

Natural rubber (*Hevea* sp.) crops have its origin in Amazon region; it belongs to the *Euphorbiáceae* family grouping several characteristic genera for latex production. *Hevea brasiliensis* is the most important specie from this genus due to high natural rubber production and the excellent physical-chemical properties which synthetic rubber cannot provide (Polhamus, 1962; Mooibroek and Cornish, 2000; Cornish, 2001).

Asia has the greatest planted area of natural rubber; this continent pioneered worldwide latex marketing. Around 7,000,000 ha have been established in Indonesia, Thailand and Malaysia, such production representing 70% of the world market for this biopolymer. Latin-America represents just 3% of such production (365,000 ha planted) despite being the center of origin of this species (IRSG, 2010). Colombia currently has 29,917 ha planted with rubber (MADR, 2010); it is hoped to broaden this to supply natural rubber needs by promoting new seeding programs, 889,674 ha

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being available which have favourable climatic conditions for the crop (Candelo and Motta, 1996).

Establishing new plantations has show that existing clone gardens usually do not have clear historical records concerning the origin of their vegetal material. Sanitary control and certification techniques must be implemented to overcome such limitation within the legal framework provided by ICA resolution 001478/2006 stating the need for follow-up, sanitary surveillance and genetic identification in natural rubber propagation nurseries to guarantee the vegetal material's source and quality, preventing low yields in adult plantations and the introduction and dissemination of diseases.

Vegetal material destined for this specie's propagation is currently identified by the isoenzyme technique (Chevallier, 1988) which has limitations related to specificity and costs. The limited number of alleles which can be detected this type of marker is one of its main disadvantages (i.e. poor discrimination power) (Vera *et al*., 1999; Belleti *et al*., 1992). This technique is linked to gene expression phenotypes which could be influenced by environmental factors and the crop's development stages, meaning that it is hardly reproducible.

Recently DNA-based molecular markers have been widely used due to their sensitivity and rapidity in varietal identification in different vegetal species (Raina *et al*., 2001; Vera *et al*., 1999; Prince *et al*., 1995), as well as leading to discriminating individuals having closely-related genotypes (Ilbi, 2003). Such markers are classified into two large groups: hybridisation-based molecular markers and the PCR-based molecular markers. The latter are more used due to the low DNA concentration required for their development, their ability to amplify genome sequences from preserved tissue and being methodologically accessible by small laboratories regarding equipment, ease of use and cost. RAPD, SSR or microsatellites, AP-PCR and AFLPs are some of the currently available PCR-based markers (Semagn, 2006).

Microsatellites or simple sequence repeats (SSR) are amongst the most efficient molecular markers due to their co-dominant multi-allele nature and their broad random distribution throughout the whole genome, thereby making them a highly polymorphic marker. SSR have been successfully used in plants in genetic diversity studies, constructing genetic maps (Lespinasse *et al*., 2000) assisted improvement by molecular markers and genotype identification studies (Okogbenin *et al*., 2006; Rajeev *et al*., 2005).

RAPD molecular markers have been used for genotyping natural rubber clones (Varghese *et al*., 1997; Hernández *et al*., 2006; Nakkanong *et al*., 2008); however, this is not an efficient methodology for mass evaluation of clones due to the technique's inherent characteristics such as low reproducibility, low discrimination power and difficulty with profile analysis. Studies have been developed using microsatellite markers. Lekawipat *et al*. (2003) reported the SSR M574 marker as being highly polymorphic after evaluating 108 *Hevea brasiliensis* accessions and obtaining 21 alleles. Saha *et al*. (2005) reported four markers (HMAC4, HMAC5, HMCT1 and HMCT5) for discriminating 27 *Hevea* sp. clones. Studies by Saha *et al*. (2007) reported that the HMGR marker was useful for studying genetic variability in wild *Hevea brasiliensis* material. Feng *et al*. (2009) have recently published a set of markers designed from EST sequences stored in GenBank for evaluating genetic diversity and SSR transferability between *Hevea* species.

Four SSR markers proposed by Saha *et al*. (2005) were used in preliminary assays in six *Hevea* sp. clones having commercial interest for Colombia; these assays showed that all of them could only be discriminated by the HMCT5 marker. However, a clear and reproducible banding pattern could not be obtained, even when modifying annealing temperatures (Tm) and using PCR adjuvants. The above work also reports an evaluation of the EST-SSR markers proposed by Feng *et al*. (2009) on 67 rubber clones and by Saha *et al*., (2007) on six clones respectively, obtained low polymorphism. The present work was aimed at identifying new microsatellite markers having greater discrimination power for genotyping *Hevea* sp. clones from genome sequences reported in GenBank databases for experimentally designing and evaluating primers leading to easy amplification and reading.

Methodology

Prior evaluation. The 12 clones evaluated (Tab. 1) in this study had been previously analysed with the HMCT1, HMCT5, HMAC1 and HMAC4 microsatellites described for identifying Asian natural rubber genotypes (Saha *et al*., 2005). These PCR and allele visualisation conditions were performed as proposed by the authors.

Oligo design. Sequences found in Genbank were manually analysed using the search word "*Hevea brasiliensis microsatellite sequence*". Sequences were initially chosen which presented two and three repeat motifs (minimum 11 and 4 repeats from the motif, respectively). It was also was verified whether there had been any redundancy in

TABLE 1. Primer sets selected using OligoAalyzer 3.1 software after analysing *Hevea* sp. microsatellite-containing sequences deposited in GenBank.

GenBank ID	Denomination	Microsatellites	Primers (5´→ 3')	Expected size (bp)	T° annealing	No. of alleles
AY486867	SSRH102	(GA) 20	CCAAGCCAATCATCAGGAAT (F) AGCAGCCCATGATACAACTG (R)	290	54°C	6
AY486866	SSRH103	(CT) 21	TCCTCTCCTCGTCAACATCC (F) TGTCATTCGAACTCCGTCAA(R)	251	55°C	7
FJ160574	SSRH132	(CTT) 11	CTGCCTTGTCTTCCTCATCC (F) CTCGGAGACCTGTCCCTGTA (R)	232	55°C	2
AY486835	SSRH134	(CT) 15	GCATGTCTTGCTGCAGAATC (F) GGAAACCAAGCCCTCAATCT (R)	251	55°C	7
FJ160571	SSRH135*	(GTT) 4	TTGGGTAGGAACAG G GTCAG (F) CAA CGCTCTCATCACGAAAA (R)	226	55°C	Non-specific
FJ160570	SSRH136*	(CTT) 17	TATGGAGAGGAGGGGCTTTTAT (F) GAGTTTGAGGGGGAGGAC (R)	204	55°C	Non-specific
AY486832	SSRH137	(TC) 26TT (CTT) 6	TCCGGTGTTATGGTGTTCTG (F) AGGAAGAAAGGGAAGAAA (R)	247	55°C	3
AY486754	SSRH358	(GA) 20	TCCGCTCTAGCTTCTTCCTG (F) GCCGCATAAGAGTGAACGA (R)	226	57°C	5
AY486708	SSRH402	(CT)16	CCATCTTTCCTCTCGCTCAG (F) GTTCGTCTGCTGGGCATAGT (R)	265	55°C	2
AY486707	SSRH403	(GA) 16	TGCCATCCTGCAGTTATCAG (F) GCACATATG AGGAAGCCACA (R)	242	57°C	8
AY486694	SSRH416	(CT) 22	GTTCCCAACTACTCGCTTTC (F) GGCATGTGCTACAGCATTGT (R)	222	55°C	4
AY486599	SSRH508	(GA) 16	TGCAGTAGGCCTCTGTGATG (F) GCTGGACCCATCTTAGGTG A (R)	253	55°C	Non-specific
AY486597	SSRH510	(CT) 23	TCAGGGCTTCAGGATGATTC (F) GGATGAAGCGCTTATGGAGA (R)	251	55°C	7
AY486589	SSRH516	(GA) 16	CCCAATTGTGCAGTA ACAC G (F) CCCTCCTTGTCTGAACCTCA (R)	183	55°C	7
AY439314	SSRH548	(GA) 4 CA (GA) 7	TGAGCAACGGAGGAGAGAA (F) CCAAACACCCAAACCCAATTC	250	57°C	5

* These pairs of oligos did not fulfil all the selection parameters; however, they were evaluated due to the presence of SSRs having domains containing three nucleotides.

the selected sequences by using alignment with the bl2seq algorithm (Altschul *et al.,* 1990). These sequences were used for designing primers with OligoPerfect (http://tools.invitrogen.com/content.cfm?pageid=9716), Primer3 (Rozen and Skaletsky, 2000) and PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/Default.aspx) software, taking the following criteria into account: 18 to 24 base pair (bp) primer size, 180 to 300 bp expected fragment size, 55 to 62°C fusion Tm and 45 to 55% GC. IDT OligoAnalyzer 3.1 software (http://www.idtdna.com/Scitools/Scitools.aspx) was used for evaluating the primers' thermodynamics. Maximum 3 bp complementariety was taken into account to avoid branch formation; homodimer and heterodimer formation was then determined (maximum 6 bp complementariety) (Montoya, 2009). NCBI Blast (Altschul *et al.,* 1990) was then used for alignments to ensure specificity and homology between primers and target sequence.

Vegetal material: 12 *Hevea brasiliensis* clones or their *Hevea benthamiana* hybrids were analysed to ascertain designed primer polymorphism (Tab. 2) using three individuals per clone. Foliar material were collected from Mavalle (Remolinos, Meta department), Vorágine (Medellín, Antioquia department) and Rincón Llanero (Puerto Gaitán, Meta department) clone gardens which had clear vegetal material source records. Four leaflets from each clone were stored in sealed hermetic bags with silica gel as preservative and then taken to the laboratory for total DNA extraction.

Nucleic acid extraction. Genomic DNA was extracted from each sample using the method proposed by Varghese *et al.* (1997). The DNA so obtained was visualised on 0.8% agarose gel and quantified by fluorometry (Quant-iT ds BR assay kit, Invitrogen).

PCR amplification and microsatellite analysis. The PCR reaction was carried out for 25 µL final volume containing 25 ng genomic DNA, 0.4 µM of each oligo, 1.5 mM $MgCl_2$, 200 mM dNTPs and 1 Taq polymerase unit. The master mix was placed in a MyCycler (BioRad) thermocycler using the following amplification programme: 94°C initial denaturing for 3 min, followed by 35 denaturing cycles at

TABLE 2. Size (in base pairs) of the alleles obtained for each of the 7 microsatellites having the greatest number of alleles in the 12 clones evaluated here, and values calculated regarding observed heterozygosity (Ho), expected heterozygosity (He) and PIC.

Clone	SSRH548		SSRH358		SSRH134		SSRH403		SSRH516		SSRH102		SSRH103	
Allele size (bp)	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 1
GT1	240	250	220	236	230	240	224	248	185	185	280	280	255	270
RRIM 600	230	243	238	238	236	236	239	248	185	210	280	280	255	250
PB 260	243	243	240	240	225	236	238	252	197	205	278	279	250	282
PB 235	227	250	235	235	238	240	224	238	185	185	280	280	265	270
IAN 710	243	250	220	238	225	236	224	248	185	185	280	280	270	270
IAN 873	243	243	222	238	230	240	224	250	185	188	279	281	255	270
IAN 713	243	243	222	238	230	240	224	250	185	188	279	281	255	270
FX 3864	243	243	222	238	230	240	224	250	185	188	282	282	255	270
FX 3899	243	247	222	238	228	238	224	250	185	188	282	282	255	270
GU 198	243	247	227	238	240	248	232	239	238	238	290	290	260	260
AVROS 1581	243	247	227	238	240	248	232	239	238	238	290	290	268	270
AVROS 2037	243	247	238	238	240	248	239	255	232	232	280	280	268	270

	SSRH548	SSRH358	SSRH134	SSRH403	SSRH516	SSRH102	SSRH103
Ho	0.667	0.667	0.917	1	0.5	0.25	0.75
He	0.611	0.733	0.792	0.826	0.722	0.747	0.729
PIC	0.77	0.76	0.78	0.83	0.83	0.81	0.72

94°C for 15 s, annealing for 1 min (temperatures reported in Tab. 1 for each set of primers), extension at 72°C for 15 s and a final 7 min extension step at 72°C.

The amplimers obtained were run on 7% polyacrylamide gels and visualised by silver staining (Bassam *et al*., 1991). The sizes of the alleles obtained by microsatellite were determined using 10 bp molecular weight ladder (Invitrogen) as reference.

Data analysis: The number of alleles detected for each microsatellite so amplified was estimated in the 12 clones evaluated. The degree of polymorphism was calculated from expected heterozygosity (He) by applying the formula $He= 1- \Sigma p_i^2$ where p was the frequency of the i^{th} allele in the genotypes examined. Observed heterozygosity (Ho) was calculated using the ratio between the number of heterozygotes regarding the amount of total genotypes analysed using GENALEX 6 software (Peakall and Smouse, 2000). Polymorphism information content (PIC) was calculated using the $PIC= 1- \Sigma f_i^2 \, i = 1$ equation where f_i^2 was the frequency of the i^{th} allele (Anderson *et al*., 1993). Similarity was analysed using NTSYS-pc 2.1 software (Rohlf, 2006); the Dice index was used for generating a similarity matrix, followed by a dendrogram using the UPGMA algorithm.

Results and discussion

Only two of the 12 clones evaluated in this study (Asian clones GT 1 and PB 235) had been analysed by Saha *et al.* (2005) whose results were confirmed for 3 of the microsatellites (HMCT1, HMAC4 and HMCT5), unlike the results obtained with microsatellite HMAC5. The authors reported two alleles for GT 1 (270/272 bp) and PB 235 (272/274 bp) with this microsatellite; a single 265 bp allele was observed in this work for both clones. It was also found that the clones being studied (FX 3899, FX 3864, IAN 873, IAN 713, GU 191 and AVROS 1981) were not discriminated using the four reported microsatellites as a set (data not shown). It is thus recommended using these microsatellites for identifying some Asian clones present in Colombian clone gardens; however, additional markers must be included for American clones to allow them to be differentiated.

The 120 of the 604 sequences found in the search for microsatellite regions contained in the *H. brasiliensis* genome in GenBank using the search word "*H. brasiliensis microsatellite sequence*" were selected for designing primers; bearing in mind repeat motifs' greater sizes and the greater number of repeats, work on other vegetal species such as *Capsicum annuun* have reported increased polymorphism in SSR sequences having the aforementioned characteristics (Yi *et al*., 2006).

The search did not include microsatellites present in *H. brasiliensis* expressed sequence tag (EST) libraries (Okogbenin *et al.,* 2006), due to them usually being more conserved between individuals from the same specie and related species in contrast to genomic SSRs, thereby implying less polymorphism (Eujay *et al*., 2002), such characteristic not being useful in this work given that vegetal material having

a reduced genetic base had to be discriminated, such as the American clones from the IAN and FX series.

The information found in GenBank for some of the selected sequences included some primer sets, together with their amplification conditions, which were not included due to them not fitting the selection criteria for the primers used in this work (lower Tm and shorter length) since using primers having low annealing Tm and short size increases the probability of non-specific amplimers (Abd-Elsalam *et al*., 2003).

The 560 pairs of primers were obtained from the selected sequences (i.e. four or five per sequence); 15 pairs were selected after analysis with OligoAnalyzer 3.1 software was they fulfilled the thermodynamic criteria descried in the methodology and were sent to be synthesised. Two of these primer sets led to amplifying microsatellites having three bp repeat domains in spite of not fulfilling all previously established criteria (Tab. 1); this parameter allowed better differentiation of the fragments obtained.

Prior knowledge of Tm obtained with the design programmes led to a better approach to establishing the PCR cycle, specific amplimers being thereby obtained for all the primers. These parameters avoided having to apply formulae for finding Tm and these values' discrepancy with PCR components, as well as using empirical thermocycling assays with touchdown (Don *et al*., 1991; Roux, 2009). PCR was optimised regarding $MgCl_2$, dNTP, primer and template DNA concentrations; hybridisation temperature based on the lower limit of the range selected for designing primers was also standardised (54 to 59°C experimental hybridisation Tm) (Tab. 1). As specific, easily interpreted amplifications were obtained with 10 of the 13 primer sets, this showed that the selection criteria defined for their design were sound.

It was found that 3 of the 15 primer sets evaluated in the 12 *Hevea* clones had non-specific band patterns (SSRH136, SSRH135 and SSRH508). Two of the markers only generated two alleles (SSRH132 and SSRH 402); the SSRH137 and SSRH416 markers generated three and four alleles, respectively and seven had 5 to 8 alleles (Tab. 2). The sizes obtained ranged from 222 to 290 bp, these being congruent with size predicted by primer design programmes (Fig. 1, Tab. 2). These diversity and polymorphism measurements calculated for microsatellites which generated more than 5 alleles ranged from 0.66 to 0.82 for He, 0.25 to 1.00 for Ho and 0.72 to 0.83 for PIC (Tab. 2).

The SSRH403 microsatellite (He=1.000, Ho=0.826 and PIC=0.830) amplified the greatest number of alleles (8) and could discriminate the 12 clones into eight different groups. The PB260 clone was differentiated from the other Asian clones with 7 of the 11 markers evaluated, this being the most divergent clone (both Asian and American). The SSRH548, SSRH358, SSRH134 and SSRH403 microsatellites discriminated Asian clones PB 235, RRIM 600 and GT-1 (Ho=0.667-1.000, He=0.611-0.826 and PIC 0.76-0.87). The GU-191 (from Guatemala) and AVROS-1981 (Asian) clones were only differentiated with microsatellite SSRH103 (Ho=0.750, He=0.729 and PIC=0.720). The American clones obtained in Brazilian genetic improvement programmes (FX 3899, FX 3864, IAN 873, IAN 710 and IAN 713) were discriminated by fewer microsatellites grouped together with most markers. The FX clones could only be differentiated with the SSRH134 microsatellite. IAN 713 and IAN 873 clones were not differentiated with any of the 10 microsatellites. The minimum number of microsatellites allowing 10 of the 12 clones to be differentiated was four (i.e. SSRH403, SSRH548, SSRH358 and SSRH103). Tab. 3 summarises the foregoing, showing the different genotypes obtained for all the clones.

Similarity analysis with the 10 most polymorphic microsatellites differentiated 10 of the 12 clones evaluated; a group clustering the five American clones (Brazilian) included in the study (0.48 similarity) was also observed,

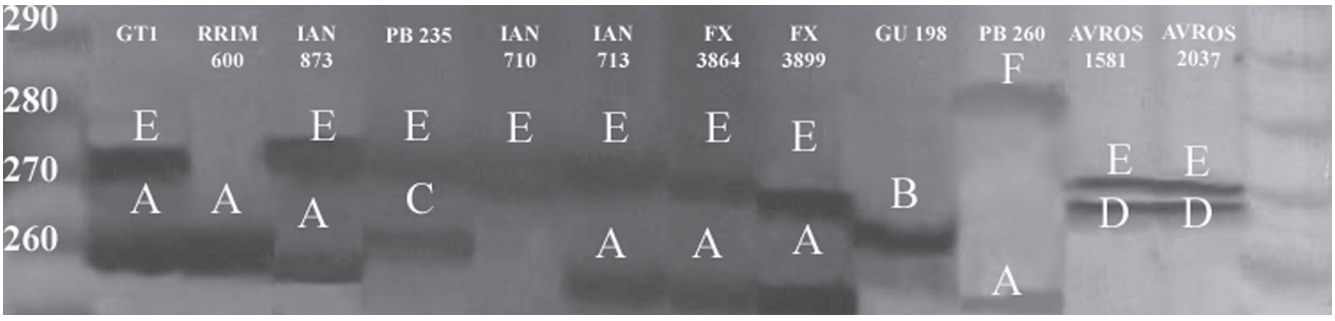

FIGURE 1. Electrophoretic profiles obtained for the 12 clones evaluated with microsatellite SSRH 103 (6 alleles).

TABLE 3. Genotypes obtained for each of the 7 microsatellites having the greatest number of alleles in the 12 clones evaluated here.

Clone	Genotype						
Allele size (bp)	SSRH548	SSRH358	SSRH134	SSRH403	SSRH516	SSRH102	SSRH103
GT1	CF	AE	CF	AE	AA	CC	AE
RRIM 600	BD	FF	DD	DE	AE	CC	AA
PB 260	DD	AA	AD	CG	CD	AB	AF
PB 235	AF	DD	EF	AC	AA	CC	CE
IAN 710	DF	AF	AD	AE	AB	CC	EE
IAN 873	DD	BF	CF	AF	AB	BD	AE
IAN 713	DD	BF	CF	AF	AB	BD	AE
FX 3864	DD	BF	CF	AF	AB	EE	AE
FX 3899	DE	BF	BE	AF	AB	EE	AE
GU 198	DE	CF	FG	BD	GG	FF	BB
AVROS 1581	DE	CF	FG	BD	GG	FF	DE
AVROS 2037	DE	FF	FG	DH	FF	CC	DE

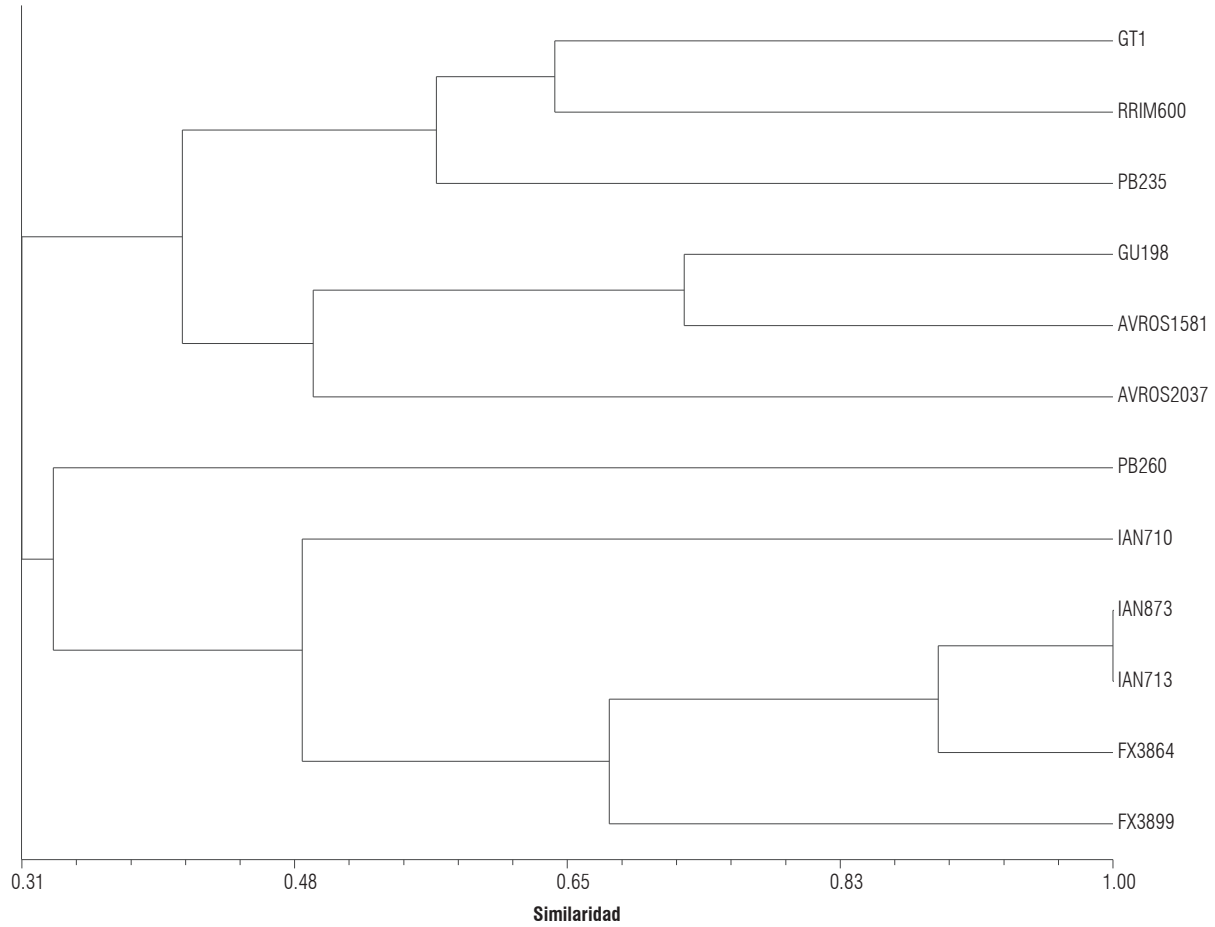

FIGURE 2. Clustering was analysed with GT1, RRIM-600, PB-260, PB-235, IAN-710, IAN-873, IAN-713, FX-3864, FX-3899, GU-198, AVROS-1581 and AVROS-2037 clones using the selected SSRH548, SSRH510, SSRH358, SSRH416, SSRH134, SSRH137, SSRH403, SSRH516, SSRH102 and SSRH103 microsatellites. A similarity matrix was constructed using the Dice similarity index; the UPGMA algorithm was then used to produce a dendrogram with NTSYS PC. 2.02i software.

thus confirming this group's reduced gene base (Fig. 2). Similar results were obtained by Hernández *et al.* (2006) who evaluated Asian and Brazilian clones using RAPD markers. The foregoing could explain the three commercial clones from the IAN series which, together with the FX 3864 clone, share the maternal parent (PB 86). The GU 198 clone was grouped with Asian AVROS clones (0.71 similarity), AVROS 1581 (0.48) and AVROS 2087 (0.34),

thereby agreeing with the results obtained by Hernández *et al*. (2006) who concluded that this clone was produced by crossing Asian parental clones; however, it is known that one of its parental clones corresponded to the American FX 16 clone. The RRIM 600, GT 1 and PB 235 clones were grouped at a 0.49 genetic distance and the PB 260 clone was grouped at 0.31 genetic distance.

The IAN 873 and IAN 713 clones could not be differentiated with any of the 10 microsatellites. It is thus considered that evaluation of microsatellite markers should be considered as this leads to discriminating genotypes of clones present in Colombian clone gardens. Recent work has offered other useful markers in this field, such as that reported by Souza *et al*. (2009) who produced 30 microsatellites which were then applied to studying the genetic diversity of 30 different *H. brasiliensis* genotypes, including genotypes from other *Hevea* species; the loci analysed presented observed and expected heterozygosity ranging from 0.13 to 0.88 and 0 to 0.89, respectively. Work by Le Guen *et al*. (2009) reported 15 microsatellites applied to studying the genetic diversity of natural rubber germplasm collections established in French Guyana, Peru and Brazil, all having high polymorphism.

It is worth stressing that the study presented here was not aimed at analysing the genetic diversity of the clones included in it; however, the results have led to us suggesting using the microsatellites evaluated here for this type of study. This work has contributed towards identifying *Hevea* sp. clones for organising clone gardens which are the commercial source of vegetal material distribution for establishing plantations, since suitable selection of clones to be planted in a determined region will greatly ensure latex performance and thus increase producers' economic benefits. Prior identification of clones is needed as they perform differently according to a particular region's characteristics. It is clear that when a plantation's genetic base can be controlled then better profitability can be expected, bearing in mind that natural rubber is a late performance crop thereby involving high management costs.

Conclusions

The preliminary evaluation of the 12 clones being studied with microsatellites reported in the pertinent literature for identifying some Asian rubber genotypes showed that they were unable to differentiate American clones present in Colombian clone gardens. This study made advances in characterising four clones which could not be differentiated by the microsatellites reported by Saha *et al*. (2005).

Implementing thermodynamic analysis programmes has led to establishing parameters for selecting and designing oligos, thereby simplifying standardising PCR amplification the conditions in the laboratory. Sets of oligos have thus been obtained which have allowed microsatellite amplification, in turn, managing to differentiate 10 of the 12 *Hevea* sp. clones evaluated in this study with a minimum number of four of these markers, thereby abbreviating identification of the 12 clones.

Analysis of clustering the clones characterised in this work has revealed the need for continuing to apply molecular tools leading to the discrimination of all the clones present in Colombia so that they can be used in varietal identification and genetic variability studies.

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