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Original Article

Analysis of genetic diversity in early introduced clones of rubber tree (*Hevea brasiliensis*) using RAPD and microsatellite markers

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Abstract

Genetic analysis in 53 early introduced clones of rubber tree (*Hevea brasiliensis*) collected from different areas in Southern Thailand was performed using RAPD (Random Amplified Polymorphic DNA) and microsatellite markers. Seventeen cultivated clones (34 samples) were also included to compare DNA patterns. DNA was isolated from leaf samples using CTAB buffer. One hundred and ninety two 10-base oligonucleotide primers for RAPD were first screened and 8 primers (OPB-17, OPN-16, OPR-02, OPR-11, OPZ-04, OPAD-01, OPAD-10 and OPAD-12) were chosen for genetic variation analysis in 87 individual plants. Seventy amplification fragments were obtained from the 8 primers with an average of 8.75 fragments for each primer. From all fragments 55 were polymorphic fragments (78.57%). One RAPD primer (OPAD-01) yielded a 700-bp fragment that was present only in the Tjir1 clone. Four microsatellite primer pairs (hmac4, hmct1, hmct5 and hmac5) produced a total of 44 amplified fragments with an average of 14.67 fragments per primer, of which 37 were polymorphic (84.09%) while hmac5 produced only monomorphic fragments. A phenogram showing genetic similarities among rubber trees was constructed based on the polymorphic bands of the RAPD and microsatellite analyses using UPGMA (Unweighted Pair-Group Method Using Arithmetic Average). Cluster analysis was performed by the NTSYS Version 2.1 program. The results from phenogram showed that the 87 rubber clones could be clustered into 6 groups with similarity coefficients ranging from 0.541-1.000. Cultivated clones revealed more narrow genetic diversity compared to the early introduced clones. The clustering was not correlated with the geographical location of the collected samples.

Keywords: *Hevea brasiliensis,* early introduced clones, RAPD, microsatellite

1. Introduction

The rubber tree (*Hevea brasiliensis*) is a tropical perennial originating from the Amazon basin in South America and has been established as the primary commercial sources of rubber over a century ago. It is now grown extensively in Southeast Asia including in Indonesia, Thailand and Malaysia. In 2003, world production of rubber was approximately 9.42 million tons, out of which Thailand produced 2.97 million tons (Boukaew *et al*., 2006). *H. brasiliensis* is an open pollinated crop which is highly heterozygous in nature. True to type of rubber can be vegetatively propagated by budding selected scions on about 2-8 month old seedlings a process known as green budding or via budding in the nursery and transferring to polybags as budded stumps. In the past, the most common rootstocks for rubber tree production in Thailand were seeds of any early introduced clones which had high heterozygosity based on cross pollination in nature. However it is believed that the rubber stock common throughout Asia has only a narrow genetic base, since almost all of this stock is thought to have originated from 22 seedlings of Wickham's original collection (Kinnarat and Rattanawong, 2002). At the present, a cultivar known as RRIM600 is the major cultivated variety of rubber plantations in Thailand (~70-75%), and it is believed that almost all of the other early introduced clones have been gradually lost, a situation which means that currently there is probably

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a high level of inbreeding. Preliminary study reported by Khonglao (2006) on this situation indicated that there was higher vigorous rooting development of seedlings from early introduced clones than in RRIM600 seedlings and suggested that it would be wise to identify some of other early introduced clones of rubber for a breeding program in the future. It is important to collect and conserve those clones to prevent potential extinction and loss of genetic diversity.

In a breeding program, clones of close genotypic similarity will not be crossed to guard against inbreeding depression. However, identification based on morphological traits in *H. brasiliensis* clones is not an exact science. Several methods of identifying molecular markers have been used with *H. brasiliensis* including assessment of genetic diversity in wild and cultivated clones by nuclear RFLPs (Besse *et al*., 1994), mitochondrial DNA (Luo *et al*., 1995), RAPD (Varghese *et al*., 1997 and Cesar *et al.,* 2006), evaluation of the genetic relatedness of wild and cultivated *Hevea* accessions with SSCP (Lekawipat *et al*., 2003) and microsatellite (Roy *et al*., 2004; Saha *et al*., 2005).

The objectives of the present study were to use RAPD and microsatellite markers to identify and assess genetic variability of early introduced clones and to evaluate the genetic relationships among early introduced and cultivated clones.

2. Materials and Methods

2.1 Plant materials and DNA extraction

Young fully expanded leaves from a total of 53 individuals representing early introduced clones of *H. brasiliensis* were collected from different places in southern part of Thailand (Table 1). Early introduced rubber clones were recognized by their big trunk indicating an age of more than 50 years and their random location outside established rubber plantation areas. Thirty four clones of cultivated were also collected from the Songkhla Rubber Research Center, the Surat Thani Rubber Research Center and the Krabi Plant and Plant Production Materials Technical Service for comparison study (Table1). Total genomic DNA was extracted from 200 mg of leaves from each clone by the cetyltrimethyl ammonium bromide protocol of Doyle and Doyle (1990).

2.2 RAPD assay

Random amplified polymorphic DNA (RAPD) analysis was performed according to the methodology of Williams *et al.* (1990). Each amplification mixture of 25 µl contained 25 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 60 ng of template DNA. The thermal profile for RAPD-PCR was started at 94°C for 2 min, followed by 41 cycles of 94°C for 30 sec. 37°C for 1 min. 72°C for 2 min. and finally 72°C for 5 min. Amplification products were then separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5X TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 min and viewed under ultraviolet light with gel documentation. One hundred and ninety two primers were first screened with individuals of 3 cultivated clones (RRIM600, PB235 and GT1) and one early introduced clone. Primers with a reproducible pattern and clear cut polymorphism were chosen to use for analysis of 87 DNA samples.

2.3 Microsatellite assay

Four microsatellite primer pairs were used for PCR amplification of DNA following a protocol from Saha *et al.* (2005). The PCR reaction was carried out in 10-µl final volume containing 20 ng of genomic DNA, 25 mM $MgCl_2$, 10x *Taq* buffer, 0.2 µM each of the forward and reverse primers, 200 µM dNTPs and 0.7 units of *Taq* polymerase. The temperature profile involved an initial denaturation step of 5 min at 95°C followed by a touch-down PCR programme. Temperature profiles of touch-down PCR for seven cycles were as follow: 94°C for 30 sec, 63°C for 1 min, decreased 1°C for seven cycles, and finally 72°C for 1 min. This was followed by a normal cycling of 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min for 23 cycles and a final extension at 72°C for 10 min. The touch-down protocol was used to eliminate stuttering and artifact bands. Amplification products were run on a 6% denaturing polyacrylamide gel containing 7 M urea using 0.5 TBE buffer at 1,000 V. DNA bands were visualized with silver nitrate.

2.4 Data analysis

Data were scored as discrete variables using 1 to indicate the presence and 0 the absence of a band. Only bands reproducible in repeated amplifications were considered for estimation of genetic distances. Genetic distance was estimated as a complement of Jaccard[§]s analysis (1908). A pairwise distance matrix between genotypes was the basis for cluster analysis by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) and relationships among clones were visualized as a phenogram using the NTSYS-pc Exeter Software version 2.1 (Rohlf, 2002).

3. Result

3.1 RAPD analysis

One hundred ninety two primers were first screened with 3 cultivated clones and one early introduced clone, of which only 8 primers (Table 2) which produced reproducible bands and polymorphic bands were chosen for genetic diversity evaluation in the individuals of a total of 87 trees including early introduced and cultivated clones (Table 1). From the 8 primers, a total of 70 fragments were obtained, of which 55 fragments or about 78.57% showed polymorphisms. The highest polymorphic fragment was found in primer OPAD-01 and the lowest was observed in primers

Table 1. List of samples with abbreviation and place of collection

Name	Place of collection	Abbreviation	Number
Early Introduced Clones			
Clone#1	The first rubber tree, Trang	(EIRf)	1
Clone#2	Phraya Rassadanupradit, Trang	(EIRhp)	3
Clone#3	Kuan-Tamnak-jan, Trang	(EIRkj)	2
Clone#4	KP&PPMTS, Krabi	(EIRnch)	2
Clone#5	Hat Yai Municipal Park, Songkhla	(EIRp)	2
Clone#6	Khuanlang Rubber plantation, Songkhla	(EIRk)	1
Clone#7	Thungwang Rubber plantation, Songkhla	(EIRt)	3
Clone#8	Numnoi Rubber plantation, Songkhla	(EIRn)	3
Clone#9	Chang Rubber plantation, Songkhla	(EIRch)	6
Clone#10	Senanarong military fort, Songkhla	(EIRm)	4
Clone#11	Khohong, Songkhla	(EIRkh)	1
Clone#12	Prince of Songkla University, Songkhla	(EIRpsu)	17
Clone#13	Boripat waterfall, Songkhla	(EIRb)	7
Clone#14	Trang plantation, Trang	(EIRtr)	1
Cultivated clones			
GT1	SRRC.	(GT1)	1
BPM24	SRRC.	(BPM24)	1
BPM24	Surat Thani	(BPM24su)	3
RRIM600	SRRC.	(RRIM600)	1
RRIM600	Surat Thani	(RRIM600su)	8
RRIT250	SRRC.	(RRIT250)	1
RRIT251	SRRC.	(RRIT251)	1
RRIT156	SRRC.	($RRIT156_1$)	1
PB235	SRRC.	(PB235)	1
PB235	Surat Thani	(PB235su)	2
PB310	SRRC.	(PB310)	1
AVROS2037	STRRC.	(AVROS2037)	1
RRIC110	STRRC.	(RRIC110)	1
Tjir1	STRRC.	(Tjir1)	1
RRIT156	STRRC.	($RRIT156_2$)	1
BPM1	STRRC.	(BPM1)	1
PR255	STRRC.	(PR255)	1
PB255	STRRC.	(PB255)	1
PB260	STRRC.	(PB260)	1
PB311	STRRC.	(PB311)	1
RRIT402	STRRC.	(RRIT402)	1
RRIT300/1	KP&PPMTS	(RRIT300/1)	1
PB51	Surat Thani	(PB51su)	2
Total			87

SRRC: Songkhla Rubber Research Center
STRRC: Surat Thani Rubber Research Center
KP&PPMTS: Krabi Plant and Plant Production Materials Technical Service

OPN-16 and OPR-2. The number of polymorphic DNA fragments per primer ranged from 3 to 14, having an average of 7 bands per primer. One DNA fragment, 700 bp amplified by primer OPAD-01 (Figure 1c), was found specific to Tjir1. Examples of the amplification of RAPD markers are shown in Figure 1.

3.2 Microsatellite analysis

Four microsatellite primer pairs (hmac4, hmac5, hmct1 and hmct5) as reported by Saha *et al*. (2005), were used for sample analysis. These primers pairs have been identified from the genomic library of *H. brasiliensis* (Roy *et al*.,

Table 2. Primers producing polymorphic DNA bands in RAPD patterns of *Hevea brasiliensis*

Primer	Sequence (5' → 3')	Amplified fragments	Monomorphic fragments	Polymorphic fragments	Polymorphim (%)
OPB-17	AGGGAACGAG	13	1	12	92.31
OPN-16	AAGCGACCTG	5	2	3	60.00
OPR-02	CACAGCTGCC	4	1	3	75.00
OPR-11	GTAGCCGTCT	7	2	5	71.43
OPZ-04	AGGCTGTGCT	10	2	8	80.00
OPAD-01	CAAAGGGCGG	15	1	14	93.33
OPAD-10	AAGAGGCCAG	6	2	4	66.67
OPAD-12	AAGAGGGCGT	10	4	6	60.00
Total		70	15	55	

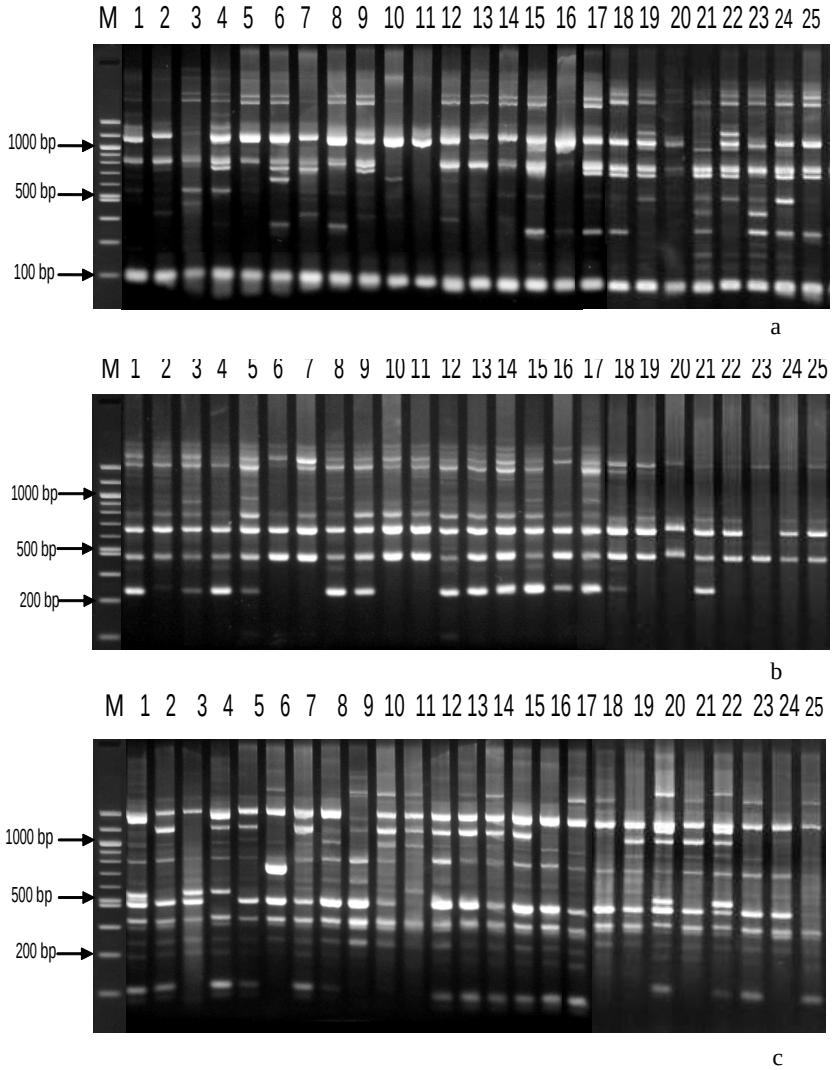

Figure 1. RAPD patterns of cultivated clones (lane1-17) consist of GT1, RRIT402, BPM1, BPM24, AVROS2037, Tjir1, RRIC110, RRIT250, RRIT251, RRIT156, PR255, PB235, PB255, PB260, PB310, PB311, RRIM600 and early introduced clones (lane 18-25) amplified by primer OPB-17 (a), OPR-02 (b), OPAD-01 (c). Lane M = 100 bp ladder.

2004). However, only 3 primer pairs (hmac4, hmct1 and hmct5) were chosen for genetic diversity evaluation in all clones (Table 1) since hmac5 showed monomorphic amplification. From 44 fragments obtained, 37 fragments or about 84.09% showed polymorphisms. The highest polymorphic fragment was found in primer hmct5 and the lowest was

observed in primer hmct1 (Figure 2).

3.3 Genetic similarities and clustering analysis

Cluster analysis of the genetic similarity values was performed to generate a phenogram illustrating the overall genetic relationships among *H. brasiliensis* clones. The similarity coefficient between the cultivated and early introduced clones varied from 0.549-1.000 and 0.441-1.000 by RAPD and microsatellite, respectively. The average similarity coefficient from both techniques ranged from 0.541-1.000. When considering the general phenogram constructed using the combined data of both sets of molecular markers, the highest similarity value (1.000) was observed among RRIM600 clones from Surat Thani (RRIM 600su). Some differences in DNA patterns were detected among the 6 clones of RRIM600 indicating genetic variation within this clone. The largest genetic distinct was found between the early introduced clone from Boripat waterfall (EIRb6) and cultivated clone PR255 with a similarity coefficient of 0.541. With the phenogram, all 87 plants could be separated into six clusters, with early introduced clones found in 5 clusters. Within the cultivated clones, genetic similarity ranged from 0.647-0.929. The highest similarity value (0.929) was observed between RRIT250 and RRIM600 and the lowest similarity value (0.647) was obtained between BPM24 and PB311. A higher range of similarity values was observed among the early introduced clones (0.600-0.965).

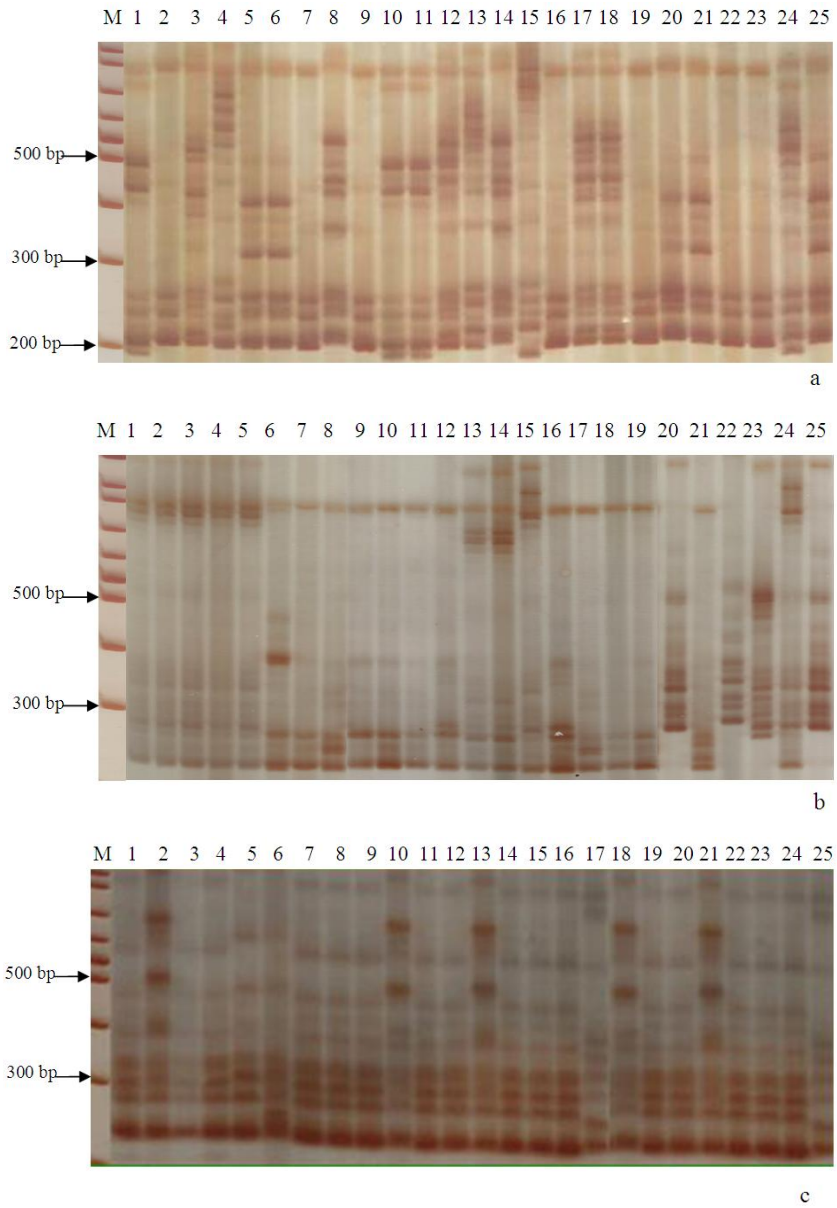

Figure 2. Microsatellite patterns of cultivated clones (lane1-17) consist of GT1, RRIT402, BPM1, BPM24, AVROS2037, Tjir1, RRIC110, RRIT250, RRIT251, RRIT156, PR255, PB235, PB255, PB260, PB310, PB311, RRIM600 and early introduced clones (lane 18-25) amplified by primer hmct5 (a), hmac4 (b), hmct1(c). Lane M = 100 bp ladder

4. Discussion

Molecular markers such as RAPD and microsatellite or SSR have been reported to provide an improved technology in assessing genetic variation among closely related species (Varghese *et al.*, 1997; Meszaros *et al*., 2007), including *H. brasiliensis* (Lespinasse *et al*., 1999; Saha *et al*., 2005). In this study, genetic discrimination among 87 clones of early introduced and current cultivars of rubber tree was assessed with 8 RAPD primers to estimate genetic diversity. A high level of polymorphisms was observed. Approximately 79% of polymorphic fragments among *H. brasiliensis* clones were obtained by using 8 primers. One specific RAPD marker was identified, a 700 bp fragment from primer OPAD-01 that was only found in the Tjir1 clone. Among 3 primer pairs used for microsatellite analysis (hmct5, hmac4 and hmct1), 84.09% of polymorphic fragments was obtained. Saha *et al*. (2005) reported that among 4 primer pairs used (hmct1, hmct5, hmac4 and hmac5) hmct5 has the highest discriminatory power and 12 genotypes from 27 samples of *H. brasiliensis* were obtained while 7 genotypes can be identified by hmac5. In this study, hmac5 produced monomorphic amplification and from this reason hmac5 was not included in future analysis.

The relatedness of the 87 clones studied was efficiently established through the use of RAPD and microsatellite markers. Both markers showed high degrees of similarity in phenogram topologies, though with some differences in the positioning of some clones at the main group. The combined phenogram from both sets of molecular markers (Figure 3) was similar to those obtained separately with each marker type (data not shown). Based on RAPD and microsatellite techniques, a total of 114 fragments and 92 polymorphic fragments were detected over all clones. Similarity coefficients of all 87 rubber clones (53 early introduced and 34 cultivated clones) in this study varied from 0.541-1.00. The comparison between both populations showed that cultivated clones are less variable than early introduced clones. However, the mean genetic similarity of more than 0.6 indicates a narrow genetic diversity in both populations. This finding was not surprising and can be explained by understanding that, most rubber breeding materials used in Asia are believed to have come from 22 seedlings surviving from Sir Henry Wickham's original collection known as the 'Wickham base', and consequently, the original genetic base of cultivated rubber in Asia is low (Annamma Varghese, 1992 and Cesar *et al*., 2006). The same finding was reported by Lekawipat *et al*. (2003) who studied wild and cultivated *Hevea* species using SSCP marker. In the current study, among different clones tested, RRIM600 displayed the highest average genetic similarity to RRIT250 with a similarity coefficient of 0.929. RRIT250 was reported to be developed from rubber tree found in rubber plantation at amphur Natawee, Songkhla province (Sookmak, 1995). It is possible that RRIT250 come from seeding of biparental crossed within an RRIM600 population or RRIM600 crosses with other clones.

As shown by the phenogram, the 87 *H. brasiliensis* clones clustered into 6 groups and early introduced clones are presented in 5 groups. The results indicated a wider genetic diversity in the early introduced than in the cultivated clones. For example, early introduced clones collected from different locations on PSU, Hat Yai campus represent 4 out of the 6 groups of the clustering. Rubber trees are a cross-pollinated tree species, and so a certain amount of genetic variability is expected when propagated by seeds. It is believed that the planting materials for the early rubber plantations were seeds, with vegetative propagation only becoming popular after the establishment of the Office of the Rubber Replanting Aid Fund in 1960 (Sookmak, 1995). Genetic variability results from gene flow among populations i.e. seed /pollen dispersal. It is also affected by local natural selection due to gene mutation, but mutation is not generally considered as a significant factor affecting the rubber population differentiation because of the low incidence rate of mutation in this species. Geographical location is another parameter that could contribute to genetic diversity but according to the clustering obtained in this study, we

Table 3. Primers producing polymorphic DNA bands in microsatellite patterns of *Hevea brasiliensis*

Primer	Sequence (5' → 3')	Amplified fragments	Polymorphic fragments	Polymorphism (%)
hmac4	GTTTTCCTCCGCAGACTCAG(L) ATCCACCAAATAAGGCATGA(R)	15	13	86.67
hmac5	TCGGTTGGTTTACCATGACA(L) ACATCACATGAGTGTATCTGATCTC(R)	3	0	0
hmct1	AACCAGAAGGGTGTCATGCT(L) GGAATCCCATGACAATCCAC(R)	13	9	69.23
hmct5	ATGTATGTGTGCGCAGGAAG(L) CTGTAGTCATGGCAGCAGGT(R)	16	15	93.75
	Total	47	37	

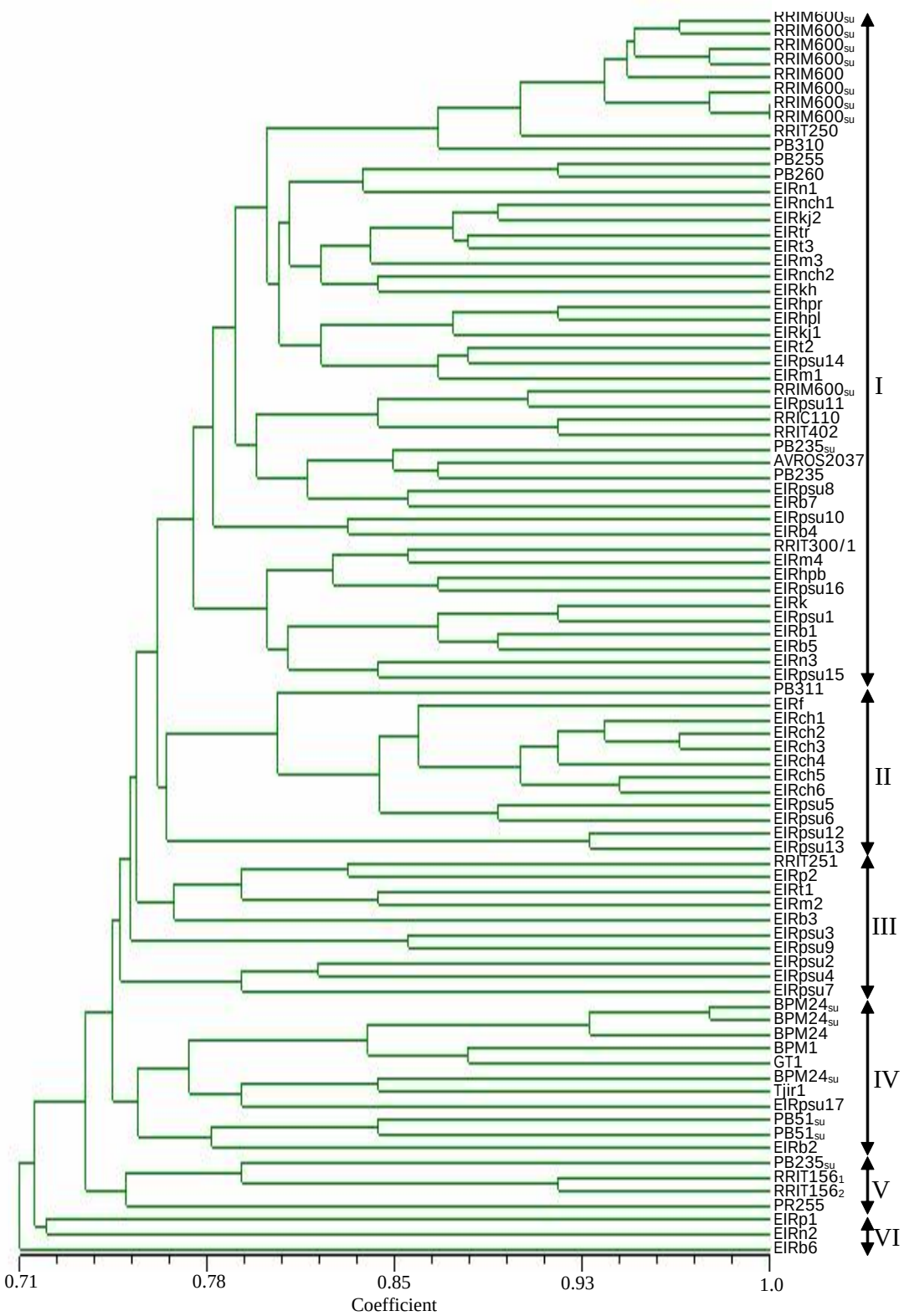

Figure 3. Phenogram showing the relationship between 87 *H. brasiliensis* based on RAPD and microsatellite analysis.

found no correlation between geographical location of collected samples and genetic clustering. This is in agreement with previous molecular studies in *H. brasiliensis* by Varghese *et al*. (1997), who did not observe any relationship between geographic correlation when grouping the species based on RAPD data. It also agrees with the generally known breeding history of *H. brasiliensis*, since all clones bred in different South-East Asian countries originated from the same

source, resulting in a sparse representation of the earliest geographic diversity. In addition, there have always been regular exchanges of materials between the different south-east Asian countries which facilitated the distributions of genes in various clones irrespective of the country in which they were first developed. However, controversial results were reported by Lekawipat *et al*. (2003) and Cesar *et al*. (2006). They reported some relationship of geographic and sample collection.

In the present study, the main rubber clones could be distinguished using eight RAPD or three microsatellite primer pairs. Both of these marker types were suitable for the identification of the clones. Polymorphic bands of a given RAPD primers may bind to many parts of the genome, so each primer may give information on the polymorphism of several chromosome regions. Microsatellite primers also provide a large quantity of polymorphic information, but this very detailed information only originates from a single hypervariable section of the genome. For this reason, microsatellite markers are ideal for distinguishing between genotypes that are genetically very similar. The joint use of various types of primers is an excellent way of identifying genotypes (Meszaros *et al*., 2007)

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