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Genetic Diversity Analysis of Wild Germplasm and Cultivated Clones of Hevea brasiliensis *Muell. Arg. by Using Microsatellite Markers*

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Twelve microsatellite markers were used to detect DNA polymorphism among 108 accessions of Hevea brasiliensis *inclusive of 40 cultivated (Wickham) clones and 68 wild accessions (1981 Amazonian accessions) collected from Amazon forest. In this study, the extent of genetic diversity among Wickham clones and wild accessions (1981 Amazonian accessions) in selecting suitable parents for enlarging genetic resource in Thailand's breeding program was evaluated. Genetic similarity values between genotypes calculated from all the microsatellite markers, were used to produce a dendrogram of the relationship among accessions, using the unweighted pair-groups method with arithmetic average. A total of 170 alleles were detected. The number of alleles ranged from 5 to 21, with an average of 14 alleles per marker. M574 marker demonstrated the highest polymorphism with 21 alleles, while M264 and M692 markers showed the lowest polymorphism with 5 alleles. The results clearly demonstrated that wild accessions were more polymorphic than cultivated Wickham clones and could be divided into three clusters, depending on the geographical origin of collection areas such as Acre, Rondonia and Mato Grosso state However, two Rondonia accessions (RO/OP/4 20/16 and RO/A/7 25/133) could not be discriminated into the suitable cluster according to the high level of specific alleles. Despite the narrow genetic basis of Wickham clones, their high level of polymorphism could be detected. The result indicated that a relatively small number of microsatellite markers could be used as molecular markers for* H. brasiliensis *genetic studies. Furthermore, this information could also be used to help in guiding the selection of suitable populations for enlarging genetic resource in breeding schemes.*

Key words: *Hevea brasiliensis*; microsatellite markers; Amazon forest; genetic diversity; Wickham clones; breeding; alleles; polymorphism

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Hevea or rubber (*Hevea brasiliensis* Muell Arg), the main source of natural rubber which is extensively cultivated in South-east Asia, is indigenous to the Amazon basin of South America The cultivated clones (known as Wickham clones) which were all derived from 22 seedlings surviving from the Wickham collection in 1876, showed a very narrow genetic base[1] Therefore, international surveys of *Hevea* wild germplasm have been conducted in the Amazon forest Exploitation of these genetic resources require information on the genetic diversity of germplasm[2-4] Traditionally, *Hevea* clones have been identified by using morphological and agronomic characters (*i e* leaves, branching, seeds characters and yield performance *etc*) Although, these characters are helpful in determining the phenotype variability among accessions, they are highly sensitive to environmental effects[5] Molecular markers have been developed to detect genetic variation of *Hevea* accessions, since they are more precise than morphological markers, unaffected by environmental effects and capable of plant genotypic characterisations at an early stage of development[5]

Isozyme electrophoresis successfully demonstrated and enabled the investigation of genetic diversity[6] and clonal identification[7] Minisatellite[8], Restriction Fragment Length Polymorphism (RFLP)[9,10], mitochondria DNA RFLP analysis[11], Random Amplified Polymorphic DNA (RAPD), DNA Amplification Finger-printing (DAF)[12] and Amplified Fragment Length Polymorphism (AFLP)[12,13] were developed and used in *Hevea* variability detection in order to improve the number of available molecular markers

Microsatellites, also known as simple sequence repeats (SSR), are based on tandem repeats of short (2 bp–6 bp) DNA sequence Microsatellites are very attractive to plant geneticists as they combine several features of utility markers, typically co-dominant, highly polymorphic and thus allow precise discrimination of even closely related individuals Also, microsatellites are abundant and uniformly dispersed in plant genome[14,15] Microsatellites have already been developed for several plants as in sorghum (*Sorghum bicolor*)[16,1], barley (*Hordeum vulgare*)[18], wheat (*Triticum dicoccoides*)[19], apple[20], cassava (*Manihot esculenta Crantz*)[21] and coconut (*Cocos nucifera L*)[22,23] In *H brasiliensis*, a number of microsatellite markers have been used to identify rubber clones[14] and construct a genetic linkage map[13]

In this study, we report on the application of microsatellite markers to evaluate genetic diversity of 108 *H brasiliensis* accessions, including 40 Wickham clones and 68 wild accessions collected from Amazon forest This study was done in order to use the information to select suitable parents for extending rubber genetic resource in future breeding programs

MATERIALS AND METHODS

Plant Material

Forty Wickham clones and sixty-eight wild accessions which were used in this study are listed in *Table 1* The wild accessions represent the 1981 Amazonian collection coming from 3 states in Brazil The 68 wild accessions could be divided as follows 26 are Acre, 20 are Rondonia and 22 are Mato Grosso accessions (details of all accessions are listed in *Table 1*) These accessions have been conserved in budwood gardens at Chachoengsoa Rubber Research Center, Chachoengsoa Province, Thailand

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TABLE 1. LIST OF WILD ACCESSIONS AND WICKHAM CULTIVATED CLONES OF *H. BRASILIENSIS*, USED IN THIS STUDY

No	Name	Type	Source	No	Name	Type	Source
1	AC/F/5	Wild	Acre / Feijo	55	MT/C/6	Wild	Mato Grosso /
2	AC/F/5	Wild	Acre / Feijo	56	MT/C/8	Wild	Mato Grosso /
3	AC/F/5	Wild	Acre / Feijo	57	MT/C/9	Wild	Mato Grosso /
4	AC/F/5	Wild	Acre / Feijo	58	MT/C/10	Wild	Mato Grosso /
5	AC/F/5	Wild	Acre / Feijo	59	MT/C/10	Wild	Mato Grosso /
6	AC/F/6A	Wild	Acre / Feijo	60	MT/IT/7	Wild	Mato Grosso /
7	AC/F/6A	Wild	Acre / Feijo	61	MT/IT/7	Wild	Mato Grosso /
8	AC/F/6A	Wild	Acre / Feijo	62	MT/IT/12	Wild	Mato Grosso /
9	AC/F/6A	Wild	Acre / Feijo	63	MT/IT/13	Wild	Mato Grosso /
1	AC/F/6A	Wild	Acre / Feijo	64	MT/IT/14	Wild	Mato Grosso /
1	AC/F/6B	Wild	Acre / Feijo	65	MT/IT/15	Wild	Mato Grosso /
1	AC/F/7	Wild	Acre / Feijo	66	MT/IT/16	Wild	Mato Grosso /
1	AC/F/1 5/4	Wild	Acre / Tarauaca	67	MT/IT/18	Wild	Mato Grosso /
1	AC/T/4	Wild	Acre / Tarauaca	68	MT/A/21	Wild	Mato Grosso /
1	AC/S/8	Wild	Acre / Sena	69	RRIT 13	Wickham	Thailand
1	AC/S/8	Wild	Acre / Sena	70	RRIT 21	Wickham	Thailand
1	AC/S/8	Wild	Acre / Sena	71	RRIT 156	Wickham	Thailand
1	AC/S/11	Wild	Acre / Sena	72	RRIT 218	Wickham	Thailand
1	AC/B/15	Wild	Acre / Brasileia	73	RRIT 225	Wickham	Thailand
2	AC/B/15	Wild	Acre / Brasileia	74	RRIT 226	Wickham	Thailand
2	AC/B/15	Wild	Acre / Brasileia	75	RRIT 250	Wickham	Thailand
2	AC/B/16	Wild	Acre / Brasileia	76	RRIT 251	Wickham	Thailand
2	AC/B/19	Wild	Acre / Brasileia	77	PB 5/51	Wickham	Malaysia
2	AC/B/19	Wild	Acre / Brasileia	78	PB 5/63	Wickham	Malaysia
2	AC/B/19	Wild	Acre / Brasileia	79	PB 217	Wickham	Malaysia
2	AC/X/20	Wild	Acre / Xapuri	80	PB 235	Wickham	Malaysia
2	RO/A/7	Wild	Rondonia /	81	PB 260	Wickham	Malaysia
2	RO/A/7	Wild	Rondonia /	82	PB 310	Wickham	Malaysia
2	RO/A/7	Wild	Rondonia /	83	PB 311	Wickham	Malaysia
3	RO/C/8	Wild	Rondonia / Calama	84	RRIM 600	Wickham	Malaysia
3	RO/C/8	Wild	Rondonia / Calama	85	RRIM 707	Wickham	Malaysia
3	RO/C/9	Wild	Rondonia / Calama	86	LCB 1320	Wickham	Indonesia
3	RO/C/9	Wild	Rondonia / Calama	87	Tjir 1	Wickham	Indonesia
3	RO/CM/10	Wild	Rondonia / Costa	88	AVROS	Wickham	Indonesia
3	RO/CM/10	Wild	Rondonia / Costa	89	AVROS	Wickham	Indonesia
3	RO/CM/11	Wild	Rondonia / Costa	90	BPM 1	Wickham	Indonesia
3	RO/CM/12	Wild	Rondonia / Costa	91	BPM 24	Wickham	Indonesia
3	RO/J/5	Wild	Rondonia / Jaru	92	GT 1	Wickham	Indonesia
3	RO/J/5	Wild	Rondonia / Jaru	93	PR 107	Wickham	Indonesia
4	RO/J/6	Wild	Rondonia / Jaru	94	PR 255	Wickham	Indonesia
4	RO/JP/3	Wild	Rondonia / Jiparana	95	PR 302	Wickham	Indonesia
4	RO/JP/3	Wild	Rondonia / Jiparana	96	PR 305	Wickham	Indonesia
4	RO/OP/4	Wild	Rondonia / Ouro	97	RRIC 7	Wickham	Sri Lanka
4	RO/OP/4	Wild	Rondonia / Ouro	98	RRIC 52	Wickham	Sri Lanka
4	RO/PB/1	Wild	Rondonia / Pimenta	99	RRIC 100	Wickham	Sri Lanka
4	RO/PB/2	Wild	Rondonia / Pimenta	10	RRIC 101	Wickham	Sri Lanka
4	MT/VB/25A	Wild	Meto Grosso / Vila	10	RRIC 110	Wickham	Sri Lanka
4	MT/VB/25A	Wild	Meto Grosso / Vila	10	Tian-ren	Wickham	China
4	MT/C/1	Wild	Meto Grosso /	10	SCATC 93-	Wickham	China
5	MT/C/2	Wild	Meto Grosso /	10	Hai ken 1	Wickham	China
5	MT/C/2	Wild	Meto Grosso /	10	Hai ken 2	Wickham	China
5	MT/C/3	Wild	Meto Grosso /	10	RRII 105	Wickham	India
5	MT/C/5	Wild	Meto Grosso /	10	RRII 118	Wickham	India
5	MT/C/5	Wild	Meto Grosso /	10	RRII 203	Wickham	India

Microsatellite Analysis

DNA samples were isolated from fresh young leaves using CTAB modified protocol[24] at DNA laboratory, Kasetsart University, Khamphaengaseng Campus, Thailand. Electrophoresis was carried out at CIRAD laboratory, Montpellier, France. The microsatellite primers used were described by Seguin *et al*[25]. Twelve primers were chosen in this analysis. Characteristics of these primers are listed in *Table 2*. PCR reactions were carried out in a 25 µL volume containing 100 ng of genomic DNA, 0.2 µM of each primer, 200 µM of each dNTP, and 1.5 U of Taq DNA polymerase, 1 × buffer (20 µM Tris HCl, 1.5 mM $MgCl_2$). PCR amplification consisted of an initial denaturation (94°C for 5 min), and 35 cycles each consisting of denaturation at 94°C for 1 min, annealing at 50°C/55°C for 45 sec, extension at 70°C/72°C for 1 min and a final extension at 70°C/72°C for 10 min. PCR amplified products were visualised following eletrophoresis on 5% denaturing polyacrylamide gels at 60 W constant power. Radiolabeled PCR products were obtained by labeling forward primers with γ [^{33}P] ATP using a T_4 polynucleotide kinase. Before loading, PCR amplified products were mixed with 1 volume of denaturing gel loading buffer [containing 10 mM EDTA, 98% formamide, 0.01% (W/V) xylene cyanol and 0.01% (W/V) bromophenol blue] and denatured at 94°C for 3 min. After electrophoresis, gels were transferred onto Whatman 3M paper, dried, and exposed to X-ray for 72 h.

TABLE 2. CHARACTERISTICS OF 12 MICROSATELLITE MARKERS

Primer (locus name)	Repeat sequence	Annealing / extension temp (°C)	Fragment size (bp)	Gene Bank accession no.
MnSOD	(GA)16	55 / 72	200	G73377
M124	(GTGGAC)33(N)12(GA) 8AA(GA)6	50 / 70	166	AF221697
M256	(GA)9	55 / 72	285	AF383930
M264	(AT)2(GT)6(AT)2	50 / 70	228	AF383931
M412	(GT)6(GA)8	50 / 70	258	AF383934
M425	(CA)6	50 / 70	187	AF383936
M574	(TA)10(GA)24	50 / 70	238	AF221706
M692	(T)12	50 / 70	247	AF383938
Ma31	(GA)22	50 / 70	154	AF383940
Ma66	(GA)18	50 / 70	275	AF383941
Mt65	(GT)18	50 / 70	196	AF383942
Mt67	(CA)21	50 / 70	120	AF383943

Data Analysis

The polymorphic information was scored based on the presence or absence of bands, and data were analysed using NTSYS-PC software version 2 01e[76] The presence or absence of each single fragment was detected visually using a light box and scored as 1 and 0 respectively, for the binary data matrix Three *Hevea* accessions (PB 260, GT 1 and RO 38), with known fragment size (Rodier-Goud, personal contact), were used as size references in this study Cluster analysis was based on similarity value using the unweighted pair-groups method with arithmetic average (UPGMA), and relationship between accessions were visualised as dendrogram A tree dendrogram was designed by using DICE coefficient from the same computer package

Genetic similarity between individuals were calculated for all according to Nei and Li[77] $GS = 2\ Nij\ /\ (Ni + Nj)$, where Nij is the number of fragments common to accession i and j, and $(Ni + Nj)$ is the total number of fragments in both accessions

RESULTS

Level of Polymorphism

A total of 170 alleles from 12 microsatellites markers were detected across all accessions The number of alleles per microsatellite marker ranged from 5 to 21 (*Table 3*) On average, 14 alleles were detected per locus For each marker, wild accessions showed higher polymorphism than the Wickham clones The most polymorphic microsatellite marker demonstrated in the Amazonian accessions was M574 with 21 alleles However, the MnSOD microsatellite marker demonstrated the highest polymorphism among Wickham clones with 10 alleles (*Table 3*) The M264 and M692 markers had the lowest polymorphism in all accessions with 5 alleles among wild accessions and 3 alleles within Wickham clones *Figure 1* shows the distribution of the 20 polymorphic allelic profiles, amplified with MnSOD primer among the 108 accessions

TABLE 3 NUMBER OF ALLELES OBSERVED IN 68 WILD ACCESSIONS AND 40 WICKHAM CLONES OF *H BRASILIENSIS*

Microsatellite marker	Number of alleles per locus		
	Wild accessions + Wickham clones	Amazonian accessions	Wickham clones
MnSOD	20	20	10
M124	16	16	4
M256	8	8	3
M264	5	5	3
M412	9	9	4
M425	18	15	9
M574	21	21	9
M692	5	5	3
Ma31	20	20	8
Ma66	19	19	7
Mt65	13	10	7
Mt67	16	15	4

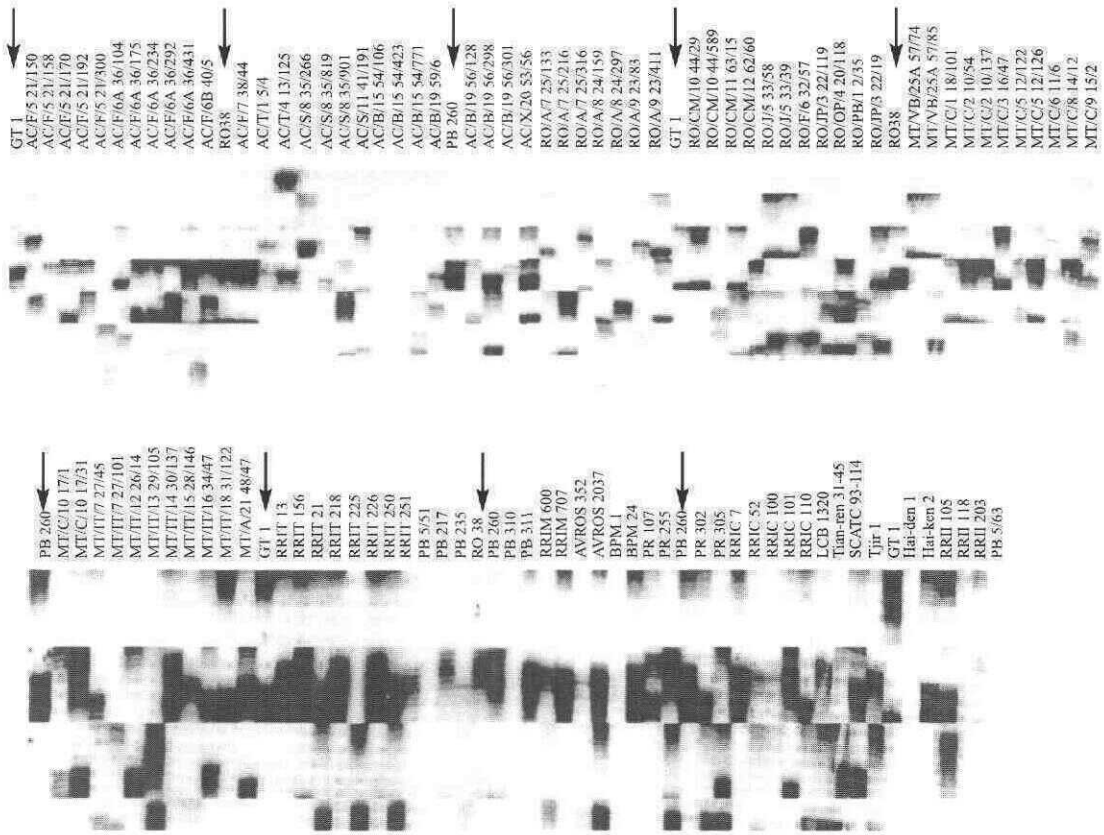

Figure 1. Polymorphism of 66 Amazonian accessions and 40 Wickham clones based on MnSOD microsatellite marker. Arrow lines are Hevea *reference clones (PB 260, GT 1 and RO 38).*

Relationship of Wild Accessions and Wickham Clones

Pair-wise comparisions were made between all accessions and average similarity values were calculated based on microsatellite polymorphic data. The genetic distance for all accessions ranged from 0.18 to 0.88 (*Figure 2*). Cluster analysis between wild accessions was carried out using UPGMA method and resulted in a dendrogram as shown in *Figure 3*. The dendrogram discriminated all of the wild accessions and revealed five distinct clusters. The first cluster comprised eighteen Acre, two Rondonia and two Mato Grosso accessions. This cluster are closely related with Cluster II, formed by sixteen Rondonia, two Acre and two Mato Grosso accessions. Cluster III consists of eighteen Mato Grosso and six Acre accessions. Finally, one Rondonia accession (RO/OP/4 20/16) formed cluster IV and was closely related with the accessions in Cluster III, while Cluster V also contained one Rodonia accession (RO/A/7 25/133) but was more

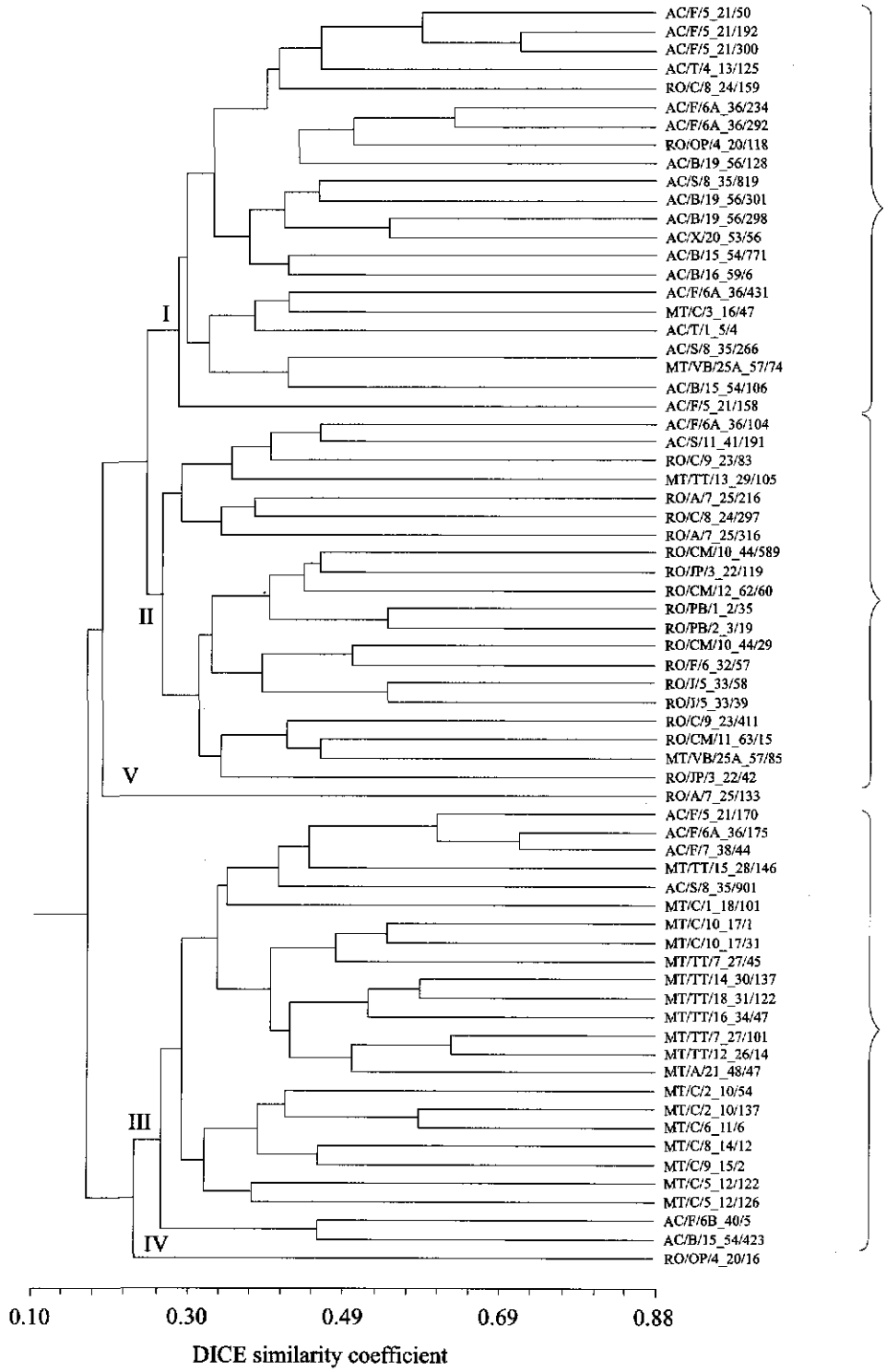

Figure 2. Similarity dendrogram of 68 H. brasiliensis *wild accessions, calculated from data of 12 microsatellite markers, using UPGMA as the clustering method.*

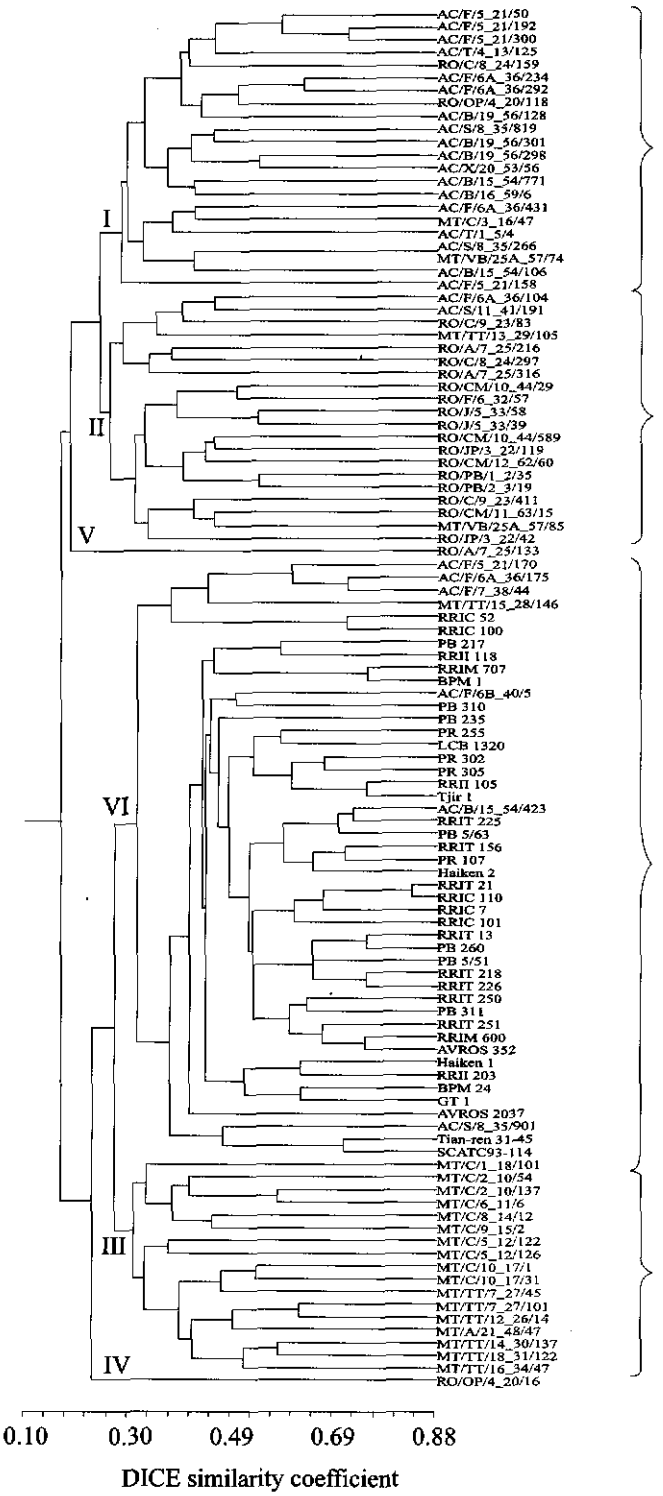

Figure 3. Similarity dendrogram of 68 H. brasiliensis *wild accessions, calculated from data of 12 microsatellite markers, using UPGMA as the clustering method.*

related with accessions Cluster I and II. The similarity between 2 accessions, AC/S/8 38/266 and MT/VB/25A 57/74 appeared interesting. Although these accessions were from different states, they were discriminated at the same level of similarity because they had the same allelic profiles after detecting with all 12 primers (data not shown), whereas the other Vila Bela district accessions of Mato Grosso state (MT/VB/25A 57/85) were separated in Cluster II.

When Wickham clones were added to the analysis, these clones were discriminated as Cluster VI (*Figure 3*). This cluster demonstrated the lowest variability with genetic distance ranging from 0.36 to 0.83 and a close relationship with Mato Grosso germplasm.

DISCUSSION

Many genetic diversity studies in plants are available based on microsatellite markers. For instance, genetic diversity analysis in soybean (*Glycine max*)[14]. sorghum (*S. bicolor*)[16,17], wheat (*T. dicoccoides*)[19], cassava (*M. esculenta Crantz.*[21]), barley (*H. vulgare*)[18]. sugar beat (*Veta vulgaris* L.)[28], apple[20], rice (*Oryza sativa* L.)[29] and coconut (*C. nucifera* L.)[22,23] For *H. brasiliensis*, several molecular markers such as isozyme[6], minisatellite[8] and RFLP[10] are used for genetic diversity analysis. Micro-satellites were also used for clonal identification[12] and construction of linkage maps in rubber genome mapping study[5,13]. The objective of this study was carried out to evaluate the extent of *H. brasiliensis* genetic diversity among wild accessions (1981 Amazonian accessions) and Wickham clones, in order to select suitable accessions for expanding genetic resource in breeding program and use the germplasm information for conservation. Twelve microsatellite primers used in this study were provided by Dr. Marc Seguin (CIRAD laboratory). Most of these markers were mainly (GA)n repeats[25,13]. All microsatellite markers are referenced in EMBL/GeneBank (*Table 2*).

The comparison between wild accessions and cultivated clones showed unambiguously that cultivated Wickham clones were less variable than wild accessions and closely related to Mato Grosso accessions. Similar results have already been detected by isozyme[6] and RFLP[10].

Genetic diversity of wild accessions were clearly separated into three clusters on the basis of geographical origin of the collection areas: Acre, Rondonia and Mato Grosso state. Acre and Rondonia accessions are the most polymorphic, according to the appearance of more specific alleles in these accessions than in Mato Grosso accessions. This result is different from the RFLP study that Rondonia and Mato Grosso states were the most polymorphic[10]. It may be the result of different mutation detection; RFLP variations are mostly due to base-change mutation, whereas with microsatellites, the polymorphism between different individuals was due to the variations in the number of repeat units and each locus could have many alleles.

Moreover, from the dendrogram, there were some heterogenous accessions in each cluster, for example, there were two Rondonia accessions (RO/C/8 24/159 and RO/OP/4 20/118) and two Mato Grosso accessions (MT/C/3 16/47 and MT/VB/25A 57/74) in Cluster I which mainly consisted of Acre accessions, due to similar allelic profiles. Increasing the large number of polymorphic microsatellite primers to investigate all accessions might give better solutions.

Two Rondonia germplasm, RO/OP/4 20/16 and RO/A/7 25/133 were individuals not belonging to any suitable clusters because of the significant difference of specific alleles, particularly, with M256, M264, M425, M692 and Mt67 microsatellite markers This indicated that both these Rondonia clones might have an interspecific origin between Mato Grosso and Rondonia states In the case of the clones AC/S/8 35/266 and MT/VB/25A 54/74, a high similarity with all 12 microsatellite markers was shown, in spite of originating from different states It is possible that there was an error during propagation in the budwood garden

Despite their narrow genetic base and high level of inbreeding, cultivated Wickham clones showed a high level of polymorphism with 9 microsatellite markers (except M256, M264 and M692) This result was also obtained in other studies by using different types of genetic markers, including isozyme[9] and RFLP analysis[10] This result contributed to the idea that Wickham clones could be identified using nine microsatellite markers, thus providing powerful information for the *Hevea* breeder However to ascertain the genetic diversity in all these accessions, much more polymorphic primers should be used to investigate polymorphism

In conclusion, the present study had clearly demonstrated that wild accessions (1981 Amazonian accessions) were more polymorphic than cultivated Wickham clones and could be divided into three groups depending on geographical location of collection areas This result could be a guide in choosing the suitable population for recurrent breeding schemes Despite the narrow genetic diversity of the Wickham clones, high level polymorphism could be detected It was proven that microsatellite markers could be used as molecular markers for *H brasiliensis* genetic studies such as clonal identification and genetic diversity study

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