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Analysis of Genetic Diversity and SSR Allelic Variation in Rubber Tree (*Hevea brasiliensis*)

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1. Introduction

Rubber tree, *Hevea brasiliensis*, belongs to the family of Euphorbiaceae, originated from Amazon Trends. The family has ten varieties (*H. brasiliensis*, *H. nitida*, *H. pauciflora*, *H. spruceana*, *H. benthamiana*, *H. camporum*, *H. microphylla*, *H. rigidifolia*, *H. guianensis*, *H. comargcana*) and four variation varieties (*H. guianensis* var. *luter*, *H. guianensis* var. *marginata*, *H. parciflora* var. *coriacea*, *H. nitida* Mart var. *toxicadendroides*). *H. brasiliensis* is the most economically important member of the genus *Hevea*, because its economic importance and its sap-like extract (as latex) can be collected and is the primary source of natural rubber. There are many rubber tree germplasm resources in Brazil, Malaysia, Indonesia, India, French, and China.

Simple sequence repeat (SSR) marker has been used as an ideal molecular marker to investigate the genetic diversity because of its multi-allelic nature, reproducibility, co-dominant inheritance, high abundance and extensive genome coverage (Gupta & Varshney, 2000) in many crops, such as rubber tree (Lekawipat et al., 2003; Saha et al., 2005; Gouvêa et al., 2010), wheat (Liu et al., 2007; Hao et al., 2006), bean (Choi et al., 1999), barley (Brantestam et al., 2007), cole (Hasan et al., 2006), jowar (Marco et al., 2007), triticale (Tams et al., 2004), rice (Song et al., 2003) and coffee (Aggarwal et al., 2007).

Several researchers have investigated the genetic diversity of rubber tree by using molecular markers (Lekawipat et al., 2003; Saha et al., 2005; Lam et al., 2009; Gouvêa et al., 2010; Oktavia et al., 2011), but there was no report about the polymorphism of lower repeats SSR markers and the loci and the flanking area variation of SSR markers in rubber tree. SSR marker used to detect the alleles by PAGE gel after PCR has slight restrictions in distinguishing the fragments as length or size homoplasy (Estoup et al., 1995; Grimaldi & Crouau-Roy, 1997; Angers & Bernatchez, 1997). However, sequencing of repeats and flanking regions can help detect the difference of the alleles exactly (Xie et al., 2006; Feng et al., 2008).

In this study, 16 primer pairs from genome and EST-SSR amplified across the popular cultivars cultivated in China, wild accessions and interspecies. There were three main objectives: (1) detect the genetic diversity and relationships between cultivars and wild accessions in rubber tree, (2) to investigate the polymorphism of low repeat SSR marker, and (3) analyze the loci variations in rubber tree.

2. Materials and methods

2.1 Plant materials and SSR markers

Forty-five cultivars which are the main cultivars in China, 11 wild accessions from Brasil and 3 related species were used in this study (Table1). Fresh leaves were collected in bronze period from Rubber Research Institute, Chinese Academy of Tropic Agricultural Science (Danzhou) and stored at -20°C after washing by pure water. Leaf genomic DNA was extracted following the CTAB protocol (Venkatachalam et al., 2002).

Sixteen SSR primer pairs (Table 2) were used, including 10 EST-SSRs (SSR from expressed sequence tag) (Feng et al., 2009; Unpublished), 6 genomic-SSRs (<http://tropgenedb.cirad.fr/en/rubbertree.html>). The four effective EST-SSR primer pairs were (1) HBE280 (gga cac ctg gag caa aat ag & tat gct tcg atg tat att cac agt: [(gaaa)₄]) ; (2) HBE301 (ggc ata caa gaa aaa aat tt & taa gga ttg acg gct acg: [(cagcaa)₅]); (3) HBE316 (cga caa cca gga act tac c & aaa caa ctg cgg agg att: [(tctgt)₄]); (4) HBE329 (cca aaa caa ggg aaa tca c & gac cga gac gct tag ttc: [(aga)₉]); All primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

No.	materials	scientific name	Pedigree/source
1	RRIM600	<i>H. brasiliensis</i>	Tjir 1×PB86
2	Zhenxuan1		PB28/59×RRIM60
3	Dafeng117		RRIM513×PR107
4	Reyan88-13		RRIM600×PilB84
5	Baoting155		RRIM600×PR107
6	Haiken2		PB86×PR107
7	Wenchang217		Haiken1×PR107
8	Reyan7-18-55		RRIM600×PR107
9	IAN873		PB86×PR107
10	PB86		original clone
11	Tianren31-45		original clone
12	Reyan7-33-97		RRIM600×PR107
13	Gunagxi6-68		original clone
14	PB5/51		PB56×PB24
15	GT1		original clone
16	PR107		original clone

No.	materials	scientific name	Pedigree/ source
17	Yunyan68-273	<i>H. brasiliensis</i>	GT1×PR107
18	Reyan217		RRIM600×PR107
19	Dafeng99		PB86×PR107
20	Reyan7-20-59		RRIM600×PR107
21	Dafeng95		PB86×PR107
22	Hekou3-11		original clone
23	Tjir 1		original clone
24	Hongxing 1		original clone
25	Haiken 1		Unknown
26	RRIM712		RRIM605×RRIM71
27	RRIM513		Pil B16×Pil A 44
28	Haiken 6		PB86×PR107
29	Baoting 3410		RRIM600×PR107
30	Wenchang 7-35-11		PB5/51×PR107
31	Baoting 911		RRIM600×PR107
32	Tianren 93-114		Tianren31-45×Hekou3-11
33	Wenchang 193		PB5/51×PR107
34	Wenchang 11		RRIM600×PR107
35	Daling 68-35		Qiaozhi42-67×PB86
36	PB 28/59		Unknown
37	PB 5/63		PB56×PB24
38	Yunyan 277-5		PB5/63×Tjir 1
39	Reyan 8-333		Reyan88-13×Reyan217
40	Baoting 235		RRIM600×PR107
41	Reyan8-79		Reyan88-13×Reyan217
42	Reken525		IAN873×RRIM803
43	Reken523		IAN873×PB260
44	Yunyan77-2		GT1×PR107
45	PB217		PB5/51×PB6/9
46	AC/T/15/114		Brazil
47	AC/S/1037/297		Brazil
48	RO/CM/1163/17		Brazil
49	RO/C/923/176		Brazil
50	MT/C/1017/88		Brazil
51	MT/IT/1634/209		Brazil
52	AC/AB/1554/16		Brazil
53	MT/C/210/28		Brazil
54	MT/IT/1634/192		Brazil
55	RO/A/725/121		Brazil
56	MT/IT/1430/118		Brazil
57	Sebao rubber	<i>H. spruceana</i>	Brazil
58	Guangye rubber	<i>H. nitida</i>	Brazil
59	Bianqin rubber	<i>H. benthamiana</i>	Brazil

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Table 1. Plant materials used for analysis of genetic biodiversity and loci variation

2.2 PCR amplification and detection of fragments

All primers were amplified in the TaKaRa PCR Thermal Cycler Dice, each PCR reaction consisted of: 2 μ l of 10 \times buffer, 0.25 μ l of 10 M dNTPs, 1 μ l each of forward and reverse primer (20 μ mol), 2 μ l of template leaf genomic DNA (20 ng/ μ l), 0.15 μ l of Taq polymerase (5 U/ μ l) (TAKARA Biotechnology (Dalian) Co. Ltd), ddH₂O added to a total reaction volume of 20 μ l. The PCR reaction profile was pre-denatured at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 sec, annealing temperature for 45 sec and 72 °C for 1 min and finally, 72 °C for an extension of 5 min. To ensure precision and reproducibility of fragments, DNA samples were amplified and analyzed at least twice from each individual sample.

2.3 Cloning and sequencing of SSR alleles

Five EST-SSR markers (HBE008, HBE063, HBE164, HBE187, and HBE199) were selected to investigate SSR loci variation. Of the five primer pairs, the repeat motif of HBE008 and HBE187 was (CT) *n*, HBE164 and HBE199 was (AG) *n*. The selected alleles were amplified, recovered, purified, cloned and sequenced.

Alleles from these SSR loci were cut from the dried PAGE gels and used as templates for a new round of PCR amplifications. Each of these alleles was directly cloned into the pGEM-T Easy Vector (Promega, USA) according to the manufacturer's instructions, and transformed into *Escherichia coli* DH5 α cell. The positive clones were sequenced using the ABI PRISM 3730 sequencer. To obtain reliable sequences, at least three clones per allele were sequenced. The nucleotide sequences were aligned using Clustal X (<http://www.ftp-igbmc.u-strasbg.fr/pub/ClustalX/>) to compare the amplified SSR alleles with the SSR-containing ESTs to investigate the loci variation.

2.4 Data analysis

Sixty-two genomic DNA of cultivars and wild accessions were amplified with 16 primer pairs, and visualized on PAGE gel with silver stain. These bands were recorded as "1" for presence, "0" for absence, "999" for missing data. Number of alleles, observed heterozygosity (*Ho*) and power of discrimination (PD) were calculated for each locus. *Ho* was calculated as the number of genotypes which were heterozygous at a given locus divided by the total number of genotypes surveyed at that locus. PD was calculated as $1 - \sum G_{ij}^2$ (Kloosterman et al., 1993), where G_{ij} is the frequency of the *j*th genotype for the *i*th locus summed across all alleles at that locus. Genetic similarity (GS) between any two pairs of the 56 cultivars and wild accessions was calculated from the alleles across the 16 SSR loci using the Jaccard similarity coefficients (Sneath & Sokal, 1973). A dendrogram was constructed with the un-weighted pair group method with arithmetic averages (UPGMA) on the basis of the similarity coefficients. All these analyses were performed with NTSYS-pc 2.10 software package.

3. Results

3.1 Polymorphic analysis of EST- and gSSR markers

Sixteen SSR primer pairs, of which ten from EST-SSRs and six from genome, could successfully amplify, expected products across 45 cultivars, 11 wild accessions and 3 related species (Table

2). A total of 43 alleles were obtained from 10 EST-SSR primer pairs across cultivars and wild accessions, with an average of 4.3 alleles and $H_o = 0.488$. Ten alleles were amplified by HBE280, followed by HBE199 with 5 alleles, and HBE164 and HBE316 with 2 alleles each, respectively. Six alleles were amplified by HBE008 and HBE280 in wild accessions, respectively, followed by HBE199 with 5 alleles and HBE316 with 2 alleles. A total of 30 alleles were obtained from 6 gSSR primer pairs, with an average of 5 alleles and $H_o = 0.743$. Six alleles were amplified by M197 and MnSOD, respectively in cultivars, 6 alleles by MnSOD as well as in wild accessions. And the other primer pairs were amplified 4 or 5 alleles in cultivars and wild accessions, respectively. HBE280, M197 and MnSOD were the most informative.

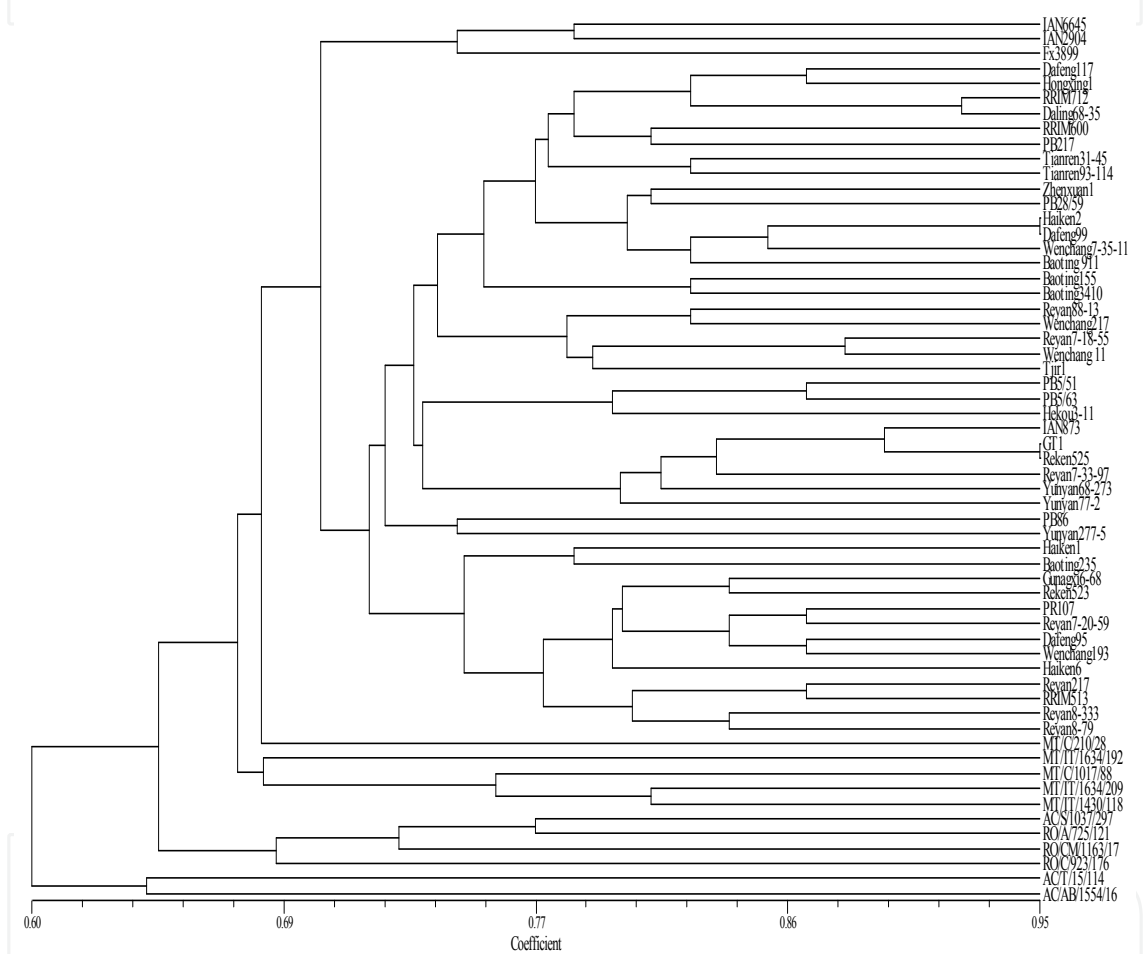


Fig. 1. UPGMA dendrogram of genetic relationship among cultivars and wild accessions of rubber tree based on analysis using ten EST- and six genomic SSR markers.

For cultivars, the ten EST-SSR markers produced a total of 40 alleles with an average of 4 alleles, $H_o = 0.495$ and $PD = 0.591$ per locus; and the six gSSRs produced a total of 30 alleles with an average of 5 alleles, $H_o = 0.739$ and $PD = 0.681$ per locus (Table 2). For the wild accessions, the ten EST-SSR markers produced a total of 38 alleles with an average of 3.8 alleles, $H_o = 0.455$ and $PD = 0.616$ per locus; and the six gSSRs produced a total of 28 alleles with an average of 4.7 alleles, $H_o = 0.758$ and $PD = 0.685$ per locus (Table 2). HBE280 was the most informative among the EST-SSRs, and MnSOD was the most informative among the genomic SSR markers.

Of the 16 primer pairs, only PD value of HBE280 was larger than 0.8 (0.834). PD values of the ten EST-SSRs ranged from 0.420 to 0.834 and the average was 0.597; and the PD values of the six genomic-SSRs ranged from 0.587 to 0.772 and the average was 0.689.

3.2 Analysis of genetic diversity

The Jaccard similarity coefficient for the 16 SSR markers was used to analysis the genetic similarities (GS) among the 45 cultivars and 11 wild accessions. In comparison with the 56 accessions, GS values of cultivars ranged from 0.51 (Reken523 and PB5/63) to 0.95 (Reken525 and GT1, Haiken2 and Dafeng99). GS values between cultivars and wild accessions ranged from 0.48 (AC/T/15/114 and Wenchang217) to 0.79 (MT/IT/1430/118 and Tianren31-45).

UPGMA cluster analysis based on GS values for comparisons among all samples was used to construct a dendrogram (Fig. 1) with the cophenetic value of 0.854, indicating a high level of reliability. The 45 cultivars and 11 wild accessions were clustered into two categories. Wild accessions were distinguished from cultivars on the level of similarity coefficient 0.68. On the level of similarity coefficient 0.73, cultivars were divided into four groups, IAN6645, IAN2904 and Fx3899 in group I, PB86 and Yunyan277-5 in group III, Haiken1, Baoting235, Gunagxi6-68, Reken523, PR107, Reyan7-20-59, Dafeng95, Wenchang193, Haiken6, Reyan217, RRIM513, Reyan8-333 and Reyan8-79 in group IV. Group II contained mostly cultivars except those in group I, III and IV. On the level of similarity coefficient 0.77, Group II can be divided into six sub-groups. Group IV can be divided into three sub-groups on the level of similarity coefficient 0.78.

All accessions could be distinguished from each other on the level of similarity coefficient 0.95 except Haiken2 and Dafeng99, GT1 and Reken525.

3.3 Detection of loci variation

Substantial sequence variation was found in the 61 sequences of 5 EST-SSR loci. The insertion, deletion, transition and transversion can be found in these sequences (Fig. 2).

The original EST sequence (the RefEST) ID of HBE008 is EC609578.1 in Genebank and the SSR repeat loci is (CT)₂₁(AT)₁₄. In comparison with EC609578.1, 1~6 bp deletions were happened at the AT repeat loci of *H. nitida*, IAN2904, Fx3899, AC/T/15/114 and MT/IT/1634/192, and transversions were also observed at the CT and AT repeat loci of Fx3899, *H. spruceana* and AC/T/15/114, the CT repeat lost 1~3 bp and C was replaced by G or A in sequences of *H. nitida*, IAN2904, MT/IT/1634/192, AC/T/15/114, *H. spruceana* and Fx3899.

The original EST sequences (the RefEST) ID of HBE063 is EC607362.1, and the SSR repeat loci is (GA)₁₆. In comparison with EC607362.1, insertions were observed from the repeat sequences of Zhenxuan1, Hekou3-11, Yunyan77-2, Reyan88-13, Baoting155 and RRIM712, and deletions were observed at all other sequences except RRIM600, and transversions were also observed from the flanking regions of Hekou3-11, Reyan88-13, Baoting155, Fx3899, RRIM712, RO/C/923/176, MT/IT/1634/209 and MT/IT/1430/118 too.

The original EST sequences (the RefEST) ID of HBE164 is EC603146.1, and the SSR repeat loci is (AG)₆. In comparison with EC603146.1, no variation was observed from all repeat

regions, and transversions were observed from the flanking regions of all sequences and insertions were found in the flanking sequences of Dafeng117 and AC/T/15/114. The original EST sequences (the RefEST) ID of HBE187 is EC601817.1, and the SSR repeat loci is (CT)₆. In comparison with EC601817.1, no variation was observed from all repeat regions, but deletions were observed from the flanking sequences of RRIM712, Dafeng99, Hongxing1, AC/T/15/114, MT/IT/1634/192, MT/IT/1430/118, Reken525, IAN873, IAN2904 and *H. nitida*. However, transversions were found in the flanking regions of RRIM712, AC/T/15/114, MT/IT/1634/192, MT/IT/1430/118, *H. nitida* and Reken525.

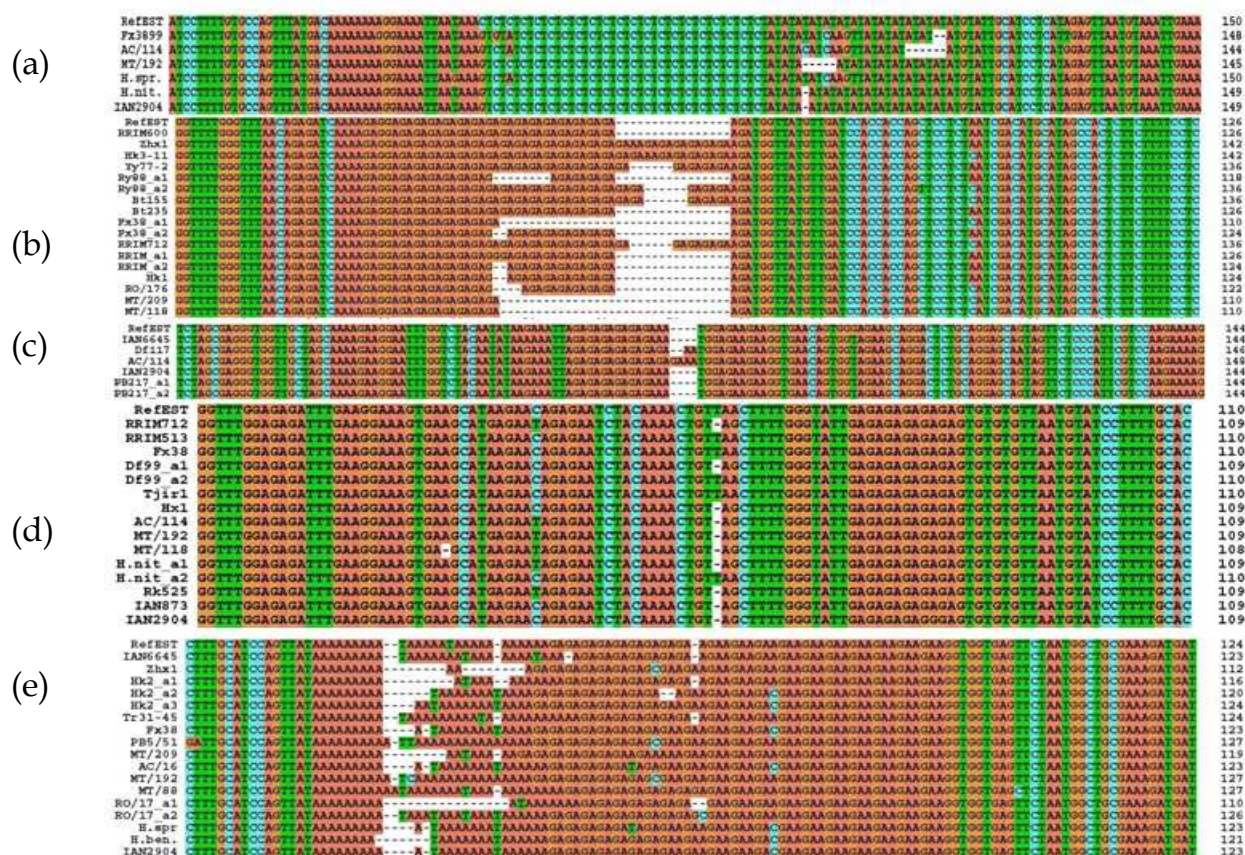


Fig. 2. Sequences obtained using 5 EST-SSRs markers amplifying across *H. brasiliensis*, wild *H. brasiliensis* and interspecies. (a), (b), (c), (d) and (e) (from the top down) represent the markers HBE008, HBE063, HBE164, HBE187 and HBE199, respectively; RefESTs in (a), (b), (c), (d), (e) represent the accession numbers: EC609578.1, EC607362.1, EC603146.1, EC601817.1 and EC600890.1 in NCBI database, respectively. The suffix 'a1', 'a2' and 'a3' represent the allele numbers. AC/114, MT/192, RO/176, MT/209, MT/118, RO/17, MT/88, AC/16, Zhx1, Ry88, Bt155, Hk3-11, Hk1, Bt235, Yy77-2, Fx38, Df117, Df99, Hx1, Rk525, Hk2, Tr31-45, RRIM, H.spr., H.nit., H.ben., represent AC/T/15/114, MT/IT/1634/192, RO/C/923/176, MT/IT/1634/209, MT/IT/1430/118, RO/CM/1163/17, MT/C/1017/88, AC/AB/1554/16, Zhenxuan1, Reyan88-13, Baoting155, Hekou3-11, Haiken1, Baoting235, Yunyan77-2, Fx3899, Dafeng117, Dafeng99, Hongxing1, Reken525, Haiken2, Tianren31-45, RRIM513, *H. Spruceana*, *H. Nitida*, *Hevea. Benthiana*, respectively.

Sequence ID	Marker	SSR motif	No of alleles			H_o			PD		
			Cultivars	Wild accessions	Total	Culti- vars	Wild access- ions	Total	Culti- vars	Wild access- ions	Total
EST-SSR markers											
EC609578.1	HBE008 ^{a,f}	(CT)21(AT)14	4	6	7	0.674	0.455	0.633	0.669	0.641	0.699
EC609548.1	HBE010 ^a	(AG)14	3	3	3	0.456	0.636	0.490	0.634	0.642	0.637
EC607362.1	HBE063 ^{a,f}	(GA)16	3	4	4	0.278	0.273	0.277	0.734	0.734	0.590
EC603146.1	HBE164 ^{a,f}	(AG)6	2	3	3	0.304	0.636	0.366	0.371	0.649	0.493
EC601817.1	HBE187 ^{a,f}	(CT)6	3	3	3	0.413	0.273	0.387	0.430	0.357	0.420
EC600890.1	HBE199 ^{a,f}	(A)11(AG)9*(AGA)11*	5	5	5	0.870	0.727	0.843	0.737	0.787	0.748
EC605179.1	HBE280 ^b	(GAAA)4	10	6	10	0.696	0.545	0.668	0.840	0.799	0.834
EC602073.1	HBE301 ^b	(CAGCAA)5	3	3	3	0.391	0.182	0.352	0.439	0.556	0.488
EC600641.1	HBE316 ^b	(TCTGT)4	2	2	2	0.348	0.364	0.351	0.427	0.391	0.429
CB376545.1	HBE329 ^b	(AGA)9	3	3	3	0.522	0.455	0.510	0.628	0.602	0.636
Average			4.0	3.8	4.3	0.495	0.455	0.488	0.591	0.616	0.597
Genomic SSR markers											
AF221698	M127 ^{c,d,e}	(GA)13	5	5	5	1.000	1.000	1.000	0.750	0.710	0.751
AF221711	M197 ^{c,d,e}	(GA)10	6	4	6	0.609	0.636	0.614	0.716	0.560	0.681
AF221705	M508 ^{c,d,e}	(TAA)21 + (TA)6	4	4	4	0.435	0.727	0.489	0.541	0.690	0.587
AF221707	M613 ^{c,d,e}	(GA)23+(GA)8	4	4	4	0.565	0.455	0.544	0.608	0.602	0.626
AF383936	mHBCIR4 ^{c,d,e}	(CA)6	5	5	5	0.826	0.727	0.808	0.711	0.744	0.719
G73377	MnSOD ^{c,d,e}	(GA)16	6	6	6	1.000	1.000	1.000	0.759	0.802	0.772
Average			5	4.67	5	0.739	0.758	0.743	0.681	0.685	0.689
Average(all markers)			4.38	4.13	4.56	0.587	0.568	0.549	0.625	0.642	0.632

^aFeng et al., 2009
^bFeng et al.,data not shown
^cSeguin et al., 1996
^dLespinasse et al., 2000
^eSeguin et al.,2002
^fUsed for amplified sequence analysis

Table 2. No. of alleles, observed heterozygosity (H_o) and power of discrimination (PD) of EST- and genomic SSR markers among *H. brasiliensis*

The original EST sequences (the RefEST) ID of HBE199 is EC600890.1, and the SSR repeat loci is (A)₁₁(AG)₉*(AGA)₁₁. In comparison with EC600890.1, deletions were observed from the AG repeat regions of IAN6645 and Haiken2, and transversions or transitions were also observed from the AG repeat regions of Zhenxuan1, PB5/51, MT/IT/1634/192, AC/AB/1554/16 and *H. spruceana*. Deletions, insertions, and transversions or transitions were found in the flanking regions of all sequences.

4. Discussion

4.1 Polymorphism of SSR marker

Feng et al. (2009) developed 87 EST-SSR primer pairs by analyzing the NCBI database, and the criteria of searching SSR-ESTs was using mononucleotide repeats ≥ 10 , dinucleotide to hexanucleotide repeats ≥ 6 . But in this study, the search criteria of EST-SSR was changed and 108 novel EST-SSR primer pairs were developed by the same method previous mentioned, of which 4 were selected to analysis the genetic diversity of cultivated varieties and wild accessions. HBE280 appeared to have the most informative despite its repeat number was 4 and the other three novel EST-SSR primer pairs (HBE301, HBE316) also showed different polymorphisms, which were similar to the results of Steinkellner et al. (1997) studied in oak. The four novel primer pairs could be useful as molecular markers in the future study for rubber tree.

Generally, higher-order repeat motifs, which refers to repeat motif more than three bases as well as the compound repeat, have lower polymorphism than lower-order repeat ones (Dreisigacker et al., 2004; Feng et al., 2009). In this study, higher polymorphism was observed in HBE280 [(gaaa)₄] which contained only four times repeats, and this may be due to more A/T content in the repeat unit GAAA/CTTT which deduced a replication origin during DNA replication, and the mismatch cannot be repaired easily because of the existence of rows of (A/T) n.

4.2 The polymorphism of EST- and gSSR

SSR molecular markers have been widely used to distinguish crop genotypes (Sun et al., 1999; Virk et al., 1999; Eujayl et al., 2001; Eujayl et al., 2002) and study genetic diversity (Song et al., 2003; Hao et al., 2006; Liu et al., 2007; Caniato et al., 2007). In recent years, a large number of EST- and gSSR molecular markers for many crops were developed (Davierwala et al., 2001; Varshney et al., 2005; Aggarwal et al., 2007). However in rubber tree study, the earliest SSR markers developed by Lespinasse et al. (2000) were from the genomic DNA. Recently, Feng et al. (2009) developed 87 EST-SSR markers from NCBI database. Because of the conservative sequences of ESTs, The level of polymorphism in EST-SSR was lower compared to that of genomic-SSR marker (Eujayl et al., 2002; Leigh et al., 2003; Gonzalo et al., 2005; Yang et al., 2005; Pinto et al., 2006). In this study, gSSRs produced more polymorphisms than EST-SSR as well as PD was slightly higher, despite HBE280 was the most informative marker which belonged to EST-SSRs.

All the SSR primer pairs could be amplified successfully through the 3 related species, and there were no significant differences for transferability between EST- and gSSR, and this was different from other reports (Liewlaksaneeyanawin et al., 2004; Feng et al. 2009). This may due to the relevant lower number of SSR primer pairs.

4.3 Diversity analysis and genetic relationship within/between cultivars and wild accessions

Crop genetic diversity is the basis of genetic improvement of crops, and the study was of great significance in the collection, preservation, evaluation and utilization of crop germ plasm resources. In the past few years, genetics investigations of *H. brasiliensis* have been

studies (Seguin et al., 1996; Chevallier et al., 1998; Seguin et al., 2002), Lekawipat et al. (2003) used twelve microsatellite markers to detect DNA polymorphism among 108 accessions of *H. brasiliensis* inclusive of 40 cultivated (Wickham) clones and 68 wild accessions (1981 Amazonian accessions) collected from Amazon forest, and they found 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. In this study, the similar results were also reached and 16 EST- and gSSR molecular markers were used to detect the genetic diversity within/between 45 cultivars planted in China and other countries, of which 33 were cultivars of China, and 11 wild accessions. The results showed that wild accessions were more polymorphic than cultivars, and the analysis of rubber tree germplasm resources by SSR markers consisted with the pedigree analysis approximately, a true reflection of genetic variation and relationships for rubber tree. For example, IAN6645, IAN2904 and FX3899 were clustered together, and they are good clones from Brazil, in which IAN6645 is a descendant from an FX43.655 [FX213 (F4542×AVROS183) ×AVROS183] ×PB86 cross, IAN2904 from FX516 (F4542×AVROS363) ×PB86, FX3899 from F4542×AVROS363, which all have the same parent F4542. The clustering result consisted with the pedigree analysis. PB5/51 and PB5/63 were clustered together, which are high-yielding clones in Malaysia, and they are the descendant from a common PB56×PB24 cross and the clustering result consisted with the pedigree analysis as well; Haiken2, Dafeng99 and Wenchang7-35-11 were clustered together, and the similarity coefficient between Haiken2 Dafeng99 was 0.95, and they are the descendant from a common PB86×PR107 cross. Wenchang7-35-11 from PB5/51×PR107, these three wind-resistant and high-yielding clone have the same paternal PR107, which is a wind-resistant and high-yielding clone, and the high-yielding maternal PB86 and PB5/51, the clustering result consisted with the pedigree analysis as well. IAN873, GT1, Reken525 and Reyan7-33-97 were clustered together; these results consisted with Feng et al. (2009). At the same time the descendants from the same cross would not be clustered in the same category or nearest position, such as Dafeng99 and Dafeng95 bred by Haiken Dafeng Farm, Hainan, from the same PB86×PR107 cross, and the similarity coefficient between them was 0.72, but they were clustered in group II and IV respectively, more variations would be in F1 hybrid generation. Baoting155, Reyan7-18-55, Reyan7-33-97, Reyan7-20-59, Baoting3410, Baoting911, Wengchang11 and Baoting235 are descendants from the same RRIM600×PR107 cross, but the similarity coefficient between them varied from 0.57 to 0.88, and they were clustered in different group or sub-group. The reasons of inconsistent with the pedigree may be due to that rubber trees are cross-pollinated crops and there is a long-term natural hybridization among the population. The variations between descendants from the different species cross may be from their parents or separation.

With the development of the forest breeding, the consistency of bred varieties increased gradually, resulting in the narrow basis of genetic breeding. Therefore, the reasonable utilization of wild germplasm resources may be an effective way to breeding improvement and widen genetic basis (Benong, 2002; Aidi-Daslin, 2002; Clement-Demange, 2002; Varghese, 2002). In our study, the genetic diversity of wild accessions was higher than the cultivars, which consisted with Besse et al. (1993, 1994). Similarity coefficient between cultivars ranged from 0.51 to 0.95, with an average of 0.73, and the variations of the descendants from those crosses with high similarity coefficient would be limited. It was difficult to breed breakthrough varieties. The similarity coefficient between the cultivars and

wild accessions was 0.48~0.81, which may provide possibility for the selection of elite parents with improved genetic basis (Varghese, 2002).

It was difficult to distinguish Haiken2 from Dafeng99, GT1 from Reken525 on the level of similarity coefficient of 0.95. Feng et al. (2009) also failed to distinguish GT1 from Reken525 on the level of similarity coefficient of 0.96 by 87 EST-SSR markers, however, according to Feng et al. (2008), there were both the same repeat number and structure in GT1 and Reken525, and the point mutations were found in the flanking regions by sequencing in the HBE156 loci.

The methods of detecting genetic diversity, whether on the level of morphology, cytology (chromosome), physiology, biochemical or even now molecular method, each had its own advantages and limitations in theory or practice, and they cannot be replaced with each other completely. Therefore, using different method reflecting by agronomic characterization or molecular data, the decision should be based on one or several methods selected in detecting and evaluation of the genetic diversity. Analysis of genetic diversity may be help to select the elite and fit parents for improving breeding efficiency.

4.4 Detection of loci variation

As exactly as most crops (Fraser et al., 2004; Jung et al., 2005; Aggarwal et al., 2007), Feng et al. (2009) reported that DNRs were the main repeat motif, and AG / TC were the predominant DNRs; In this study, 61 sequences were recovered from 5 loci, of which 44 sequences belonged to AG / TC repeats.

The changes of flanking regions might lead to SSR loci variation, Feng et al. (2009) found that there were point mutations and deletions occurred in the flanking regions in rubber tree, and allelic variations were due to the most frequent InDels (insertions and deletions) in maize flanking regions (Matsuoka et al., 2002). On the contrary, Xie et al. (2006) pointed out that there were no insertion or deletion mutation occurred in AG/CT repeat loci flanking regions for almond, and the similarity results were found in *A. thaliana* (Symonds & Lloyd, 2003). In this study, of the four AG/CT repeat loci in flanking regions, no insertions or deletions mutation occurred in HBE008, however in HBE164, Dafeng117 and AC/T/15/114 were found AA and GAAA insertion respectively, and deletions occurred in HBE187 and HBE199.

Gutierrez et al. (2005) reported that variation was mainly due to the change of the repeat number and insertion, deletion mutation and base substitution in *Medicago truncatula*, insertion and deletion mutation also led to sequence variation (Feng et al., 2009); Symonds & Lloyd (2003) pointed out that interruptions in the repeat regions of most SSR loci were associated with shortening of the original repeat length in *A. thaliana*. In rubber tree, substitutions can be found in many alleles, and a complete and long-repeat sequence was divided into several smaller repeats or become relatively short. For example, CT repeats were shortened for C was replaced by G or A in HBE008, and AT repeats were divided into smaller ones for A was replaced by C as well, in HBE199, A was replaced by C or T led the repeat units into several smaller ones.

Dieringer & Schlötterer (2003) assumed that a length-independent mutation process operates on short SSR loci; and Xie et al. (2006) believed that the point mutation in flanking

sequence might lead to a new SSR repeat unit. But in rubber, interestingly, point mutations frequently occurred in flanking sequence of different loci, in HBE063, A was replaced by C, in HBE187, A by G, in HBE87, C by T, and in HBE199, A/T and T/A were replaced with each other. Long repeat sequences are more frequently targets for mutation (Johannsdottir et al., 2000), Symonds & Lloyd (2003) and Xie et al., (2006) have proved that a SSR motif with more repeats should provide an even more efficient substrate for rapid mutation rate in comparison with SSR motifs containing fewer repeats. In rubber tree, more variations occurred in HBE063 supported the above view.

5. Conclusion

A total of 43 alleles were obtained from 10 EST-SSR and 30 alleles from 6 genomic SSR (gSSR) primer pairs across cultivated and wild accessions; and HBE280, M197 and MnSOD were the most informative SSR markers. All the cultivated and wild accessions were clustered into two big groups. On the level of similarity coefficient 0.68, wild accessions were distinguished from cultivars. Sixty one sequences were sequenced from 5 EST-SSR loci. In comparison with the original EST sequences, insertion, deletion, conversion and transversion mutation occurred in SSR repeats and flanking regions, and long repeat sequences had more variations, and point mutation frequently occurred in flanking regions indicated the new SSR loci in rubber tree.

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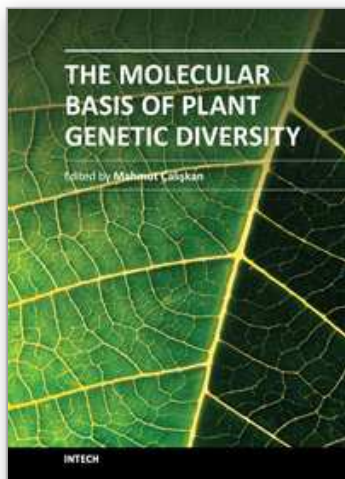
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The Molecular Basis of Plant Genetic Diversity

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The Molecular Basis of Plant Genetic Diversity presents chapters revealing the magnitude of genetic variations existing in plant populations. Natural populations contain a considerable genetic variability which provides a genomic flexibility that can be used as a raw material for adaptation to changing environmental conditions. The analysis of genetic diversity provides information about allelic variation at a given locus. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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