



UNIVERSITI PUTRA MALAYSIA

**THE APPLICATION OF DNA MOLECULAR MARKER TECHNIQUES
IN HEVEA BRASILIENSIS**

SAFIAH BT. ATAN

FSMB 2001 28

THE APPLICATION OF DNA MOLECULAR MARKER TECHNIQUES
IN *HEVEA BRASILIENSIS*

By

SAFIAH BT. ATAN .

Thesis Submitted in Fulfillment of Requirement for
the Degree of Master of Science in the Faculty of
Food Science and Biotechnology,
Universiti Pertanian Malaysia

February 1997



ACKNOWLEDGMENTS

I would like to thank my mother for being extremely patient throughout my graduate study. Special thanks to the following:

Dr. Low Fee Chon, who has invested a lot of her time in explaining numerous scientific theories, genetic nomenclature and grammar. Her patience in reading this thesis is greatly appreciated. Her encouragement throughout this whole experience will be fondly remembered

Drs. Norihan Salleh, Suhaimi Napis and Harikrishna whose input throughout the writing of this thesis is greatly appreciated

Dr. Cheong Kay Fong, who even though had retired and moved to Australia was still keen in reading this thesis

Dr. Ahmad Ikram Abd. Jalil, who taught me to 'shoot' myself (with a camera!)

Finally, to my late father who provided his children with love, education and knowledge of life. He taught us to be modest and humble, to have a positive outlook in life and to have a sense of humour.



TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF PLATES	xi
LIST OF ABBREVIATIONS	xv
ABSTRACT	xix
ABSTRAK	xxi
 CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	5
Molecular Markers	5
Restriction Fragment Length Polymorphism (RFLP) Markers	6
Utility	7
Application	8
Efficiency of Technique	12
Disadvantages	17
PCR-Based Genetic Markers	18



Random Amplified DNA Polymorphism (RAPD)	19
DNA Amplification Fingerprinting (DAF)	23
Sequence-Tagged Sites (STSs)	24
Other Genetic Markers	27
Molecular Marker Studies in <i>Hevea brasiliensis</i>	34

III MATERIALS AND METHODS

Equipments	43
Chemicals	46
Commonly Used Buffers	46
Media	48
Extraction of DNA	49
Plant Materials	49
Isolation of DNA	51
Statistical Analysis	54
Concentration Measurements and Integrity Check	55
DNA Digestions with Restriction Enzymes	56
Electrophoresis	57
Southern Transfer	57



Preparation of Probes	58
Concatemerizing Synthetic Fingerprinting Probes	58
M13 Minisatellite Fingerprinting Probe	61
Labelling of Probes	61
Simple Sequence Repeats (SSRs)	61
M13 DNA Fragments	62
Hybridization Conditions and Washing of Filters	63
Simple Sequence Repeats Fingerprinting	63
M13 DNA Fragments	65
Autoradiography	66
Filter Stripping	66
PCR-Based Molecular Marker Techniques	67
Random Amplified Polymorphic DNA (RAPD)	67
DNA Amplification Fingerprinting (DAF)	69
Sequence-Tagged-Microsatellite Sites (STMS)	70
Construction of Microsatellite Library	72
Restriction Enzyme Digestion and Elution of DNA	72
‘Filling in’ of Ends of DNA Fragments	73



Preparation of pBluescript KS+	74
Ligation	74
Preparation of <i>E. coli</i> DH5 α Competent Cells ...	75
Transformation	76
Plating of Transformants	77
Maintenance of Transformed Cells	77
Minipreparation Isolation of Recombinant Plasmid	77
Digestion of Inserts from Recombinant Plasmids	78
Amplification of Insert DNAs by PCR	79
Screening of Positive Clones with SSRs	79
Sequencing of Microsatellite Library	80
DNA Sequence Determination	80
Preparation of Glass Plates for Sequencing	81
Polyacrylamide Preparation	82
Sequencing Gel Electrophoresis	82
Silver Staining of the Sequencing Gels	83



IV RESULTS AND DISCUSSION

Extraction of gDNA	86
Methylation Studies	91
Statistical Analysis	95
Construction of Microsatellite Library and Screening	96
gDNA and Vector Preparation	96
Ligation	100
Transformation	102
Screening of Colonies	103
PCR Amplification and Library Screening	106
Hybridization Studies	111
pTa71 RFLP Probe	111
M13 DNA Fragments	114
Simple Sequence Repeats (SSRs)	122
Microsatellite Library	125
PCR-Based Marker Techniques	129
Random Amplified DNA Polymorphism (RAPD)	130
DNA Amplified Fingerprinting (DAF)	141



Sequence-Tagged-Microsatellite Sites (STMS)	152
Sequencing of Microsatellite Insert	158
V CONCLUSIONS.....	166
BIBLIOGRAPHY.....	169
PUBLICATIONS.....	183
BIOGRAPHICAL SKETCH.....	185



LIST OF TABLES

Table		Page
1	Characteristics of Various DNA Markers ...	32
2	Potential Application of DNA Fingerprinting	34
3	DNA Content of Some Representative Organisms and Organelles	38
4	Clones of <i>Hevea brasiliensis</i> and Other <i>Hevea</i> Species Used as Sources of gDNA	50
5	Analysis of Variance of Girth Measurements	55
6	Simple Sequence Repeats and Their Corresponding Hybridization Temperatures	64
7	List of Sequence-Tagged-Sites Primers	71
8	Comparison between Two gDNA Isolation Protocols	87
9	List of Methylation Sites	93
10	Percentage of Clones Positive to SSRs	107



LIST OF FIGURES

Figure		Page
1	An Example of Inheritance of a RFLP Marker Compared to the Inheritance of a Conventional Single Gene Marker Controlling Flower Colour	10
2	The pBluescript Phagemid is a 2.9 kb Phagemid Derived from pUC 19	108
3	The Complete Nucleotide Sequence of a Putative Microsatellite Clone #76	160



LIST OF PLATES

Plate		Page
1	Amplified Concatemers Appear as Smears after Separation Through Agarose Gel Electrophoresis	60
2	Integrity Check on gDNA Extracted from <i>In Vitro</i> Culture Plants	88
3	Restriction Enzyme Digestion of gDNA	89
4	Comparison of Colour between Two gDNA Extractions	90
5	Comparison of DNAs Digested by Four Methylation Sensitive Restriction Enzymes	94
6	Quantification of Restricted DNA Fragments DNA (300-600 bp) against Standard λ DNA	98
7	Quantification of Linearized pBluescript against Standard λ DNA	99
8	Comparing the Success of Ligation Reaction against the Control Ligation Reaction	101
9	Comparing the Success of Recombinant Plasmid against Control Transformant	105
10	DNA Inserts Amplified with SK/KS Primer Set	109
11	Amplified DNA Inserts Hybridized with (GC) _n Repeats	110



12	Southern Hybridization of RFLP Probe pTa71 with DNAs Digested with <i>AluI</i>	113
13	<i>HaeIII</i> Restricted M13 mp8 DNA	115
14	Hybridization of M13 DNA Fragments with DNA from Bulked <i>In Vitro</i> Culture Plants ...	116
15	M13 DNA Fragments a : 309 bp and b : 849 bp Hybridized with DNAs from Individual <i>In Vitro</i> Culture Plants	117
16	M13 Fragment 309 bp Hybridized with Inter- and Intraspecific DNAs Restricted by <i>DpnII</i>	119
17	M13 DNA Fragments a : 849 bp and b : 309 bp were Hybridized with Inter- and Intraspecific DNAs Restricted by <i>DpnII</i>	121
18	Simple Sequence Repeats (SSRs) were Hybridised with <i>AluI</i> -Digested DNAs from <i>In Vitro</i> Plants	124
19	Southern Hybridisation of SSR (SSRs) Clones #80 and #76 with <i>DpnII</i> -Digested DNA from <i>In Vitro</i> Cultured Plants	127
20	Southern Hybridization of SSR Clone #76 with <i>DpnII</i> -Restricted Interspecific DNAs	128
21	Southern Hybridization of SSR Clone #76 with <i>DpnII</i> -Restricted Interspecific DNAs	129
22	Monomorphic Pattern of Bulked DNA from <i>In Vitro</i> Culture	131
23	An Illustration of the Higher Resolution Power of dPAGE	132



24	Comparison of Products Amplified By Two RAPD Protocols	134
25	Reproducibility of RAPD Technique	135
26	Inheritance Pattern Observed in RRIM 600 (Tjir 1 X PB 86)	137
27	Polymorphic RAPD Patterns of DNA Samples from Various <i>Hevea</i> Species	140
28	Monomorphic DAF Pattern from <i>In Vitro</i> Culture DNA	143
29	Monomorphic DAF Pattern of Amplified DNA of Ovule-Derived Plants	144
30	Polymorphic DAF Pattern of Amplified DNA from Anther-Derived Plants	145
31	(Tjir 1 X PB 86) Cross was Amplified with a: OpA-04/OpA-19 b: OpA-05/OpA-08 and c: OpA-07/OpA-07 Primer Pairs	148
32	RRIM 600 (Tjir 1 X PB 86) Cross and Six F ₂ Generation DNA Samples were Amplified with OpA-04/OpA-19 Primer Pair	149
33	DNAs from Two Genetic Crosses were Amplified with OpA-04/OpA-19 Primer Pair	150
34	Polymorphic DAF Patterns of Amplified DNA from the Interspecific <i>Hevea</i> Sources	151
35	Monomorphic Pattern of <i>In Vitro</i> Culture DNAs Amplified with STS Primers	154
36	Amplified Products of <i>In vitro</i> STMS were Separated Through 15% Native PAGE	155



37	(Tjir 1 X PR 107) Genetic Cross Amplified with Hev-1/Hev-4 Primer Pair	157
38	Clone #76 Amplified with SK/KS Primer Set	162
39	Concentrated i: Insert of Clone #76 Compared against Standard λ DNA	163
40	Comparisons of Sequenced Insert at Different Concentrations	164
41	Illustration of Part of the Sequenced Insert	165



LIST OF ABBREVIATIONS

APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
bp	Base-pair
BPB	Bromo-phenol blue
BSA	Bovine serum albumin
cm	Centimeter
cpm	Counts per minute
DAF	DNA Amplified Fragments
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EDTA Na	Sodium salt of EDTA
EMBL	European Molecular Biology Library
EtBr	Ethidium bromide
EtOH	Ethanol
g	Gram
GL 1	Clone Glensheld 1 of <i>Hevea brasiliensis</i>
hr	Hour



HCl	Hydrochloric acid
HMGR-1	Hydroxymethylglutaryl coenzyme A reductase-1
IPTG	Isopropyl- β -D-Thiogalacpyranoside
kb	Kilobase
L	Litre
M	Molar
m	meter
min.	Minutes
M13	A filamentous lysogenic bacteriophage containing a single-stranded DNA
mM	Millimolar
mm	Millimeter
M.W.	Molecular weight
Na citrate	Sodium citrate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
ng	Nanogram
OD ₂₆₀	Optical density measured at 260 nm wavelength
OD ₂₈₀	Optical density measured at 280 nm wavelength
PAGE	Polyacrylamide gel electrophoresis



[α - ³² P]dCTP	Phosphorous-32 alpha labelled deoxyCTP
[γ - ³² P]ATP	Phosphorous-32 gamma labelled ATP
PP	Polypropylene
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
RF	Replicating form
RNase	Ribonuclease
rpm	Revolutions per minute
RRIM	Rubber Research Institute of Malaysia
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
sec	Second
ss	Single stranded
STS	Sequence-tagged-sites
T4 PNK	T4 polynucleotide kinase
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	N,N,N',N',-Tetramethylethylenediamine
TE	Tris EDTA buffer
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultra violet light



μg	Microgram
μl	Microlitre
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside, a lactose analogue



Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia as fulfillment of the requirements for the degree of Master of Science.

**THE APPLICATION OF DNA MOLECULAR MARKER TECHNIQUES
IN *HEVEA BRASILIENSIS***

By

SAFIAH BT. ATAN

FEBRUARY 1997

Chairman : Dr. Suhaimi Napis

Faculty : Food Science and Biotechnology.

DNA was extracted from several *Hevea* sources; namely, various *Hevea* species, several cultivars from within the *Hevea brasiliensis* species such as clones and *in vitro* cultured *H. brasiliensis*. Four DNA molecular marker techniques were used to analyze the DNA. These techniques included a hybridization-based marker technique called restriction fragment length polymorphism (RFLP) and three polymerase chain reaction (PCR)-based techniques *viz.* random amplified polymorphic DNA (RAPD), DNA amplified fingerprinting (DAF) and sequence-tagged microsatellite sites (STMS). In the RFLP study, a wheat ribosomal DNA, pTa71 (rDNA) probe was able to detect a reduction in rDNA loci number in DNA from *in vitro* cultured plants compared to DNA from control plants. Hybridization with M13 DNA fragments revealed inter- and intraspecific variations among the DNA samples. Neither of these



hybridization probes could detect somaclonal variation within a sample of *in vitro* cultured plants. On the other hand, RAPD and DAF were able to detect somaclonal variation within the *in vitro* cultured plants. The polymorphic patterns produced by RAPD could be neither correlated with any particular morphological trait nor the source of calli i.e. anther or ovule. Meanwhile, DAF proved to be more sensitive as it was able to detect a high degree of variation in the DNA extracted from anther derived calli. STMS could not detect any variation nor insertion/deletion mutation at the HMGR-1 gene within the *in vitro* culture DNA. RAPD and DAF molecular markers were found to be dominant while RFLP and STMS markers were co-dominant in all of the *H. brasiliensis* crosses tested in this study. No change in the methylation sites for both *in vitro* culture and control plants were detected when the DNAs were digested with both isoschizomeric restriction enzymes *HpaII* and *MspI*. A microsatellite enriched library was constructed and was found to be enriched with (GA)_n repeats (39%). Hybridization with one of these clones revealed inter- and intraspecific variations with *DpnII*-restricted DNAs. This clone was subsequently sequenced and found to be an imperfect repeat.



Abstrak thesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains.

**APLIKASI TEKNIK PENANDA-PENANDA MOLEKUL DNA
KE ATAS *HEVEA BRASILIENSIS***

Oleh

SAFIAH BT. ATAN

FEBRUARI 1997

Pengerusi : Dr. Suhaimi Napis

Fakulti : Sains Makanan dan Bioteknologi.

DNA telah diekstrak daripada beberapa spesis *Hevea*, kultivar dan kultur *in vitro Hevea brasiliensis*. Empat teknik penanda molekul DNA telah digunakan untuk menganalisis DNA tersebut. Teknik tersebut termasuk teknik yang berasaskan penghibridan iaitu 'restriction fragment length polymorphism' (RFLP) dan tiga teknik berasaskan 'tindakbalas polimeras berangkai' (PCR) iaitu 'random amplified polymorphic DNA' (RAPD), 'DNA amplified fingerprinting' (DAF) dan 'sequence-tagged microsatellite sites' (STMS). Dalam kajian RFLP, DNA ribosom gamdum, pTa71, (rDNA) telah digunakan sebagai prob dan telah dapat mengesan pengurangan jumlah loci rDNA pada DNA yang didapati dari pokok yang telah dikultur secara *in vitro* berbanding kepada DNA dari pokok kawalan. Fragmen DNA M13 pula telah dapat menunjukkan variasi inter- dan intraspesifik dan diantara sampel-sampel DNA yang digunakan. Teknik RFLP gagal



mengesan variasi somaklonal dalam suatu populasi pokok yang telah dikultur secara *in vitro* dengan menggunakan kedua-dua prob tersebut diatas. Sementara itu, teknik-teknik RAPD dan DAF telah dapat mengesan variasi somaklonal dalam populasi pokok-pokok yang dikultur secara *in vitro*. Corak polimorfik yang dihasilkan oleh RAPD tidak boleh dikaitkan dengan mana-mana ciri morfologikal atau sumber kalli (anter atau ovul). DAF terbukti lebih peka, oleh kerana ia dapat mengesan variasi pada kadar yang tinggi dalam DNA yang diekstrak daripada kalli anter. Walaubagaimanapun, STMS tidak berjaya mengesan apa-apa variasi ataupun mutasi sisipan/pemadaman pada gen HMGR-1 dalam DNA daripada pokok yang dikultur secara *in vitro*. Penanda molekul RAPD dan DAF didapati adalah penanda dominan sementara penanda-penanda RFLP dan STMS adalah kodominan pada semua kacukan *H. brasiliensis* yang dikaji. Tidak ada perubahan pada tapak-tapak metilasi untuk DNA dari pokok yang dikultur secara *in vitro* mahupun pada pokok kawalan dapat dikesan apabila sampel DNA tersebut dicerna dengan enzim-enzim isoskitzomer *HpaII* dan *MspI*. Satu koleksi klon-klon yang diperkaya dengan mikrosatelit telah dibina dan didapati mempunyai banyak jujukan DNA ulangan-ulangan (GA)_n (39%). Satu klon terpilih telah digunakan sebagai prob dan telah dapat menunjukkan variasi inter- dan intraspesis apabila dihibridisasikan kepada DNA yang telah dicernakan dengan enzim *DpnII*. Klon ini telah ditentukan jujukan DNANYA dan didapati ianya adalah ulangan jujukan DNA yang tidak sempurna.



CHAPTER I

INTRODUCTION

Marker assisted selection (MAS) is a strategy that enables one to follow a selected trait utilizing a linked genetic marker in a breeding programme. It also facilitates the selection of heritable traits that may not be expressed among individuals at any particular time. Such a programme has yet to be implemented in the breeding programme of *Hevea brasiliensis*. Unlike crop plants such as tomato and lettuce where genetic linkage maps were not only constructed (Helentjaris *et al.*, 1986a; Landry *et al.*, 1987), but also had genetic markers linked to quantitative trait loci in high density genetic maps, the genetic studies of *H. brasiliensis* are still at its infancy.

Cultivated *H. brasiliensis* is a perennial plant that has a long generation time as well as a narrow genetic base. It is believed that with a narrow genetic base, there is a low variability among the cultivated clones due to high levels of inbreeding. However, Chevallier (1988) and Besse *et al.* (1993;1994) found these to be otherwise based on isozyme and restriction fragment length polymorphism (RFLP) techniques, respectively. Variability of the Wickham material as demonstrated by



the isozyme technique was thought to be dependent upon the number and choice of loci sampled e.g. the loci for esterases and phosphatases (Chevallier, 1988). Thus, the variability that was encountered was overestimated whereas that of the germplasm was under estimated. The same argument could be said of the RFLP study.

Several new DNA molecular marker techniques that were successfully developed and used in other plants like maize and tomato were also applied to *Hevea* studies. These new techniques are polymerase chain reaction (PCR)-based which include (a) random amplified polymorphic DNA (RAPD) (Low, 1991), (b) sequence-tagged sites (STS) or sequence-tagged microsatellite sites (STMS) (Low *et al.*, 1994 a, b), (c) DNA amplified fingerprinting (DAF) (Low and Safiah, 1995; Low *et al.*, 1995 a, b) and (d) simple sequence repeats (SSRs) (Safiah *et al.*, 1996).

PCR-based marker techniques promise to be more versatile and robust than RFLP markers as only minute amounts or degraded DNA samples can be used for analysis (Li *et al.*, 1988). These techniques are also much faster than RFLP. Therefore screening of a large number of samples e.g. for mapping a large population, is more feasible with these techniques than with RFLP analysis. PCR-based techniques are less labourious and in the long run much more economical especially when this programme is extended (Ragot and Hoisington, 1993).

Cultivated rubber plants were initially seedling progeny from clonal parents that were randomly crossed. Grafted clones of selected seedlings were later established (Whycherley, 1976). *Hevea* is still propagated today by grafting and breeding programs that are based on hand pollination. But clonal assessment of