

信州大学審査学位論文

A Dissertation  
Presented to  
The Graduate School, Shinshu University

Analysis of Genetic Variation in *Morus* spp. Derived from  
Tropical Zone and Asia Monsoon Zone Cultivated under a  
Common Environmental Condition

March, 2012

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DEDICATED  
TO MY DEAREST PARENTS,  
UDOM AND SA-NGIUM CHUMCHUEN,  
IN TOKEN OF  
AFFECTION AND GRATITUDE

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## ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AP-PCR	arbitrarily primed-polymerase chain reaction
bp	base pairs
CTAB	cetyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
DNJ	deoxynojirimycin
EDTA	ethylenediamine tetra acetic acid
ESI-MS	electrospray ionization mass spectrometer
g	gram
GS	genetic similarity
HCl	hydrochloric acid
HILIC	hydrophilic intereaction chromatography
HILIC-MS	hydrophilic intereaction chromatography - mass spectroscopy
ISSR	inter simple sequence repeat
kb	kilobase pair
KCl	potassium chloride
l	liter
m	milli
M	mole
MgCl <sub>2</sub>	magnesium chloride
n	nano
NIAS	National Institute of Agrobiological Science
p	pico
PCR	polymerase chain reaction
rpm	revolutions per minute
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
s	seconds
TAE	Tris-acetate, EDTA
Tris	2-amino-2-(hydroxymethyl) propane 1:3 diol
TE	10 mM Tris, 1mM EDTA
T <sub>m</sub>	melting temperature
U	unit
UPGMA	unweighted pair group method using arithmetic average
UV	ultraviolet
V	volt
°C	degrees Celsius
μ	micro

## MULBERRY VARIETIES USED IN THIS STUDY

### *Morus alba* (white mulberry)

*Hayatesakari*  
*Ichinose*  
*Iran2*  
*Kairyo-ichinose*  
*Kairyo-nezumigaeshi*  
*Kokuso-dai21*  
*Nezumigaeshi*  
*Rosiyaguwa*  
*Shin-ichinose*  
*Enasiguwa*

### *Morus acidosa*

*Okinawa (Shimaguwa)*

### *Morus atropurpurea*

*Amoi No.1*

### *Morus bombycis* (Japanese local mulberry)

*Okushiritou Inaho2* (wild type)  
*Negoyatakasuke*  
*Goroujiwase*  
*Akagi*  
*Aizuujyujima*  
*Enasiguwa*  
*Yukishirazu*  
*Kenmochi*  
*Mitsushigeri*

### *Morus formosensis*

*Taisou2*  
*Taisou3*

### *Morus kagayamae*

### *Mikurajima24* (wild type)

*Hachijouguwa*

### *Morus latifolia*

*Fukkoguwa*  
*Kosou199*  
*Ooshimaguwa*  
*Shiromerosou*  
*Tsukasaguwa*  
*Naganuma*  
*Kokuso-dai21*  
*Roguwa*

### *Morus microphylla* (Texas mulberry)

*Beikoku13*

### *Morus rotunbiloba* (Thai local mulberry)

*Chiangkum*  
*HarnGPLalod*  
*Tadam*  
*Som*  
*Soi*  
*Poo*  
*Pai*  
*Noi*  
*Maeluk-on* (♀, ♂)  
*Keekai*  
*Keawstuk*  
*Keaw* (♂)  
*Bai poe*

### Classification Unknown

*Hayoshino-ookuwa*  
*Usuneno-ookuwa*



## SUMMARY

The mulberry (*Morus spp.*) is a perennial tree belonging to the family Moraceae, a taxonomic group showing great genetic variability and adaptability to different environmental conditions. Mulberry leaves are an exclusive food source of silkworms (*Bombyx mori*).

Mulberry domestication began several thousand years ago, and the origins of most cultivated varieties are believed to be in China, Japan, and the Himalayan foothills. Although mulberry includes mainly diploid cultivars with 28 chromosomes, natural polyploids are also cultivated (Machii *et al.*, 2001).

Today, the mulberry germplasm collection of countries, such as China, Japan, India, Thailand and other countries, are maintained both in the field and the greenhouse. A large number of morphological and agronomical characteristics are being evaluated for utilization in breeding programs. Today, plant breeders are able to use simple techniques involving molecular markers in breeding programs. There are numerous types of molecular markers available for genome analysis; however, the choice of an appropriate genetic marker depends on its suitability to answer a particular ecological question, on the type of plants, and on laboratory facilities.

This study of molecular markers in mulberry is divided into six main chapters, summarized below.

Chapter 1 clarifies the development of markers in plant breeding, including the choice of a suitable marker system, such as RFLP, RAPD, ISSR, and AFLP. This chapter includes

an overview of currently available molecular markers in the field of mulberry plant breeding. In addition, morphological (and other) characteristics of mulberry are summarized.

Chapter 2 clarifies the genetic diversity and relationships among Thai and Japanese mulberry local varieties, as revealed by AP-PCR and ISSR marker assays. This chapter attempts to compare genetic diversity of mulberries from Thailand and Japan based on AP-PCR and ISSR marker methods. Six pairs of AP-PCR primers generated 61 clear amplification bands, of which 35 were polymorphic (57.35%). Three sets of ISSR primers generated 30 bands, of which 14 were polymorphic (46.66%). Dendrograms were constructed by UPGMA from AP-PCR and ISSR amplification products. To the best of our knowledge, this is the first attempt to use molecular markers to investigate the genetic relationships among Thai and Japanese local varieties grown under the same temperate agro ecological conditions. In this report, the analyses were done on a limited number of mulberry varieties; therefore, the analytical results should be enriched in the future by increasing the number of mulberry varieties. The information generated in the study will be useful for mulberry plant breeding. Higher productivity and good resistance to high temperature and high humidity are the most important characteristics requested for mulberry plants in Thailand.

Chapter 3 details the AFLP-based transcript profiling for genetic relationships of mulberry germplasm. This chapter was supported by the National Institute of Agrobiological Science (NIAS), Tsukuba, Japan for collected mulberry samples, including Japanese mulberry varieties, Thai mulberry varieties, Chinese mulberry varieties, exotic mulberry varieties, and wild-types. AFLP analysis was used to measure the genetic diversity of *Morus* spp. among cultivated mulberry plants of different origins and naturally occurring wild-types.

One hundred and five clearly visible amplified bands were separated on a polyacrylamide gel, of which 27 bands (24.58%) were polymorphic. The genetic similarity coefficient based on AFLP ranged from 0.556 to 0.992 between the mulberry cultivars themselves, and from 0.603 to 0.865 between the mulberry cultivars and the wild-type mulberries. The cophenetic correlation coefficient was  $r = 0.9606$ , indicating a high statistical agreement between the dendrogram clusters and the similarity matrix. Cluster analysis of the AFLPs using the UPGMA revealed a genetic distance between the mulberry cultivars and wild-type accessions, supporting the UPGMA clustering. The results of this study showed that AFLP analysis is a useful tool for investigating the genetic diversity of the genus *Morus*.

Chapter 4 details the application of amplified fragment length polymorphism (AFLP) for the isolation of sex-specific markers in *Morus* spp. At present, there is no effective way to identify the sex of mulberry trees during the early stage, which lasts a relatively long time. Thus, the aim of this study was to find a genetic sex marker using nine Japanese mulberry varieties and amplified fragment length polymorphism (AFLP) analysis. Among the 16 selective combination primers tested, 20 bands were generated by the selective combination primer, *EcoRI*+TC / *MseI*+CTC, one of which, at around 250 bp, was present in females and absent in males. To confirm this observation, this combination primer was re-tested with male and female individuals from the nine Japanese mulberry cultivars: the band was completely absent in the male individuals tested.

Chapter 5 examines the effect of environmental conditions on the  $\alpha$ -glucosidase inhibitory activity of mulberry leaves. Mulberry leaves have been used as the sole food for silkworms in sericulture, and as a traditional medicine for diabetes prevention. Mulberry leaf

components, e.g. 1-deoxynojirimycin (1-DNJ), inhibit the activity of  $\alpha$ -glucosidase and prevent increased blood glucose levels, and they are highly toxic to caterpillars other than silkworms. The  $\alpha$ -glucosidase inhibitory activity of mulberry leaves changes with the season, but it is not known which environmental conditions influence the  $\alpha$ -glucosidase inhibitory activity, especially temperature and photoperiod. The objective of this study was to establish the effect of environmental conditions on mulberry leaf components. The results showed that  $\alpha$ -glucosidase inhibitory activity is regulated by temperature rather than photoperiod; cultivars with high inhibitory activity were harvested during May to August and then decreased from August to October.

The analysis of genetic variation derived from environmental effects between the tropical zone and Asia monsoon zone, established that young leaves of mulberry plants from the tropical zone could produce organic compounds, such as 1-deoxynojirimycin, whose  $\alpha$ -glucosidase inhibitory activity was better than those from mulberry plants from the Asia monsoon zone. In addition,  $\alpha$ -glucosidase inhibitory activity content varies among mulberry varieties. The genes that participate in the biosyntheses of 1-deoxynojirimycin will be identified by examining the inherited genetic background.

In conclusion, the genetic diversity and relationships analysis of mulberry varieties can be performed using molecular markers (AP-PCR, ISSR and AFLP techniques). AFLP provided a rapid method for the identification of a sex-specific marker in *Morus* spp. and could be applied to breeding programs, conservation, or be used to develop new DNA markers in the future.

## CHAPTER 1 General Introduction

### 1.1 MOLECULAR MARKERS IN PLANT GENOMES

Since Mendel's discoveries in the 1860s, advances in genetics have established that the units of inheritance (genes) can be transferred from one generation to the next and actually determine the inheritance of traits.

Since then, researchers and breeders have been monitoring, inducing, and mapping single genes in higher plants to make them more suitable for human needs.

Conventional plant breeding, which involves crossing of the best plants possessing the most desirable traits, has helped in achieving this target to a considerable extent.

A glance at the well-populated linkage maps for crops such as tomato or maize, provides convincing evidence of the amount of work that has accumulated in this area of research. Most of the single gene markers used in higher plant genetics are those affecting morphological characters (dwarfism, chlorophyll deficiencies, or altered leaf morphology). Though these markers have been useful in various types of basic and applied research, their use in the field of plant breeding has been limited. Recently developed molecular markers, especially DNA-based markers, have been extensively used in many areas, such as gene mapping and tagging (Karp and Edwards, 1997; Kliebenstein *et al.*, 2001), characterization of sex (Martinez *et al.*, 1999; Flachowsky *et al.*, 2001), analysis of genetic diversity (Godt and Hamrick, 1999; Lerceteau and Szmidt, 1999; Palacios *et al.*, 1999; Erschadi *et al.*, 2000), and genetic relatedness (Brookfield, 1992; Roa *et al.*, 1997; Mace and Lester, 1999). In

population genetics, protein and DNA markers offer the possibility of developing new approaches to breeding procedures.

The most popular markers employed in plant molecular breeding studies and research include:-

- i) Restriction fragment length polymorphisms (RFLPs)
- ii) Random amplification of polymorphic DNA (RAPD) or arbitrarily-primed PCR (AP-PCR)
- iii) Inter simple sequence repeat markers (ISSRs) or micro-satellites
- iv) Amplified fragment length polymorphism (AFLP).

## 1.2 TYPES AND DESCRIPTION OF DNA MARKERS

A new generation of markers has been introduced over the last two decades. The choice of appropriate genetic markers depends on their suitability for answering a particular ecological question and, to a lesser extent, on laboratory facilities (McCartney *et al.*, 2003).

This indicates that there is no unique and ideal marker suitable for all studies undertaken by the plant breeder or geneticist; however, these markers can often complement each other.

### 1.2.1 Hybridization-based markers

Restriction fragment length polymorphism (RFLP) markers, introduced by Botstein *et al.* (1980), were the first DNA markers, and result from differences in the sequences of restriction enzyme cleavage sites in the DNA of different plants. However, this analysis

requires large amounts of high quality DNA (about 50-200 µg) for restriction digestion and Southern blotting. It is also a lengthy procedure and involves radioactive isotopes, making the analysis hazardous (Fig. 1.1).

### 1.2.2 PCR-based markers

PCR is a molecular biology technique that enzymatically replicates (amplifies) small quantities of DNA *in vitro*. It is used to amplify short (usually up to 10 kb), well-defined regions of a DNA strand from a single gene or a part of a gene.

Random amplified polymorphic DNA (RAPD) markers and arbitrarily primed PCR (AP-PCR) were the first PCR-based markers, and were developed simultaneously by Welsh *et al.* (1990) and William *et al.* (1991). These markers are used to amplify the template DNA without prior knowledge of the sequence of the fragment. This analysis is easy and quick to perform; hence, a large number of markers can be screened in a very short period. It has most frequently been used to find polymorphisms, such as taxonomic markers in population studies of a variety of organisms. RAPD and AP-PCR have been used in the molecular genetics of plants. For example, Sobrel and colleagues placed 200 markers on the sugarcane genetic map (Sobrel *et al.*, 1994), while similar projects have been performed in several laboratories on many other plants (Pooler and Hurting, 1995; Prince *et al.*, 1995; Stammers *et al.*, 1995; Tanhuanpaa *et al.*, 1995; Wachira *et al.*, 1995). The method generates a fingerprint using arbitrarily selected primers, under conditions where the primer will initiate synthesis on template DNA, even when the match with the template is imperfect. Some of these priming events occur on opposite strands. The most efficiently primed of these pairs of priming events

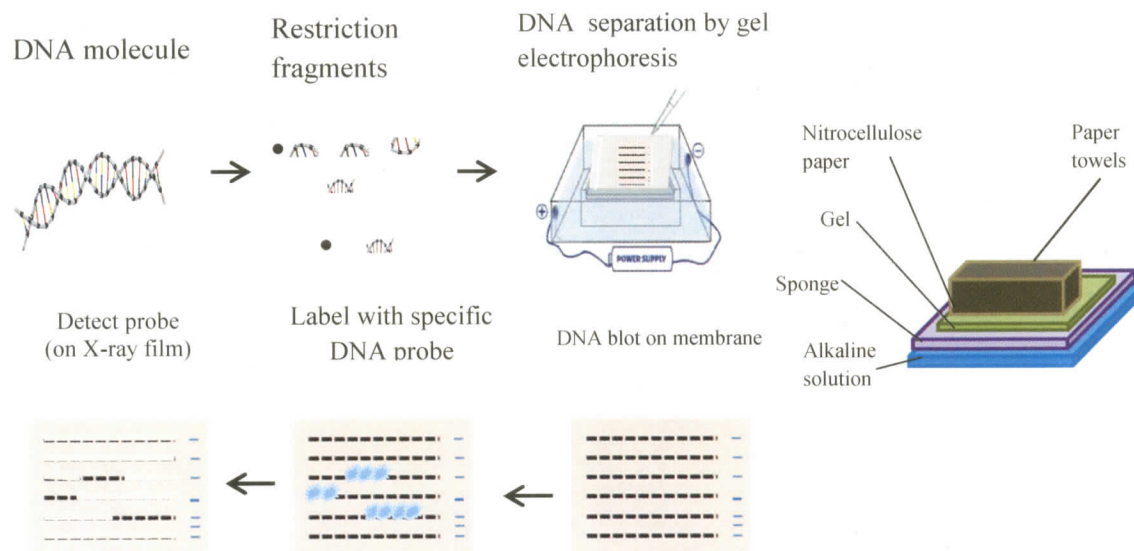


Fig. 1.1. Outline of restriction fragment length polymorphism (RFLP) marker analyses. Double stranded DNA fragments generated by cleavage with restriction enzymes are separated according to fragment length by gel electrophoresis. A sheet of membrane of either nitrocellulose or nylon is laid over the gel, to transfer the separated DNA fragments by blotting (Southern transfer) for subsequent detection with labeled probes.

compete with each other during amplification to produce a fingerprint of a few to more than 100 prominent PCR products (Fig. 1.2). Analysis of the products has been simplified by separating the fragments on agarose gels and using ethidium bromide staining for visualization.

Inter simple sequence repeats (ISSRs) are widely used PCR-based markers. Similar to minisatellites, microsatellites are short sections of DNA where a simple motif, generally 1-5 bp long, such as (AC)<sub>n</sub>, (AAT)<sub>n</sub>, or (GATA)<sub>n</sub>, is repeated in tandem up to ~60 times (Fig. 1.3). Microsatellite markers are highly polymorphic, abundant, and evenly distributed throughout eukaryotic genomes. They are also co-dominant markers, as heterozygotes can be discriminated from homozygotes, because alleles at a particular microsatellite locus vary in the number of tandem repeats and can be differentiated on the basis of the resulting differences in sequence length. Such characteristics mean that since they were first isolated and amplified from humans in 1989 (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989), microsatellite loci have become the molecular markers of choice in many biological fields, including conservation genetics. Microsatellites have been used in a variety of studies, for example the construction of linkage maps in *Arabidopsis thaliana* (Bell and Ecker, 1994), to establish paternities (Houlden *et al.*, 1997), and to identify species and individuals from small amounts of non-invasively collected tissue (Ernest *et al.*, 2000; Sloane *et al.*, 2000).

Amplified fragment length polymorphism (AFLP) are fragments of DNA that have been amplified using directed primers from restriction digestion of genomic DNA, and were developed by Vos *et al.* (1995) and Zabeau and Vos (1993). AFLPs combine both the restriction digestion of genomic DNA and the power of PCR technology. AFLP refers to

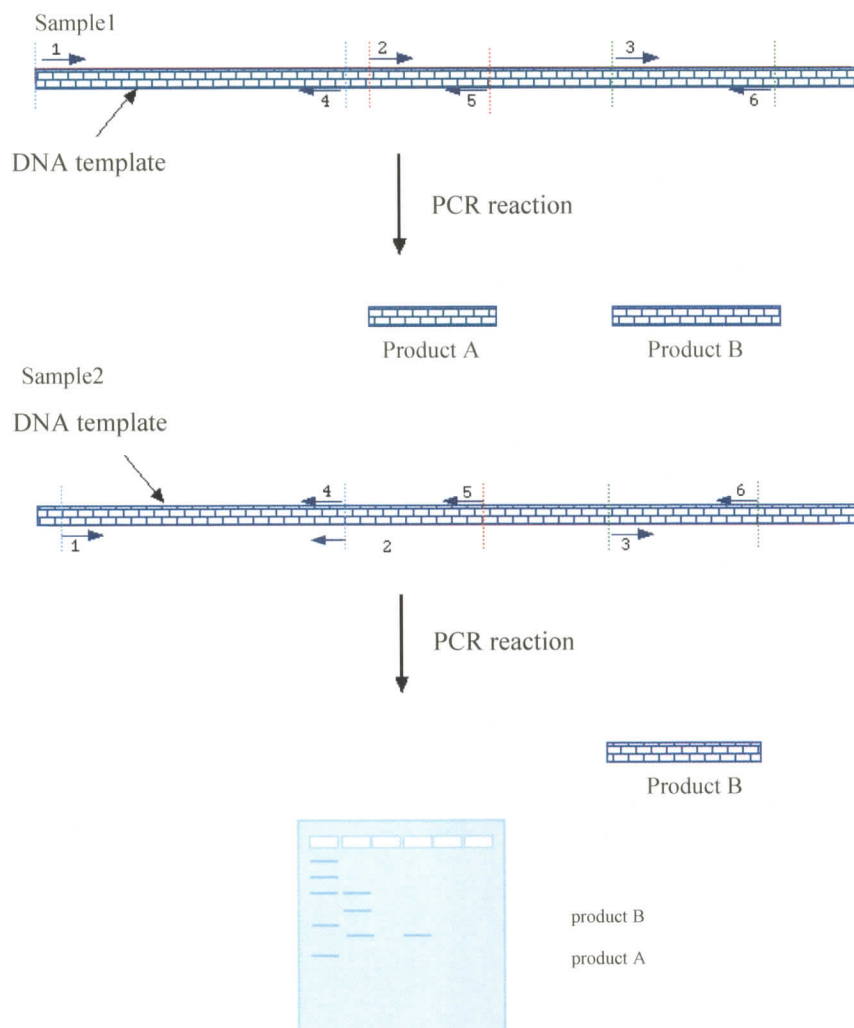


Fig.1.2. Schematic representation of the reaction conditions for random amplified polymorphic DNA (RAPD). The primers must anneal in a particular orientation and within a reasonable distance to one another. The arrows represent multiple copies of a single primer and the direction of the arrow indicates the direction in which DNA synthesis will occur. The numbers in the figure represent primer annealing sites on the DNA template. In sample 1, for example, primers anneal at sites 1, 2, and 3 on the top strand of the DNA template and at sites 4, 5, and 6 on the bottom strand of the template. In this example, only two RAPD products are generated from sample 1. Here, product A is generated by PCR amplification of the DNA sequence that lies between the primers bound at positions 2 and 5. On the other hand, product B is generated by amplification of the DNA sequence between the primers bound at positions 3 and 6. No PCR product is produced by the primers bound at positions 1 and 4, because these primers are too far apart to allow completion of the PCR reaction. The primers bound at positions 4 and 2 or positions 5 and 3 are not oriented towards each other; therefore, no PCR products are produced. For sample 2, the primer failed to anneal at position 2 and a PCR product was obtained only for primers bound at positions 3 and 6.

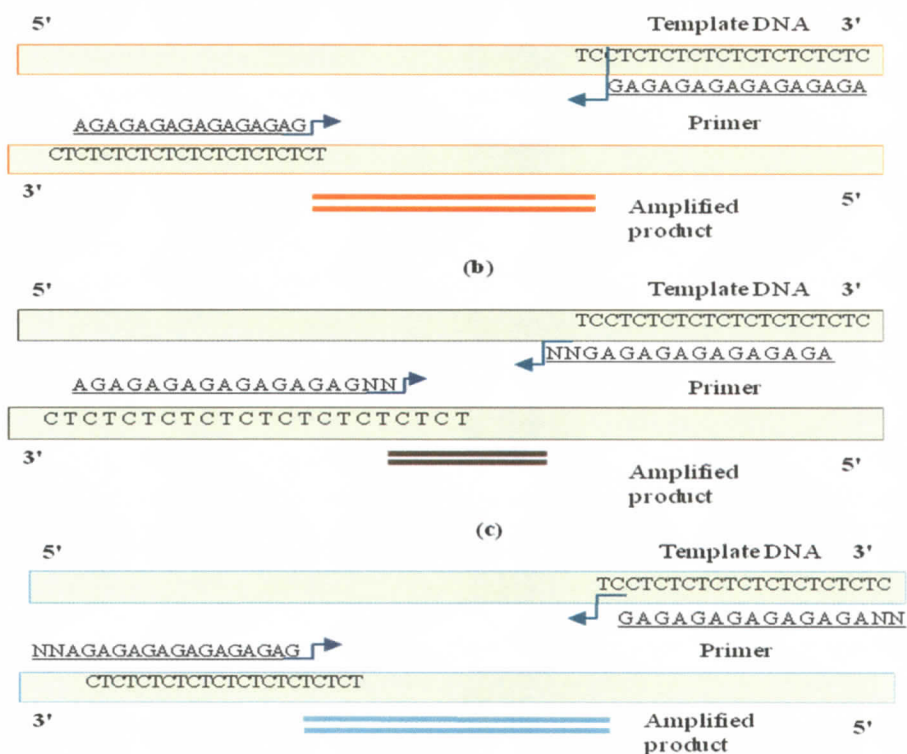


Fig. 1.3. Schematic representation of ISSR-PCR with a single primer (AG)<sub>8</sub>, unanchored (a), 3'-anchored (b), and 5'- anchor (c) targeting a (TC)<sub>n</sub> repeat used to amplify an inter simple sequence region flanked by two inversely oriented (TC)<sub>n</sub> sequences. (a), Unanchored (AG)<sub>n</sub> primer can anneal anywhere in the (TC)<sub>n</sub> repeat region on the template DNA leading to slippage and, ultimately, smear formation. (b), (AG)<sub>n</sub> primer anchored with two nucleotides (NN) at the 3' end anneals at specific regions on the template DNA and produces clear bands. (c), (AG)<sub>n</sub> primer anchored with two nucleotides (NN) at the 5' end anneals at specific regions and amplifies a part of the repeat region also leading to clear bands. Schematic illustration from Reddy *et al.* (2002).

molecular markers obtained by selective PCR amplification of restriction fragments. The fingerprint of any DNA can be obtained, regardless of its source, and without prior knowledge of its DNA sequence. This technique involves a restriction digestion with a combination of a rare cutter and a frequent cutter. A subset of the total fragments is then ligated to oligo-nucleotide adaptors. The ligation fragments are then subjected to PCR amplification under exacting conditions, with primers that are complementary to the adaptors. In general, 75 to 150 fragments are amplified with each selective primer combination set (Fig.1.4).

The principal attraction of AFLP analysis is that it generates a large number of polymorphisms. Its ability to differentiate individuals in a population makes the technique useful for paternity analyses (Krauss, 1999). Other advantages of the AFLP technique are: no sequence information is required, and the PCR technique is fast, with a high multiplying ratio. Obtained data are readily compared between different laboratories (Rafalski *et al.*, 1996).

The idea of using gene markers for a variety of purposes in applied genetics and breeding research is not new. However, until the advent of molecular markers, many of the proposals were technically unfeasible. Today, plant breeders are fortunate to have simple techniques available for utilizing protein markers in breeding programs. These markers, coupled with the promise of DNA markers, will permit the rapid development of new breeding methodologies that take advantage of these modern techniques.

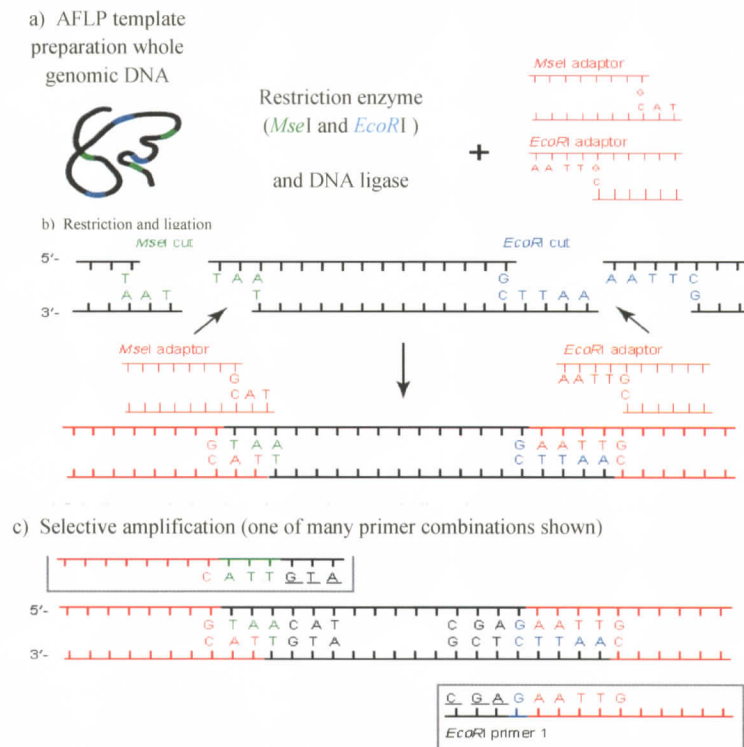


Fig. 1.4. Schematic representation of the procedure in generating AFLP markers. a), Digestion with two restriction enzymes; b), ligation of adapters onto the digested DNA fragments; c) specific amplification of some of the fragments of DNA using selective amplification primers to minimize the possible occurrence of unspecific noises. Schematic illustration from Mueller and Wolfenbarger (1999).

### 1.3 MORPHOLOGY OF MULBERRY PLANTS

The mulberry (*Morus* spp.) is a perennial tree belonging to the family Moraceae, Magnoliophyta division, Magnoliopsida class, Urticales order, which comprises 37 genera and approximately 1,100 species. The most important mulberry species are *Morus alba* (white mulberry), *Morus australis* (Chinese mulberry), *Morus indica* (Indian mulberry), *Morus microphylla* (Texas mulberry), *Morus nigra* (Black mulberry), *Morus rubra* (Red-purple mulberry), and *Morus serrata* (Himalayan mulberry).

The mulberry is a tree and shrub, which can reach over 20 m in height. In their cultivated form, mulberry plants are normally dioecious, but they can also be monoecious on different branches of the same plant. The pendulous pistillate (female) and staminate (male) catkins are arranged on spikes, and appear in April and May (Rehder, 1956).

The pistillate catkins of mulberry are 0.5 to 2 cm long and the staminate catkins are 2.5 to 4 cm long (Radford *et al.*, 1968; FNAEC, 1997). The green females have four sepals, one pistil that is two-parted at the top, and a two-locular ovary positioned above the floral organs. The ovary is about 2mm long (Radford *et al.*, 1968). All mulberries have hairy stigmas. On average, 44% of the pistillate inflorescences are parthenocarpic, with seedless fruits being somewhat smaller than seeded fruits (Griggs and Iwakiri, 1973), as shown in the illustrations of Moraceae (Fig. 1.5).

For this dissertation, mulberry material from several geographical locations (tropical zone and Asia monsoon zone) and genotypes was used to investigate genetic diversity and to establish the relationships among them. The species used were *M. rotundiloba* from Thailand, *M. bombycis* and *M. latifolia* from Japan, *M. formensis* and *M. atropurpurea* from China, *M.*

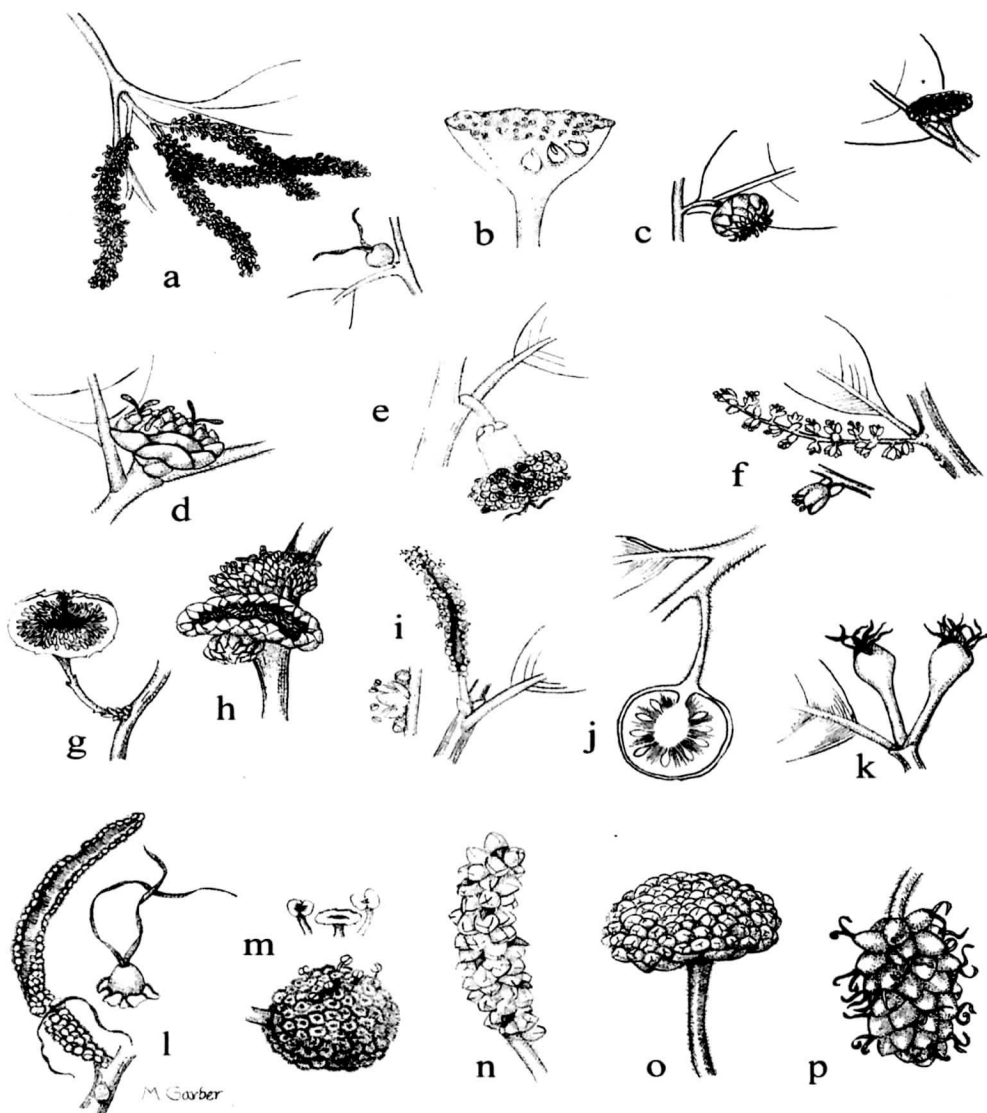


Fig. 1.5. Illustrations of Moraceae inflorescence character states. Magnification shown in parentheses. a-c: Generalized arrangement of Moraceae flowers. a, *Clarisia biflora* staminate (left;  $\times 0.75$ ) and pistillate (right;  $\times 0.75$ ) inflorescence, dioecious species with dissimilar staminate and pistillate inflorescences; b, *Dorstenia choconiana* ( $\times 2$ ), monoecious species with a bisexual inflorescence; c, *Antiaropsis decipiens* pistillate (left;  $\times 1$ ) and staminate (right;  $\times 1$ ) inflorescences, dioecious species with similar male and female inflorescences. d-k: Inflorescence architectures of Moraceae. d, Pistillate *Naucleoopsis guianensis* ( $\times 3$ ), disc shaped receptacle; e, bisexual *Trymatococcus amazonicus* ( $\times 2.5$ ), turbinate receptacle; f, staminate *Sorocea affinis* ( $\times 1.5$ ), raceme; g, bisexual *Ficus wassa* ( $\times 1$ ), syconium; h, staminate *Castilla elastic* ( $\times 2$ ), bivalvate receptacle; i, staminate *Trophis racemosa* ( $\times 2$ ), spike; j, pistillate *Artocarpus vrieseanus* ( $\times 0.5$ ), globose receptacle; k, pistillate *Sparattosyce dioica* ( $\times 1$ ), urceolate receptacle. l-p: Interfloral bract character states. l, *Streblus elongates* ( $\times 0.75$ ), interfloral bracts pellate on a spike; m, *Brosimum rebescenes* ( $\times 2.5$ ), interfloral bracts pellate on a globose inflorescence; n, *Trophis scandens* ( $\times 1.5$ ), interfloral bracts not pellate; o, *Helicostylis fomentosa* ( $\times 3.5$ ), interfloral bracts absent on a disc shaped receptacle; p, *Morus alba* ( $\times 4$ ), interfloral bracts absent on spike. Schematic illustrations from Clement and Weiblen (2009).

*microphylla* from America, *M. alba* from Iran, Japan, and Russia, and two wild-types from Japan (*M. kagayamae* and *M. bombycis*). To achieve the above mentioned aims, studies were conducted under the research protocols presented in this dissertation with the following chapters:

Chapter 2: Genetic diversity and relationships among Thai and Japanese mulberry varieties as revealed by AP-PCR and ISSR marker assays.

Chapter 3: AFLP-based transcript profiling for determining genetic relationships of mulberry (genus *Morus*) germplasm

Chapter 4: Application of amplified fragment length polymorphism (AFLP) as sex-specific markers in *Morus* spp.

Chapter 5: Effect of environmental conditions on the  $\alpha$  - glucosidase inhibitory activity of mulberry leaves

Chapter 6: General discussion

## CHAPTER 2 Genetic diversity and relationships among Thai and Japanese mulberry varieties as revealed by AP-PCR and ISSR marker assays

### 2.1 INTRODUCTION

The mulberry (*Morus spp.*) has a wide range of uses, the most important being food for the silk producing caterpillar, *Bombyx mori* L. Mulberry is believed to have originated at the foothills of the Himalayas and later spread into many countries in tropical, subtropical, and temperate zones (Vijayan, 2004).

Therefore, the genetic varieties of mulberry have been classified into many species and subspecies. In the past, only the morphological traits, such as those based on floral characteristics, were used to classify of the genetic diversity of mulberry plants (Linnaeus, 1753; Koidzumi, 1917; Hotta, 1954; Katsumata, 1972).

However, morphological traits are easily affected by environmental conditions and growth process. In recent years, a number of molecular markers, such as RAPD (Xiang *et al.*, 1995; Feng *et al.*, 1996; Zhao and Pan, 2004), ISSR (Vijayan and Chatterjee, 2003), and AFLP (Sharma *et al.* 2000) markers have been used to study the genetic relationships among mulberry plants. While previous studies focused on the identification and genetic variation among different species of *Morus*, little is known about the genetic variation in local mulberry varieties. In this study, AP-PCR and ISSR markers were used to investigate the genetic relationships among Thai and Japanese local mulberry varieties. The results obtained

are expected to contribute to the elucidation of genetic diversity in mulberry plants. Such comprehensive information would be a valuable genetic resource for the breeder, geneticist, or conservationist.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Plant materials

Fifteen mulberry varieties were selected for this study including five Thai mulberry varieties (Fig. 2.1) and 10 Japanese varieties (Table 2.1). They were obtained from the “Gene Resource Stock Yard”, Laboratory of Field Science, Shinshu University (Ueda, Nagano, Japan).

### 2.2.2 DNA extraction

Genomic DNA was isolated from approximately 1.5 g of young leaves of mulberry by the cetyltriethyl ammonium bromide (CTAB) extraction method, according to the procedure of Doyle and Doyle (1990), with slight modifications. Young mulberry leaves were ground into a fine powder in liquid nitrogen, using a mortar and pestle. The obtained powder was extracted with 7 ml of CTAB extraction buffer (1% CTAB, 100 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8.0), 700 mM NaCl, 4% polyvinylpyrrolidone (PVP) and 0.2% of  $\beta$ -mercaptoethanol) in a 15 ml polypropylene tube. Extraction was performed with gentle shaking under 65°C for 60 min. Subsequently, extracted solutions were washed with an equal volume of chloroform/octanol (24:1) and centrifuged at 13,000 rpm for 10 min at room temperature. The aqueous layer was poured into a new 15 ml polypropylene centrifuge tube

Table 2.1. Information on the *Morus* germplasm, used for the AP-PCR and ISSR analysis in this chapter

No.	Varieties	Symbol	Species <sup>a</sup>	Polyploidy <sup>b</sup>	Country origin
1	<i>Chiangkum</i>	(CHK)	<i>Morus rotunbiloba</i> Koidz.	2x	Thailand
2	<i>Harnngplalod</i>	(HPL)	<i>Morus rotunbiloba</i> Koidz.	2x	Thailand
3	<i>Keaw stuk</i>	(KST)	<i>Morus rotunbiloba</i> Koidz.	2x	Thailand
4	<i>Maeluk-on</i>	(MLO)	<i>Morus rotunbiloba</i> Koidz.	2x	Thailand
5	<i>Soi</i>	(SOI)	<i>Morus rotunbiloba</i> Koidz.	2x	Thailand
6	<i>Kenmochi</i>	(KMT)	<i>Morus bombycis</i> Koidz.	2x	Japan
7	<i>Kairyo-nezumigaeshi</i>	(KNZ)	<i>Morus alba</i> Linn.	2x	Japan
8	<i>Enasiguwa</i>	(ENS)	<i>Morus bombycis</i> Koidz.	2x	Japan
9	<i>Ichinose</i>	(INS)	<i>Morus alba</i> Linn.	2x	Japan
10	<i>Roguwa</i>	(ROG)	<i>Morus latifolia</i> Poir.	2x	Japan
11	<i>Yukishirazu</i>	(YAS)	<i>Morus bombycis</i> Koidz.	2x	Japan
12	<i>Hachijouguwa</i>	(HAJ)	<i>Morus kagayamae</i> Koidz.	2x	Japan
13	<i>Fukkoguwa</i>	(FKK)	<i>Morus latifolia</i> Poir.	2x	Japan
14	<i>Shimaguwa</i>	(SIM)	<i>Morus acidoca</i> Griff.	2x	Japan
15	<i>Rosiyaguwa</i>	(ROS)	<i>Morus alba</i> Linn.	2x	Japan

<sup>a</sup>, Classified according to the original labeling provided by NIAS, Tsukuba, if known.

<sup>b</sup>, Polyploidy according to the original labeling provided by NIAS, Tsukuba, if known.

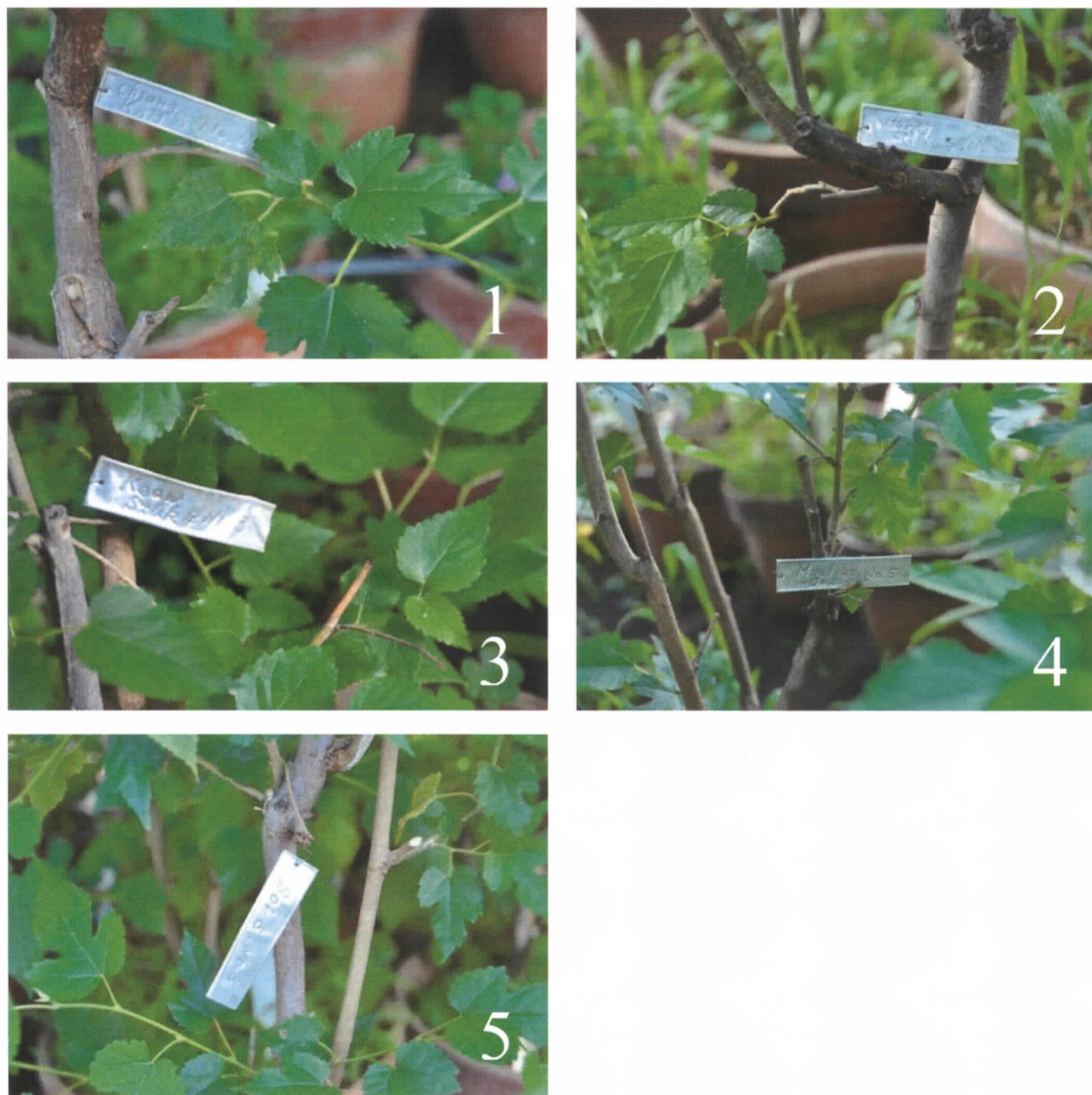


Fig. 2.1. Photographs showing Thai mulberry varieties (*Morus rotundiloba* Koidz.) used in this study. 1, Chiangkum; 2, Harnplalod; 3, Keawstuk; 4, Maeluk-on; 5, Soi. Plants were grown in a greenhouse of the Laboratory of Field Science, Shinshu University.

and re-extracted with 4.5 ml chloroform/octanol (24:1), followed by centrifugation at 13,000 rpm for 10 min. The DNA preparation was treated with RNase at a final concentration of 10 µg/ml, at 37°C for 30 min. The DNA precipitate was washed with washing buffer (76% ethanol, 0.2 mM sodium acetate, 10 mM ammonium acetate and 76% ethanol) and dissolved in TE buffer (10 mM Tris-HCl (pH 8.0) with 1mM EDTA). The extracted genomic DNA was stored at -20°C until use. The yield and purity of the genomic DNA samples were estimated spectrophotometrically at 260 nm and the quality of the genomic DNA was determined by electrophoresis on a 0.7% agarose gel.

### 2.2.3 AP-PCR analysis

AP-PCR analysis was carried out using 10 mers of long primers (Invitrogen, Tokyo, Japan; Table 2.2) using the method of Welsh *et al.* (1990), with some modifications. PCR was performed in an 18 µl volume, containing 10 mM Tris-HCl (pH 9.0), 2.0 mM MgCl<sub>2</sub>, 50 mM KCl, 200 µM of each dNTP, 25 pmoles of primer (Operon, Tokyo, Japan), 1.0 U *Taq* DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan), and 10 ng of template DNA.

The amplification reactions were carried out in a thermal cycler (ThermoGen, Nagano, Japan) using the following thermal profile: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 55°C for 40 s, extension at 72°C for 1 min (45 cycles); final extension at 72°C for 5 min. The PCR samples were stored at 15°C.

#### 2.2.4 ISSR method

Three ISSR markers were screened using 15 mulberry DNA samples. Three primers were chosen for ISSR analysis of genetic diversity based on their ability to reproducibly produce bands (Table 2.2). PCR reactions were carried out in a 15 µl volume containing 50 ng total DNA, 1.0 × PCR buffer (200mM Tris-HCl pH8.0, 2.0mM MgCl<sub>2</sub>, 100 mM KCl), mixture of dNTP 2.5 mM each, and 1U *Taq* polymerase.

PCR cycling conditions for all varieties were 2 min initial denaturation step (94°C); followed by 28 cycles of 30 s at 94°C, 30 s for annealing temperature at 55°C and 120 s at 72°C; ending with a final extension step of 10 min at 72°C.

#### 2.2.5 Electrophoresis

The AP-PCR and ISSR amplified products were analyzed by electrophoresis at 8-10 V/cm in 1 × TAE buffer on a 1.0% Agarose gel (TaKaRa Bio Inc.). Gels were stained with ethidium bromide. The gels were placed on a UV transilluminator and photographed.

#### 2.2.6 Scoring and data analysis

AP-PCR and ISSR amplified bands were scored as present (1) or absent (0). Data analysis was performed using NTSYS-pc (Numerical taxonomy system, version 2.1, Rohlf 2002). Similarity matrices based on simple matching coefficients were calculated with NTSYS pc 2.1 and used to construct dendrograms by UPGMA, then used the cophenetic correlation coefficient to confirm the cluster data.

Table 2.2. Primers employed for AP-PCR and ISSR

Primer Code for AP-PCR	Sequence of primer 5' → 3'	Primer Code for ISSR	Sequence of primer 3' → 5'
AP01	TGGTGGCAGA	CHC835-2	AGAGAGAGAGAGAGATC
AP04	AGGCAGAGCA	CHC864	TGATGATGATGATGATGA
AP05	AGCGTCCTCC	CHC807	CTCTCTCTCTCTCTCTA
AP06	ACAGGTGCTG		
AP07	GGTGACGTT		
AP12	CTGAGACGGA		

## 2.3 RESULTS

Prescreening assays with 15 varieties of Thai and Japanese mulberries using six AP-PCR primers generated clear amplification bands and polymorphisms as shown in Fig.2.2 and 2.3. Out of 61 bands generated, 35 bands were judged to be polymorphic (57.37%). Of the 30 bands produced by the three ISSR primers obtained from 15 mulberry varieties, 14 bands were polymorphic (46.66%: Fig. 2.4 and 2.5).

### 2.3.1 Genetic variation

The genetic similarity matrix using the “simple matching coefficient” showed the range of coefficient generated by the AP-PCR markers varied from 0.458 to 0.979, with an average of 0.718. The highest genetic similarity (0.979) was found between *Soi* and *Maeluk-on*. The lowest genetic similarity coefficient (0.458) was found between *Soi* and *Rosiyaguwa*. Among Thai mulberry varieties, the similarity coefficient varied from 0.854 to 0.975, with an average of 0.914. The highest genetic similarity was found between *Maeluk-on* and *Soi*. Among Japanese varieties, the genetic similarity coefficient varied from 0.708 to 0.917 (average=0.812). The highest genetic similarity was found between *Roguwa* and *Hachijouguwa*, and between *Kenmochi* and *Yukishirazu*. The lowest coefficient was found between *Enasiguwa* and *Roguwa* (Table 2.3).

For the ISSR markers, the range of coefficients varied from 0.525 to 0.925, with an average of 0.725. The highest genetic similarity (0.925) was found between *Soi* and *Kenmochi*. The lowest genetic similarity coefficient (0.525) was found between *Chiangkum* and *Yukishirazu*. Among Thai mulberry varieties, the similarity coefficient varied from 0.700

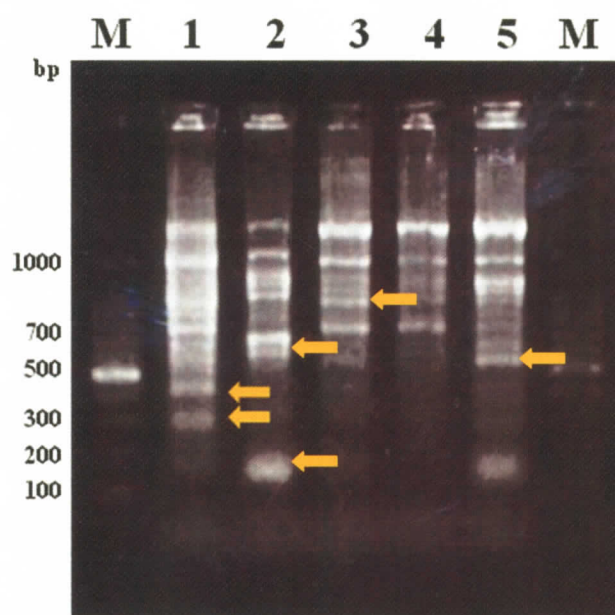


Fig. 2.2. Agarose gel electrophoresis of AP-PCR patterns obtained with primer AP06 from five Thai mulberry varieties. Lane M, DNA molecular size marker combined 100 bp / 1 kb DNA ladder; 1, *Chiangkum*; 2, *Harnngplalod*; 3, *Keawstuk*; 4, *Maeluk-on*; 5, *Soi*. Arrows indicate polymorphic bands.

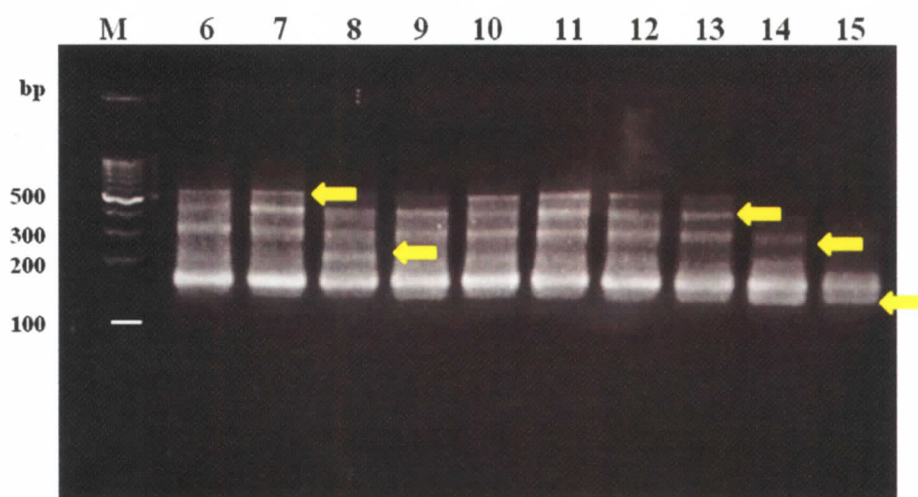


Fig. 2.3. AP-PCR bands obtained by amplification with primer AP 06. M, Molecular size marker 100 bp; 6, *Kenmochi*; 7, *Kairyo-nezumigaeshi*; 8, *Enasiguwa*; 9, *Ichinose*; 10, *Roguwa*; 11, *Yukishirazu*; 12, *Hachijouguwa*; 13, *Fukkoguwa*; 14, *Shimaguwa*; 15, *Rosiyaguwa*. Arrows indicate polymorphic bands.

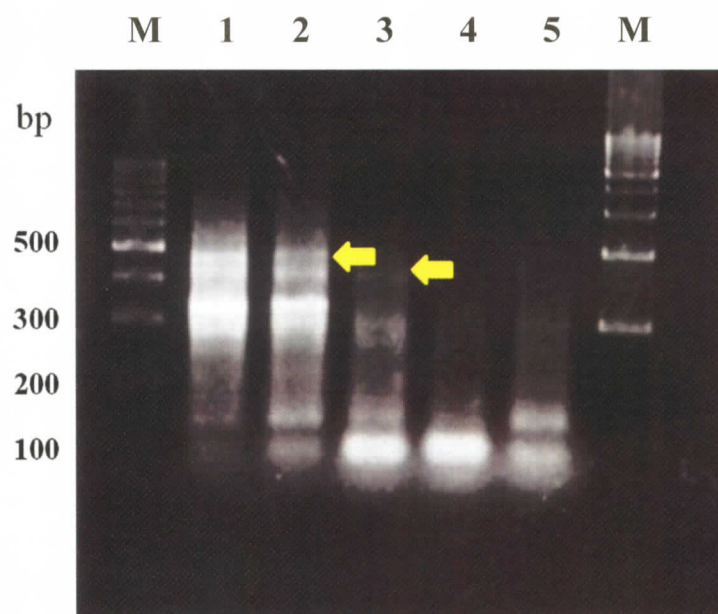


Fig. 2.4. ISSR-PCR bands obtained by amplification with primer CHC-835-2. M, Molecular size marker 100 bp; 1, *Chiangkum*; 2, *HarnGPLalod*; 3, *Keawstuk*; 4, *Maeluk-on*; 5, *Soi*; Arrows indicate polymorphic bands.

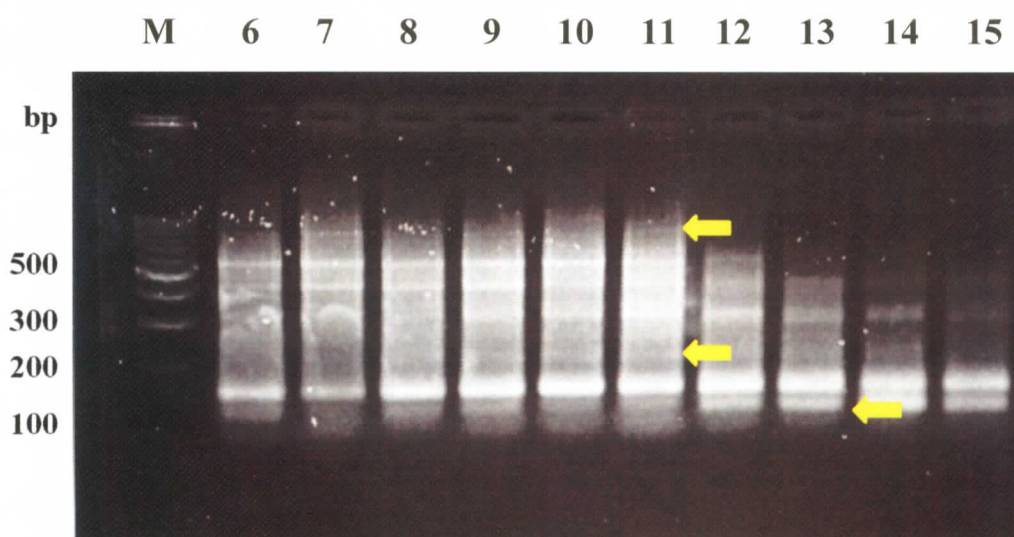


Fig. 2.5. ISSR-PCR bands obtained by amplification with primer CHC-835-2. M, Molecular size marker 100 bp; 6, *Kenmochi*; 7, *Kairyo-nezumigaeshi*; 8, *Enasiguwa*; 9, *Ichinose*; 10, *Roguwa*; 11, *Yukishirazu*; 12, *Hachijouguwa*; 13, *Fukkoguwa*; 14, *Shimaguwa*; 15, *Rosiyaguwa*. Arrows indicate polymorphic bands.

to 0.875, with an average of 0.787. The highest genetic similarity was found between *Keawstuk* and *Soi*. Among Japanese varieties, the genetic similarity coefficient varied from 0.575 to 0.975 with an average 0.775). The highest genetic similarity was found between *Shimaguwa* and *Rosiyaguwa*. The lowest coefficient was found between *Kenmochi* and *Shimaguwa* (Table 2.4).

### 2.3.2 Cluster analysis

Two dendrograms were generated from the results of AP-PCR and ISSR marker assay by the UPGMA method (Fig. 2.6 and 2.7). The 15 mulberry varieties were grouped into two main clusters, A and B. The dendrogram generated from the AP-PCR results shows that all the Japanese mulberry varieties were separated into cluster A, and cluster B consisted of all the Thai mulberry varieties. The dendrogram constructed by UPGMA from the ISSR amplified bands also grouped the data into two clusters, A and B (Fig.2.7). Almost all the Japanese mulberry varieties comprised cluster B. However, some Japanese mulberry varieties (*Kenmochi*, *Enasiguwa* and *Kairyo-nezumigaeshi*) were grouped with Thai mulberry varieties in cluster A. The cophenetic correlation values for the AP-PCR dendrogram and ISSR dendrogram were 0.944 and 0.748, respectively (Fig. 2.8 and 2.9).

## 2.4 DISCUSSION

The genetic distance within a crop species provides a measure of the average genetic divergence among cultivars of the species. Relationships derived from agronomic traits proved to be useful for the analysis of variability (Smith, 1984), for the selection of parents

Table 2.3. Genetic similarity of Thai and Japanese mulberry varieties based on AP-PCR marker analysis between 15 mulberry varieties

Mulberry genotype	CHK	HPL	KST	MLO	SOI	KMT	KNZ	ENS	INS	ROG	YAS	HAJ	FKK	SIM	ROS
CHK	1.000														
HPL	0.792	1.000													
KST	0.792	0.917	1.000												
MLO	0.729	0.854	0.938	1.000											
SOI	0.750	0.875	0.917	0.979**	1.000										
KMT	0.563	0.563	0.563	0.583	0.563	1.000									
KNZ	0.625	0.667	0.667	0.688	0.667	0.813	1.000								
ENS	0.583	0.667	0.625	0.646	0.625	0.771	0.875	1.000							
INS	0.604	0.604	0.604	0.625	0.604	0.833	0.896	0.813	1.000						
ROG	0.583	0.542	0.500	0.521	0.500	0.813	0.792	0.708	0.854	1.000					
YAS	0.604	0.604	0.563	0.583	0.563	0.917	0.813	0.771	0.833	0.896	1.000				
HAJ	0.542	0.625	0.583	0.604	0.583	0.854	0.875	0.792	0.896	0.917	0.896	1.000			
FKK	0.583	0.625	0.583	0.563	0.542	0.813	0.750	0.792	0.813	0.792	0.771	0.833	1.000		
SIM	0.500	0.583	0.542	0.521	0.500	0.771	0.750	0.750	0.771	0.750	0.771	0.833	0.875	1.000	
ROS	0.500	0.500	0.500	0.479	0.458*	0.854	0.792	0.750	0.854	0.792	0.771	0.833	0.875	0.833	1.000

CHK, Chiangkum; HPL, Harnngplalod; KST, Keaw stuk; MLO, Maeluk-on; SOI, Soi; KMT, Kenmochi; KNZ, Kairyo-nezumigaeshi; ENS, Enasiguwa; INS, Ichinose; ROG, Roguwa; YAS, Yukishirazu; HAJ, Hachijouguwa; FKK, Fukkoguwa; SIM, Shimaguwa; ROS, Rosiyaguwa.

\*\*, highest similarity coefficient;

\*, lowest similarity coefficient

Table 2.4. Genetic similarity of Thai and Japanese mulberry varieties based on ISSR marker analysis between 15 mulberry varieties

	CHK	HPL	KST	MLO	SOI	KMT	KNZ	ENS	INS	ROG	YAS	HAI	FKK	SIM	ROS
CHK	1.000														
HPL	0.825	1.000													
KST	0.825	0.700	1.000												
MLO	0.775	0.700	0.850	1.000											
SOI	0.800	0.775	0.875	0.825	1.000										
KMT	0.825	0.750	0.800	0.750	0.925	1.000									
KNZ	0.750	0.675	0.825	0.725	0.800	0.825	1.000								
ENS	0.725	0.800	0.750	0.750	0.875	0.850	0.725	1.000							
INS	0.650	0.725	0.625	0.625	0.700	0.725	0.700	0.625	1.000						
ROG	0.575	0.600	0.600	0.600	0.575	0.600	0.675	0.600	0.725	1.000					
YAS	0.525*	0.600	0.600	0.600	0.625	0.650	0.675	0.650	0.775	0.850	1.000				
HAI	0.625	0.550	0.600	0.550	0.575	0.650	0.575	0.600	0.675	0.750	0.750	1.000			
FKK	0.600	0.625	0.625	0.575	0.650	0.625	0.600	0.725	0.650	0.825	0.825	0.825	1.000		
SIM	0.550	0.625	0.625	0.675	0.600	0.575	0.650	0.625	0.700	0.875	0.775	0.725	0.850	1.000	
ROS	0.575	0.650	0.625	0.650	0.625	0.600	0.625	0.650	0.725	0.850	0.750	0.750	0.875	0.975**	1.000

CHK, Chiangkum; HPL, Harnngplalod; KST, Keaw stuk; MLO, Maeluk-on; SOI, Soi; KMT, Kenmochi; KNZ, Kairyo-nezumigaeshi; ENS, Enasiguwa; INS, Ichinose; ROG, Roguwa; YAS, Yukishirazu; HAI, Hachijouguwa; FKK, Fukkoguwa; SIM, Shimaguwa; ROS, Rosiyaguwa.

\*\*, highest similarity coefficient;

\*, lowest similarity coefficient

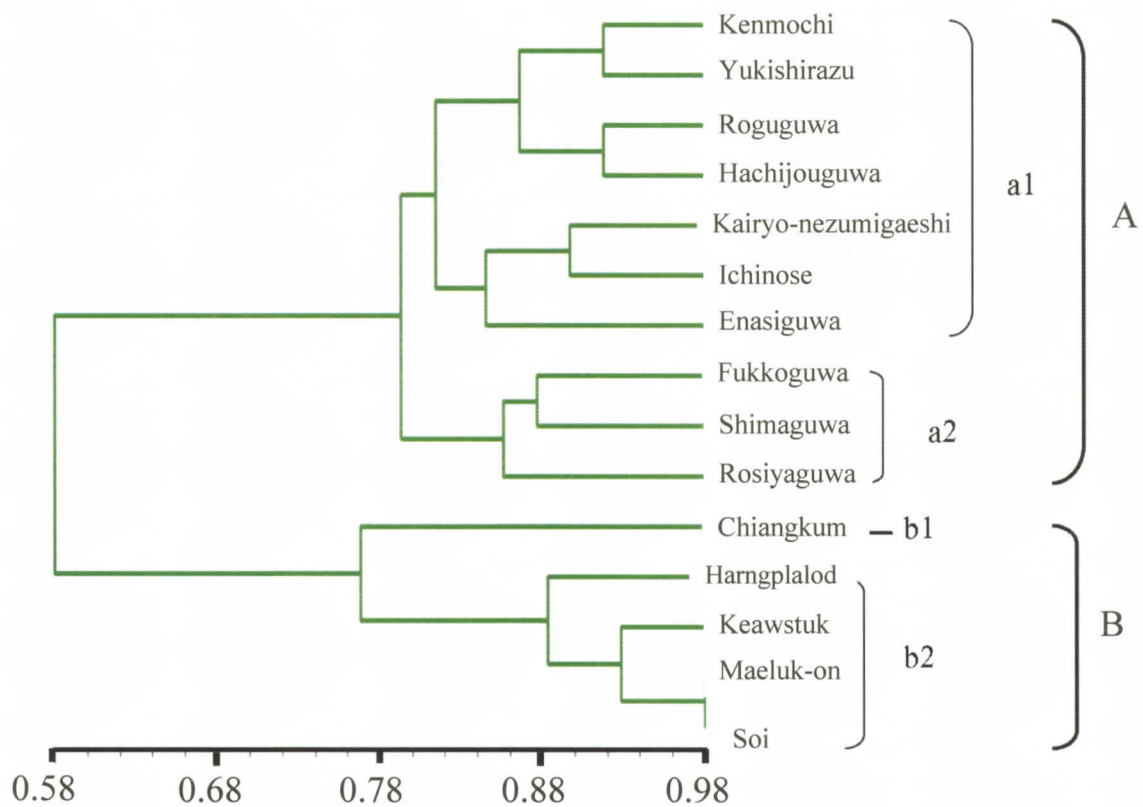


Fig. 2.6. UPGMA dendrogram presenting the relationships of 15 mulberry varieties analyzed by AP-PCR markers.

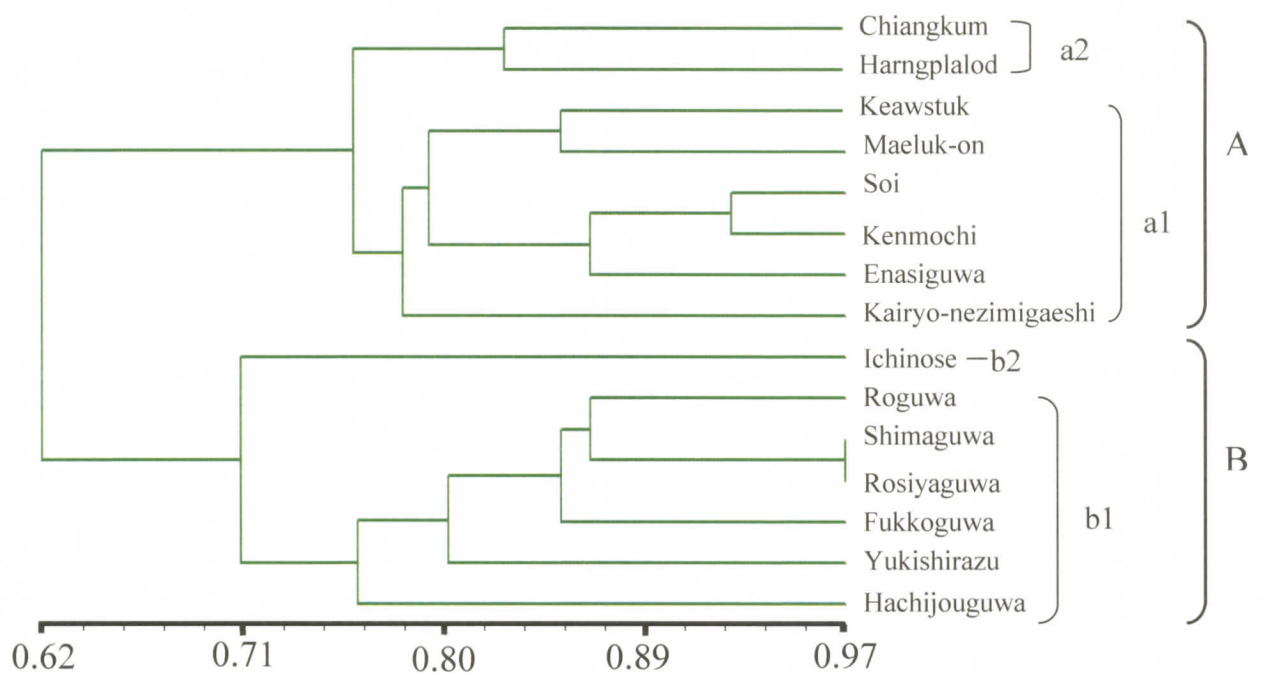


Fig.2.7. UPGMA dendrogram presenting the relationships of 15 mulberry varieties analyzed by ISSR-PCR markers.

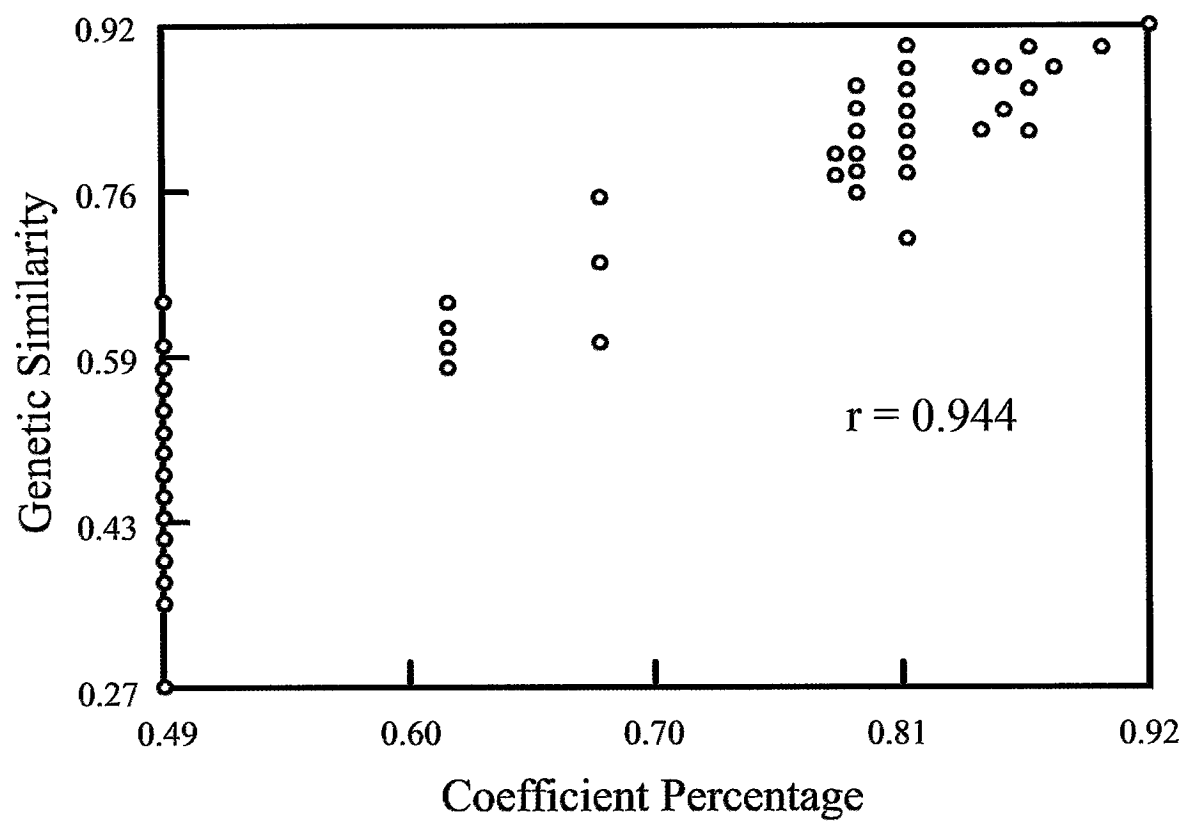


Fig.2.8. Matrix comparison of 15 mulberry varieties based on AP-PCR analyzed by the NTSYS pc 2.1 program.

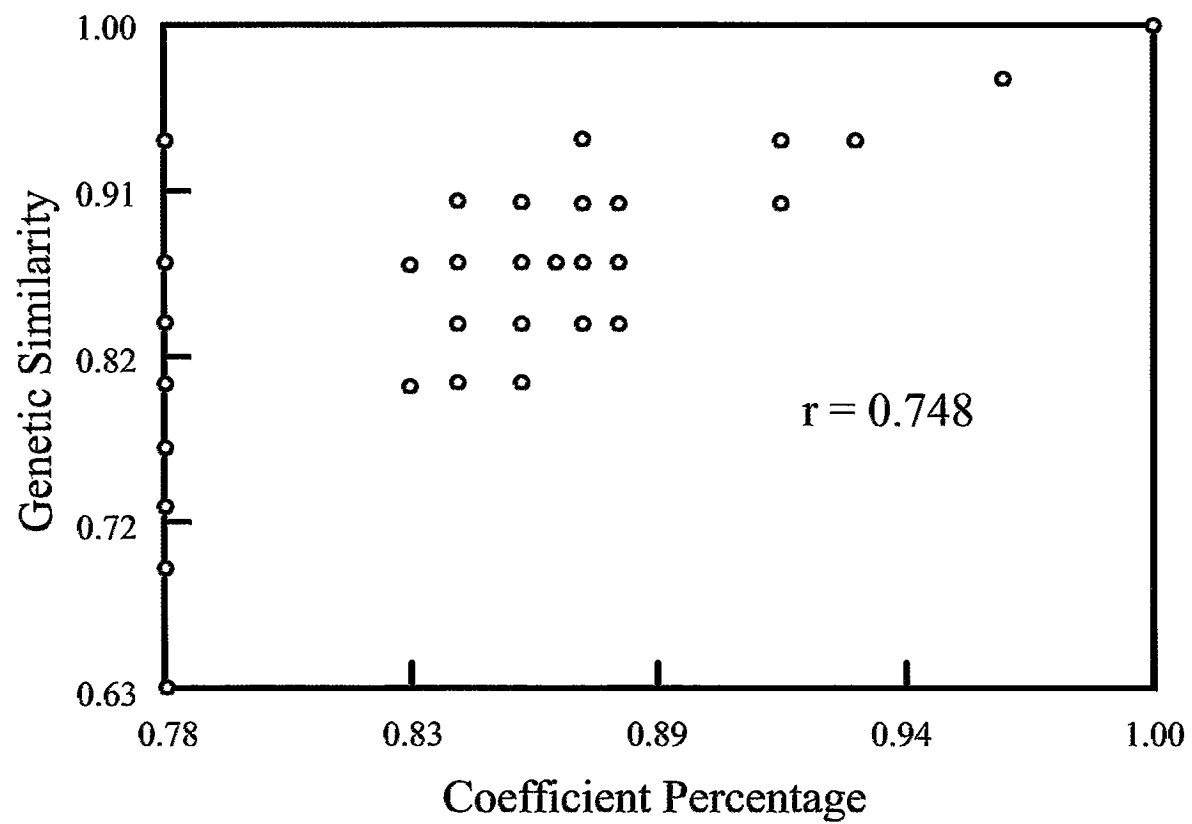


Fig.2.9. Matrix comparison of 15 mulberry varieties based on ISSR marker analyzed by the NTSYS pc 2.1 program.

for hybridization (Frei *et al.* 1986), and for the prediction of progeny performance (Grafius, 1956). However, with recent advances in molecular biology, analysis of genetic variability with molecular markers is preferred to that with agronomic traits, as it is not influenced by environmental factors.

Among the various molecular markers, AP-PCR (Welsh *et al.*, 1991) and the ISSR assay developed by Vijayan *et al.* (2006) are probably the most reliable tools used in crop plants to investigate genetic diversity, and to identify genes associated with many economically important characteristics, like disease resistance (Bridge *et al.*, 2003). Genetic variations in mulberry have been studied mainly on the basis of morphological and phenotypic characteristics (Mala *et al.*, 1997). Most studies concentrated on determining the genetic diversity among different species, cultivar types, and wild-types and indigenous genotypes together. Vijayan *et al.* (1999) used ISSR marker to estimate the genetic diversity among Indian mulberry genotypes. However, there has been no study comparing AP-PCR and ISSR markers for assessing genetic diversity of Thai and Japanese mulberry varieties.

The results presented here suggest that AP-PCR and ISSR are both well suited to DNA fingerprinting. As no prior sequence knowledge is required, they are more rapidly applied. The AP-PCR, however, has some disadvantages, such as somewhat inferior reproducibility because of mismatches in annealing on the genome DNA. On the other hand, ISSR is more robust than AP-PCR, possibly because the primers are longer, and hence PCR conditions are more stringent.

To the best of our knowledge, this is the first attempt to use molecular markers to investigate the genetic relationships among Thai and Japanese local varieties that have been

grown under the same temperate agro-ecological conditions. In this report, analyses were done on a limited number of mulberry varieties. Thus, the analytical results should be enriched by increasing the number of mulberry varieties studied. Nonetheless, the information generated in the study will be useful in the field of mulberry plant improvement. For example, high productivity and resistance to high temperature and a high humidity are the most important characteristics requested for mulberry plants in Thailand.



## CHAPTER 3 AFLP-based transcript profiling for determining genetic relationships of mulberry (genus *Morus*) germplasm

### 3.1 INTRODUCTION

The mulberry (genus *Morus*), which belongs to the Moraceae family, is one of the most interesting taxonomic groups because of its large genetic variability, and is of commercial importance in the sericultural industry. Mulberry leaves are the exclusive food source for the domestic silkworm, *Bombyx mori*. Mulberry is believed to have originated in the foothills of the Himalayas and to have later spread into many countries in tropical, subtropical, and temperate zones (Vijayan, 2004). Mulberry is particularly prevalent in China and Japan, while it is barely represented in Africa, Europe, and the Middle East. Mulberry, a perennial plant, has a wide geographical distribution and adapts easily to different agro-climatic conditions. It can be propagated both asexually and sexually, and easily hybridizes both naturally and artificially. These features have generated a complex genetic background in the genus *Morus*.

Many attempts have been made to classify *Morus* into various species and subspecies, primarily based on their floral characteristics (Linnaeus, 1753; Koidzumi, 1917; Hotta, 1954; Katsumata, 1972). However, the high fertility observed in the interspecific hybridization of this genus (Das and Krishnaswami, 1965; Dandin *et al.*, 1987), together with results obtained from morpho-biochemical traits (Mala *et al.*, 1997; Fotedar and Dandin, 1998; Machii *et al.*, 1999; Vijayan *et al.*, 1999; Tikader and Roy, 2001), indicate that the demarcation between

different species of mulberry is quite ambiguous. Thus, the species status of many mulberry genotypes, especially those from tropical zones, remains a matter of debate.

The genetic diversity of the species is an important trait and cannot be overlooked in the initial steps towards the selection and breeding of superior genotypes. Evaluation of the genetic diversity of this genus using molecular techniques could provide a useful basis for breeding programs. Recently, molecular markers have been used to study the genetic relationship between plants, based on the principle that molecular markers are not influenced by environmental factors, and can be evaluated using DNA from any growth stage. Therefore, molecular markers are very useful for assessing the genetic diversity of plant species. Various molecular approaches based on polymerase chain reaction (PCR) using arbitrary primers, such as arbitrarily primed PCR (AP-PCR) (Welsh *et al.*, 1991), randomly amplified polymorphic DNA (RAPD) (William *et al.*, 1990), inter simple sequence repeats (ISSR) (Zietkiewicz *et al.*, 1994; Prevost and Wilkinson, 1999) and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), have been widely used to study genetic relationships and diversity in plant populations and cultivars. There have been studies on the molecular genetic diversity of the genus *Morus* using PCR-based techniques, such as RAPD (Xiang *et al.*, 1995; Feng *et al.*, 1996; Zhao *et al.*, 2000; Esha and Shirish, 2001), AFLP (Sharma *et al.*, 2000), and ISSR approaches (Vijayan and Chatterjee, 2003).

The present study was carried out to determine the genetic variability within and among Japanese and Thai mulberry cultivars, exotic varieties, and wild-types of mulberry through amplified fragment length polymorphism (AFLP) fingerprinting. The genotyping results were then analyzed using UPGMA. The information provided by this approach will

benefit breeders, geneticists, and conservationists who use selective markers to study the phylogenetic relationships in *Morus* spp.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plant materials

Forty-two mulberry varieties were used in this study, representing nine species of the *Morus* genus, including 40 cultivated mulberry varieties and two wild mulberry varieties (Table 3.1). All of the mulberry samples used in this study are currently maintained at the “Gene Resource Stock Yard”, Laboratory of Field Science, Shinshu University (Ueda, Nagano, Japan) and the National Institute of Agrobiological Science (NIAS), Tsukuba, Ibaraki, Japan. Young mulberry leaves were harvested separately and stored immediately at -80°C before DNA extraction.

### 3.2.2 Genomic DNA extraction and quantification

Genomic DNA was isolated from approximately 1.5 g of young leaves of mulberry by the cetyltriethyl ammonium bromide (CTAB) extraction method, according to the procedure by Doyle and Doyle (1990) with slight modifications (see “MATERIALS AND METHODS” in Chapter 2).

### 3.2.3 AFLP analysis

The AFLP analysis was performed according to Vos *et al.* (1995) with some modifications.

### 3.2.3.1 Digestion using restriction enzymes

Approximately 100 ng of genomic DNA was digested with *EcoRI* and *MseI* at 37°C for 2 hr in a final volume of 25 µl, containing 5µl of 5× reaction buffer, 4.0 U *EcoRI*, 2.5 U *MseI* and distilled water.

### 3.2.3.2. Ligation of adapters

An *EcoRI* adapter (5 pmole/µl) and an *MseI* adapter (25 pmole/µl) were ligated with the restricted DNA fragments in ligation buffer (5 × T4 ligase buffer and 1.0 U of T4 DNA ligase) and incubated at 16°C for either 10 hr or overnight. All buffers were purchased from TaKaRa Bio Inc. and the enzymes were purchased from New England Biolabs, Tokyo, Japan.

### 3.2.3.3 Pre-amplification

Five microliters of the diluted ligated DNA were added to a 50 µl PCR reaction mixture containing PCR buffer, 0.2 mM of dNTP, 1U Taq polymerase, and 50 ng each of the *EcoRI*-0 and *MseI*-C pre-amplification primers (Table 3.2). The PCR program used was 3 minutes denaturing at 94°C; followed by 20 cycles of 30s at 94°C, 60s at 56°C, 60s at 72°C; ending with a 20 minute final elongation stage at 72°C in a thermocycler (ThermoGen, Nagano, Japan). The samples were then diluted 20-fold with the TE buffer and used as templates for subsequent selective amplification.

#### 3.2.3.4 Selective amplification

The selective amplification was carried out with six selective primer combinations of *EcoRI* (*EcoRI*+2) and *MseI* (*MseI*+3) primers (Table 3.2) in a total volume of 20 µl. The PCR program consisted of two segments: the first segment was a touch-down program comprising 12 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, with a continuous reduction in the annealing temperature of 0.7°C after each cycle during the first 12 cycles. The second segment consisted of 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; ending with a 20 minute final elongation stage at 72°C.

#### 3.2.4 Electrophoresis and silver staining of amplified products

After completion of the PCR reaction, 10 µl of AFLP loading buffer (98% formamide, 10 mM EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol) was added to the reaction mixture and incubated at 90°C for 3 min, followed by immediate transfer onto ice. A 10 µl aliquot was loaded onto a 10% e-PAGEL (ATTO Corporation, Tokyo, Japan). Electrophoresis was conducted at constant power (200 V, 20 mA) for 80 min. The gel was then visualized with *EzStain Silver* (AE-1360, ATTO Corporation). All chemicals, unless otherwise stated, were purchased from Wako Pure Chemicals (Osaka, Japan).

#### 3.2.5 Data analysis

##### 3.2.5.1 Genetic similarity estimation

Only bright and clearly distinguishable AFLP bands ranging from 100 to 500 bp were scored for the analysis. The presence or absence of bands was scored as 1 or 0, respectively.

Table 3.1. Information on the *Morus* germplasm, used for the AFLP analysis in this study

No.	Varieties	Species <sup>a</sup>	Ploidy <sup>b</sup>	Country origin <sup>c</sup>
1	<i>Beikoku13</i>	<i>Morus microphylla</i> Loud.	2×	America
2	<i>Iran2</i>	<i>Morus alba</i> Linn.	2×	Iran
3	<i>Kosou199</i>	<i>Morus latifolia</i> Poir.	2×	China
4	<i>Amoi No.1</i>	<i>Morus atropurpurea</i> Roxb.	2×	China
5	<i>Mikurajima24 (wild type)</i>	<i>Morus kagayamae</i> Koidz.	unknown	Japan
6	<i>Okushiritou Inaho2 (wild type)</i>	<i>Morus bombycis</i> Koidz.	unknown	Japan
7	<i>Tsukasaguwa</i>	<i>Morus latifolia</i> Poir.	2×	Japan
8	<i>Shiromerosou</i>	<i>Morus latifolia</i> Poir.	2×	Japan
9	<i>Ooshimagawa</i>	<i>Morus latifolia</i> Poir.	2×	Japan
10	<i>Nezumigaeshi</i>	<i>Morus alba</i> Linn.	2×	Japan
11	<i>Negoyatakasuke</i>	<i>Morus bombycis</i> Koidz.	2×	Japan
12	<i>Naganuma</i>	<i>Morus latifolia</i> Poir.	2×	Japan
13	<i>Kokuso-dai21</i>	<i>Morus latifolia</i> Poir.	2×	Japan
14	<i>Goroujiwase</i>	<i>Morus bombycis</i> Koidz.	2×	Japan
15	<i>Akagi</i>	<i>Morus bombycis</i> Koidz.	3×	Japan
16	<i>Aizujyujima</i>	<i>Morus bombycis</i> Koidz.	2×	Japan
17	<i>Okinawa</i>	<i>Morus acidosa</i> Griff.	2×	Japan
18	<i>Taisou3</i>	<i>Morus formosensis</i> Hotta	unknown	China
19	<i>Taisou2</i>	<i>Morus formosensis</i> Hotta	unknown	China
20	<i>Tadam</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
21	<i>Som</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
22	<i>Soi</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
23	<i>Poo</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
24	<i>Pai</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
25	<i>Noi</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
26	<i>Maeluk-on (♂)</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
27	<i>Maeluk-on (♀)</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
28	<i>Keekai</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
29	<i>Keaw stuk</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
30	<i>Keaw (♂)</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
31	<i>Chiangkum</i>	<i>Morus rotunbiloba</i> Koidz.	2×	Thailand
32	<i>Bai poe</i>	<i>Morus rotunbiloba</i> Koidz.	unknown	Thailand
33	<i>Rosiyaguwa</i>	<i>Morus alba</i> Linn.	unknown	Russia
34	<i>Shimagawa</i>	<i>Morus acidosa</i> Griff.	2×	Japan
35	<i>Fukkoguwa</i>	<i>Morus latifolia</i> Poir.	2×	Japan
36	<i>Hachijouguwa</i>	<i>Morus kagayamae</i> Koidz.	2×	Japan
37	<i>Yukishirazu</i>	<i>Morus bombycis</i> Koidz.	2×	Japan
38	<i>Roguwa</i>	<i>Morus latifolia</i> Poir.	2×	Japan
39	<i>Ichinose</i>	<i>Morus alba</i> Linn.	2×	Japan
40	<i>Enasiguwa</i>	<i>Morus bombycis</i> Koidz.	2×	Japan
41	<i>Kairyo-nezumigaeshi</i>	<i>Morus alba</i> Linn.	2×	Japan
42	<i>Kenmochi</i>	<i>Morus bombycis</i> Koidz.	2×	Japan

<sup>a</sup>, Classified according to original labeling provided by NIAS, Tsukuba.<sup>b</sup>, Ploidy according to original labeling provided by NIAS, Tsukuba, if known.<sup>c</sup>, Geographical origin.

Table 3.2. Pre-amplification and selective amplification primers used in this study

Primer		Primer sequence (5' - 3')
<i>Eco</i> RI – adapter top strand	making adapter	CTCGTAGACTGAGTACC
<i>Eco</i> RI – adapter bottom strand	„	CATCTGACGCATGGTTAA
<i>Mse</i> I – adapter top strand	„	GACGATGAGTCCATGAG
<i>Mse</i> I – adapter bottom strand	„	TACTCAGGACTCAT
<i>Eco</i> RI – 0	Pre-amplification primer	GACTGCGTACCAATTC
<i>Mse</i> I – C	„	GATGAGTCCTGAGTAAC
<i>Eco</i> RI – TA	Selective-amplification primer	GACTGCGTACCAATTCTA
<i>Eco</i> RI – AT	„	GACTGCGTACCAATTCAT
<i>Eco</i> RI – AG	„	GACTGCGTACCAATTCAG
<i>Mse</i> I – CAA	„	GATGAGTCCTGAGTAACAA
<i>Mse</i> I – CAC	„	GATGAGTCCTGAGTAACAC
<i>Mse</i> I – CTA	„	GATGAGTCCTGAGTAACTA
<i>Mse</i> I – CTG	„	GATGAGTCCTGAGTAACTG

The % polymorphism (%*P*) was calculated using the following equation:  $P = (Pb/Nb) \times 100\%$ , where *Pb* is the number of polymorphic bands and *Nb* is the total number of amplified bands.

The estimate of genetic similarity (GS) among all of the genotypes was calculated according to the simple matching coefficient (Sneath and Sokal, 1973) as follows:  $GS_{ij} = a + d / (a + b + c + d)$ , where  $GS_{ij}$  is the measurement of the genetic similarity between individuals *i* and *j*, *a* is the number of polymorphic bands present in both individuals, *b* is the number of bands present in *i* and absent in *j*, *c* is the number of bands present in *j* and absent in *i*, and *d* is the number of bands absent in both individuals (but present in at least one other genotype using the same primers).

The matrix of similarity was analyzed using UPGMA, as suggested by Sneath and Sokal (1973). The cophenetic value based on the GS was calculated as a quantitative indication of the grouping analysis performance. All of the analyses were performed using NTSYS pc.2.1p (Rohlf, 2002).

### 3.3 RESULTS

#### 3.3.1 AFLP fingerprinting and the degree of polymorphism in mulberry varieties

AFLP analyses were performed according to Vos *et al.* (1995), with some modifications. In the present study, 12 primer combinations of three *Eco*RI primers and four *Mse*I primers were used. Among them, six primer combinations, E-TA/M-CAA, E-TA/M-CAC, E-AT/M-CAA, E-AT/M-CAC, E-AG/M-CTA, and E-AG/M-CTG effectively generated AFLP bands from the 42 *Morus* varieties (Table 3.2). The primer combinations

Table 3.3. Results of the AFLP analysis obtained from 6 AFLP primer combinations among 42 mulberry genotypes

Primer pairs	Nb	Pb	%P
E – TA/M – CAA	19	9	47.37
E – TA/M – CAC	17	3	17.64
E – AT/M – CAA	15	2	13.33
E – AT/M – CAC	16	2	12.50
E – AG/M – CTA	20	8	40.00
E – AG/M – CTG	18	3	16.66
Total	105	27	147.50
Mean	17.50	4.5	24.58

Nb, total number of detected bands;

Pb, number of polymorphic bands;

%P, proportion of polymorphic bands.

produced at least 105 bands, with many faint bands that were omitted from the analysis. Among the 105 bands, 27 bands (24.58%) were detected as polymorphic (Fig. 3.1).

Here, a “polymorphic band” is defined as PCR product that is not observed in every mulberry strain. As shown in Fig. 3.2A, band “a” is found in lanes 3 (*Roguwa*), 6 (*Kairyo-nezumigaeshi*), and 7 (*Kenmochi*), whereas the band “b” is found in lanes 3 (*Roguwa*), 4 (*Ichinose*), 5 (*Enasiguwa*), 6 (*Kairyo-nezumigaeshi*), and 7 (*Kenmochi*). In Fig. 3.2A, PCR was performed using the primer combination of E-TA and M-CAA, while in Fig. 3.2B, the primer combination used was E-AA and M-CAG. Every DNA fragment in Fig. 3.2B was observed in all the mulberry strains; thus, they are designated as “non-polymorphic bands”.

The total number of bands generated by each primer combination ranged from 15 for E-TA/M-CAA to 20 for E-AG/M-CTA, with an average of 17.50. The number of polymorphic bands ranged from two for E-TA/M-CAA and E-AT/M-CAC to nine for E-TA/M-CAA, with an average of 4.5. The highest degree of polymorphism (47.37%) was found for the E-TA/M-CAA primer set, followed by E-AG/M-CTA (40.00%), E-AT/M-CAC (17.64%), E-AG/M-CTG (16.66%), and E-AT/M-CAC (12.50%), as shown in Table 3.3.

### 3.3.2 Genetic relationships

The genetic similarity matrix for all mulberry varieties was obtained from the similarity coefficients (data not shown). The values of genetic-similarity ranged from 0.556 to 0.992, with an average of 0.774. The lowest genetic similarity (0.556) was found between *Nezumigaeshi* (10) and *Taisou3* (18), *Nezumigaeshi* (10) and *Taisou2* (19), and *Nezumigaeshi* (10) and *Tadam* (20). The highest genetic similarity (0.992) was found between *Chiangkum*

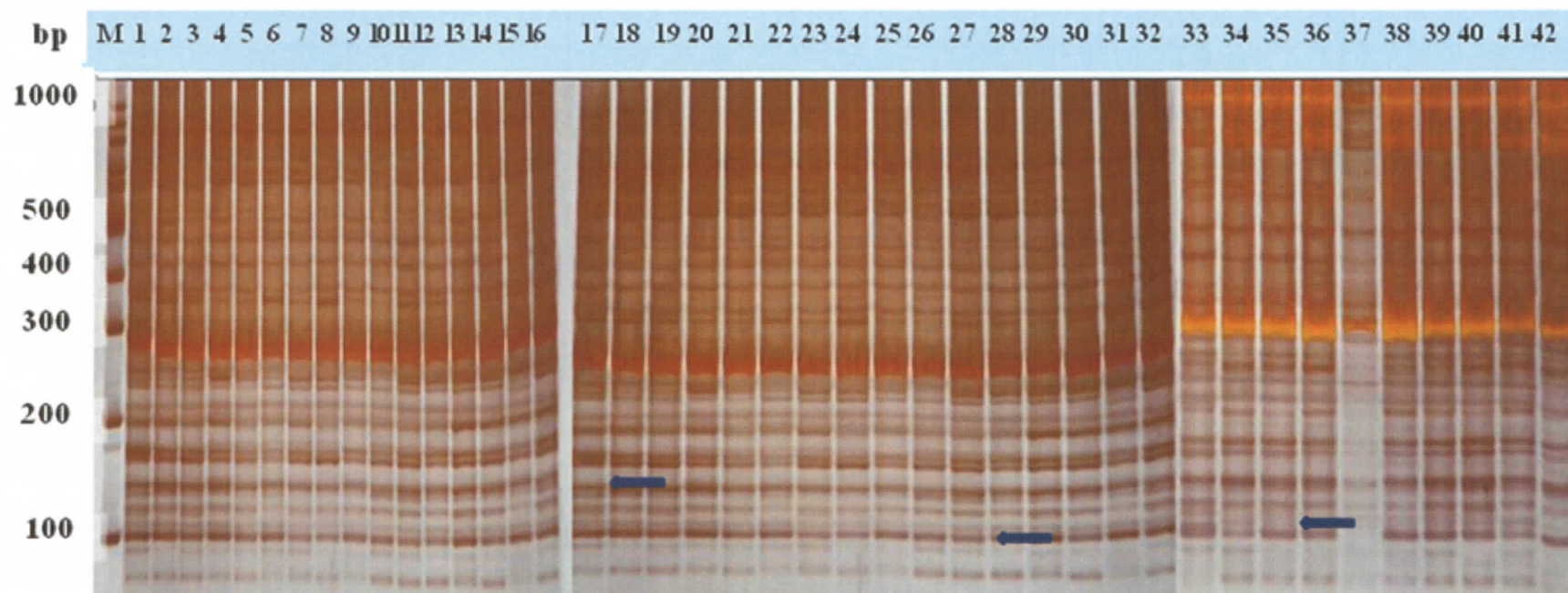


Fig. 3.1. PAGE pattern of AFLP fingerprints using primer combination E-AG/M-CTG in 42 mulberry varieties. The AFLP method was carried out following the standard procedure described by Vos *et al.* (1995). Genomic DNA was digested with a pair of restriction enzyme (*EcoRI* / *MseI*), then ligated to double stranded *EcoRI* and *MseI* adapters. Electrophoresis was performed using e-PAGEL under constant power 200V, 20mA for 80 min. The gel was visualized by silver staining. Lanes 1-32 are mulberry varieties collected from NIAS. Lanes 5-6 are wild-type mulberries. Lanes 20-32 are Thai mulberry varieties grown in a greenhouse. Lanes 33-42 were collected from the Experimental Farm of Textile Science and Technology, Shinshu University. M, DNA molecular size marker 100 bp DNA ladder. The mulberry variety codes are the same as those shown in Table 4.1.

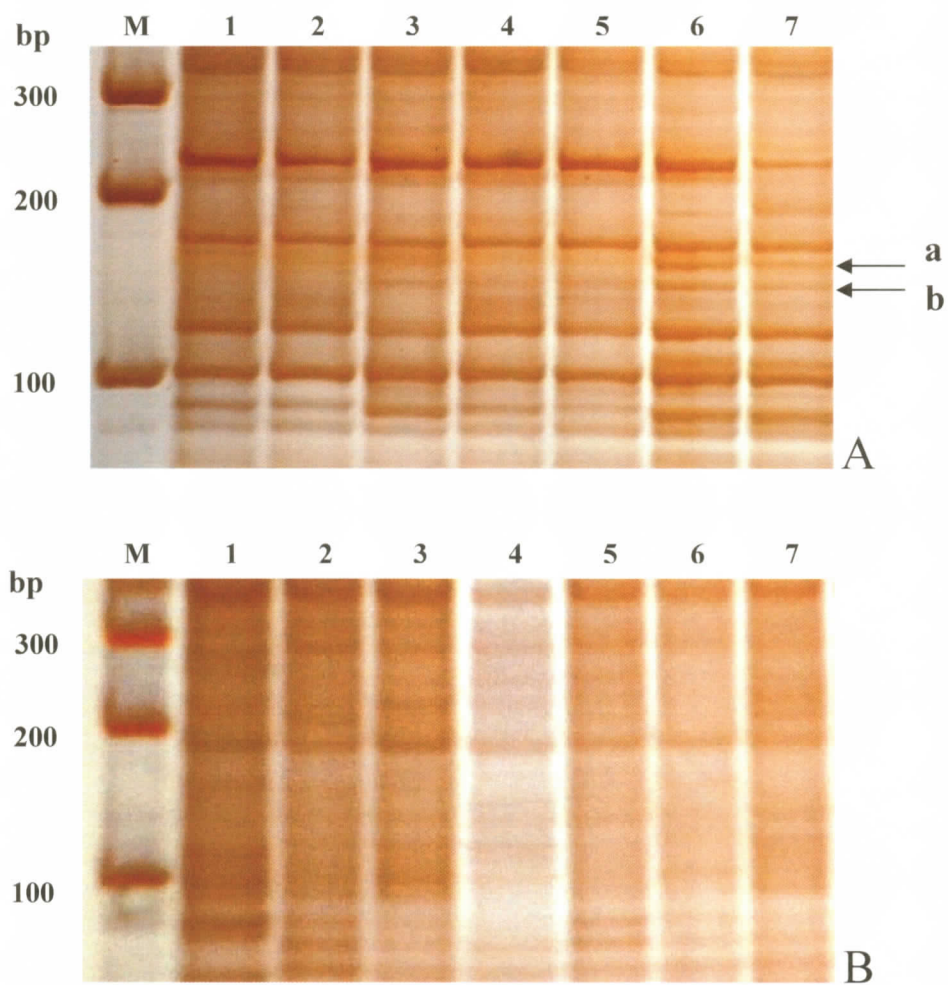


Fig. 3.2. Polyacrylamide-gel electropherograms of PCR products showing: A, an example of a primer combination that generated “polymorphic bands” and B, an example that generated only “non-polymorphic bands”. Primer combination for PCR in A, E-TA and M-CAA and B, E-AA and M-CAG. ← a and ← b, polymorphic bands. Each electropherogram shows a restricted range of the degree of polymerization between 100 and 500 bp. The mulberry variety codes are the same as those shown in Table 3.1.

(31) and *Bai poe* (32). The same value was also found between *Maeluk-on* (♀) (27) and *Keekai* (28). For the wild-type mulberries, *Mikurajima24* (5) and *Tsukasaguwa* (7), the highest genetic similarity (0.865) was found with *Shiromerosou* (8). The lowest genetic similarity (0.603) was found between *Okushiritou Inaho2* (6) and *Tadam* (20), suggesting that they are relatively remote in terms of their genetic relationship.

### 3.3.3 Cluster analysis

Cluster analysis based on the polymorphic AFLP bands separated the 42 mulberry genotypes into two main clusters, A and B (Fig. 3.3). Cluster A was further divided into three sub-clusters. The first sub-cluster (a1) is the largest sub-cluster of cluster A and comprises 10 genotypes (*Beikoku13* (1), *Kosou199* (3), *Amoi No. 1* (4), *Naganuma* (12), *Kokuso-dai21* (13), *Tsukasaguwa* (7), *Shiromerosou* (8), *Ooshimaguwa* (9), *Mikurajima24* (wild-type) (5), and *Okushiritou Inaho2* (wild-type) (6)). The second sub-cluster (a2) is the smallest group, with two genotypes, *Iran2* (2) and *Nezumigaeshi* (10). The third sub-cluster (a3) comprises four genotypes (*Negoyatakasuke* (11), *Goroujiwase* (14), *Akagi* (15), and *Aizuujyujima* (16)).

Cluster B is composed of 26 genotypes exhibiting high similarity coefficients (more than ~0.8) and was further divided into two sub-clusters. Sub-cluster b1 comprises 16 genotypes, where four genotypes (*Okinawa* (17), *Taisou3* (18), *Taisou2* (19), and *Tadam* (20)) are grouped together with 12 genotypes (*Som* (21), *Keawstuk* (29), *Soi* (22), *Keaw* (♂) (30), *Poo* (23), *Pai* (24), *Noi* (25), *Maeluk-on* (♀) (27), *Keekai* (28), *Maeluk-on* (♂) (26), *Chiangkum* (31), and *Bai poe* (32)). Sub-cluster b2 comprises 10 genotypes, where nine genotypes (*Rosiyaguwa* (33), *Shimaguwa* (34), *Hachijyouguwa* (36), *Ichinose* (39), *Kairyo-*

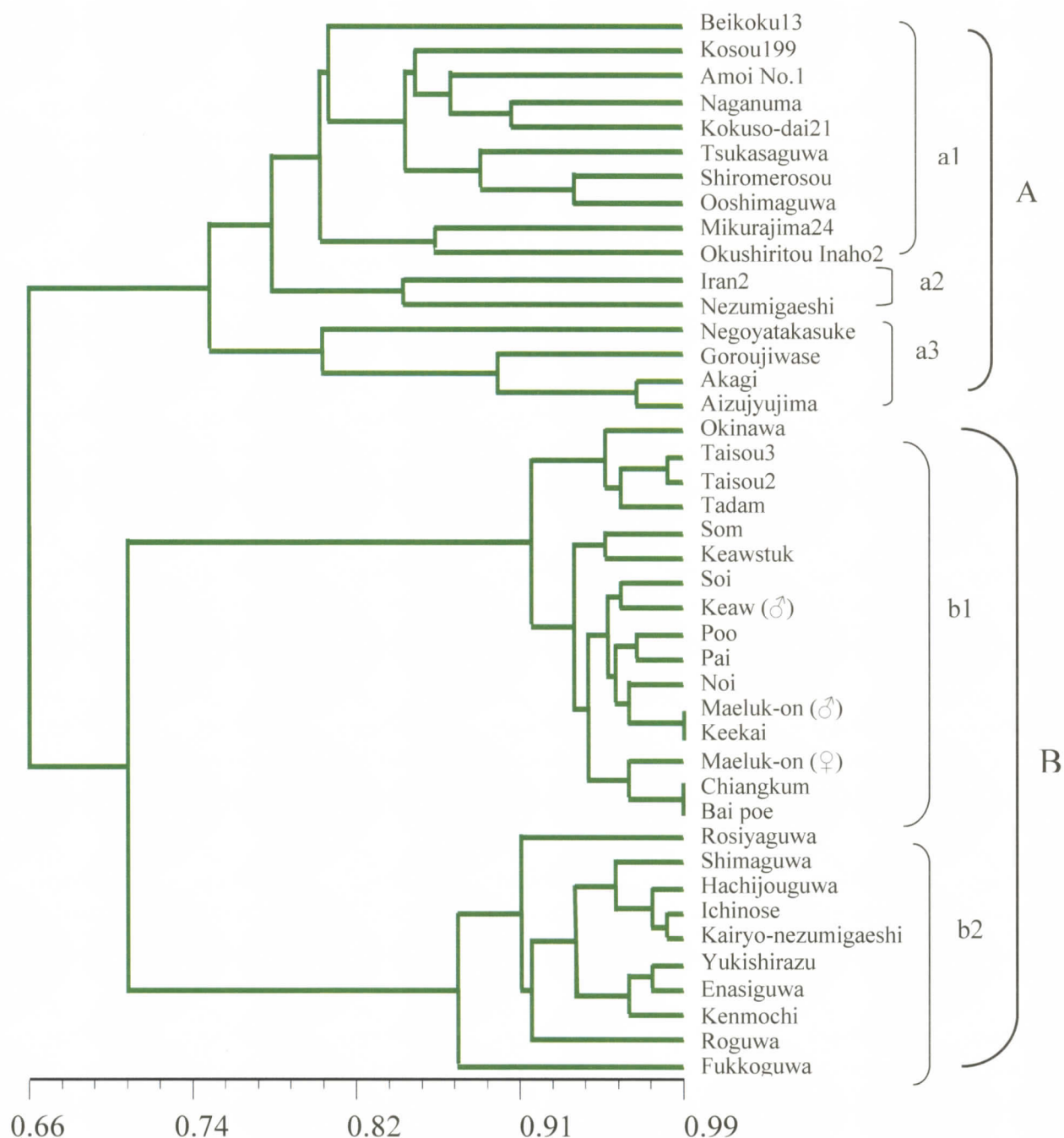


Fig. 3.3. Dendrogram of *Morus* accessions based on AFLP data using the simple matching coefficient UPGMA. A UPGMA dendrogram based on genetic distance as determined by AFLP markers. Pairwise similarity matrices were generated by simple matching coefficient analysis. To construct the dendrograms, NTSYS pc 2.10 (Rolf, 2000) was used. Cluster methods and similarity coefficients were tested by applying SIMQUAL and TREE in NTSYS pc.2.10. The SAHN module was used as the clustering method.

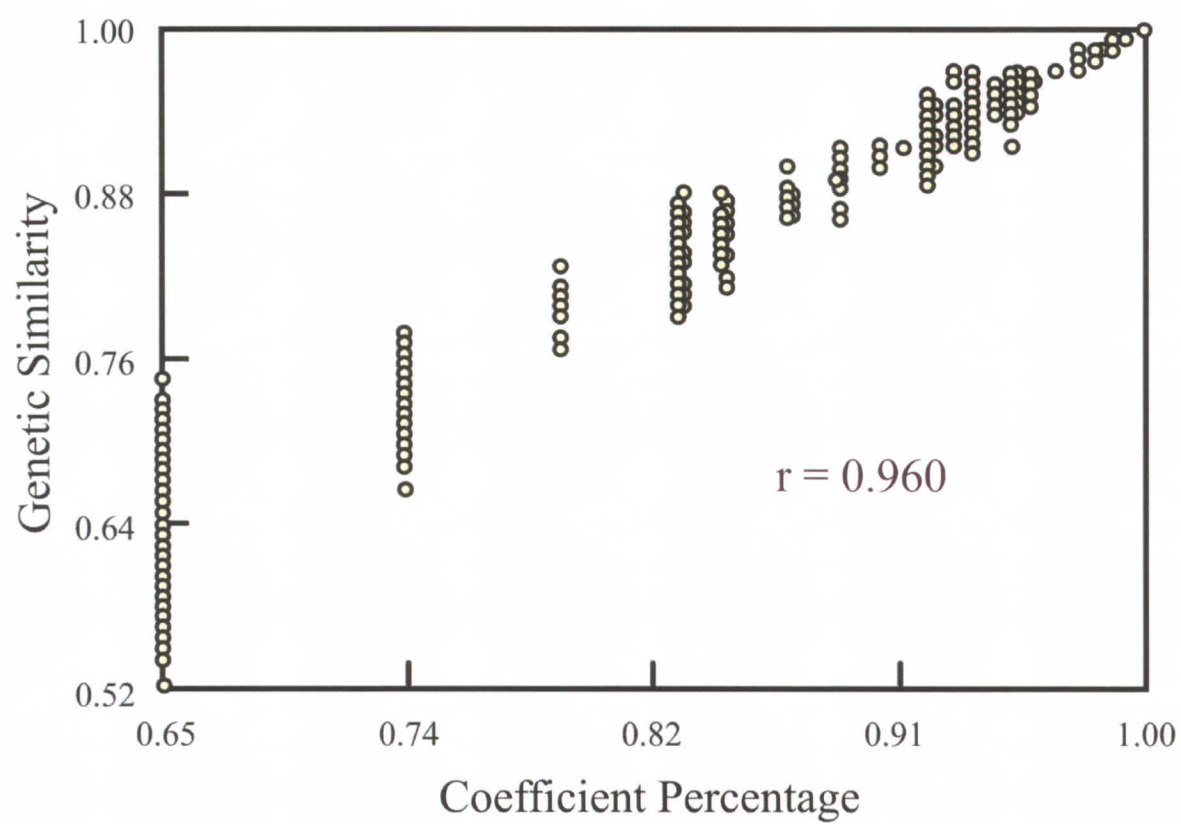


Fig. 3.4. Matrix comparison of 42 mulberry varieties based on AFLP analyzed by the NTSYS pc 2.1 program.

*nezumigaeshi* (41), *Yukishirazu* (37), *Enasiguwa* (40), *Kenmochi* (42), and *Roguwa* (38)) are grouped together with *Fukkoguwa* (35). The cophenetic matrix correlation coefficient (Mantel, 1967) was highly significant ( $r = 0.960$ ), indicating strong statistical agreement between the dendrogram clusters and the similarity matrix, as shown in Fig. 3.4.

### 3.4 DISCUSSION

The mulberry is a perennial and economically important plant that has traditionally been cultivated as food for silkworms. It is important to evaluate the genetic relationships among varieties of mulberry for the long-term improvement of leaf yield, quality, and resistance to various diseases, as well as for germplasm conservation and identification. In this study, we successfully used AFLP to analyze the genetic diversity among 42 mulberry accessions comprising various cultivars and wild-types. Such an analysis is particularly useful in the selection of genetically divergent parents for breeding purposes. For example, the wild species exhibit drought-tolerance characteristics, such as leaf rolling, abundant xylem, less stomata per unit area, and slow growth in response to moisture stress, which are useful breeding traits for the development of stress-resistant varieties (Tikader and Thangavelu, 2003). In the dendrogram shown in Fig. 3.3, the large genetic distance between wild-types and local Thai varieties (*M. rotundiloba*) may indicate an opportunity for the selective breeding of mulberry varieties for sericulture in water-stressed areas. In this study, more than 20 combinations of *EcoRI* and *MseI* primers were examined, of which only six primer combinations produced clear polymorphic marker profiles for the 40 cultivars and two wild-types of *Morus* spp. (Table 3.3).

The average number of polymorphisms per primer combination was low (24.58%). However, the primer sets clearly generated AFLP fragments and the small number of bands was convenient for this analysis, for both the cultivated and wild-type mulberries. The size range of the bands that were employed for scoring in this analysis was rather short (100-500 bp), although they were longer than those scored in a previous study, which applied bands with sizes between 35-500 bp on AFLP electropherograms of *Morus* sub-groups (Sharma *et al.*, 2000). However, the genetic similarity estimated in our study ranged from 0.556 to 0.992, which is not significantly different compared with the study by Sharma *et al.* (2000), who used different primer combinations in their AFLP analysis. They reported that the use of five AFLP primer combinations (*Eco*RI+3 carrying three selective nucleotides in combination with *Mse*I+3 primers carrying three selective nucleotides) identified a genetic similarity ranging from 0.58 to 0.99. Sharma *et al.* (2000) investigated genetic diversity using 45 mulberry accessions from different eco-geographic regions, including Japan. In that study, an alteration in the DNA sequence was detected by sequencing or DNA analysis (fingerprint profiling). Their analysis of polymorphisms focused mainly on the genetic relationship between genotypes from the different eco-geographic regions. Here, we report the results from a similar AFLP analysis using 42 mulberry genotypes. In this study, the detailed genetic relationships among cultivar species and between wild species and cultivar species were analyzed, which provides novel information regarding the genetic relationships of mulberry plants.

The dendrogram of the genotype grouping was based on the simple matching (SM) coefficient between mulberry cultivars and wild-type, which ranged from 0.603 [*M. bombycis*

(wild-type) and *M. rotunbiloba*] to 0.865 [*M. kagayamae* (wild-type) and *M. latifolia*]. This result implies a correlation with a morphological characteristic of *M. kagayamae* and *M. latifolia* plants, which has been described in a previous study (Machii *et al.*, 1999; Machii *et al.*, 2001). Among the species of cultivated mulberry, the genetic similarity matrix distance indicated that *M. alba* was far from *M. rotunbiloba*, and the lowest SM value between the two species was 0.556, which agrees with the morphological classification of the genus *Morus* (Koidzumi, 1917). Koidzumi (1917) grouped mulberry species into two clusters according to the length of the styles of the female flowers, with Dolichostylae belonging to *M. rotunbiloba* and Macromorus belonging to *M. alba*. In the present study, the highest SM value (0.984) was found between *M. alba* Linn. and *M. bombycis* Koidz, further supporting the view that accessions identified as *M. alba*, *M. latifolia*, and *M. bombycis* are not genetically differentiated and share a common gene pool.

The cophenetic correlation for the resulting AFLP dendrogram in this study was 0.960 ( $0.5 < x \leq 1.0$ ), indicating a highly credible statistical agreement between the dendrogram clusters and the similarity matrix.

The results from this study are in agreement with the genetic relationships of mulberry varieties and indicated that AFLP fingerprints are a reliable and reproducible method for establishing a molecular reference system for the genus *Morus*, especially for four of the species (*M. alba*, *M. bombycis*, *M. latifolia* and *M. rotunbiloba*). Furthermore, our results indicate that AFLP analysis is suitable for evaluating the extent of natural hybridization that can occur among populations.

In conclusion, AFLP markers can be successfully employed to assay the level of polymorphism and diversity in mulberry. Using modern techniques, such as PCR-based DNA amplification, one can discriminate Japanese mulberry varieties from Thai mulberry varieties, which is difficult based on morphology alone. Thus, we suggest that estimates of genetic similarity based on molecular markers provide more accurate information for plant breeders than estimates based on morphological methods, allowing breeders to make reliable crosses on a short-term basis or to formulate a breeding program on a long-term basis.



## CHAPTER 4 Application of amplified fragment length polymorphism (AFLP) as sex-specific markers in *Morus* spp.

### 4.1 INTRODUCTION

In most of the regularly dioecious plants, pistils and stamens develop on separate individuals, which are distinguished as “pistillate plants” (female plants) and “staminate plants” (male plants), respectively. Such sex separation (dioecy) is found in some 15,000 species, in 1,300 genera, and 60 families (Parker, 1990).

The mulberry (*Morus* spp.) is a typical dioecious plant. Mulberries are a group of small trees or shrubs belonging to the family Moraceae. The Moraceae are characterized by simple inflorescences, such as racemes or spikes, with flower parts in four (Fig. 1.5). Many Moraceae have fixed stamens, which are structurally supported by pistillodes. The stamens spring back and eject pollen into the air at anthesis (Clement and Weiblen, 2009). Moraceae comprises 37 genera and approximately 1,100 species (Burger, 1962), distributed in a wide area of temperate, tropical, subtropical, and sub-arctic zones (Sharma *et al.*, 2000). The sex chromosome system of mulberry is  $n=12+XX$  in females and  $n= 12+XY_1 Y_2$  in males. Cytologically, in addition to normal diploid mulberry with  $2n=28$  chromosomes, natural polyploids are also cultivated (Machii *et al.*, 2001), such as *M. alba*, *M. bombycis*, *M. latifolia*, and *M. rotundiloba*.

In many agriculturally important plants, such as Hemp (*Cannabis sativa* L.), male plants usually have a higher fiber content and better qualities than female (Bosca and Karus,

1998; Mandolino *et al.*, 1999). In *Asparagus*, male plants provide better product qualities than female plants, whereas in other plants, such as kiwi fruit, papaya, and mulberry (for fruit), the females are the commercial harvest. Therefore, identification of the sex of such plants at their early stage of growth can be of great economic benefit. Sex-related markers have been identified successfully in a number of dioecious plant species, such as *Pistacia vera* (Hormaza *et al.*, 1994) or *Salix viminalis*, (Alstrom-Rapaport *et al.*, 1998).

In Chapter 3, we used amplified fragment length polymorphism (AFLP) analysis to study the genetic relationships of mulberry strains. During that research, we identified some polymorphic bands amplified by certain combinations of AFLP primers that may correspond with sex expression of mulberry trees. Based on this, we attempted to extend our experiment to other mulberry varieties whose sexualities are not yet known. Our results and modified methods could provide valuable data on the mechanisms of sex determination, and is suitable for a precise, early, and rapid identification of female mulberry plants during breeding programs in the future.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Plant materials

Nine selected Japanese mulberry varieties were obtained from the National Institute of Agrobiological Science (NIAS), Tsukuba, Ibaraki, Japan (Table 4.1). Young mulberry leaves (2-3 leaves from the top) were harvested separately and stored immediately at -80° C before DNA extraction.

Table 4.1. Information on the *Morus* germplasm, used for the AFLP analysis in this study

No.	Varieties	Species <sup>a</sup>	Polyploidy <sup>b</sup>	Sex expression <sup>c</sup>
1	<i>Shin-ichinose</i>	<i>Morus alba</i> Linn.	2x	Male
2	<i>Shiromerosou</i>	<i>Morus latifolia</i> Poir.	2x	Male
3	<i>Hayatesakari</i>	<i>Morus alba</i> Linn.	2x	Male
4	<i>Kairyo-ichinose</i>	<i>Morus alba</i> Linn.	2x	Female
5	<i>Kokuso-dai21</i>	<i>Morus alba</i> Linn.	2x	Male
6	<i>Kenmochi</i>	<i>Morus bombycis</i> Koidz.	2x	Female
7	<i>Mitsushigeri</i>	<i>Morus bombycis</i> Koidz.	3x	Female
8	<i>Kairyo-nezumigaeshi</i>	<i>Morus alba</i> Linn.	2x	Male
9	<i>Ichinose</i>	<i>Morus alba</i> Linn.	2x	Female
10	<i>Usuneno-ookuwa</i>	unknown	unknown	unknown
11	<i>Okushiritou Inaho2</i>	<i>Morus bombycis</i> Koidz.	unknown	unknown
12	<i>Hayoshino-ookuwa</i>	unknown	unknown	unknown

<sup>a</sup>, Classified according to the original labeling provided by NIAS, Tsukuba, if known;

<sup>b</sup>, Polyploidy according to the original labeling provided by NIAS, Tsukuba, if known;

<sup>c</sup>, Sex expression according to the original labeling provide by NIAS, Tsukuba, if known.

Table 4.2. Pre-amplification and selective amplification primers used in this study

Primer		Primer sequence (5' → 3')
<i>Eco</i> RI – adapter top strand	making adapter	CTCGTAGACTGAGTACC
<i>Eco</i> RI – adapter bottom strand	„	CATCTGACGCATGGTTAA
<i>Mse</i> I – adapter top strand	„	GACGATGAGTCCATGAG
<i>Mse</i> I – adapter bottom strand	„	TACTCAGGACTCAT
<i>Eco</i> RI – 0	Pre-amplification primer	GACTGCGTACCAATTC
<i>Mse</i> I – C	„	GATGAGTCCTGAGTAAC
<i>Eco</i> RI – AT	Selective-amplification primer	GACTGCGTACCAATTTCAT
<i>Eco</i> RI – TC	„	GACTGCGTACCAATTCTC
<i>Eco</i> RI – TG	„	GACTGCGTACCAATTCTG
<i>Eco</i> RI – TT	„	GACTGCGTACCAATTCTT
<i>Mse</i> I – CAG	„	GATGAGTCCTGAGTAACAG
<i>Mse</i> I – CTC	„	GATGAGTCCTGAGTAACTC
<i>Mse</i> I – CTG	„	GATGAGTCCTGAGTAACTG
<i>Mse</i> I – CTT	„	GATGAGTCCTGAGTAACTT

#### 4.2.2 DNA extraction

Genomic DNA extraction was performed as described in “MATERIALS AND METHODS” in Chapter 2.

#### 4.2.3 AFLP analysis

The AFLP analysis was performed according to Vos *et al.* (1995) with some modifications (see “MATERIALS AND METHODS” in Chapter 3)

#### 4.2.4 Electrophoresis and silver staining of AFLP products

All procedures were done as described in “MATERIALS AND METHODS” in Chapter 3.

### 4.3 RESULTS

Sixty-four AFLP primer combinations were used to identify genetic relationships among the mulberry genus *Morus* and were able to amplify fragments by AFLP. Sixteen AFLP primer combinations were selected to produce AFLP bands in the genomic DNA of male *Shiromerosou* and female *Mitsushigeri* mulberry individuals. Out of more than 21 clear bands in the range of 100 to 500 bp, a single band of approximately 240 bp was identified using the *EcoRI*+TC / *MseI*+CTC primer combination that showed a consistent sex-specific pattern: it is present in all females, but in none of the males (Fig.4.1). To verify that the (*EcoRI*+TC / *MseI*+CTC)<sub>240</sub> band was linked to sex, seven other Japanese varieties, including three females and four males, were tested independently (Table 4.1). The result

showed that primer combination *EcoRI*+TC / *MseI*+CTC produced a DNA band at 240 bp in all female samples (lanes 4, 6, and 9; Fig. 4.2) with a clear sex, but was absent in all males (lanes 1, 2, 3, 5 and 8; Fig.4.2). On the other hand, other combinations of AFLP primers used in this study could have amplified non-polymorphic and polymorphic bands that did not correspond to the sex of mulberries. Thus, the primer (*EcoRI*+TC / *MseI*+CTC)<sub>240</sub> could be recognized as a putative sex-linked marker for nine Japanese mulberry varieties.

To the best of our knowledge, this is the first report to successfully provide a sex-specific DNA marker in Japanese mulberry varieties. The marker (amplified DNA fragment) is specific to females, and is not amplified from males. The primer combination *EcoRI*+TC/*MseI*+CTC can be used as a gender determination tool.

Other species and types of mulberry were examined using the *EcoRI*+TC/ *MseI*+CTC primers to detect the sex of mulberry correctly, according to original labeling by NIAS, as shown in Table 4.1. For example, three Japanese mulberry wild-types (*Okushiritou Inaho2*, *Hayoshino-ookuwa* and *Usuneno-ookuwa*) were examined with the *EcoRI*+TC / *MseI*+CTC primers. A single band, with length around 240 bp, generated for *Okushiritou Inaho2* and *Hayoshino-ookuwa*, was observed on the polyacrylamide gel (Fig. 4.3). In the author's opinion, *Okushiritou Inaho2* and *Hayoshino-ookuwa* should be designated as female; however, this opinion requires further verification. Therefore, the sex determination method detailed in this chapter remains tentative.

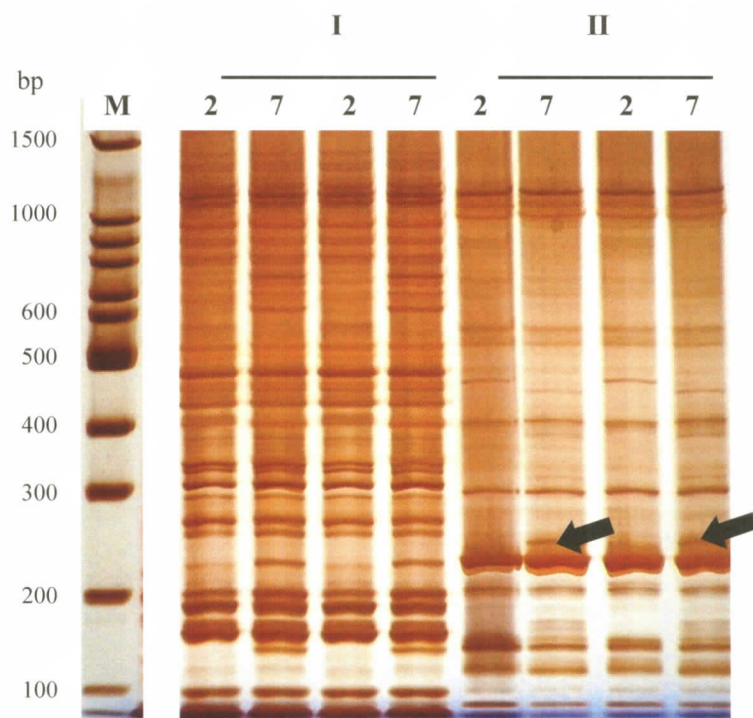


Fig. 4.1. Amplified fragment length polymorphism (AFLP) polyacrylamide gel pattern with female and male samples of two Japanese mulberry varieties. The gel was visualized by silver staining. The selective nucleotide of the primer combinations are: (I), *Eco*RI+TG / *Mse*I+CTG and (II), *Eco*RI+TC / *Mse*I+CTC. The order of the bulks within the primer combination is No. 2, (*Shiromerosou*) male and No. 7, (*Mitsushigeri*) female. M, DNA molecular size marker 100 bp ladder. Putative positive polymorphisms are indicated with black arrows.

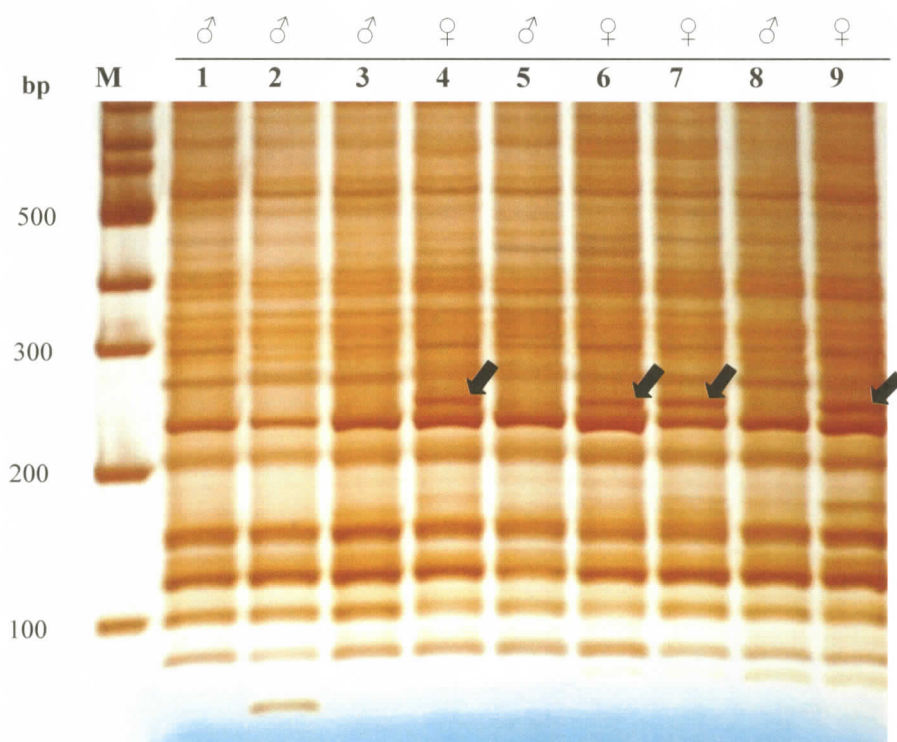


Fig. 4.2. Amplified fragment length polymorphism (AFLP) polyacrylamide gel pattern generated from the bulked female and male DNA samples of nine *Morus* cultivars using selective combination primer *Eco*RI+TC/*Mse*I+CTC. M, DNA molecular size marker 100 bp ladder. The mulberry variety codes are the same as those shown in Table 1. Black arrows indicate polymorphic bands in female samples at ~240 bp.

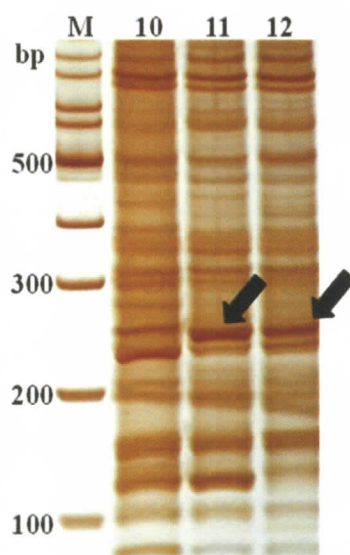


Fig. 4.3. Amplified fragment length polymorphism (AFLP) polyacrylamide gel pattern generated from the bulked female and male DNA samples of 3 *Morus* wild varieties using selective combination primer *Eco*RI+TC / *Mse*I+CTC. M, DNA molecular size marker 100 bp ladder. The mulberry variety codes are the same as those shown in Table 1. Black arrows were indicated polymorphism bands of female ~240 bp.

#### 4.4 DISCUSSION

In many sexually reproducing plants and animal species, all individuals are essentially alike in their gender condition. Only a minority of plant species is 'sexually monomorphic', including dioecious species, with separate males and females. Many dioecious species with hermaphrodite relatives have rudimentary opposite sex structures in the flowers of plants of each sex, suggesting the recent evolution of unisexual flowers (Darwin, 1877). In previous studies, DNA analyses using PCR techniques have proved to be a reliable strategy. For example, in the identification of markers for the sex-specific Y chromosome in animals (Charlesworth, 1991), the isolation W and Z sex chromosome in the ostrich, (*Struthio camelus*) (Griffiths and Orr, 1999), or the isolation of sex specific markers in the Spotted Halibut (*Veraper variegates*) (Ma *et al.*, 2010). In addition, several studies have identified sex-specific markers in dioecious plants, such as the use of the RAPD technique to identify possible markers of sex determination in *Asparagus* (Jiang and Sink, 1997), *Carica papaya* (Urasaki *et al.*, 2002; Chaves-Bedoya and Nunenz, 2007), and *Eucommia ulmoides* Oliv. (Xu *et al.*, 2004).

The present study could be of great help in identifying the sex of Japanese mulberry cultivars at an early stage of growth, based on the AFLP method. At present, we are sequencing the female polymorphic band(s) (240 bp) not only from Japanese mulberry cultivars, but also from Thai mulberry local varieties and wild-types. The author also believes that our results, and the modified methods, contribute to the understanding of the mechanisms of sex determination, and are suitable for a precise, early, and rapid identification of female mulberry plants during future breeding programs.

## CHAPTER 5 Effect of environmental conditions on the $\alpha$ -glucosidase inhibitory activity of mulberry leaves

### 5.1 INTRODUCTION

Mulberry leaves have been historically used as food for domestic silkworms. Mulberry leaves are also consumed as an ingredient of beverages or health food for humans. Certain mulberry leaf components inhibit the activity of digestive enzymes associated with sugar absorption,  $\alpha$ -glucosidases, and promote slow absorption of glucose into the blood vessels (Asano *et al.*, 1994; Asano *et al.*, 1995; Asano *et al.*, 2001). The most important substance in mulberry leaves, 1-deoxynojirimycin (1-DNJ), is known to be a strong intestinal  $\alpha$ -glucosidase inhibitor (Kimura *et al.*, 2004), leading to the commercialization of mulberry leaf products as health foods.

In contrast, mulberry leaves are highly growth suppressing to caterpillars other than the silkworm, *Bombyx mori*, because they contain 1-DNJ and other  $\alpha$ -glucosidase inhibitors that affect the growth of common insects (Konno *et al.*, 2006; Hirayama *et al.*, 2007). The silkworm has evolved adaptive enzymes to circumvent the growth suppressing effects of  $\alpha$ -glucosidase inhibitors, and is thus able to feed on mulberry leaves (Hirayama *et al.*, 2007; Daimon *et al.*, 2008). 1-DNJ and the other  $\alpha$ -glucosidase inhibitors from mulberry leaves are therefore used to both improve the symptoms of diabetes mellitus, and inhibit the growth of caterpillars.

Mulberry plants are distributed throughout the world, with over 1,000 mulberry cultivars in Japan alone. These cultivars match the climatic characteristics in various areas. The typical cultivars of mulberry plants are maintained and administered in Japan at the National Institute of Agrobiological Science (Tsukuba, Ibaraki, Japan) and the “Gene Resource Stock Yard”, Laboratory of Field Science, Shinshu University (Ueda, Nagano, Japan). It has been reported that the  $\alpha$ -glucosidase inhibitory activity and 1-DNJ concentration of mulberry leaves differ among various cultivars and seasons (Yatsunami *et al.*, 2003; Kimura *et al.*, 2007; Yatsunami *et al.*, 2008; Nakanishi *et al.*, 2011), but it is unknown which environmental conditions influence the  $\alpha$ -glucosidase inhibitory activity. We therefore investigated the relationship between environmental conditions and  $\alpha$ -glucosidase inhibitory activity.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Chemicals

Standard 1-DNJ, acetonitrile, ethanol, and ammonium acetate were purchased from Wako Pure Chemicals (Osaka, Japan).

### 5.2.2 Plant materials

The mulberry cultivars used in this study were *Akameyanagida* (*M. bombycis* Koidz.), *Ichinose* (*M. alba* L.), *Fukkoguwa* (*M. latifolia* Poir.), and *Harnngplalod* (*M. rotunbiloba* Koidz.). The largest glossy leaves from the top of the branches of the *Akameyanagida*, *Ichinose*, and *Fukkoguwa* cultivars were harvested twice a week between May 19 and

October 19, 2009 from an outdoor field at the “Gene Resource Stock Yard”, Laboratory of Field Science, Shinshu University (Ueda, Nagano, Japan). The “largest glossy leaves” means fully extended young leaves that are usually used as a starting food for newly hatched silkworm larvae, because they are both sufficiently tender and contain certain nutritional factors. Scission was conducted by cutting all branches at a height of 60-90 cm from the ground at the end of June. The largest glossy leaves from the top of branches of *Harngplalod* were harvested on June 9, July 13, August 7, September 11, and October 2, 2009 from a greenhouse and the outdoor field. The cultivation conditions in the greenhouse were similar to the natural conditions, except for the temperature. Windows of the greenhouse were kept open whenever the temperature was high to ventilate and maintain an approximately ambient temperature, while a thermostatic heater was used whenever the temperature was low to maintain 25°C. Only natural light was used for illumination in the greenhouse. Branches of approximately 50 cm in length from *Akameyanagida*, *Ichinose*, and *Fukkoguwa* were collected on May 19, August 25, September 24, and October 13, 2010 from the outdoor field, and the mulberry leaves were collected from these branches.

### 5.2.3 Preparation of the sample solution

The samples were prepared from one plant of each cultivar. The leaves were dried in hot air or at room temperature, crushed into a powder, and then stored in desiccators until needed. Fifty mg of mulberry leaf powder was extracted with 1.0 ml of 70% methanol in a microtube. After sonicating for 15 minutes, the mixture was centrifuged at 15,000 g for 10

min. The supernatant was passed through a polytetrafluoroethylene (PTFE) filter with a pore size of 0.45  $\mu\text{m}$ .

#### 5.2.4 Measurement of $\alpha$ -glucosidase inhibitory activity

The inhibition of sucrose degradation was measured as the  $\alpha$ -glucosidase inhibitory activity. Fifty  $\mu\text{l}$  of a diluted sample solution was mixed with 500  $\mu\text{l}$  of 1.0% sucrose containing an M/7.5 phosphate buffer solution (pH7.0) and 500  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , and the mixture was preincubated for 10 min at 30°C. The reaction was started by adding 50  $\mu\text{l}$  of  $\alpha$ -glucosidase from yeast (25 U/ml; Wako Pure Chemicals, Osaka, Japan) to the reaction mixture, and this mixture was incubated for 10 min at 30°C. The glucose released in the reaction mixture was determined using a glucose test kit (Glucose C-II Test Wako; Wako Pure Chemicals, Osaka, Japan). Each sample solution was diluted to 1/2 (*Akameyanagida*), 1/5 (*Ichinose*) or 1/50 (*Fukkoguwa* and *HarnGPLalod*).

#### 5.2.5 Quantification of 1-DNJ from mulberry leaves

Mulberry 1-DNJ was determined by hydrophilic interaction chromatography (HILIC) – mass spectroscopy (HILIC-MS) as previously reported by Nakagawa *et al.*, 2007), with some modifications. The HILIC-MS system consisted of an LC-10AD pump (Shimadzu, Kyoto, Japan), a DGU-14AM degasser, a CTO-10A column oven, a SIL-10AD auto injector, and an LCMS-2010 electrospray ionization mass spectrometer (ESI-MS). A TSK gel AMIDE-80 column (2.0 mm  $\times$  150 mm; Tosoh, Tokyo, Japan) was used, with a mobile phase composed of acetonitrile and 6.5 mmol/L of ammonium acetate (72 : 28. v/v, to pH 5.5). The

flow rate was adjusted to 0.2 ml/min, and the column temperature was maintained at 40°C. ESI-MS was carried out in the positive ion measurement mode. The mass spectrum of 1-DNJ was detected at a retention time of 9.2 min via single-ion monitoring (SIM) at  $m/z$  164.0  $[M+H]^+$ .

## 5.3 RESULTS

### 5.3.1 $\alpha$ -glucosidase inhibitory activity from different seasons

The  $\alpha$ -glucosidase inhibitory activity of mulberry leaves varied according to the environmental conditions. To identify the factors that regulated the  $\alpha$ -glucosidase inhibitory activity, we measured this activity in the *Akameyanagida*, *Ichinose*, and *Fukkoguwa* samples from different seasons. The  $\alpha$ -glucosidase inhibitory activity of the largest glossy leaves on the shoot that had not been subjected to scission increased from May to August, and decreased from August to October in the three cultivars (Fig.5.1A). These results are similar to the effects of temperature change (Fig.5.1B). Furthermore, the  $\alpha$ -glucosidase inhibitory activities of the three cultivars subjected to scission increased more than those without scission. This result indicates that new branch growth increased the  $\alpha$ -glucosidase inhibitory activity.

### 5.3.2 Influence of the photoperiod

Although the foregoing result suggests that the  $\alpha$ -glucosidase inhibitory activity was mostly controlled by the surrounding temperature, the influence of the photoperiod needed to be evaluated. Therefore, we measured the difference in  $\alpha$ -glucosidase inhibitory activity of

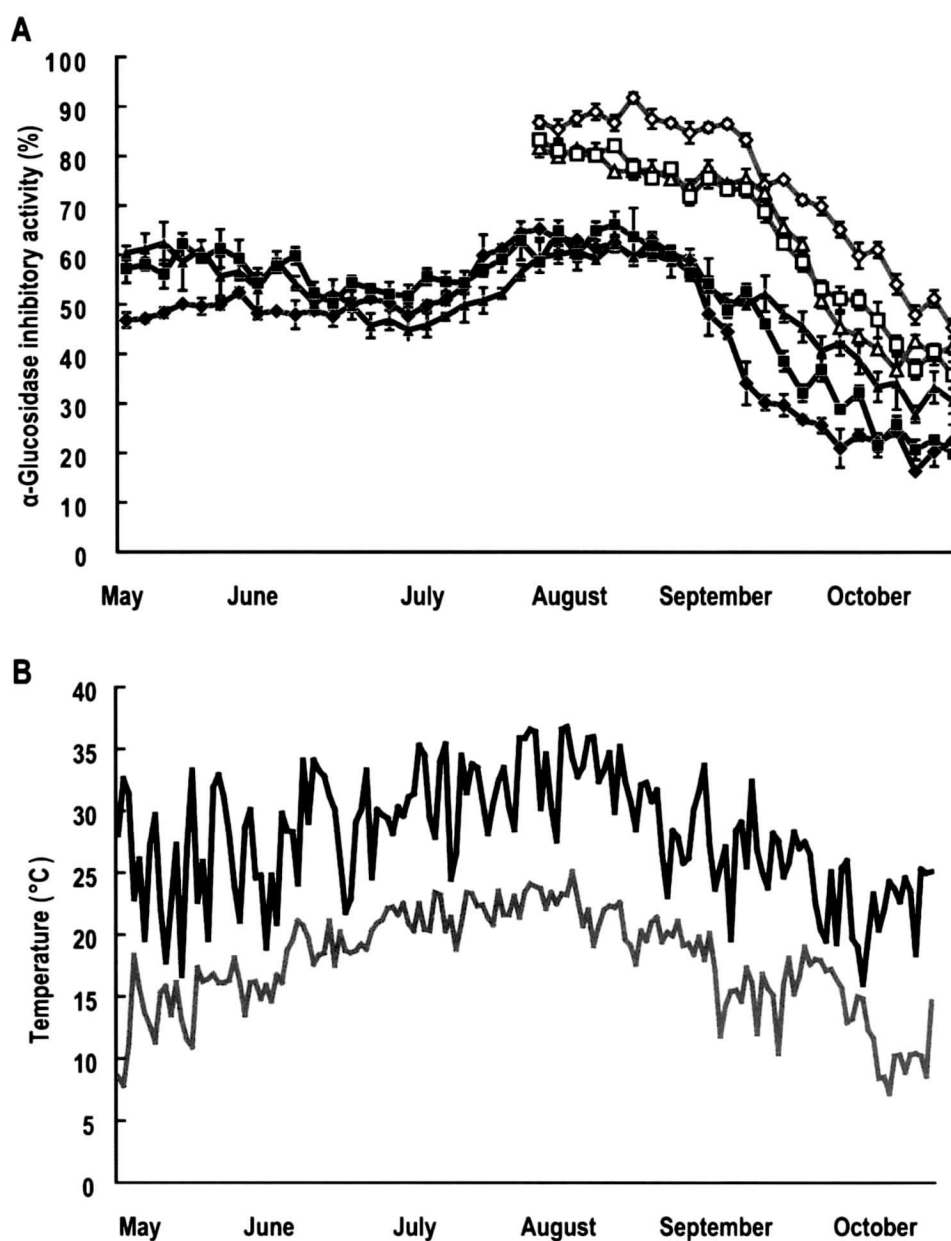


Fig. 5.1. Effect of seasonal temperature change on the  $\alpha$ -glucosidase inhibitory activity. A, The  $\alpha$ -glucosidase inhibitory activities were measured from continuously collected largest glossy leaves harvested from the field in 2009. Diamonds, *Akameyanagida*; triangles, *Ichinose*; squares, *Fukkokuwa*. The cultivars with and without scissions of the branches are indicated by open symbols with gray lines and closed symbols with black lines, respectively. Each sample solution of the three cultivars was adjusted to give a similar levels (see Materials and Methods). The data represent the means  $\pm$  standard deviation of three independent experiments. B, The highest and lowest temperature were measured at the "Gene Resource Stock Yard", Laboratory of Field Science, Shinshu University. Black line, the highest temperature; gray line, the lowest temperature.

the *Harnngplalod* between greenhouse cultivation at a constant temperature and outdoor cultivation at varying temperatures (Fig.5.2). Greenhouse cultivation of *Harnngplalod* maintained consistently high  $\alpha$ -glucosidase inhibitory activity. However, with outdoor cultivation, the  $\alpha$ -glucosidase inhibitory activity of *Harnngplalod* decreased from August to October, similar to *Fukkoguwa*. This result indicated that the  $\alpha$ -glucosidase inhibitory activity was influenced by temperature.

### 5.3.3 Relationship between the $\alpha$ -glucosidase inhibitory activity and the amount of 1-DNJ

We next measured the amount of 1-DNJ from the three cultivars (*Akameyanagida*, *Ichinose* and *Fukkoguwa*), which showed different  $\alpha$ -glucosidase inhibitory activities (Fig. 5.3). The  $\alpha$ -glucosidase inhibitory activity of *Fukkoguwa* was the highest among them when the three cultivar extracts were diluted at the same ratio. In contrast, the amount of 1-DNJ in *Ichinose* was the highest among them. These results indicate that the  $\alpha$ -glucosidase inhibitory activity was not consistent with the amount of 1-DNJ. The  $\alpha$ -glucosidase inhibitory activity must depend on other materials besides 1-DNJ.

## 5.4 DISCUSSION

This study demonstrated that the  $\alpha$ -glucosidase inhibitory activity was remarkably decreased by low temperature. Moreover, the induction of newly grown shoots by scission of the branches induced a significant rise in the  $\alpha$ -glucosidase inhibitory activity. These results suggest that the  $\alpha$ -glucosidase inhibitory activity is related to the defense mechanism of

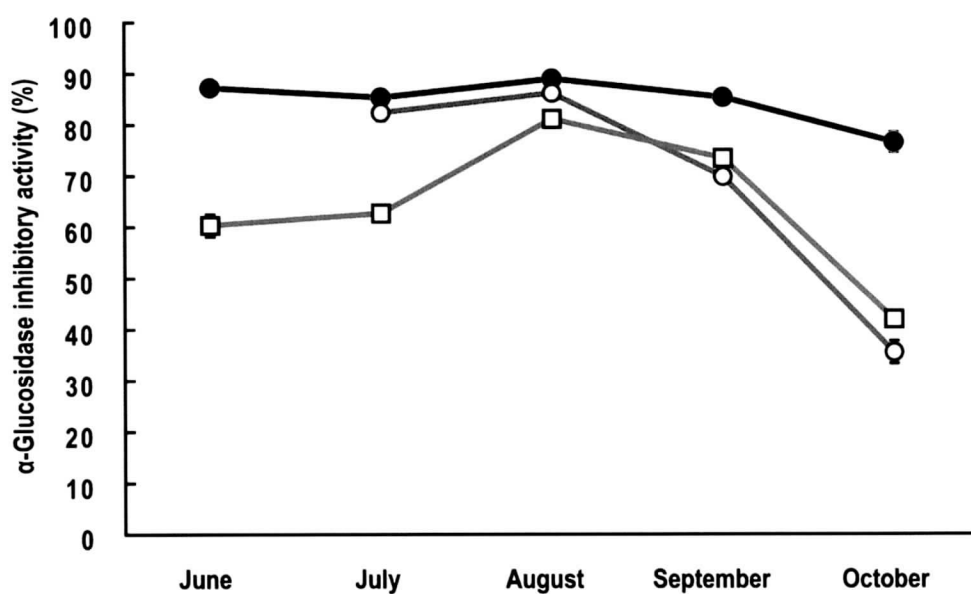


Fig. 5.2.  $\alpha$ -Glucosidase inhibitory activity in *Harngplalod* and *Fukkoguwa*. The  $\alpha$ -glucosidase inhibitory activities in *Harngplalod* (Circles) and *Fukkoguwa* (squares) were measured from continuously collected largest glossy leaves harvested in 2009. The cultivars grown in the greenhouse and outdoors are indicated by the black lines and the gray lines, respectively. Each test sample solution was used in the enzyme assay at a 1/50 dilution. The data represent the means  $\pm$  standard deviation of three independent experiments.

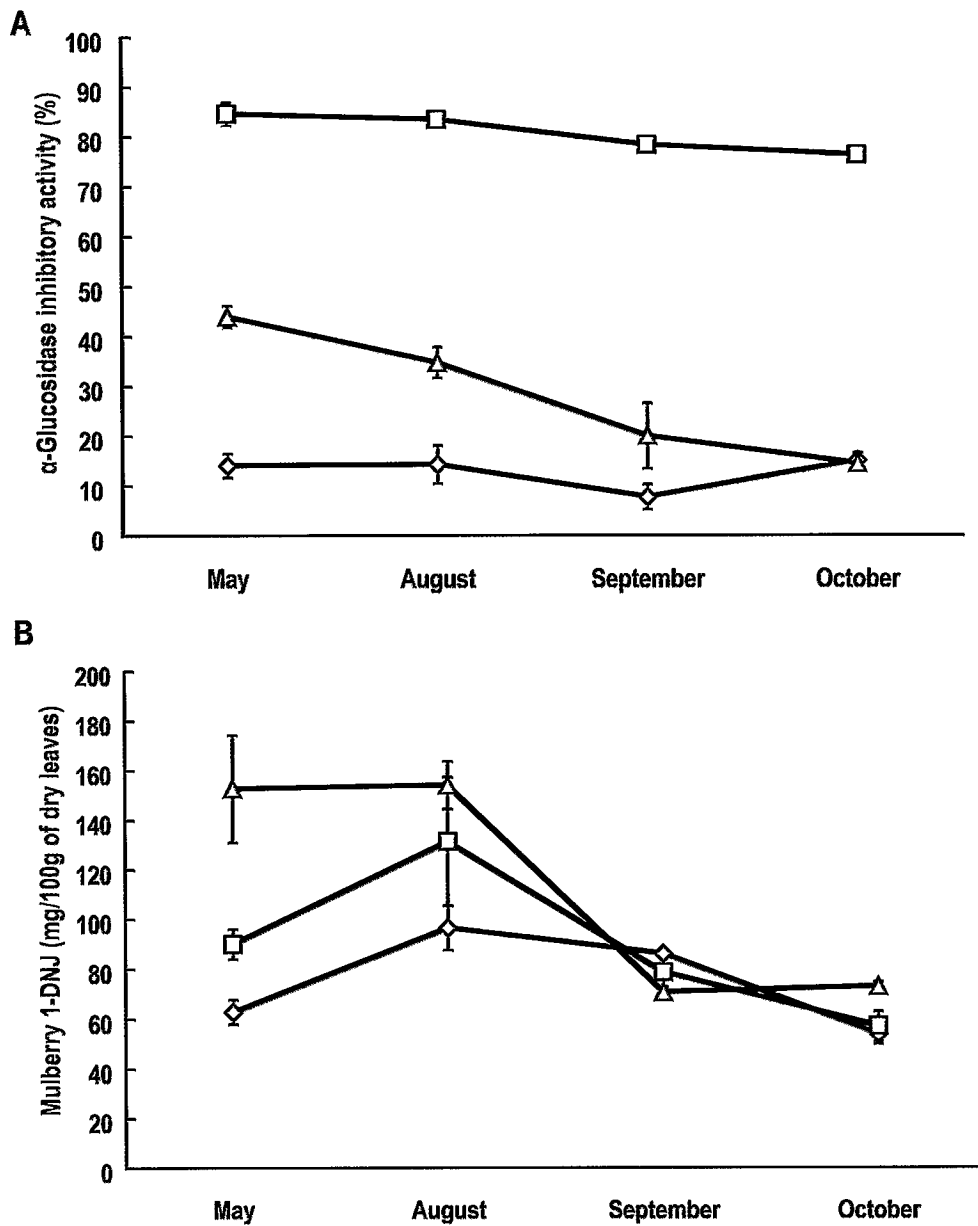


Fig. 5.3. Monthly changes in  $\alpha$ -glucosidase inhibitory activity and 1-DNJ amount. A, The  $\alpha$ -glucosidase inhibitory activities in *Akameyanagida* (diamonds), *Ichinose* (triangles) and *Fukkoguwa* (squares) were measured from continuously collected largest glossy leaves from branches of approximately 50 cm in length harvested from the field in 2010. The test sample solutions were used for the enzyme assay at a 1/50 dilution for the three cultivars. The data represent the means  $\pm$  standard deviation of three independent experiments. B, The amount of mulberry 1-DNJ in *Akameyanagida* (diamonds), *Ichinose* (triangles), and *Fukkoguwa* (squares) were continuously measured. The data represent the means  $\pm$  standard deviation of 3 independent experiments.

mulberry plants against insect herbivores (Konno *et al.*, 2006; Hirayama *et al.*, 2007). It is reasonable for the  $\alpha$ -glucosidase inhibitory activity to be increased in young leaves during the summer, because insect activity is high during the high-temperature season, and insects would preferentially eat soft, young leaves. Furthermore, it is believed that several cultivars utilize other  $\alpha$ -glucosidase inhibitors in addition to 1-DNJ, such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) and 1,4-dideoxy-1,4-imino-D-ribitol (Asano *et al.*, 2001; Konno *et al.*, 2006), for effective defense against insect herbivores.

Previous studies have reported the comparative  $\alpha$ -glucosidase inhibitory activities and 1-DNJ concentrations from various cultivars in different harvest seasons (Yatsunami *et al.*, 2003; Kimura *et al.*, 2007; Yatsunami *et al.*, 2008; Nakanishi *et al.*, 2011). Yatsunami *et al.* (2008) reported the relationship between the  $\alpha$ -glucosidase inhibitory activity and 1-DNJ concentrations from a group of species, but not enough is known about  $\alpha$ -glucosidase inhibitors other than 1-DNJ from these cultivars. The  $\alpha$ -glucosidase inhibitory activity was not consistent with the 1-DNJ concentrations in several cultivars, indicating that other  $\alpha$ -glucosidase inhibitors, in addition to 1-DNJ, are involved in the defense against insect herbivores by these mulberry cultivars.

In this study, low temperature was shown to induce a decrease in the  $\alpha$ -glucosidase inhibitory activity; however, the relationship between the photoperiod and  $\alpha$ -glucosidase inhibitory activity requires further examination. Examining the  $\alpha$ -glucosidase inhibitory activity at a fixed temperature with a varying photoperiod would clarify the influence of photoperiod. Despite this, the  $\alpha$ -glucosidase inhibitory activity was shown to be regulated by environmental conditions to protect against insect herbivores. Furthermore, identification and

analysis of the other  $\alpha$ -glucosidase inhibitors would clarify the defense mechanism in mulberry plants against insect herbivores.



## CHAPTER 6 General Discussion

In the general introduction to this dissertation, the author attempted to compile detailed protocols and applications of DNA markers from across the currently available range. The author has highlighted the specific attributes and methodology of DNA markers and has discussed the possible limitations or inherent problems of each method, and some proposed modified procedure are presented in Chapters 2 and 3.

First, the relative efficiencies of markers were analyzed and the most suitable marker for mulberry diversity was determined in Chapter 2. Fifteen mulberry varieties were evaluated using the arbitrarily-primed PCR (AP-PCR) and ISSR methods and AFLP analysis was used in Chapter 3. In this case, the material samples were obtained from mulberries that originated from different geographical zones, i.e. Japanese strains from Asia monsoon zone and Thai mulberry strains from the tropical zone. The dominant feature of the AP-PCR, ISSR, and AFLP techniques is that they do not require any sequence information of the genome under investigation. The methods are suitable for rapid identification and classification in animals and plants. Moreover, they need only a small amount of sample material.

However, these molecular markers have technical differences in terms of cost, speed, amount of DNA required, and technical labor. In addition, these methods of analysis have different abilities to detect the degree of polymorphism, differ in their precision of estimation of the genetic distance, and the statistical power of each test differs.

In author's opinion, the AP-PCR technique is simple and well suited to DNA fingerprinting, although it does suffer from a certain lack of reproducibility because of mismatch annealing. However, occasionally, the PCR amplification process that produces AP-PCR involves competition among fragments that can be affected by small variations in the reaction conditions.

As explained in Chapter 1, the ISSR markers technique is a PCR based method that involves amplification of a DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide, or penta-nucleotide. The primer can be either unanchored or, more usually, anchored at the 3' or 5' end with one to four degenerated bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994). In this dissertation, di-nucleotides (CHC835-2 and CHC 807) and tri-nucleotides (CHC 864) were used to anchor the 5' end (Table 2.2).

It was also observed DNA sequence matching the tri-nucleotide primers occurred less frequently than those matching di-nucleotide primers. In addition, ISSR markers are more robust than AP-PCR markers, mainly because of the method of detection, and, possibly, because the primers are longer, and hence the PCR conditions are more stringent. However, ISSR markers have not proven to be as polymorphic or as efficient as AFLP analysis. Nevertheless, in most plant species tested, ISSR markers can be applied to most situations.

In chapter 3, AFLP based transcript profiling was used to examine the genetic relationships among mulberry germplasm. The AFLP technique shares some characteristics with RFLP, RAPD and AP-PCR analysis. It combines the specificity of restriction analyses

with PCR amplification. AFLP can be used to distinguish between closely related individuals at the sub-species level. Moreover, from a technical point of view, AFLP also requires no prior DNA sequence information, is analyzed in a short time, and a high number of polymorphisms are detected. In Chapter 3, AFLP successfully assayed the level of polymorphism and diversity in mulberry, which agreed with the morphological classification of the genus *Morus* by Koidzumi (1917).

To analyze the results of Chapter 2 and 3, a graphical output known as a dendrogram or tree that shows the clustering structure was produced. A variety of methods of analysis for genetic relationships can be used to determine in which order clusters may be assembled. The nearest neighbor method is based on the elements of two clusters that are most similar, whereas the farthest neighbor method is based on the elements that are most dissimilar. Before clustering, each sample is considered as a group, albeit of a single sample. Clustering begins by finding the two groups that are most similar, based on the distance matrix, and merging them into a single group. The characteristics of this new group are based on a combination of all the samples in that group. This procedure of combining two groups and merging their characteristics is repeated until all the samples have been joined into a single large cluster.

The following deductions concerning the three DNA marker methods could be made. The AP-PCR technique is relatively simple, whereas both AFLP and ISSR protocols are technically demanding. Both require competent users with experience in molecular biology techniques, although the requirements for such skills are decreasing because several convenient kits have been developed. However, we believe that AFLP markers are the best

choice for evaluating diversity and assessing the genetic relationships of mulberry plants between wild varieties and cultivar varieties.

AFLP analysis is also a useful tool for investigating the genetic diversity in a large number of applications, including the localization of mulberry germplasms. The results of chapter 3 are readily applicable to breeding new mulberry varieties. The parents with high genetic distances between Japanese and Thai varieties should be selected for breeding, the offspring of which are expected to show high hybrid vigor.

During the AFLP analysis in Chapter 3, it was observed that a single band of approximately 240 bp was amplified using the *EcoRI*+TC/ *MseI*+CTC combination of primers. The 240 bp band showed a consistent sex-specific pattern; it was present in all females, but in none of the males. Thus, Chapter 4 focused on determining whether the 240 bp AFLP band could be developed as a genetic sex marker using nine Japanese mulberry varieties (*Morus* spp.). Of the 16 selective combination primers tested, 20 bands from selective combination primer, *EcoRI*+TC / *MseI*+CTC, were produced, of which one, at around 240 bp, was present in females and absent in males. To confirm this observation, the primer combination was re-tested with known male and female individuals of the nine Japanese mulberry cultivars; the band was completely absent in all the male individuals tested. We believe that this is the first report of a molecular marker permitting the determination of the sex of mulberry cultivars at an early stage.

Chapter 5 examined the effect of environmental conditions on the  $\alpha$ -glucosidase inhibitory activity of mulberry leaves.

Generally, the objective of plant breeding is for higher yield, improved quality, biotic resistance, abiotic resistance, earliness, or wider adaptability. In tropical zones, the environmental temperature is high all year round. In addition, many types of insects that could eat mulberry leaves would have a serious effect on sericulture. It is not unreasonable to suppose that mulberry trees possess some anti-feeding activities against these sorts of insects. Chapter 5 demonstrated that low temperature decreased the  $\alpha$ -glucosidase inhibitory activity and the scissoring of branches induced increased  $\alpha$ -glucosidase inhibitory activity. Moreover, that the high  $\alpha$ -glucosidase inhibitory activities of mulberry varieties from Thailand imply a strong anti-feeding activity against insects other than silkworms.

The results presented in this dissertation are useful for future breeding programs, particularly the AFLP-determined sex-specific marker in Chapter 4. Leaf productivity and leaf quality can be evaluated at their “non-reproducing stage.” If the sexuality of mulberry strains could be defined at the stage of “vegetative growth stage,” then an appropriate Japanese strain could be selected for crossing. Thus, the results from Chapters 3 and 4 could be useful for selecting parents with high genetic distances between Japanese and Thai varieties for breeding new mulberry varieties. For example, *Ichinose* and *Kairyo-nezumigaeshi* are widely cultivated in Japan and *Kenmochi*, a variety intensively cultivated in cold areas, could be selected as parents for breeding with Thai varieties like *Noi*, which can grow in water-stress conditions. In terms of insect resistance, Japanese strain *Fukkoguwa*, which is 1-DNJ-rich, could be selected as parents with *HarnGPLalod* or another Thai local variety. Their offspring would be expected to maintain strong  $\alpha$ -glucosidase inhibitory activity from 1-DNJ all year round. In this study, *HarnGPLalod* was not used in the AFLP

experiment. However, *Harnngplalod* has a close genetic relationship with *Chiangkum*, which has a significant genetic distance from *Fukkoguwa*, as seen in Fig. 2.6 and Fig 2.7. Thus, the results of this series of experiments will prove useful for developing region-specific high-yielding mulberry varieties for promoting multivoltine sericulture, especially in Thailand.

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## ACKNOWLEDGEMENTS

This work was supported by the Scholarship program of the "Agricultural Research Development Agency (ARDA)", Thailand and in part by Grant-in-Aid for Global COE Program by the Ministry of Education, Culture, Sports, Science, and Technology.

The author wishes to express her sincere thanks to Dr. Akio Koyama, NIAS, Tsukuba, Japan, for providing several mulberry samples and much valuable advice. The author also wishes to express her gratitude to Dr. Tomoaki Horie, Shinshu University, for frequent stimulating and helpful discussions. Thanks are due to the hospitality and encouragement of Dr. Rensuke Kanekatsu and all the young students of his laboratory.

The reviewers of this dissertation, Dr. Masuhiro Tsukada, Dr. Hideo Matsumura, Dr. Goro Taguchi and Dr. Hirokaki Machii, provided advice that helped make this dissertation more substantial.

Nearly four years of the author's student life was consistently supported by numerous persons who encouraged her at several times during these years. Their names are listed with sincere thanks:

Dr. Masao Yajima

Dr. Jin Xudong

Dr. Huang Lanxiang

Mr. Zhang Lei

Mrs. Asumi Kanekatsu

Mrs. Nittaya Thongpana



## LIST OF PUBLICATIONS

### Original Articles

Hiromitsu NAKANISHI, Shinji ONOSE, Eriko KITAHARA, Sukunya CHUMCHUEN, Midori TAKASAKI, Hajime KONISHI and Rensuke KANEKATSU (2011): Effect of Environmental Conditions on the  $\alpha$ -Glucosidase Inhibitory Activity of Mulberry Leaves. *Biosci. Biotechnol. Biochem.*, **75**, 2293-2296.

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### Proceeding

Sukunya CHUMCHUEN, Masao YAJIMA, Rensuke. KANEKATSU and Prateep MEESILP (2009): Genetic Diversity and Relationship in Thai and Japanese Mulberry Local Varieties as Revealed by AP-PCR and ISSR Marker Assays. *The 3rd International Symposium on High-Tech Fiber Engineering, 2009*, 111-116. (Chonbuk National University, Jeonju, Chonbuk, Korea)

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