A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Citrus Breeding using Polyembryonic Varieties

Suhyun Yun

Department of Agricultural Chemistry

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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CONTENTS

SUMMARY IN KOREAN

감귤에서 다배성은 주심배 발생으로 인해 하나의 종자 안에 여러 개의 배가 형성되는 현상으로 유성배의 출현비율을 매우 낮아지게 하거나 없게 하는 경우가 많다. 이러한 특성은 교배 육종에서는 교잡 실생의 획득을 어렵게 하여 문제가 되지만 품종을 균일하게 증식할 때 이용되는 대목에서는 필수 형질이 되며, 주심배실생을 이용한 품종개량에도 유용하게 이용되고 있다. 이와 같이 감귤의 다배성은 품종육성 및 대목 개발에서 육종 효율에 영향을 미치는 주요한 요인이 되고 있으며, 이에 대한 다양한 연구가 진행되어 왔다.

본 연구에서는 다배성 품종을 모본으로 이용한 교배육종에서 엽 형태를 이용한 형태적인 방법과 PCR 기법에 바탕을 둔 RAPD 및 SRAP 방법을 이용하여 교잡실생의 효율적인 식별방법을 개발하고 다배성(또는 단배성) 형질에 연관된 SCAR 마커를 개발하여 육종효율을 높이고자 하였다. 그리고 주심배를 이용하여 궁천조생과 흥진조생 등 현재 가장 많이 재배되고 있는 조생계 온주밀감을 대체할 수 있는 품종을 선발하고자 하였다.

1. 다배성 품종을 종자친으로 한 교배육종에서 한개의 종자에서 유래한 교잡실생과 주심배 실생을 효과적으로 식별할 수 있는 마커와 다배성 또는 단배성 형질과 연관된 마커를 개발하고 이의 이용성에 대하여 시험을 수행하였으며 주요 결과는 다음과 같다.

엽형질을 이용한 교잡실생 식별 시험을 수행 한 결과, '성전온주' × '병감' 조합에서는 44.2%, '성전온주'×'Lee' 조합에서는 31.8%, '궁천조생' × 'Orlando' 조합에서는 7.0%의 교잡실생을 식별할 수 있었다. RAPD 기법을 이용하였을 때는 각각 52.6%, 51.1% 및 7.0%의 교잡실생을 식별할 수 있었으며, SRAP 기법을 이용하였을 때는 51.6%, 46.6% 및 4.7%의 교잡실생을 각각 식별할 수 있었다. 상기의 세 가지 방법을 모두 이용하면 '성전온주' × '병감', '성전온주' × 'Lee', '궁천조

생' × 'Orlando' 조합에서 각각 52.6%, 51.1% 및 7.0%의 교잡실생을 식별할 수 있었다.

한편, '성전온주' × '병감', '성전온주' ×'Lee' 두 조합에서 주심배로 판명된 모든 실생들은 교잡실생에 비하여 현저히 생육이 부진한 왜화현상을 보였다. 이것은 감귤에서 보고된 적이 없는 특성으로 '성전온주'에서만 특이적으로 발생하는 것으로 판단되며, 다배성 종자친('성전온주')을 사용하는 교잡육종에서 교잡실생과 주심배 실생을 구별할 수 있는 유용한 지표로 사용될 수 있을 것으로 판단된다. 주심배 실생의 왜화성을 지표로 '성전온주' × '병감' 조합과 '성전온주' × 'Lee' 조합의 전체 실생을 조사한 결과, 약 90%가 주심배 실생인 것으로 판별되었다.

다배성 품종을 종자친으로 한 교배육종에서 RAPD와 SRAP 등 분자마커를 이용하는 방법이 엽 형질 등 형태적인 특성을 이용하는 방법에 비하여 효율적으로 교잡실생을 식별 할 수 있었다.

'청견'(단배성) × '진귤'(다배성) 교배조합 F1 후대의 다배성(또는 단배성) 형질을 갖고 있는 개체의 비율을 조사한 결과 1:1의 분리비를 나타내었다. 각각의 집단을 이용한 BSA-RAPD 분석을 실시하여 단배성 후대 집단에 특이적인 밴드를 선발한 후 이를 바탕으로 단배성 개체를 선발할 수 있는 pMono-U/p468D SCAR 마커를 개발하였다.

2. 다배성 품종을 종자친으로 사용한 교배육종에서 발생한 주심배실생을 이용하여 "하례조생'을 육성하였다.

1992년 '입간조생'에 '하귤'을 교배하여 얻은 주심배 실생에서 선발, 육성한 '하례조생'은 밝은 등황색의 과일은 과중이 80~90g, 과형은 편평형이며, 과피두께는 약 2mm이다. 숙기는 11월 상순으로 성숙기 당도는 10-11°Bx, 산함량은 1-1.1%이며 과육은 등황색을 띠고 있다, 이것은 제주도에서 가장 많이 재배되고 있는 궁천조생에 비하여 당도는 약 1°Bx 높고, 산 함량은 0.1%정도 낮은 것이다. 수세

는 강한 편이며 개장성이나 초기에는 다소 직립성을 보인다. 해거리 정도는 궁천조생과 비슷하다. 또한, 토양멀칭 재배 시 11월 상순의 당도는 13.8°Bx, 산함량은 1.0% 로서 궁천조생에 비하여 당도는 1.2°Bx 높고, 산 함량은 0.1% 낮다.

상기 결과를 종합해 볼 때, 하례조생은 궁천조생 및 흥진조생의 대체품종으로서 유망할 것으로 기대된다.

ABBREVIATIONS

A	Acute
BSA	Bulked segregant analysis
LBAS	Leaf blade apex shape
LBBS	Leaf blade base shape
LBS	Leaf blade shape
LBW	Leaf blade wave
PA	Pointed acute
PO	Pointed obtuse
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
SCAR	Sequence Characterized Amplified Region
SRAP	sequence-related amplified polymorphism
SS	Spindle-shaped
V	Vestigial
WS	Wing shape

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SUMMARY

The trait of polyembryony has long been a major challenge to citrus breeding. Polyembryony is associated with nucellar embryony, which results in the development of multiple embryos in seeds and often leads to the production of few or no sexual progeny during conventional cross breeding. Polyembryony is essential for the production of uniform rootstocks , and it also provides an opportunity to improve cultivars via nucellar selection. However, polyembryony has been a serious problem in conventional cross breeding, which requires sexual progenies. As a result, many studies have been conducted to control this trait.

The present study consisted of 2 parts. Part Ⅰ-1 was conducted to evaluate the ability of various identification methods, including visual observation of the phenotypic characteristics and molecular techniques based on PCR such as RAPD and SRAP, to distinguish zygotic and nucellar seedlings of satsuma mandarin, a polyembryonic female parent. Part Ⅰ-2 was conducted to develop a monoembryony-specific SCAR marker and evaluate its utilization in citrus breeding. Part Ⅱ was conducted to develop a new nucellar selection of a satsuma mandarin cultivar capable of growth on Jeju Island for replacement of the 'Miyagawa Wase' and 'Okitsu Wase' cultivars.

Part Ⅰ: The use of morphological markers (leaf shape) to identify putative zygotic seedlings had a selection efficiency of 44.2%, 31.8% and 7.0% when used to evaluate crosses of 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando', respectively. When molecular techniques were utilized, RAPD analysis selected zygotic seedlings with an efficiency of 52.6%, 51.1% and 7.0% when crosses of 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando' were

evaluated, respectively, while SRAP selected zygotic seedlings of these crosses with an efficiency of 51.6%, 46.6% and 4.7%, respectively. Furthermore, when a combination of these methods was used, zygotic selection rates of 52.6%, 51.1% and 7.0% were observed for the 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando' crosses, respectively.

When 'Morita unshiu' × 'Ponkan' and 'Morita unshiu' × 'Lee' crosses were conducted, all nucellar seedlings showed inferior growth (dwarfism) when compared to zygotic seedlings. In addition, dwarfism-based selection was conducted, approximately 90% of the seedlings of both crosses were nucellar. This finding indicates that the dwarfism originated from the 'Morita unshiu' cultivar and could be a useful tool for obtaining sexual hybrids from satsuma progenies.

The RAPD and SRAP markers were more efficient than morphological markers such as leaf shape for differentiating zygotic and nucellar seedlings when satsuma mandarins were used as a female parent.

RAPD-based bulked sergeant analysis conducted using the monoembryoic and polyembryonic F1 populations of the 'Kiyomi' and 'Jinkyul' cross enabled development of a sequence characterized amplified region (SCAR) marker, pMono-U/p468D, for selection of monoembryonic seedlings. The pMono-U/p468D amplified a 500 bp single fragment from the monoembryonic seedlings.

Part Ⅱ: A new early maturing satsuma mandarin, 'Haryejosaeng' (*Citrus unshiu* Marc.), was developed from a nucellar seedling produced by a cross of 'Tachima Wase' (*C. unshiu* Marc.) and *C. natsudaidai* Hayata that was cultivated at the Citrus Research Station on Jeju Island in 1992. This strain produced seedless fruit that matured in early November and had higher soluble solids (10 to 11 °Bx) and lower acidity (1 to 1.1%) than the fruit

produced by 'Miyagawa Wase', the leading early-maturing satsuma mandarin cultivar grown on Jeju Island. The fruit was approximately 80 - 90 g and composed of compressed-oblate globose with a light orange color and a rind of approximately 2 mm that was easy to peel. The tree showed vigorous growth, spreading of thornless twigs, and alternate bearing similar to 'Miyagawa Wase'. The tree was susceptible to citrus scab disease and melanose, but resistant to citrus canker. In addition, subjecting the 'Haryejosaeng' cultivar to a mulching experiment resulted in the production of fruit that had better soluble solid contents and acidity than fruit produced by 'Miyagawa Wase' that had also been subjected to mulching. The soluble solid and acid content of the 'Haryejosaeng' fruit were 13.8 °Bx and 1.0%, respectively, in early November. In addition, the °Bx and acid content of the 'Haryejosaeng' fruit were greater than 1.2 higher and 0.1% lower, respectively, than these values in 'Miyagawa Wase'. The 'Haryejosaeng' cultivar shows promise for use as an alternative to 'Miyagawa Wase' and 'Okitsu Wase' that can enhance the profits of citrus farmers on Jeju Island.

RESEARCH BACKGROUND

Satsuma mandarins are a primary citrus crop in northeastern Asia including Japan and China. In Korea, satsuma mandarins have been a major citrus product for several decades. Citrus fruit production on Jeju Island represented 25% of the domestic fruit production in Korea and 55% of the agricultural production in Jeju province in 2006. Nearly all of the citrus crops grown in this area were satsuma mandarins, which represented 95.6% of the annual tonnage and 93.0% of the cultivation area reported for Jeju Island during 2006.

Recently, several mandarin hybrids cultivars including 'Shiranuhi' ((*C. unshiu* × *C. sinensis*) × *C. reticulata*), which are also known as 'Hanlabong', and 'Setoka' (((*C. unshiu* × *C. sinensis*) × *C. reticulata*) × *C. reticulata*) have been produced. These cultivars have a good fruit quality, rich-flavor and high levels of soluble solids. However, these cultivars are still a minor fruit crop that account for less than 10% of the citrus production on the island. Although the growth of these cultivars is expected to increase in the future due to their high market price, their high cost of production may prevent their widespread cultivation on the island.

As a result, satsuma mandarins are expected to remain as the primary crop on Jeju Island unless alternative cultivars that can produce fruit before winter replace them. However, no such hybrids have been developed to date. Accordingly, development of breeding programs designed to produce a new satsuma hybrid has been a primary objective of the citrus breeding industry in Korea.

Conventional fruit breeding generally faces the following problems: there is often a long juvenile period and generation time from the time that the

cross is made to flowering or selection; it is necessary to evaluate large scale populations to develop new hybrids or rootstocks with useful target genes due to the highly heterozygous nature of fruit genotypes (Roose, 2008). Accordingly, the development of fruit breeding programs is expensive, requires a great deal of effort and is time-consuming. Moreover, many commercially-important citrus crops such as satsuma mandarins, some tangerines and oranges are polyembryonic, which results in their seeds containing a zygotic embryo and one or several adventitious embryos of nucellar origin (Hamilton, 1936; Koltunow et al., 1996). Furthermore, polyembryony is a heritable trait that occurs during conventional cross breeding. As a result, polyembryony has long been a problem in traditional citrus breeding because it results in difficulty in the identification of sexual hybrids for new varieties or nucellar hybrids for the development of rootstocks. Generally, more than two seedlings are germinated when polyembryonic varieties are used as a mother plant (Fig. 1), and it is difficult to identify such embryos during the early stages of development when there are mixed seedlings from seeds produced by a zygotic plant.

Consequently, the demand for methods capable of separating nucellar and zygotic embryos has increased. Several studies have been conducted to evaluate the effectiveness of various techniques at enabling such separation, including thin-layer chromatography (Tatum et al., 1978), isozyme analysis (Moon and Ko, 1991; Luro et al., 1995; Elisiario et al., 1999), and gas chromatography (Weinbaum et al., 1982). However, the metabolic products evaluated by these techniques can be influenced by the age of the plant and environmental conditions therefore, the results are unreliable for identification purposes (Luro et al., 1995 Yun et al., 2007). Recently, molecular markers have become a useful tool for the direct analysis of DNA without any interference from the environment or tissue age (Tansksley et al., 1989). It is

generally recognized that DNA marker-based selection can be beneficial to the plant breeding field (Hospital et al., 1992), and such techniques have been widely used in the cultivation of a variety of plants (Oliveira et al., 2002; Ruiz et al., 2000; Sun et al., 2006; Li and Quiros, 2001). Polymerase chain reaction (PCR) has been especially useful as an inexpensively method of genomic DNA analysis.

Several PCR marker systems with different complexities, reliability, and information generating capacity are currently available. These include random amplified polymorphic DNA analysis (RAPD), simple sequence repeat polymorphism analysis (SSR), amplified fragment length polymorphism analysis (AFLP), and a few other methods (Lee, 1995; Lin et al., 1995; Rafalski et al., 1996; Del Rio and Bamberg, 2000; Negi et al., 2000; Ruiz et al., 2000; Oliveria et al. 2002). Each system has its own advantages and disadvantages. For example, RAPD is a simple, fast, and inexpensive technique that has been widely used for the development of genetic markers (Fang et al., 1997; Paran and Michelmore, 1993; Yen et al., 1997), but poor consistency and low multiplexing output limits its use. Recently, the use of a new marker-related technique known as sequence-related amplified polymorphism (SRAP), which combines simplicity, reliability, a moderate through-put ratio, and facile sequencing of selected bands, was proposed (Li and Quiros, 2001; Sun et al., 2006). SRAP targets coding sequences in the genome, which results in a moderate number of co-dominant markers. SRAP molecular markers can be extensively applied in the construction of genetic linkage maps (Li and Quiros, 2001), genetic diversity analysis (Ferriol et al., 2003; Lin et al., 2004), and comparative genetics (Li et al., 2003) of different species. Application of the SRAP technique to identify zygotic seedlings in citrus crops also has economic benefits similar to those of the RAPD system. Furthermore, the SRAP system only requires a small amount of DNA, takes

little time, and is relatively simple. However, to date, the SRAP system has not been used for the identification of zygotic seedlings in citrus crops.

In contrast to cross breeding, apomixis is an important trait in traditional citrus breeding programs designed to improve citrus rootstocks. Apomictic embryos arising from nucellar tissue give rise to seedlings that are genetically identical to the seed source tree, which enables citrus to be propagated by budding the desired scion onto the rootstock seedlings. As a result, the rootstock is a major contributor to the tree performance and longevity, and influences tree size, yield, and fruit quality (Davies and Albrigo, 1994). Therefore, nucellar embryony is essential to the development of citrus rootstocks because it allows nurseries to propagate trees that are highly heterozygous, but genetically uniform, which results in the production of uniform rootstocks for propagation and planting (Frost, 1943; Xiang and Roose, 1988; Garcia et al., 1999; Ruiz et al., 2000; Kepiro and Roose, 2007).

Several studies have been conducted to evaluate the inheritance of nucellar embryony. Spiegel-Roy and Goldschmidt (1996) reported that monoembryonic traits in citrus crops resulted from a single homo recessive gene. Additionally, Parlevliet and Cameron (1959) suggested that nucellar embryony is controlled by a single major dominant gene that is heterozygous in trifoliate and absent from the 'Chandler' pummel, and that minor genes may control the level of expression in these cultivars. It has also been suggested that several genes control nucellar embryony and that polyembryony is an independent trait (Garcia et al., 1999; Asins et al., 2002). Finally, Hong et al. (2001) reported that two complementary dominant genes control apomixis.

Marker assisted selection (MAS) involves molecular markers as well as phenotypic or biochemical markers, which require less labor, money and time than traditional selection that usually includes a examination of fruit trait.

Furthermore, DNA-based polymorphic markers that utilize traits that are linked to a single gene, especially agriculturally valuable characteristics, can increase the efficiency at which hybrids that contain a target gene are selected during an early stage. Although PCR-based marker selection techniques are considered to be useful for MAS, an additional process is often required to enable PCR-based markers to be more informative. Because arbitrary marker techniques such as RAPD and AFLP provide reproducible results (Agarwal, 2008), a method for PCR-based identification of sequence characterized amplified regions (SCAR) linked to specific qualitative and quantitative trait loci was developed based on specific primer pairs designed from the sequence of the RAPD marker (Barzen et al., 1997; Brahm et al., 2000; Cao et al., 2001; Gill et al., 1998; Kim et al., 2000; Paran and Michelmore, 1993; Vidal et al., 2000). SCAR markers have the following characteristics: low abundance, high reproducibility, medium degree of polymorphism and the requirement for a low quantity of DNA. SCAR markers can also facilitate the development of a dominant marker co-dominant, which has advantages for genetic analysis (Plomion et al., 1996).

The citrus breeding strategy generally involves the selection of a nucellar seedling from polyembryonic citrus plants. This strategy has also been adopted as a breeding program for the development of new satsuma varieties. Because nucellar seedlings are genetically identical to the mother plant , they enable selection of plants with valuable horticultural characteristics such as high tree vigor, freedom from virus disease, and higher yields (Spiegel-roy and Goldschmidt, 1996). Since the 1950s, many citrus varieties have been cultivated using nucellar seedlings, including 'Okitsu Wase', 'Miho Wase' and 'Okitsu Wase', which were developed in Japan (Iwamasa, 1988). These cultivars are now major crops throughout northeast Asia.

'Miyagawa Wase' and 'Okitsu Wase' are early maturing forms of the

satsuma mandarin. The production of these cultivars has increased for 4 decades, and they have encompassed nearly 80% of the total citrus area on Jeju Island since the 1990s. However, these cultivars now often produce low-quality fruit when compared to newer high-quality citrus cultivars such as 'Shiranuhi', which is also known as 'Hanlabong', 'Setoka', as well as improved apples, pears and grapes. For that reason, alternative varieties are needed to replace these older satsuma mandarin varieties.

PART Ⅰ. Development and Utilization of Markers in Polyembryonic Citrus Breeding

Abstract

This study was conducted to evaluate the ability of selection based on morphological markers such as leaf shape and molecular marker-based selection methods such as RAPD and SRAP analysis, as well as to develop a PCR-based SCAR marker for the selection of monoembryonic seedlings.

The use of morphological markers (leaf shape) enabled the selection of putative zygotic seedlings with an efficiency of 44.2%, 31.8% and 7.0% from crosses of 'Morita unshiu' ×'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando', respectively. When molecular techniques were utilized, RAPD analysis selected zygotic seedlings with an efficiency of 52.6%, 51.1% and 7.0% from crosses of 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando', respectively, while SRAP selected zygotic seedlings with an efficiency of 51.6%, 46.6% and 4.7%, respectively. Furthermore, when a combination of these methods was used, zygotic selection rates of 52.6%, 51.1% and 7.0% for 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' ×'Orlando', respectively, were observed.

When 'Morita unshiu' × 'Ponkan' and 'Morita unshiu' × 'Lee' crosses were conducted, all nucellar seedlings showed inferior growth (dwarfism) when compared to zygotic seedlings. When dwarfism-based selection was conducted, approximately 90% of the seedlings for the entire population of both crosses were nucellar. The RAPD and SRAP markers were adequate for

the identification of zygotic and nucellar seedlings from 'Morita unshiu', although visual selection based on dwarfism also enabled the identification of nucellar seedlings from this cultivar.

RAPD-based bulked sergeant analysis conducted using the monoembryoic and polyembryonic F1 populations of the 'Kiyomi' and 'Jinkyul' cross enabled development of a sequence characterized amplified region (SCAR) marker, pMono-U/p468D, for the selection of monoembryonic seedlings. The pMono-U/p468D primer amplified a 500 bp single fragment from the monoembryonic seedlings

We expect that the selected markers can be used to increase the selection efficiency in polyembryonic citrus breeding.

Introduction

Satsuma mandarins are a major citrus crop that occupy greater than 90% of the total acreage used for crops on Jeju Island. In addition, they have been the only species harvested before winter for several decades in this region. As a result, they have become an important breeding resource in Korea.

Conventional fruit breeding generally encounters the following problems: there is often a long juvenile period and generation time from the time that the cross is made to flowering or selection; it is necessary to evaluate large scale populations to develop new hybrids or rootstocks with useful target genes due to the highly heterozygous nature of fruit genotypes (Roose, 1990). Accordingly, the development of fruit breeding programs is expensive, requires considerable effort and is time-consuming. Moreover, many commercially-important citrus crops such as satsuma mandarins, some tangerines and oranges are polyembryonic, which results in their seeds containing a zygotic embryo and one or several adventitious embryos of nucellar origin (Hamilton, 1936; Koltunow et al., 1996).

There are two large barriers to cross breeding satsuma mandarins. One is that plants have a weak seed formation ability due to female sterility (Nesumi et al., 2000) and the other is that polyembryony is often caused by undesirable nucellar embryogenesis of the female parent (Soost and Roose, 1996). Polyembryony has been especially difficult to overcome in citrus breeding programs because it makes the identification of hybrids difficult. Generally, two or more seedlings emerge from polyembryonic varieties of citrus plants such as the satsuma mandarin (Fig. 1) (Yun, 2007, unpublished data), and it is difficult to determine which one is zygotic, even during the

early stages of growth. Despite this problem, satsuma mandarins are commonly used as a female parent in the citrus breeding program because they provide benefits to new hybrids such as cold tolerance, early maturity, seedlessness, production of a moderate tree size and easy peeling. As a result, the demand for methods that enable separation of the nucellar and the zygotic cultivars has increased.

Accordingly, several studies have been conducted to evaluate the ability of various techniques to identify nucellar and zygotic cultivars. These techniques include thin-layer chromatography (Tatum et al., 1978), isozyme analysis (Moon and Ko, 1991; Luro et al., 1995; Elisiario et al., 1999), and gas chromatography (Weinbaum et al., 1982). However, because the metabolic products evaluated by these techniques can beinfluenced by the age of the plant and environmental conditions, the results are unreliable for identification purposes (Luro et al., 1995 Yun et al., 2007).

Many DNA marker technologies have been used in commercial plant breeding programs since the early 1990s. Recently, PCR-based molecular marker systems have been used to identify zygotic seedlings when polyembryonic varieties are used as the mother plant. These techniques include random amplified polymorphic DNA (RAPD), simple sequence repeat polymorphism (SSR), amplified fragment length polymorphism (AFLP), and a few others (Lee, 1995; Rafalski et al., 1996; Ruiz et al., 2000; Oliveria et al., 2002). Recently, the use of a new marker-related technique known as sequence-related amplified polymorphism (SRAP), which combines simplicity, reliability, a moderate throughput ratio, and facile sequencing of selected bands, was proposed (Li and Quiros, 2001; Sun et al., 2006). SRAP requires only a small amount of DNA and less time than other methods. In addition, SRAP is a simple and inexpensive process. However, to date, SRAP molecular markers have not been used to identify zygotic seedlings in citrus crops.

Marker assisted selection (MAS) involves molecular markers as well as phenotypic or biochemical markers, which require less labor, money and time (Gmitter et al., 2007). Furthermore, DNA-based polymorphic markers that utilize traits that are linked to a single gene, especially agriculturally valuable characteristics, can increase the efficiency at which hybrids that contain a target gene are selected during an early stage. PCR-based markers such as RAPD, AFLP and SSR have been used in MAS however, Sequence Characterized Amplified Region (SCAR) markers are needed because arbitrary marker techniques such as RAPD and AFLP are not sensitive enough to obtain reproducible results (Agarwal, 2008).

Therefore, this study was conducted to evaluate the efficiency of phenotypic based identification methods and molecular identification methods employing RAPD and SRAP technique to identify zygotic or nucellar origin. In addition, a monoembryo-specific band was identified for use as a SCAR marker, and its usefulness for citrus breeding was subsequently evaluated.

Fig. 1. Appearance of seedlings germinated from polyembryonic seed. A: 'Aoshima unshiu' × 'Ponkan', B: 'Hamlin' × 'trifoliate orange', C: 'Sasaki Wase' × 'Ponkan'

Materials and methods

Identification of zygotic hybrid from the satsuma mandarin cross

Plant Materials

Crosses between 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando' were conducted at the Citrus Research Station (CRS) in May of 2004. The seeds were harvested from the mature fruit of each of these crosses and sown in a seedbed that contained artificial soil in December of the same year. Almost all seeds germinated within 3 weeks. Two months later, the seedlings were transplanted to plastic pots at a density of one seedling per pot and then grown in a greenhouse that had no set temperature controls.

A total of 226 pots were then randomly selected for subsequent identification of zygotic seedlings: 95 pots containing seedlings produced by the 'Morita unshiu' × 'Ponkan' cross, 88 pots containing seedlings produced by the 'Morita unshiu' × 'Lee' cross, and 43 pots containing seedlings produced by the 'Miyagawa Wase' × 'Orlando' cross.

Table 1. Scientific name of cultivars used in this experiment (Tanaka system)

No.	Common name	Scientific name
1	Morita unshiu	*Citrus unshiu* Marc.
2	Miyagawa Wase	*C. unshiu* Marc.
3	Ponkan (Batangus tangerine)	*C. reticulata* Blanco.
4	Lee	*C. reticulata* Blanco.
5	Orlando	*C. paradisi* × *C. reticulata*

Genomic DNA extraction

Young leaves were collected from the seedlings and used for total genomic DNA (gDNA) extraction. When a seed had three or more seedlings, two leaf samples were prepared, one from a leaf from the most vigorous seedling and another from the remaining seedlings. The DNA extraction was conducted using an Automated Purification Maxwell 16 Instrument (Promega, Madison, WI, USA) and the isolated gDNA was stored at 4℃ until use.

Morphological analysis of zygotic or nucellar seedlings

To determine the origin of the seedlings, the leaf morphology of 2-year-old seedlings and their parents (Table 2) were compared for leaf blade shape, leaf blade apex shape, leaf blade base shape, leaf blade wave and leaf wing shape (Fig. 2). Tree vigor among seedlings was also compared based on tree height. Seedlings with morphologies that differed from those of their seed parents or both parents were putatively classified as zygotic.

Table 2. Leaf morphological characteristics of parental varieties used in this study.

Parental varieties	LBS[Z]	LBAS	LBBS	LBW	WS
Morita unshiu	SS	PA	PA	None	None
Ponkan	SS	PO	A	None	None
LEE	SS	PO	A	None	V
Miyagawa Wase	SS	PA	A	None	None
Orlando	SS	PO	PA	None	SS

[Z]LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

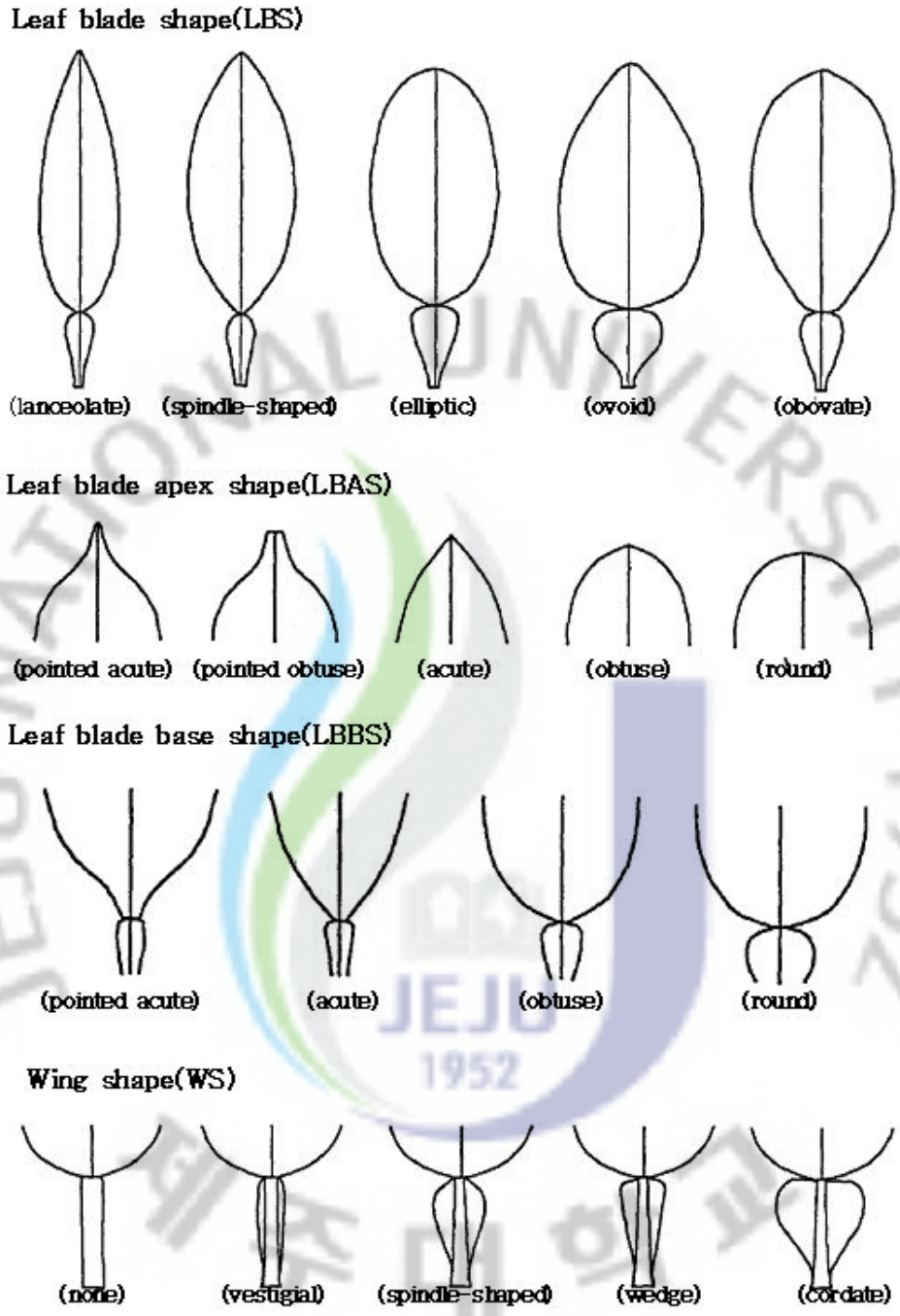


Figure 2. Morphological characteristics and its terminology.

RAPD analysis

The DNA polymorphic pattern analysis by RAPD was conducted to evaluate seedlings produced by crosses of 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando'. A total of 199 arbitrary RAPD primers (10 base) were used to identify unique polymorphic DNA bands from the parents of the crosses. Of the 199 RAPD primers (Bioneer, Korea) that were initially screened in preliminary test or previously screened, 3 primers (Table 1) were selected to identify various citrus seedlings.

PCR was conducted using a Takara PCR Thermal cycler (Takara, Japan) and a reaction mixture with a total volume of 20 μL that consisted of AccuPower PCR Premix (Bioneer, Korea) [250 μM dNTP, 1.5 mM MgCl2, 1.0 unit Taq DNA polymerase, 10 mM Tris-HCl (pH9.0), 40 mM KCl] and 50 pmole of eachprimer and template DNA.

PCR was conducted by subjecting the samples to the following conditions: initial denaturation at 94℃ for 5 min, followed by 40 cycles of denaturation at 94℃ for 1 min, annealing at 38℃ for 1 min, and extension at 72℃ for 2 min, with a final extension for 10 min at 72℃. The mixture was then cooled to 4℃, after which the PCR products were separated by electrophoresis in 1.2% (w/v) agarose gel and then stained with 0.5 μg/ml ethidium bromide.

SRAP analysis

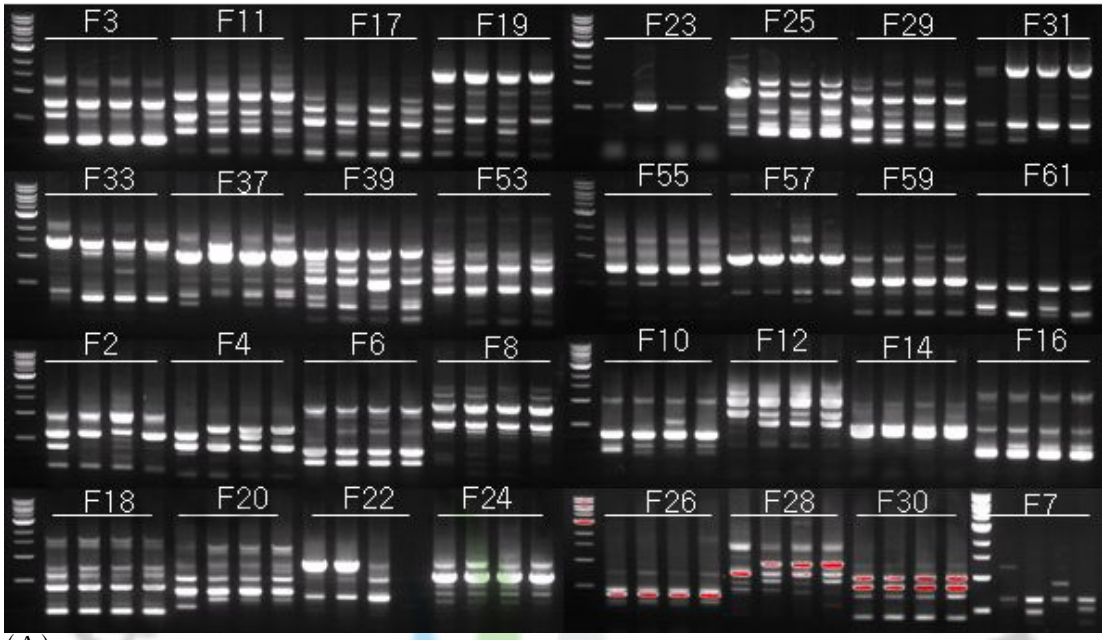
DNA polymorphic pattern analysis by SRAP was utilized to evaluate the same crosses that were evaluated by the RAPD analysis. To accomplish this, primer sets composed of 32 forward and 32 reverse primers were used to amplify samples from 5 parental varieties. Primers that revealed DNA

polymorphism between parental varieties were then selected (Fig. 2, Table 3), after which the combinations of forward and reverse primers were tested again against the same 5 varieties (Fig. 3). Finally, 3 primer sets were selected for identification of zygotic and nucellar seedlings from the crosses used in this study.

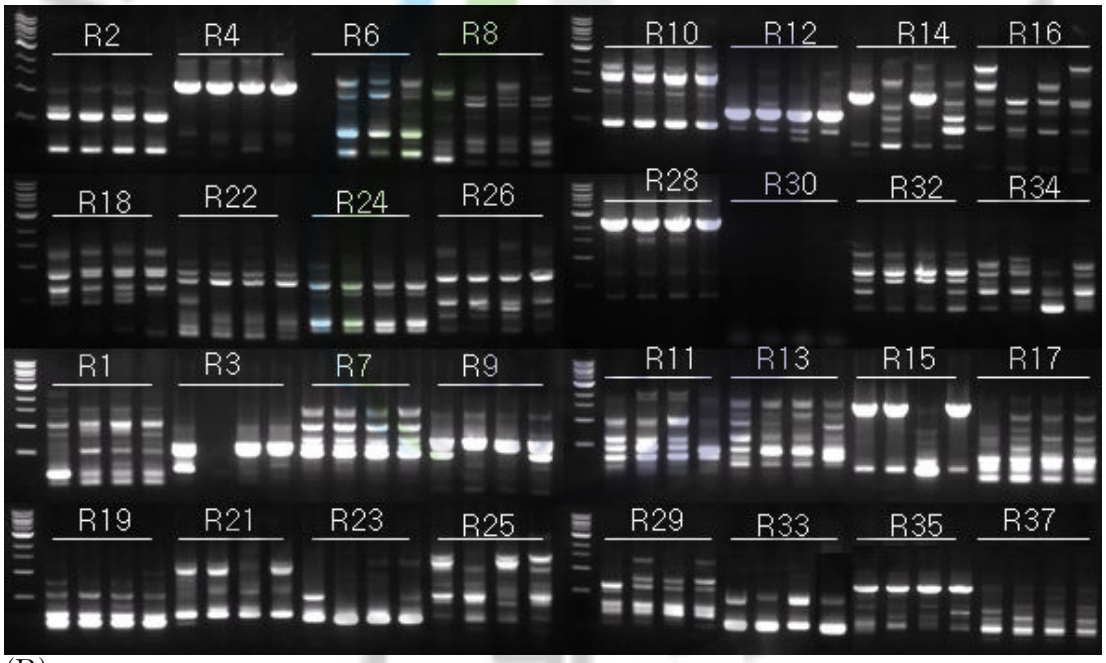
SRAP samples were analyzed using the same volume of PCR reaction mixture that was used for RAPD amplification; however, the samples were subjected to different reaction conditions, which were as follows: initial denaturation at 94℃ for 5 min, 5 cycles 1 min of denaturation at 94℃, 1 min of annealing at 35℃, and 2 min of elongation at 72℃ followed by 35 cycles with increasing annealing temperature to 50℃ and final elongation for 10 min at 72℃ (Sun et al. 2006). The PCR products were then separated and visualized as described above.

Table 3. List of primers used in this study and their sequences.

Primers		Base sequences (5'-3')
RAPD	OPO14	5'-AGCATGGCTC-3'
	UBC27	5'-TTTGGGGGGA-3'
	UBC229	5'-CCACCCAGAG-3'
SRAP	F4	5'-TGAGTCCAAACCGGAAT-3'
	F7	5'-TGAGTCCAAACCGGACG-3'
	F11	5'-TGAGTCCAAACCGGAGG-3'
	R14	5'-GACTGCGTACGAATTATC-3'
	R29	5'-GACTGCGTACGAATTCTA-3'



(A)



(B)

Fig. 3. Polymorphic DNA band patterns of 4 varieties as determined by SRAP analysis using 32 forward (A) and 32 reverse (B) SRAP primers. From left to right, 'Morita unshiu', 'Lee', 'Orlando' and 'Ponkan'.

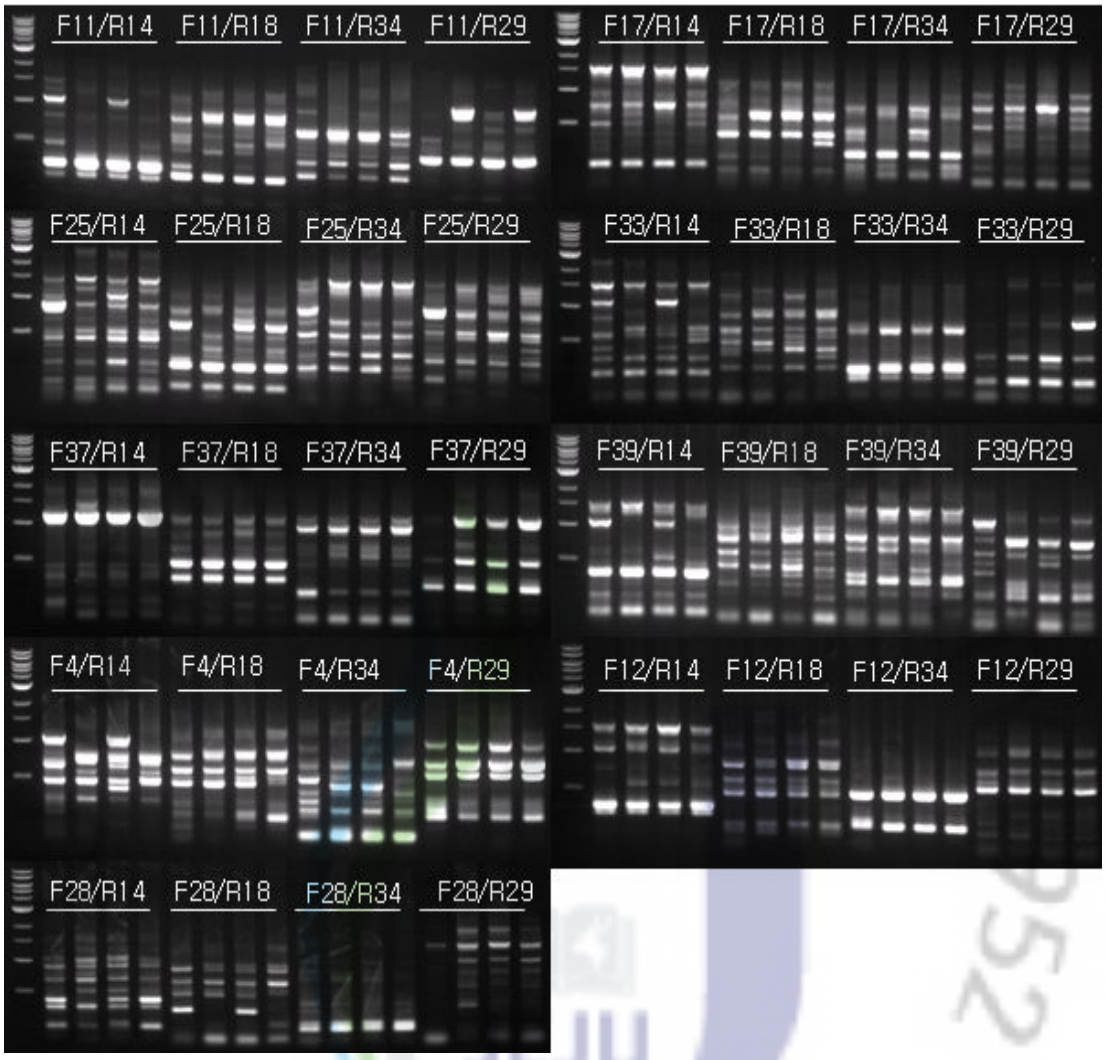


Fig. 4. Screening of polymorphic SRAP primer sets by using combinations of forward and reverse primers to evaluate the 4 varieties included in this study. From left to right, 'Morita unshiu', 'Lee', 'Orlando' and 'Ponkan'.

Development of a SCAR Marker Linked to Monoembryony

Plant materials

One hundred seedling of ten year old from the cross 'Kiyomi' (C. *unshiu* × *C. sinensis*) as a monoembryonic seed parent and 'Jinkyul' (*C. sunki*) as a polyembryonic male parent were used for development of molecular markers related to a polyembryony. The polyembryony of the seedlings was determined by counting the embryo number of their seeds. The crossing and growing its seedlings were done at Citrus Research Station located in Jeju Island in Korea.

Genomic DNA extraction

Total genomic DNA (gDNA) was extracted in young leaves from the seedlings using Automatic Nucleic Acid Extractor (Compacbio Sciences Co.) and stored at 4℃. Amplified products were visualized and measured with Lambda DNA which has various DNA concentrations after electrophoresis on 1.0% (w/v) and 0.5 ug ml^{-1} of ethidium bromide staining.

BSA-RAPD primer selection

For BSA-RAPD analysis of the 'Kiyomi' × 'Jinkyul' population, two bulks of DNAs were prepared: the monoembryonic bulk having an equal amount of DNAs from the fifteen monoembryonic seedlings and the polyembryonic bulk having an equal amount of DNAs from the fifteen polyembryonic seedlings.

A total of 800 (UBC 1 - 800) random primers were used to produce polymorphism. Total 20 μL of PCR reaction mixture was obtained from

AccuPower PCR Premix (Bioneer, Korea) [250 μM dNTP, 1.5 mM $MgCl_2$, 1.0 unit Taq DNA polymerase, 10 mM Tris-HCl (pH9.0), 40 mM KCl], and 50 pmole primer and template DNA were added for reaction.

PCR was performed using Takara PCR Thermal cycle (Takara, Japan) under the following conditions: preheating at 94℃ for 5 min; 40 amplification cycles at 94℃ for 1 min, at 38℃ for 1 min, and 72℃ for 2 min, and elongation was completed by final extension at 72℃ for 10 min. The mixture was cooled to 4℃, and PCR products were analyzed by electrophoresis in 1.2% (w/v) agarose gel and stained with 0.5 μg/ml of ethidium bromide.

The primers generating polymorphism between the monoembryonic and polyembryonic bulk DNAs on BSA-RAPD analysis were subsequently tested on each DNAs from the fifteen monoembryonic and polyembryonic individuals. The primers maintaining the polymorphism were finally used to make a monoembryonic linkage RAPD marker.

Cloning of monoembryony-specific DNA fragments

Monoembryony-specific polymorphic RAPD fragment from BSA-RAPD analysis was excised from a 1.2% (w/v) low-melting point agarose gel and purified using the Wizard PCR Prep DNA purification system (Promega, USA). The purified DNA fragment was cloned into the Topo Cloning kit pCR 2.1-Topo vector (Invitrogen Co.) and transformed into Topo Supercompetent Cells (E. coli) by electroporation. Positive (White) colonies were incubated at 2XYT media. A plasmid DNA was then purified using the Wizard Plus SC Minipreps DNA Purification system (Promega, USA). After purification, a insert on the plasmid DNA was verified by *Eco*R Ⅰ.

The nucleotide sequencing was performed with the Bigdye Terminator Cycle Sequencing kits (PE Biosystems, Foster City, CA, USA) by using an

automated DNA sequencer (ABI 3730XL, Applied Biosystems, Foster, CA, USA).

SCAR marker design and analysis

The DNA sequences of the cloned fragment were searched for the similarity using the Blast algorithm within National Center for Biotechnology Information (NCBI) and the Multiple alignments program Clustal W programs. The sequences were used to design SCAR primers by Bioneer Co. (Korea). The size of designed primer is 19-22 bases (Table 1).

PCR amplifications were performed in total 20 μL of PCR reaction mixture of AccuPower PCR Premix (Bioneer, Korea) [250 μM dNTP, 1.5 mM $MgCl_2$, 1.0 unit Taq DNA polymerase, 10 mM Tris-HCl (pH9.0), 40 mM KCl], and 50 pmole primer and template DNA were added for reaction.

The PCR amplification program consisted of an initial denaturalization at 95℃ for 5 min followed by 35 cycles of 95℃ for 50 sec, annealing at 53℃ for 1 min 30 sec, and 70℃ for 1 min 30 sec with a final extension at 70℃ for 5 min. Amplification products were separated in 1.2% agarose gels in TAE buffer, stained with ethidium bromide, visualized and photographed as for the SCAR markers.

Results

Identification of zygotic hybrid from the satsuma mandarin cross

Morphological analysis of zygotic or nucellar seedlings

Seeds were obtained from crosses of 'Morita unshiu' × 'Ponkan', 'Morita unshiu' × 'Lee' and 'Miyagawa Wase' × 'Orlando' (Table 4). Among them, 226 randomly selected seedlings were examined for of the presence of putative zygotic seedlings by morphological analysis.

Because nucellar seedlings are genetically identical to their seed parents, it is possible to identify hybrids morphologically based on characteristics donated by the male parent that the female parent does not possess. When the morphological characteristics of seedlings are different from their seed parent or both parents, the seedlings can be classified as putative zygotes. In this study, a total of 73 seedlings were putatively classified as zygotic based on their morphological characteristics. Specifically, 42 of the 'Morita unshiu' × 'Ponkan' seedlings, 28 of the 'Morita unshiu' × 'Lee' seedlings, and 3 of the 'Miyagawa Wase' × 'Oralndo' seedlings were zygotic (Table 5, 6, 7, 8).

When crosses of 'Morita unshiu' × 'Ponkan' and 'Morita unshiu' × 'Lee' were considered, only a few seedlings were evaluated because the remainder had a very small tree size, low leaf number and abnormally shaped leaves. These dwarf trees were not observed when the seedlings produced by 'Miyagawa Wase' × 'Oralndo' were evaluated (Fig. 5). The implications of this dwarfism are discussed later in the paper.

Table 4. Artificial pollination and seed-gathering

Cross combination	No. of fruit (a)	No. of seedy fruit	No. of seeds per fruit
'Morita unshiu' × 'Ponkan'	558	293	2.7 b[z]
'Morita unshiu' × 'Lee'	207	165	4.6 a
'Miyagawa Wase' × 'Orlando'	435	49	1.3 c

[z]Different letters denote a significant difference at $p \leq 0.001$ as determined by Duncan's multiple range test.

Table 5. Identification of putative zygotic seedlings based on a comparison of leaf morphological characteristics between parents and their progeny.

Cross	No. of seeds	No. of obtained seedlings	No. of examined seedlings	Total No. of putative zygotic seedlings	Leaf Morphological Characteristic				
					LBS[z]	LBAS	LBBS	WS	LBW
Morita unshiu×Ponkan	95	135	56	42	0[y]	32	32	16	4
Morita unshiu×Lee	88	148	50	28	0	16	16	6	3
Miyagawa Wase×Orlando	43	86	76	3	0	0	2	3	0

[z]LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shape; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

[y]No. of putative zygotic seedlings identified based on the concerned leaf morphological characteristic

A B

C D

Fig 5. Growth state of nucellar (left) and zygotic (right) seedlings produced by 'Morita unshiu' × 'Ponkan' (A), 'Morita unshiu' × 'Lee' (B) and 'Miyagawa Wase' × 'Orlando' (C). D potted zygotic (left) and nucellar (right) seedlings germinated from one seed produced by a 'Morita unshiu' × 'Ponkan' cross.

Table 6. The morphological characteristics of seedlings produced by 'Morita unshiu' × 'Ponkan' cross.

No.	[z]Plants(No.)	[y]LBS	LBAS	LBBS	WS	LBW
1	2	SS	PO	PO	None	None
2	2	SS	PO	PO	None	None
3	1	SS	PO	PO	None	None
4	2	SS	PA	PA	None	None
5	1	SS	PO	PO	None	None
6	2	SS	PO	PO	None	None
7	1	SS	PA	PA	None	None
8	3	SS	PO	PO	None	None
9	1	SS	PO	PO	None	None
10	2	SS	PA	PA	None	○
11	1	SS	PO	PO	None	None
12	1	SS	PO	PO	None	None
13	1	SS	PO	PO	SS	None
14	1	SS	PO	PO	None	None
15	2	SS	O	O	SS	None
16	2	SS	PO	PO	V	None
17	1	SS	PO	PO	SS	None
18	2	SS	PO	PO	None	None
19	1	SS	PO	PO	V	None
20	3	SS	PO	PO	None	None
21	2	SS	PA	PA	None	None
22	2	SS	PA	PA	None	None
23	1	SS	PA	PA	V	None
24	3	SS	PA	PA	V	None
25	1	SS	PA	PA	SS	None
26	2	SS	PA	PA	SS	None
27	1	SS	PO	PO	V	None
28	2	SS	A	A	SS	None
29	1	SS	PA	PA	V	None
30	1	SS	PO	PO	None	None
31	1	SS	PA	PA	None	○
32	1	SS	PO	PO	V	None
33	1	SS	PA	PA	None	None
34	1	SS	PA	PA	None	None
35	1	SS	PA	PA	None	○

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

Table 6. continued

No.	[z]Plants(No.)	[y]LBS	LBAS	LBBS	WS	LBW
36	1	SS	PA	PA	None	None
37	2	SS	A	A	V	None
38	2	SS	PA	PA	None	None
39	3	SS	PA	PA	None	None
40	2	SS	PA	PA	None	None
41	1	SS	A	A	None	None
42	2	SS	PA	PA	V	None
43	1	SS	PA	PA	None	None
44	2	SS	PO	PO	None	None
45	2	SS	PO	PO	None	None
46	1	SS	A	A	None	None
47	2	SS	PO	PO	None	None
48	2	SS	PA	PA	None	None
49	3	SS	PA	PA	None	○
50	3	SS	PA	PA	V	None
51	2	SS	PO	PO	None	None
52	1	SS	PA	PA	None	None
57	2	SS	PO	PO	None	None
61	2	SS	PO	PO	None	None
70	2	SS	PO	PO	None	None
82	2	SS	PO	PO	None	None

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

Table 7. The morphological characteristics of seedlings produced by 'Morita unshiu' × 'Lee' cross.

No.	[z]Plants(No.)	[y]LBS	LBAS	LBBS	WS	LBW
1	2	SS	PO	A	None	○
2	2	SS	PA	PA	SS	None
3	2	SS	PO	A	None	None
4	2	SS	ND	ND	None	None
5	2	SS	PO	A	SS	None
6	2	SS	PO	A	None	None
7	1	SS	PO	PA	None	None
8	1	SS	A	PA	None	None
9	1	SS	PA	PA	None	None
10	1	SS	PA	PA	None	None
11	2	SS	A	A	W	None
12	1	SS	PA	PA	None	None
13	1	SS	PA	PA	None	None
14	2	SS	PO	PA	None	None
15	2	SS	PO	A	W	None
16	2	SS	PO	PA	None	None
17	1	SS	PA	PA	None	None
18	2	SS	PO	PA	None	None
19	1	SS	PA	PA	None	None
20	2	SS	PO	A	None	None
21	2	SS	PO	PA	None	None
22	1	SS	PO	PA	W	None
23	2	SS	PA	A	None	None
24	1	SS	PA	A	None	None
25	1	SS	PA	A	None	None
26	1	SS	PA	PA	None	None
27	1	SS	PO	A	None	None
28	1	SS	PA	PA	SS	None
29	1	SS	PA	PA	None	None
30	1	SS	PA	PA	None	None
31	2	SS	PA	PA	None	None
32	4	SS	PA	A	None	None
33	2	SS	PA	PA	None	None
34	1	SS	PA	PA	None	None
35	1	SS	PA	PA	None	None

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

Table 7. continued

No.	[z]Plants (No.)	[y]LBS	LBAS	LBBS	WS	LBW
36	2	SS	PA	PA	None	None
37	2	SS	PA	A	None	None
38	4	SS	PA	PA	None	None
39	2	SS	PA	PA	None	○
40	2	SS	PA	PA	None	None
41	2	SS	PA	PA	None	None
42	1	SS	PA	PA	None	None
43	1	SS	PO	PA	None	None
44	4	SS	PA	A	None	None
45	3	SS	PA	A	None	None
46	2	SS	PA	PA	None	○
47	3	SS	PA	A	None	None
52	2	SS	PA	PA	None	None
58	2	SS	PA	PA	None	None
75	2	SS	PA	PA	None	None

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

Table 8. The morphological characteristics of seedlings produced by 'Miyagawa Wase' × 'Orlando' cross.

No.	[z]Plants(No.)	[y]LBS	LBAS	LBBS	WS	LBW
1	2	SS	PA	PA	None	None
2	2	SS	PA	PA	None	None
3	1	SS	ND	PA	None	None
4	2	SS	PA	PA	None	None
5	2	SS	PA	PA	None	None
6	2	SS	PA	PA	None	None
7	2	SS	PA	PA	None	None
8	2	SS	PA	PA	SS	None
9	3	SS	ND	PA	None	None
10	2	SS	PA	PA	None	None
11	2	SS	PA	PA	None	None
12	2	SS	PA	PA	None	None
13	1	SS	PA	PA	None	None
14	1	SS	PA	PA	None	None
15	2	SS	PA	PA	None	None
16	2	SS	PA	PA	None	None
17	3	SS	PA	PA	None	None
18	3	SS	PA	A	SS	None
19	2	SS	PA	PA	None	None
20	2	SS	PA	PA	None	None
21	2	SS	PA	PA	None	None
22	2	SS	PA	PA	None	None
23	2	SS	PA	PA	None	None
24	2	SS	PA	PA	None	None
25	1	SS	PA	PA	None	None
26	2	SS	PA	PA	None	None
27	3	SS	PA	PA	None	None
28	2	SS	PA	PA	None	None
29	2	SS	PA	PA	None	None
30	3	SS	PA	PA	None	None
31	2	SS	PA	PA	None	None
32	2	SS	PA	PA	None	None
33	2	SS	PA	A	SS	None
34	2	SS	PA	PA	None	None
35	1	SS	PA	PA	None	None

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

Table 8. continued

No.	[z]Plants(No.)	[y]LBS	LBAS	LBBS	WS	LBW
36	2	SS	PA	PA	None	None
37	2	SS	PA	PA	None	None
38	2	SS	PA	PA	None	None
39	3	SS	PA	PA	None	None
40	1	SS	PA	PA	None	None
41	2	SS	PA	PA	None	None
42	2	SS	PA	PA	None	None
43	2	SS	PA	PA	None	None

[z] Number of seedlings produced by one seed

[y] LBS: Leaf blade shape; LBAS: Leaf blade apex shape; LBBS: Leaf blade base shape; LBW: Leaf blade wave; WS: Wing shape; SS: Spindle-shaped; PA: Pointed acute; PO: Pointed obtuse; A: Acute; V: Vestigial

RAPD and SRAP analysis

Three RAPD primers, OPO14, UBC27 and UBC229, were selected for identification and data analysis. In the preliminary SRAP analysis, which was conducted using 62 primers, 10 forward primers and 4 reverse primers showed parent-related polymorphic amplification. After analysis, three forward/reverse primer sets, F7/R14, F11/R29 and F4/R14, were selected for identification and data analysis.

Two types of polymorphic DNA bands, dominant and recessive, were observed among the progenies. A dominant marker indicates that a band is present in the male plant, but not the female parent. A recessive marker is present in female plants, but absent from the hybrids. During RAPD or SRAP analysis, seedlings showing a pollen parent-specific band (dominant) or no female parent-specific band (recessive) were considered to be zygotic (Fig. 6). Dominant and recessive DNA amplification profiles amplified by the RAPD and SRAP primers are shown in Fig. 7, Fig. 8, Fig. 9 and the appendix.

RAPD analysis resulted in the amplification of 13 dominant markers. Specifically, six dominant markers were observed among the offspring produced by the 'Morita unshiu' × 'Ponkan' cross, while four were observed among the offspring produced by the 'Morita unshiu' × 'Lee' cross, and three were observed among the offspring produced by the 'Miyagawa Wase' ×'Orlando' cross (Table 9). The identified markers were then used for a consecutive analysis. When the offspring of the 'Morita unshiu' × 'Lee' cross were evaluated, all 4 dominant markers were amplified by the OPO14 primer. However, 2 markers with sizes of 2700 bp and 750 bp were excluded from the consecutive RAPD analysis due to their unstable reproduction.

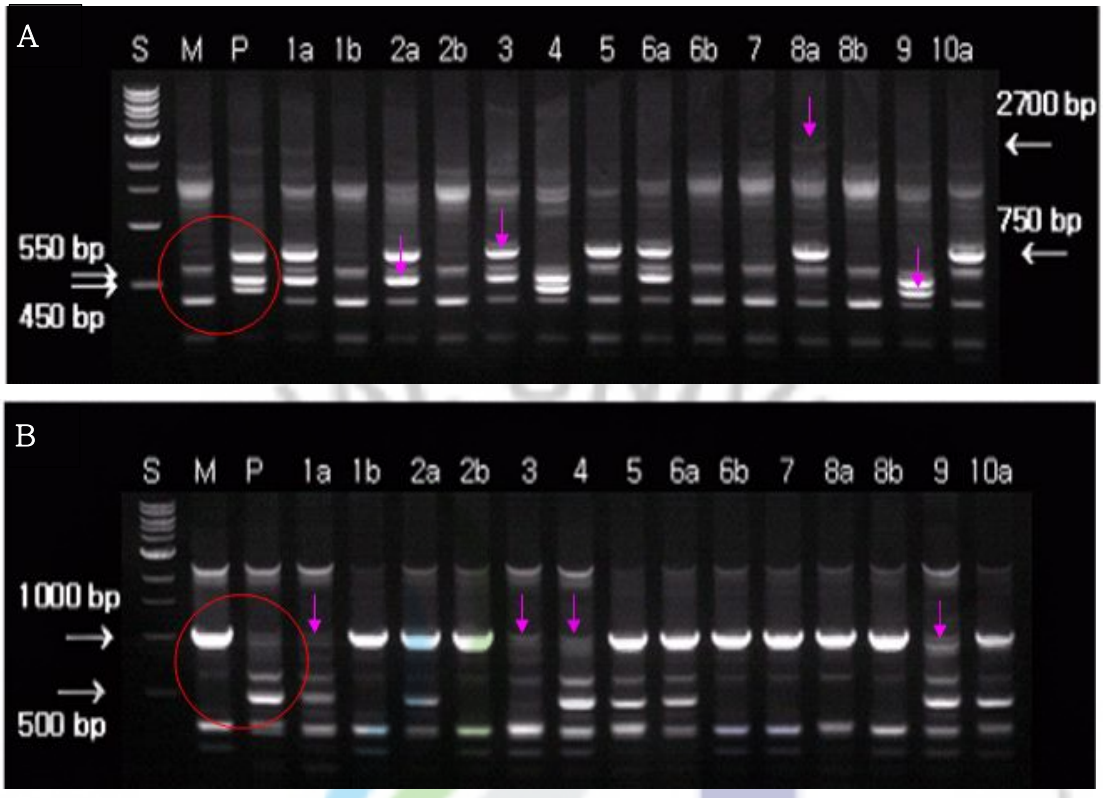


Fig. 6. Polymorphic DNA amplification in response to RAPD (A) and SRAP (B) analysis of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The arrows indicate dominant (A) bands produced by the OPO14 primer and recessive (B) bands produced by the F7/R14 primer. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedling produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'

Table 9. Zygotic seedlings identified using RAPD and SRAP primers.

Cross	No. of seedlings (seeds)	Total no. of zygotic seedlings	Primers	Markers	No. of zygotic seedlings	
Morita unshiu × Ponkan	135 (95)	50	[z]OPO14	[y]2700	20	50
				750	24	
				550	26	
				450	25	
			UBC27	250		39
			UBC229	1200		13
			F7/R14	500	29	38
				[x]1000	20	
			F11/R29	1050		23
			F4/R14	800	29	40
				[x]650	24	
Morita unshiu × Lee	148 (88)	45	OPO14	550	24	45
				450	44	
			UBC27	800	27	45
				250	44	
			F11/R29	1050	23	28
				[x]450	14	
			F4/R14	800	19	28
				[x]650	15	
Miyagawa Wase × Orlando	86 (43)	3	OPO14	550	3	3
				450	3	
			UBC27	200		2
			F4/R14	800		2

[z]OPO14, UBC27 and UBC229 are RAPD primers; F7/R14, F11/R29 and F4/R14 are SRAP primers.

[y]Dominant marker

[x]Recessive marker

When the SRAP analysis was conducted, 3 dominant and 2 recessive markers were identified among the offspring produced by the 'Morita unshiu' × 'Ponkan' cross, while 2 dominant and 2 recessive markers were observed among the offspring produced by the 'Morita unshiu' × 'Lee' cross and only one dominant marker was observed among the offspring produced by the 'Miyagawa Wase' ×'Orlando' cross (Table 9).

The dominant markers were as follows: OPO14-2700, OPO14-750, OPO14-550, OPO14-450, UBC27-250, UBC229-1200, F7/R14-500, F11/R29-1050 and F4/R14-800 for the 'Morita unshiu' × 'Ponkan' cross; OPO14-550, OPO14-450, UBC27-800, UBC27-250, F11/R29-1050 and F4/R14-800 for the 'Morita unshiu' × 'Lee' cross; OPO14-550, OPO14-450, UBC27-250 and F4/R14-800 for the 'Miyagawa Wase' × 'Orlando' cross. In addition, the following recessive markers were identified: F7/R14-1000 and F4/R14-650 for the 'Morita unshiu'× 'Ponkan' cross; F11/R29-450 and F4/R14-650 for the 'Morita unshiu' × 'Lee' cross.

When RAPD analysis was conducted, a total of 98 seedlings were identified as zygotic. Specifically, 50 of 95 'Morita unshiu' × 'Ponkan' seedlings (52.6%), 45 of 88 'Morita unshiu' × 'Lee' (51.1%) seedlings, and 3 of 43 'Miyagawa Wase' × 'Orlando' (7.0%) seedlings were identified as zygotes (Table 9).

When SRAP analysis was conducted using primers specific for the 3 dominant and 2 recessive markers, 92 seedlings were classified as zygotic. Specifically, 49 of 95 'Morita unshiu' × 'Ponkan' seedlings (51.6%), 41 of 88 'Morita unshiu' × 'Lee' seedlings (46.6%), and 2 of 43 'Miyagawa Wase' × 'Orlando' (4.7%) seedlings were found to be zygotic.

The OPO14 primer was the most efficient one used in both analyses. When the 'Morita unshiu' × 'Ponkan' seedlings were evaluated using this

primer, 4 male parent-specific dominant markers amplified by the OPO14 primer enabled detection of all of the zygotic seedlings that had been identified based on the presence of 11 markers amplified by 6 primer sets. When the 'Morita unshiu' × 'Lee' seedlings were evaluated, the OPO14 primer amplified 2 male parent-specific dominant markers in all 45 zygotic seedlings that had been identified using 8 markers from 4 primers (Table 9, Fig. 7).

The UBC27 primer showed the second highest identification efficiency of zygotic seedlings. When the seedlings produced by the 'Morita unshiu' × 'Ponkan' cross were evaluated using the UBC27 primer alone, 1 dominant marker related to the pollen parent enabled identification of 78.0% (39/50) of 50 zygotic seedlings that had been identified by 11 markers from 6 primer sets. When the 'Morita unshiu' × 'Lee' hybrids were evaluated, the use of UBC27 primer alone amplified 2 dominant markers related to the pollen parent, which enabled detection of 100% (45/45) of the 45 zygotic seedlings that were identified based on the presence of 8 markers amplified by 4 primer sets (Table 9, Fig. 8).

When the efficiency of the dominant and recessive markers were evaluated, the pollen parent-specific dominant markers utilized for both the RAPD and SRAP analysis effectively identified the zygotic seedlings, while the recessive markers efficiently identified zygotes when used for SRAP analysis (Table 9, Fig. 9).

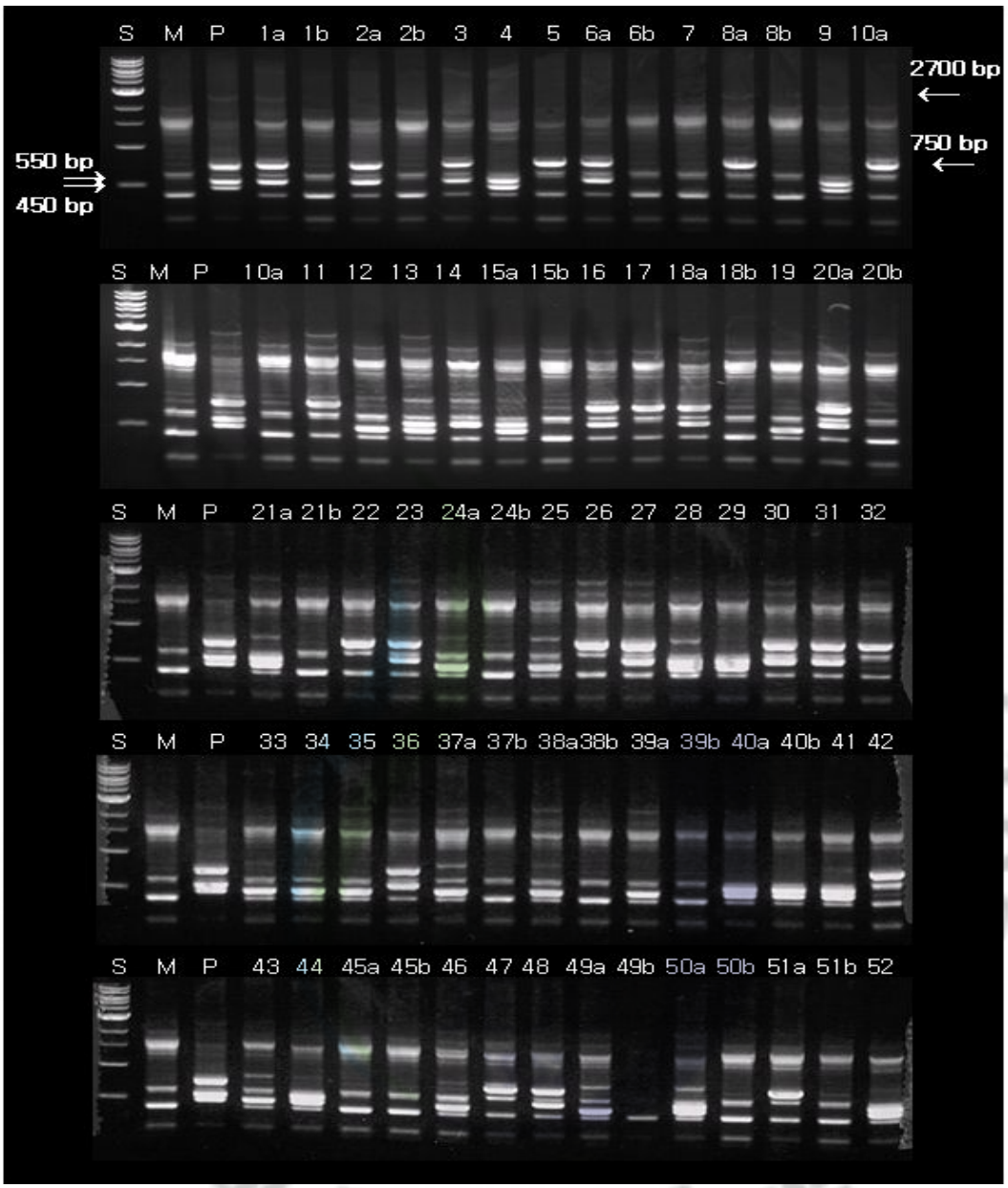


Fig 7. DNA amplification profiles obtained using the OPO14 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'

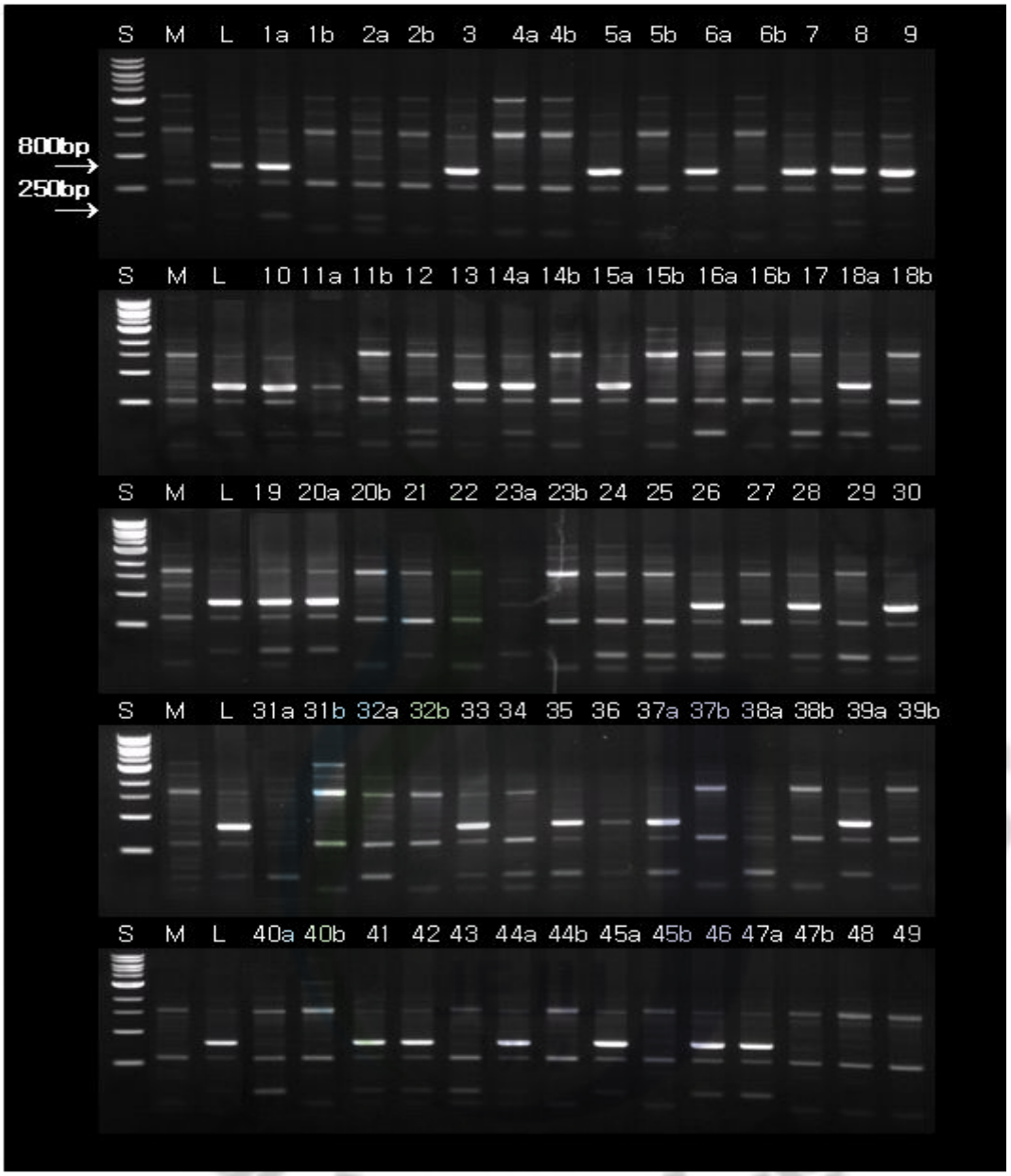


Fig 8. DNA amplification profiles obtained using the UBC27 primers to analyze samples of seedlings produced by the 'Morita unshiu' × 'Lee' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; L: 'Lee'

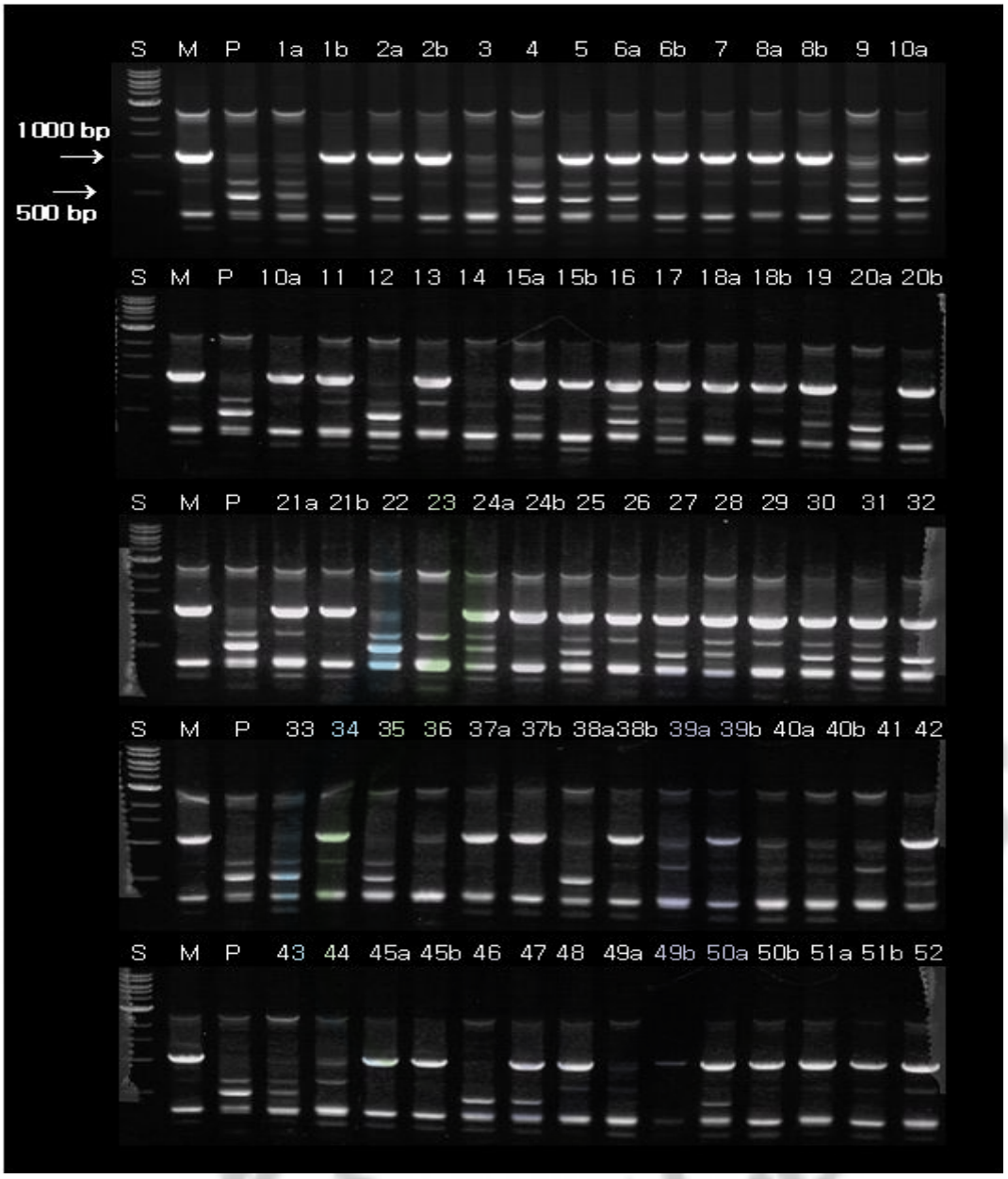


Fig 9. DNA amplification profiles obtained using the F7/R14 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'.

The paired identification efficiency of markers from three primer sets was also investigated (Table 10). The OPO14-750 and OPO14-450 showed 1:1 segregation in the zygotic seedlings, but appeared in all of the seedlings. The presence of these markers enabled detection of 98% of the zygotic seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. Conversely, the OPO14-2700 and OPO14-750 markers only identified 68.0% of the zygotic seedlings. The efficiency of the remaining markers used in this study ranged from 70-78%. Furthermore when three primer sets were used together, the identification rate ranged from 83.0 to 100.0%. When the UBC27-250 marker, which identified genes with a 3:1 segregation, was included, the markers were capable of identifying more than 90% of the zygotic seedlings in all crosses (Table 7). The segregation of dominant markers identified by RAPD and SRAP are described in table 11. Generally, the markers showed segregation ratios of 1:1 or 3:1 (or 1:3) without exception. The OPO14-450 and UBC27 250 markers, which were present in almost all of the 'Morita unshiu' × 'Lee' hybrids, were apparently homozygous alleles.

Table 10. Identification efficiency of zygotic seedlings as determined using combinations of two or three dominant markers to evaluate the progeny produced by 'Morita unshiu' × 'Ponkan' crosse.

Markers	OPO14-2700	OPO14-750	OPO14-550	OPO14-450	UBC27-250	F7/R14-500	OPO14-750 + OPO14-550	OPO14-550 + OPO14-450	F7/R14-500 + UBC27-250	UBC27-250 + OPO14-750
OPO14-2700		[z]34(68.0%)	39(78.0%)	35(70.0%)	43(86.0%)	40(80.0%)	[y]44(88.0%)	43(86.0%)	49(98.0%)	46(92.0%)
OPO14-750			37(74.0%)	**49(98.0%)**	46(92.0%)	38(76.0%)			49(98.0%)	48(98.0%)
OPO14-550				39(78.0%)	44(88.0%)	42(84.0%)			49(98.0%)	48(98.0%)
OPO14-450					42(84.0%)	40(80.0%)			48(98.0%)	**50(100%)**
UBC27-250						47(94.0%)	48(98.0%)	46(92.0%)		
F7/R14-500							46(92.0%)	45(90.0%)		47(94.0%)

[z] No. of zygotic seedlings identified by two markers

[y] No. of zygotic seedlings identified by three markers

A total of 50 zygotic hybrids were identified by 11 markers by RAPD and SRA

Table 11. Segregation of dominant markers in male parental 'Ponkan' and 'Lee' plants.

Cross combination	No. of zygotic seedlings	Markers	Pollen parent specific band		Expected ratio	χ^2
			Present	Absent		
Morita unshiu × Ponkan	50	OPO14-2700	20	30	1:1	2.000
		OPO14-750	24	26	1:1	0.080
		OPO14-550	26	24	1:1	0.080
		OPO14-450	25	25	1:1	-
		UBC27-250	39	11	3:1	0.240
		UBC229-1200	13	37	1:3	0.027
		F7/R14-500	29	21	1:1	1.280
		F11/R29-1050	23	27	1:1	0.320
		F4/R14-800	29	21	1:1	1.280
Morita unshiu × Lee	45	OPO14-550	24	21	1:1	0.200
		OPO14-450	44	1	1;0	-
		UBC27-800	27	18	1;1	1.800
		UBC27-250	44	1	1;0	-
		F11/R29-1050	23	22	1:1	0.022
		F4/R14-800	19	26	1:1	1.089

5% critical value: 3.841

Relationship between nucellar and dwarfism

When seedlings produced by the 'Morita unshiu' × 'Ponkan' cross and the 'Morita unshiu' ×'Lee' cross were evaluated, we found that nucellar seedlings identified by RAPD and SRAP analysis tended to exhibit dwarfism (Fig. 5). Specifically, 2-year-old zygotic seedlings were at least 50cm tall, while the nucellar seedlings were only 10-12cm tall (Table 12). Accordingly, the 337 seedlings produced by the 'Morita unshiu' and 'Ponkan' cross, and the 285 seedlings produced by the 'Morita unshiu' and 'Lee' cross were classified as a zygotic or nucellar based on tree height, and the results were then compared to the results obtained by the DNA marker analysis (Table 13, 14). No zygotic dwarf seedlings were identified when the RAPD and SRAP analysis of the 'Morita unshiu' x 'Ponkan' seedlings were evaluated, and only 2 of the 23 seedlings that displayed medium vigor were nucellar. All of the vigorous seedlings were zygotic. Similarly, when the seedlings produced by the 'Morita unshiu' and 'Lee' cross were evaluated, there were no zygotic seedlings that exhibited dwarfism and only one of the 22 seedlings that exhibited medium vigor was nucellar. Among the vigorous seedlings, 25 zygotic seedlings and one nucellar seedling were observed. Hence, most vigorous seedlings, including those that displayed medium vigor, could be identified as zygotic.

The parental origin of a randomly selected group of 135 seedlings (95 seeds) produced by the 'Morita unshiu' × 'Ponkan' cross, which consisted of 29 vigorous, 23 medium, and 83 dwarf seedlings, was determined using the DNA markers identified in this study. The results revealed that all dwarf seedlings were nucellar, while the remainder of the seedlings, with the exception of 2 that showed medium vigor, were zygotic (Table 13). When the 'Morita unshiu' × 'Lee' seedlings were evaluated (Table 14), all of the dwarf seedlings were identified as nucellar by DNA marker-based analysis. These

findings indicate that observation of tree vigor can be used effectively to select nucellar seedlings in crosses in which 'Morita unshiu' is used as a female parent. Indeed, it is expected that approximately 90% of seedlings can be identified as nucellar (Table 15).

Table 12. Tree height of zygotic seedlings and nucellar seedlings produced by crosses of 'Morita unshiu' ×'Ponkan' and 'Morita unshiu' × 'Lee'.

Cross	Tree height (cm)	
	Zygotic	Nucellar
Morita unshiu × Ponkan	118.0±43.4***	13.4±10.2
Morita unshiu × Lee	102.7±26.9***	11.2±5.1

***significantly different at $p \leq 0.001$ as determined by a student's t test

Table 13. Relationship between tree vigor and parent origin of seedlings in of the progeny produced by crosses of 'Morita unshiu' and 'Ponkan'.

Parent origin[Y]	No. of obtained seedlings (No. of seeds)	Tree vigor[Z]		
		Vigorous	Medium	Dwarf
Zygotic	135 (95)	29	21	-
Nucellar		-	2	83

[Z] Vigorous: ≥ 100cm, Medium: < 100cm and ≥ 50cm, Dwarf: < 50cm

[Y] Determined on the basis of molecular marker analysis

Table 14. Relationship between tree vigor and parent origin of seedlings produced by crosses of 'Morita unshiu' and 'Lee'.

Parent origin[Y]	No. of obtained seedlings (No. of seeds)	Tree vigor[Z]		
		Vigorous	Medium	Dwarf
Zygotic	148 (88)	25	20	-
Nucellar		1	1	101

[Z] Vigorous: ≥ 100cm, Medium: < 100cm and ≥ 50cm, Dwarf: < 50cm

[Y] Determined on the basis of molecular marker analysis

Table 15. Tree vigor distributions of seedlings produced by crosses in which 'Morita unshiu' was used as a female parent.

Cross	No. of seedlings (No. of seeds)	Tree vigor		
		Vigorous	Medium	Dwarf
'Morita unshiu' × 'Ponkan'	528 (337)	29	23	476
'Morita unshiu' × 'Lee'	513 (285)	30	21	462

Development of a SCAR Marker linked to Monoembryony

The 48 monoembryonic seedlings and the 52 polyembryonic seedlings were obtained from 'Kiyomi' tangor × 'Jinkyul' cross. The segregation ratio of monoembryonic to polyembryonic seeds in the progenies was 1:1 and X^2 value was 0.160 (< X^2 0.05 (1) = 3.84).

The monoembryonic individuals had only one embryo in their seeds, while the polyembryonic individuals showed various embryo numbers from 1 to 14. A total of 915 seeds from 52 polyembryonic individuals were investigated on their embryo number. Seeds of 22.7% had 3 and more embryos and 14.3% of the seeds did a single embryo. Totally, 85.7% of seed was polyembryonic (Fig. 10), showing the 3.6 of mean embryo number.

Total 800 UBC primers were tested to produce polymorphism between 'Kiyomi' tangor and 'Jinkyul', and 285 designated primers were primarily selected (Fig. 11). Then, BSA-RAPD on two bulked DNA samples, monoembryonic and polyembryonic plants, was conducted, and 67 primers with polymorphism between monoembryonic and polyembryonic progenies were selected. Finally, UBC55 primer, which showed a polymorphic band of about 750 bp only in monoembryonic individuals, was selected (Fig. 12).

The fragment of 750 bp was cloned into the TOPO-TA cloning vector, and examined on its size and DNA sequences (Fig. 12, 13). It was 742 base pair in size and did not show a homology with any data on Gene Bank. In the future, whether this fragment is directly linked to a polyembryony or not must be examined.

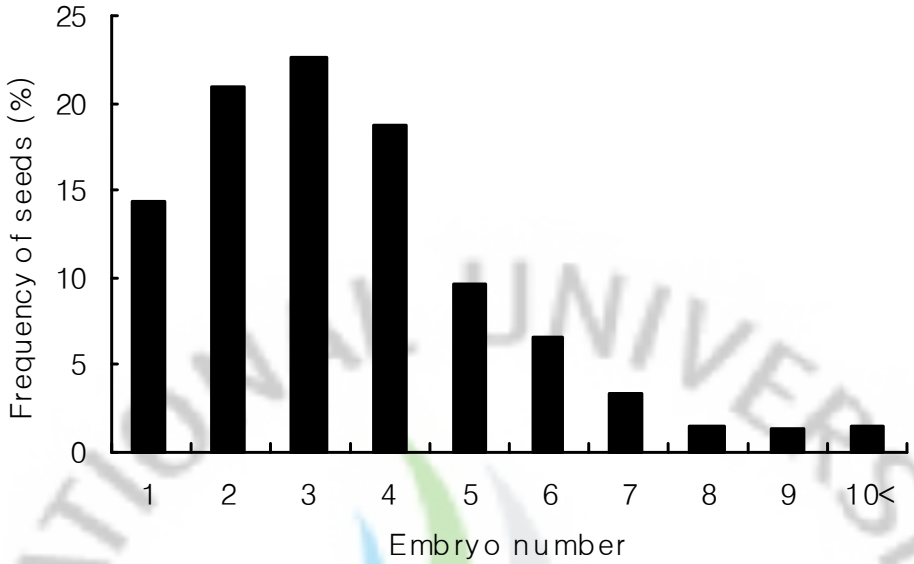


Fig. 10. Distribution of embryo number in seeds of 'Kiyomi' (*Citrus spp.*) × 'Jinkyul' (*C. sunki*) hybrid seedlings.

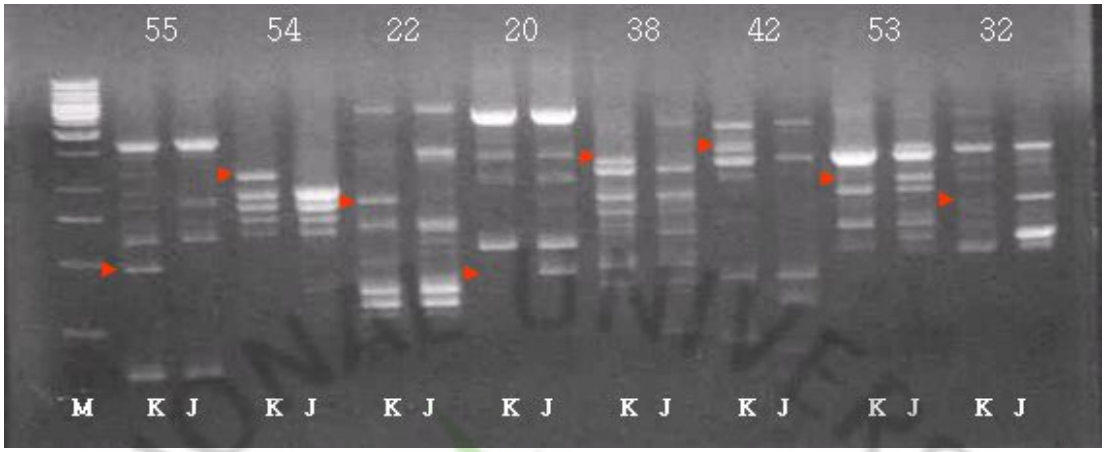


Fig. 11. DNA amplifications of *Citrus spp.* 'Kiyomi' (K) and *C. sunki* 'Jinkyul' (J) using UBC primers. M: 1kb ladder marker; Numbers denote the corresponding UBC primer number. Arrows indicate polymorphic bands.

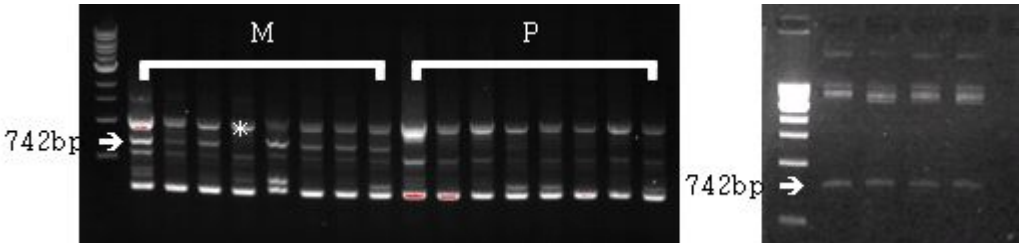


Fig. 12. Selected RAPD marker linked to the polyembryony which was amplified with UBC 55 primer (left) and polymorphic band cloning into TOPO TA cloning vector (right). Cloning vector was cut with EcoR Ⅰ. M, Monoembryonic seedlings P, Polyembryonic seedlings *, Recombinant individuals.

From the 742 bp fragment, we generated 6 forward and 6 reverse primers for development of monoembryony-specific primer (Table 16). Among the 36 combinations of forwards and reverse primer, the pMono-U (forward primer) and p468D (reverse primer) primer set amplified 500 bp fragments in almost all monoembryonic progenies with an exceptional one individual, probably because of a recombinant (Fig. 14).

Fig. 13. Nucleotide sequences of monoembryony specific DNA fragment amplified with UBC 55 primer. Squares are PCR specific primer sequences of pMono-U and p468D.

Table 16. Primers used for direct amplification of SCAR markers.

Primer		Sequence
Forward	pMono-U	5′-TCCCTCGTGCCCCTCCAAATA-3′
	p1U	5′-CGTGCCCCTCCAAATACTTA-3′
	p122U	5′-ACCCCTTTTCCCACAACTCT-3′
	p222U	5′-TTGCATGCTCTGTTTTCAGC-3′
	p484U	5′-ACCTTCTCGCATCAGCAACT-3′
	p561U	5′-CGTGGGAATGAAATGGTAGG-3′
Reverse	pMono-D	5′-TCCCTCGTGCTGTCATGCATT-3′
	p334D	5′-AGGTTTCGACCAGAAGCTGA-3′
	p468D	5′-GGTTGAGAAAAAGAGATAGTT-3′
	p682D	5′-TCTTCGTCGCTGTTTCACTG-3′
	p750D	5′-CGTGCTGTCATGCATTACAT-3′
	p802D	5′-GCTCGCGTGTTACTGAGATG-3′

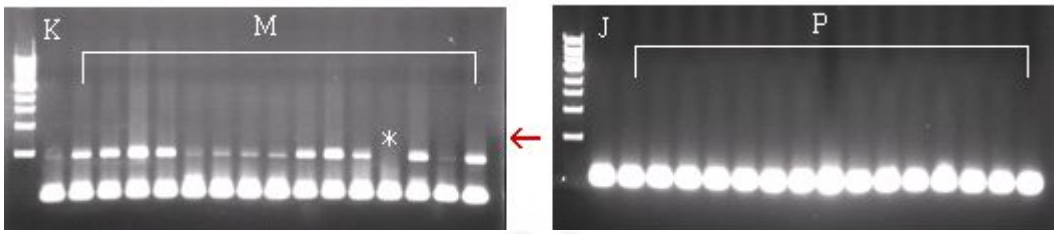


Fig. 14. Polymerase chain reaction of *Citrus spp*. 'Kiyomi' (K) × *C. sunki* 'Jinkyul' (J) hybrid seedling using pMono-U/p468D primer set. M, Monoembryonic seedlings P, Polyembryonic seedlings *, Recombinant individuals.

Discussion

Identification of zygotic hybrid from the satsuma mandarin cross

Many studies have been conducted to evaluate methods of identifying polyembryony using markers such as morphological characteristics, the presence of isozymes and DNA-based markers to identify zygotic or nucellar individuals. However, to date, it has been difficult balance the identification efficiency and the cost of various methods. For example, the use of leaf characteristics such as the ratio of the total leaf length to width has been used to differentiate zygotes and nucellar offspring produced by King mandarin and orange crosses, as well as tangelos and tangerines (Teich and Spiegel-Roy, 1972). Furthermore, the width of the leaf petiole of seedlings produced by crosses between satsuma mandarins and 'Natal' sweet oranges, 'Rio' and 'Carvo' tangerines, or 'Murcott' tangors (Donadio, 1979) has been used to differentiate zygotic and nucellar individuals, and the width of the leaf petiole wing (Ballve et al., 1997) has been used to differentiate the sexual progenies of seedlings produced by a polyembryonic female parent. In addition, the use of dominant characteristics as morphological markers to evaluate sexual citrus hybrids, such as the trilobate leaf trait that is observed in *Poncirus trifoliata* (L.) Raf., can enable easy identification of the zygotic progeny produced by a cross between this species and other citrus species (Cooper et al., 1962). However, morphological methods are often not sufficient to distinguish zygotic hybrids in polyembryonic citrus crosses.

Moore and Castle (1988) reported that most zygotes were morphologically distinct in Swingle citrumelo, but not in Volkamer lemons. Similarly, Xiang and Roose (1988) found that nearly all zygotes could be recognized based on the morphological characteristics of citrumelos, but not in Taiwanica or

Volkamer lemons. In the present study, morphological analysis enabled the identification of 7 - 44% of putative zygotic seedlings that had features that differed from that of the mother plant or had a characteristic found in the father plant. However, it was not easy to differentiate morphological traits related to leaf shape among hybrids because the parents had similar genetic backgrounds; therefore, it was presumed that their parental leaf shape differed only slightly. Kepiro and Roose (2007) explained that if the parents were fairly closely related (e.g. two mandarins), it would be more difficult to identify zygotic off-types than if the parents were distantly related. Oliveira et al. (2002) also reported that, even though the LAMI (leaf apex morphometric index) could increase the identification efficiency of zygotic seedlings produced by a cross between 'Murcott' (*Citrus reticulata* Blanco) and 'Pera' sweet oranges, the use of LAMI alone was not enough to identify hybrids without confirmation using molecular markers.

Recently, many studies have been conducted to evaluate the development of zygotic seedling selection markers using advanced methods such as AFLP, SSR, RAPD, isozyme identification (Anderson et al., 1991) and flow cytometry for use as complementary morphological markers. Scarano et al. (2003) reported that evaluation of the use of flow cytometry, SSR and modified AFLP markers for the identification of zygotic plantlets produced by backcrosses between Femminello lemon cybrids and a diploid clone of Femminello lemons was evaluated revealed that flow cytometry was only useful for the identification of interploid crosses, while the SSR technique appeared to be more suitable than modified AFLP. In a study conducted to evaluate the same population, Tusa et al. (2002) reported that among flow cytometry, isozyme analysis and ISSR-PCR, ISSR-PCR was the most efficient and reliable technique for the identification of zygotic plantlets. Furthermore, Ruiz et al. (2002) reported that SSR markers were more efficient

than isozymic markers for the identification of sexual origin in a cross of *Poncirus trifoliata* (L.) Raf. var. and tangor 'Ortanique' (*Citrus reticulata* Blanco × *C. sinensis* Osb.), as well as in selfing progenies produced by *Fortunella crassifolia* Swing.

As the aforementioned studies indicate, the use of a single technique alone is often not sufficient for the identification of zygotic individuals among mixed seedlings. In many cases, multiple markers identified using several techniques, including morphological identification, were used concurrently. However, Nageswara Rao et al. (2008) reported that the use of RAPD alone was a less expensive and more broadly available method capable of identifying zygotic and nucellar individuals when compared to EST-SSR, and that EST-SSR should only be considered when the use of RAPD alone is inadequate. Therefore, in the present study, we attempted to identify a DNA-based marker that enabled the early identification of zygotic seedlings from the offspring of satsuma offspring by RAPD and SRAP. According to Yun et al. (2007), 20 of 149 (13.4%) seedlings produced by a cross of 'Miyagawa Wase' and 'Ponkan' mandarins were zygotic. In addition, Bastianel et al. (1998) found that 54 of 202 (26.7%) seedlings produced by a cross of 'King' and 'Montenegrina' were zygotic. The percentages of zygotic progenies in various citrus hybrids have been found to depend on the seed parent used (Spiegel-Roy et al., 1977), the pollen origin (Cameron and Soost, 1969), and the environmental influences (Moore and Castle, 1988). In addition, the percentage of zygotic offspring has been found to vary considerably among species, years and locations (Xiang and Roose, 1988).

In this study, approximately 50% of the seedlings produced by crosses between 'Morita unshiu' and 'Ponkan' and 'Morita unshiu' and 'Lee' were found to by zygotic by RAPD and SRAP analysis. However, this does not indicate that there was a high percentage of sexual progeny produced by

artificial fertilization between 'Morita unshiu' and 'Ponkan' or 'Lee'. It is more likely that these findings were caused by a small statistical population (95 or 88) or sampling error because many individuals were often too small to obtain leaf samples from (Fig. 5). Conversely, evaluation of hybrids of 'Miyagawa Wase' and 'Orlando' revealed that only 7.0% of the seedlings were zygotic, and that the zygotic seedling occurrence was lower than that of 'Morita unshiu' × 'Ponkan' and 'Morita unshiu' × 'Lee' crosses.

RAPD analysis conducted using the OPO 14 primer, which was capable of identifying all zygotic seedlings when used alone, had the highest efficiency and produced multi dominant markers. Specifically two dominant markers amplified by OPO14, OPO14-750 and OPO14-450, which were detected in 'Morita unshiu' × 'Ponkan' cross progenies, appear to be co-dominant markers that are trans located in repulsion. Williams et al. (1993) proposed that a pair of dominant markers tightly linked in repulsion provided almost the same information as a single co-dominant marker however, this case is rare in RAPD. At our institute, the use of OPO14 primer alone identified approximately 20% of the zygotic seedlings produced by crosses of 'Miyagawa Wase' × 'Winking' (*Citrus reticulata*) and 'Aoshima ushiu' × 'Ponkan' (data not shown). These findings indicate that the OPO14 primer detected a tangerine-specific dominant allele that was not present in satsuma mandarins. Therefore, we expect that this primer will be useful in the evaluation of crosses between satsuma mandarins and tangerines or their hybrid varieties.

Dominant markers are commonly used in RAPD, while co-dominant markers are generally identified by SRAP (Agarwal et al., 2008). Li and Quiros (2001) reported that SRAP polymorphism results from fragment size changes due to insertions and deletions, which results in co-dominant markers. However, there were no co-dominant markers produced by SRAP in

the present study, and all markers related to the pollen parent had one dominant marker type. Conversely, the recessive marker from SRAP was also useful for the identification of zygotic hybrids when compared to RAPD. Indeed, RAPD produced only a few recessive bands, while the SRAP recessive marker, F4/R14-650, enabled the identification of 24 (48%) zygotic seedlings produced by 'Morita unshiu' × 'Ponkan'. Because seedlings without female-specific bands amplified by RAPD analysis can be zygotic (Andrade-Rodriguez et al., 2004; Bastianel et al., 1998), the recessive marker produced by SRAP in the present experiment can also be considered a useful tool for the identification of zygotic seedlings.

The identification efficiency between RAPD and SRAP did not differ greatly in this study. These findings indicated that the use of RAPD or SRAP alone would enable accomplishment of our goals regarding many of the crosses conducted at our institute. Furthermore, both RAPD and SRAP are less expensive, require less DNA and provide results more rapidly than other methods of identification.

Agarwal et al. (2008) and Li et al. (2001) reported that random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) were simple, inexpensive and efficient methods of identifying markers that are widely used for a variety of purposes. Although co-dominant markers produced by SSR, RFLP, and the high multiplexing ratio produced by AFLP provide greater efficiency when used to identify zygotic individuals, these methods are relatively complex, time-consuming and expensive. Therefore, it is difficult to adapt these PCR marker systems to the numerous populations that must be evaluated during citrus cross breeding.

In the present study, we also found that nucellar seedlings produced by crosses between 'Morita unshiu' 'Ponkan' and 'Morita unshiu' 'Lee' showed very weak growth. Many varieties of citrus plants and its related genera are

polyembryonic and contain two or more embryos with zygotic or nucellar origins (Soost and Roose 1996). In such cases, the zygotic embryos usually compete with the nucellar embryos for space and nutrients (Kobayashi et al., 1978). Weinbaum et al. (1982) reported that90 to 95% of the seedlings produced by 'Satsuma' mandarins were of nucellar origin. The results of their study indicate that most zygotic embryos did not grow normally in the egg cells. However, even though a few zygotic embryos germinated successfully, there were no considerable differences in tree vigor observed between zygotic and nucellar individuals in their study. Conversely, Xiang and Roose (1988) reported that zygotic seedlings were generally shorter than nucellar seedlings, and that use of height as a marker of zygotic seedlings was generally more variable than its use as a marker of nucellar seedlings in 12 rootstocks. As a result, it is generally difficult to differentiate zygotic and nucellar individuals produced by crosses in which the female parent is a polyembryonic citrus cultivar based on phenotypic characteristics such as tree vigor. However, in the present study, nucellar progenies of 'Morita unshiu' were found to be remarkably shorter than zygotic seedlings (Fig. 5), which enabled the identification of zygotic seedlings based on visual identification of dwarfism.

There have been several studies conducted to evaluate dwarfism in citrus crops. For example, Cheng and Roose (1995) reported that a single dominant gene is responsible for dwarfism in a citrus rootstock, *Poncirus trifoliata* ('Flying Dragon'). 'Flying Dragon' trifoliate oranges greatly reduced tree size when used as a rootstock for any citrus scion cultivar (Bitters et al., 1979; Roose, 1990). However, to date, no studies have reported dwarfing in nucellar citrus seedlings. It was true in many crosses using a wide variety of satsuma mandarins at our institute, with an exceptional cross using 'Morita unshiu' as a female parent. These findings indicate that dwarfism probably

originates from female parent 'Morita unshiu' trees.

The dwarfism could be used effectively in the breeding programs using satsuma mandarins as a female parent, because 'Morita unshiu' is the mutant selection of 'Miyagawa Wase', the leading variety in Korea; 'Miyagawa Wase' and 'Morita unshiu' are very similar in their genetic backgrounds in terms of fruit quality, maturation time and cultivation.

In our experiment, the combinatory screening using molecular markers using RAPD, SRAP analysis and visual observation could indicate that 15-17% of the seedlings produced by 'Morita unshiu' × 'Ponkan' and 'Morita unshiu' × 'Lee' crosses were zygotic. It indicates that RAPD or SRAP analysis can improve the breeding efficiency by early selection of sexual hybrids in satsuma mandarin cross breeding program, although large scale of primer screening is at cost. Some primers selected in our experiment and the dwarfism also could save the money and time. Especially, the dwarfism could be powerful tool for identification of zygotic and nucellar progenies in the crosses using 'Morita unshiu' as a female parent. The dwarfism has never been reported in any researches for nucellar embryony in citrus. So, additional researches must make it clear that whether nucellar progeny is genetically identical to mother plants in citrus.

The results of the present study are expected to aid in the development of satsuma mandarin introgression hybrids for use in a breeding program focused on the genetic improvement of open field citrus scion cultivars to produce plants with a rich aroma, high soluble solid content, and resistance against scab disease, which is a major disease on Jeju Island. Because satsuma mandarins are susceptible to scab disease in areas that receive large amounts of rain, they must be protected from this disease by agro-chemicals. Therefore, the introduction of a resistant trait against scab disease from 'Ponkan' or 'Lee' could lead to a labor-saving and eco-friendly form of citrus agriculture on Jeju Island.

Development of a SCAR Marker Linked to Monoembryony

Polyembryony is very common in the genus Citrus and its related genus. It is a specific seed propagation system caused by somatic embryogenesis in nucellus tissue. It was reported that nucellar embryony is controlled by a single major dominant gene that is heterozygous in trifoliate and absent in 'Chandler' pummelo, and also minor genes may control the level of expression (Parlevliet and Cameron, 1959). On the contrary, there were other reports that several genes control nucellar embryony and that polyembryony is an independent trait (Garcia et al., 1999; Ains et al., 2002). Further, Hong et al. (2001) reported that two complementary dominant genes are controlling apomixis. On the other hand, monoembryonic trait in citrus has a single homo recessive gene (Spiegel-Roy and Goldschmidt, 1996).

In the present cross of 'Kiyomi' and 'Jinkyul', the segregation of polyembryonic and monoembryonic progenies was 1:1. Because the monoembryonic cultivar, 'Kiyomi', has a homo recessive gene controlling the monoembryony, it is highly probable that 'Jinkyul' has a hetero gene controlling the monoembryony.

In the present study, 85.7% of seed had several embryos. Andrade-Rodriguez et al. (2005) reported that the polyembryonic seed appeared 79.4~90.1 percent, and Prates and Pompeu Jr. (1981) also reported over 60 percent of polyembryonic seeds in *Citrus reshni*. Similar results have been reported in other seeds with polyembryo (Soares Filho, 1995; Prates and Pompeu Jr., 1981). The variation of polyembryony among fruits may be influenced by type of pollinator, amount of viable pollen, plant nutrition, air temperature, air and soil humidity, and wind speed. Therefore, factors affecting pollination, fertilization or seed development will also affect the percentage of polyembryony and embryo number per seed.

We made a SCAR marker, pMono-U/p468D, related to monoembryony. However, we did not advance research about this marker through other crosses because we have not another fruiting F1 population yet. It will be needed to test availability of this SCAR marker in the future.

A necessity of marker assistant selection using poly- or monoembryonic trait could be different according to goal in citrus breeding. Because there have been no reports that this trait is related to fruit characteristics, resistance, and tree growth, this would be confused whether it is a necessary and sufficient condition in citrus scion breeding program. However, the marker related to polyembryony is important in rootstock development because it is essential having a trait of polyembryo (Frost, 1943; Xiang and Roose, 1988; Garcia et al., 1999; Ruiz et al., 2000; Kepiro and Roose, 2007).

In conclusion, a SCAR marker related to monoembryonic trait was developed from the seedlings of 'Kiyomi' and 'Jinkyul', and it was the first case that the marker linked to monoembryony in citrus was developed. Even though the SCAR marker needs an additional research about its universality, it could help develop marker assisted selection (MAS) system on monoembryonic trait in citrus breeding.

PART Ⅱ. Development of new Satsuma mandarin 'Haryejosaeng' by nucellar selection

Abstract

A new early maturing satsuma mandarin (*Citrus unshiu* Marc.), which was named 'Haryejosaeng', was developed from a nucellar seedling produced by a cross of 'Tachima Wase' (*C. unshiu* Marc.) with *C. natsudaidai* Hayata at the Citrus Research Station on Jeju Island in 1992.

'Haryejosaeng' produced seedless fruits that matured in early November and had higher levels of soluble solids (10 to 11 °Bx) and lower acidity (1% to 1.1%) than 'Miyagawa Wase', which is the leading early-maturing satsuma mandarin cultivar on Jeju Island. In addition, 'Haryejosaeng' produced fruit that weighed 80 to 90 g, was compressed-oblate globose with a light orange color and had a rind of approximately 2 mm that was easy to peel. The 'Haryejosaeng' trees showed vigorous growth, spreading of thornless twigs, and alternate bearing similar to that of 'Miyagawa Wase' trees. 'Haryejosaeng' was susceptible to citrus scab disease and melanose, but resistant to citrus canker.

Introduction

The citrus fruit production on Jeju Island accounted for 25% of the total domestic fruit production in Korea and 55% of the agricultural production in Jeju province in 2006. Of these crops, satsuma mandarins accounted for 95.6% of the total tonnage and 93.0% of the total acreage used by the citrus industry on Jeju Island, Korea (Jeju Special-Governing Province 2007; MAF 2007)

Satsuma mandarins are a major crop on Jeju Island for several reasons. Satsuma mandarins are more cold-hardy (Swingle et al., 1967) than other varieties in the region that must be cultivated in green houses. In addition, satsuma mandarins are well adapted to environmental conditions on Jeju Island such as a volcanic ash soil and the rainy summer season. The early maturing satsuma mandarins, 'Miyagawa Wase' and 'Okitsu Wase', are cultivated on 80% of the acreage used for citrus production on Jeju Island. However, the quality of these cultivars has recently become relatively low when compared to that of other newer varieties such as 'Shiranuhi' and 'Setoka'. Therefore, mulching has been employed to improve the quality of fruit produced by the satsuma mandarin cultivar (Hyun et al., 1993). Although the mulching system has been found to effectively improve the quality of the fruit, the rate at which high quality is produced is generally not high and the effects of the system differ among regions.

Accordingly, it is necessary to develop a new form of early maturing satsuma mandarin to address these problems. Despite this need, no new early maturing cultivars of satsuma suitable for the replacement of 'Miyagawa Wase' and 'Okistu Wase' on Jeju Island have been developed in the last two decades. Furthermore, although many varieties of very-early maturing

mandarins from Japan, including 'Pungbok Wase' and 'Hinoakebono', were introduced in the early 2000's, most of these strains did not express their own traits in their new environment. Therefore, this study was conducted to develop a new satsuma mandarin that produces high quality fruit and is adaptable to the environment of Jeju Island to replace 'Miyagawa Wase' and 'Okitsu Wase'.

Materials and methods

Cross combinations

Eight satsuma mandarin cultivars were crossed with the pollen from 'Natsudaidai' (*Citrus natsudaidai* Hay) in 1992 at the Citrus Research Station (CRS). The seed parents of the satsuma mandarin (*Citrus unshiu*) were 'Shinikjosaeng', 'Okitsu Wase', 'Miho Wase', 'Tachima Wase', 'Miyagawa Wase', 'Sigeta unshiu', 'Nankan No. 20' and 'Aoshima unshiu'. Anther of 'Natsudaidai' was collected in early May, incubated at 25℃ for 24 - 48 hours, and then stored at -4℃ until use. The crosses were performed when the petals on approximately 50% of the flowers on the tree opened. Pollination was conducted using flowers with petals that had not yet opened. To accomplish this, one petal was removed using tweezers, after which the pollen from 'Natsudaidai' was carefully smeared onto the stigma. The flower was then wrapped with an oilpaper envelope to protect it from open pollination.

Germination of polyembryonic seeds and culture

Fruit produced by the crosses was harvested in December each year, at which time the seeds were collected. The gathered seeds were then stored in tap water for 24 hours to exclude viscous substances from their surface, after which they were dried at room temperature for 24 hours. To separate the embryos from each respective seed, which generally contained polyembryos (Frost and Soost, 1968), the testa was carefully removed with tweezers. Next, the embryos were cultured in Petri dishes that contained filter paper at 25℃ for one month. The young seedlings that germinated from each embryo were then planted in a plastic pot in a green house. Each pot was covered with

vinyl for 2 months to maintain the moisture. After cultivation for 2 years, the seedlings were planted in an open field in March, and the seedling numbers were then scored.

Investigation of growth and fruit characteristics

The characteristics of tree growth and leaves produced by the seedlings were investigated. Specifically, the tree height (cm), leaf width (cm), leaf length (cm), presence of thorns, one-year-old shoot length and internodes were evaluated. In addition, the characteristics of fruit produced by the seedlings in 2001 were investigated. The fruit characteristics that were evaluated included thefruit weight (g), fruit width (cm), fruit length (cm), fruit shape (width/length×100), peel thickness (mm), soluble solids content (oBx), acid content (%), soluble solid content/acid content ratio and rind and juice color. The soluble solid and acid content were evaluated using an analyzer (Horiba NH-2000), while the rind and juice color were examined visually.

Field evaluation

The 'Haryejosaeng' cultivars were also investigated by comparison with 'Miyagawa Wase' (*Citrus unshiu* Marc.) in field evaluation trials conducted at three locations, Namwon up, Hamduk ri and Bomok ri. For the field trials, scions were collected from the seedlings in early March of 2002. The scions were then washed with tap water and subsequently wrapped with vinyl, after which they were stored at 4℃ for one month. In early April, the scions were grafted onto 3 satsuma mandarin trees that were approximately 25 years old at each location. In addition, 'Miyagawa Wase' scions were grafted onto the same trees.

Field evaluation trials were also conducted at two locations on Jeju Island that were used to produce commercial produce between 2005 and 2008, Wimi and Daejung. At each of these locations, scions of 'Haryejosaeng' were grafted onto Satsuma mandarin trees that were approximately 25 years old. The characteristics of the fruit produced by 'Haryejosaeng' and 'Miyagawa Wase' from 2007 to 2008 were then compared.

Mulching experiment

A mulching experiment that utilized polyethylene film (Tybek, DuPont Co.) was also conducted at 'Wimi' in 2008. In these experiments, polyethylene film was used to cover the entire soil surface in early July. The treatment was applied after three days of continually clear weather. The fruit characteristics were then investigated from late August to early November at an interval of approximately 15 days.

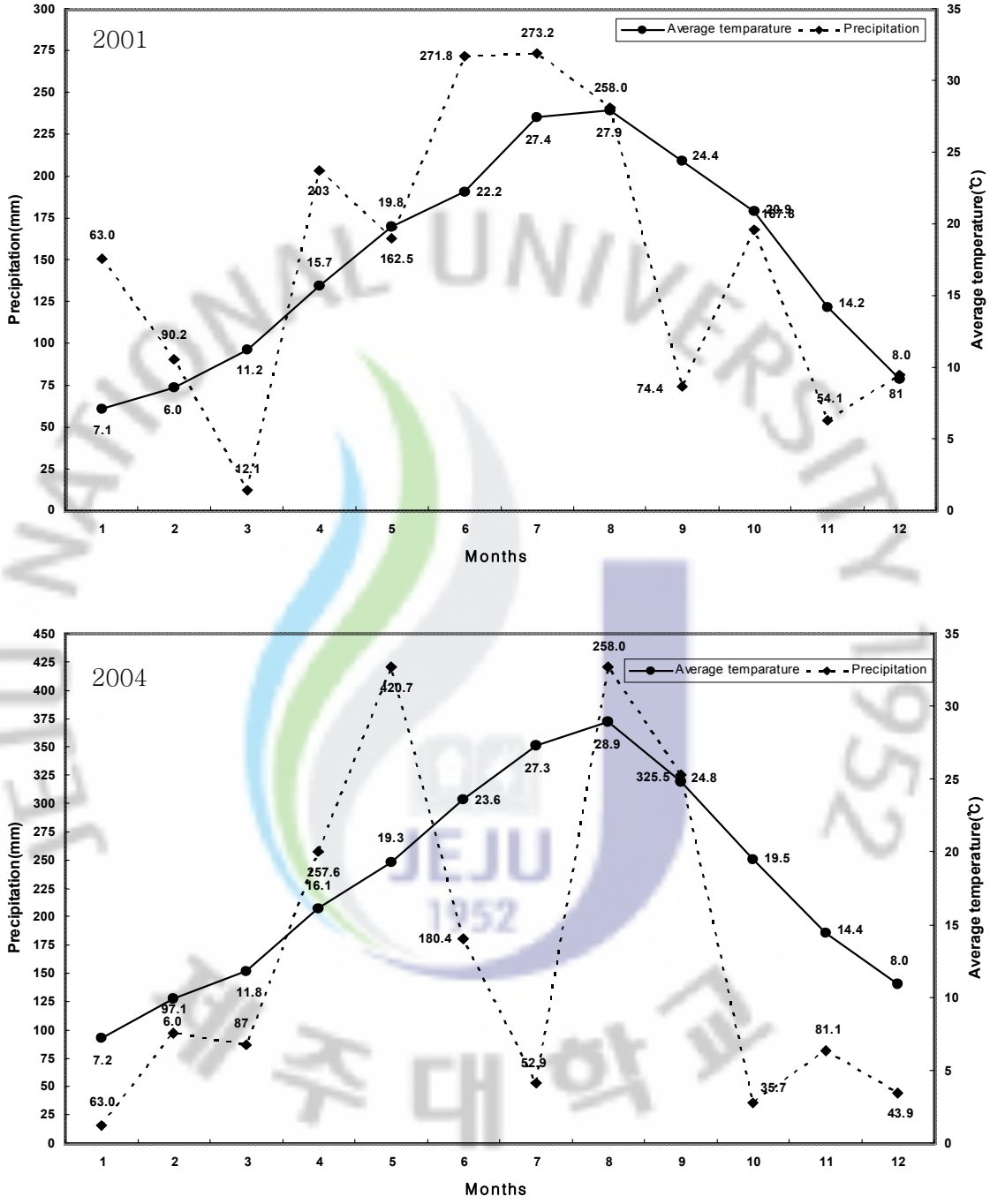


Fig. 15. Precipitation (mm) and average temperature (℃) in Seogwipo

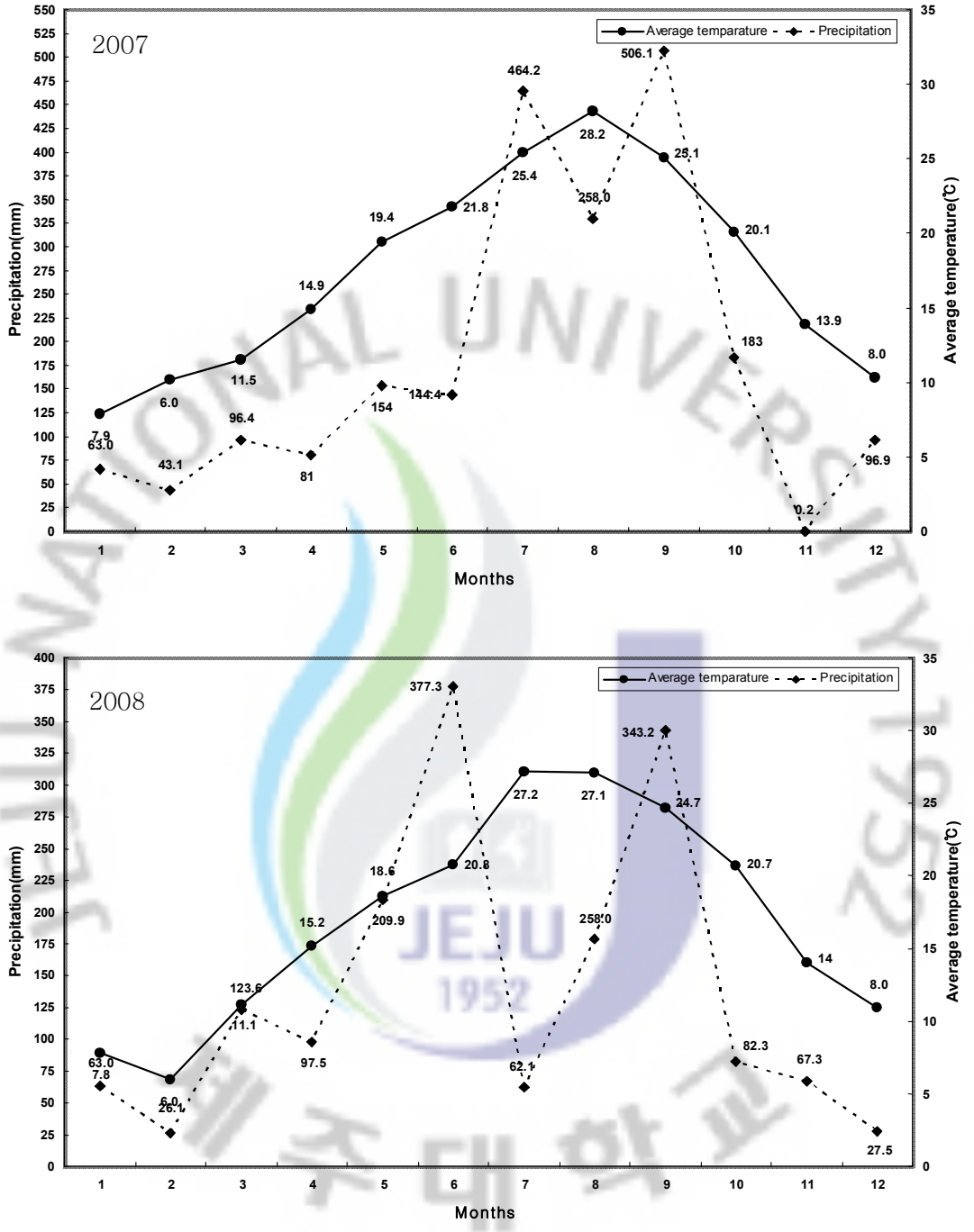


Fig. 15. continued.

Results

Selection of nucellar seedlings with high fruit quality

Two hundred ninety-six nucellar seedlings were obtained from eight satsuma mandarin crosses in 1993 (Table 17). Flowering began in a few seedlings in 2000. In 2001, most of the seedlings produced fruit therefore, the characteristics of the fruit were investigated. The criterion of selection was based on the characteristics of the fruit shape, soluble solid content, acid content and the ratio of soluble solid to acid content. Among the characteristics, soluble solid content was a priority factor for selection.

Two seedlings, 92-Na-T-8 and 92-Na-T-22, were preliminarily selected from the 'Tachima Wase' nucellar progeny in 2001 because they were found to have higher soluble solid contents than the other progeny (Table 18).

Table 17. Cross combinations and seedlings used in this experiment.

Cross	No. of seedlings
Shinkijosaeng × Natsudaidai	51
Okitsu Wase × Natsudaidai	8
Miho Wase × Natsudaidai	36
Tachima Wase × Natsudaidai	25
Miyagawa Wase × Natsudaidai	23
Sigeta unshiu × Natsudaidai	12
Nankan No. 20 × Natsudaidai	65
Aoshima unshiu × Natsudaidai	76
Total	296

Table 18. Characteristics of fruit produced by nucellar seedlings of 'Tachima Wase'.

Seedlings	Weight (g)	Fruit shape	Rind thickness (mm)	Soluble solids (°Bx)	Acid (%)	Soluble solid /Acid
[z]92-Na-T-2	[y]101	130	1.62	11.0	1.08	10.1
92-Na-T-3	91	128	2.23	9.8	1.17	8.3
92-Na-T-7	84	147	1.52	10.3	1.44	7.1
92-Na-T-8	**76**	**127**	**1.36**	**11.4**	**1.26**	**9.0**
92-Na-T-12	102	125	2.20	9.9	1.33	7.4
92-Na-T-14	88	124	1.63	11.2	1.23	9.1
92-Na-T-15	84	119	1.71	10.9	1.19	9.1
92-Na-T-17	122	134	2.30	10.4	1.52	6.8
92-Na-T-20	70	135	1.54	10.4	1.18	8.8
92-Na-T-22	**98**	**137**	**1.51**	**11.6**	**1.15**	**10.1**
92-Na-T-25	84	128	1.96	10.5	1.15	9.1

[z]92: year of cross; Na: nucellar; T: Tachima Wase; numeral: seedling number

[y]Data were obtained using 10 fruit samples collected on 2 Nov. 200

The 'Jegam ga No. 2', first selection name of 92-Na-T-22, was also compared to 'Miyagawa Wase' (*Citrus unshiu* Marc.) in a field evaluation trial (Table 19, 20) from 2003 to 2004 in three locations on Jeju Island. After 2 years, 'Jegam ga No. 2' was submitted to the judging committee of new variety (JCNV) of the rural development administration (RDA) for evaluation. The JCNV judged 'Jegam ga No. 2' as a new citrus selection of the RDA based on its superior traits, high soluble solid and low acid content, when compared to the 'Miyagawa Wase' cultivar, after which 'Jegam ga No. 2' was named 'Haryejosaeng'.

Table 19. Tree growth characteristics of 'Jegam ga No. 2' and 'Miyagawa Wase' as determined by a field evaluation at three locations on Jeju Island.

Cultivar	Location	Germinating	Flowering	Maturation period
Jegam ga No. 2	Namwon	[z]9 Apr.	18 May	Ear-Nov.
	Bomok	2 Apr.	7 May	Ear-Nov.
	Humduk	8 Apr.	13 May	Ear-Nov.
Miyagawa Wase	Namwon	9 Apr.	17 May	Ear-Nov.
	Bomok	2 Apr.	7 May	Ear-Nov.
	Humduk	8 Apr.	13 May	Ear-Nov.

[z]Data shown are the means for 2 years (2003 to 2004)

Table 20. Fruit characteristics of 'Jegam ga No. 2' and 'Miyagawa Wase' as determined by a field evaluation at three locations on Jeju Island.

Cultivar	Location	Weight (g)	Soluble solids (°Bx)	Acid (%)	Soluble solids /Acid
Jegam ga No. 2	Namwon	[z]123	10.0	1.37	7.3
	Bomok	89	10.0	1.03	9.7
	Humduk	91	9.2	1.09	8.4
Miyagawa Wase	Namwon	92	9.6	1.34	7.2
	Bomok	89	9.6	1.14	8.4
	Humduk	83	9.2	1.10	8.4

[z]Sampling date: 4 Nov. 2004

Description of 'Haryejosaeng'

Origin

A new early maturing satsuma mandarin designated 'Haryejosaeng' (*Citrus unshiu* Marc.) was developed as a nucellar seedling (Fig. 16) produced by a cross of 'Tachima Wase' (*Citrus unshiu* Marc.) with *C. natsudaidai* Hayata at the Citrus Research Station (CRS) on Jeju Island in 1992. This nucellar seedling was initially selected in 2001, at which time it was named 'Jegam ga No. 2'. Field evaluation trials of this cultivar were then conducted at three locations on Jeju Island from 2003 to 2004, after which it was finally selected. The name 'Haryejosaeng' was assigned based on the location of the Citrus Research Station.

Characteristics of growth

The 'Haryejosaeng' tree shows vigorous and somewhat upright growth. In addition, the tree has strong thorns during its younger period that disappear as it ages. The tree germinates in early April and flowers in mid-May at the CRS. Fruit coloration begins in mid-October and is completed by mid-November, when the fully matured fruit can be harvested. Similar to the early-maturing satsuma mandarin 'Miyagawa Wase', 'Haryejosaeng' shows moderate alternate bearing (Table 21).

Fig 16. Seedling of 'Haryejosaeng'

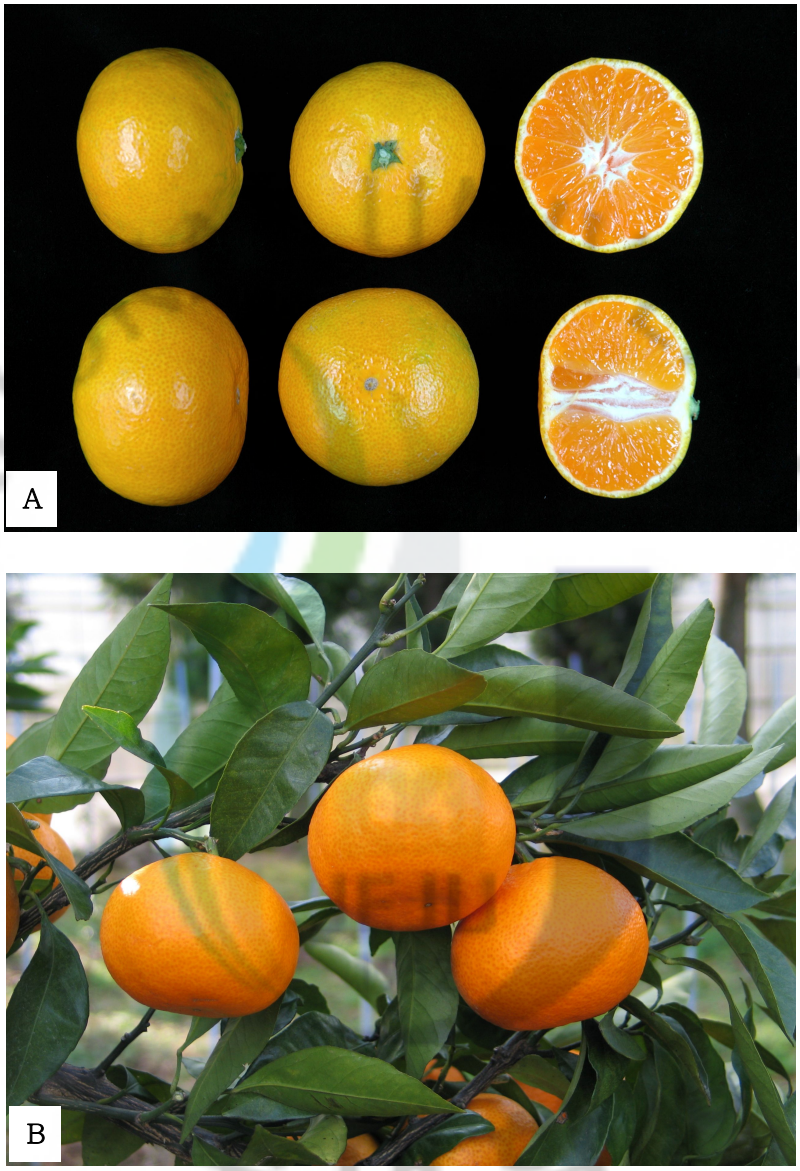


Figure 17. Fruit morphology (A) and fruiting (B) of a new early maturing satsuma mandarin *Citrus unshiu* 'Haryejosaeng'

Table 21. Tree growth characteristics of 'Haryejosaeng' and 'Miyagawa Wase' grown at the Citrus Research Station on Jeju Island.

Cultivar	Tree vigor	[z]Tree habit	Germi-nating	Flower-ing	Maturation period	Alternate bearing
Harye josaeng	Strong	Medium	Early Apr.	Mid.-Mar.	Early-Nov.	Medium
Miyagawa Wase	Medium	Spreading	Early Apr.	Mid.-Mar.	Mid.-Nov.	Medium

[z] Identified by upright, medium and spreading

Characteristics of fruit

At maturity, the fruit has a rich and sweet taste, soluble solids of 10 to 11 °Bx and 1 to 1.1% acidity. The fruit shows a stable seedlessness, is approximately 80 - 90g, is compressed-oblate globose in shape (128 in fruit shape index), and has flesh that is orange in color. The rind of the fruit is approximately 2mm in thickness, easy to peel and light-orange in color (Table 22, Fig. 17).

As described in Table 24, the fruit produced by 'Haryejosaeng' was larger, sweeter and less acidic than the fruit produced by 'Miyagawa Wase', the leading satsuma mandarin produced on Jeju Island. In addition, other characteristics such as skin thickness and fruit shape were similar between the two cultivars.

Culture

The strong vigor of 'Haryejosaeng' when it is young may cause poor fruiting; therefore, pruning of a strong shoot (watershoot) is needed. When the tree reaches the time of fruiting, it is necessary to spread the tree shape using a prop. The disease and pest control and nutrition management are generally similar to that of early maturing satsuma mandarins. The long term storage of fruit is not recommended because its acid content decreases rapidly after harvest.

Table 22. Fruit characteristics of 'Haryejosaeng' and 'Miyagawa Wase' grown at the Citrus Research Station on Jeju Island.

Cultivar	Weight (g)	Fruit[z] shape	Skin thickness (mm)	Soluble solids (°Bx)	Acid (%)	Soluble solids/acid
Harye josaeng	[y]92	128	1.8	10.6	1.15	9.2
Miyagawa Wase	80	130	1.7	9.8	1.24	7.9

[z](fruit width /fruit length) x 100

[y]Mean for 5 years from 2001 to 2004 at the CRS

Field evaluation

The field evaluation of 'Haryejosaeng' was conducted at commercial citrus orchards located in Wimi and Daejung (Fig. 18, Table 23).

In 2007, there was no difference in the soluble solid content of fruit produced by 'Haryejosaeng' and 'Miyagawa Wase' grown at Wimi, but 'Haryejosaeng' showed a soluble solid content that was 1 °Bx higher than that of 'Miyagawa Wase' when the cultivars grown at Daejung were compared. In addition, the acid content of 'Haryejosaeng' declined to less than 1% by Mid- to late-November. The acid content of both 'Haryejosaeng' and 'Miyagawa Wase' were less than 1% at Daejung.

In 2008, 'Haryejosaeng' showed a soluble solid content that was 0.5-1 °Bx higher than that of 'Miyagawa Wase' produced at Wimi and Daejung. Furthermore, the acid content of 'Haryejosaeng' was higher than that of 'Miyagawa Wase' when grown at Wimi, but lower at Daejung. Despite this difference, the acidity level of the fruit produced in both regions was less than 1%.

Figure 18. Field evaluation trials in Daejung (A) and Wimi (B) for commercial production of citrus orchard.

Table 23. Fruit characteristics of 'Haryejosaeng' and 'Miyagawa Wase' obtained during commercial production trials at two locations on Jeju Island.

Location	Sample date	cultivar	Fruit wt (g)	Soluble solids (°Bx)	Acid (%)	Soluble solids /acid
Wimi	22 Nov. 2007	Haryejosaeng	[z]88.4.0±9.88	10.7±0.32	0.87±0.07*	12.3
		Miyagawa Wase	103.1±7.80	10.7±0.22	1.04±0.18	10.5
	10 Nov. 2008	Haryejosaeng	90.3±13.31	10.3±0.51***	0.94±0.09	11.0
		Miyagawa Wase	96.1±13.59	9.9±0.44	0.87±0.09***	11.4
Daejung	22 Nov. 2007	Haryejosaeng	106.7±18.21	10.9±0.64	0.75±0.06*	14.5
		Miyagawa Wase	99.5±9.05	9.9±0.50	0.91±0.10	11.0
	10 Nov. 2008	Haryejosaeng	88.5±9.24	10.7±0.64***	0.69±0.09***	15.5
		Miyagawa Wase	86.8±11.49	9.4±0.53	0.83±0.15	11.3

[z]Data shown are the means ± Standard error

*, *** Significantly different between columns within the same location at $p \leq 0.05$, 0.001 as determined by a student's t test

Effect of mulching on fruit quality

The effect of mulching on 'Haryejosaeng' was evaluated at Wimi on Jeju Island in 2008. The soluble solid and acid content of the fruit produced by 'Haryejosaeng' cultivars were 13.8 °Bx and 1.00% during early November after 4 months of mulching treatment (Table 24). These soluble solid and acid contents were 1.2 °Bx higher and 0.1% lower than those of 'Miyagawa Wase'. Furthermore, the acid content was lower when the fruit had soluble solid levels that ranged from 11.0 - 11.9 °Bx, but there was no difference observed when the soluble solids were 12.0 °Bx or higher (Fig 19).

The change in soluble solid content was examined from late August to early November. The soluble solid content of 'Haryejosaeng' and 'Miyagawa Wase' increased at a similar rate until late September. However, from early October, the soluble solid content of 'Haryejosaeng' increased more rapidly than that of 'Miyagawa Wase'. Furthermore, the acid content of the 'Haryejosaeng' was higher than that of 'Miyagawa Wase' during the early stages, but lower in early November (Fig 20, Fig 21). The rate of the fruit that had a soluble solid content greater than 12 °Bx was 98% while that of 'Miyagawa Wase' was 84% (Fig 22).

Table 24. Effect of polyethylene film mulching on fruit quality characteristics of 'Haryejosaeng' and 'Miyagawa Wase' produced during a commercial production trial at Wimi in 2008.

Cultivar	Weight (g)	Soluble solids (°Bx)	Acid (%)	Soluble solids/acid
Haryejosaeng	[z]72.8±11.39	13.8±0.81***	1.00±0.14***	13.8
Miyagawa Wase	72.3±12.54	12.6±0.65	1.10±0.12	11.5

[z]Data shown are the means ± Standard error

*** Significantly different within columns at $p \leq 0.001$ as determined by a student's t test

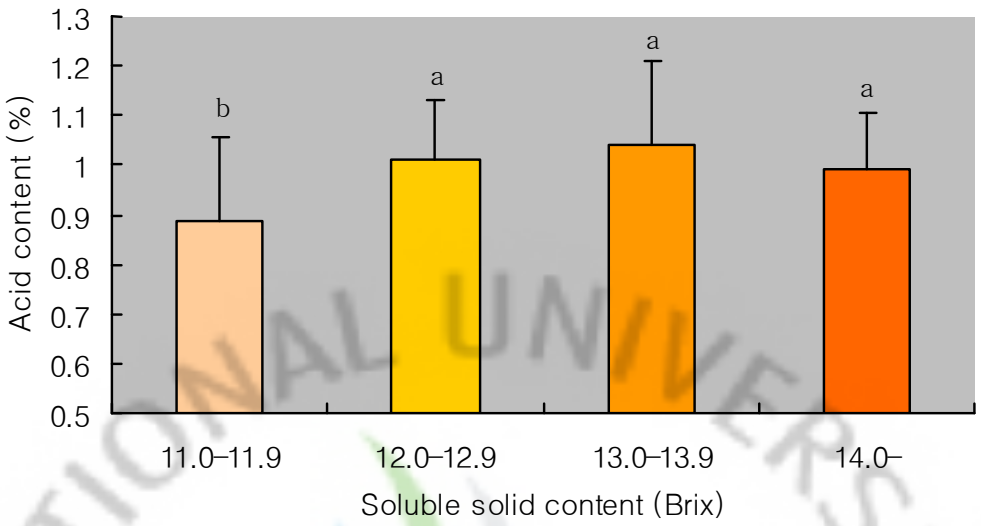


Fig. 19. Relationship between acidity and soluble solid contents of fruit produced by 'Haryejosaeng' that had been subjected to mulching. The different letters above the columns indicate significant differences in acidity at $p \leq 0.05$ as determined by Duncan's multiple range test.

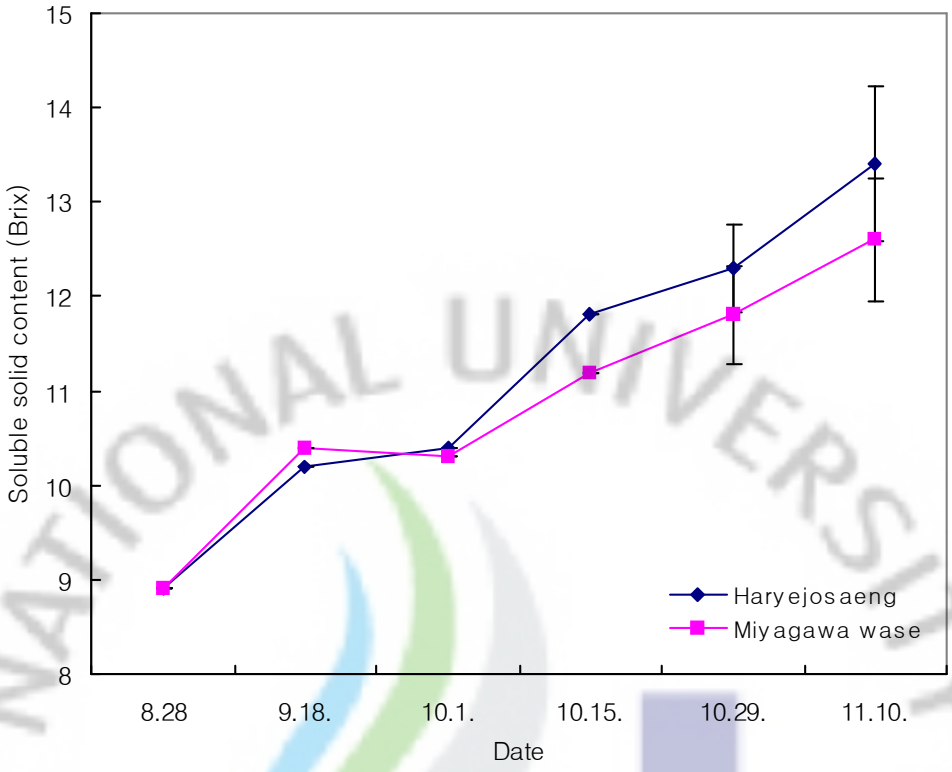


Fig. 20. Seasonal changes in the soluble solid content of 'Haryejosaeng' and 'Miyagawa Wase' that had been subjected to mulching.

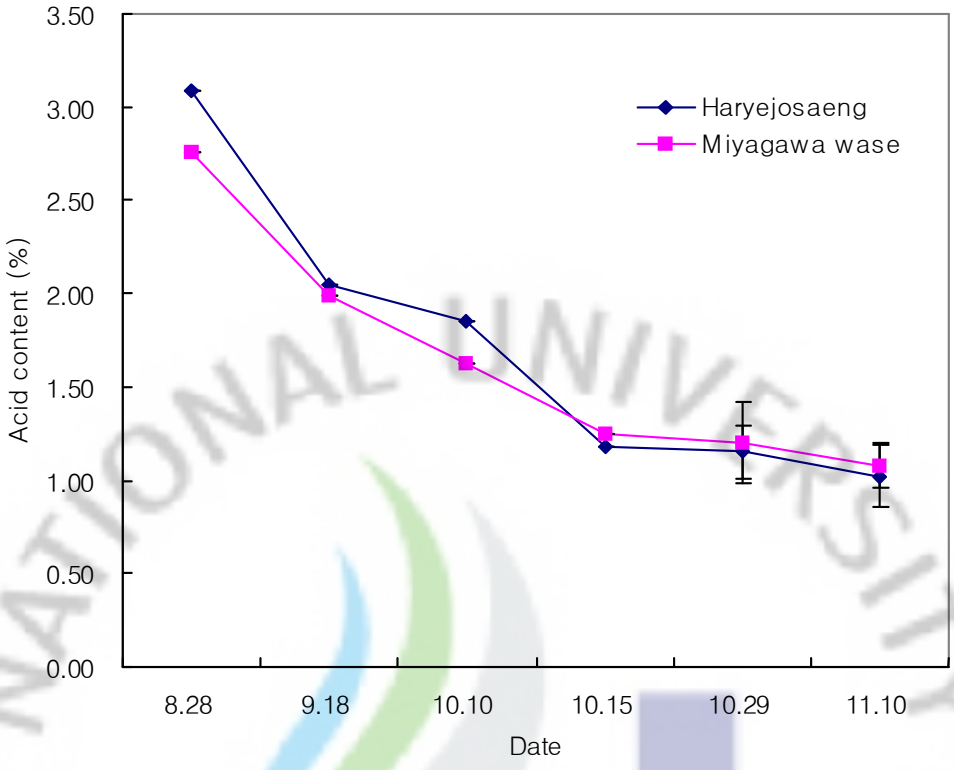


Fig. 21. Seasonal changes in the acid content of 'Haryejosaeng' and 'Miyagawa Wase' that had been subjected to mulching.

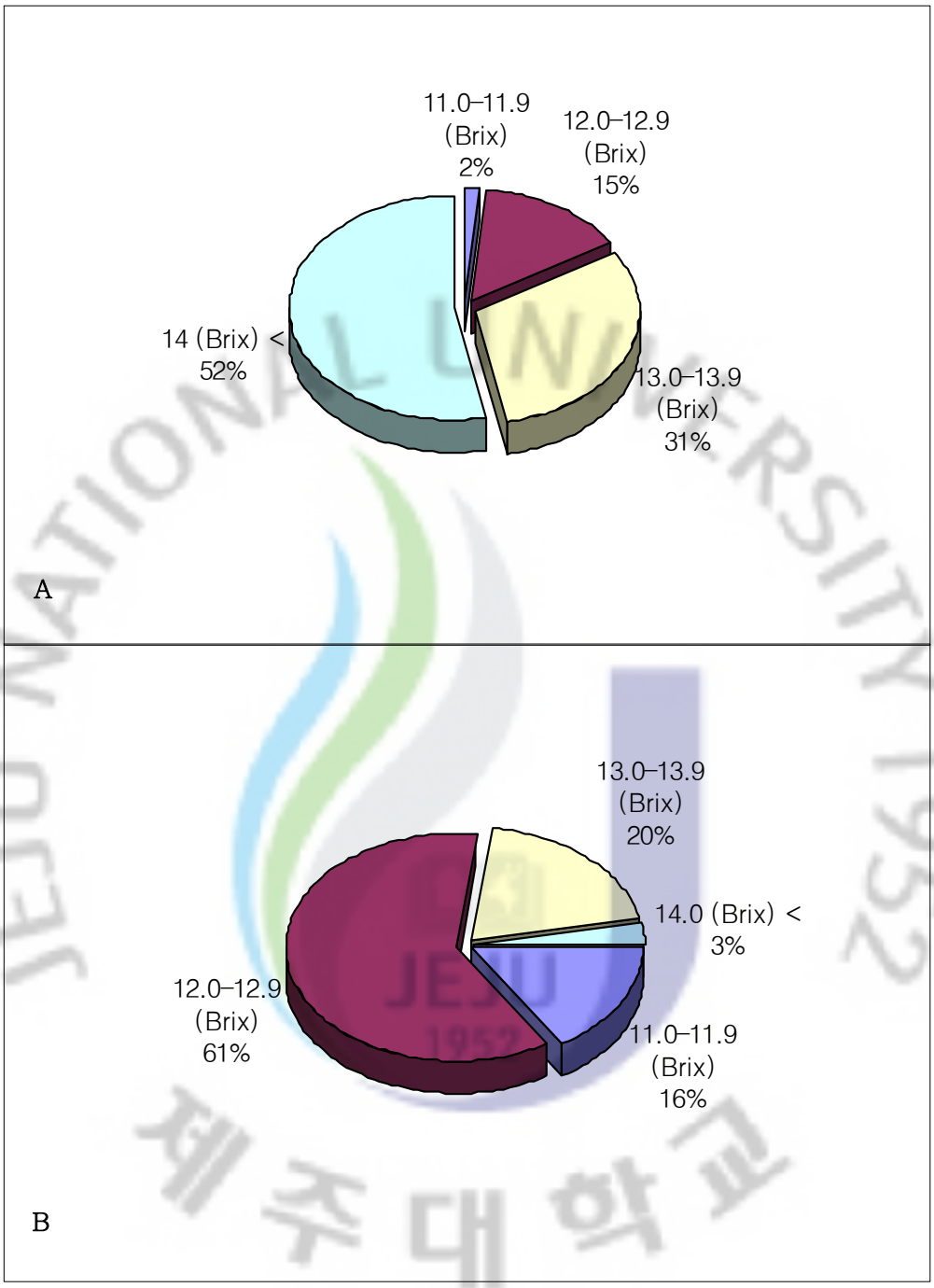


Fig. 22. Distribution of the fruit based on the soluble solid content of 'Haryejosaeng' (A) and 'Miyagawa Wase' (B) that had been subjected to mulching.

Discussion

With the exception of acid content, the characteristics of the 'Haryejosaeng' cultivar were almost identical to those of 'Miyagawa Wase'. However, the acid content of 'Haryejosaeng' was lower than that of 'Miyagawa Wase'. Ten years ago, low acid content was considered to be disadvantageous in terms of the storage of fruit. However, the consumer's definition of high quality satsuma mandarin fruit has shifted, and they now prefer fruit with soluble solid contents greater than 10 oBx and acidity lower than 1%. As a result, citrus fruit farmers on Jeju Island have made an effort to produce fruit that meet these criteria.

Mulching is considered to be the most powerful method of producing high quality fruit from satsuma mandarins. Some reports have indicated that mulching with polyethylene film results in an increased soluble solid content in mandarins (Roh et al., 2002; Baek et al., 1992). Therefore, the number of farmers adopting this system has increased since the early 2000s. The results of the present study indicated that, although mulching facilitated the development of high quality fruit, the production rate of trees that were subjected to mulching was generally not high and the effect of this system differed among regions.

In addition, there are other obstacles to the production of good fruit by early-maturing satsuma mandarin such as 'Miyagawa Wase' and 'Okitsu Wase' due to their genetic backgrounds. The fruit produced by these plants generally has a high soluble solids content concomitant with a high acid content in mid-November, which results in the harvest being delayed to mid-December. However, this can lead to chilling injury to fruit produced on Jeju Island. Conversely, 'Haryejosaeng' can produce fruit with qualities that

are considered good by the consumer in mid- or late November.

The 'Haryejosaeng' trees that were subjected to mulching showed higher soluble solid contents and lower acid contents than 'Miyagawa Wase' that were subjected to mulching ($p < 0.001$) (Table 24). Generally, early maturing satsuma mandarin cultivars such as 'Miyagawa Wase' and 'Okitsu Wase' require water irrigation when they are subjected to mulching to lower the acid content to below 1%. However, this results in increased cost and has prevented increased use of the mulching technique. Conversely, the acid content of the 'Haryejosaeng' reached 1% in early November without irrigation. Moreover, the soluble solid content at that time was greater than 13 oBx, which would enable an early harvest. Therefore, it is expected that a mulching system that is as labor intensive or expensive as currently used mulching cultivation systems can be used to produce high quality fruit through 'Haryejosaeng'.

In conclusion, 'Haryejosaeng' is recommended as an alternative cultivar to replace 'Miyagawa Wase' and 'Okitsu Wase'. This cultivar is expected to provide increased profits to citrus farmers on Jeju Island, Korea.

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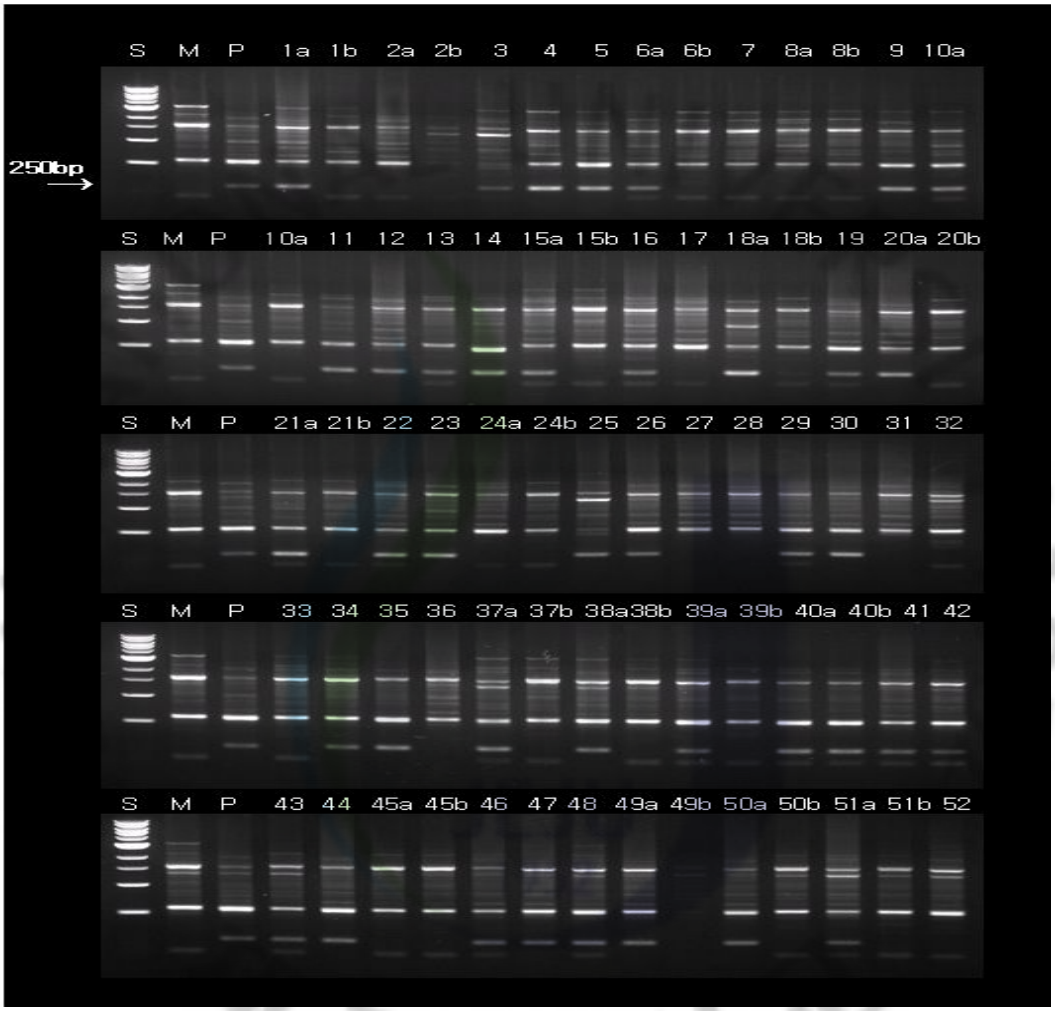
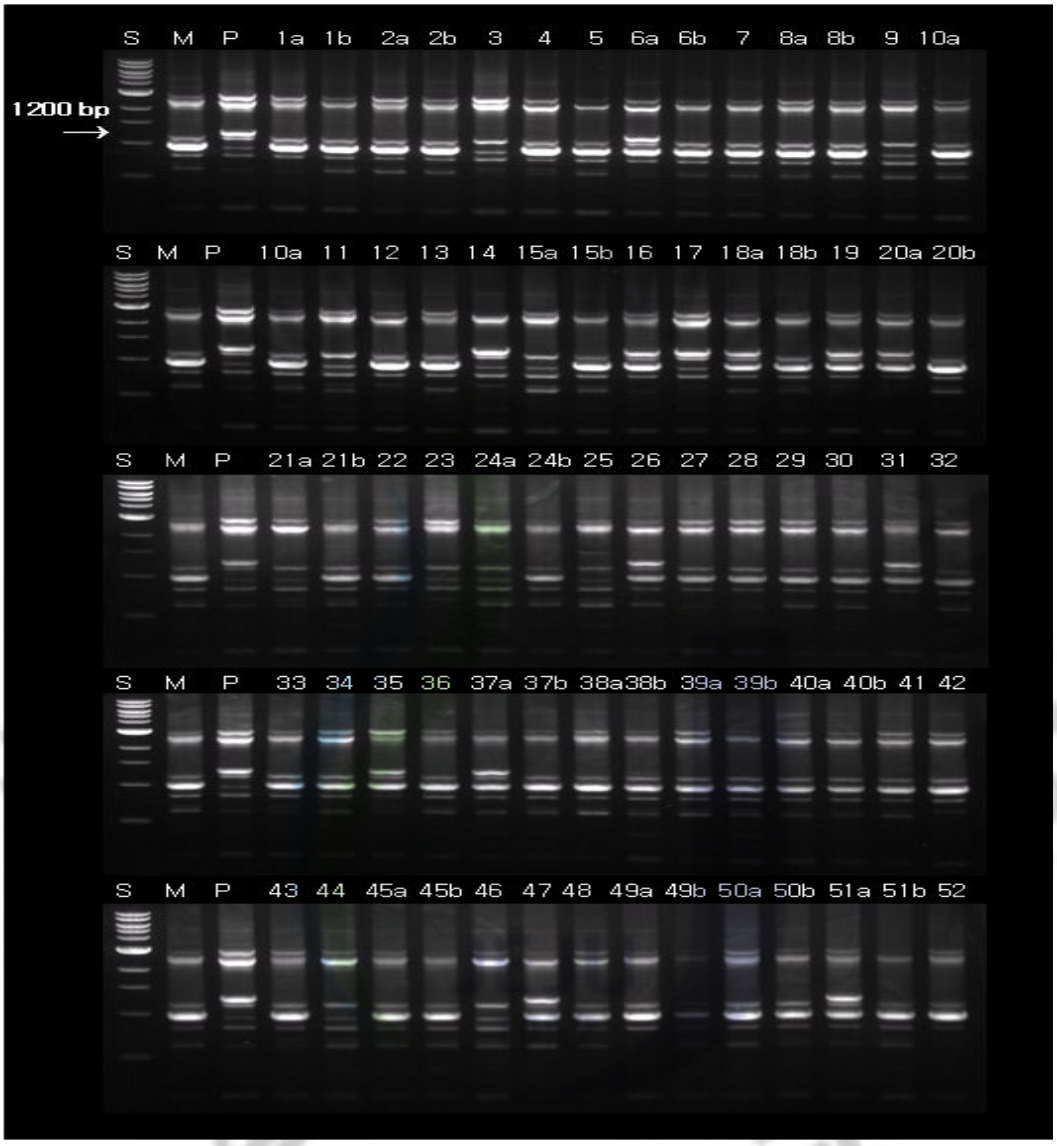
APPENDIX

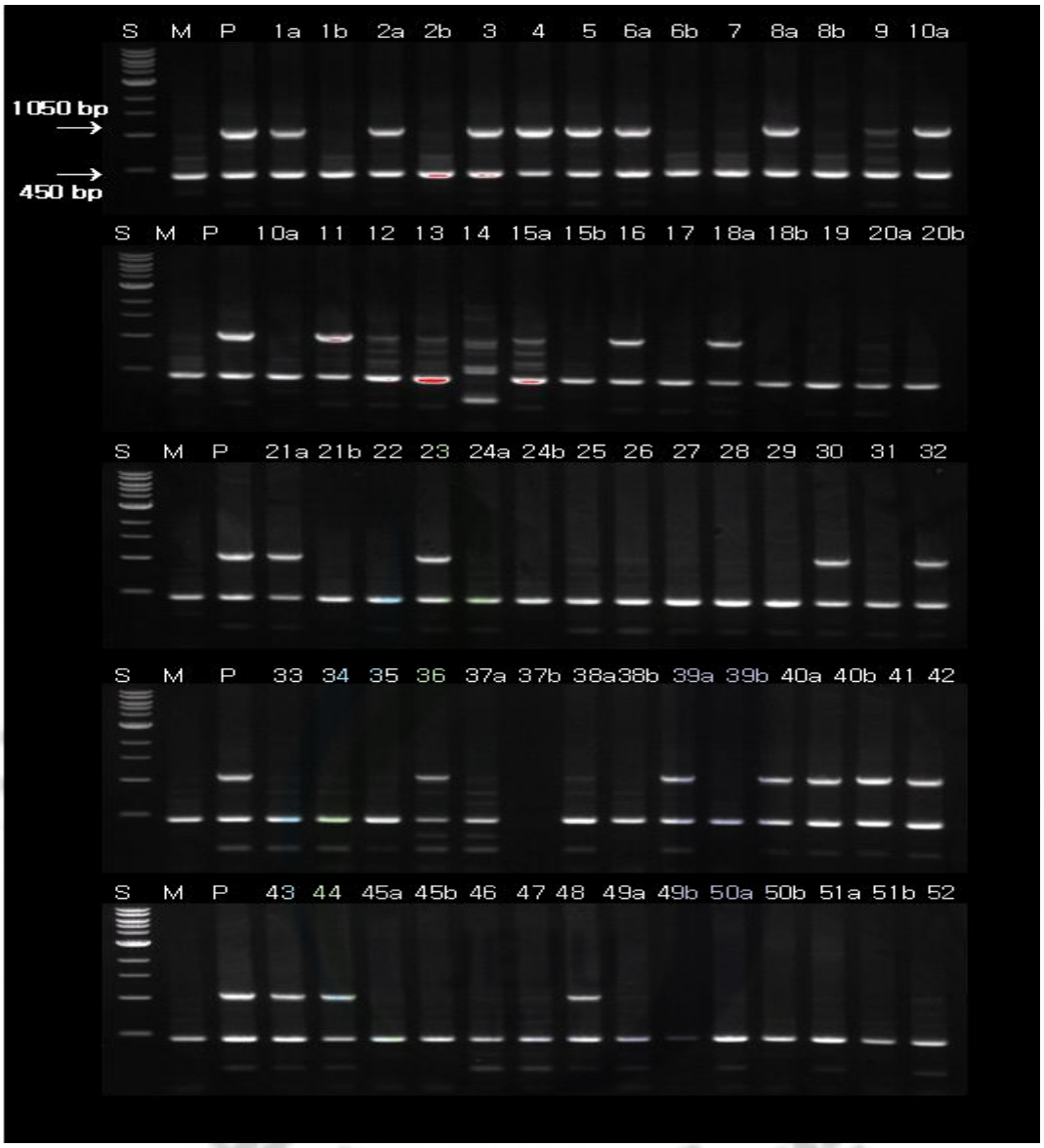
1. DNA amplification profiles by RAPD and SRAP primers



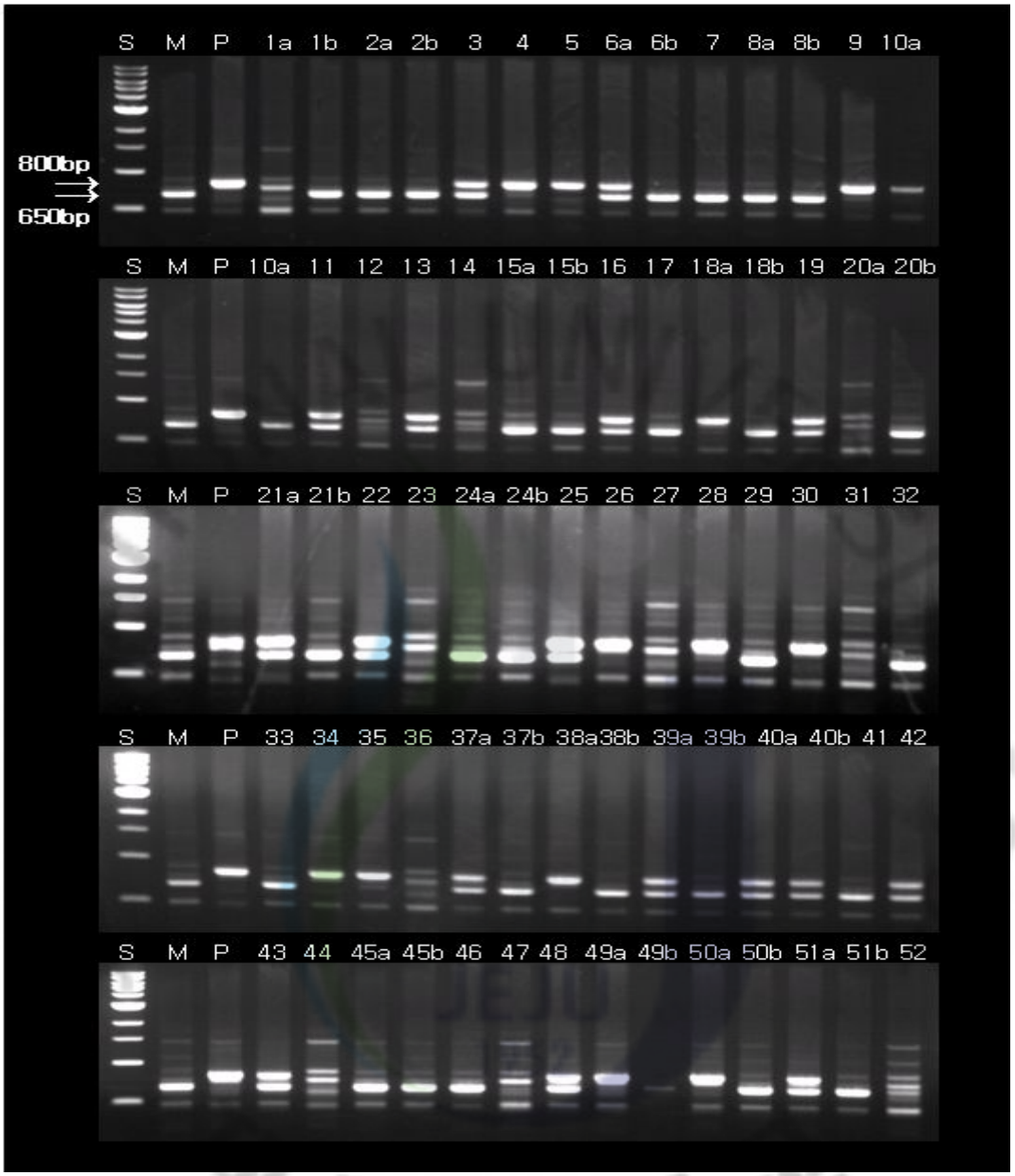
Appendix 1-1. DNA amplification profiles obtained using the UBC27 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'



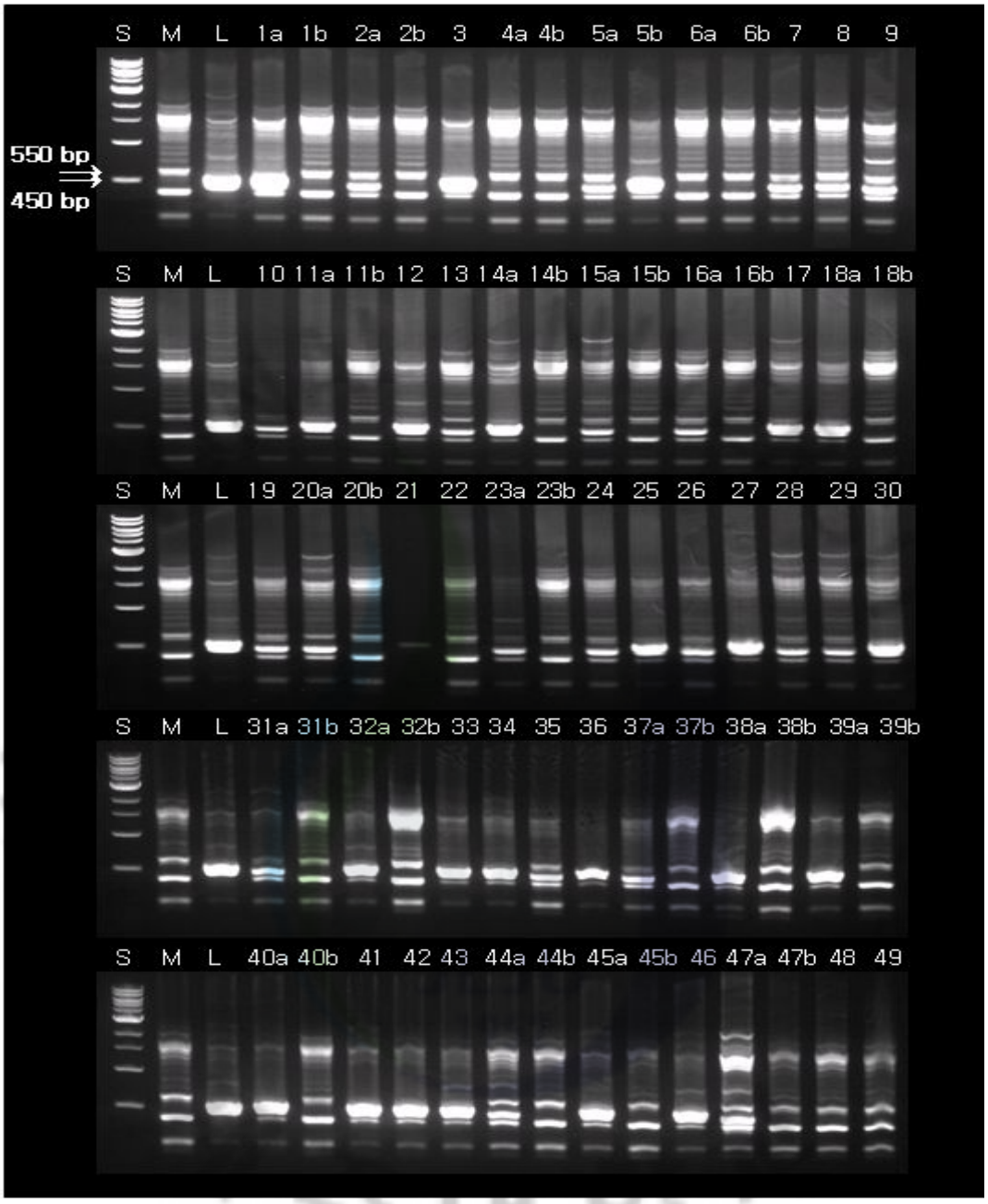
Appendix 1-2. DNA amplification profiles obtained using the UBC229 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'



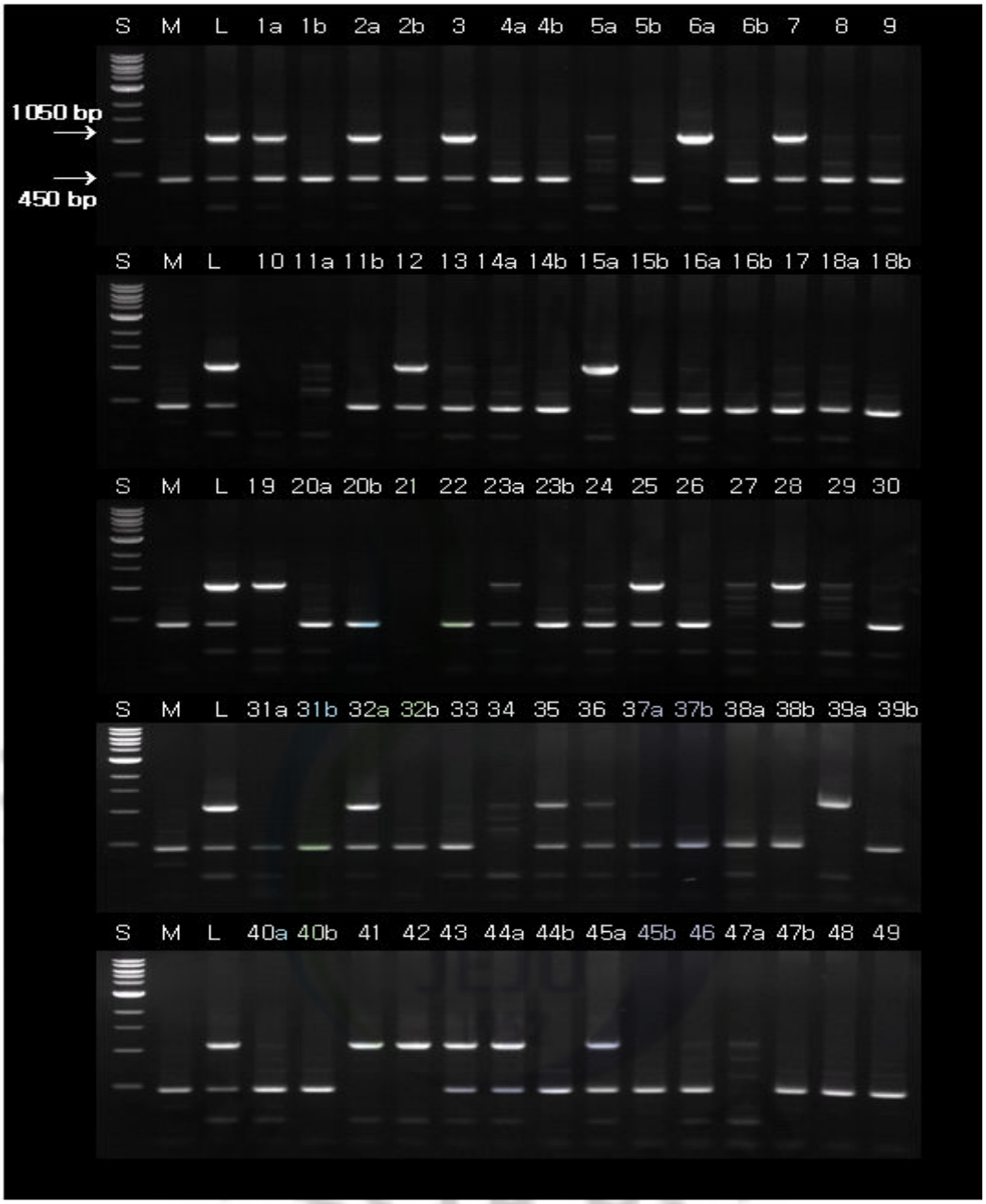
Appendix 1-3. DNA amplification profiles obtained using the F11/R29 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'



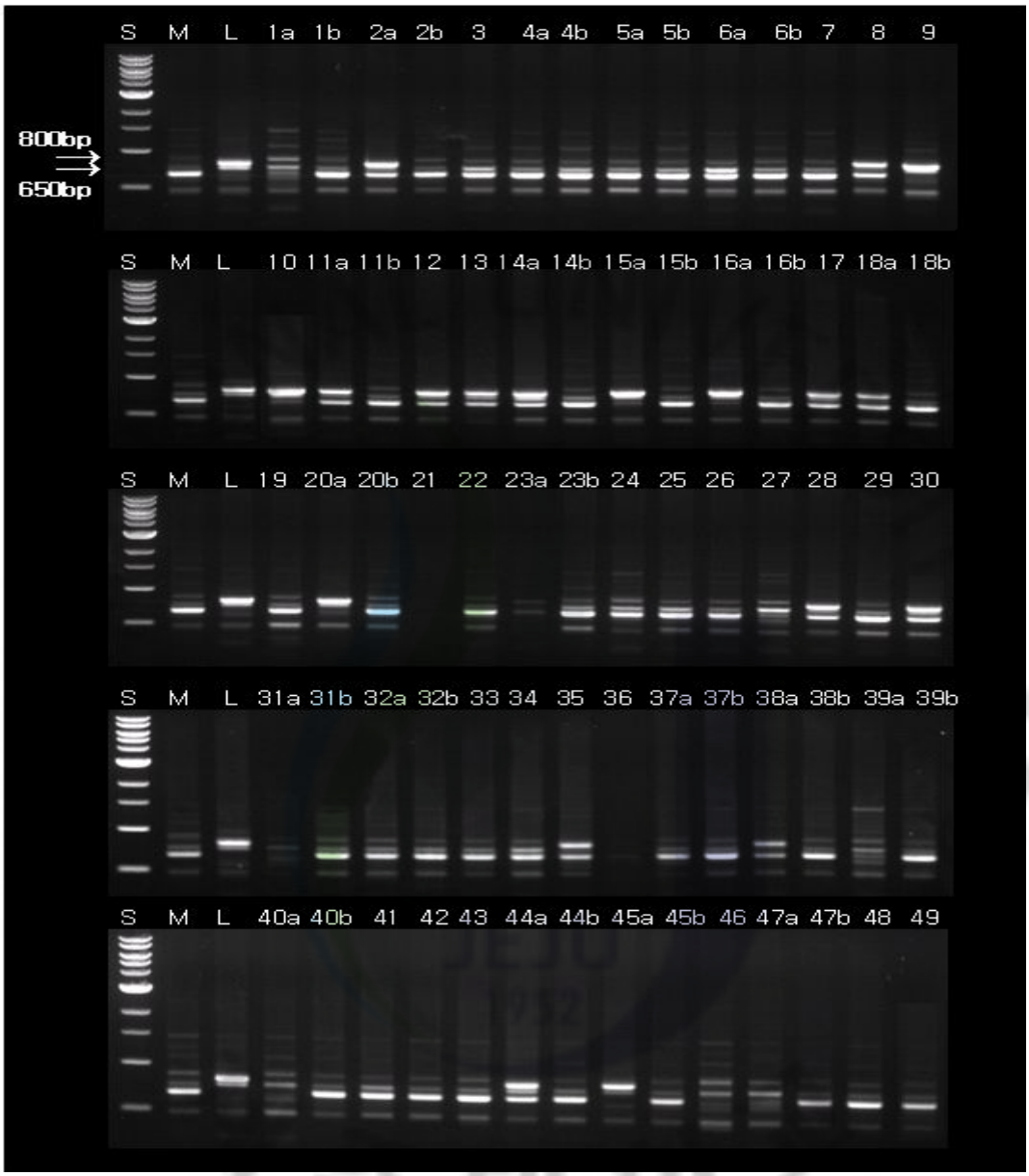
Appendix 1-4. DNA amplification profiles obtained using the F4/R14 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Ponkan' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; P: 'Ponkan'



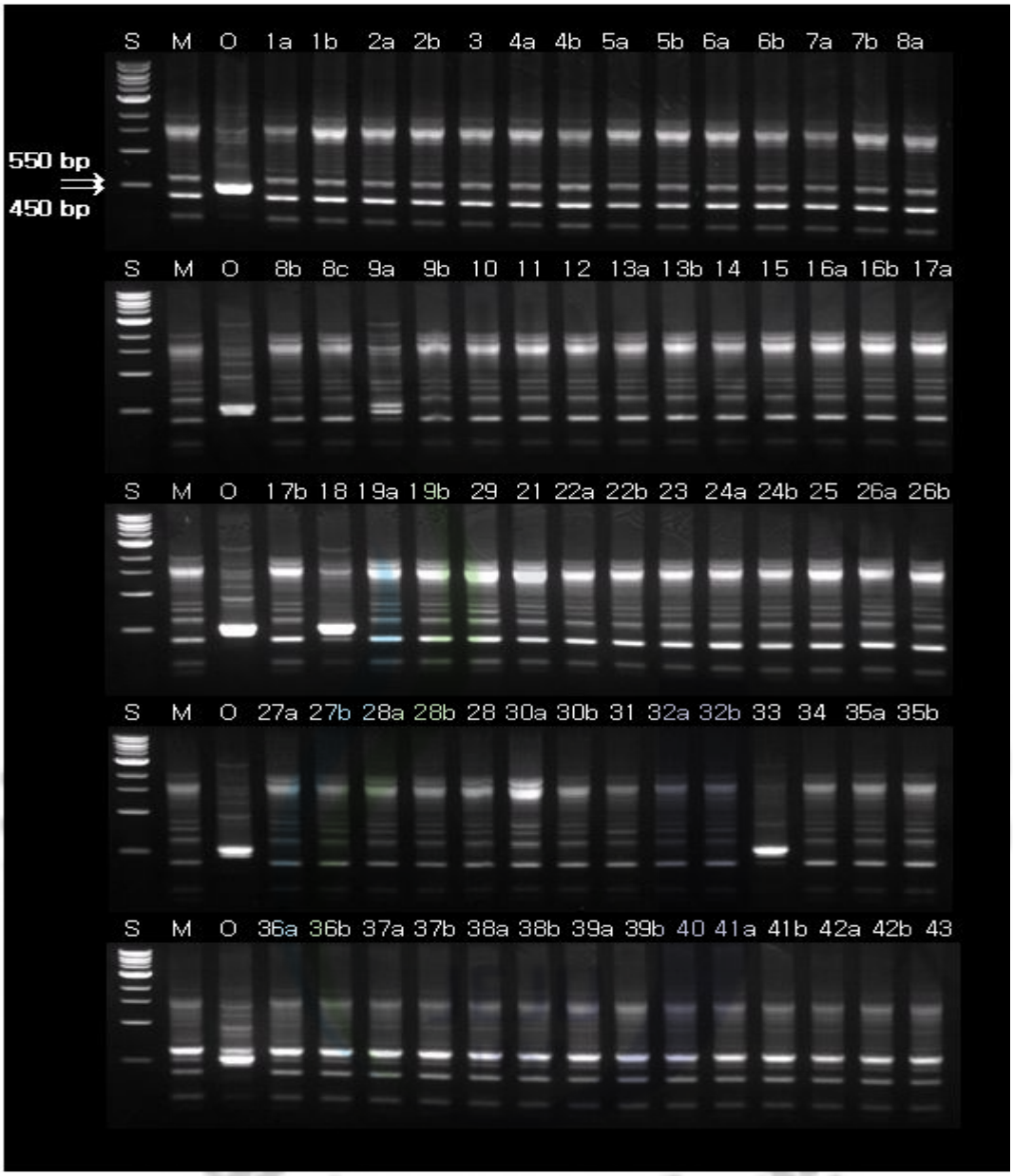
Appendix 1-5. DNA amplification profiles obtained using the OPO14 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Lee' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; L: 'Lee'



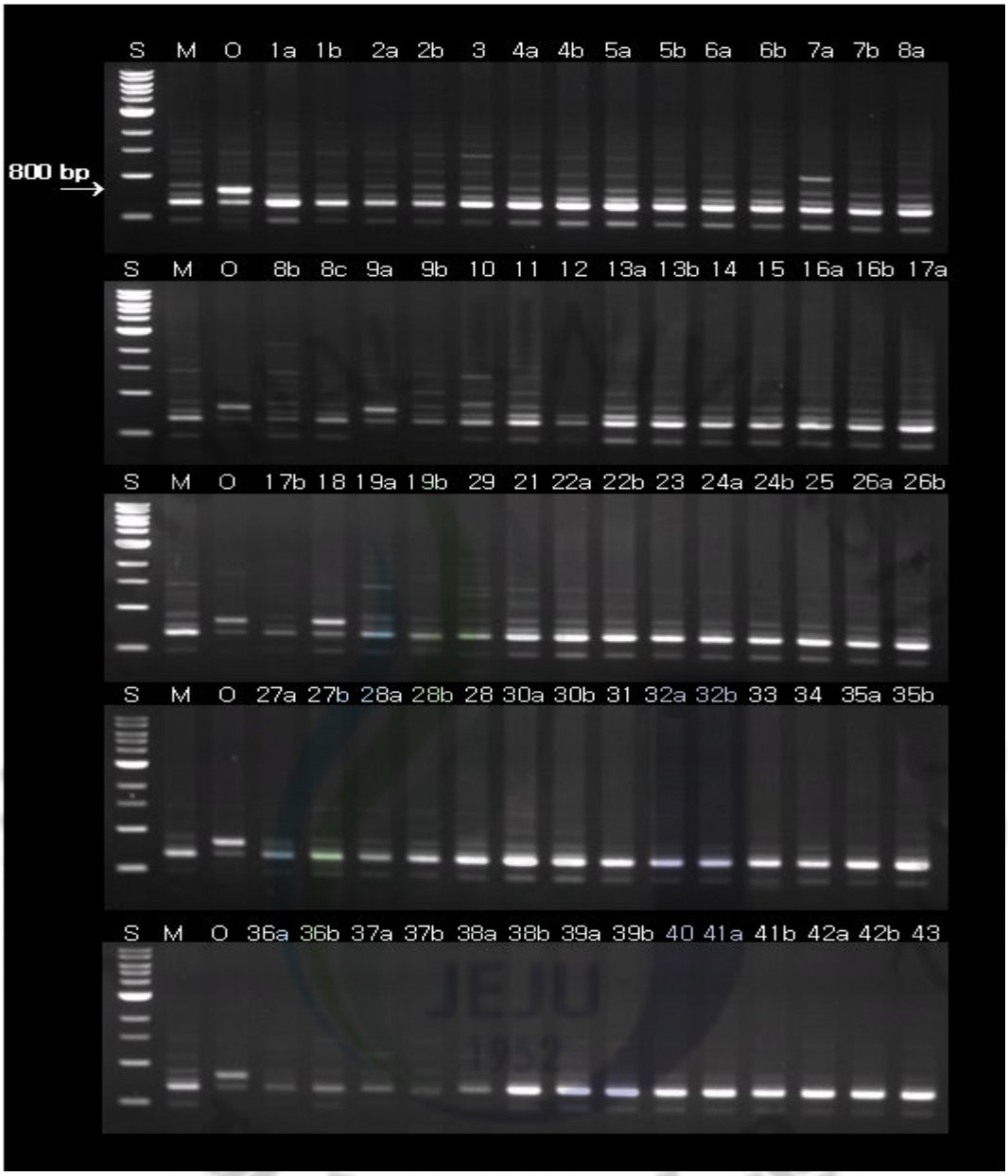
Appendix 1-6. DNA amplification profiles obtained using the F11/R29 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Lee' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; L: 'Lee'



Appendix 1-7. DNA amplification profiles obtained using the F4/R14 primer to analyze samples of seedlings produced by the 'Morita unshiu' × 'Lee' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Morita unshiu'; L: 'Lee'



Appendix 1-8. DNA amplification profiles obtained using the OPO14 primer to analyze samples of seedlings produced by the 'Miyagawa Wase' × 'Orlando' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Miyagawa Wase'; O: 'Orlando'



Appendix 1-9. DNA amplification profiles obtained using the F4/R14 primer to analyze samples of seedlings produced by the 'Miyagawa Wase' × 'Orlando' cross. The figure at the top of each lane is the seed number. The "a" represents the first seedling and "b" represents the second seedlings produced by one seed, the third and fourth seedlings were combined with b. S: molecular size markers (1kb DNA Ladder); M: 'Miyagawa Wase'; O: 'Orlando'

2. Table of distribution of zygotic seedlings in the crosses.

Appendix 2-1. Zygotic or nucellar origin of 'Morita unshiu' × 'Ponkan' cross as determined RAPD and SRAP analysis.

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers						SRAP markers				
			OPO14				UBC27	UBC299	F7/R14		F11/R29	F4/R14	
			2700bp	750bp	550bp	450bp	250bp	1200bp	500bp	1000bp	1050bp	800bp	650bp
1	2	z	[z]o	o	o	–	o	–	o	*	o	–	*
2	2	z	–	o	o	–	–	–	o		o	–	
3	1	z	–	o	o	–	o	–	–	*	o	o	
4	2	z	–	–	o	o	o	–	o	*	o	o	*
5	1	z	–	o	–	–	o	–	o		o	o	*
6	2	z	–	o	o	–	o	o	o		o	o	
7	1	n	–	–	–	–	–	–	–		–	–	
8	3	z	o	o	–	–	–	–	–		o	–	
9	1	z	–	–	o	o	o	–	o	*	–	o	*
10	2	z	–	o	–	–	o	–	o		o	o	*
11	1	z	o	o	–	–	o	–	–		o	o	*
12	1	z	–	–	–	o	o	–	o	*	–	–	*
13	1	z	o	–	o	o	o	–	–		–	o	
14	1	z	–	–	o	–	o	o	–	*	–	–	*
15	2	z	–	–	o	o	o	–	o		–	–	
16	2	z	–	o	o	–	o	o	o		o	o	
17	1	z	–	o	–	–	–	o	o		–	–	*
18	2	z	o	o	o	–	o	o	–		o	o	
19	1	z	–	–	–	o	o	o	o		–	o	
20	3	z	–	o	o	–	o	o	o	*	–	–	*

[z]o: detected by dominant marker(pollen specific band); –: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-1. continued

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers						SRAP markers				
			OPO14				UBC27	UBC29	F7/R14		F11/R29	F4/R14	
			2700bp	750bp	550bp	450bp	250bp	1200bp	500bp	1000bp	1050bp	800bp	650bp
21	2	z	[z]o	–	o	o	o	–	–		o	o	
22	2	z	–	o	–	–	o	–	o	*	–	o	
23	1	z	o	o	o	–	o	–	–	*	o	–	*
24	3	z	–	–	–	o	–	–	o		–	–	
25	1	z	o	–	–	o	o	–	o		–	o	
26	2	z	o	o	–	–	o	o	–		–	o	*
27	1	z	o	o	o	–	–	–	o		–	–	*
28	2	z	–	–	o	o	–	–	o		–	o	*
29	1	z	–	–	o	o	o	–	–		–	–	
30	1	z	o	o	o	–	o	–	o		o	o	*
31	1	z	–	o	o	–	–	o	o		–	–	
32	1	z	o	o	–	–	–	–	o		o	–	
33	1	z	–	–	–	o	–	–	o	*	–	–	
34	1	z	–	–	–	o	o	–	–		–	o	*
35	1	z	o	–	–	o	o	o	o	*	–	o	*
36	1	z	o	o	o	–	–	–	–	*	o	–	*
37	2	z	o	–	–	o	o	o	–		–	o	
38	2	z	o	–	–	o	o	–	o	*	–	o	*
39	3	z	o	–	–	o	o	–	–	*	o	o	
40	2	z	–	–	o	o	o	–	–	*	o	o	

[z]o: detected by dominant marker(pollen specific band); –: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-1. continued

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers						SRAP markers				
			OPO14				UBC27	UBC29	F7/R14		F11/R29	F4/R14	
			2700bp	750bp	550bp	450bp	250bp	1200bp	500bp	1000bp	1050bp	800bp	650bp
41	1	z	[z]–	–	o	o	o	–	–	*	o	–	
42	2	z	–	o	–	–	o	–	o		o	o	
43	1	z	–	–	–	o	o	–	o	*	o	o	
44	2	z	–	–	o	o	o	–	–	*	o	–	*
45	2	n	–	–	–	–	–	–	–		–	–	
46	1	z	o	–	–	o	o	–	o	*	–	–	
47	2	z	–	o	–	–	o	o	o		–	–	*
48	2	z	–	o	o	–	o	–	–		o	o	
49	3	z	o	–	–	o	o	–	–	*	–	o	*
50	3	z	o	–	o	o	o	–	o		–	o	*
51	2	z	–	o	–	–	o	o	–		–	o	
52	1	z	–	–	o	o	–	–	–		–	–	*
53	2	n	–	–	–	–	–	–	–		–	–	
54	2	n	–	–	–	–	–	–	–		–	–	
55	2	n	–	–	–	–	–	–	–		–	–	
56 ~ 95		n	–	–	–	–	–	–	–		–	–	
Total		50	20	24	26	25	39	13	29	20	23	29	24
			50				39	13	38		23	40	

[z]o: detected by dominant marker(pollen specific band); –: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-2. Zygotic or nucellar origin of 'Morita unshiu' × 'Lee' cross as determined RAPD and SRAP analysis.

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers				SRAP markers			
			OPO14		UBC27		F11/R29		F4/R14	
			550bp	450bp	800bp	250bp	1050bp	450bp	800bp	650bP
1	2	z	[z]o	o	o	o	o		–	*
2	2	z	–	o	–	o	o		o	
3	2	z	o	o	o	o	o		–	
4	2	n	–	–	–	–	–		–	
5	2	z	–	o	o	o	–	*	–	
6	2	z	o	o	o	o	o	*	–	
7	1	z	–	o	o	o	o		–	
8	1	z	–	o	o	o	–		o	
9	1	z	–	o	o	o	–		o	*
10	1	z	–	o	–	o	–	*	o	*
11	2	z	o	o	o	o	–	*	o	
12	1	z	o	o	–	o	o		o	
13	1	z	–	o	o	–	–		o	
14	2	z	o	o	o	o	–		o	
15	2	z	–	o	o	o	o	*	o	*
16	2	z	–	o	–	o	–		o	*
17	1	z	o	o		o	–		o	
18	2	z	o	o	o	o	–		o	
19	1	z	–	o	o	o	o	*	–	
20	2	z	–	o	o	o	–		o	*

[z]o: detected by dominant marker(pollen specific band); –: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-2. continued

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers				SRAP markers			
			OPO14		UBC27		F11/R29		F4/R14	
			550bp	450bp	800bp	250bp	1050bp	450bp	800bp	650bP
21	2	z	[z]o	–	–	o	–	*	–	*
22	1	n	–	–	–	–	–		–	
23	2	z	–	o	–	o	o		–	*
24	1	z	–	o	–	o	–		–	
25	1	z	o	o	–	o	o		–	
26	1	z	–	o	o	o	–		–	
27	1	z	o	o	–	o	o	*	–	
28	1	z	–	o	o	o	o		o	
29	1	z	–	o	–	o	o	*	–	
30	1	z	o	o	o	o	–		o	
31	2	z	–	o	–	o	–		–	*
32	4	z	o	o	–	o	o		–	
33	2	z	o	o	o	o	–		–	
34	1	z	o	o	–	o	o	*	–	
35	1	z	–	o	o	o	o		o	
36	2	z	o	o	–	o	o		–	*
37	2	z	–	o	o	o	–		–	
38	4	z	o	o	–	o	–		o	
39	2	z	o	o	o	o	o	*	–	*
40	2	z	o	o	–	o	–		–	*

[z]o: detected by dominant marker(pollen specific band); –: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-2. continued

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers				SRAP markers			
			OPO14		UBC27		F11/R29		F4/R14	
			550bp	450bp	800bp	250bp	1050bp	450bp	800bp	650bP
41	2	z	[z]o	o	o	o	o	*	-	
42	1	z	o	o	o	o	o	*	-	
43	1	z	o	o	-	o	o		-	
44	4	z	-	o	o	o	o		o	
45	3	z	o	o	o	o	o		o	*
46	2	z	o	o	o	o	-		-	*
47	3	z	-	o	o	o	-	*	-	*
48		n	-	-	-	-	-		-	
49		n	-	-	-	-	-		-	
50		n	-	-	-	-	-		-	
51~88		n	-	-	-		-		-	
Total		45	24	44	27	44	23	14	19	15
			45		45		28		28	

[z]o: detected by dominant marker(pollen specific band); -: no amplification; *: detected by recessive marker(female specific band absent)

Appendix 2-3. Zygotic or nucellar origin of 'Miyagawa Wase' × 'Orlando' cross as determined RAPD and SRAP analysis.

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers			SRAP markers
			OPO14		UBC27	F4/R14
			550bp	450bp	200bp	800bp
1	2	n	[z]–	–	–	–
2	2	n	–	–	–	–
3	1	n	–	–	–	–
4	2	n	–	–	–	–
5	2	n	–	–	–	–
6	2	n	–	–	–	–
7	2	n	–	–	–	–
8	2	n	–	–	–	–
9	**3**	**z**	**o**	**o**	**o**	**o**
10	2	n	–	–	–	–
11	2	n	–	–	–	–
12	2	n	–	–	–	–
13	1	n	–	–	–	–
14	1	n	–	–	–	–
15	2	n	–	–	–	–
16	2	n	–	–	–	–
17	3	n	–	–	–	–
18	**3**	**z**	**o**	**o**	**o**	**o**
19	2	n	–	–	–	–
20	2	n	–	–	–	–
21	2	n	–	–	–	–

[z]o: detected by dominant marker(pollen specific band); –: no amplification

Appendix 2-3. continued

Seed No.	Plants /seed	Zygotic or Nucellar	RAPD markers			SRAP markers
			OPO14		UBC27	F4/R14
			550bp	450bp	200bp	800bp
22	2	n	[z]–	–	–	–
23	2	n	–	–	–	–
24	2	n	–	–	–	–
25	1	n	–	–	–	–
26	2	n	–	–	–	–
27	3	n	–	–	–	–
28	2	n	–	–	–	–
29	2	n	–	–	–	–
30	3	n	–	–	–	–
31	2	n	–	–	–	–
32	2	n	–	–	–	–
33	**2**	z	**o**	**o**	–	**o**
34	2	n	–	–	–	–
35	1	n	–	–	–	–
36	2	n	–	–	–	–
37	2	n	–	–	–	–
38	2	n	–	–	–	–
39	3	n	–	–	–	–
40	1	n	–	–	–	–
41	2	n	–	–	–	–
42	2	n	–	–	–	–
43	2	n	–	–	–	–
Total		3	3	3	2	2

[z]o: detected by dominant marker(pollen specific band); –: no amplification

감사의 글

논문을 다 쓰고 보니, 그 내용엔 17년간의 세월이 있었습니다. 그런데, 그 긴 시간만큼의 흔적이라 하기엔 한없는 아쉬움과 자책감이 짙게 남습니다. 그러나, 아직도 진행 중인 이 흔적들이 머지않은 미래 농민의 얼굴에 웃음으로 돌아올 수 있으리란 희망으로 부끄럽지만 제 논문을 세상에 내 놓고자 합니다.

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나를 보면 항상 눈가에 눈물짓는 어머니, 이 자식의 논문에 살아온 세월이 위안이 되고 기꺼이 조금이나마 희생적 삶에 맘 적으로나마 보상이 되었으면 합니다. 사랑합니다.

그리고, 아버지, 전 당신께서 걸어가신 삶의 궤적은 닮고자 하지 않았으나, 감히 짐작코자 세상을 보고자 했던 본질은 따르고자 노력하였습니다. 이 논문은 제가 아버지께 쓰는 두 번째 편지입니다. 그러고 보니 우연히도 이글을 쓰는 순간 아버지가 이 세상에 흔적을 남긴 딱 그만큼의 세월만큼 제 나이가 들었다는 걸 알게 되네요.

끝으로 나의 동행자 아내 이경미, 든든한 나의 원군이여 고맙다. 그대에 기대어 세상에 나와 외롭지 않게 살아가는 것에 한없이 감사합니다. 눈물로 낳은 수정아, 제진아 건강하게 크고, 이제 너희들 마음속에 넉넉한 친구가 되어줄 아빠의 생활을 기대해라. 그리고 제진아 어린이집 에서처럼 고자질 하지마라, “아빠 사모실만 가이말고 나랑 가치 노아요”라고 하지마, 음,, 맴이 쪼매 아팠다. 이제 많이 놀아줄게 ! ^---------^

감사합니다.