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THESIS

DEVELOPMENT OF EST-SSR MARKERS FOR ORCHID IN *DORITIS* FAMILY AND GENETIC DIVERSITY ASSESSMENT OF *DORITIS* SPECIES IN THAILAND

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Savitree Ritchuay 2012: Development of EST-SSR Markers for Orchid in *Doritis* Family and Genetic Diversity Assessment of *Doritis* Species in Thailand. Master of Science (Genetics), Major Field: Genetics, Department of Genetics. Thesis Advisor: Assistant Professor Chatchawan Jantasuriyarat, Ph.D. 105 pages.

Doritis is a wild terrestrial orchid, originated in the Northeastern part of Thailand. With its good floral structure and very diverse color, it has high potential to develop into commercial ornamental plant or to use as a hybridizer with other orchid species. Unfortunately it has not been study in detail for its potential of development. In this study we developed SSR markers from EST database and examine the transferability of genomic SSR markers from other orchids in *Doritis*. 8,009 ESTs belong to family *Orchidaceae* were collected and analyzed for microsatellite. A total of 195 EST-SSR were identified. Twenty-three EST-SSR and seven genomic SSR primers (from *Dendrobium* and *Serapias*) were used for genetic diversity assessment of *Doritis* germplasm. 142 alleles were generated in which, 92 alleles (64.8%) show polymorphism among 30 *Doritis* accessions (*D. regnieriana*, *D. pulcherrima* and *D. pulcherrima* var. *buyssoniana*.). The polymorphic information content (PIC) average at 0.6076 and ranged from 0.1244 to 0.8439. The phylogenetic tree based on UPGMA showed three major clades based on their taxonomy indicating that SSR markers developed in this study can be used for identification, conservation and selection of appropriate parents for the *Doritis* hybrid production in future.

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LIST OF ABBREVIATIONS

bp	=	base pair
CTAB	=	hexadecyltrimethylammonium bromide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
EDTA	=	ethylene diamine tetraacetic acid
EST	=	expressed sequence tag
EtBr	=	ethidium bromide
EtOH	=	ethanol
HCl	=	hydrochloric
kb	=	kilobasepair (1,000 base pair)
KCl	=	potassium chloride
M	=	molar
mg	=	milligram
$MgCl_2$	=	magnesium chloride
mM	=	millimolar
mRNA	=	messenger ribonucleic acid
NaCl	=	sodium chloride
ng	=	nanogram
nt	=	nucleotide
PCR	=	polymerase chain reaction
SSR	=	simple sequence repeat
Taq	=	*Thermus aquaticus*
TBE	=	Tris-Borate-EDTA
TE	=	Tris-EDTA buffer
T_m	=	melting temperature
Tris	=	2-amino-2-hydroxymethyl-propane-1,3-diol
UV	=	ultraviolet

LIST OF ABBREVIATIONS (Continued)

µg	=	microgram
µl	=	microliter
µM	=	micromolar

DEVELOPMENT OF EST-SSR MARKERS FOR ORCHID IN *DORITIS* FAMILY AND GENETIC DIVERSITY ASSESSMENT OF *DORITIS* SPECIES IN THAILAND

INTRODUCTION

Doritis is a wild orchid, closely related to *Phaleanopsis*, originated in Southeast Asia and distributed throughout Indochina, Burma, Thailand, Malaysia and Sumatra (Christenson, 2001). In Thailand, two *Doritis* species found in Northeastern region are *D. pulcherrima* and *D. pulcherrima* var. *buyssoniana* (Kamemoto and Sa, 1975). *D. pulcherrima* has chromosome number of 2n = 2x = 38 while *D. pulcherrima* var. *buyssoniana* has 2n = 4x = 76 which the later one has larger flower, larger leave and longer inflorescence. Generally, *Doritis* can grow in a relatively dry environment. The characteristic of *Doritis* is quite remarkable when compare with other orchids because of its flower shape and variation of flower's color. These characteristics of tree shape and flower are appropriate to develop for the commercial ornament. Unfortunately, it has not been study in detail for its potential of development into high value export ornament. In order to reach its maximum potential for developing new high value ornament hybrids and species, the prior knowledge about its genetic diversity is needed.

The use of molecular technologies will help to speed up this process including using molecular markers in the selection of parents for hybrid production. Determining of genetic diversity of *Doritis* is an important step in generating the information for breeders to breed for *Doritis* new hybrids and species.

OBJECTIVES

1. To develop microsatellite markers by using EST database of other orchid species available in public database and examine the transferability of genomic SSR markers from other orchids in *Doritis*

2. To determine genetic diversity of *Doritis* in Thailand

LITERATURE REVIEW

Doritis

Doritis, a genus closely related to *Phaleanopsis,* comprises only a few species. The exact number has not been established due to the confusion which exists in its taxonomy. The highly variable *Doritis pulcherrima,* which abound in Thailand, was first described by Lindley in 1833. It has undergone several changes in nomenclature over the year. In 1874 Reichenbach, without knowledge of Lindley's earlier work, named it *Phaleanopsis esmeralda.* Then in 1917, Rolfe came across Lindley's original painting and, because of priority and sufficient differences, retained the separate genus and accepted the name *Doritis pulcherrima.* Later, J.J. Smith and Holttum treated it as a member of the *Phaleanopsis* genus. Meanwhile, orchidists had adopted the name *Phaleanopsis esmeralda,* and this orchid was so designated in Sander's *List of Orchid Hybrids* for many years. Finally in 1958, the name was changed to *Doritis pulcherrima,* and its hybrids with *Phaleanopsis* are known as Doritaenopsis. Holttum, in 1963, accepted the genus *Doritis* and recognized two species, *D. pulcherrima* and *D. regnieriana*. (Kamemoto and Sakarik, 1975)

Doritis pulcherrima Ldl.

The orchid is widely distributed in Indochina, Burma, Thailand, Malaysia and Sumatra. In Thailand, it had been found in just about every phytogeographical region. It often grows in sandy soils in the shade of bushes or shrubs. The growth habit is erect. Side shoots are common, and a plant undisturbed in its natural habitat often forms a huge clump consisting of 10 or more growths. The leathery leaves are long and pointed or rounded, green to purplish green, and measure 2 to 6 inches long and 1 to 2.5 inches wide. (Figure 1) The flowering season is from June to December, but some plants will continue to flower for nearly a year. The erect to sub-erect inflorescences are sometimes branched and measure up to 20 inches, often bearing 20-30 flowers. A great many more flowers are borne if the flowering period is prolonged. Individual flower measure about 1 inch across. The broad base of the

lateral sepal is attached to the column foot, and the three-lobe labellum is hinged to the column foot. A pair of narrow appendages appears near the base of the side lobe. (Kamemoto and Sakarik, 1975)

The color of the sepals and the petals ranges from white to light lavender to amethyst-purple. The mid lobe also varies in intensity of color but is invariably darker than sepals and petals. The side lobes vary from light lavender to yellow to brown. The sepals and petals may be greatly reflexes, moderately reflexes, or nearly flat, and the dorsal sepals may even be hooded. (Figure 2) Plants inhabiting Prachuab and Hua Hin, Thailand are generally highly variable in flower color, ranging from near white to dark purple. On the other hand, plants from certain areas of Prachinburi in eastern Thailand are more uniformly dark purple, and the petals and sepals are not reflexes. (Kamemoto and Sakarik, 1975)

Plants are relatively easy to cultivate and flower. Since in nature they are often found in sandy, well-drained soil with an accumulation of organic debris, such materials as crushed rock, crushed brick, charcoal, fern fiber and other organic media, either alone or mixed in various combinations, can serve as planting media. A mixture of small charcoal pieces and crushed brick in equal amounts has proved satisfactory in Bangkok. As long as the medium is well drained and well aerated, liberal watering during the dry season will keep the plants in a healthy state. It is important, however, to provide good aeration and drainage during the rainy season to prevent bacterial soft rot and the fungus crown rot. Frequent applications of fungicides should minimize the incidence of troublesome diseases. (Kamemoto and Sakarik, 1975)

Doritis pulcherrima* var. *buyssoniana

From Northeastern of Thailand, near the bank of the Mekong River in Ubon Ratchathani province, there is a larger type of *D. pulcherrima* which the Thai refer to as *Daeng Ubon* (Ubon Red). This is Reichenbach's *Pha. buyssoniana*, which is now considered a variation of *D. pulcherrima* distinctive enough to warrant a botanical

variety status. It appears to be endemic to the Ubon Ratchathani area. (Kamemoto and Sakarik, 1975)

This variety has been established as a tetraploid with 2n = 4x = 76 chromosomes. It has the “gigas” characteristics common to tetraploids. The large, thick, leathery leaves are often spotted with purple on the upper surfaces and suffused with purple on the lower surfaces. (Figure 1) The inflorescence is sturdy and erect and attains a height of about 45 inches. The flowers are almost twice the size of diploid variety and are not generally reflexes. The petals and sepals are of heavy substance, broad, and sometimes overlapping to form a round, full flower. Color ranges from light lavender to dark purple. (Figure 2) The flowering season is usually short, from June to August, with the peak in July, but occasionally a few stragglers are seen as late as October, and even later in Chiang Mai province. (Kamemoto and Sakarik, 1975)

This orchid flower grows among grasses, in the open among rocks or in sandy soils rich in humus. Ubon Ratchathani, in northeast Thailand, has a relatively low rainfall and a long dry period. The last rain come in late October and, except for an occasional thunderstorm, it is dry from November to mid-May, the plants often shed their leaves. With the advent of the rainy season, they begin active growth and immediately send out of flowering spike. Brought down to the lowland, they do not fare well. They are highly susceptible to the bacterial soft-rot disease. Flowering is sparse unless the winter temperatures drop to the fifties.

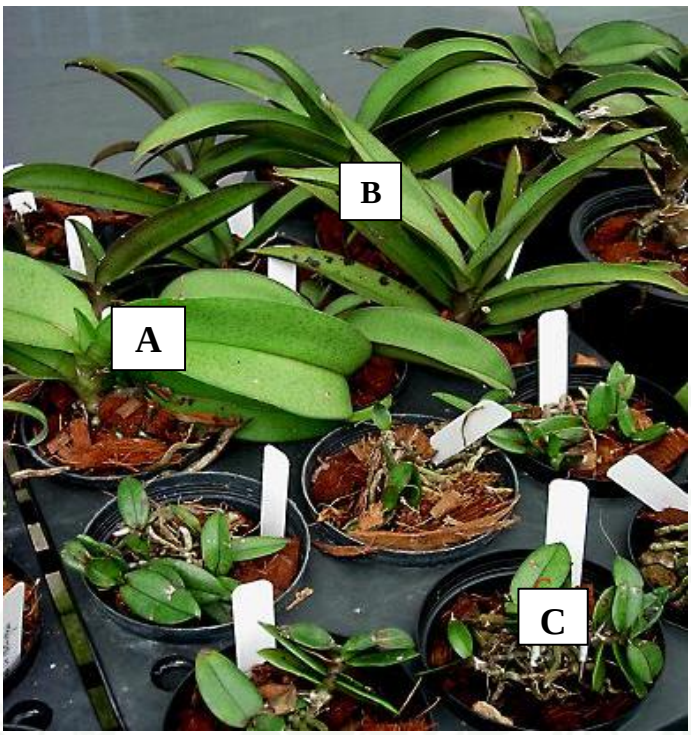

Figure 1 Leaf's morphology of *Doritis* (A: *Doritis pulcherrima* var.*buyssoniana* B: *Doritis pulcherrima* C: *Doritis regnieriana*)

Source: Bloggang (2007b)

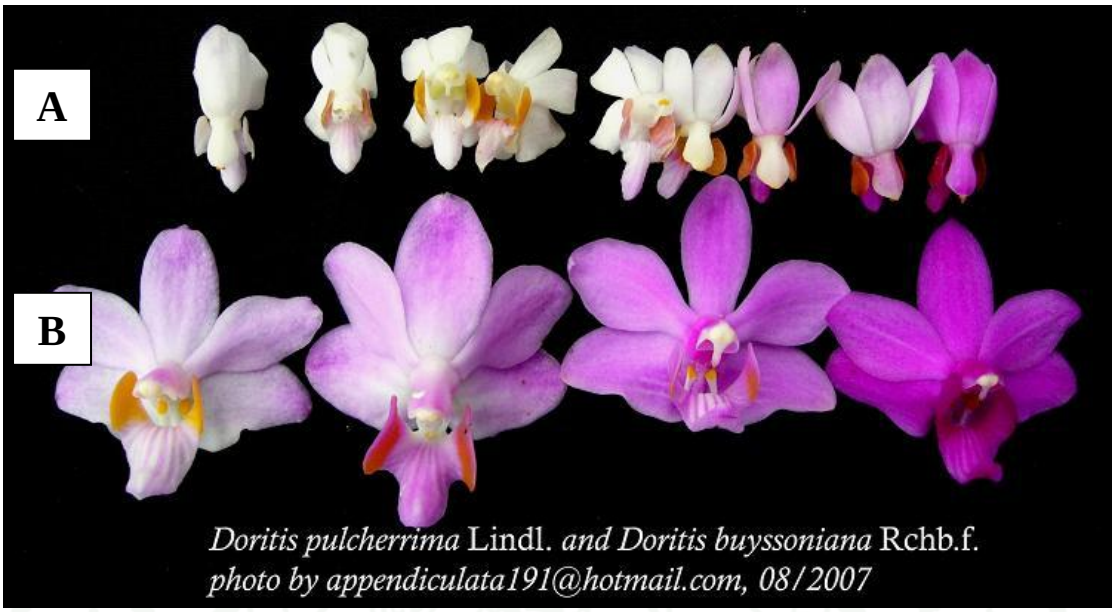

Figure 2 Comparison of flowers between *Doritis pulcherrima* var. *buyssoniana* (A) and *Doritis pulcherrima* (B).

Source: Bloggang (2007a)

Hybrids of *Doritis*

The first hybrid of *D. pulcherrima* was registered as *Pha. asahi* by Iwasaki of Hawaii as early as 1923. The parents were *D. pulcherrima* and *Pha. lindeni*. It was not until 30 years later, in 1955, that another *Doritis* hybrid was appeared. Since then, however, numerous hybrids have been introduced. The majority of these have been registered within the last decade. Several hybrids have been shown excellent horticultural qualities. Among them, *Doritaenopsis* Red Coral, a result of crossing *D. pulcherrima* var. *buyssoniana* and *Pha. Doritis*, both tetraploids, has been outstanding, as attested to by several award-winning plants of this progeny.

Primary, or first generation, diploid Doritaenopsis hybrids are sterile or of very low fertility, due to the difference in the chromosomal makeup. Although, the basic chromosome number of the parental species is identical (2x = 2n = 38), the chromosome size of *Doritis* are nearly three times of those of the *Euphalaenopsis*

group. Paring between *Doritis* and *Phalaenopsis* chromosome at meiosis is poor and impaired fertility result (Kamemoto and Sakarik, 1975).

Variations in chromosome numbers are common in advanced generation *Phalaenopsis* hybrids. If the diploid *D. pulcherrima* is crossed with a diploid *Phalaenopsis,* diploid hybrid progenies will be obtained. If the diploid *D. pulcherrima* is crossed with a tetraploid *Phalaenopsis,* triploid hybrid progenies will be generated. Both diploid and triploid hybrids are low in fertility because of lack of chromosome homology in the diploid hybrids and lack of homology and chromosome imbalance in the triploid hybrids. If the tetraploid *D. pulcherrima* var. *buyssoniana* is crossed with tetraploid *Phalaenopsis,* the fertility in the hybrid will be enhanced. For the tetraploid hybrids, they consist of two set of chromosomes, one set from *Phalaenopsis* and the other set from *Doritis.* This has been borne out by the success in obtaining second-generation hybrids utilizing *Doritaenopsis* Red Coral (Kamemoto and Sakarik, 1975).

An understanding of the cytology of the two groups of orchid is of great value in a hybridization program, particularly since hybrid sterility is often encountered. The circumvention of this sterility may be possible through obtaining two sets of chromosomes each of *Doritis* and *Phalaenopsis*. (Kamemoto and Sakarik, 1975)

Genetic Diversity

Genetic diversity is important priori information in a study of any plant species because it amount and distribution are likely to affect the evolutionary potential of species (Futuyma, 1986). It is particularly useful in the characterization of individuals, accessions and cultivars in determining duplications in germplasm collection and for the choice of parental genotypes in breeding program (Xie *et al.*, 2010).

When a new allele appears in a population, it has the potential to change the genetic make-up of successive generations. Harmful mutations will likely not persist because the affected individual will either not survive, or will have limited

reproductive success. However, some mutations may be passed on to successive generations because an organism with that allele is better equipped to survive in its environment, that is, it has a selective advantage. Those individuals that produce a greater number of offspring that survive are said to be more fit (Sangsiri, 2009). Other mutations may have no effect on phenotype, and may persist simply by chance (genetic drift). It is the selective advantage that drives evolution, albeit momentarily, in one direction or another (Russell, 2003; Sangsiri, 2009)

Molecular markers

Molecular markers or DNA markers have become important in the genetic characterization and improvement of many crop species. They have been used to identify the genetic region or different alleles of loci on chromosomes. They have contributed to and greatly expanded the assessment of biodiversity and understanding of phylogenetic relations.

DNA markers can be classified into two categories: hybridization-based and polymerase chain reaction (PCR) based markers.

Hybridization-based markers

Hybridization-based markers include techniques that use labeled DNA molecules as hybridization probes, either short synthetic oligonucleotide probes or longer DNA. DNA is digested with restriction endonucleases and the resulting fragments are separated by electrophoresis and transferred to membrane by Southern blotting. Restriction fragments are hybridized to probes representing single copy genomic segment or repeated sequences such as mini and microsatellites. The most well known hybridization-based marker is RFLP. Because of their co-dominant properties, and thus can distinguish between homozygosity and heterozygosity. RFLP analysis utilizes the difference in nucleotide sequences at specific site recognized and cut by restriction enzymes, and then separated according to size under electrophoresis. Individual DNA fragment is identified by labeled probe specific to certain sequence.

The presence of particular allele at this locus is detected by length polymorphism caused by mutation that has led to loss or gain of a restriction site between genotypes. However, a limitation of RFLP is that it requires a large amount of good quality DNA for the analysis. This technique is also time-consuming and expensive, making it less suitable for large-scale screening in plant breeding program (Taiji *et al.*, 2002).

PCR-Based Markers

PCR-based markers use amplification of target DNA sequences by the polymerase chain reaction (PCR) in vitro. In 1985, Saiki *et al*. described that PCR-based markers require DNA template and complementary DNA fragment with a free 3'OH. The template is provided by DNA sample to be amplified and the free 3'OH is provided by site specific oligonucleotide primer. The primers are complementary to each of the ends of the sequence that is to be amplified. Three steps of the PCR reaction are:

1. Denaturation: the DNA template is heated usually at 95°C to generate single stranded DNA.

2. Annealing: the two primers bind to denatured DNA templates at the appropriate locations based on the complementary sequences between DNA template and primers. The temperature for this step varies depending on the size of the primer and its homology to the target DNA.

3. Primer extension: DNA polymerase extends the primer by its polymerase activity.

This is done at a temperature optimal for the particular polymerase that is used.

Currently the most popular enzyme for this step is *Taq* DNA polymerase, the DNA polymerase from the thermophilic bacteria *Thermus aquaticus*. The extension is usually performed at 72˚C (Maison, 2005)

PCR based markers can be generated by random or specific primer and are dependent on the stringency of the PCR condition and the method of fragment separation and detection. PCR based markers have advantages over RFLP as the assay requires comparatively little DNA. It can generate a large number of polymorphic markers quickly without the need to develop libraries (Sangsiri, 2009). PCR techniques that are commonly used for genetic diversity such as; random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR) and single strand conformation polymorphism (SSCP).

Microsatellite or simple sequence repeats (SSRs)

Microsatellites are simple sequence repeats (SSRs) of 2-6 nucleotides, detected within the genomes of every eukaryotic organism**.** Microsatellite marker is based on PCR amplification of tandem repeat using unique DNA sequences flanking the repeat as oligonucleotide primer. Polymorphisms are detected as length variations of fragments, usually on polyacrylamide gels. Decreasing and increasing amount of single or multiple number of repeats due to slipped strand mispairing during DNA replication, indicated the variation of microsatellite loci (Wright and Bentzen, 1995; Na-Nakorn,1998); in which led to extensive allelic variation (Antoro, 2004). Initial studies in rice indicated that these markers were highly polymorphic. While, RFLPs usually only exhibit 2 or 3 alleles per locus, as many as 25 alleles have been observed at a single microsatellite locus among a diverse set of cultivars (Yang *et al.,* 1994; Jangsutthivorawat, 2008). Microsatellites can be divided into three types; perfect, imperfect and compound microsatellites. Perfect microsatellite has only one repeat sequences such as $(AC)_n$ and $(AT)_n$, imperfect microsatellite has certain number of nucleotide bases between the repeat sequences such as; $(AC)_nCT(AC)_n$ and compound microsatellite has different sequence repeat such as; $(AC)_n(TG)_n$. Microsatellites are

abundant, dispersed throughout the genome, show higher level of polymorphism than most of DNA markers and they are considered locus specific markers. These features, coupled with their ease of detection, have made them a useful molecular marker. Their potential for automation and their inheritance in a co-dominant manner are additional advantages when compared with other types of molecular markers (Goldstein and Schlötterer, 2001; Holton, 2001). Microsatellite marker has been developed in many plant species such as; melon (Ritschel *et al*., 2004), sugarcane (Pinto *et al*., 2004), cotton (Qureshi *et al*., 2004), *Dendrobium* (Boonsrangsom *et al*., 2008), *Gymnadenia conopsea* (Campbell *et al*., 2002) and *Ophrys fusca* (Cotrim *et al*., 2008).

Nevertheless, despite their advantages over other genetic markers, microsatellite loci have several disadvantages and limitations. The appearance of shadow or stutter bands is always a problem of di-nucleotide microsatellites and lead to the miscoding alleles. They are generally thought to be due to slipped-strand mispairing during PCR. Another limitation is the presence of null alleles which are not amplified due to unidentified mutation at primer sites. If exist, the null alleles cause miscoding of heterozygotes for homozygotes and parent offspring incompatibility in the pedigree analysis (Antoro, 2004).

Development of microsatellite marker is quite complicated and expensive but compared with other type of molecular markers, SSR markers have become one of the most important due to their co-dominant inheritance, relative abundance, multi-allelic nature, extensive genome coverage and ease of detection by polymerase chain reaction (PCR). Furthermore, microsatellites are the most successful and powerful PCR-based DNA markers for resolving genetic diversity and phylogenetic relationships in a wide range of taxonomic groups as found in rose (Park *et al.*, 2010) and for marker-assisted selection in modern breeding program such as the study of diversity and genetic relationship in orchardgrass (Xie *et al*., 2010) and cymbidium (Moe *et al.*, 2010). However, widespread use of SSR markers is often limited by the time and cost involved in their development, which require DNA library construction, sequencing, identification of SSR containing clones, and SSR transferability, which is

generally limited to closely related species. The number of transferred markers would likely improve by using markers developed from expressed sequences (Li *et al.,* 2011)

Expressed sequence tag (EST) studies have generated a vast amount of publicly available sequence data from many plant species, these data can be mined for simple sequence repeats (SSRs) (Pashley *et al.,* 2006). EST-SSR markers are different from traditional SSR (genomic-SSR) markers, which have some intrinsic advantages over genomic SSRs in their direct association with transcribed genes, lower expense of development, and high level of transferability to related species (Mian *et al.,* 2005). Polymorphisms derived from EST-SSRs are associated with the coding regions of the genome.

With large increase in EST sequences, more EST-SSRs have been identified and used extensively for comparative mapping, DNA fingerprinting, and genetic diversity and transferability. For example such as in 2008, Zhuang *et al*. developed chromosome mapping of new wheat EST-SSR markers and the application for characterizing Rye chromosome added in Wheat. In 2008, Yu *et al.* studied genetic evaluation using EST-SSR derived from *Gossypium herbaceum*, the development of EST-SSR in graminous species and their chromosome location on wheat by Li *et al*., 2008, studied of genetic diversity analysis and transferability of cereal EST-SSR markers to orchardgrass by Xie *et al.,* 2010 and assessment of genetic diversity and population structure of Chinese wild almond, *Amygdalus nana*, by using EST-SSR by Omirshat *et al*., 2009 etc.

MATERIALS AND METHODS

Materials

Plant materials

Thirty accessions of *Doritis* were collected from Northeastern part of Thailand and Sarabury province (Figure 3) including three accessions of *D. regnieriana*, twelve accessions of *D. pulcherrima* and fifteen accessions of *D. pulcherrima* var. *buyssoniana* (Table 1 and Figure 4). All *Doritis* samples were cultured in the greenhouse at Faculty of Agriculture, Ubon Ratchathani University.

Figure 3 Map of Thailand showing the collection sites of *Doritis* from Northeastern part of Thailand (Red circle: *D. pulcherrima*, Blue square: *D. pulcherrima* var.*buyssoniana* and Green triangle: *D. regnieriana*)

Methods

Genomic DNA Extraction

Genomic DNA was extracted from young leaves using a modified CTAB method (Doyle and Doyle, 1990). 0.3 gram of young leaf was grinded in liquid Nitrogen, powder was collected into 1.5 ml tube that contains 750 µl of 2X CTAB extraction buffer. The extraction buffer contained 100 mM Tris buffer pH 8.0, 25 mM EDTA, 2 M NaCl and 4% CTAB. Then mixed sample by vortexing and incubated at 65 ºC for approximately one hour. Add 850 µl of chloroform: isoamylalchohol (24:1), mix by inverting the tube and centrifuge at 12,000 rpm at 4 ºC for 30 minutes. After that transfer upper aqueous phase into new tube and add equal volume of isopropanal then mix gently. Incubate sample at 40 ºC for 1 hour and centrifuge at 12,000 rpm for 30 minutes. The supernatant was discarded, carefully not to lose the pellet then add 1 ml of 95% cold ethanol to wash pellet, centrifuge at 12,000 rpm at 4 ºC for 10 minutes. Wash the pellet again with 70% cold ethanol, centrifuge at 12,000 rpm at 4 ºC for 10 minutes then dry the pellet for 15 minutes at 50 ºC and dissolve in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0), store at -20 ºC.

Measurement of quality and quantity of samples

Each DNA sample is separated on 1% agarose gels in 0.5X TBE buffer (Tris-base, Boric acid, 0.5 M EDTA pH 8.0) by electrophoresis at 100 voltage for 30 minutes and gene ruler DNA mix is used for standard marker, staining with Ethidium bromide and DNA bands are visualized under ultraviolet light.

Table 1 The morphological characteristics and collection sites of *Doritis* accessions used in this study

No.	ID.	Name	Leaves' characteristic/ Spot	Flowers' morphology	Color of flowers	Color of lips	Color of pedicel	Origin
1	S2	*D. regnieriana*	Short, rounded/purple	-	-	-	-	Sarabury
2	S3	*D. regnieriana*	Short, rounded/purple	-	-	-	-	Sarabury
3	S4	*D. regnieriana*	Short, rounded/dark green	-	-	-	-	Sarabury
4	M2	*D. pulcherrima*	Long, pointed/ purple	-	-	-	-	Sarabury
5	M5	*D. pulcherrima*	Long, pointed/ purple	-	-	-	-	Sarabury
6	Mah	*D. pulcherrima*	Long, pointed/ dark green	-	-	-	-	Chiang Rai
7	RO4	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Roi et
8	RO10	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown		-	-	-	Roi et
9	DB6	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Chongmex Amphoe Sirindhon Ubon Ratchathani

Table 1 (Continued)

No.	ID.	Name	Leaves' characteristic/ Spot	Flowers' morphology	Color of flowers	Color of lips	Color of pedicel	Origin
10	DB8	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	Broad	Lavender	Light orange	Green	Chongmex Amphoe Sirindhon Ubon Ratchathani
11	MDD2	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ dark green	-	-	-	-	Indochaina market Mukdahan
12	MDD3	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
13	MDD6	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
14	MDD10	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
15	MDD11	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
16	MDD15	*D. pulcherrima* var. *buyssoniana*	Long, rounded/dark green	-	-	-	-	Indochaina market Mukdahan

Table 1 (Continued)

No.	ID.	Name	Leaves' characteristic/ Spot	Flowers' morphology	Color of flowers	Color of lips	Color of pedicel	Origin
17	MDD20	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
18	MDD22	*D. pulcherrima* var. *buyssoniana*	Long, rounded/dark green	Broad	Light lavender	Yellowish orange	Green	Indochaina market Mukdahan
19	CM8	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	-	-	-	Chongmex Amphoe Sirindhon Ubon Ratchathani
20	CM11	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	-	Purple	Dark purple	Yellowish green	Chongmex Amphoe Sirindhon Ubon Ratchathani
21	CM16	*D. pulcherrima* var. *buyssoniana*	Long, pointed/ brown	pointed	Lavender	Yellow	Green	Chongmex Amphoe Sirindhon Ubon Ratchathani
22	MV4	*D. pulcherrima*	Long, pointed/-	pointed	Lavender	Dark orange	Yellowish green	Chongmex Amphoe Sirindhon Ubon Ratchathani

Table 1 (Continued)

No.	ID.	Name	Leaves' characteristic/ Spot	Flowers' morphology	Color of flowers	Color of lips	Color of pedicel	Origin
23	MVP2	*D. pulcherrima*	Long, pointed/ brown	pointed	White	Dark orange	Yellowish green	Amphoe Pho Sai Ubon Ratchathani
24	MVP26	*D. pulcherrima*	Long, pointed/ brown	pointed	Lavender	Dark purple	Yellowish green	Amphoe Pho Sai Ubon Ratchathani
25	MVP27	*D. pulcherrima*	Long, pointed/ brown	-	-	-	-	Amphoe Pho Sai Ubon Ratchathani
26	PT1	*D. pulcherrima*	Long, pointed/ brown	pointed	-	Dark yellow	Yellowish green	Phu Pha Thoep National park Mukdahan
27	PT2	*D. pulcherrima*	Long, pointed/ brown	pointed	Lavender	Dark orange	Yellowish green	Phu Pha Thoep National park Mukdahan
28	MDM14	*D. pulcherrima*	Long, pointed/-	pointed	Lavender	purple	Green, purple	Indochaina market Mukdahan

Table 1 (Continued)

No.	ID.	Name	Leaves' characteristic/ Spot	Flowers' morphology	Color of flowers	Color of lips	Color of pedicel	Origin
29	MDM18	*D. pulcherrima*	Long, pointed/ brown	-	-	-	-	Indochaina market Mukdahan
30	MDM21	*D. pulcherrima*	Long, pointed/-	-	-	-	-	Indochaina market Mukdahan

Polymerase chain reaction (PCR)

One sample was randomly selected to amplify with 135 primer pairs that previously designed. PCR reactions were carried out in a final volume of 20 µl, containing with approximately 20 ng of genomic DNA, 0.5 units of DreamTaq™ DNA Polymerase (Fermentas), 1X DreamTaq™ DNA Polymerase buffer, 5 µM of each primer and 200 µM of dNTP.

Amplification reactions were carried out on a Bioer GenePro Thermal Cycler (USA) using the following cycling profile: 94 °C for 5 minutes followed by 30 cycles at 94 °C for 30 seconds, 48-62 °C (gradient) for 30 seconds, 72 °C for 60 seconds, final extension step at 72 °C for 10 minutes and the PCR products were stored at 4 °C before analysis.

The PCR products are separated on 2.0% agarose gel electrophoresis at 75 Voltage for 1.5 hours and visualized under UV light after staining with Ethidium bromide. The detection of polymorphism from the PCR products analyze with HAD-GT12^{TM} capillary gel electrophoresis system (Qiagene, Germany).

Data analysis

Genetic diversity and phylogenetic analysis

For data analysis, the polymorphic of EST- and genomic-SSR alleles were scored for the presence (1) and absence (0) for all the individuals. The number of polymorphic band (PB), percentage of polymorphic band (PPB), observed number of alleles per locus (A_o), effective number of alleles per locus (A_e), expected heterozygosity (H_e) and Shannon's information index (H_o) were calculated using POPGENE v.1.31 (Yeh *et al.*, 1999). Polymorphism information content (PIC) was calculated by applying the formula given by Anderson *et al* (1993). The NTSYS pc version 2.1 software package (Rohlf, 2000) was used to calculate Dice genetic similarity coefficient (Nei and Li, 1979) for cluster analysis using unweighted pair

group method with arithmetic average (UPGMA). Principal Coordinate Analysis (PCA) was also performed using NTSYS-pc software to resolve the pattern of clustering among genotypes. AMOVA was used to investigate genetic diversity among and within species (Excoffier *et al.*, 1992; Missio *et al.*, 2009) using Arlequin3.5 software program (http://cmpg.unibe.ch/software/arlequin3).

1. Number of allele per locus (N)

N = Total alleles / Total number of loci studied

2. Effective number of allele

A_e is estimation of the reciprocal of homozygosity (Kimura and Crow, 1964). The data were calculated using the software POPGENE version 1.31 (Yeh *et al.*, 1999).

$$N_e = 1/\sum p_i^2$$

Where p_i = frequency of the i^{th} allele for the studied locus

3. Allele frequencies

Allele frequencies were calculated using the software POPGENE version1.31 (Yeh *et al.*, 1999).

$$X_i = (2H_A+H_B)/2N$$

Where X_i = the frequency of the i^{th} allele

H_A = number of homozygous

H_B = number of heterozygous

N = number of all samples

4. Polymorphism information content (PIC)

PIC is refered to the value of a marker for detecting polymorphism within a population (Bostein *et al.*, 1980) and depends on the number of detectable alleles and the distribution of their frequency (Anderson *et al.*, 1993).

$$PIC_i = 1-\sum_{j=1}^{n} P_{ij}^{2}$$

Where PIC_i = the polymorphic information content of a marker i

P_{ij} = the frequency of the j pattern for marker i and the summation extends over n patterns

5. Heterozygosity

Heterozygosity is the state of having two different alleles of the same gene. It was calculated using the program POPGENE version 1.32 (Yeh *et al.*, 1999).

5.1 Observed heterozygosity (H_o), was calculate as (Nei, 1978)

H_o = Number of heterozygous individuals / Total number of genotypes per locus

5.2 Expected heterozygosity (H_e), were calculated according to the formula (Nei, 1978).

$$h_k = 2n(1-\sum x_i^2)/2n-1$$

$$He = \sum h_k/r$$

Where h_k = the value of h for the k^{th} locus

X_i = the frequency of the i^{th} allele

N = number of the k^{th} allele

r = number of study allele

H_e = average heterozygosity per locus

6. Genetic distance

Genetic distance (Nei, 1978), standard genetic distance is defined as

$$D = -\ln[G_{XY}/\sqrt{GxGy}]$$

Where G_X = the means of $\sum p_i^2$ all over loci

G_Y = the means of $\sum q_i^2$ all over loci

G_{XY} = the means of $\sum p_i q_i$ all over loci

7. Phylogenetic tree

Phylogenetic tree was produced by clustering the data with the unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) using NTSYS pc version 2.1 software package (Rohlf, 2000)

8. Principal Coordinate Analysis (PCA) (Anderson, 2003)

PCA is a method to represent on a 2 or 3 dimensional chart objects described by a square matrix containing that contains resemblance indices between

objects. PCA is a computer program that calculates a principal coordinate analysis of any symmetric distance matrix in the manner of Gower (1966).

D = (d_{ij}) be an (nxn) distance matrix

A = $(a_{ij}) = -1/2\ (d_{ij}^2)$,

then calculate Gower's centered matrix (G) by centering the elements of A, i.e.

G = $(I - 1/n 11')A(I - 1/n 11')$

where 1 = column of 1's of length n

I = the identity matrix

Matrix G is then decomposed into its component eigenvalues and eigenvectors. The eigenvectors, standardized by dividing by the square root of their corresponding eigenvalues, are output as the principal coordinate axes. This analysis is also called metric multi-dimensional scaling. It is useful for ordination of multivariate data on the basis of any distance function.

Place

Department of Genetics, Faculty of Science, Kasetsart University, Bangkhen campus, Bangkok

Duration

March 2009 to September 2011

RESULTS AND DISCUSSION

DNA Extraction

A total of 30 *Doritis* accessions were collected from the different sites, mostly from Ubon Ratchathani province in Northeastern Thailand, as described in materials. DNA was extracted using a manual method and DNA extraction kits. Leave and root tissues of *Doritis* samples were used to compare the quality of DNA from each method. The result of DNA extraction showed that CTAB method produced higher DNA quality than the manual method. The extracted DNA showed clear genomic DNA band at the top of the 1% agarose gel (Figure 5).

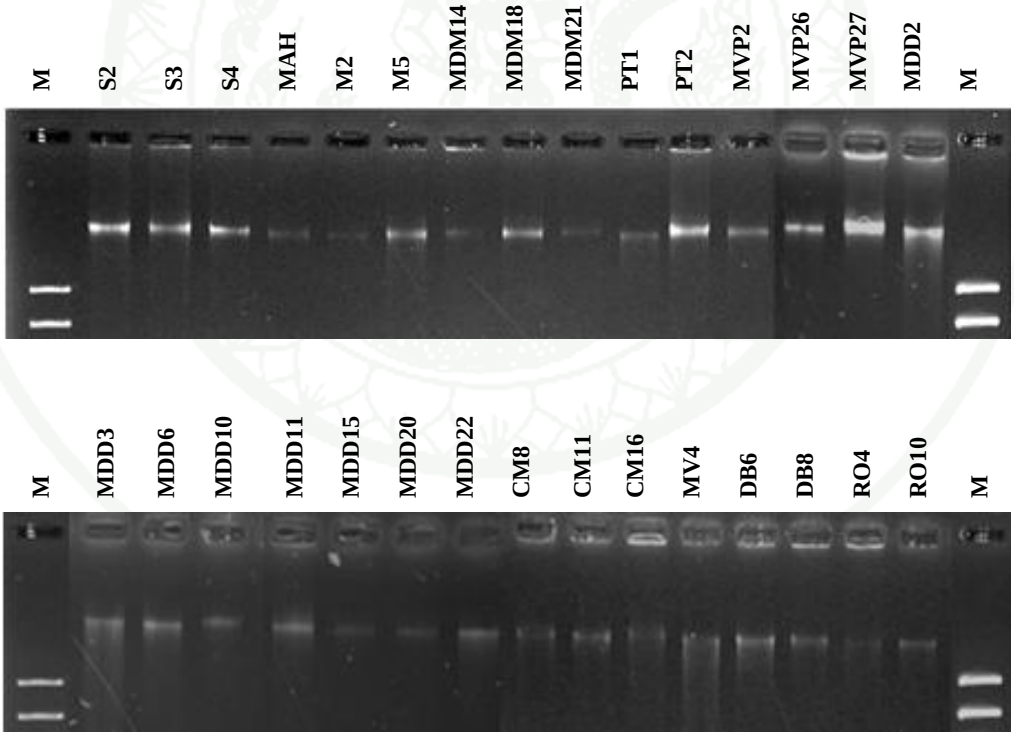

Figure 4 genomic DNA from 30 *Doritis* samples.

EST-SSR identification, development and characterization

EST sequences were obtained from the EST database at the NCBI (http://www.ncbi.nlm.nih.gov/). A total of 8,018 EST-sequences (5.4 Mb) belonging to family orchidaceae were obtained for this study on February, 2010. All EST sequences were preceded by trimming off the vector sequences and low complexity sequences, clustered and assembled using the TGIL software (http://sourceforge.net/projects/tgicl/) to remove redundant sequences. After processing, the number of unique sequences is 1,765 sequences consisting of 695 contiqs and 1,070 singletons. Then, unique sequences were used for searching to identify SSR sequence using the Troll software (http://wsmartins.net/websat/). The software was set to detect repeat motifs which minimum repeat of 10, 6, 5, 4, 4 and 4 base pairs for mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats, respectively. Finally, EST primers were designed from conserved sequences flanking repeats where the main parameters were melting temperature of primer ranges between 57-68˚C, GC content ranges between 40-80%, primer size ranges between 18-27 base pairs and PCR products range between 100-400 base pairs in length. All of unique sequences can design 195 EST-SSR primer pairs, based on described criteria. From all identified EST-SSR primer, there are 67 (34.36%) mono nucleotide repeats, 76 (38.98%) di-nucleotide repeats, 39 (20%) tri-nucleotide repeats, 6 (0.38%) tetra-nucleotide repeats, 1 (0.51%) penta-nucleotide repeat and 6 (3.08%) hexa-nucleotide repeats. (Table 2) (Figure 6) The TC/GA motif was the most common among di-nucleotide repeats accounting for 14.87% followed by AC/CT (12.82%), AT (5.13), TA (4.1%) and AC/GT (1.54%) but GC/CG was not found because GC/CG motif repeat is rare in most plant's EST (Kunkeaw *et al*., 2011). Similarly, significant numbers of tandem repeats were also identified in many recent studies such as Kunkeaw *et al* (2011), found TC/GA motif was the most common among di-nucleotide repeat follow by TA/AT, AC/TG and GC/CG motifs, in cassava respectively. The similar results were also found in many EST sequence studies in plants including Iris (Tang *et al*., 2009), lettuce (Simko, 2008), cotton (Qureshi *et al*., 2004) and cucumber (Hu *et al*., 2010). For tri-nucleotide repeats, AAG/CTT motif was found the most which is similar to the results studies in other plant species. The relative abundance of AG/AAG observed in

Doritis ESTs is in agreement with earlier reports of other plant species (Kunkeaw *et al*., 2011, Tang *et al*., 2009, Simko, 2008), suggesting it maybe a common feature of the dicot species (Hu *et al*., 2010). Then one hundred EST-SSR primer pairs were randomly selected and synthesized for evaluating the applicability of developed EST-SSR in *Doritis*.

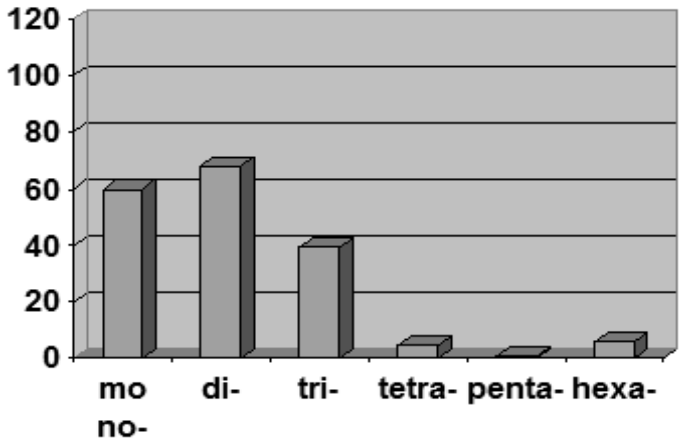

Figure 5 The numbers of repeat motif in EST-SSR markers.

Table 2 The frequency of repeat motifs in EST SSR markers.

Repeat unit	Motif	Number	Frequency
mono-nucleotide	A/T	64	32.8205
	G/C	3	1.53846
di-nucleotide	TC/GA	29	14.8718
	AG/CT	25	12.8205
	AT	10	5.12821
	TA	8	4.10256
	AC/GT	3	1.53846
	CA	1	0.51282

Table 2 (Continued)

Repeat unit	Motif	Number	Frequency
tri-nucleotide	CGG/CCG	8	4.10256
	GCC/GGC	7	3.58974
	GCG/CGC	3	1.53846
	GTG/CAC	3	1.53846
	AAG/CTT	3	1.53846
	CAA/TTG	3	1.53846
	AGG/CCT	2	1.02564
	ATC/GAT	2	1.02564
	TTC/GAA	2	1.02564
	AGA/TCT	1	0.51282
	ATA/TAT	1	0.51282
	GAC/GTC	1	0.51282
	CAG/CTG	1	0.51282
	TGA/TCA	1	0.51282
	TGG/CCA	1	0.51282
tetra-nucleotide	AAAC/GTTT	5	2.5641
	AATT/TTAA	1	0.51282
penta-nucleotide	TTGGT/ACCAA	1	0.51282
hexa-nucleotide	CGGCAG/CTGCCG	3	1.53846
	CCCAAA/TTTGGG	2	1.02564
	TAACCC/GGGTTA	1	0.51282
Total		195	100

EST-SSR amplification

Expressed sequence tag (EST) studied have generated a vast amount of publicly available sequence data from many plant species (Li *et al*., 2011). EST-SSRs are different from traditional SSR (genomic-SSR) marker, which have some intrinsic advantages over genomic SSRs in their direct association with transcribed genes, lower expense of development, and high level of transferability to related species (Mian *et al*., 2005). In present study, EST-SSRs were developed from orchid species and used for genetic diversity assessment in *Doritis*. Twenty three primer pairs from a total of 100 primer pairs (23%) were successfully amplified genomic DNA of *Doritis*.(Figure 7)(Table 3) This percentage was low when compared with the previous reports such as 952 successfully amplified markers from 994 markers (95.8%) in hop (Koelling *et al*., 2011), 31 from 35 markers (88.6%) in cucumber (Hu *et al*., 2010), 44 from 50 markers (88%) in sesame (Wei *et al*., 2008), 251 from 290 markers (86.5%) in peanut (Liang *et al*., 2009), 10 from 12 markers (83%) in oil palm (Singh *et al*., 2008), 170 from 239 markers (71.13%) in Japanese chestnut (Nishio *et al*., 2011) and 54 from 97 (55.7%) in tea (Ma *et al*., 2012). One explanation for the low percentage of SSR product amplification might be due to the principle attributable to natural sequence variations existing within the primer binding sites of different orchid species, since the majority of EST sequences were obtained from *Dendrobium* and *Phaleanopsis* (only few sequences from *Doritis*). Another reason might be the poor quality sequence of the ESTs from which the SSR primers were designed (Park *et al*., 2010). However, 13 from 23 (56.5%) of these developed EST-SSR primer pairs shown polymorphism was acceptable as shown by previous studied, Liang *et al*., 2009 reported that 26 from 251 (10.3%) developed EST-SSR markers showed polymorphism in cultivated peanut which naturally has narrow genetic gene pool and Wei *et al*., 2008 reported that 27 from 44 markers (61.4%) showed polymorphism in sesame.

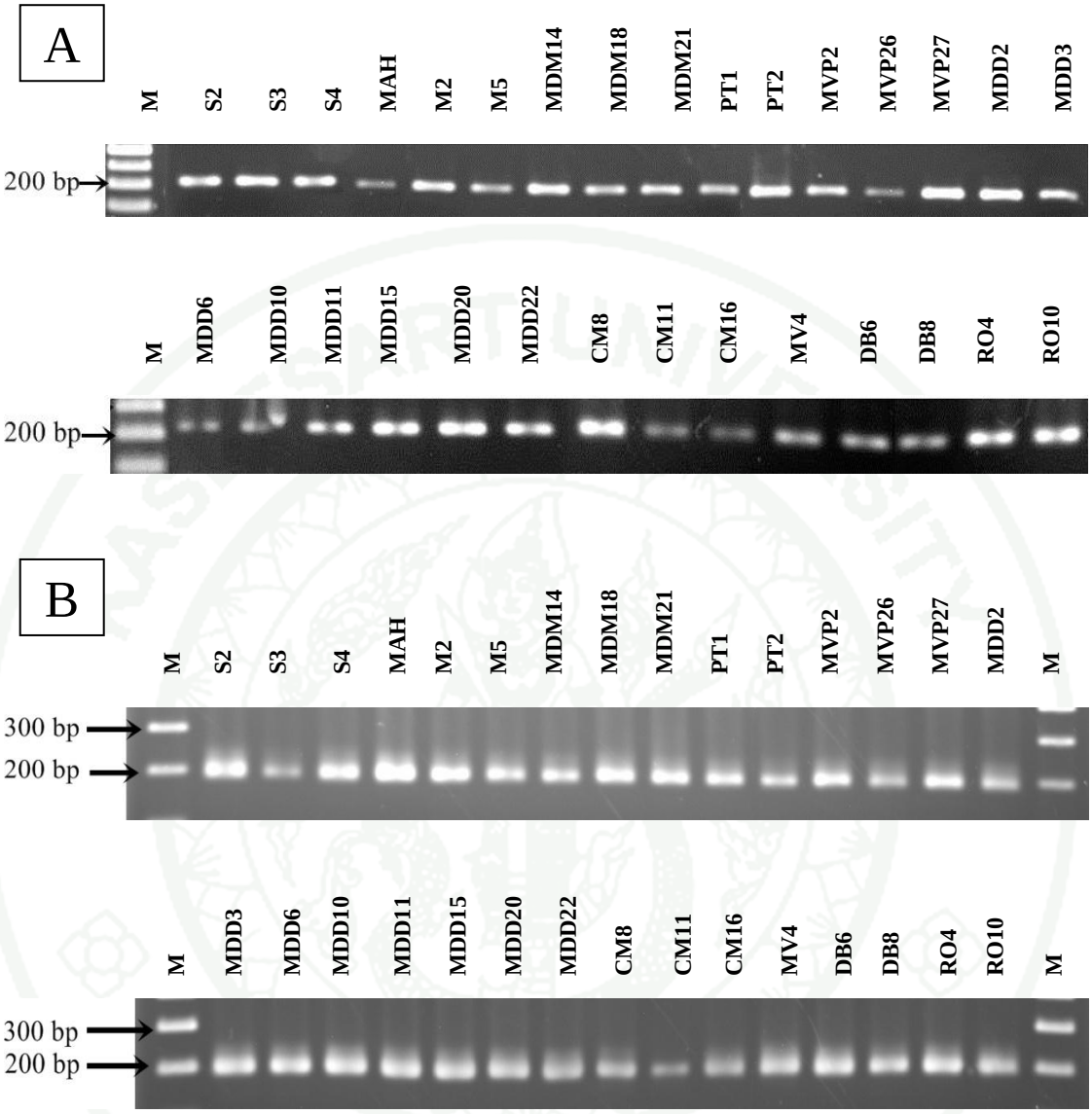

Figure 6 PCR products of some primers on agarose gel (A; Primer A11, B; Primer A26, C; Primer A32).

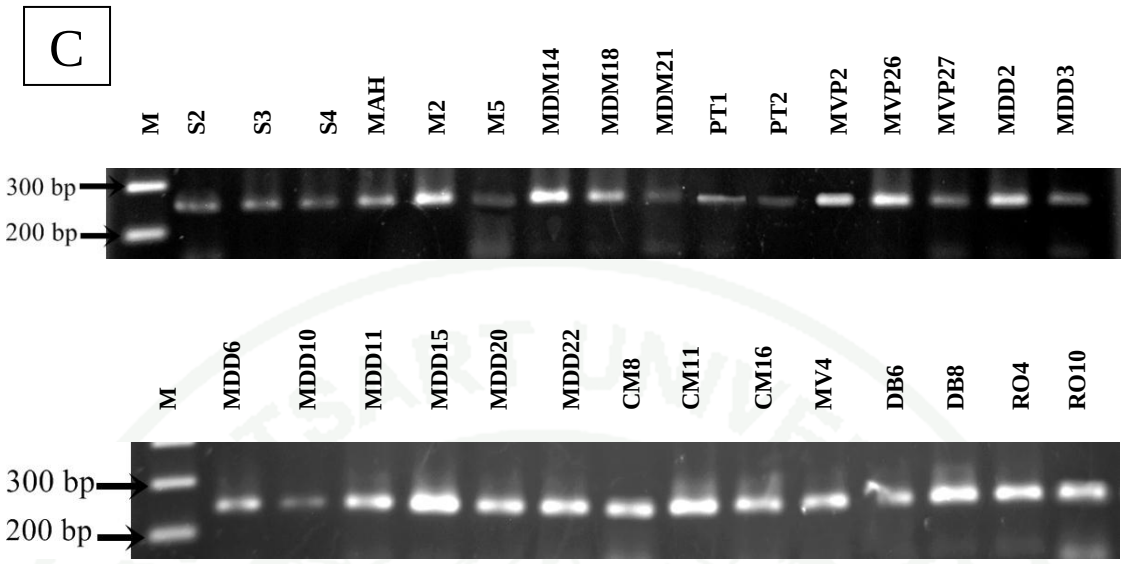

Figure 6 (Continued)

Table 3 List of EST-SSR primers that were successfully to amplified

No.	Primers name	Forword / Reverse	T_a	Product size	Mg^{2+} concentration
1	7A	TTCTTCTGTGCATCTTTTGCTC	54	120	2 mM
		CACAAACCCAATTTAACACCCT			
2	11A	CTTGATGAATGTGAGGAAACCA	62	200	2 mM
		AAACCTTACTGTTGGGCACACT			
3	15A	ACCCTTTTCTTGGCTTCTAACC	58	150	2 mM
		ATGGAGTGGATCTCTTGCTCTC			
4	26A	TGGAAATGACAAGGTTTCTGTG	62	230	2 mM
		TTGAGCTTAGGAGGCAAATAGG			
5	32A	GTCTTGGTCTGATGAACATGGA	58	250	2 mM
		TTGTTGTTGTTATTGCTACCGC			
6	47A	ATTGACGATGAAACCATGACTG	62	400	2 mM
		AGAAATACTCACCACTGCCCTG			
7	48A	TCTTTTCTCCCTCTCCATTCAG	58	350	2 mM
		AGAAGCAGCAGAACTAGAACCG			

Table 3 (Continued)

No.	Primers name	Forword / Reverse	T_a	Product size	Mg^{2+} concentration
8	2B	TGGTATGTATGTAAATGCCCCA AGACGGGCTTAATGATGTCATT	56	250	2 mM
9	9B	AAAGGAATTGGAAGAGTTGCTG AAGTCGTTGCTTCTCCCTCTC	58	250	2 mM
10	11B	GTACTCCACTCCGAACGAAAAC AGGTGAAGGCTGGATTCTTGT	52	230	3.25 mM
11	15B	CCAGGACAACCCATTTGATAAC AGCTGAGGTGGTAGAGACGAAG	56	350	3.25 mM
12	24B	GGAAGTCCAGAAAGGAAGAACA CCATCACGGTCACGATCTAAT	56	350	2 mM
13	27B	TACAAATCAAAGTCATCGTCGG CATTCCATTCATCTCTCATCCA	56	150	3.25 mM
14	28B	GCTTTCGACTCCATTACTCTGAA GAAGCAGCAGCAGAAGAGGT	56	150	3.25 mM

Table 3 (Continued)

No.	Primers name	Forword / Reverse	T_a	Product size	Mg^{2+} concentration
15	29B	AACGGAAATCCTTGAACCAGT AGCACAGAAAACAAAGCATGAG	58	100	3.25 mM
16	33B	TAATAACTGGGCAGCAAGTCG CCCCAACTCATCTCCACCT	56	200	3.25 mM
17	34B	TAATTCCAGCCACAGTCTCCTT AATCCAAATCCAACACCAACTC	58	180	3.25 mM
18	35B	AGTATCGTCAAAGCCAGGGATA TTTCCATCCTCCTCACTTCATT	50	180, 250	3.25 mM
19	36B	AGTCCCTCTCCGTCCTATGATT CTTCTTTCCAGTCATGTCCAGC	52	150	3.25 mM
20	37B	TCCATTACTCTGAAACCGTGCT ATGGCAGCAGTTGAGGTAGAAT	48	400	2 mM
21	39B	GGAAGATGATGGGTACGTCATT GCACGAGGATCAATTTAGCATA	48	150, 200	3.25 mM

Table 3 (Continued)

No.	Primers name	Forword / Reverse	T_a	Product size	Mg^{2+} concentration
22	41B	TGCCTTCTTCGGTCTTAGTCTC ATGGCAGCAGTTGAGGTAGAAT	58	180	2 mM
23	42B	AGCAATGGGAAGAAACACATCT CTCTCTCCTAACCCTCTCCCTC	48	230	3.25 mM

Transferability of genomic SSR

The 35 genomic SSR markers from *Serapias* (Pellegrino *et al*., 2001) and *Dendrobium* (Yoocha *et al*., 2006.; Yue *et al*., 2006) were check for amplification in *Doritis* using PCR gradient as described in method protocol. Then selected primers that can amplify clear bands in *Doritis* were used for genetic diversity assessment. The optimization of PCR condition showed that 7 primer pairs from a total 35 primer pairs (20%) were successful in *Doritis* DNA amplification (Figure 8) and were used for genetic diversity assessment. One primer pair belongs to *Serapias* (20C) and six pairs belong to *Dendrobium* (1C, 5C, 19C, 23C, 32C, 33C). (Table 4) One explanation for low number of successful primers may due to the long evolutionary distance between *Dendrobium* and *Serapias* and *Doritis*. The two genera are not the closely related to *Doritis*. The transferability of SSR markers depends on the genetic relatedness among species examined (including difference in DNA sequence, genome size and evolution rate) (Xie *et al*., 2010). The other explanation may due to inconsistency in the amplification of the locus for given PCR conditions and due to low intensity in the amplification of the targeted locus in some of the genotype or the amplification of unspecific fragments complicating the identification of the corresponding SSR locus (Wunsch, 2009).

Previous reports of the transferable of genomic SSR such as the studied of cross-transferable polymorphic SSR loci in *Prunus* species by Wunsch, 2009 showed that a set of 18 SSR primers were conserved or polymorphic in different *Prunus* species, and also found 13 primers can amplify. From previous studies reported that the transferability of SSR loci in different specie of the genus has been observed to range from 57% to 100% in closely related species and range from 20% to 30% in a more distantly related species. Pellegrino *et al*., 2001 reported the transferability of 6 SSR primers from *Serapias vomeraceae* (*Orchidaceae*) to other *Serapias* species and found all of six primers were successful to amplify with 4 species of *Serapias*, that shown a high transferability of genomic SSR to closely related species. Kuleung *et al*., 2004, were reported the transferability of 148 SSR markers from wheat to rye and triticale found only 17% of markers can amplified with rye samples but shown 58% in

triticale samples because of their genome relationship between wheat, rye and triticale, wheat and rye is not closely related species while triticale is a hybrid between wheat and rye, so they have close relationship in genome.

In this study, when compared between EST-SSR and genomic SSR markers, the level of transferability of EST-SSR marker (23%) was higher than genomic SSR marker (20%). A similar difference in marker transferability was observed in other plant taxa such as lettuce (Simko, 2008) almond (Tahan *et al*., 2009), cassava (Sraphet *et al*., 2011), and Helianthus (Pashley *et al*., 2006). As EST-SSR markers are developed from the coding regions of genome, they are likely more conserved in related species than genomic SSRs.(Simko, 2008) and the mutation frequency of EST sequences is lower than genomic DNA sequence (Wen *et al*., 2010).

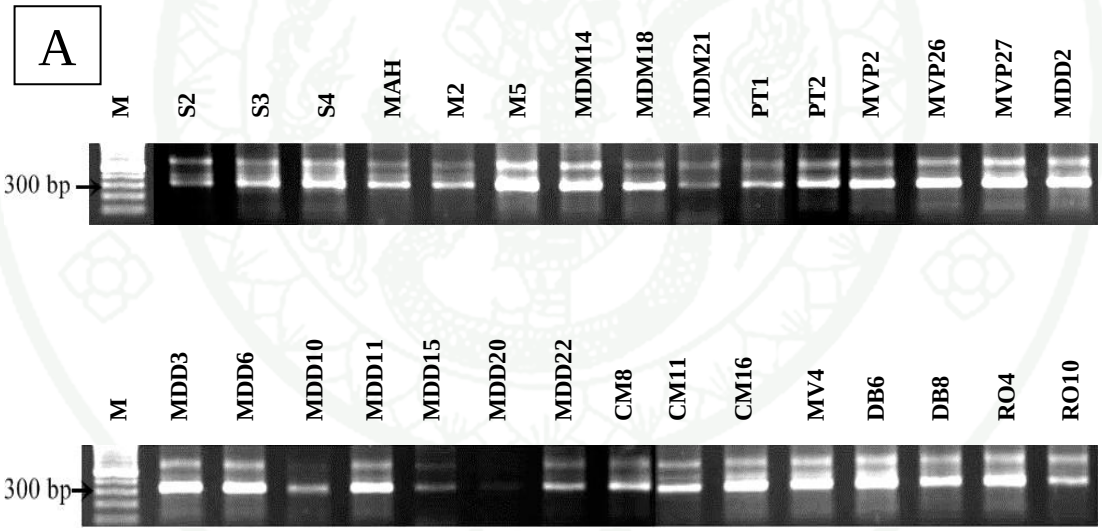

Figure 7 PCR products from primer 5C (A), 20C (B), 32C (C).

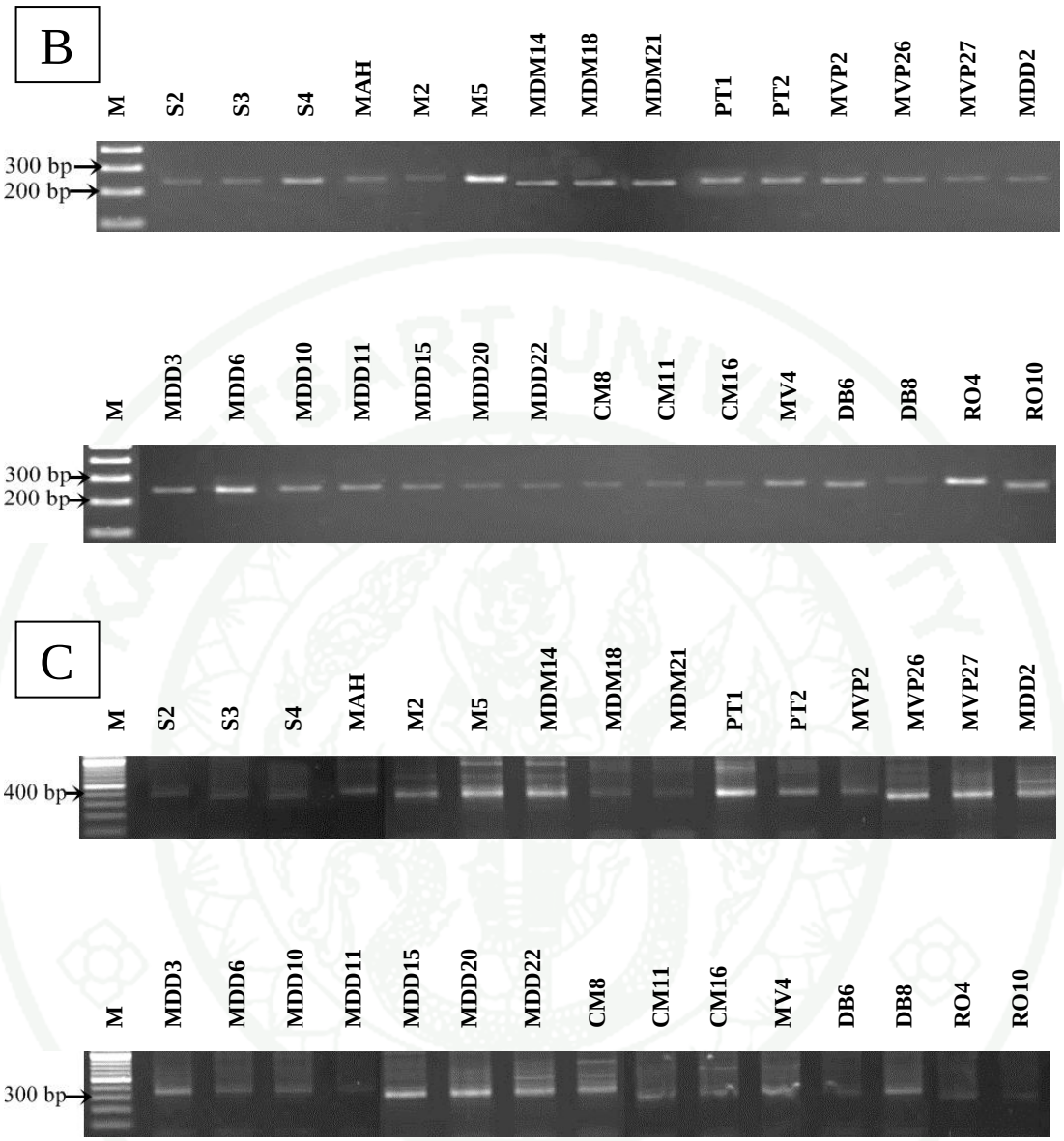

Figure 7 (Continued)

Table 4 The list of genomic SSR primers from other orchids

No.	Primers name	Forword Reverse	T_a	Product size	Mg^{2+} concentration
1	ORCA40(1C)	CAAGCTACTTCAGACTCATC GAAGACACTGCTCATATTTC	46	200	2 mM
2	ORCT6(5C)	CTCCTTGCTTGGATTTGCTTC CACCTCATCACATCTTGCCAT	42	300, 350	2 mM
3	OA20(19C)	CATATATTAGCCACTTCACTCTC ATGTCCACCTCCCTAAAATAGTA	42	200, 300	2 mM
4	SV04(20C)	CTGGTCTCTTCTTTCTGGAT AACACCAACACACATATACAT	42	250	2 mM
5	OA08(23C)	AGGCAAAATATAACATACCTCAAT AATCAAGCCATTTATCTCCTCT	42	250, 400	2 mM
6	S122(32C)	GTGACTCGAGCCTTGGAATACG ACGCCGGTGAAAGAAGAAGAG	42	350	2 mM
7	OA19(33C)	AAGGCAAACTTGAGTGCTTTATT CTTGTGATTTCTTTGCCTTTCTT	42	250	2 mM

Polymorphism of SSR markers

Thirty SSR primer pairs (23 EST-SSRs and 7 genomic SSRs) were used for genetic diversity assessment of 30 *Doritis* accessions using capillary gel electrophoresis, then score polymorphism from a presence and absence peak of graph result. The results showed that 13 of 23 EST-SSR primers (56.5%) showed polymorphism among 30 *Doritis* accessions, including 7A, 11A, 26A, 2B, 27B, 28B, 29B, 34B, 35B, 36B, 37B, 39B and 41B. (Table 6) The percentage of polymorphism from EST-SSR primers were lower than cucumber (93%) (Hu *et al*., 2010) and sesame (61.4%) (Wei *et al*., 2008) but higher than cultivated peanut (10.4%) (Liang *et al*., 2009) and Liriope (32.8%) (Li *et al*., 2011). From this studied and previous studied indicating that the transferability rate of EST-SSRs may be related to evolutionary distance between genera. The transfer rate increase slightly in more closely related species but decrease lower in distantly related species. The polymorphic rate of EST-SSR due to the conservation of coding sequences and low of mutation in transcribe region.

The polymorphic rate of genomic SSR was 100%. All 7 genomic SSR primers were revealed polymorphism among *Doritis* accessions, including 1C, 5C, 19C, 20C, 23C, 32C and 33C. (Table 6) A high percentage of polymorphic of genomic SSR was found in many plants such as 92.3% in *Prunus* species (Wunch, 2009), 78.6% in vanilla (Bory *et al*., 2008), 100% in almond (Tahan *et al*., 2009) and 100% in cucumber (Hu *et al*., 2011).

In generally, the level of transferability of EST-SSR marker was higher than genomic SSR marker because of the conserved nature of coding sequences compared with non-coding genomic DNA (coding sequences will always more conserved than non coding sequences) and the mutation frequency of EST sequences is lower than genomic DNA sequence (Wen *et al*., 2010). Thus, the polymorphic rate of genomic SSR (100%) was higher than EST-SSR (56.5%) due to the conserved sequence in transcribed region of genes (Hu *et. al.,* 2011; Wen *et al*., 2010), the low of mutation in

coding sequences and the frequencies of tandem repeat in whole genome compared with coding region of genes.

A total of 20 primers (13 EST-SSRs and 7 genomic SSRs) were generated 142 SSR and 92 alleles (64.8%) showed polymorphism among *Doritis* accessions. The number of polymorphic band was average at 4.6 alleles per locus (ranged from 2-8). The genetic diversity was measured by the polymorphic information content (PIC) and the polymorphism rate (P). The loci polymorphism can be considered high, medium or low if PIC>0.5, 0.5>PIC>0.25 and PIC<0.25 (Xie *et al*., 2010). In present study the result varied from 0.1244 (2B) to 0.8439 (20C), showed a medium PIC in EST-SSR (0.6154) and genomic SSR (0.5064) which average at 0.6076. The PIC values indicate the usefulness of DNA marker for gene mapping, molecular breeding and germplasm evaluation (Wei *et al*., 2008). The observed number of alleles per locus (A_o) ranged from 2.25 to 4.15 (mean=3.33) and the effective number of alleles per locus (A_e) ranged from 1.9227 to 3.0805 (mean=2.49). The percentage of polymorphic loci (PPB) per species was relatively high, ranged from 90% in *D. regnieriana* to 100% in *D. pulcherrima* var. *buyssoniana*) (Table 5).

These results suggested that the EST-SSR and genomic SSR markers, developed from other orchid genera, were successfully employed to measure genetic diversity and relationships within *Doritis* collection.

Table 5 Genetic diversity of 3 *Doritis* species, PB : number of polymorphic band, PPB : percentage of polymorphic band, A_o : observed number of alleles per locus, A_e : effective number of alleles per locus, H_e : expected heterozygosity, H_o : Shannon's information index.

Species	No. of SSR locus	No. of PB[a]	PPB[b]	Ao[c]	Ae[d]	He[e]	Ho[f]
D. regnieriana	20	18	90%	2.25	1.9227	0.5233	0.3500
D. pulcherrima	20	19	95%	3.60	2.4675	0.5317	0.2250
D. pulcherrima var. buyssoniana	20	20	100%	4.15	3.0805	0.6144	0.3036

Table 6 Sequences of 20 polymorphic primers, their accession numbers, repeat motif, annealing temperature, size range of products, number of alleles, PIC and protein description.

Name	Genbank accession no.	Forward / Reword	Repeat motif	T_a	size range	no. of alleles	PIC	protein description
7A	CB034531.1	TTCTTCTGTGCATCTTTTGCTC CACAAACCCAATTTAACACCCT	(A)11	54	74-85	5	0.6794	Fiber protein Fb11AAN77150.1
11A	CB032260	CTTGATGAATGTGAGGAAACCA AAACCTTACTGTTGGGCACACT	(CAA)5	62	116-119	2	0.495	Not hit
26A	C0742605.1	TGGAAATGACAAGGTTTCTGTG TTGAGCTTAGGAGGCAAATAGG	(GGAGCA)4	62	183-189	2	0.4528	Not hit
2B	CK859111.1	AGACGGGCTTAATGATGTCATT TGGTATGTATGTAAATGCCCCA	(TGG)5	56	244-247	2	0.1244	Unknown protein ACR34461.1
27B	CB031838.1	CATTCCATTCATCTCTCATCCA TACAAATCAAAGTCATCGTCGG	(CGG)5	56	127-183	6	0.6672	Abscisic stress ripening ACZ50749.1
28B	CK857691.1	GAAGCAGCAGCAGAAGAGGT GCTTTCGACTCCATTACTCTGAA	(CGC)5	56	122-129	3	0.6128	Not hit
29B	CB0343051	AGCACAGAAAACAAAGCATGAG AACGGAAATCCTTGAACCAGT	(CAC)5	58	89-98	4	0.6397	Stress responsive protein NP001149550.1

Table 6 (Continued)

Name	Genbank accession no.	Forward / Reword	Repeat motif	T_a	size range	no. of alleles	PIC	protein description
34B	CK855825.1	AATCCAAATCCAACACCAACTC TAATTCCAGCCACAGTCTCCTT	(GGC)5	58	136-165	7	0.8278	Unnamed protein product NP001174118.1
35B	CB855825.1	TTTCCATCCTCCTCACTTCATT AGTATCGTCAAAGCCAGGGATA	(AC)7	50	157-178	7	0.7836	LLA-115 ABI4885901
36B	CK856598.1	AGTCCCTCTCCGTCCTATGATT CTTCTTTCCAGTCATGTCCAGC	(GAA)6	52	100-114	6	0.7511	Hypothetical protein XP002443940.1
37B	CK859137	ATGGCAGCAGTTGAGGTAGAAT TCCATTACTCTGAAACCGTGCT	(TC)6	48	393-402	4	0.63	Not hit
39B	CK857960.1	GCACGAGGATCAATTTAGCATA GGAAGATGATGGGTACGTCATT	(AC)7	48	145-212	6	0.7328	Cleavage dioxygenase Q48K96.1
41B	CB034609	ATGGCAGCAGTTGAGGTAGAAT TGCCTTCTTCGGTCTTAGTCTC	(CCG)6	58	194-200	3	0.6039	Not hit

Table 6 (Continued)

Name	Genbank accession no.	Forward / Reword	Repeat motif	T_a	size range	no. of alleles	PIC	protein description
1C	ORCA40 (Yoocha *et al*., 2006)	CAAGCTACTTCAGACTCATC GAAGACACTGCTCATATTTC	F(TG)8CA(TGCG)12, R(TG)10(TGCG)6	46	200-209	4	0.6639	-
5C	ORCT6 (Yoocha *et al*., 2006)	CTCCTTGCTTGGATTTGCTTC CACCTCATCACATCTTGCCAT	(TC)5(GT)18	42	250-262	6	0.6533	-
19C	OA20 (Yue *et al*., 2006)	CATATATTAGCCACTTCACTCTC ATGTCCACCTCCCTAAAATAGTA	data not shown	42	321-419	4	0.5828	-
20C	SV04 (Pellegrino *et al*., 2001)	CTGGTCTCTTCTTTCTGGAT AACACCAACACACATATACAT	(CT)8AC(CT)5	42	284-305	8	0..8439	-
23C	OA08 (Yue *et al*., 2006)	AGGCAAAATATAACATACCTCAAT AATCAAGCCATTTATCTCCTCT	data not shown	42	200-289	3	0.2886	-
32C	S122 (Yue *et al*., 2006)	GTGACTCGAGCCTTGGAATACG ACGCCGGTGAAAGAAGAAGAG	F(TC)9(AC)4 ,RAT(AC)8	42	333-358	6	0.7889	-
33C	OA19 (Yue *et al*., 2006)	AAGGCAAACTTGAGTGCTTTATT CTTGTGATTTCTTTGCCTTTCTT	(GA)31	42	260-374	4	0.5672	-

Genetic diversity and phylogenetic analysis

The expected heterozygosity (H_e) of *Doritis* samples ranged from 0.5233 to 0.6194 (mean=0.558), which *D. pulcherrima* var. *buyssoniana* showed the highest level of H_e and *D. regnieriana* showed the lowest. *D. pulcherrima* var. *buyssoniana* exhibits the greatest level of variability (PPB: 100%, H_e: 0.6194, H_o: 1.1235, respectively), whereas the *D. regnieriana* exhibit the lowest level of variability (PPB: 90%, H_e: 0.5233, H_o: 0.6768, respectively) as shown in Table 5. The AMOVA result revealed 10.59% and 89.408% variation presented among and within species (*D. regnieriana, D. pulcherrima and D. pulcherrima* var. *buyssoniana*) (Table 7) respectively and fixation indices from AMOVA was 0.106 ($P<0.001$).

A UPGMA dendrogram, inferred from the shared allele frequencies, based on dice's similarity, for generated genetic relationship among 30 accessions (Figure 9), revealed 3 main groups distinguished by species and varieties. Three accessions of *D. regnieriana* were grouped together, twelve accessions of *D. pulcherrima* were grouped together and fifteen accessions of *D. pulcherrima* var. *buyssoniana* were also grouped together. *D. regnieriana* group was placed closer to *D. pulcherrima* than to *D. pulcherrima* var. *buyssoniana*. This result was in agreement with their chromosome studied as both *D. regnieriana* and *D. pulcherrima* are diploid species, 2n=2x=38 but *D. pulcherrima* var. *buyssoniana* is a tetraploid species, 2n=4x=76. *D. pulcherrima* and *D. pulcherrima* var. *buyssoniana* were placed next to each other as they both are the same species (Christenson, 2001). Both species have similar characteristics with the tetraploid exhibit larger structure (Kamemoto and Sakarik, 1975). Also inconsistent with Nei's unbiased genetic distance between species showed the highest level between *D. regnieriana* and *D. pulcherrima* (0.3036) and the lowest level between *D. pulcherrima* and *D. pulcherrima* var. *buyssoniana* (0.1739) (Table 8), the result showed that *D. pulcherrima* and *D. pulcherrima* var. *buyssoniana* was closed to each other (0.1739) while both were more distance with *D. regnieriana*. The principle coordinate analysis (PCA) was separated 30 accessions into 3 major groups based on their taxonomy, which was consistent with the result of phylogenetic analysis (Figure 10).

Table 7 Analysis of molecular variance (AMOVA) of *Doritis* germplasm

source of variation	sum of squares	variance component	percentage variation	P-value
Among species	35.2420	0.68184	10.5900	<0.001
within species	328.092	5.75590	89.4082	<0.001
Total	363.333	6.43790		

Table 8 Nei's unbiased genetic distance among 3 *Doritis* species

Species	*D. regnieriana*	*D. pulcherrima*	*D. pulcherrima var. buyssoniana*
D. regnieriana	0.0000		
D. pulcherrima	0.3036	0.0000	
D. pulcherrima var. buyssoniana	0.2623	0.1739	0.0000

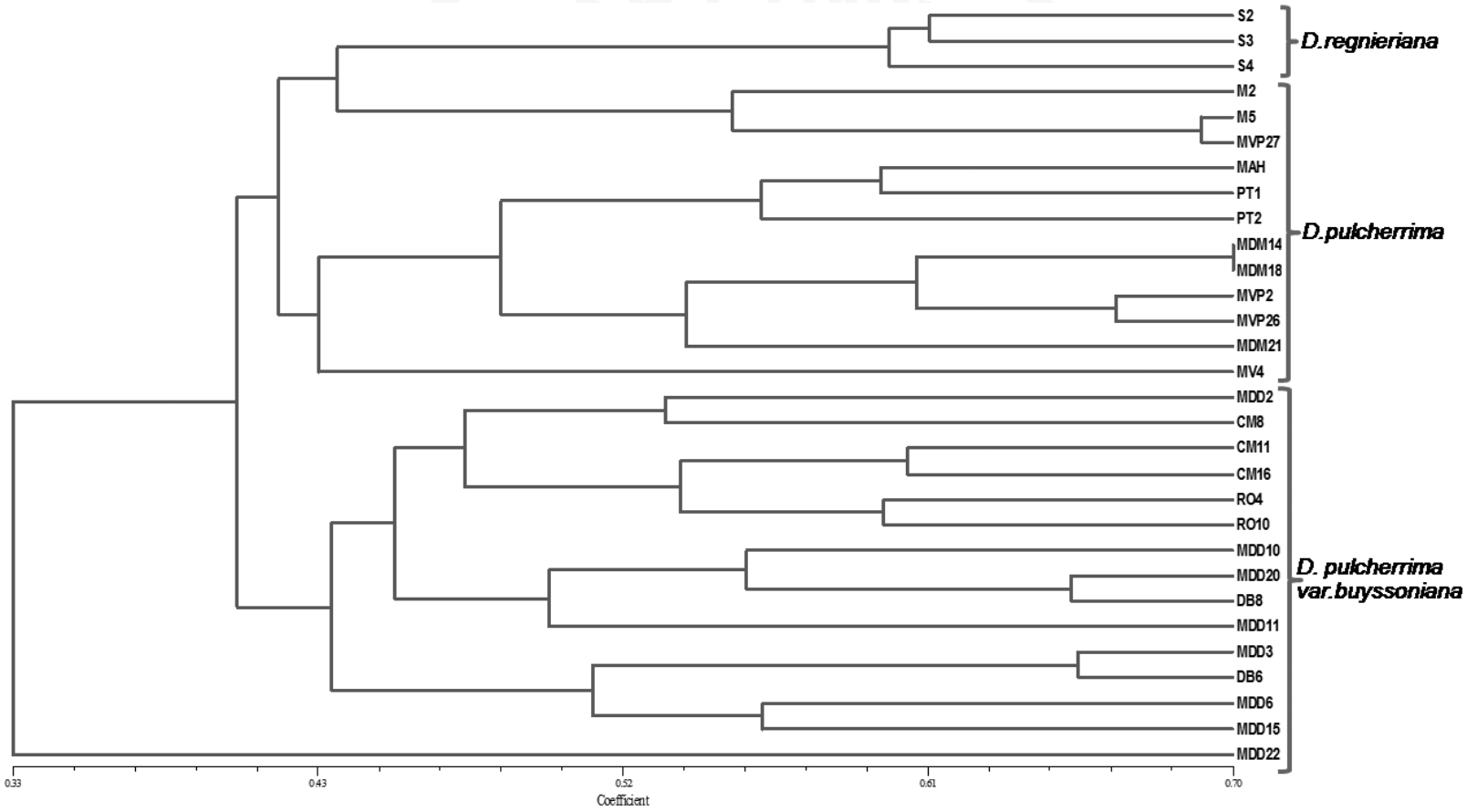

Figure 8 Phylogenetic trees of 30 *Doritis* accessions based on Dice's similarity

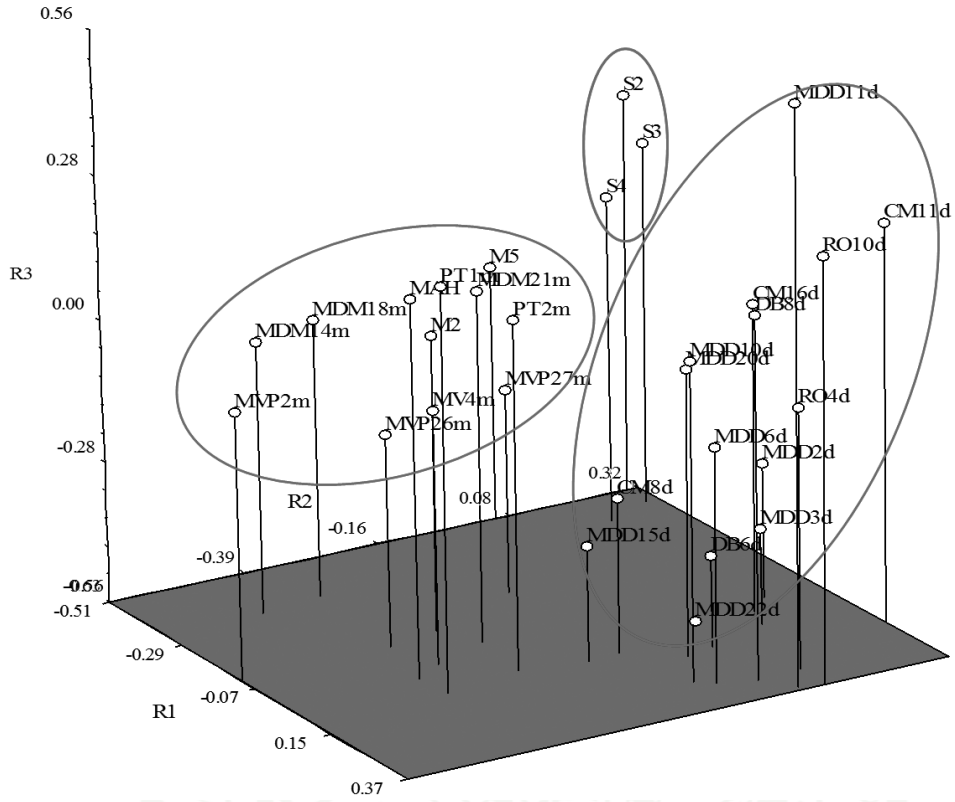

Figure 9 Principles coordinate analysis for SSR markers using the genetic similarity matrix for 30 *Diritis* accessions

CONCLUSION

The development of EST-SSR marker and determine genetic diversity for *Doritis* was started by extracted genomic DNA from their leave tissue. CTAB method showed the highest DNA quality, and then amplified with two groups of primers; first group was the genomic SSR primers of other orchid species and second group was the EST-SSR primers, developed from orchid EST database. The results showed the transferability rate of EST-SSR (23%) was higher than genomic SSR (20%) but the polymorphic rate of genomic SSR (100%) was relatively higher than EST-SSR (56.5%). Because of the nature of coding sequences are more conserved than non coding sequences and conserve in closely related species.

The genetic diversity and relationships within 30 *Doritis* accessions were generated using UPGMA method. The results revealed 3 main groups distinguished by species and variet. Three accessions of *D. regnieriana* were grouped together, twelve accessions of *D. pulcherrima* were grouped together and fifteen accessions of *D. pulcherrima* var. *buyssoniana* were also grouped together.

The result revealed that EST-SSR and genomic SSR markers, developed from other orchid genera, were successfully employed to measure genetic diversity and relationships within *Doritis* collection. Future studies with these SSR markers could be useful for identification, conservation and will selection of appropriate parents for the hybrid produce

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APPENDIX

Appendix Table 1 Primer sequences of 100 EST-SSR markers

No.	Names	Sequences
1	ORVDM1-F	CCACGGTTGTATGTTCCTGTTT
2	ORVDM1-R	AACAAAGCACTAGGCAAAGGC
3	ORESTM5-F	CAAACCATAGAGCGGTGGAT
4	ORESTM5-R	CCTTAGCCCAATTTATCGAA
5	ORESTM17-F	TTTCCATCTCTACTTCGTGCG
6	ORESTM17-R	ATTCTACCTCAACTGCTGCCAT
7	ORESTM32-F	GGTGGATGTTAACTTTGGTTGC
8	ORESTM32-R	CACGTTTAAACCCGCCAA
9	ORESTM45-F	CTTTCAATGGGGATAAAAGCAC
10	ORESTM45-R	AAACCAGAAACATTTCACCCC
11	ORESTM49-F	TTTGTTGGTGTTTTGCAGTAGG
12	ORESTM49-R	TGGGATTTGATACAGTGAGAGC
13	ORESTM59-F	TTCTTCTGTGCATCTTTTGCTC
14	ORESTM59-R	CACAAACCCAATTTAACACCCT
15	ORESTM94-F	CTGCTTCCCAAGTGATTCTTCT
16	ORESTM94-R	GGTCAGGCATTTCAACAAAAC
17	ORESTM109-F	CGGTCTTCTTCTATCGCATTTC
18	ORESTM109-R	AATTCCCTTGTCATGTTGGTCT
19	ORESTM115-F	AGGAGGAGATGGAAGAGTTGAT
20	ORESTM115-R	GGCAAGAAACAAAGCAAAAG
21	ORESTM127-F	CTTGATGAATGTGAGGAAACCA
22	ORESTM127-R	AAACCTTACTGTTGGGCACACT
23	ORESTM129-F	CAGAACCCTCTTTCCCCTACTT
24	ORESTM129-R	AAACTGCATAACTCCCACACAA
25	ORESTM130-F	GAGGCCTCGTGCCGAATT
26	ORESTM130-R	CGAGAATGTACCGGAAAACCTG
27	ORESTM133-F	AAGCGAGAGAGAGAGAGAACGA
28	ORESTM133-R	ATGAGGAGAAGGTAATGGGGAA

Appendix Table 1 (Continued)

No.	Names	Sequences
29	ORESTM136-F	ACCCTTTTCTTGGCTTCTAACC
30	ORESTM136-R	ATGGAGTGGATCTCTTGCTCTC
31	ORESTM140-F	CACTGAGAGGAAGAAAGGTACAAAG
32	ORESTM140-R	ATAACAGAGGAGGATTGCGAGA
33	ORVAESTM2-F	AATTCGATTAGTCAGCCACCAA
34	ORVAESTM2-R	ATTTGAGCGTCCGTTGAAGTC
35	ORVAESTM1-F	CATGGGCAACAAGAGTGAAAC
36	ORVAESTM1-R	AAATAGGCAAACACTGGCCTT
37	ORDBM5-F	AAAAGAAGCTGAGAGAGAAGTGG
38	ORDBM5-R	GAGTGGAGAAGACAATTAGGGC
39	ORPHESTM1-F	TCTTCCATCATCGCACATACTC
40	ORPHESTM1-R	GCACAAGACCAATAAGGAAAGG
41	ORPHESTM3-F	GCGGTATCGAACTTGAATGG
42	ORPHESTM3-R	AAGAATAACAAGCAGTCGAGCC
43	ORPHESTM9-F	CATTGCAATGCCTCCACTATAA
44	ORPHESTM9-R	CCTTTAAGCCCATTTACCCCTA
45	ORPHESTM12-F	CTAGCCTGCAAGAAGGGC
46	ORPHESTM12-R	CAAGAGGCACTAAATCCAGACA
47	ORPHESTM13-F	CACGAGGAACAAAAGAAGGAA
48	ORPHESTM13-R	CTAAATCTCAACAAAGCCCTGC
49	ORPHESTM14-F	CCCACCGACAAATTCATACTT
50	ORPHESTM14-R	TAAGAGAGGTTGGAGAGAAGGG
51	ORPHESTM16-F	TGGAAATGACAAGGTTTCTGTG
52	ORPHESTM16-R	TTGAGCTTAGGAGGCAAATAGG
53	ORPHESTM23-F	CTAGCCTGCAAGAAGGGC
54	ORPHESTM23-R	CGGTACAACAAGAGGCACTAAA
55	ORPHESTM27-F	AGTGAAGGTTTTGAGGAAAGGC
56	ORPHESTM27-R	CATAGGGATTGGTCCGGC

Appendix Table 1 (Continued)

No.	Names	Sequences
57	ORPHESTM30-F	TTTCCATCTCTACTTCGTGCG
58	ORPHESTM30-R	GTGGGGTTTCTAGGGTTTCTTC
59	ORPHESTM44-F	TCTACTGGATTTTGGATGGGCA
60	ORPHESTM44-R	GGGTTCAAGGGAAGCCCC
61	ORDBM8-F	GCCTCTCTCTTTCTCTCACTGC
62	ORDBM8-R	GAAGAATCCCCAGTTCTCACAG
63	ORESTM8-F	GTCTTGGTCTGATGAACATGGA
64	ORESTM8-R	TTGTTGTTGTTATTGCTACCGC
65	ORESTM19-F	GACTAAGACTGAAGAAGGCAGCA
66	ORESTM19-R	ATTTCCAGAAGCCAATACTCCA
67	ORESTM24-F	TAAAACCAGATCCTCGTCCATA
68	ORESTM24-R	CCCCAACATTAAAAGGGAAT
69	ORESTM28-F	CTAACCATGCCAAACGATGATA
70	ORESTM28-R	AACGTCCTTCCCAATAGCATAA
71	ORESTM30-F	CGAATTCGGCACGAGGAG
72	ORESTM30-R	GAAGAAGAACCTTGTAATGGCG
73	ORONM1-F	AGTAACATCAGGCGCTTCAACT
74	ORONM1-R	GCTTGGGAAATGACTAAACTGG
75	ORCAM1-F	CGACCATTCTAAGCAGCAACTA
76	ORCAM1-R	GCCGGTAATTTTCTTTGTTCAC
77	ORDBM2-F	CTTATTGTCGGCATCATCCTTT
78	ORDBM2-R	ATCATTTGACACCGCTAAACCT
79	ORDBM3-F	AAGTTAGCGGGGATGCTGAT
80	ORDBM3-R	GCCTTAGCTGTTCAAATTCTTCC
81	ORDBM4-F	CCTTACTTGTCCCGACTCCTTA
82	ORDBM4-R	GGAAGAGAAGACACAGCCAAAG
83	ORDBM7-F	AGATAAATTGCTCCCTTCCCC
84	ORDBM7-R	GAGGTCGTCTTCTTCCTCTGAA

Appendix Table 1 (Continued)

No.	Names	Sequences
85	ORESTM4-F	CCATGAGATCCCCATAGTTGTT
86	ORESTM4-R	CCGGGTAACCAGGGTTTT
87	ORESTM7-F	ATGAGTTTATGCTTCATCGGGT
88	ORESTM7-R	GCGGCCAGTAATTGTATAGCTC
89	ORESTM22-F	TCAGTTGGTTCCTTAATTGGCT
90	ORESTM22-R	GCAAGGTATGCCAGAGACTTTT
91	ORESTM26-F	CACTGGATCACCTATCACGGTA
92	ORESTM26-R	TAGTTCCAAATAAAAGGCCCC
93	ORESTM27-F	ATTGACGATGAAACCATGACTG
94	ORESTM27-R	AGAAATACTCACCACTGCCCTG
95	ORESTM35-F	TCTTTTCTCCCTCTCCATTCAG
96	ORESTM35-R	AGAAGCAGCAGAACTAGAACCG
97	ORESTM38-F	AGATTTCCCAAGACATGCAGTT
98	ORESTM39-R	CGGGATAAATTCCTACATCCAC
99	ORESTM42-F	GGTCTTCAACAATAGGCAGTGA
100	ORESTM42-R	TGGTAGCTCGCATTTAAGGAAT
101	CL244CONTIG1-R	TCTCTGAAACGAAAAGCGAAG
102	CL244CONTIG1-F	CATCGGTGGAGAAAACAGAAG
103	CL296CONTIG1-R	AGACGGGCTTAATGATGTCATT
104	CL296CONTIG1-F	TGGTATGTATGTAAATGCCCCA
105	GI|110664761-R	CCCCAAAATCTGGGATCTC
106	GI|110664761-F	GGTTGAACGGAACTTGATAAGC
107	GI|110663516-R	ATCTGAAGCCATTGATAACCGA
108	GI|110663516-F	AGAAATACTCACCACTGCCCTG
109	GI|110664470-R	TACGAGTCATTTTGCCCTTTG
110	GI|110664470-F	TTCGGATAAAAGCGAAGGAA
111	GI|109154407-R	TCAGATTCTTCTTGGTCGTGAG
112	GI|109154407-F	GCTGGTGGAGACCCTGAA

Appendix Table 1 (Continued)

No.	Names	Sequences
113	GI|109151992-R	CCAGGATCAAATCTTCCTTGAG
114	GI|109151992-F	CAACACATAGACGAACTCCATCA
115	GI|109151206-R	GCAAATGAACTGAAGTGGGTTA
116	GI|109151206-F	CAAGCATATAGCACACCACACA
117	CL195CONTIG1-R	AAGTCGTTGCTTCTCCCTCTC
118	CL195CONTIG1-F	AAAGGAATTGGAAGAGTTGCTG
119	CL141CONTIG1-R	TCGAGATATGTTCTGGGGTCTT
120	CL141CONTIG1-F	AGACTGCCCTCATATATAACAGCA
121	CL491CONTIG1-R	AGGTGAAGGCTGGATTCTTGT
122	CL491CONTIG1-F	GTACTCCACTCCGAACGAAAAC
123	GI|109154284-R	GTCTGCAACATACTCAAGCATAGC
124	GI|109154284-F	CTTTTGGTCTTTCCCATCCTTT
125	GI|109152173-R	ATCACGGTCTTTGGCGAT
126	GI|109152173-F	GGAAGTCCAGAAAGGAAGAACA
127	GI|109153360-R	AAAGGAACTATTATTGGCGGCT
128	GI|109153360-F	ATATGGCTTGGACTGATCGC
129	GI|109152185-R	AGCTGAGGTGGTAGAGACGAAG
130	GI|109152185-F	CCAGGACAACCCATTTGATAAC
131	CL140CONTIG1-R	TGAGGAATTCCGCACGAG
132	CL140CONTIG1-F	CAAATAGGCGGCGATTTATTAG
133	CL201CONTIG1-R	GCAAATGAACTGAAGTGGGTTAG
134	CL201CONTIG1-F	GCATAACAGTCGGAAGCATACA
135	CL258CONTIG1-R	GGAGCATCTAGGAGTTGGAAGA
136	CL258CONTIG1-F	AGACGAGTTCATTTGCATTGG
137	GI|110663306-R	GAAGCAGCAGCAGAAGAGGT
138	GI|110663306-F	GACTCCATTACTCTGAAACCGTG
139	GI|110663752-R	TGGCGGGTCTTCAACAATA
140	GI|110663752-F	GCCCTGTTTGGATCACTTTCTA

Appendix Table 1 (Continued)

No.	Names	Sequences
141	CL11CONTIG3-R	GATTTGGTTTTGCTGATAAGGC
142	CL11CONTIG3-F	TTTACTCGCCAGAGAGAGCAG
143	CL128CONTIG1-R	GCATACCCAGAATGAAGAGGAC
144	CL128CONTIG1-F	ACACATCAGCAACAAGGTCAAT
145	CL163CONTIG1-R	TTTGAGAGGAAGTTTGTGTCCC
146	CL163CONTIG1-F	GGTTTGAAGAACAGCAACATGA
147	CL186CONTIG1-R	CCATCACGGTCACGATCTAAT
148	CL186CONTIG1-F	GGAAGTCCAGAAAGGAAGAACA
149	CL295CONTIG1-R	TAAAAGGAGCAAGGCGAGATAG
150	CL295CONTIG1-F	GGCTGTAGATGACTGGGAGAAG
151	CL340CONTIG1-R	CAAATCTTAACTTCTGTCCCCG
152	CL340CONTIG1-F	CATGCTATCCACTCCACCATTA
153	CL455CONTIG1-R	CATTCCATTCATCTCTCATCCA
154	CL455CONTIG1-F	TACAAATCAAAGTCATCGTCGG
155	GI|109151933-R	GAAGCAGCAGCAGAAGAGGT
156	GI|109151933-F	GCTTTCGACTCCATTACTCTGAA
157	GI|109153057-R	AGCACAGAAAACAAAGCATGAG
158	GI|109153057-F	AACGGAAATCCTTGAACCAGT
159	GI|109152891-R	GGACCTTCGTCACTCACTCTTT
160	GI|109152891-F	TCTTCCCCTGTACCTTCTTTGA
161	GI|110663257-R	GAAGCAGCAGCAGAAGAGGT
162	GI|110663257-F	GACTCCATTACTCTGAAACCGTG
163	GI|110663905-R	CGGGTCTTCAACAATAGGCA
164	GI|110663905-F	GCCCTGTTTGGATCACTTTCTA
165	GI|109153889-R	CCCCAACTCATCTCCACCT
166	GI|109153889-F	TAATAACTGGGCAGCAAGTCG
167	GI|109154341-R	AATCCAAATCCAACACCAACTC
168	GI|109154341-F	TAATTCCAGCCACAGTCTCCTT

Appendix Table 1 (Continued)

No.	Names	Sequences
169	CL106CONTIG1-R	TTTCCATCCTCCTCACTTCATT
170	CL106CONTIG1-F	AGTATCGTCAAAGCCAGGGATA
171	CL275CONTIG1-R	AGTCCCTCTCCGTCCTATGATT
172	CL275CONTIG1-F	CTTCTTTCCAGTCATGTCCAGC
173	CL10CONTIG1-R	ATGGCAGCAGTTGAGGTAGAAT
174	CL10CONTIG1-F	TCCATTACTCTGAAACCGTGCT
175	CL96CONTIG1-R	TGAAACCCTAACTAGCCGCC
176	CL96CONTIG1-F	TATACCGCCTACCAATCAATCG
177	CL106CONTIG1-R	GCACGAGGATCAATTTAGCATA
178	CL106CONTIG1-F	GGAAGATGATGGGTACGTCATT
179	CL117CONTIG1-R	TGCAAGAAACCTTAGAAGCTCA
180	CL117CONTIG1-F	CTAGGAGAACAAAAGGGGAGGT
181	CL4CONTIG1-R	ATGGCAGCAGTTGAGGTAGAAT
182	CL4CONTIG1-F	TGCCTTCTTCGGTCTTAGTCTC
183	CL32CONTIG1-R	CTCTCTCCTAACCCTCTCCCTC
184	CL32CONTIG1-F	AGCAATGGGAAGAAACACATCT
185	CL39CONTIG2-R	CGCTGAGGAGAAGCACTATTTT
186	CL39CONTIG2-F	GTGGACGGTGTGGTCATAATC
187	CL61CONTIG1-R	AATCCAAATCCAACACCAACTC
188	CL61CONTIG1-F	AGCACACAGAACGAAAGAAACA
189	CL62CONTIG1-R	CAGCACAGAAAAGAAAGCATGA
190	CL62CONTIG1-F	TTCGGTCCACAGTATAGATTCGT
191	CL138CONTIG1-R	AATTTCAGTACCATTGCGATCC
192	CL138CONTIG1-F	TCCGTATTTTGAGCCTTCAGTT
193	CL295CONTIG1-R	CGCCAATCGTCTCTCTTCTATT
194	CL295CONTIG1-F	GTTTTCCTCTTCTTGCCTCTCC
195	GI|110663752-R	TGGCGGGTCTTCAACAATA
196	GI|110663752-F	TGGTAGCTCGCATTTAAGGAAT

Appendix Table 1 (Continued)

No.	Names	Sequences
197	CL17CONTIG3-R	GTGAGGAAACCACCATGTATGA
198	CL17CONTIG3-F	AAACCATCCAGCAAAGACAAAC
199	GI|109154809-R	CCTCGTGCCGAATTCGGC
200	GI|109154809-F	AAACTTGGGCTTCGCCTTTTCC

Appendix Table 2 Bands scoring of 92 loci of 30 *Doritis* accessions

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
1	0	1	0	1	1	1	1	0	0	1	1	0	0	1	0
2	1	0	1	0	0	0	1	1	1	0	0	1	1	0	1
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	0	0	0	0	1	1	1	1	1	1	1	0	1	0
5	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
6	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1
7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
9	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1
10	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
13	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0
14	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	1	1	0	1	1	1	1	0	1	1	1	0
17	1	1	1	0	0	1	0	0	0	0	1	0	0	0	1

Appendix Table 2 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
20	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1
23	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
24	1	0	0	1	0	0	1	0	0	1	1	1	0	1	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
27	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
28	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	1	1	1	0	1	1	0	0	0	1	1	0	0
31	1	1	0	0	0	1	0	0	0	0	0	0	0	1	1
32	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Appendix Table 2 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
35	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
36	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1
37	0	1	0	0	0	1	0	0	0	0	1	1	0	1	0
38	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0
39	0	1	1	0	0	0	1	1	1	0	1	1	1	1	0
40	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1
41	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0
42	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0
43	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1
44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0
46	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
47	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
50	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
51	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1

Appendix Table 2 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
52	0	0	1	0	0	0	1	1	1	0	0	1	1	0	1
53	1	1	0	0	1	1	0	0	0	0	0	0	0	1	0
54	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0
57	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
58	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0
59	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
60	1	1	1	1	0	0	1	0	1	0	0	0	0	0	0
61	0	1	0	1	0	0	1	1	1	1	1	1	1	1	0
62	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0
63	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0
64	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
65	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0
66	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1
67	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0
68	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix Table 2 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
69	1	1	1	0	0	1	0	1	0	0	0	0	0	0	0
70	0	0	0	1	0	0	0	0	1	1	1	1	1	0	0
71	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1
72	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0
73	0	0	0	1	0	0	1	1	1	0	0	1	0	0	0
74	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1
75	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
76	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
77	0	0	0	0	1	1	1	1	1	0	0	0	0	1	0
78	1	1	1	1	0	0	0	0	0	1	1	0	0	0	1
79	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
81	1	1	1	0	0	1	0	0	1	0	1	0	0	1	0
82	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0
83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
84	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0

Appendix Table 2 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26	MVP27	MDD2
85	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
86	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
87	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
88	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1
89	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0
90	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
92	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
1	0	0	1	1	0	1	0	1	1	1	0	0	1	1	1
2	1	1	0	0	1	0	1	0	0	0	1	1	0	0	0
3	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
4	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1
5	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
6	0	0	1	1	0	1	0	1	1	0	0	0	1	1	1
7	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0
8	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
9	1	1	1	0	1	1	0	1	0	1	0	0	1	1	1
10	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
11	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
12	1	0	0	0	0	1	1	1	1	1	0	0	1	0	0
13	0	1	0	0	1	0	0	0	0	1	1	1	0	1	0
14	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1
15	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
16	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0
17	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0
20	1	1	0	0	0	0	0	0	1	0	0	1	1	1	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
22	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1
23	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
24	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
25	1	0	0	0	1	0	0	0	0	0	0	1	0	1	1
26	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
27	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
29	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
30	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0
31	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0
32	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
33	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
34	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36	1	1	1	1	0	1	0	0	1	1	1	0	1	1	1
37	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0
40	0	0	0	1	0	0	0	0	1	1	0	0	0	1	1
41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	0	1	0	1	1	1	0	0	0	0	0	1	1	0	1
43	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0
44	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0
45	0	0	1	1	0	1	1	0	0	1	0	1	1	0	0
46	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1
47	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
49	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
52	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1
53	1	0	0	1	0	1	0	0	1	1	1	1	0	1	0
54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
55	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0
56	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
59	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
61	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64	1	1	1	0	0	0	1	0	1	1	0	1	1	1	1
65	0	0	1	0	0	0	0	1	1	1	0	0	1	1	1
66	1	1	0	0	1	1	0	0	0	0	0	1	1	1	0
67	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
68	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	1	0	1	1	0	0	0	0	0	1	0	1	1	1
71	1	0	1	0	0	1	1	1	1	1	0	1	0	0	0
72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
73	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
74	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0
75	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
76	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
77	0	1	1	1	0	0	1	0	0	1	0	1	0	0	1
78	1	0	0	0	0	0	0	1	1	1	0	1	1	1	1
79	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0
80	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
81	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
82	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
83	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0
84	0	1	0	1	0	0	0	1	1	1	0	0	0	1	0
85	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Appendix Table 2 (Continued)

No.	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4	DB6	DB8	RO4	RO10
86	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
87	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
88	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0
89	1	0	0	0	0	0	0	0	1	1	0	1	1	1	1
90	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
91	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
92	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0

Appendix Table 3 Dice's similarity matrix of 30 Doritis accessions generated from SSR analysis.

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26
S2	1												
S3	0.61	1											
S4	0.59	0.61	1										
MAH	0.41	0.40	0.37	1									
M2	0.38	0.30	0.35	0.46	1								
M5	0.55	0.57	0.40	0.27	0.53	1							
MDM14	0.45	0.44	0.45	0.59	0.58	0.51	1						
MDM18	0.42	0.45	0.56	0.49	0.43	0.48	0.70	1					
MDM21	0.38	0.42	0.50	0.38	0.22	0.29	0.54	0.51	1				
PT1	0.39	0.35	0.39	0.60	0.44	0.33	0.47	0.48	0.40	1			
PT2	0.38	0.53	0.35	0.54	0.35	0.41	0.42	0.39	0.43	0.58	1		
MVP2	0.37	0.33	0.41	0.64	0.46	0.39	0.67	0.60	0.54	0.60	0.58	1	
MVP26	0.33	0.36	0.53	0.44	0.42	0.35	0.57	0.58	0.56	0.48	0.42	0.67	1
MVP27	0.47	0.54	0.33	0.39	0.57	0.69	0.62	0.48	0.41	0.46	0.53	0.51	0.48
MDD2	0.38	0.38	0.54	0.38	0.30	0.45	0.35	0.35	0.26	0.31	0.30	0.33	0.37
MDD3	0.41	0.37	0.52	0.37	0.46	0.40	0.48	0.42	0.42	0.35	0.35	0.41	0.53

Appendix Table 3 (Continued)

No.	S2	S3	S4	MAH	M2	M5	MDM14	MDM18	MDM21	PT1	PT2	MVP2	MVP26
MDD6	0.33	0.32	0.33	0.31	0.37	0.35	0.36	0.41	0.41	0.42	0.49	0.39	0.43
MDD10	0.30	0.44	0.34	0.49	0.47	0.36	0.49	0.42	0.38	0.48	0.51	0.49	0.36
MDD11	0.33	0.36	0.24	0.36	0.28	0.22	0.24	0.29	0.51	0.48	0.33	0.31	0.25
MDD15	0.30	0.44	0.30	0.33	0.26	0.44	0.38	0.31	0.43	0.39	0.55	0.57	0.45
MDD20	0.27	0.42	0.38	0.38	0.39	0.33	0.42	0.47	0.35	0.40	0.48	0.42	0.33
MDD22	0.26	0.33	0.37	0.28	0.25	0.20	0.37	0.42	0.46	0.21	0.25	0.40	0.36
CM8	0.25	0.43	0.47	0.47	0.37	0.35	0.51	0.44	0.29	0.50	0.49	0.51	0.48
CM11	0.48	0.44	0.52	0.48	0.38	0.35	0.37	0.34	0.25	0.43	0.38	0.32	0.31
CM16	0.53	0.52	0.46	0.45	0.51	0.52	0.46	0.50	0.31	0.56	0.47	0.38	0.42
MV4	0.38	0.29	0.30	0.42	0.44	0.41	0.38	0.39	0.39	0.50	0.34	0.51	0.47
DB6	0.38	0.44	0.34	0.41	0.35	0.44	0.45	0.49	0.50	0.39	0.42	0.41	0.41
DB8	0.33	0.46	0.44	0.55	0.37	0.31	0.44	0.44	0.49	0.50	0.57	0.47	0.48
RO4	0.41	0.40	0.41	0.51	0.38	0.43	0.41	0.34	0.30	0.50	0.42	0.40	0.48
RO10	0.32	0.32	0.39	0.38	0.40	0.30	0.39	0.29	0.52	0.41	0.44	0.35	0.38

Appendix Table 3 (Continued)

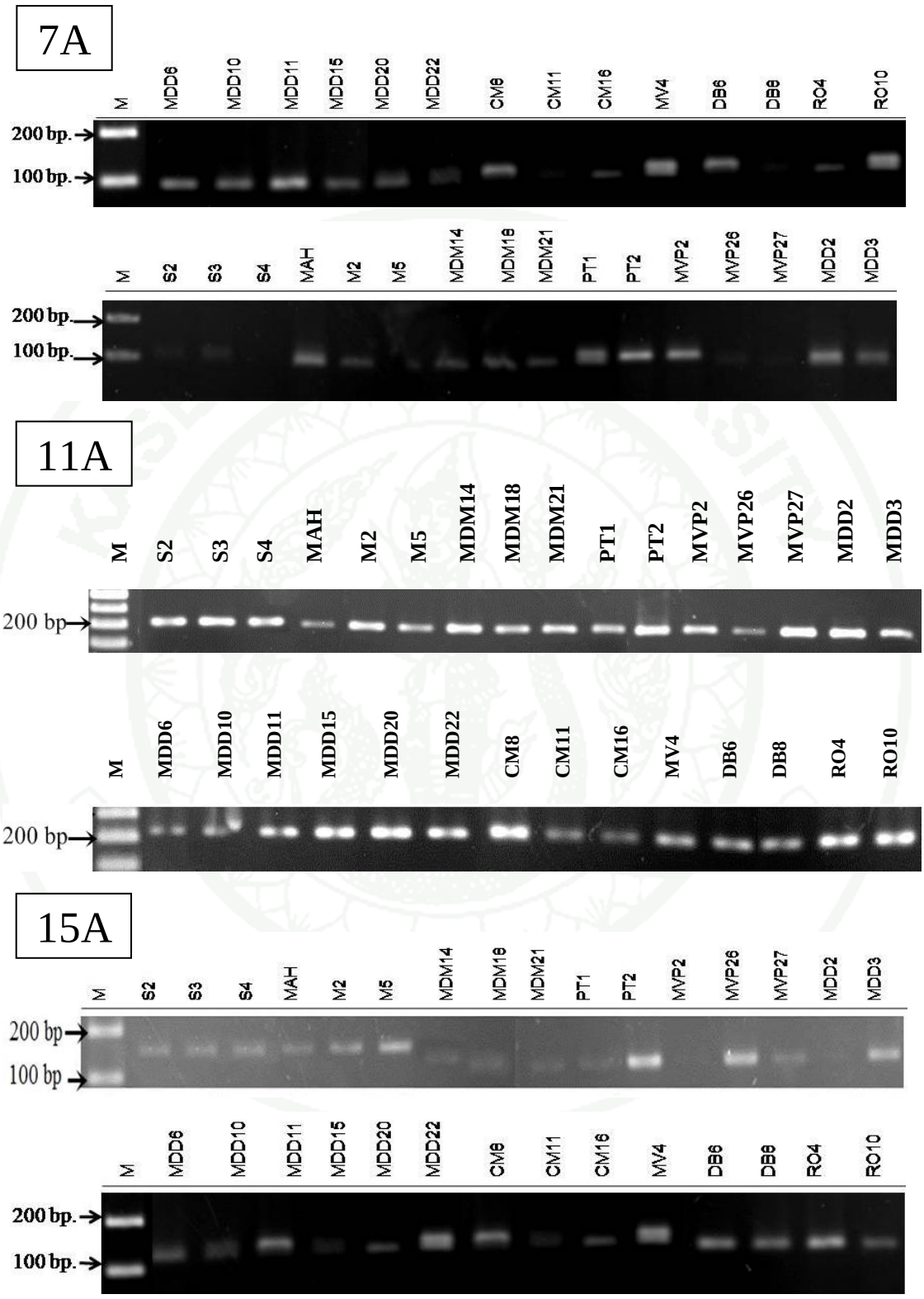
No.	MVP27	MDD2	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4
S2													
S3													
S4													
MAH													
M2													
M5													
MDM14													
MDM18													
MDM21													
PT1													
PT2													
MVP2													
MVP26													
MVP27	1												
MDD2	0.37	1											
MDD3	0.55	0.50	1										
MDD6	0.42	0.37	0.51	1									

Appendix Table 3 (Continued)

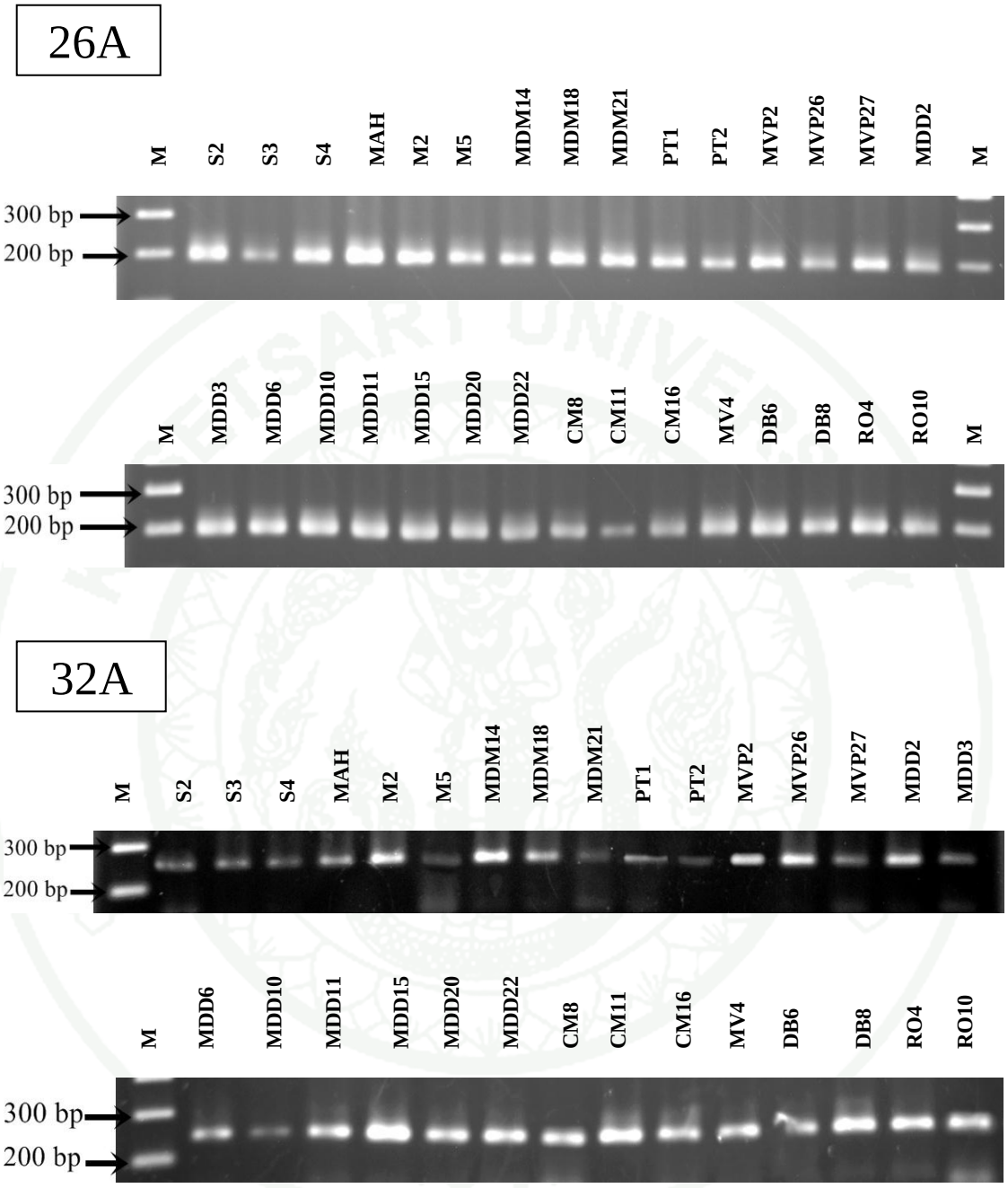
No.	MVP27	MDD2	MDD3	MDD6	MDD10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4
MDD10	0.52	0.43	0.45	0.52	1								
MDD11	0.30	0.19	0.24	0.43	0.5	1							
MDD15	0.48	0.51	0.45	0.56	0.46	0.27	1						
MDD20	0.49	0.43	0.50	0.37	0.51	0.47	0.38	1					
MDD22	0.35	0.38	0.44	0.35	0.41	0.22	0.33	0.33	1				
CM8	0.54	0.53	0.44	0.27	0.52	0.26	0.40	0.57	0.47	1			
CM11	0.39	0.46	0.52	0.43	0.49	0.44	0.24	0.46	0.28	0.43	1		
CM16	0.56	0.51	0.53	0.48	0.54	0.42	0.35	0.47	0.34	0.48	0.60	1	
MV4	0.32	0.34	0.34	0.41	0.24	0.32	0.38	0.24	0.28	0.27	0.28	0.43	1
DB6	0.55	0.42	0.66	0.55	0.49	0.33	0.53	0.42	0.48	0.40	0.41	0.56	0.38
DB8	0.46	0.41	0.58	0.50	0.60	0.52	0.40	0.65	0.35	0.46	0.59	0.52	0.27
RO4	0.46	0.53	0.51	0.46	0.41	0.40	0.44	0.34	0.25	0.54	0.55	0.59	0.46
RO10	0.38	0.44	0.46	0.38	0.47	0.51	0.35	0.32	0.27	0.38	0.50	0.51	0.27

Appendix Table 3 (Continued)

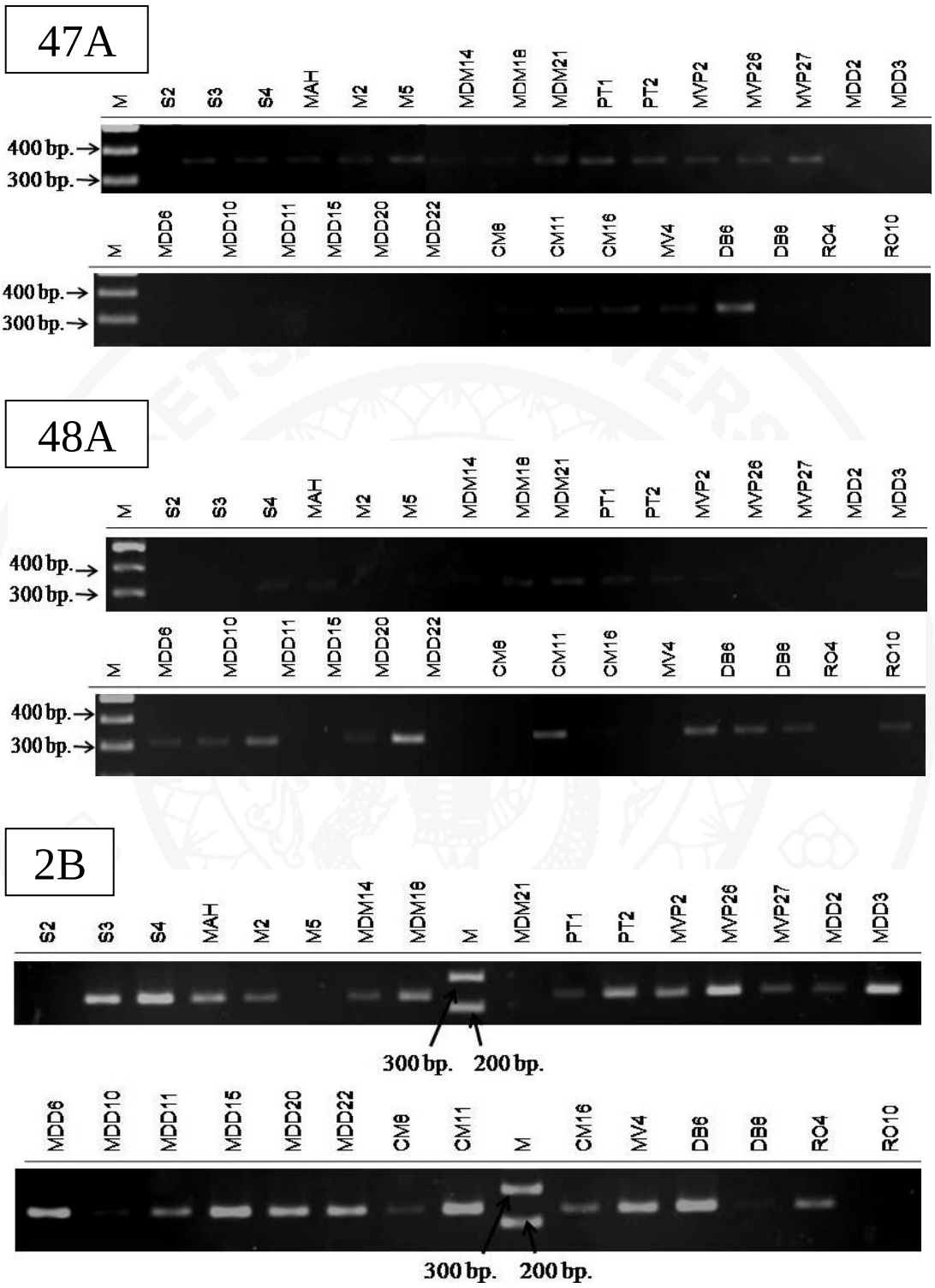
No.	DB6	DB8	RO4	RO10	MDD11	MDD15	MDD20	MDD22	CM8	CM11	CM16	MV4
DB6	1											
DB8	0.51	1										
RO4	0.51	0.57	1									
RO10	0.39	0.57	0.60	1								



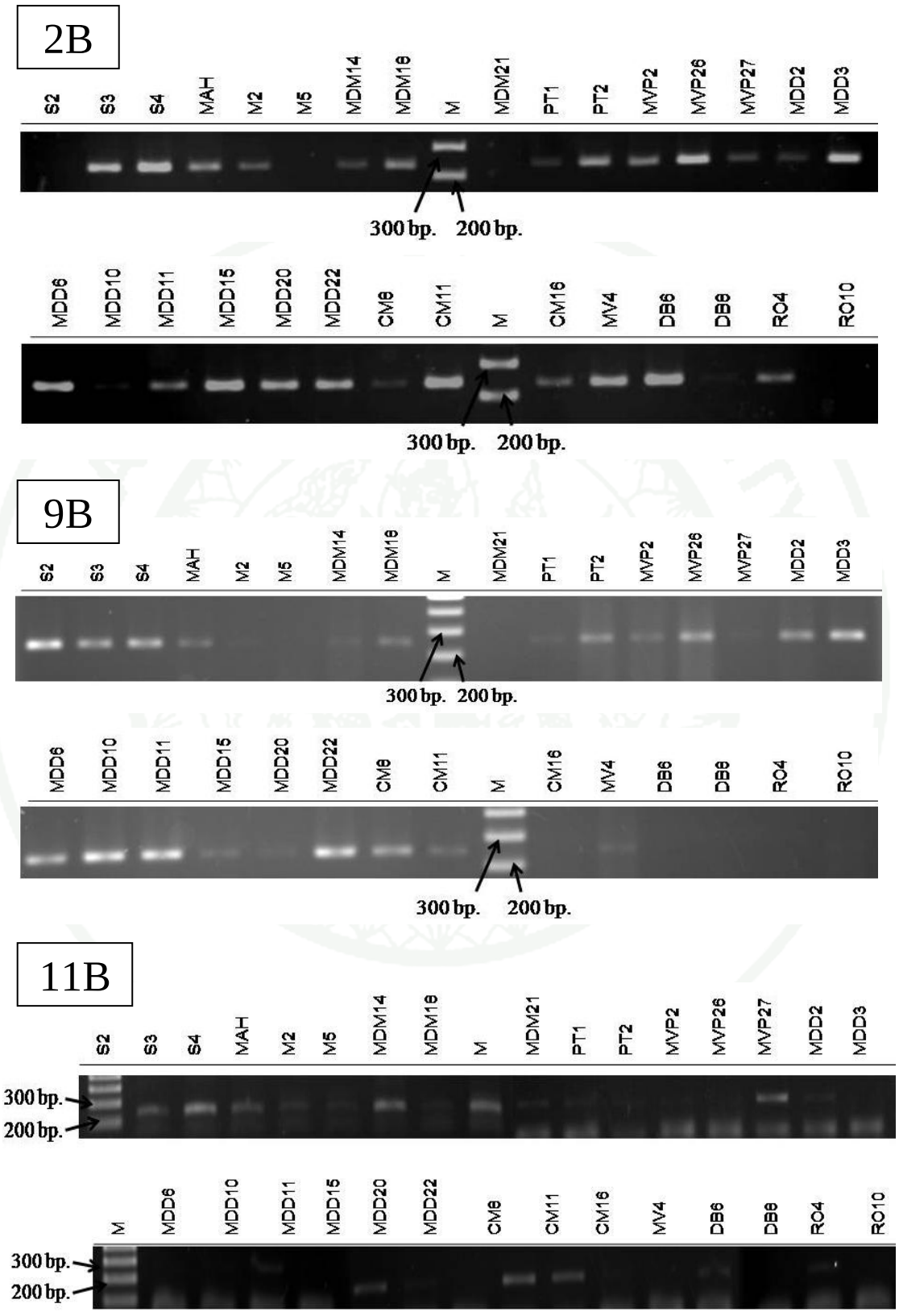
Appendix Figure 1 PCR products of 30 primers on agarose gel



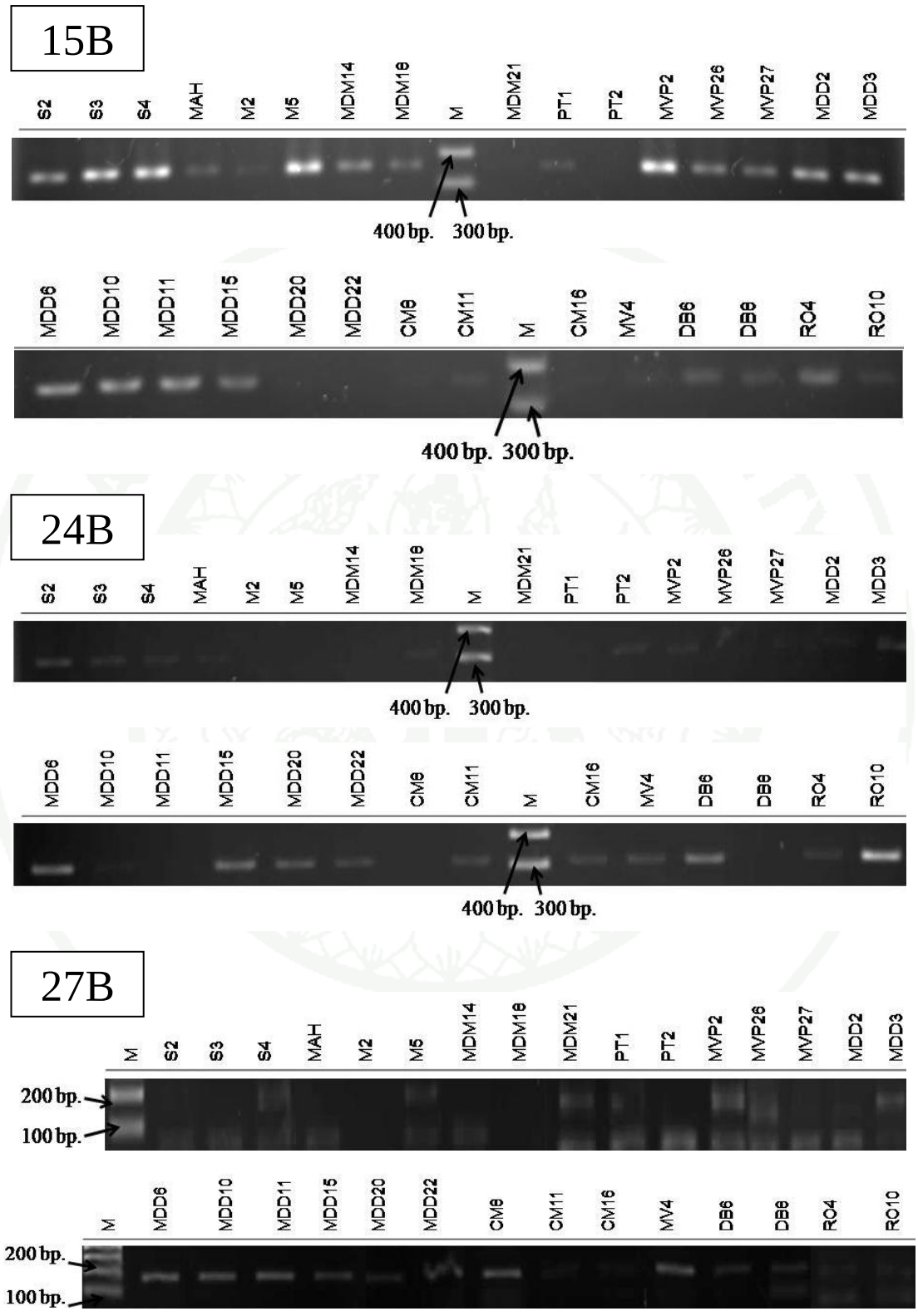
Appendix Figure 1 (Continued)



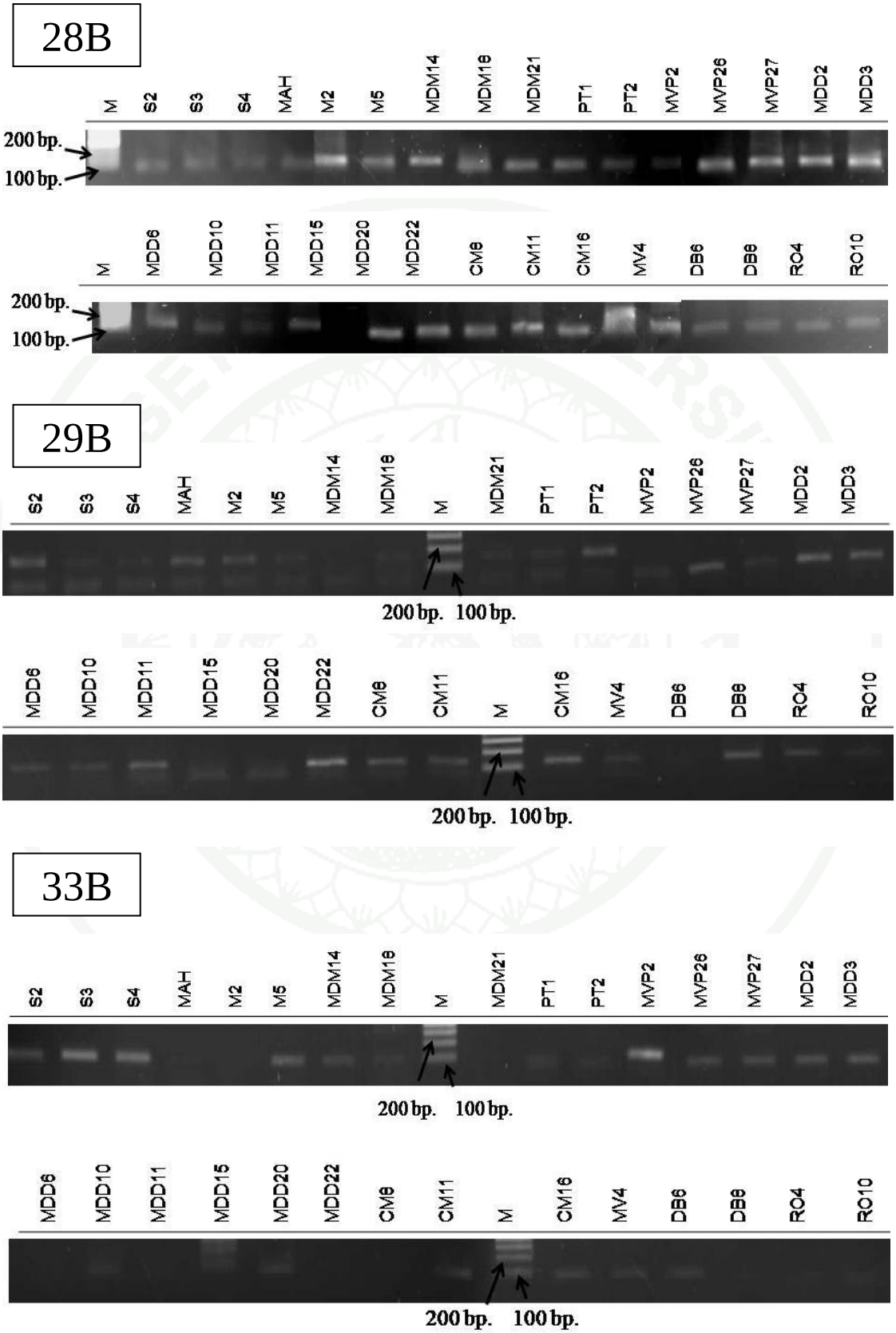
Appendix Figure 1 (Continued)



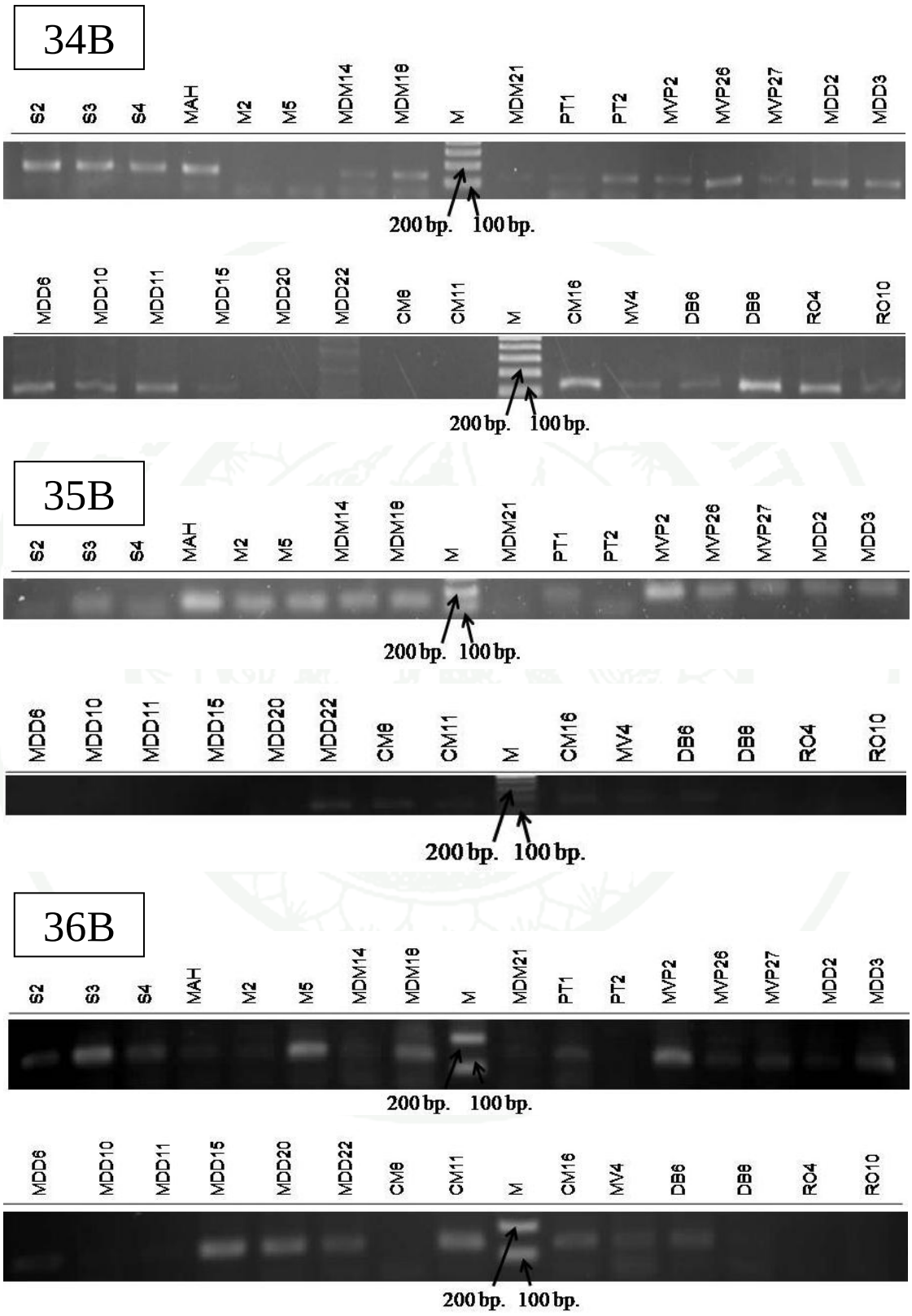
Appendix Figure 1 (Continued)



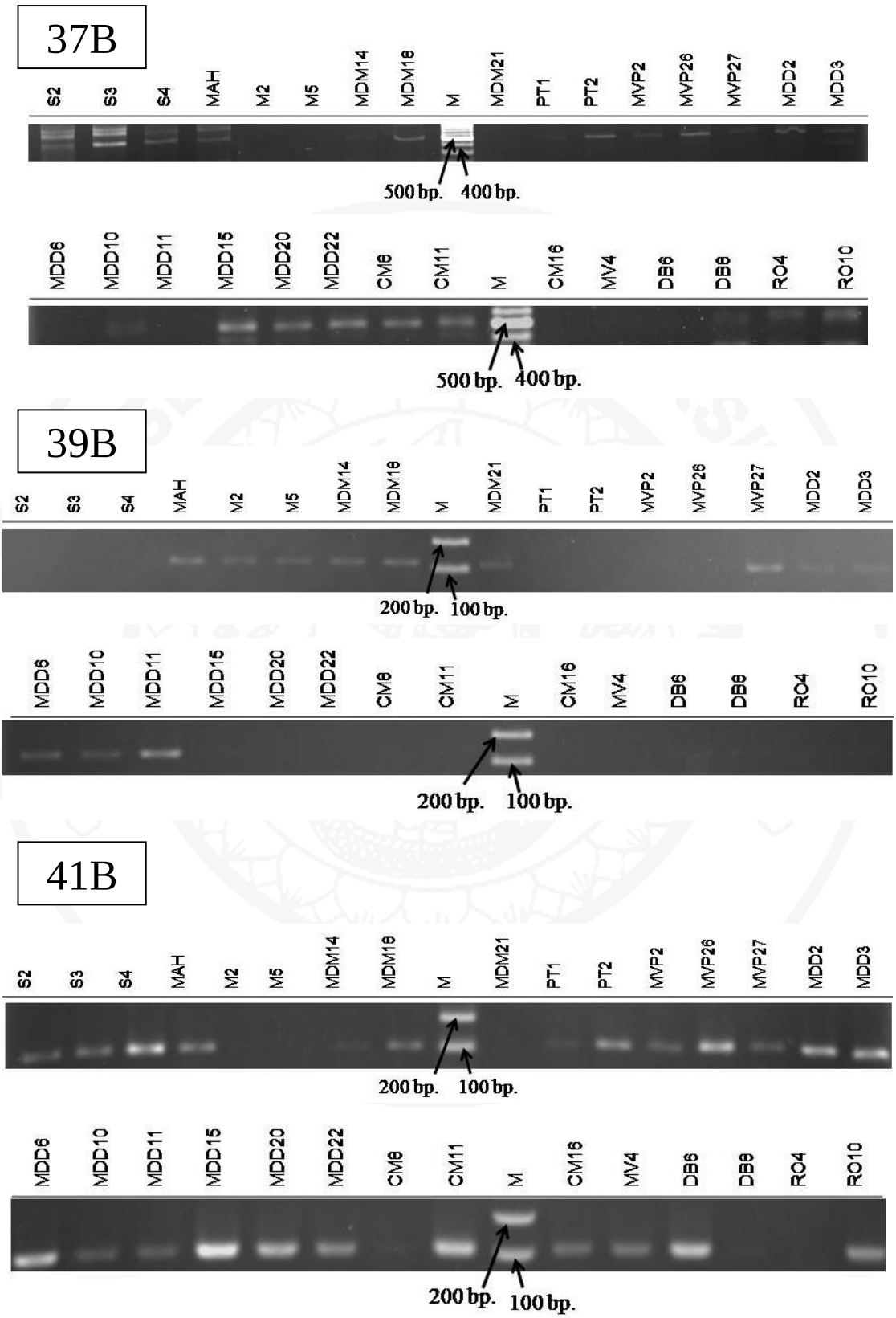
Appendix Figure 1 (Continued)



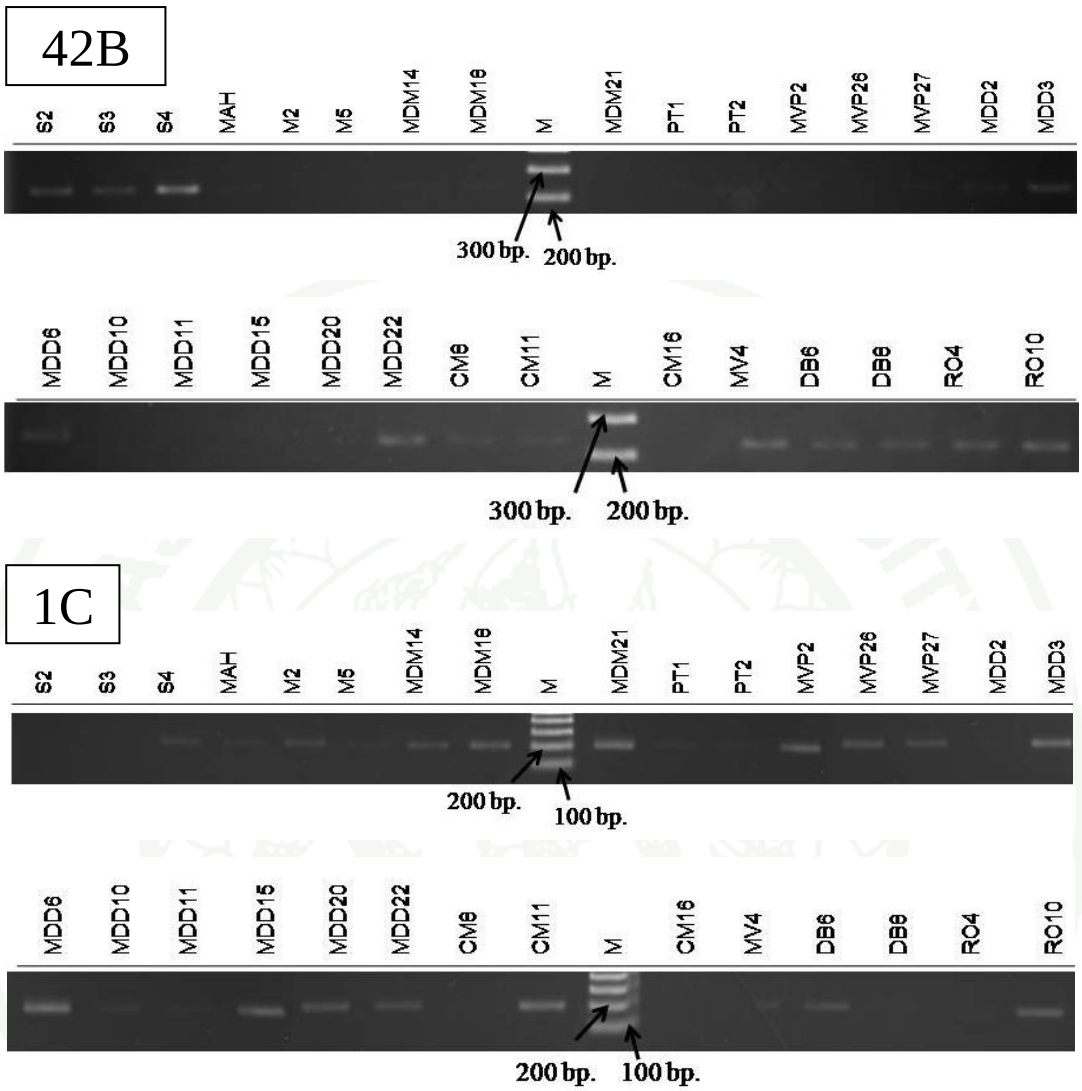
Appendix Figure 1 (Continued)



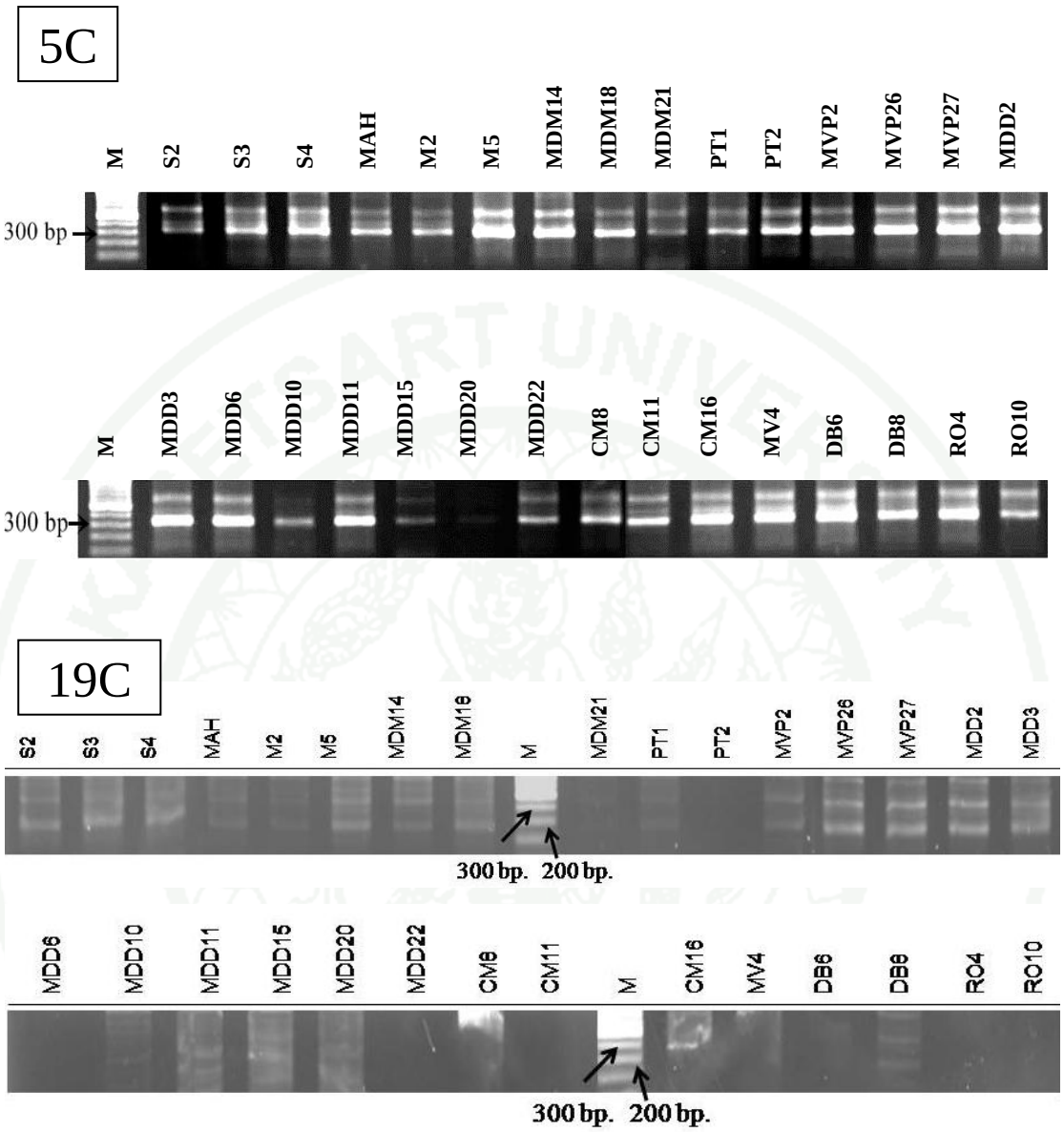
Appendix Figure 1 (Continued)



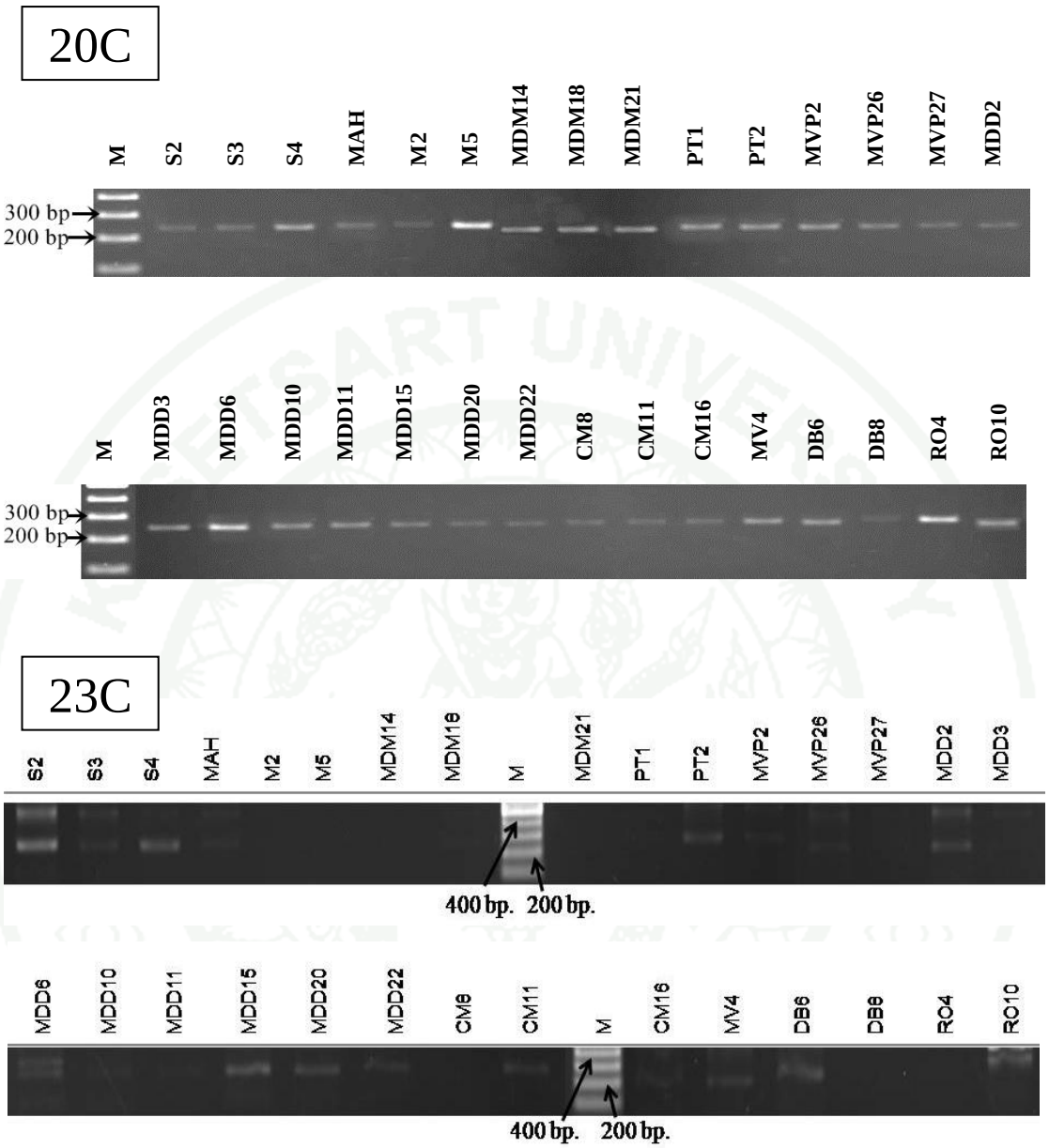
Appendix Figure 1 (Continued)



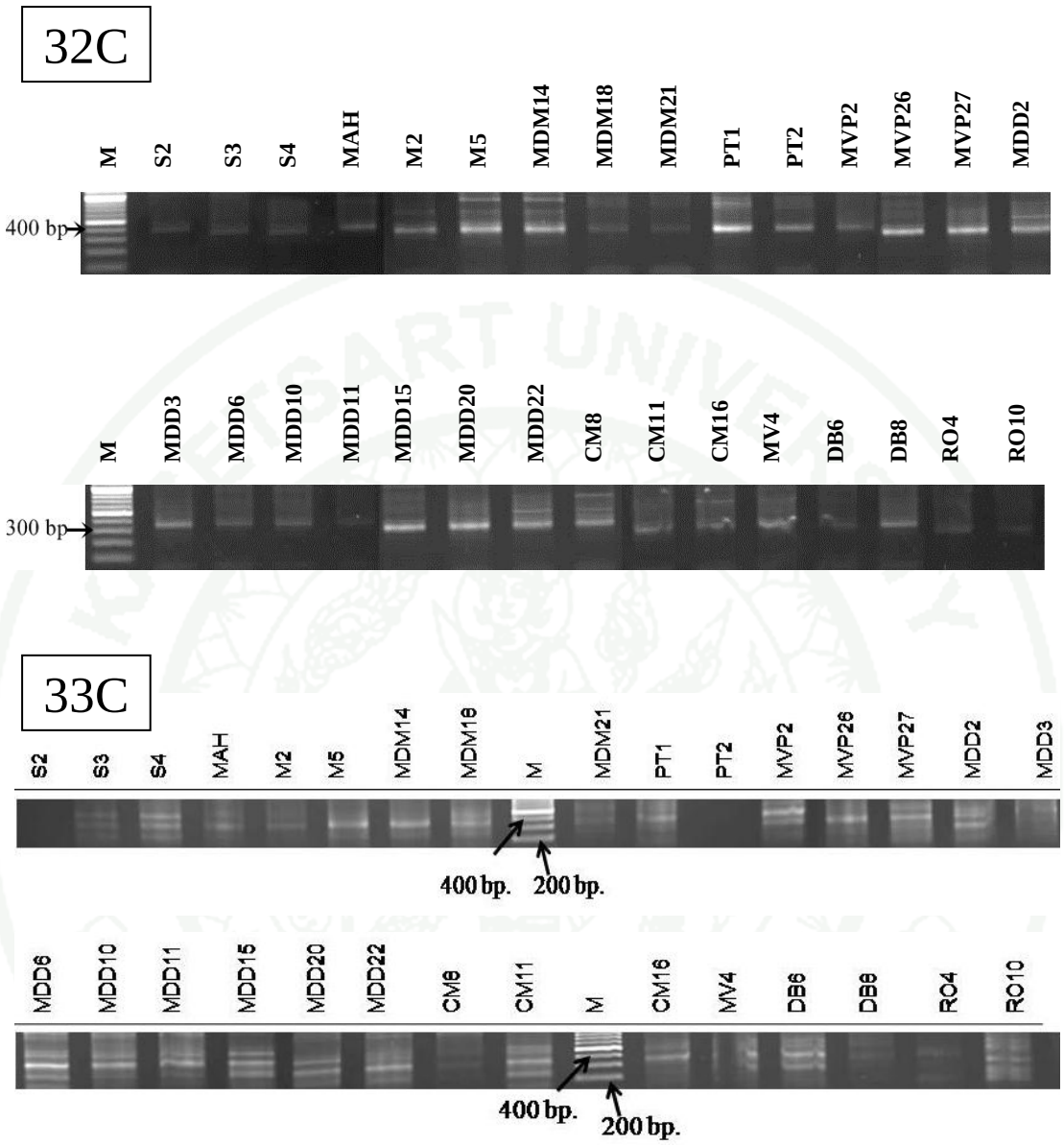
Appendix Figure 1 (Continued)



Appendix Figure 1 (Continued)

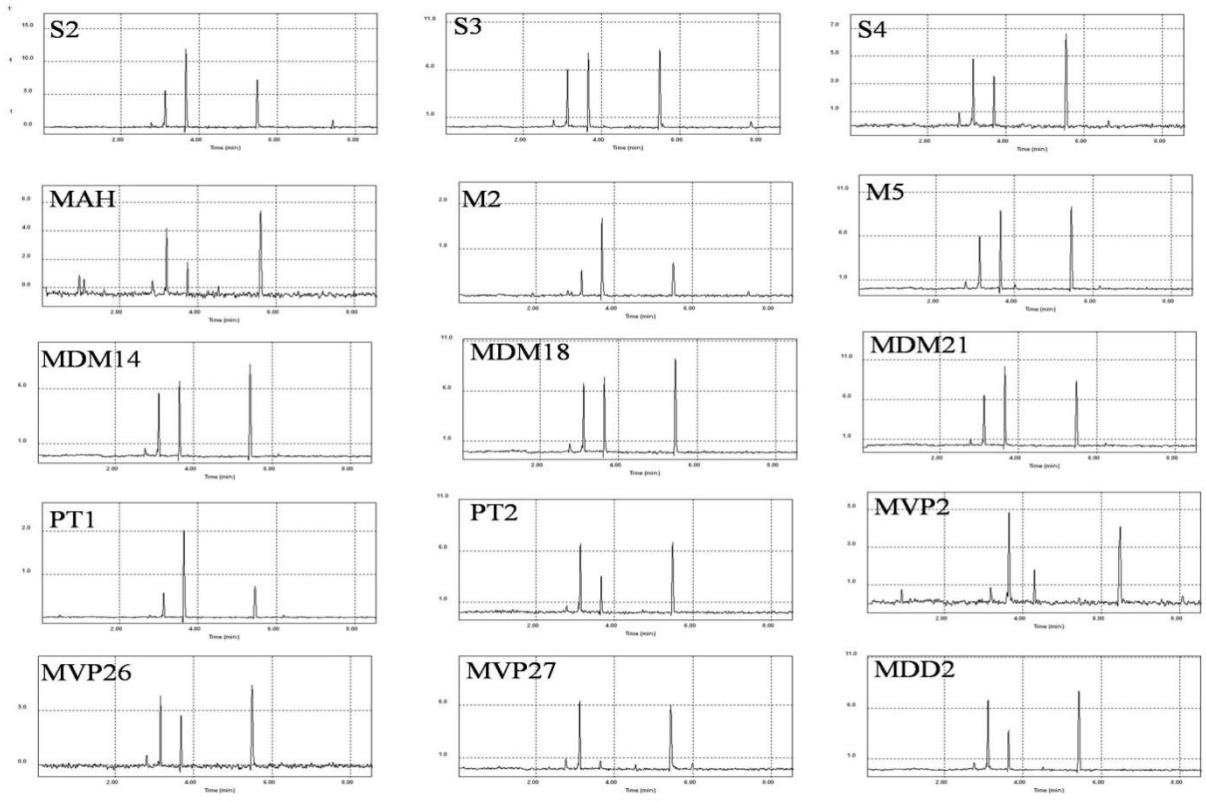


Appendix Figure 1 (Continued)



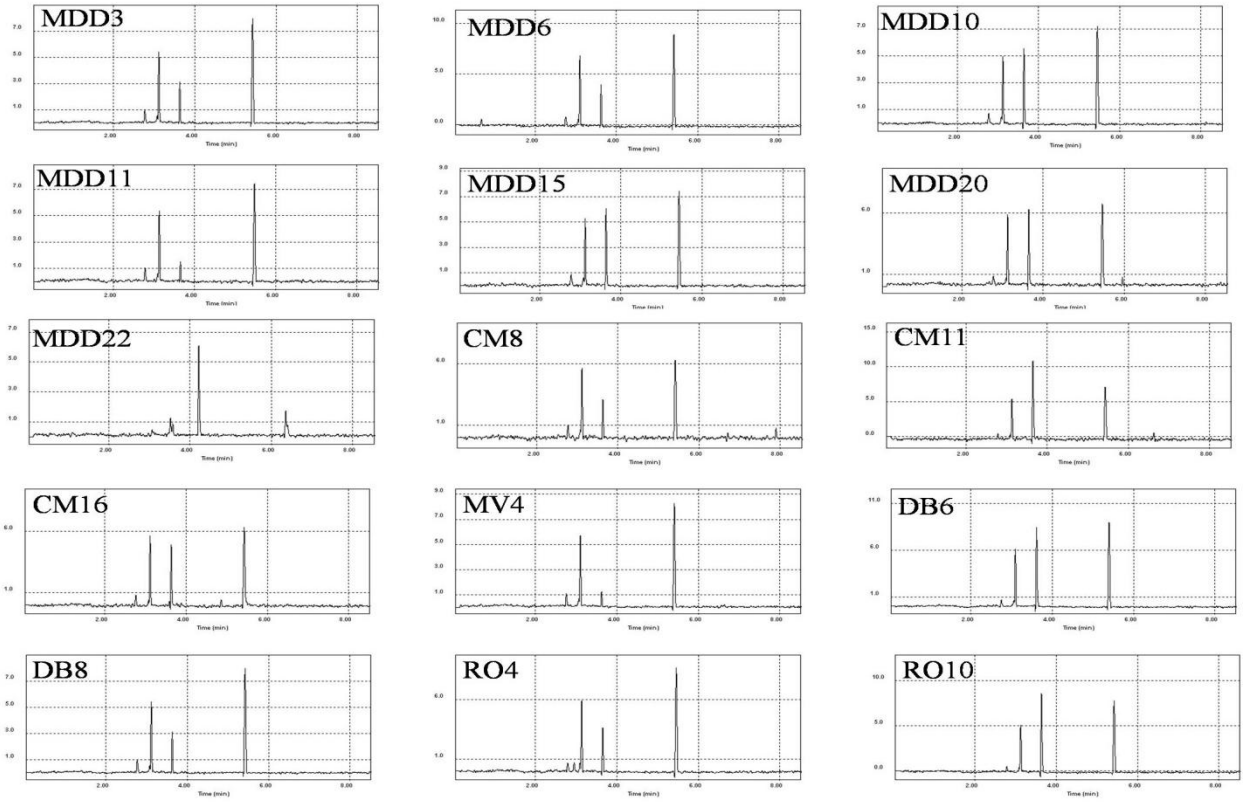
Appendix Figure 1 (Continued)

7A



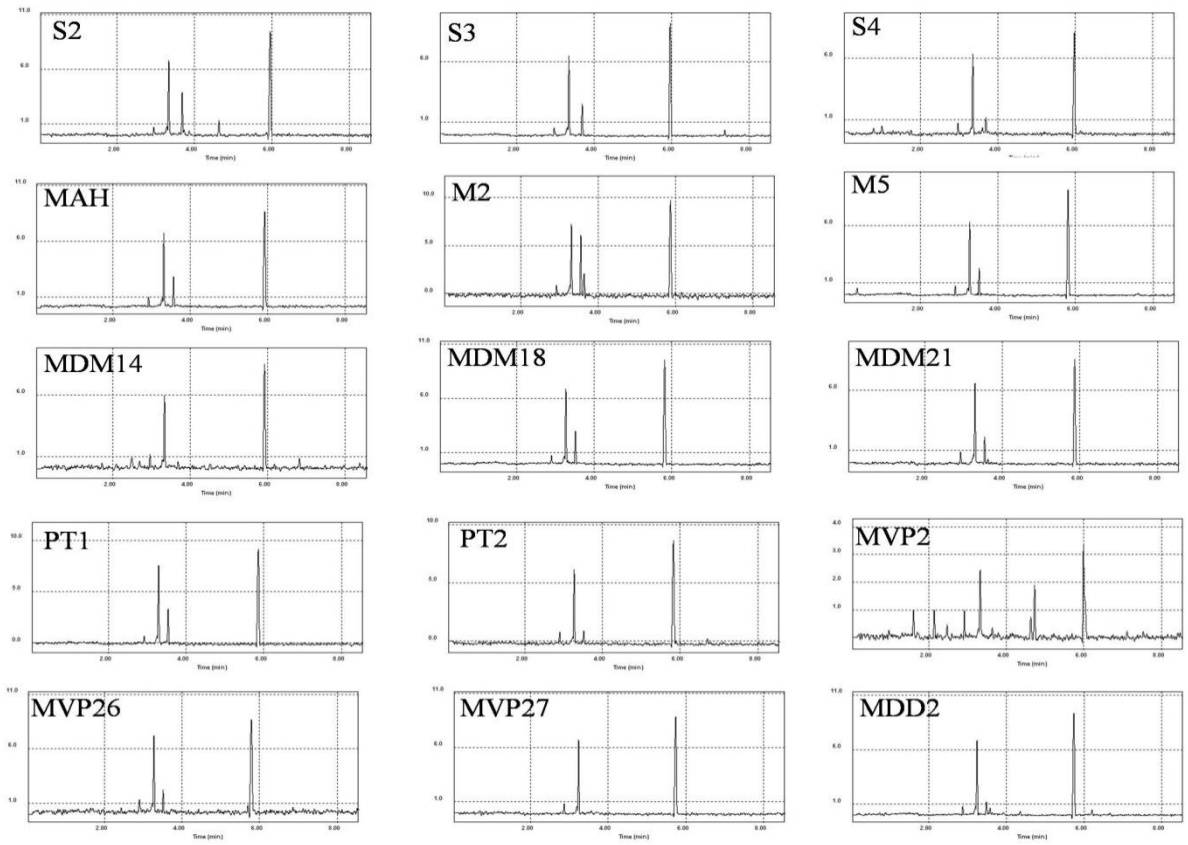
Appendix Figure 2 Graph result of some primers generated by capillary gel electrophoresis

7A



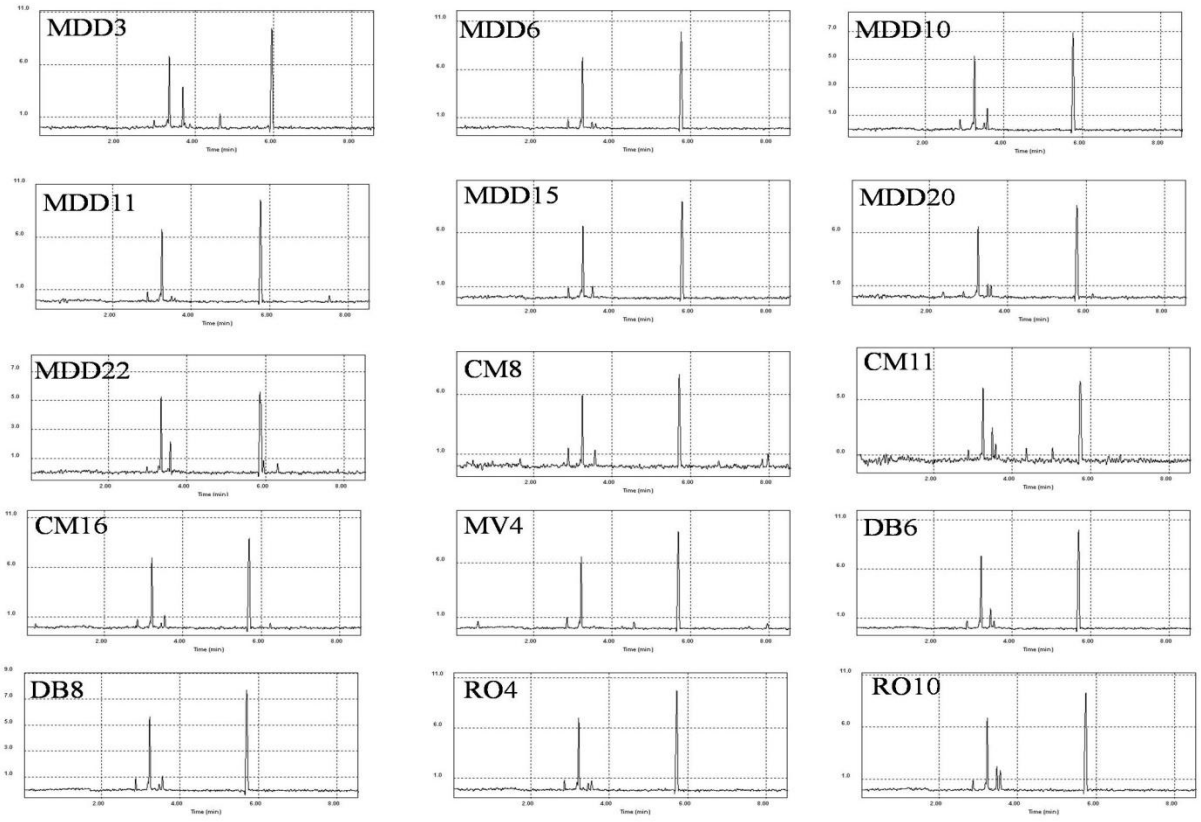
Appendix Figure 2 (Continued)

11A



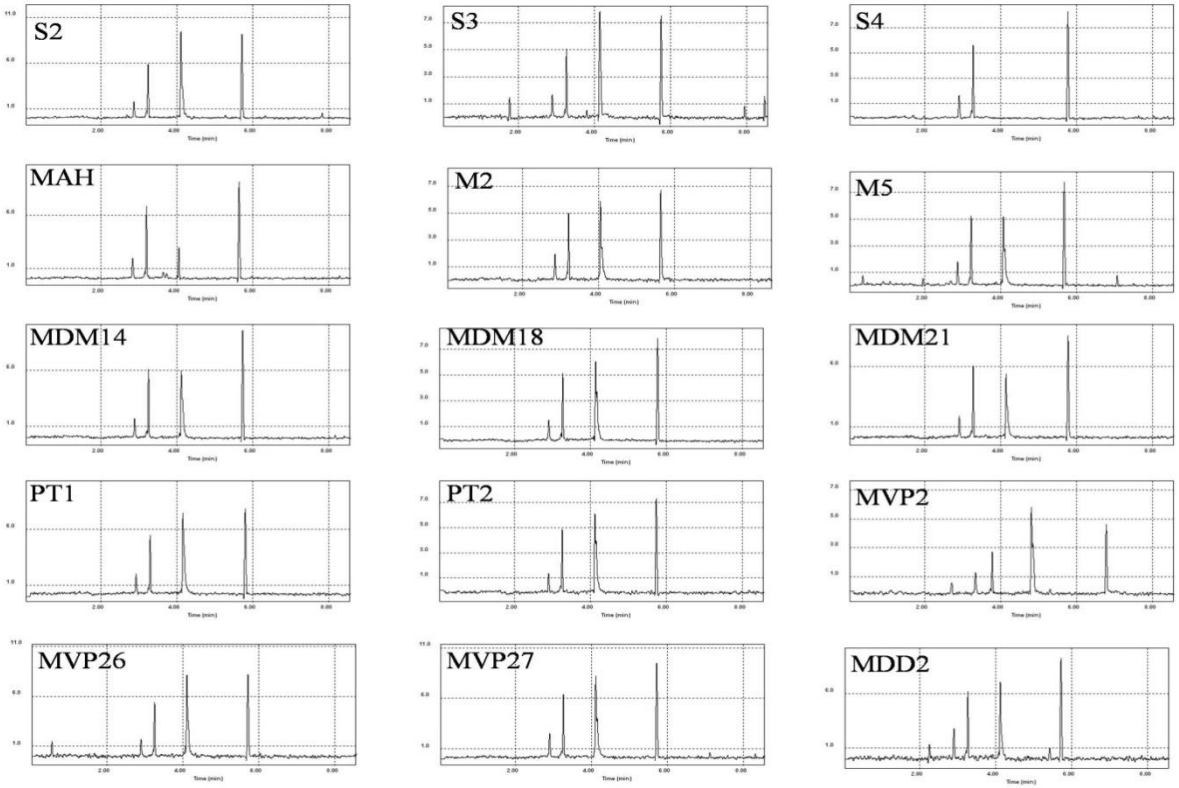
Appendix Figure 2 (Continued)

11A



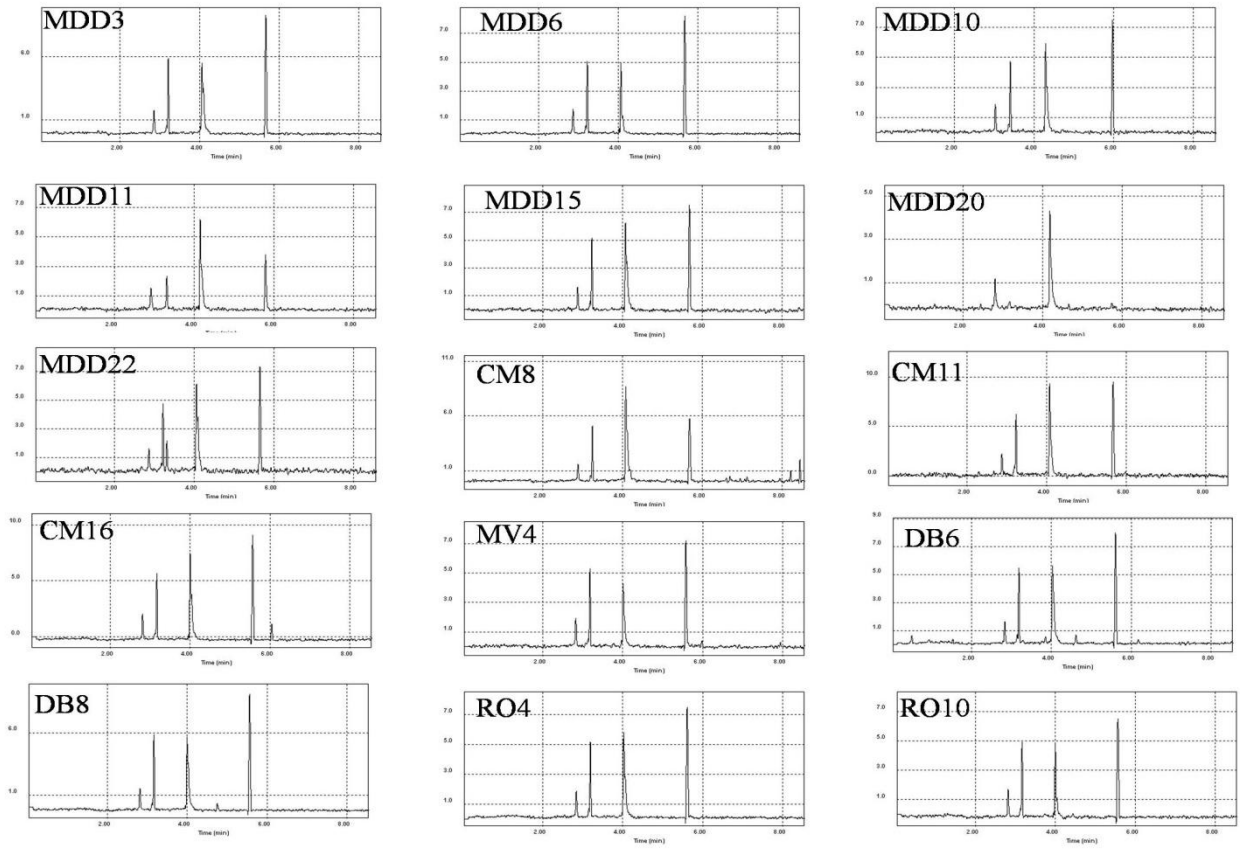
Appendix Figure 2 (Continued)

26A



Appendix Figure 2 (Continued)

26A



Appendix Figure 2 (Continued)

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