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Interspecific hybridization in *Lonicera caerulea* and *Lonicera gracilipes*

—The occurrence of green / albino plants by reciprocal crossing—

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Abbreviations

DAP	days after pollination
DAPI	4', 6-diamidino-2-phenylindole
MS	Murashige and Skoog (1962)
dNTP	deoxyribonucleoside triphosphate
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism

Abstract

Lonicera caerulea L. var. *emphyllocalyx* (Maxim.) Nakai is a berry crop cultivated in cold regions. So far, commercial cultivars have been mainly introduced from selection of wild plants. Therefore, fruit traits and other agricultural characteristics have been limited. In this study, interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* Miquel were examined to increase genetic variability of *L. caerulea* var. *emphyllocalyx*. Seedlings were obtained from reciprocal crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. The hybrid nature of seedlings was confirmed with random amplified polymorphic DNA analysis. Viable green plants were obtained efficiently from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx*. In contrast, all plants produced from *L. caerulea* var. *emphyllocalyx* × *L. gracilipes* var. *glabra* were albino. These albino plants were very weak and only survived in culture condition. The chlorophyll deficiency was unilaterally observed, suggesting the occurrence of nuclear–cytoplasmic incompatibility. Viable F₁ hybrids obtained from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* are amphidiploid ($2n = 4x = 36$) as showing same to both parents. The hybrid plans are expected to increase the variability of fruits traits, and may have heat tolerance from *L. gracilipes* var. *glabra*.

Keyword: Albino, Interspecific hybridization, *Lonicera caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra*, Nuclear–cytoplasmic incompatibility

1. Introduction

The genus *Lonicera* belongs to the family Caprifoliaceae and comprises more than 200 species (Naužemys et al., 2007). One of them, namely, *Lonicera caerulea* L., belongs

to the section *Isika*, subsection *Caeruleae* (Rehder, 1903). It is a deciduous shrub with edible fruits and is distributed in the northern regions of Eurasia to North America (Thompson and Chaovanalikit, 2003). This plant is commonly known as blue honeysuckles. In Japan, *L. caerulea* L. var. *emphylocalyx* (Maxim.) Nakai grows in cold regions from alpine in middle mainland to all over the Hokkaido (Miyashita et al., 2010; Sato, 1985), and is known as ‘Haskap’ in the Ainu language used by the indigenous Ainu people of Hokkaido. Fruits of *L. caerulea* var. *emphylocalyx* are blue-black in color, sour to sweet taste, and rich in nutrients, such as anthocyanins, minerals and vitamins (Anetai et al., 1996; Tanaka and Tanaka, 1998; Terahara et al., 1993). This plant species has been cultivated as a horticultural crop since 1970s in Hokkaido. Recently, it was introduced to North America as a new berry crop (Thompson, 2006). The commercial cultivars have been mainly introduced from selection of wild plants. Therefore, fruit traits and other agricultural characteristics have been limited. A major problem in the cultivation of *L. caerulea* var. *emphylocalyx* is that fruits of wild plants are small and thin pericarp. Therefore, harvesting is laborious. In addition, cultivation of *L. caerulea* var. *emphylocalyx* is restricted to cold region. Fruit yield and other traits also must be improved to increase commercial production. To breed this berry crop, Takada et al. (2003) evaluated the eating qualities and some horticultural characteristics of wild species, and made some elite selections. Breeding program by cross pollination between elite strains and by producing polyploid plants are in progress (Miyashita et al., 2009; Suzuki et al., 2007). Interspecific hybridization is useful for increasing the genetic variability. However, it has not been reported in *L. caerulea* var. *emphylocalyx*.

Lonicera gracilipes var. *glabra* Miquel is a deciduous shrub and belongs to the

section *Isika*, subsection *Purpurascentes* (Rehder, 1903) or subsection *Monanthae* (Hara, 1983). This species is endemic to Japan (Theis et al., 2008). It is found in deciduous forest in altitude 20-600 m of southernmost Hokkaido, mainland, and Shikoku (Hara, 1983), which is a warm region compared to the habitat of *L. caerulea* var. *emphyllocalyx*. Fruits of *L. gracilipes* var. *glabra* are red in color and have sweet taste. This species has been planted in gardens for its edible fruits (Hara, 1983). It is expected to be a new breeding material of *L. caerulea* var. *emphyllocalyx*.

In this study, we investigated the characteristics of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. Furthermore, interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* were examined.

2. Materials and Methods

2.1. Plant materials

Two strains of *L. caerulea* var. *emphyllocalyx* (Lc-Y47 and Lc-T39) and *L. gracilipes* var. *glabra* (Lg-A and Lg-N) were used in this study. All plants were grown at the Experiment Farms in Hokkaido University.

2.2. Characterizations of plant materials

The characteristics of the plant materials were investigated as flower characters (color and size), fruit characters (color, size, fresh weight, total soluble solids, pH, seed number and seed size), and chromosome number. For measurement of flower size and fruit traits, 10 flowers or fruits were used. The total soluble solids (Brix) and pH of fruits were measured using refractmeter (BRX-242, TOKYO GARASUKIKAI Co. Ltd., Tokyo, Japan) and pH meter (B-212, Horiba, Ltd., Kyoto, Japan), respectively.

Statistical tests were performed using the SPSS 16.0 J program. The differences were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test, with $P < 0.05$ as the level of statistical significance.

2.3. Interspecific hybridization and seed culture

Interspecies crosses between *L. caerulea* var. *emphylocalyx* and *L. gracilipes* var. *glabra* were performed. The cross combinations are listed in Table 3. Flowers were emasculated prior to anthesis and then hand-pollination was carried out. All pollinated flowers were covered with paper bags to prevent pollination by other plants. Mature fruits were harvested at more than 40 DAP. The seeds were sterilized with 1% sodium hypochlorite solution containing 1–2 drops of polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 20 min, and then rinsed three times with sterile-distilled water. These seeds were cultured on a half-strength MS medium containing 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum (Wako Pure Chemical Industries). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The seeds cultured on the medium were maintained in a controlled growth environment chamber at 20°C under 24 h photoperiod (35 $\mu\text{mol m}^{-2} \text{s}^{-1}$) provided by 40 watt fluorescent tubes.

2.4. Confirmation of hybrid nature with RAPD analysis

Hybrid nature was investigated with RAPD analysis. Total genomic DNA was isolated from fresh leaves using the DNeasy Plant mini kit (Qiagen, Inc., Valencia, CA, USA). PCR was carried out in a volumes of 25 μl containing 25 ng of genomic DNA, 2.5 μl of 1 \times buffer for KOD-plus-Ver.2 (Toyobo, Co. Ltd., Osaka, Japan), 0.2 mM of each dNTP,

1.5 mM MgSO₄, 0.6 µM primer and 0.5 U KOD-plus-Ver.2 DNA polymerase. A total of 11 RAPD primers reported by Naugžemys et al. (2007) were used in the PCR reactions. Amplification was carried out using a BioRad iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with one cycle at 94 °C for 2 min and 40 cycles of 10s at 98 °C, 30 s at 36°C, 2min at 68 °C according to the manufacturer's instructions of KOD-plus-Ver.2 DNA polymerase. PCR products were separated by electrophoresis in 3% agarose gels (Certified Low Range Ultra Agarose, Bio-Rad, USA). Gels were stained with ethidium bromide, visualized on UV light and photographed. Wide-Range DNA ladder (Takara Bio Inc., Shiga, Japan) was used as a size marker.

2.5. PCR–RFLP analysis of plastid DNA inheritance

PCR–RFLP analysis was performed for confirming the inheritance pattern of plastid DNA in hybrids plants. Region of Plastid DNA, *atpβ–rbcL*, was amplified from total genomic DNA isolated from fresh leaves using primers 377 (F) (5'-GTGGAAACCCCGGGACGAGAAGTAGT-3') and Z346 (R) (5'-AAATACGTTACCCACAATGGAAGTAAATAT-3') described in the report of Crayn and Quinn (2000). PCR was carried out in a volumes of 50 µl containing 10 ng of genomic DNA, 2.5 µl of 1× buffer for KOD-plus-Ver.2 (Toyobo, Japan), 0.2 mM of each dNTP, 1.5 mM MgSO₄, 0.3 µM each primer and 1U KOD-plus-Ver.2 DNA polymerase. Amplification was carried out using a Bio-Rad iCycler Thermal Cycler (Bio-Rad, USA) with one cycle at 94 °C for 2 min, and 35 cycles of 10s at 98 °C, 30 s at 53 °C and 1min at 68 °C according to the manufacturer's instructions of KOD-plus-Ver.2 DNA polymerase. The PCR products were digested with 10 unit of *Hae*III at 37 °C for 3 h. The restriction fragments were separated by electrophoresis in

2% agarose gels (Agarose S, Nippon gene Co. Ltd., Tokyo, Japan). Gels were stained with ethidium bromide, visualized on UV light and photographed.

2.6. Ploidy analysis using flow cytometry

The relative DNA content of plants of *L. caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra* and their hybrids were determined using flow cytometry (Partec PA; Partec GmbH, Münster, Germany) according to the protocol of Miyashita et al. (2010). Fresh leaves of *Capsicum annuum* (cv. 'Kyonami') were used as the internal standard. Fresh leaves were chopped with a 0.2 ml of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After filtration through a 30- μ m nylon mesh, crude nuclear samples were stained with 0.8 ml DAPI solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM MgCl₂, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, and 2 mg l⁻¹ DAPI (pH 7.5) (Mishiba et al., 2000). After incubation for 5 minutes at room temperature, the relative DNA content was measured with flow cytometry. In this study, description of genome size is expressed as C_x-value, which is designated as DNA content of a monoploid genome with chromosome number *x*, according to Greilhuber et al. (2005).

2.7. Chromosome analysis

For counting the chromosome number, actively growing root tips were utilized for chromosome observation. According to the procedure of Miyashita et al. (2010), chromosomes were observed as follows. The root tips were pretreated with ice water for 24 h and fixed with acetic acid:ethanol (1:3) at 4°C overnight. The fixed root tips were treated with an enzyme mixture of 2% (w/v) Cellulase Onozuka RS (Yakult

Pharmaceutical Co. Ltd., Tokyo, Japan) and 0.5% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Chiba, Japan) (Shibata and Hizume, 2002) in the citrate buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate), pH 4.5, at 37°C for 20 min. After treatment with the enzyme mixture, the root tips were rinsed with distilled water. Then, the root tips were squashed in a drop of 45% acetic acid on slide glass, covered, and squashed again. Cover glasses were removed by freezing glass slides in liquid nitrogen, and slides were dried at 37°C. For staining, a drop of DAPI solution [0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 ml 0.2 M Tris-HCl, pH 8.0, 9 ml glycerol, 0.5 µg ml⁻¹ of DAPI] (Sahara et al., 2003) was added on the slide glass, and covered with a cover glass. Preparations were observed under a fluorescence microscope (Axio Imager M1, Carl Zeiss, Oberkochen, Germany). For each sample, at least 10 clear chromosome images were recorded.

3. Results

3.1. Characteristics of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*

Comparative characteristics of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* were summarized in Table 1. The specific differences of two species were described as followings.

Flower characters— Flowering season of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* was late April to late May in Sapporo, Hokkaido. Flower color of *L. caerulea* var. *emphyllocalyx* was light yellow (Fig. 1A). On the other hand, that of *L. gracilipes* var. *glabra* was pink (Fig. 1B). Flowers were two in pair in *L. caerulea* var. *emphyllocalyx*, single or rarely two in pair in *L. gracilipes* var. *glabra*. The average corolla lengths were 18.6 mm and 19.4 mm in *L. caerulea* var. *emphyllocalyx* and *L.*

gracilipes var. *glabra*, respectively.

Fruit characters— Fruiting season of *L. caerulea* var. *emphyllocalyx* was early June to late July, and that of *L. gracilipes* var. *glabra* was early June to mid-July in Sapporo, Hokkaido. Fruits were blue-black in color and were covered with bloom in *L. caerulea* var. *emphyllocalyx* (Fig. 1C). On the other hand, fruits of *L. gracilipes* var. *glabra* were red and bloomless (Fig. 1D). A fruit of *L. caerulea* var. *emphyllocalyx* was consisted of two ovaries enclosed by pericarp. In *L. gracilipes* var. *glabra*, a fruits was consisted of single ovary, rarely two separated ovaries. Fruits size, Brix (%) and pH of both species were expressed in Table 2. The fruit size of *L. caerulea* var. *emphyllocalyx* was greater than that of *L. gracilipes* var. *glabra*. The average fruit fresh weight was 1.3 g and 0.6 g in *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*, respectively. The average value of total soluble solids (Brix) of *L. caerulea* var. *emphyllocalyx* was 12.8%, while that of *L. gracilipes* var. *glabra* was 12.1%. The average value of pH was much lower in *L. caerulea* var. *emphyllocalyx* than that of *L. gracilipes* var. *glabra*, 2.6 and 5.1, respectively. The fruit taste was sour-sweet in *L. caerulea* var. *emphyllocalyx*, while sweet in *L. gracilipes* var. *glabra*. The average seed size of *L. gracilipes* var. *glabra* was approximately 2-fold larger than that of *L. caerulea* var. *emphyllocalyx*. Average seed number in a fruit derived from natural pollination was 16.5 and 4.0 in *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*, respectively.

Statistical analysis revealed that significant differences were found in fruit sizes, fresh weight and pH (Table 2).

Ploidy level— All strains of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* used in this study were tetraploid ($2n = 4x = 36$) (Fig. 2A, Fig. 2B).

3.2. Interspecific hybridization

In the reciprocal crosses, a total of 15 and 20 flowers were pollinated in *L. caerulea* var. *emphylocalyx* \times *L. gracilipes* var. *glabra* and *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphylocalyx*, respectively. As a result, all pollinations were successful to set fruits (Table 3), and mature seeds were obtained from reciprocal crosses.

In the cross using *L. gracilipes* var. *glabra* as a seed parent, high percentage of germination (83.3% to 100%) was observed (Table 3). In cross of Lg-N \times Lc-Y47, a total of 18 green plants were obtained. The survival rate of seedlings after acclimation was 55.6% in Lg-N \times Lc-Y47, and the acclimated 10 seedlings grew vigorously (Fig. 3A). The seedlings obtained from Lg-A \times Lc-T39 and Lg-A \times Lc-Y47 showed slow growth, and several seedlings were pale green (Table 3). In cross of Lg-A \times Lc-T39 and Lg-A \times Lc-Y47, the survival rate of seedlings after acclimation was 20% and 10%, respectively. Totally, 73 plants were obtained from *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphylocalyx* through seed culture (Table 3), and 20 plants survived after acclimation (Table 3).

In the cross using *L. caerulea* var. *emphylocalyx* as a seed parent, the percentage of germination was 83.1%, 76.7% and 50.6% in Lc-T39 \times Lg-A, Lc-Y47 \times Lg-A, and Lc-Y47 \times Lg-N, respectively (Table 3). Totally, 127 plants were obtained from *L. caerulea* var. *emphylocalyx* \times *L. gracilipes* var. *glabra* (Table 3). However, all plants were albino (Table 3, Fig. 3B). These albino plants were very weak and only survived in culture condition. Most albino plants died in the cotyledon stage. Some albino plants survived for more than one year in culture condition. However, they remained albino.

3.3. Confirmation of hybrid nature by RAPD markers

All 11 RAPD primers used in this study could produce bands after PCR. One of them, the primer 380-06 (5'-CCCGACTGCC-3') showed clear polymorphisms between the two species. DNA fragments of 600 bp and 1200 bp were specific to *L. caerulea* var. *emphyllocalyx*, while those of 500 bp and 700 bp were specific to *L. gracilipes* var. *glabra* (Fig. 4). The survived 20 progenies from the cross between *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* had both specific fragments, indicating that they were interspecific hybrids. The RAPD analysis with primer 380-06 was also performed for representative six albino plants derived from *L. caerulea* var. *emphyllocalyx* × *L. gracilipes* var. *glabra*. The hybrid nature of albino plants examined was confirmed.

3.4. Flow cytometric analysis and chromosome number of hybrid plants

Flow cytometric analysis showed relative DNA contents evaluated by fluorescence intensity stained with DAPI (Fig. 5). The parent plants were tetraploid ($2n = 4x = 36$) and their the relative DNA contents could be shown as 4Cx DNA contents by flow cytometry. This flow cytometric result revealed that *L. caerulea* var. *emphyllocalyx* and *L. garacilipes* var. *glabra* had almost same relative DNA contents in comparison with the internal standard, indicating that ratios were 0.521 and 0.513, respectively (Fig. 5A, B). Flow cytometry showed that total 20 hybrid plants from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* had same ploidy level to both parents indicating 4Cx DNA contents (Fig. 5C). This revealed that these hybrids were allotetraploid with the similar ratio of the parents (0.510) by comparing the internal standard, suggesting that low possibility of the partial genome deletion.

The results of the flow cytometric analysis were confirmed by determining the chromosome number. The chromosome number was counted for representative 5 hybrid plants, and it revealed a tetraploid ($2n = 4x = 36$) number of chromosomes same to both parents (Fig. 2C, D). This result indicated that these hybrids were amphidiploid consisting of diploid genomes derived from both parents.

3.5. Inheritance of plastid DNA

Chloroplast-encoded *atpβ-rbcL* intergenic spacer noted for the high frequency of insertion–deletion mutations was used to analyze the composition of plastid DNA in the hybrid plants. DNA fragment of the *atpβ-rbcL* region amplified with primers of 377 (F) and Z346 (R) was approximately 1200 bp in *L. caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra* and their hybrids. The DNA fragment was digested by *HaeIII*, and polymorphisms of restricted fragments were observed between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. Restricted fragments of 600 bp and 400 bp were specific to *L. caerulea* var. *emphyllocalyx*, while that of 1000 bp was specific to *L. gracilipes* var. *glabra* (Fig. 6). The size of restricted fragments of 20 green hybrid plants from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* was same to a female parent (Fig. 6). The size of restricted fragments of albino hybrids from *L. caerulea* var. *emphyllocalyx* × *L. gracilipes* var. *glabra* was also same to a female parent (Fig. 6). These results indicated that plastid DNA was inherited maternally in the both cross combination.

4. Discussion

The interspecific hybrids were obtained from reciprocal crosses between *L. caerulea* var.

emphylocalyx and *L. gracilipes* var. *glabra*. Viable green plants were obtained efficiently from *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphylocalyx*. Interestingly, hybrid plants from reverse cross showed albinism. Thus, the chlorophyll deficiency was unilaterally observed, suggesting the occurrence of nuclear–cytoplasmic incompatibility.

Chlorophyll deficiency has been reported in interspecific hybrids in some plants, such as genera *Hibiscus* (Van Laere et al., 2007), *Lychnis* (Godo et al., 2009), *Oenothera* (Kirk and Tilney–Bassett, 1978), *Primula* (Kato and Mii, 2000), *Rhododendron* (Michishita et al., 2002; Ureshino et al., 1999), *Trifolium* (Sawai et al., 1990) and *Zantedeschia* (Kubo et al., 2006; Yao et al., 1994; Yao et al., 1995). The chlorophyll deficiency has been considered to be caused by plastome–nuclear genome incompatibility (Kirk and Tilney–Bassett, 1978; Michishita et al., 2002; Ureshino et al., 1999; Yao et al., 1994). In *Rhododendron*, maternal, paternal or biparental plastid DNA inheritance was observed in interspecific hybridization (Michishita et al., 2002; Ureshino et al., 1999), and clear relationship between leaf color of progenies and pattern of plastid DNA inheritance was reported (Ureshino et al., 1999). According to Ureshino et al. (1999), albino plants contained maternal plastid DNA, while green and pale-green plants contained paternal plastid DNA, and chimeric plants contained both maternal and paternal plastid DNA in interspecific three-way crosses (*Rhododendron kiusianum* \times *R. eriocarpum*) \times *R. japonicum* f. *flavum*. In 10 genera of Caprifoliaceae, including genus *Lonicera*, potential biparental plastid inheritance was demonstrated by visually confirming the presence of plastid DNA in male reproductive cells (Hu et al., 2008). Therefore, inheritance pattern of plastid DNA was investigated to analyze the relationship between albino/green progenies and pattern of plastid DNA inheritance in

interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. As a result, F₁ progenies exhibited only maternal inheritance of plastid DNA in both albino and green plants in the present study. In the cross using *L. caerulea* var. *emphyllocalyx* as a seed parent, all hybrid plants were albino, suggesting the incompatibility between hybrid nuclear genome and plastome of *L. caerulea* var. *emphyllocalyx*.

Sterility of the hybrids is problem of interspecific hybridization in various horticultural crops, such as *Cosmos*, *Kalanchoe* (Mii, 2009) and *Dianthus* (Nimura et al., 2006). In most cases, the hybrid sterility is considered to be due to abnormal meiosis because of structural differences between the parent chromosomes (Mii, 2009). Hence, chromosome doubling of hybrid plants is effective for restoring the fertility (Mii, 2009; reviewed by Rieseberg, 2001). In the present study, ploidy level of both *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* was tetraploid ($2n = 4x = 36$). Therefore, their F₁ hybrids are amphidiploid and are expected to have fertility without chromosome doubling.

At present, these juvenile hybrid plants are being cultivated in the field. It will take several years for flowering. Additional evaluations will be examined at flowering stage. The morphological characteristics are greatly different between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*, especially in flower and fruit color, and fruit taste. The fruit characters of *L. gracilipes* var. *glabra* such as red color and sweet taste will be useful for increasing the variability in fruit traits of *L. caerulea* var. *emphyllocalyx*. In addition, hybrid plants may have heat tolerance from *L. gracilipes* var. *glabra*.

5. Conclusion

Interspecific hybrids were successfully obtained from reciprocal crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. Green plants were obtained from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx*, while hybrid plants from reverse cross showed albinism. This result might suggest the plastome–nuclear genome incompatibility. The hybrid plants from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* vigorously grow in a greenhouse, and expected to be breeding materials for further improvement of *L. caerulea* var. *emphyllocalyx* by backcrossing.

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1 Table 1 Characterization of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*.

Species	Habitat environment ^a	Tree characters		Flower characters			Fruit characters					Ploidy level
		Height	Shape	Flowering season ^b	Color	Morphology	Fruiting season ^b	Color	Pericarp	Morphology	Taste	
<i>L. caerulea</i> var. <i>emphyllocalyx</i>	Cold regions. Alpine, subalpine, around lowland mire	0.5-2 m	Upright to spreading dwarf	Late April-late May	Light-yellow	Two in pair	Early June-late July	Blue-black	Covered with bloom	Pericarp encloses two ovaries	Sour-sweet	$2n = 4x = 36$
<i>L. gracilipes</i> var. <i>glabra</i>	Warm regions. Deciduous forest in altitude 20-600m	1-3 m	Upright	Late April-late May	Pink	Single or rarely two in pair	Early June-mid-July	Red	Bloomless	Single ovary or rarely two in pair	Sweet	$2n = 4x = 36$

2 ^a The habitat environment was referenced by the reports of Hara (1983) and Sato (1985).

3 ^b The flowering and fruiting seasons were investigated in Sapporo, Hokkaido.

4

5 Table 2 Fruit characters of *L. caerulea* var. *emphylocalyx* and *L. gracilipes* var. *glabra*.

Species / strain	Fruit characters				
	Length (mm)	Width (mm)	Fresh weight (g)	Brix (%) ^a	pH
<i>L. caerulea</i> var. <i>emphylocalyx</i>					
Lc-T39	14.1 ± 0.7 b	11.7 ± 0.7 a	1.4 ± 0.2 a	13.2 ± 0.9 a	2.6 ± 0.1 b
Lc-Y47	16.0 ± 1.1 a	10.1 ± 0.5 b	1.1 ± 0.1 b	12.4 ± 0.9 ab	2.6 ± 0.1 b
Average	15.0 ± 1.3	10.9 ± 1.0	1.3 ± 0.2	12.8 ± 1.0	2.6 ± 0.1
<i>L. gracilipes</i> var. <i>glabra</i>					
Lg-A	11.3 ± 1.1 d	7.9 ± 0.8 c	0.5 ± 0.1 c	13.1 ± 1.4 a	5.1 ± 0.1 a
Lg-N	12.7 ± 1.0 c	8.3 ± 0.5 c	0.6 ± 0.1 c	11.2 ± 0.5 b	5.2 ± 0.2 a
Average	12.0 ± 1.3	8.1 ± 0.7	0.6 ± 0.1	12.1 ± 1.4	5.1 ± 0.2

6 ^a Brix (%) indicates total soluble solids.

7 Data represent means ± standard deviation of 10 fruits.

8 Means ± standard deviation followed by the same letter are not significantly different (Bonferroni's test, $P < 0.05$).

9

10 Table 3 Seed production, germination, and leaf colors of the progenies in interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *L.*
11 *gracilipes* var. *glabra*.

Cross combination	No. of flowers pollinated ^a	No. of set fruits	No. of total seeds obtained	No. of seeds / fruit	No. of seeds germinated	% of seed germination	Leaf color (No. of seedlings)			No. of seedlings survived
							Green	Pale green	Albino	
<i>L. caerulea</i> var. <i>emphyllocalyx</i> × <i>L. gracilipes</i> var. <i>glabra</i>										
Lc-T39 × Lg-A	5	5	59	11.8	49	83.1	0	0	49	0
Lc-Y47 × Lg-A	5	5	43	8.6	33	76.7	0	0	33	0
Lc-Y47 × Lg-N	5	5	89	17.8	45	50.6	0	0	45	0
<i>L. gracilipes</i> var. <i>glabra</i> × <i>L. caerulea</i> var. <i>emphyllocalyx</i>										
Lg-A × Lc-T39	10	10	45	4.5	45	100.0	39	6	0	9
Lg-A × Lc-Y47	3	3	12	4.0	10	83.3	8	2	0	1
Lg-N × Lc-Y47	7	7	19	2.7	18	94.7	18	0	0	10

12 ^a Pollination was performed on one mother plant.

13

Figure captions

Fig. 1. Flowers and fruits of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*.

(A) Flowers of *L. caerulea* var. *emphyllocalyx*. (B) A flower of *L. gracilipes* var. *glabra*. (C) Fruits of *L. caerulea* var. *emphyllocalyx*. (D) Fruits of *L. gracilipes* var. *glabra*.

Fig. 2. Chromosomes of *L. caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra*, and their hybrids (A) *L. caerulea* var. *emphyllocalyx* ($2n = 4x = 36$). (B) *L. gracilipes* var. *glabra* ($2n = 4x = 36$). (C, D) Different hybrid plants from *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphyllocalyx* (Lg-N \times Lc-Y47) ($2n = 4x = 36$). Bars = 5 μ m.

Fig. 3. Hybrid plants obtained from crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. (A) Green hybrid plants from *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphyllocalyx* (Lg-N \times Lc-Y47). (B) Albino seedlings from *L. caerulea* var. *emphyllocalyx* \times *L. gracilipes* var. *glabra* (Lc-Y47 \times Lg-N).

Fig. 4. RAPD profiles using the primer 380-06. M: DNA size marker. Lc: *L. caerulea* var. *emphyllocalyx*. Lg: *L. gracilipes* var. *glabra*. Lanes 1–3: Albino seedlings from *L. caerulea* var. *emphyllocalyx* \times *L. gracilipes* var. *glabra* (Lc-Y47 \times Lg-N). Lanes 4–6: green hybrid plants from *L. gracilipes* var. *glabra* \times *L. caerulea* var. *emphyllocalyx* (Lg-N \times Lc-Y47). White and black arrowheads indicate specific bands to *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*, respectively.

Fig. 5. Histograms of the relative fluorescence intensity of nuclei isolated from the leaves of *L. caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra*, and their hybrids. (A) *L. caerulea* var. *emphyllocalyx* (Lc-Y47) (B) *L. gracilipes* var. *glabra* (Lg-N) (C) Hybrid plants from *L.*

39 *gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* (Lg-N × Lc-Y47). Fresh leaves of
40 *Capsicum annuum* (cv. 'Kyonami') were used as the internal standard.

41

42 Fig. 6. PCR–RFLP analysis of plastid *atpβ*–*rbcL* region digested by *Hae*III.

43 M: DNA size marker. Lc: *L. caerulea* var. *emphyllocalyx*. Lg: *L. gracilipes* var. *glabra*. Lanes
44 1–3: Albino seedlings from *L. caerulea* var. *emphyllocalyx* × *L. gracilipes* var. *glabra*
45 (Lc-Y47 × Lg-N). Lanes 4–6: green hybrid plants from *L. gracilipes* var. *glabra* × *L. caerulea*
46 var. *emphyllocalyx* (Lg-N × Lc-Y47). White and black arrowheads indicate specific fragments
47 to *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*, respectively.



Figure 1

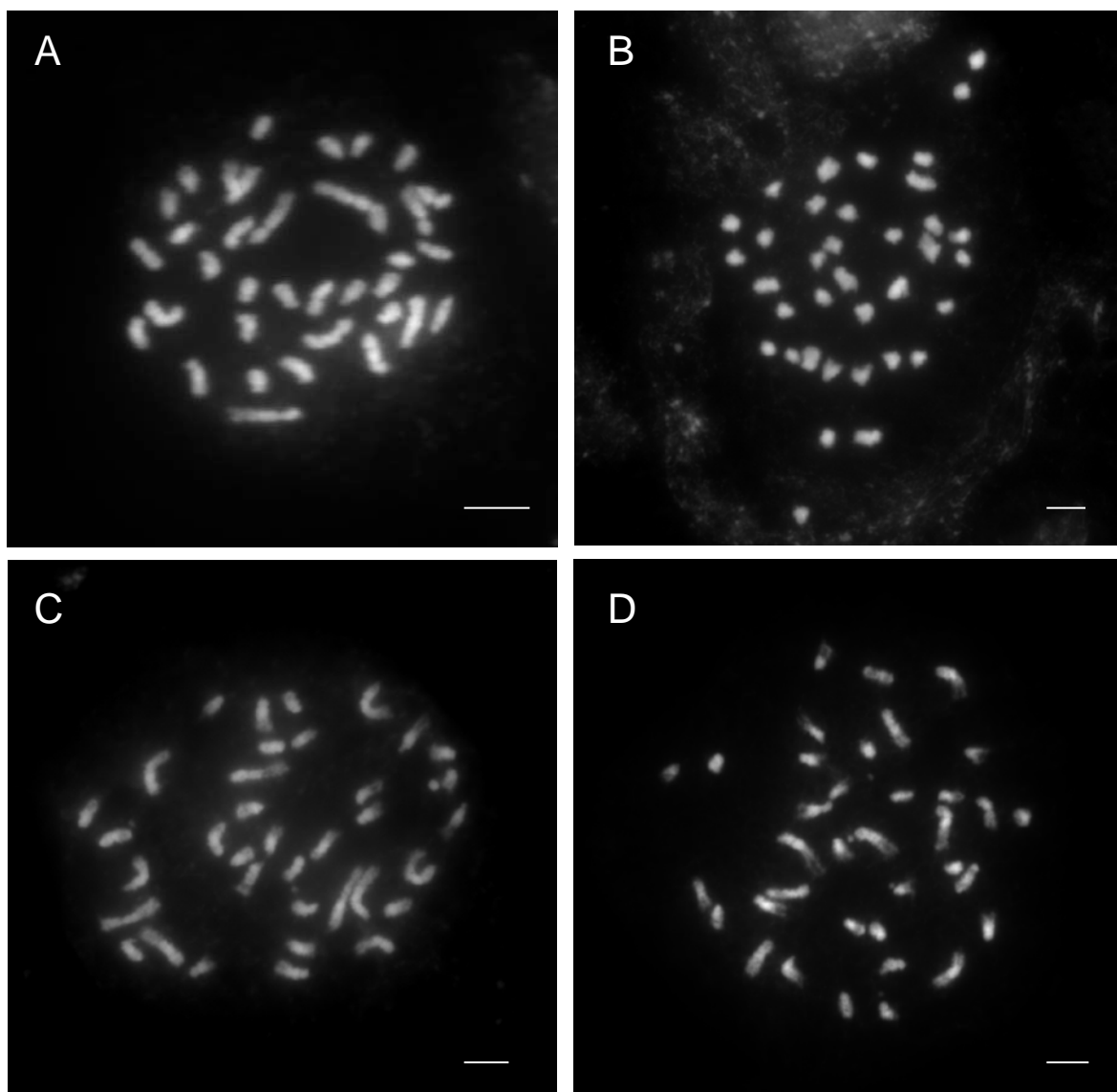


Figure 2

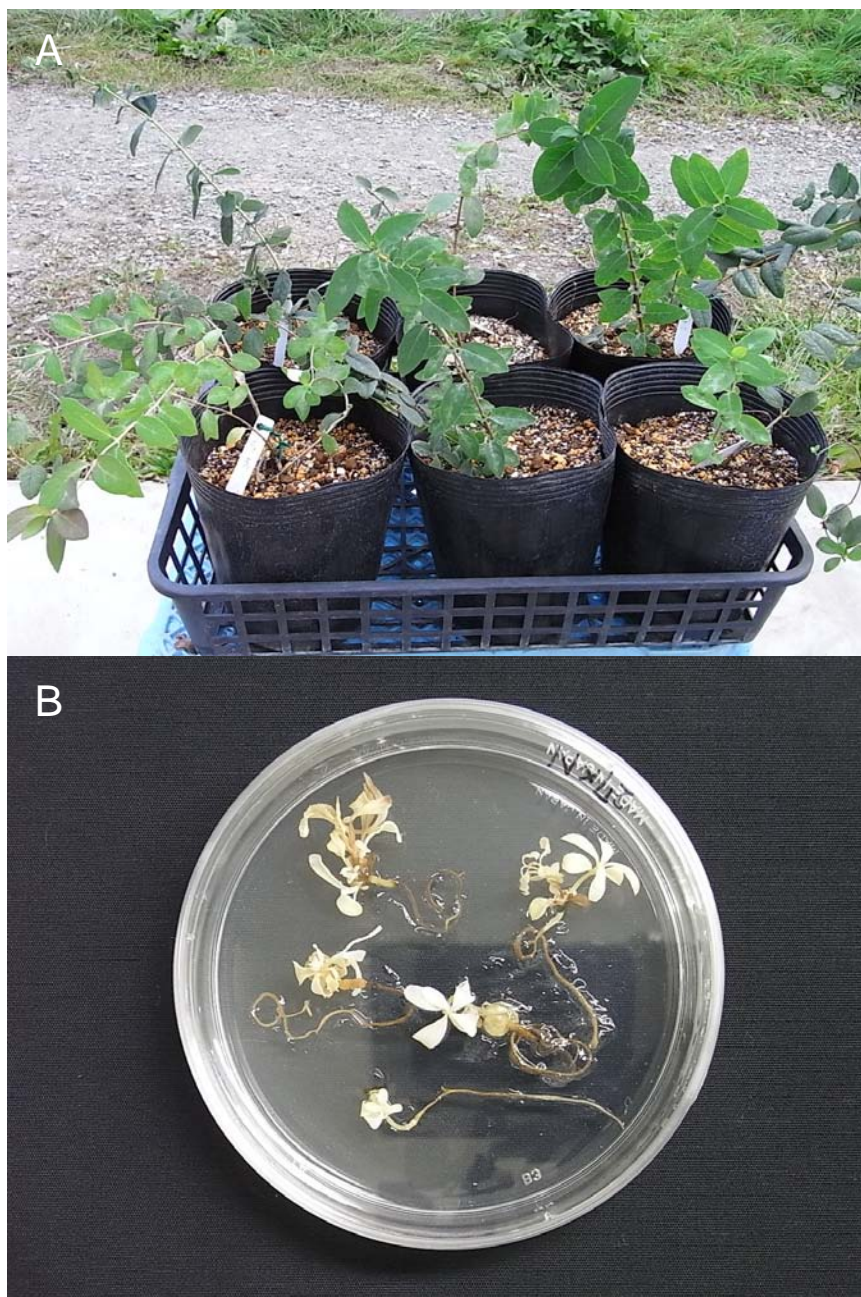


Figure 3

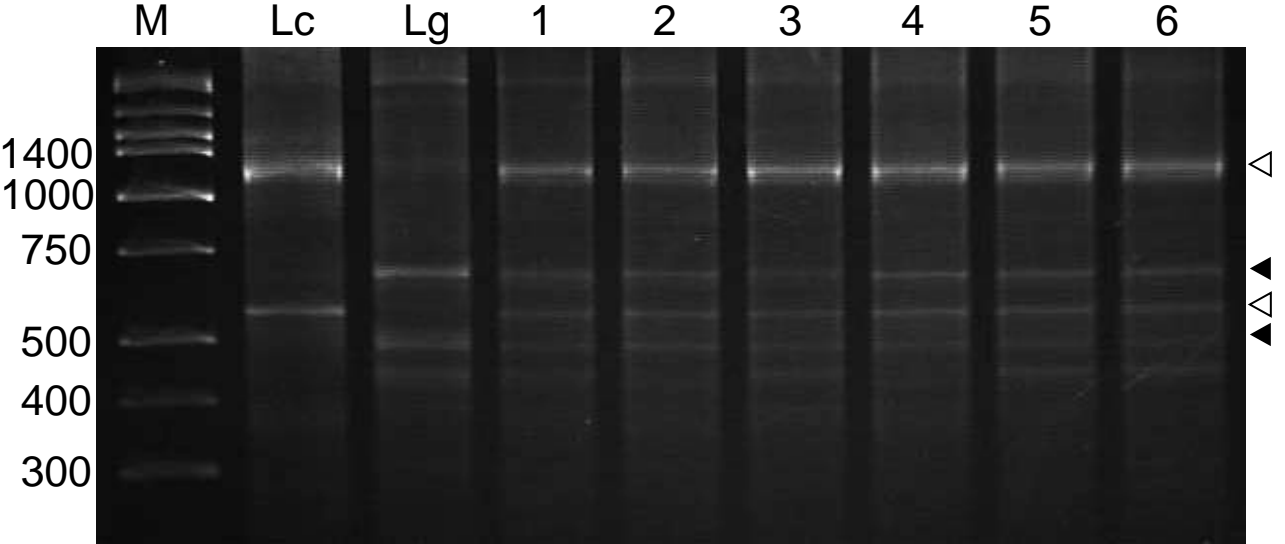


Figure 4

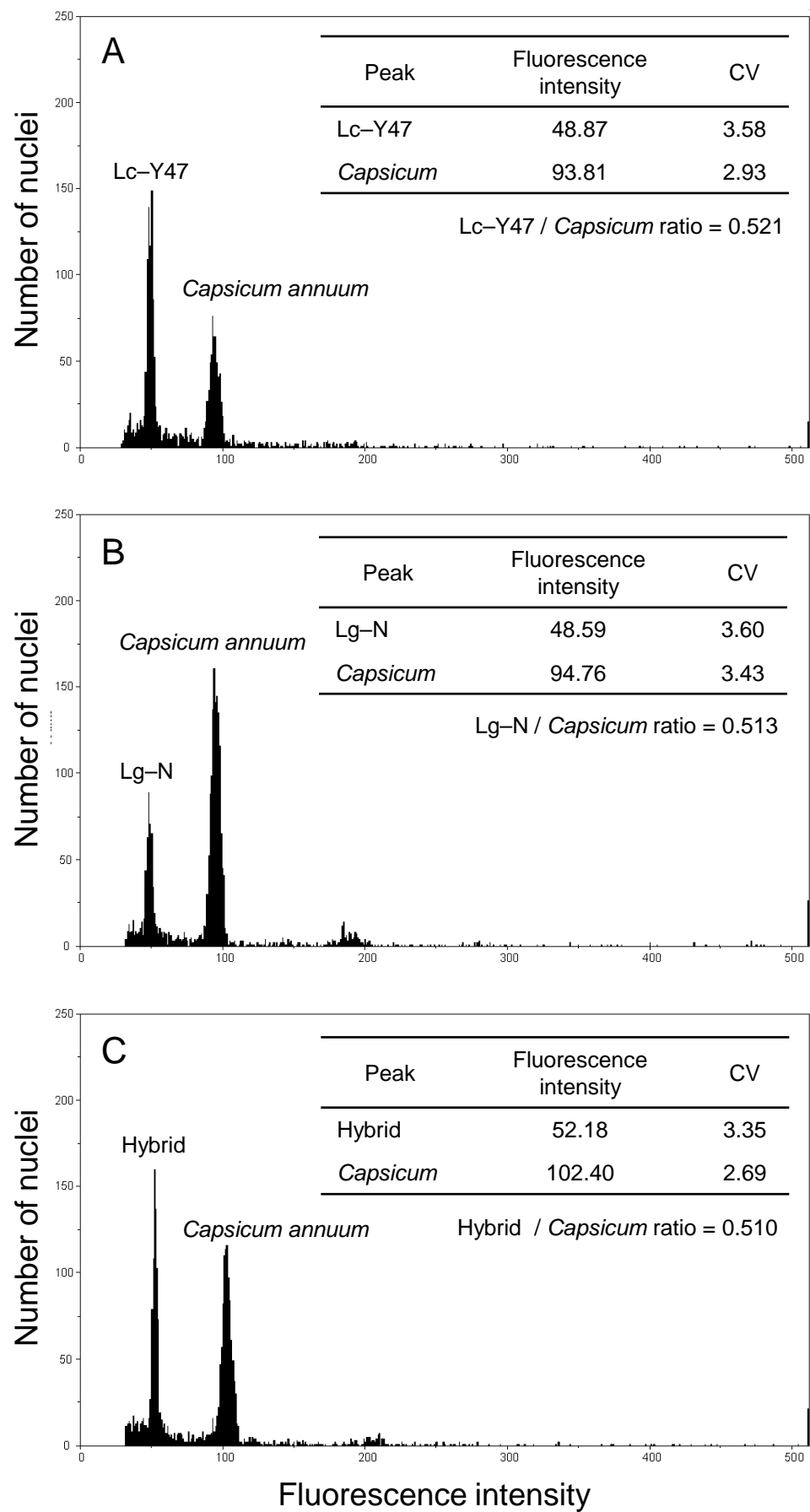


Figure 5

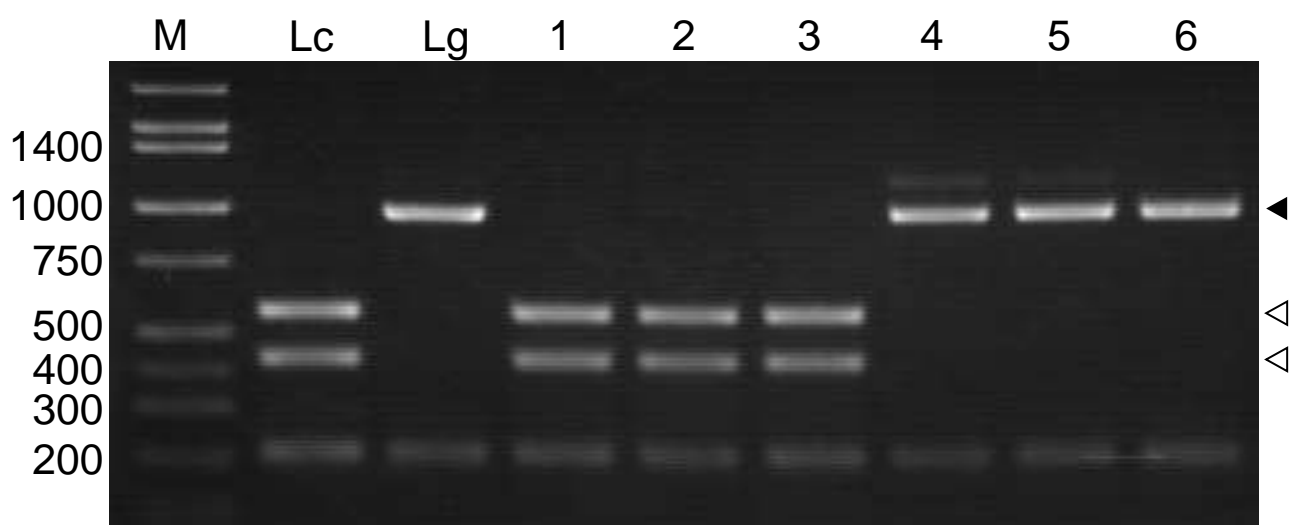


Figure 6