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Detection of section-specific random amplified polymorphic DNA (RAPD) markers in *Lilium*

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Abstract Random amplified polymorphic DNA (RAPD) markers were utilized for the identification of *Lilium* species and inter-specific hybrids. The optimum annealing temperature of the polymerase chain reaction (PCR) for the RAPD assay in *Lilium* was 54 °C, which is relatively higher than the temperature used for other genera reported by previous researchers. Among 76 primers used to amplify genomic DNA by PCR, 18 primers (24%) generated polymorphic DNA fragments in *Lilium* species and hybrids. Cultivars were also identified by RAPD markers. Some amplified fragments were unique to species of each section and to hybrids derived from these species; that is, they were the section-specific DNA markers. Sections, Sinomartagon, Leucolirion b, Leucolirion a and Archelirion could be identified by 6 section-specific markers amplified with five primers. Seven inter-section hybrids showed the section-specific bands of both parental sections, indicating that these markers would be useful for identifying the parental sections of inter-section hybrids.

Key words Cultivar identification · Hybridism determination · Lily · Random amplified polymorphic DNA (RAPD) · Section-specific DNA marker

Introduction

The genus *Lilium* consists of approximately 96 species (Asano 1989), which have been classified into six sections (Smyth et al. 1989). Many hybrid lilies have originated from inter-specific crosses, and the breeding of inter-section hybrids has been carried out (Asano 1978; Okazaki et al. 1994). The horticultural classification of lilies published by the Royal Horticultural Society (Leslie 1982) divides hybrid lilies into eight divisions.

The identification of species and hybrids and the determination of hybridism in *Lilium* have been done using morphological traits and isozyme markers. However, morphological traits are unstable because of environmental influences and the use of isozyme markers is limited by the number of informative markers. On the other hand, DNA markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers may be useful tools for these purposes, although there have been no previous reports on the application of DNA markers in *Lilium*.

The objectives of this study were: (1) to optimize polymerase chain reaction (PCR) conditions for performing the RAPD assay in *Lilium*, (2) to develop RAPD markers for distinguishing *Lilium* species and hybrids and for cultivar identification and (3) to identify section-specific RAPD markers that are able to determine the hybridism of inter-section hybrids.

Materials and methods

Plant materials

Thirteen *Lilium* species, nine intra-section hybrids and seven inter-section hybrids were used (Table 1). *L. longiflorum* and *L. speciosum*, respectively, contained two cultivars. *L. japonicum*, a wild species, contained seven individuals collected from native habitats.

DNA isolation and PCR amplification

DNA samples were isolated from fresh leaves using a CTAB method (Rogers and Bendich 1988). PCR was carried out in a 10-μl reaction mixture containing 10 ng of template DNA, 3 m*M* $MgCl_2$, 200 n*M* of primer, 0.2 units of *Taq* DNA polymerase (Wako Pure Chemical Industries and Toyobo, Japan), 200 μ*M* each of dCTP, dGTP, dATP and dTTP (Takara Shuzo, Japan), 1 × reaction buffer. A program temperature control system (PC-700, Astec, Japan) was used for DNA amplification. The cycling program consisted of three

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Table 1 *Lilium* species and hybrids used in this study

Code no.	Name of species andn hybrids	Classification[a]
1	'Connecticut King' (hybrid)	Division I
2	*L. lancifolium*	Sect. Sinomartagon
3	*L. leichtlinii*	Sect. Sinomartagon
30	*L. concolor* cv Tyousen-aka-hime	Sect. Sinomartagon
4	*L. candidum*	Sect. Lilium
6	*L.* × *formolongi* cv Hakuho (hybrid)	Division V
7	*L. longiflorum* cv Hinomoto	Sect. Leucolirion b
8	*L. longiflorum* cv Georgia	Sect. Leucolirion b
9	*L. formosanum*	Sect. Leucolirion b
10	'Aurelian Yellow' (hybrid)	Division VI
11	*L. regale*	Sect. Leucolirion a
12	*L. henryi*	Sect. Leucolirion a
13	'Pink Pearl' (hybrid)	Division VII
14	'Brushing Pink' (hybrid)	Division VII
15	'Rosario' (hybrid)	Division VII
16	'Trance' (hybrid)	Division VII
17	'Le Reve' (hybrid)	Division VII
18	'Star Gazer' (hybrid)	Division VII
19	*L. speciosum* cv Uchida	Sect. Archelirion
20	*L. speciosum* cv Mine-no-yuki	Sect. Archelirion
21	*L. auratum*	Sect. Archelirion
22	*L. rubellum*	Sect. Archelirion
23	*L. japonicum* individual 1	Sect. Archelirion
24	*L. japonicum* individual 2	Sect. Archelirion
25	*L. japonicum* individual 3	Sect. Archelirion
26	*L. japonicum* individual 4	Sect. Archelirion
27	*L. japonicum* individual 5	Sect. Archelirion
28	*L. japonicum* individual 6	Sect. Archelirion
29	*L. japonicum* individual 7	Sect. Archelirion
30	*L. nobilissimum*	Sect. Archelirion
5	'Rotehorn'[b] (hybrid)	Division VIII[c], Le.b × S.[d]
31	'Loyal Victory' (hybrid)	Division VIII[c], Le.b × S.[d]
32	'Loyal Highness' (hybrid)	Division VIII[c], Le.b × S.[d]
33	'Dianna' (hybrid)	Division VIII[c], Le.b × S.[d]
34	'Prima' (hybrid)	Division VIII[c], Le.b × S.[d]
35	'Aries' (hybrid)	Division VIII[c], Le.b × A.[d]
36	'Yuki-no-hikari' (hybrid)	Division VIII[c], Le.b × A.[d]

[a] Hybrids in dividions I, V, VI and VII are derived from crosses among species in sections Sinomartagon, Leucolirion b, Leucolirion a and Archelirion, respectively (Leslie 1982)
[b] Asano (1978)
[c] Hybrids not provided for in any other division (Leslie 1982)
[d] Inter-section hybrids. Le.b × S.: section Leucolirion b × section Sinomartagon, Le.b × A.: section Leucolirion b × section Archelirion

steps: 1 cycle of 94 °C 5 min, 54 °C 1 min and 72 °C 2 min; 40 cycles of 94 °C 1 min, 54 °C 1 min and 72 °C 2 min; 1 cycle of 94 °C 1 min, 54 °C 1 min and 72 °C 2 min, 1 cycle of 94 °C 1 min, 54 °C 1 min and 72 °C 5 min, followed by maintaining the mixture at 4 °C. The heating and cooling rates of the temperatures were 1 °C/6.5 s and 1 °C/5.3 s, respectively. Amplified samples were electrophoresed on 3% gels of NuSieve 3:1 Agarose (FMC BioProducts-Takara Shuzo) in TAE buffer (40 m*M* Tris-acetate and 1 m*M* EDTA, pH 8.0) at a constant 50 V. After staining in 2 μg/ml of ethidium bromide, the gels were photographed on a UV transilluminator. All of the reactions were repeated in separate experiments at least twice.

Primer

Two kinds of primers were used: random primers consisting of 10-base arbitrary nucleotide sequences and known DNA sequences of 10–18 bases (Table 2). The latter primers were named semi-random primers after Weining and Langridge (1991). Semi-random primers are expected to anneal at many regions of template DNA. Of a total of 76 primers, 40 random primers (OPA01–OPA20 and OPB01–OPB20) were purchased from Operon Technologies (USA), and the other 18 random primers and 18 semi-random primers were synthesized using a DNA/RNA synthesizer (model 394, Applied Biosystems).

Southern blotting

To investigate common bands from different template DNAs, sequence homology between these bands was confirmed by Southern analysis using DNA from isolated bands as probes. Amplified DNA fragments were transferred from 3% gels of NuSieve 3:1 Agarose onto nylon membranes (Hybond-N, Amersham) by the alkaline method (Reed and Mann 1985). Amplified fragments used as probes were excised from gels and purified using a centrifuged tube with filter (Suprec-01, Takara Shuzo). Southern analysis was carried out with digoxigenin labelling and AMPPD detection system (Boehringer Mannheim) according to the supplier's instructions. After hybridization, blots were washed at low stringency [2 × SSC, 0.1% (W/V) SDS at room temperature twice for 5 min] and at high stringency [0.1 × SSC, 0.1% (W/V) SDS at 68 °C twice for 15 min], followed by detection.

Table 2 List of informative primers that resulted in a large number of amplified DNA fragments. Seventy-six primers are screened in total, and only informative primers are shown

Primer code	Nucleotide sequence 5′ to 3′ (base)
Random primer	
Y24	AAC CGC GCT C (10)
Y29	TTC GGG CCG T (10)
OPA09[a]	GGG TAA CGC C (10)
OPA10[a]	GTG ATC GCA G (10)
OPA11[a]	CAA TCG CCG T (10)
OPA18[a]	AGG TGA CCG T (10)
OPB05[a]	TGC GCC CTT C (10)
OPB06[a]	TGC TCT GCC C (10)
OPB07[a]	GGT GAC GCA G (10)
Semi-random primer	
Y3[b]	GAG GGT GGC GGT TCT (15)
Y7[c]	TCG TGG CTG ACT TAC CTG (18)
Y8[d]	TGC $T^{G}_{T}T$ $^{G}_{T}T^{G}_{T}$ $T^{G}_{T}T$ GCA GGT (18)
Y14[e]	AGA GCC ACC ACC CTC (15)
Y35[f]	TGG TAT CAG AGC C (13)
Y37[g]	GTC AAG CGC G (10)
Y38[g]	TAA CCG CGC C (10)
Y41[h]	GCG TCC TGG G (10)
Y45[i]	GTC GTC GTC GTC (12)

[a] Purchased from Operon Technologies (USA)
[b] Consensus sequence of tandem repeats presented in the protein III gene of M13 (Vassart et al. 1987; Matsuyama et al. 1992)
[c] Consensus sequence of exon-intron splicing junction of plant (R1) (Weining and Langridge 1991)
[d] Consensus sequence of exon-intron splicing junction of plant (R2) (Weining and Langridge 1991)
[e] Antisense of consensus sequence of tandem repeats presented in protein III gene of M13 (Vassart et al. 1987)
[f] Sequence of primer binding site of retrotransposones in rice (Hirochika et al. 1992)
[g] Part of a tandemly repeated sequence in the *Orizae* family (Kochko et al. 1991)
[h] Part of the spacer region of wheat 5S-rDNA (Cox et al. 1992)
[i] One of the repeated unit sequences of clone SPL309, which consists of a short, highly repeated, interspersed DNA sequence in rice (Zhao and Kochert 1992)

Results

Determination of optimum PCR conditions

Annealing temperature

The effect of the annealing temperatures on amplification was estimated by testing 18 primers (Table 2) at three different temperatures; 35 °C, 54 °C and 60 °C. Amplification with 5 primers (Y3, Y7, Y8, Y14 and Y35) resulted in a smear pattern at 35 °C and many bands at 54 °C. Another 13 primers could amplify many products at 35 °C and 54 °C and the numbers of distinct bands increased as the annealing temperature increased (Fig. 1). Amplification with primers Y7 and Y14 produced more distinct band patterns at 60 °C than at 54 °C. However, the use of other primers resulted in the production of no or fewer bands at 60 °C. Consequently, 54 °C was determined to be the optimum annealing temperature of the RAPD assay in *Lilium*. In subsequent tests, template and primer DNAs were annealed at 54 °C.

Identification of informative primers

Seventy-six primers were screened using the template DNA from *L. lancifolium*, *L.* × *formolongi* and *L. japonicum* (individual 5) to identify informative primers that generated polymorphic patterns of PCR products. In total, 18 primers (24%) (Table 2) were informative, 11 primers (14%) generated a smear pattern and the remainder failed to amplify (62%). Nine semi-random primers out of 18 (50%) were informative in contrast with purely random primers of which 9 out of 58 (16%) were informative. Thus, semi-random primers were more efficient for obtaining informative primers.

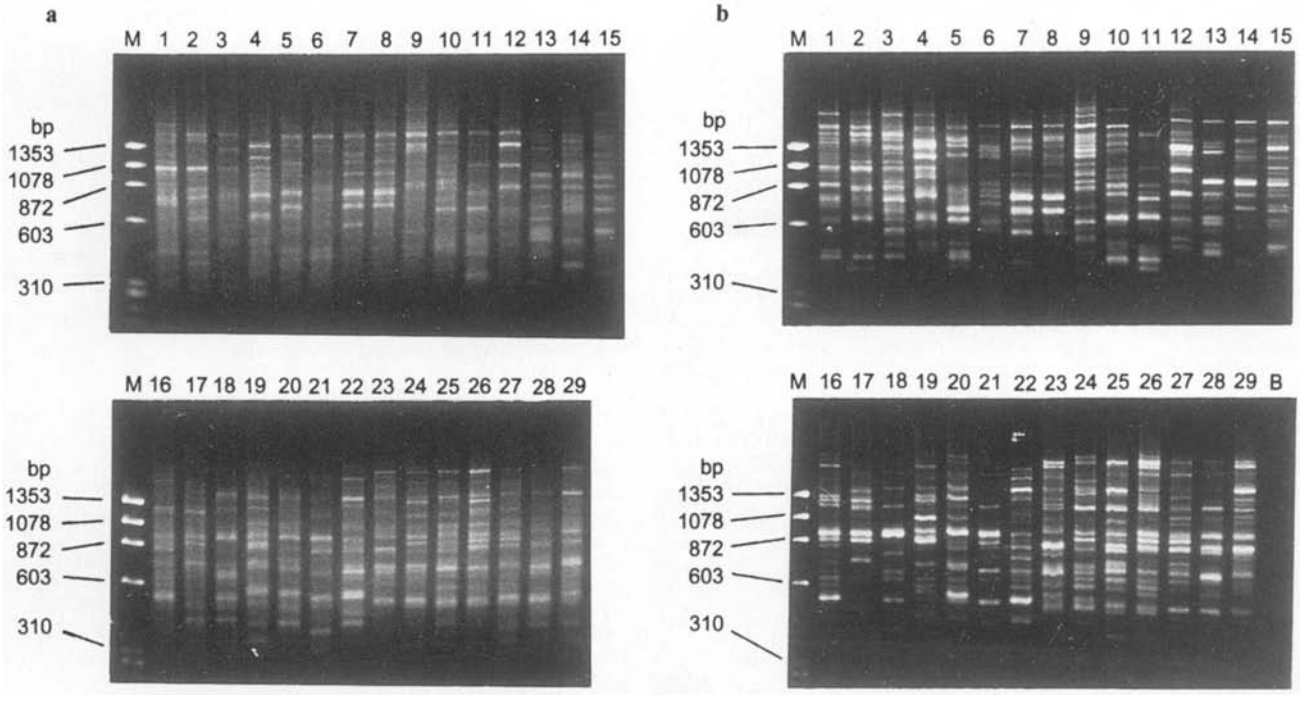
Reproducibility of RAPD patterns

Two distinct template DNAs of each of hybrids Trance and Pink Pearl were prepared from two separate plants on different days. The use of these template DNAs and primer Y37 ensured that an identical band pattern to another sample of the same hybrid was produced in each hybrid (data not shown). All reactions were repeated at least twice and most of the bands appeared at each repetition with several exceptions of a few number of low intensity bands. These results imply that differences in amplified fragment patterns reflect DNA sequence diversity in *Lilium*.

Discrimination of *Lilium* species and hybrids and cultivar identification

Thirty-seven materials (Table 1) were assayed with the 18 informative primers (Table 2). Examples of RAPD patterns amplified with primer Y24 are shown in Fig. 1b. Each of the 18 primers resulted in unique patterns for each species and hybrids, indicating that species and hybrids could be easily distinguished by the RAPD markers.

Fig. 1a, b Effects of annealing temperatures of 35 °C (**a**) and 54 °C (**b**) on amplification products from genomic DNAs of *Lilium* using primer Y24. *Lane number* corresponds to the number given in Table 1. *M* and *B* indicate the DNA size marker (*Hae*III-digested ϕ X174 DNA) and blank (template DNA was eliminated from the reaction mixture), respectively



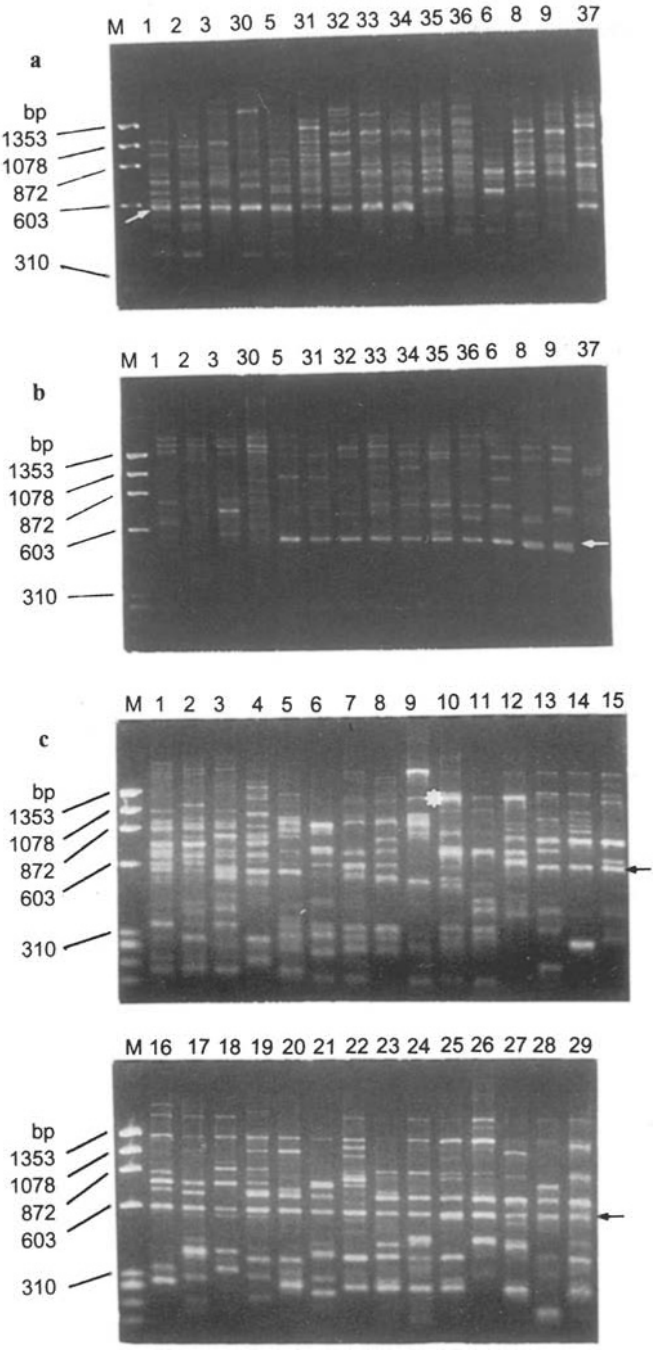


Fig. 2a–c Sectioin-specific markers generated by primer Y35 (**a**), OPB06 (**b**) and Y37 (**c**). The *arrows* and *asterisk* indicate the specific markers; that is, the 600-bp band in panel **a** is specific to section Sinomartagon, the 550-bp band in panel **b** is specific to section Leucolirion b, the 600-bp band in panel **c** is specific to section Archelirion and the 1,200-bp band in panel **c** (asterisk) is specific to section Leucolirion a. *Lane number* corresponds to the code number given in Table 1. *M* indicates the DNA size marker (*Hae*III-digested ϕX174 DNA)

Two cultivars of *L longiflorum* (code 7 and 8) produced different patterns with each of the 14 single primers except for Y3, Y7, Y8 and Y14. In *L. speciosum* (19 and 20), each of the 17 primers produced distinct patterns between the two cultivars, except for primer Y8.

Table 3 Survey of section-specific RAPD markers in *Lilium* species and hybrids[a]

Section-specific RAPD marker		Code number of species and hybrids, and their classification[b]						Section identified	Note
Primer	Marker size (bp)	1 to 3, 30 Division 1 and section Sinomartagon	4 Section Lilium	6 to 9 Division V and section Leucolirion[a]	10 to 12 Division VI and section Leucolirion[b]	13 to 29,37 Division VII and section Archelirion	5,31 to 34 Intersection hybrid, Le.b × S.[c]	35, 36 Intersection hybrid, Le.b × A.[d]		
Y35	600	+	−	−	−	−	+	−	Sinomartagon	Fig. 2a
Y29	800	−	−	+	−	−	+	+	Leucolirion b	
OPB06	550	−	−	+	−	−	+	+	Leucolirion b	Fig. 2b
OPB07	1,500	−	−	+	−	−	+	+	Leucolirion b	
Y37	1,200	−	−	−	+	−	−	−	Leucolirion a	Fig. 2c
Y37	600	−	−	−	−	+	−	+	Archelirion	Fig. 2c

[a] + and − indicate presence and absence, respectively
[b] Refer to Table 1
[c] Section Leucolirion b × section Sinomartagon
[d] Section *Leucolirion* b × section *Archelirion*

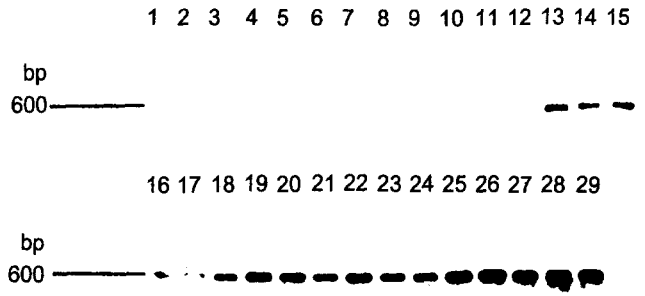


Fig. 3 Southern blot analysis of section Archelirion-specific RAPD marker. The 600-bp band from lane 27 (*L. japonicum*, individual 5) was used to probe a Southern blot of amplified fragments generated by primer Y37 (**Fig. 2c**). *Lane number* corresponds to the code number given in Table 1

All seven individuals of *L. japonicum* (23–29) resulted in a distinct fingerprint with single primers Y7, Y24, Y29, Y35, Y37, Y38, OPA09, OPA10, OPA11, OPB05 and OPB06, and several individuals were distinguishable with the other primers. These results indicate that the RAPD assay is also useful for the identification of *Lilium* cultivars and individuals using these informative primers.

Section-specific RAPD markers

Some amplified fragments were identified as section-specific DNA markers. Examples of the markers are shown in Fig. 2. Of a total of six such markers amplified with 5 primers, one was specific to section Sinomartagon, three to section Leucolirion b, one to section Leucolirion a and one to section Archelirion (Table 3).

In the lanes of inter-section hybrids in Le.b × S. (5 and 31–34), markers specific to both of the parental sections, Sinomartagon and Leucolirion b, appeared (Fig. 2a and b, Table 3). When genomic DNA of inter-section hybrids in Le.b × A. was used (35 and 36), Leucolirion b- and Archelirion-specific markers were amplified (Fig. 2b and Table 3).

Homology between sequences representing section-specific markers from different species and hybrids was confirmed by Southern analysis of RAPD gels using DNA from isolated bands as probes. An example of the results is shown in Fig. 3. All six markers proved to be section-specific.

Discussion

In a RAPD assay, relatively low annealing temperatures are usually used to permit amplification from targets that may have only poor homology to the primers (Williams et al. 1990) and the number of low intensity bands has tended to decrease as annealing temperatures increased (Caetano-Anollés et al. 1992). In the RAPD assay for *Lilium*, however, the optimal annealing temperature (54 °C) for the amplification of many products was relatively high, and the number of distinct bands increased with increasing annealing temperature (Fig. 1). That is, the PCR condition is unique. This may be due to the nature of *Lilium* genomes that have large sizes (30–40 millioin kbp) and contain many repeated DNA sequences (Leeton and Smyth 1993).

A large number of polymorphic markers were amplified with each of the informative primers (Fig. 1b). All of the species and hybrids and most cultivars and individuals could be identified with a single amplification by PCR. The RAPD assay for *Lilium* will provide useful tools for studying the genetic variation in *Lilium* because of the large number of polymorphic markers.

Six section-specific markers that were common in both species in a section and hybrids derived from intra-section crosses were identified. In inter-section hybrids, markers specific to both of the parental sections appeared, which reflects cross combination of the hybrids (Fig. 2 and Table 3). This indicates that the section-specific markers are useful in determination of their hybridism.

In the case of hybridism determination, the RAPD method can be applied for in vitro culture of bulblets derived from immature embryos because only nanogram quantities of template DNA are required. This method can also be used in screening authentic hybrids rapidly from a large number of putative hybrids because of the simple and fast experimental procedure. Therefore, the RAPD method is more advantageous than other methods such as morphological observation, isozyme and RFLP, as reported previously (Welsh et al. 1991; Baird et al. 1992; Xu et al. 1993).

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