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Full Length Article

Identification of Lily Hybrids by Sequence-Related Amplified Polymorphism Analysis

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Abstract

It is difficult to identify the authenticity of lily hybrids from distant crosses during the early growth stage by traditional morphological traits. In the study, Sequence-related amplified polymorphism (SRAP) was used to identify the authenticity of lily hybrids. Eight SRAP primer pairs were screened according to polymorphism between male and female parents in 10 cross combinations and used to identify true lily hybrids. Among 550 bands produced using selected primer pairs, 233 of them showed polymorphism. A total of 41 progenies with paternal specific markers were identified as true hybrids. All of progenies from cross-combination H5 and H9 were considered as false hybrids because no male parent-specific band was amplified from them using all screened SRAP primer pairs. The results obtained in this paper indicate that SRAP is effective to identify the authenticity of lily hybrids. © 2018 Friends Science Publishers

Keywords: Lily; SRAP; Hybrid identification; Paternal specific marker; True hybrids; Cross breeding

Introduction

Lily (*Lilium* spp.) is one of the most famous ornamental flowers in the world. The genus *Lilium* with more than 90 species belongs to the family Liliaceae (Yamagishi *et al*., 2002). So far, 2,785 lily cultivars from interspecific crosses have been registered in Royal Horticultural Society (https://www.rhs.org.uk/). They are classified into three main groups of lily hybrids: Longiflorum (L), Asiatic (A) and Oriental hybrids (O). These groups originate from taxonomic Section Leucolirion, Sinomartagon and Archelirion, respectively (Lim *et al*., 2002).

Interspecific and intraspecific crosses have proved to be the most important tools for lily breeding. New cultivars can be obtained by combination of favorable genetic traits and selection of targeted phenotype in fields (Tuyl and Holsteijn, 1996). In contrast to interspecific crosses within the same section, hybridization between lily species or cultivars from different sections is generally difficult because of pre- and post-fertilization barriers (Luo *et al*., 2014). And some crosses among Asiatic lily cultivars are incompatibility (Jiao *et al*., 2010).

A range of methods, such as cut-style, ovary-slice and embryo culture, are applied to remove fertilization barriers of intersectional hybrids (Georgi, 1985; Nassar *et al*., 1998). The embryo culture is an effective method to shorten the period of vegetative growth of lily hybrids. However, not all offspring from interspecific crosses are real hybrids. They may come from unfertilized maternal cells (Georgi, 1985; Nassar *et al*., 1998; Marasek *et al*., 2004). Therefore, it is necessary to identify whether plants from hybridization are indeed hybrids or not.

It is difficult to identify the authenticity of hybrids from distance crosses during the early growth stage by traditional morphological traits. Some alternative technologies, such as isozyme analysis, fluorescence in situ hybridization (FISH), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR), based on biochemical, cytological or molecular markers, are developed to quickly and efficiently discriminate true hybrids from false ones, and speed up the breeding process (Hulya, 2003; Marasek *et al*., 2004; Gu *et al*., 2012; Zhao *et al*., 2017). Compared with other technologies, SRAP has proven to be a new, reproducible, less expensive useful molecular marker system, which can generate more polymorphic fragments than SSR, ISSR or RAPD markers (Budak *et al*., 2004). SRAP is a PCR-based marker system that preferentially recognizes coding sequences randomly distributed throughout genome (Li and Quiros, 2001). Forward and reverse primers with core sequences of 13 to 14 nucleotides are used in SRAP analysis. The first 10 or 11 bases at 5'-end of core sequence are used as filler sequences, and the sequence CCGG or AATT is added to the 5'-end of filler sequence in the forward or reverse primer, respectively. The core sequence is followed by three selective nucleotides at

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the 3'-end of each primer (Li and Quiros, 2001). Forward and reverse primers can amplify polymorphic sequences that reflect variations in the length of introns, spacers and promoters among different genotypes and populations (Mishra *et al*., 2011). SRAP has been widely applied to research genetic diversity (Ferriol *et al*., 2003; Budak *et al*., 2004; Merotto *et al*., 2009), map constructions (Li and Quiros, 2001; Lin *et al*., 2003), comparative genomics (Li and Quiros, 2001), and character gene marker (Pan *et al*., 2003). Recently, SRAP has been used to identify hybrid offspring (Liu *et al*., 2007; Chen *et al*., 2008; Hao *et al*., 2008; Mishra *et al*., 2011).

So far, some methods, such as isozyme, FISH and RAPD, have been applied to identify lily hybrids (Hu *et al*., 2009; Yang *et al*., 2011; Gu *et al*., 2012). However, there are not any researches about lily hybrid identification using SRAP molecular markers. Considering these advantages of SRAP technology, we applied SRAP marker to identify lily hybrids in this study.

Materials and Methods

Plant Materials

Fifty-one lily hybrid progenies from 10 cross-combinations by cut-style and embryo rescue, and their parents were used in this study (Table 1). All these plant materials were conserved in the greenhouse of Beijing University of Agriculture.

DNA Extraction

The lily genome DNA was prepared according to Chen's method (2013). About 0.2 g leaf was frozen in liquid nitrogen, grounded to powder and then transferred to 1.5 mL tube. Six hundreds microliters 2 × CTAB extraction buffer with 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 4% beta-Mercaptoethanol and 100 mM Tris HCl (pH 8.0), were preheated at 65^{o}C and added to the tube with lily powder. The suspension was incubated at 65^{o}C for 60 min and shaken once every several minutes. After that, the sample was cooled down to room temperature and centrifuged at 12000 rpm for 10 min at 4^{o}C. The supernatant was transferred into a new tube and extracted twice using chloroform-isoamylalcohol (24:1). The supernatant was precipitated using 1/3 volume 5 M NaCl and 3/4 volume isopropanol in a new tube at -20^{o}C for 30 min and centrifuged at 10000 rpm for 5 min. The pellet was washed 3 times with 70% (V/V) ethanol and dissolved in 30 µL TE buffer.

SRAP Analysis

Eighty SRAP primer combinations derived from 8 forward and 10 reverse primers were used in this study (Table 2). All primers were designed according to the rule of Li and Quiros (2001). SRAP primer combinations, which produced unambiguous, reproducible and polymorphism bands, were selected and used for the analysis of lily hybrids.

The SRAP-PCR reaction mixture contained 0.4 µL of dNTPs (10.0 mM), 2.0 µL of 10 × Taq buffer, 4.0 µL of DNA template (50µM), 0.6 µL of each primer (10.0 mM), and 0.5 µL of Taq DNA polymerase (2.5 Units µL^{-1}) and topped to 20 µL volume by adding sterile super pure water (Millipore, US). The thermal cycling profile was used for amplification as follow: 95^{o}C for 3 min; 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min; and 72^{o}C for 1.5 min; then 72^{o}C for 10 min. The SRAP PCR products were run on 8% non-denaturing polyacrylamide gel electrophoresis and detected by staining with $AgNO_3$ solution. The gel profiles were scanned and documented using the UMAX software program. 100 bp LadderII (Dingguo biotech, China) was used as size markers.

Results

SRAP Markers Polymorphism

Eight primer pairs were screened from 80 SRAP primer combinations according to polymorphism between male and female parents in every cross-combination, and used for subsequent analysis of hybrids (Table 2). The number of applicable SRAP primer pairs for every cross combination ranged from 3 to 6. Among 550 bands amplified from male and female parents of 10 cross combinations using these primer pairs, 233 (42.4%) bands were polymorphism (Table 3). The number of bands amplified by every primer pair ranged from 2 (Me3/Em7 for cross-combination H5) to 30 (Me2/Em5 for H8). The average number of bands amplified for every cross combination using one primer pair ranged from 5.2 (H9) to 20.3 (H2), with an average of 12.3. The percentage of polymorphic bands produced by primer pairs in different cross combinations ranged from 19.2% (H10) to 77.8% (H3), with an average of 40.6%. The percentages of polymorphic bands from cross types of A × A and O × O were higher than that from other types of crosses.

True Hybrids Identification

Fifty-one lily hybrids from 10 cross-combinations were identified using eight SRAP primer pairs (Table 4). SRAP markers obtained from cross progenies were classified into two types according to the appearance of paternal specific bands. The SRAP markers with paternal specific bands belong to Type I, and the hybrids with Type I could be identified for true hybrids (Fig. 1). The SRAP markers without any paternal specific band belong to Type II, and the hybrid offspring with Type II grow up from maternal cells. All of the progenies from cross-combination H1, H2, H3, H4, H6, H7, H8 and H10 were identified to be true hybrids (Table 4). One to three paternal specific bands were obtained from each true hybrid (Fig. 1).

Table 1: Parents and hybrids of lily analyzed using SRAP marker

Code of cross	Parents		Type of cross*
	Female	Male	
H1	Orange Matrix	Fancy	A×A
H2	Orange Matrix	Golden Joy	A×A
H3	Mount Duckling	Monte Negro	A×A
H4	Dynasty	*L. pumilum*	A×W
H5	Courier	Couplet	LA×LA
H6	Fabgio	Esprit	LA×LA
H7	Red Alert	Esprit	LA×LA
H8	Golden Joy	Tropical	A×O
H9	Red Latin	*L.davidii*	A×W
H10	Esprit	Tresor	LA×A

*A: Asiatic hybrid, O: Oriental hybrid, LA: Longiflorum × Asiatic hybrid, W: wild lily

Table 2: Sequences of primers and primer pairs used for SRAP analysis

Forward primers		Reverse primers		Primer pairs screened for crosses	
No.	Sequence(5'-3')	No.	Sequence(5'-3')	Primers	Code of cross
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT	Me1/Em4	H1,H7
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC	Me2/Em5	H1,H2,H3,H5,H6,H7, H8,H9,H10
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC	Me3/Em7	H1,H2,H3,H4,H5, H6,H8,H9,H10
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA	Me4/Em4	H1,H2,H3,H4,H8
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC	Me5/Em8	H1,H7
Me6	TGAGTCCAAACCGGTAA	Em6	GACTGCGTACGAATTGCA	Me7/Em2	H2,H3,H6,H8,H9
Me7	TGAGTCCAAACCGGTCC	Em7	GACTGCGTACGAATTGAA	Me7/Em7	H1,H3,H4,H5,H7, H8,H9,H10
Me8	TGAGTCCAAACCGGTGC	Em8	GACTGCGTACGAATTCTG	Me7/Em10	H3,H4,H8,H9
		Em9	GACTGCGTACGAATTCGA		
		Em10	GACTGCGTACGAATTCAG		

Table 3: SRAP markers characteristics in lily hybrids

Code of cross	Total primer pairs	Total bands	Number of bands per primer pair (range)	Average of bands per primer pair	Total Number of polymorphic bands	Percentage of polymorphic bands (%)
H1	6	101	14-23	16.8	46	45.5
H2	4	81	7-23	20.3	43	53.1
H3	5	27	5-7	5.4	21	77.8
H4	4	73	16-22	18.3	39	53.4
H5	3	29	2-14	9.7	6	20.7
H6	3	22	3-16	7.3	9	40.9
H7	4	47	5-16	11.8	18	38.3
H8	6	118	11-30	19.7	40	33.9
H9	5	26	3-15	5.2	6	23.1
H10	3	26	5-12	8.7	5	19.2
Total		550			233	

The efficiency of primer pairs for true hybrids identification was different. Some primer pairs were able to recognize all of the true hybrids in detected progenies, such as Me3/Em7 (for H1, H2, H4, H6 and H8), Me2/Em5 (for H2, H3 and H10), Me5/Em8 (for H1 and H7), and Me7/Em7 (for H1 and H8). However, these primer pairs could identify only part of the true hybrids in other cross combinations, for example Me3/Em7 for H3 and Me2/Em5 for H8 (Table 4). No any paternal specific band was obtained in these progenies from H5 and H9 using all screened primer pairs, so we considered that ten progenies from H5 and H9 were not true hybrids (data not shown).

Discussion

Cross progenies of lily plants are not always true hybrids because they can arise as a result of apomixes (Marasek *et al*., 2004). Therefore, the rapid and accurate identification of hybrid authenticity is very important for lily crossing-breeding. Traditionally, morphological parameters have been widely used to discriminate true hybrids from false ones. However, the identification based on morphological characteristics is labor-consuming, and needs a lot of field to grow hybrid progenies. Furthermore, lily is perennial plant and needs more than 2 years to show vegetative and reproductive characteristics. And these characteristics may be changed in different growing conditions. Therefore, it is necessary to build an effective method to identify hybrid authenticity at the early growth stage. Recently, some molecular markers, such as RAPD, amplified fragment length polymorphism (AFLP), SSR, ISSR and SRAP, have been applied to identify hybrids

Table 4: Identification results of true hybrids

Code of cross	Number of progenies	Primer pairs	Codes of true hybrids	Total number of true hybrids
H1	4	Me1/Em4	1-8	4
		Me2/Em5	1-8, 1-10	
		Me3/Em7	1-6, 1-7, 1-8, 1-10	
		Me4/Em4	1-6, 1-7, 1-8	
		Me5/Em8	1-6, 1-7, 1-8, 1-10	
		Me7/Em7	1-6, 1-7, 1-8, 1-10	
H2	4	Me2/Em5	2-1, 2-2, 2-3, 2-4	4
		Me3/Em7	2-1, 2-2, 2-3, 2-4	
		Me4/Em4	2-1, 2-2	
		Me7/Em2	2-1, 2-2, 2-3, 2-4	
H3	4	Me2/Em5	3-4,3-5,3-7,3-8	4
		Me3/Em7	3-4,3-8	
		Me7/Em10	3-7,3-8	
H4	12	Me3/Em7	4-1, 4-3, 4-5, 4-11, 4-14, 4-25, 4-27, 4-29, 4-30, 4-32, 4-34, 4-36	12
		Me4/Em4	4-1, 4-3, 4-5, 4-11, 4-14, 4-25, 4-27, 4-29, 4-30, 4-32, 4-34, 4-36	
		Me7/Em7	4-1, 4-3, 4-5, 4-11, 4-25, 4-27, 4-29, 4-30, 4-32, 4-34	
		Me7/Em10	4-1, 4-3, 4-5, 4-11, 4-25, 4-27, 4-29, 4-30, 4-32, 4-36	
H5	6			0
H6	4	Me3/Em7	6-19, 6-47, 6-48, 6-56	4
		Me6/Em2	6-47, 6-56	
H7	6	Me1/Em4	7-44, 7-50, 7-51, 7-55	6
		Me2/Em5	7-55, 7-57	
		Me5/Em8	7-44, 7-50, 7-51, 7-55, 7-57, 7-71	
H8	2	Me2/Em5	8-1	2
		Me3/Em7	8-1, 8-2	
		Me7/Em2	8-1, 8-2	
		Me7/Em7	8-1, 8-2	
H9	4			0
H10	5	Me2/Em5	10-13, 10-14, 10-20, 10-22, 10-23	5
		Me3/Em7	10-14, 10-20, 10-22, 10-23	
		Me7/Em7	10-13, 10-22, 10-23	

(Hulya, 2003; Wu *et al*., 2006; Lubell *et al*., 2008; Chiaralo *et al*., 2011; Mishra *et al*., 2011). However, molecular markers applied for identifications of lily hybrids have rarely been reported.

In this study, SRAP markers have proved to be high efficient, rapid and reproducible on identifying lily hybrid authenticity. The authenticity of lily hybrid could be tested using SRAP when they are still culture seedling. Therefore efforts and resources can be focused on the cultivation of true hybrids, such as rooting, hardening seedlings, greenhouse transplanting, and promote them to blossom as soon as possible. The SRAP technology will effectively shorten the time of breeding the true lily hybrid with good characters into new cultivar.

Here we found that some SRAP primer pairs can only identify part of the true hybrids in a cross combination. This phenomenon may have resulted from DNA recombination, mutant and random segregation of chromosomes in meiosis during hybridization, which could bring on the loss of binding site of polymorphism SRAP primer in hybrids (Darnell *et al*., 1990; Huckett and Botha, 1995; Smith *et al*., 1996). And recombination and/or mutational events during hybridization are also responsible for the hybrid specific bands (Darnell *et al*., 1990; Huckett and Botha, 1995; Dabkevičiene *et al*., 2008). Therefore, the hybrid nature of the progenies could not be judged by one SRAP primer pair.

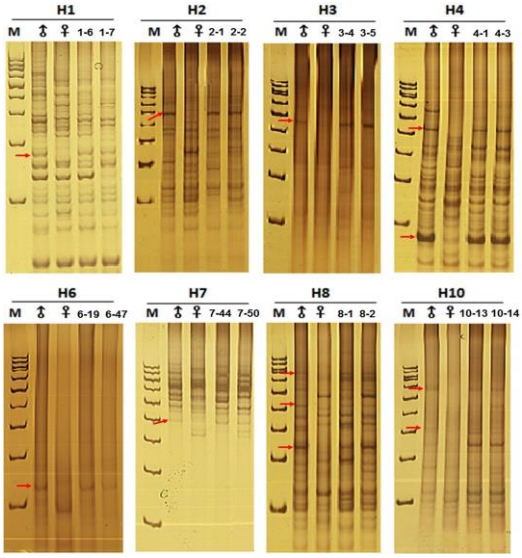

Fig. 1: Inheritance of specific bands from male parent in lily hybrids

Specific bands from cross-combination H1, H2, H3, H4, H6, H7, H8 and H10 were amplified by primer pair Me5/Em8, Me3/Em7, Me2/Em5, Me4/Em4, Me3/Em7, Me5/Em8, Me3/Em7 and Me2/Em5 respectively. In every cross two hybrids were given and located in two left lanes. Red arrow shows the position of paternal specific band in parent and hybrids. M: 100 bp Ladder Ⅱ ranged from 0.1 to 1kb, ♂: male parent; ♀, female parent

For cross-combination H5 and H9, no paternal specific band could be detected from any progenies using all of screened primer pairs (Table 2 and 4), therefore, none of detected progenies from H5 and H9 was considered as true hybrid under our experiment condition. It suggests that crosses between Courier × Couplet and Red Latin × *L. davidii* are unsuccessful in this study.

Conclusion

We obtained 233 polymorphic bands between male and female parents in 10 lily cross combinations using 8 SRAP primer pairs. Forty one true lily hybrids from 51 progenies of 10 cross combinations were successfully identified using selected SRAP primer pairs. Our research shows that SRAP molecular marker technology is effective to identify the authenticity of lily hybrids.

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