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STUDY ON GERMPLASMIC RESOURCES OF *LYCORIS LONGITUBA* USING RAPD AND ISSR

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Key words: *Lycoris longituba;* RAPD; ISSR; Germplasmic resources

Abstract: The perianth DNA extraction methods were discussed as L*ycoris longituba* for example. By means of RAPD and ISSR, germplasmic resources of *Lycoris longituba* were primarily studied. The results were as follow: by RAPD, a total of 77 discernible loci were obtained using 12 primers, of which 53 loci were polymorphic (PPB = 68.8%); by ISSR, 67 discernible loci were got using 9 primers, of which 62 loci were polymorphic (PPB = 92.5%). So, genetic diversity of *Lycoris longituba* was abundant, whose germplasmic resources could be stored for breeding. From UPGMA dendrogram of *Lycoris longituba* using RAPD or ISSR method, three *Lycoris longituba* types were supported with molecular evidence, which were originally distinguished by flower color. Therefore, in the future use of *Lycoris longituba* germplasmic resources, different varieties of *Lycoris longituba* could be cultivated.

INTRODUCTION

Lycoris longituba is a perennial, bulbiferous, herbaceous plant in the genus of *Lycoris*, *Amaryllidaceae* family. A unique biological characteristic of this plant is that its leaves come out in spring and die in early summer, followed by flowering. Its vegetative growth and reproduction are discrete, which is rare in angiosperm. With a vigorous and long scape, its flowers are diverse in both color and floral form, and fragrant in smell, having a high ornamental value. With a reputation for Chinese tulip, this species is ideal for ikebana. We collected more than 70 variants of this species in our laboratory with different floral colors and forms. In order to understand the reasons behind the variation, ISSR and RAPD markers were applied to study germplasmic resources of *Lycoris longituba*.

Inter-simple sequence repeats (ISSR) is a type of molecular marker recently proposed by Zietkiewicz *et al.* (1994) for fingerprinting. The ISSR method applies the principle of simple sequence repeat (SSR)-anchored polymerase chain reaction (PCR) amplification by designed primers that can randomly amplify DNA fragments of the inter-repeat region. This method does not need DNA sequence information prior to amplification. Therefore it is particularly useful in studying those species without available sequence information. The ISSR method usually generates more stable PCR products as compared with the RAPD technique, but it is as simple as RAPD in operation (Zou *et al.*, 2001). The ISSR fingerprinting has been commonly used in studies, such as population genetics, taxonomy and phylogeny of many plant species (Ajibades SR *et al.*，2000；Huang JC *et al.*，2000；Blair MW *et al.*，1999；Moreno *et al.*，1998；Fang DQ *et*

al. ,1998 ;Akagi H *et al.* ,1996). The objectives of this study are: (1) discussion on the perianth DNA extraction methods of *Lycoris longituba*; (2) a comparison of RAPD and ISSR markers for the molecular characterization of *Lycoris longituba*; (3) discussion on the mechanism of controlling the variation of *Lycoris longituba* flora color and form.

MATERIALS AND METHODS

Plant material and DNA extraction

Perianth of *Lycoris longituba* was sampled from natural population in Jurong in Jiangsu province, Xuyi in Jiangsu province and Chuzhou in Anhui province. 12 *Lycoris longituba* samples chosen from different flora color and form were collected (Table 1). The materials were conserved in room temperature by drying with silica gel.

Total genomic DNA of a single leaf was extracted from silica-gel-dried perianth tissue with a modified CTAB method (Zou et al., 2001) and silica gel suspended method (Shi et al., 2001) .

Table 1 Material and characters

Number	Perianth color	Character
1	White	Big flower
2	White	Floweret with more than 10 flower
3	White	Yellow stripe on perianth
4	White	Red stripe on perianth
5	Pink	Big flower
6	Pink	Floweret
7	Pink	Floweret with thin scape
8	Yellow	Deep yellow
9	Yellow	Reddish perianth
10	Yellow	Floweret
11	Yellow	Milk yellow
12	Yellow	Red stripe on perianth

RAPD amplification

Two hundred RAPD primers obtained from Operon Technologies were tested and twelve were used. The amplifications were performed in a 20-μl reaction volume containing 1×*Taq* polymerase buffer, 3.0 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 μM of primer, 0.75 units of *Taq* polymerase (Sangon promega, China) and 10 ng of genomic DNA. Initial denaturation was for 3 min at 94 °C, followed by 40 cycles of 30 second at 94 °C, 30 second at 36 °C, 90 second at 72 °C and a 7-min final extension step at 72 °C. PCR products were analyzed on 1.5% agarose gels and stained with ethidium

bromide, isualized with ultraviolet light and photographed.

ISSR amplification

A total of 36 ISSR primers, purchased from Nanjing sunshine biotechnology company, were screened using a few DNA samples to select the appropriate primers suitable for *Lycoris longituba* studies. Nine ISSR primers that showed a clear and reproducible band pattern were chosen for this study.

PCR amplification was performed in a 20-µl reaction volume, containing 1×*Taq* polymerase buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP , 0.5 µM of primer, 0.5 units of *Taq* polymerase (Sangon promega, China), and 10 ng of template genomic DNA.

The mixture was overlaid with mineral oil and subjected to PCR on a Perkin Elmer 9600 thermal cycler programmed for an initial step of 3 min at 94 °C, followed by 38 cycles of 30 second at 94 °C, 30 second at 58 °C, 90 second at 72 °C, and a 7-min final extention step at 72 °C.

PCR products were analyzed on 2% agarose gels, then stained with ethidium bromide, isualized with ultraviolet light and photographed.

Data analysis

RAPD and ISSR amplified fragments, named by the primer code and the molecular weight (bp), were scored for band presence (1) or absence (0) and two binary qualitative data matrices were constructed.

The number of polymorphic loci (*Npb*), percentage of polymorphic loci (*PPB*) and Shannon's Information index (*I*) of the 12 *Lycoris longituba* variation were calculated by the POPGENE program version 1.32 (Yeh and Boyle, 1997), used for the estimation of genetic diversity of *Lycoris longituba*. TFPGA version 1.3 (Mark P. Miller, 1997) program was also used to calculate the Nie’s (1978) genetic distance. Two dendrograms using unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) were constructed for estimating the genetic similarity based on Nei’s coefficients.

RESULTS

DNA extraction

For silica-gel-dried perianth materials of *Lycoris longituba*, DNA could be extracted using CTAB or silica gel suspended method (Fig. 1).

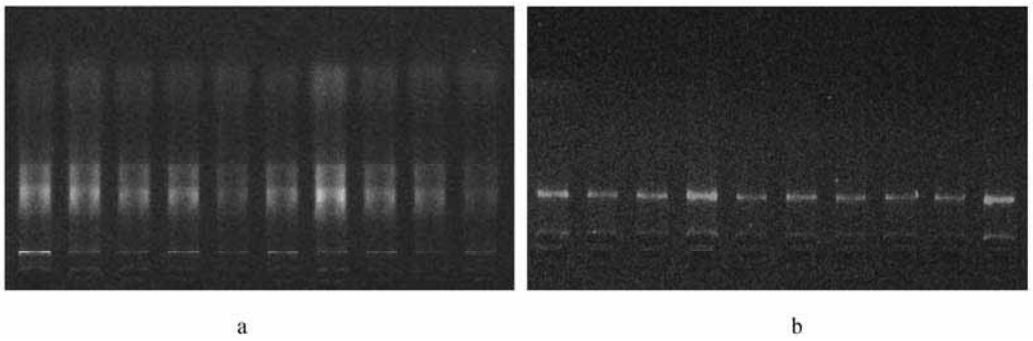

Fig. 1 Two different perianth DNA extraction methods of *Lycoris longituba* (a. DNA extracted by CTAB method; b. DNA extracted by silica gel suspended method)

Products of RAPD amplification

Twelve selected RAPD primers produced 77 reproducible and clear amplification bands, which were employed for analyzing (Fig. 2). The average number of fragments per primer was 6.4. The size of the major amplified bands varied between 400 ~ 3000 bp. 53 loci were polymorphic (PPB = 68.8%). When using Shannon's information index (*I*) to evaluate the genetic diversity of *Lycoris longituba*, the numerical value per primer was different, ranging from 0.1273 ~ 0.5895 (Table 2).

Products of ISSR amplification

A total number of 67 clear and reproducible bands were amplified using the 9 selected ISSR primers (Fig. 3). The average number of fragments per primer was 7.4. The size of the major amplified bands varied between 400 ~ 3000 bp. 62 loci were polymorphic (PPB = 92.5%). To evaluate the genetic diversity of *Lycoris longituba*, the numetical value per primer was also different, ranging from 0.4127 ~ 0.6120 (Table 3).

Table 2 RAPD amplification of *Lycoris longituba*

Primer	Primer sequence	*Nb*	*Npb*	*PPB*(%)	*I*
OPA-10	5'GTGATCGCAG 3'	6	4	66.7	0.3135
OPA-16	5'AGCCAGCGAA 3'	6	6	100.0	0.5895
OPC-04	5'CCGCATCTAC 3'	5	1	20.0	0.1273

OPD-20	5’ACCCGGTCAC 3’	6	3	50.0	0.2166
OPE-17	5’CTACTGCCGT 3’	5	3	60.0	0.2599
OPE-19	5’ACGGCGTATG 3’	5	3	60.0	0.2971
OPF-05	5’CCGAATTCCC 3’	11	8	72.7	0.4403
OPG-08	5’TCACGTCCAC 3’	5	3	60.0	0.3756
OPG-12	5’CAGCTCACGA 3’	6	3	50.0	0.2476
OPS-01	5’CTACTGCGCT 3’	8	7	87.5	0.4752
OPX-02	5’TTCCGCCACC 3’	7	6	85.7	0.4911
OPX-04	5’CCGCTACCGA 3’	7	6	85.7	0.4274
Total		77	53		
Mean		6.4	4.4	68.8	0.3551

Table 3 ISSR amplification of *Lycoris longituba*

Primer	Primer sequence	*Nb*	*Npb*	*PPB*(%)	*I*
ISSR-03	5’ACACACACACACACACTT 3’	10	9	90.0	0.4720
ISSR-04	5’ACACACACACACACACAG 3’	8	7	87.5	0.4127
ISSR-05	5’ACACACACACACACACTG 3’	9	7	77.8	0.4354
ISSR-23	5’ACACACACACACACACTA 3’	6	6	100.0	0.4826
ISSR-30	5’TGTGTGTGTGTGTGTGTC 3’	7	6	85.7	0.4146
ISSR-44	5’ACACACACACACACACGA 3’	8	8	100.0	0.5122
ISSR-45	5’ACACACACACACACACGC 3’	6	6	100.0	0.6120
ISSR-47	5’ACACACACACACACACGT 3’	8	8	100.0	0.4754
ISSR-59	5’AGAGAGAGAGAGAGAGGC 3’	5	5	100.0	0.4625
Total		67	62		
Mean		7.4	6.9	92.5	0.4283

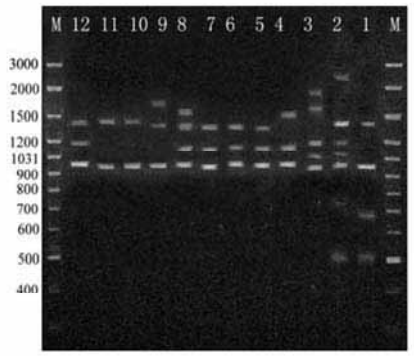

Fig. 2 RAPD amplification products using primer OPA-10

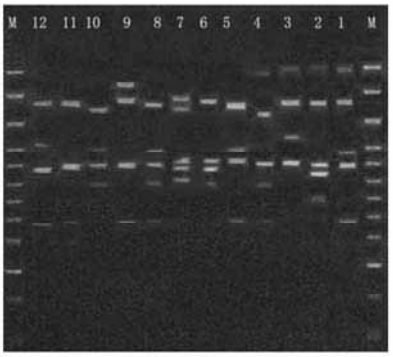

Fig. 3 ISSR amplification products using primer ISSR-05

UPGMA results

Two UPGMA dendrograms, both illustrating the genetic relationships among *Lycoris longituba* different flora colors, were identical in topology (Fig. 4, 5).

We used PAUP4.0b10 software to test genetic distance and genetic identity among twelve *Lycoris longituba* variations (Table 4, 5).

Table 4 genetic identity (above diagonal) and genetic distance (below diagonal) using RAPD amplification

No.	1	2	3	4	5	6	7	8	9	10	11	12
1	*****	0.7013	0.6494	0.7532	0.7662	0.7273	0.6623	0.6623	0.6623	0.7143	0.7143	0.6623
2	0.3548	*****	0.6883	0.7403	0.7792	0.7143	0.6494	0.6494	0.6753	0.7273	0.7013	0.6234
3	0.4318	0.3735	*****	0.7143	0.6753	0.7143	0.7532	0.7273	0.7532	0.7792	0.7532	0.7013
4	0.2834	0.3008	0.3365	*****	0.8312	0.7922	0.7532	0.7792	0.7792	0.7532	0.8052	0.7273
5	0.2663	0.2495	0.3926	0.1849	*****	0.7792	0.6883	0.7403	0.7403	0.7143	0.7143	0.6883
6	0.3185	0.3365	0.3365	0.2329	0.2495	*****	0.8312	0.7532	0.7792	0.7273	0.7792	0.6753
7	0.4120	0.4318	0.2834	0.2834	0.3735	0.1849	*****	0.7662	0.7403	0.6623	0.7143	0.6104
8	0.4120	0.4318	0.3185	0.2495	0.3008	0.2834	0.2663	*****	0.7662	0.7922	0.7403	0.7662
9	0.4120	0.3926	0.2834	0.2495	0.3008	0.2495	0.3008	0.2663	*****	0.7662	0.7403	0.6883
10	0.3365	0.3185	0.2495	0.2834	0.3365	0.3185	0.4120	0.2329	0.2663	*****	0.7662	0.7662
11	0.3365	0.3548	0.2834	0.2167	0.3365	0.2495	0.3365	0.3008	0.3008	0.2663	*****	0.7922
12	0.4120	0.4726	0.3548	0.3185	0.3735	0.3926	0.4937	0.2663	0.3735	0.2663	0.2329	*****-

Note: 1-12 stands for sample number

Table 5 genetic identity (above diagonal) and genetic distance (below diagonal) using ISSR amplification

No.	1	2	3	4	5	6	7	8	9	10	11	12
1	*****	0.7869	0.6557	0.6418	0.6269	0.7015	0.7015	0.6567	0.5522	0.5522	0.6716	0.5821
2	0.2397	*****	0.7705	0.6066	0.7049	0.6885	0.7377	0.6230	0.6066	0.5246	0.6230	0.5902
3	0.4220	0.2607	*****	0.6066	0.6721	0.6557	0.7377	0.5902	0.5410	0.4590	0.6230	0.5902
4	0.4435	0.5000	0.5000	*****	0.6866	0.7015	0.6716	0.6866	0.4925	0.6418	0.5821	0.5821
5	0.4670	0.3497	0.3973	0.3761	*****	0.7761	0.8060	0.7313	0.6269	0.6567	0.7463	0.7164
6	0.3545	0.3732	0.4220	0.3545	0.2534	*****	0.7612	0.7164	0.6716	0.6119	0.7015	0.6716
7	0.3545	0.3042	0.3042	0.3980	0.2157	0.2729	*****	0.7164	0.6716	0.6418	0.7313	0.7015
8	0.4205	0.4733	0.5274	0.3761	0.3129	0.3335	0.3335	*****	0.5970	0.7164	0.7463	0.7164
9	0.5938	0.5000	0.6144	0.7082	0.4670	0.3980	0.3980	0.5158	*****	0.5522	0.6418	0.6716
10	0.5938	0.6451	0.7787	0.4435	0.4205	0.4911	0.4435	0.3335	0.5938	*****	0.6119	06418
11	0.3980	0.4733	0.4133	0.5411	0.2927	0.3545	0.3129	0.2927	0.4435	0.4911	*****	0.7910
12	0.5411	0.5274	0.5274	0.5411	0.3335	0.3980	0.3545	0.3335	0.3980	0.4435	0.2344	*****

Note: 1-12 stands for sample number

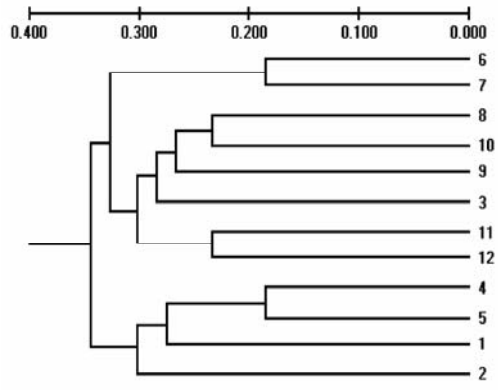

Fig. 4 Dendrogram of 12 *Lycoris longituba* varieties by RAPD

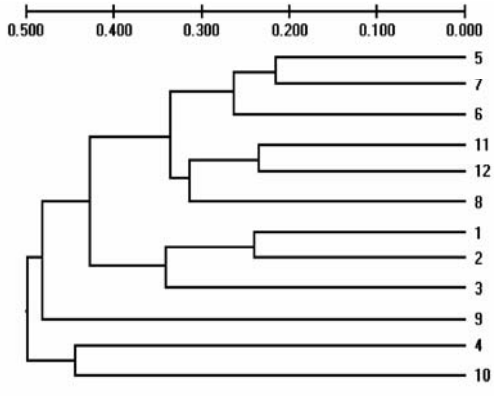

Fig. 5 Dendrogram of 12 *Lycoris longituba* varieties by ISSR

DISCUSSION

Perianth DNA extraction

For *Lycoris longituba*, leaves start growing in early spring not to die back until spring / early summer. A leafless flowering stalk arises from the ground level in July. It has different flora form and flora color, but there was no difference between leaf characters. So, in this study, perianth

DNA extraction methods were discussed. The result showed silica-gel-dried perianth DNA could be extracted by CTAB or silica gel suspended method.

Comparison on products of RAPDs and ISSRs

A number of total, polymorphic and discriminant fragments were higher for ISSRs than RAPDs in this study. A total number of 67 clear and reproducible bands were amplified using the 9 selected ISSR primers, an average number of 7.4 fragments per primer. However, twelve selected RAPD primers only produced 77 reproducible and clear amplification bands, an average number of 6.4 fragments per primer. In fact the inter-simple sequence repeats are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism and offer great potential to determine intragenomic and intergenomic diversity as compared to other arbitrary primers, like RAPDs (Zietkicwicz et al. 1994).

Analysis on genetic diversity of *Lycoris longituba*

Lycoris longituba flowers are diverse in both color and floral form. Our results were coherent with those morphological characters. In other words, morphological polymorphism was approved by genetic polymorphism. By RAPD, a total of 77 discernible loci were obtained using 12 primers, of which 53 loci were polymorphic (PPB = 68.8%); By ISSR, 67 discernible loci were got using 9 primers, of which 62 loci were polymorphic (PPB = 92.5%). So, genetic diversity of *Lycoris longituba* was abundant.

Analysis on genetic correlation among *Lycoris longituba*

Two UPGMA dendrograms showed almost identical topology. White, pink or yellow color was clustered together among *Lycoris longituba*. So these three types were supported with molecular evidence, which were originally distinguished by flower color. Therefore, in the future use of germplasmic resources, different varieties of *Lycoris longituba* could be cultivated.

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