JARQ 40 (4), 317 – 326 (2006) http://www.jircas.affrc.go.jp

REVIEW

Studies on Speciation in Genus *Lycoris* Using Interspecific Hybrids and Selfed Plants Produced through Embryo Rescue

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Abstract

This study was carried out to overcome the problems that impede genetic improvement of *Lycoris*. An improved ovule culture method was developed to rescue the abortive embryo in order to overcome the poor fertility in hybridization of *Lycoris*. Using this method, a large number of selfed and crossed plants in several species were produced, and the interspecific hybrids which used *L. incarnata* (sterile) as a cross parent were obtained for the first time. Cytological studies of the S_1 plants indicated that polyploidization and aneuploid reduction caused by self-pollination should be among the major factors affecting karyotype evolution in some *Lycoris* species. Allozyme segregations in polymorphic loci of APT, GOT and EST were observed in S_1 of five diploid species, suggesting that each of the species is originally heterozygous. The investigation using genomic *in situ* hybridization (GISH) was done to clarify the chromosome constitution in *Lycoris*. The distinction between M + T and A type chromosomes at the DNA sequence level demonstrated that genome differentiation has occurred in the genus *Lycoris*.

Discipline: Plant breeding / Horticulture
Additional key words: allozyme, Amaryllidaceae, genomic *in situ* hybridization (GISH), karyotype, ovule culture, polyploid

Introduction

The genus *Lycoris*, a member of the family Amaryllidaceae, contains about 20 species. Most of the species are commonly cultivated in China, Japan, and the United States as bulbous plants[2,7]. Recently, the demand for cut flowers of *Lycoris* has increased with diversification of flower consumption, so breeding of varieties with new flower forms and/or colors has become desirable for *Lycoris*[15].

At present, the hybridization between *Lycoris* species is most promising for improving and diversifying certain traits. However, the breeding efficiency in interspecific hybridization is low mainly due to the difficulty of obtaining F_1 plants caused by incongruity[4], inefficient embryo rescue technique[39] and so on. Moreover, little knowledge of the genetic background of *Lycoris* species as well as the inheritance of characters is available. For elucidating the genetic relationship among species and karyotype evolution, cytological studies on selfed plants and interspecific hybrids have been made by some authors, such as Kihara & Koyama[16], Koyama[17–19], Takemura[35–37], and Shii et al.[34]. However, more information is needed to clarify the genetic background of *Lycoris* species. Therefore, the authors studied the embryo rescue[25,27,28] and speciation[25,26,29,30,33] in *Lycoris*. This report deals with ovule culture for obtaining more progeny plants via self-pollination and interspecific hybrids, and with cytological, allozyme and genomic *in situ* hybridization (GISH) studies for revealing specific relationships in *Lycoris*.

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Received 14 October 2005; accepted 16 January 2006.

Production of interspecific hybrids and selfed plants in *Lycoris*

Interspecific and intergeneric crosses have been made to introduce new genetic variation into cultivated plants. For ornamental bulbs such as *Lilium* and *Narcissus*, interspecific hybridization has been frequently used to improve flower shape and color and to extend the flower period[24,39]. However, in general, the incongruity that occurs before and after pollination often becomes a barrier for producing interspecific hybrids.

In *Lycoris*, incongruity and sterility prevent successful hybridization. In many cases, although pollens of *Lycoris* species are fertile and the fertilization is normally done[8–10,34], the embryos after pollination are generally weak and prone to abort[8–10,18]. Several methods have been developed to solve the problems. The 'Mizusashi method'[38] of keeping the scapes in a flask of water after pollination was usually employed to mature seeds[16–18,34–37], and the immature seeds developed by the Mizusashi method were then cultured *in vitro*[10,15]. However, the seed formation rates through them were still low and the hybridity of F_1 plants was not confirmed. Moreover, in many 'sterile' *Lycoris* species caused by irregular meiotic division and nonhomologous pairing[8,9,32], interspecific hybrids have not been produced. Thus, the authors developed the efficient embryo rescue method[27] and produced interspecific hybrids and selfed plants[25,26,28] in various *Lycoris* species including the sterile species *L. incarnata*.

1. An ovule culture for improving the efficiency of seedling formation

Scapes of *L. sanguinea*, a 'fertile' species (2n = 22A) as defined only by its normal meiotic division[32], were cut just before anthesis and kept in flasks filled with tap water according to the Mizusashi method[18]. The ovules were isolated 20 days after self-pollination as in Ma et al.[27], and were imbedded in several culture mediums. The modified components such as sucrose, pH and casein hydrolysate (CH), resulted in a MS medium containing 3% sucrose at pH 5.8 which was satisfactory for ovule culture. A modified MS medium (mMS2) containing macro-salts, adopted from Monnier's medium[31] containing 3% sucrose at pH 5.8, induced higher germination

Table 1. Relative efficiency of casein hydrolysate (CH) supplement on obtaining seedlings from ovules obtained 20 days after self-pollination in *L. sanguinea* (Sucrose: 3%, pH: 5.8)

Medium	No. of ovules inoculated	% of ovules germinated [a)]	% of seedlings obtained [b)]
1/2 MS	18	44.4	20.2
1/2 MS + CH [c)]	19	63.2	26.3
mMS2 [d)]	12	50.0	25.0
mMS2 + CH	17	88.2	70.6

a): % of ovules germinated = No. of ovules germinated / No. of ovules inoculated.
b): % of seedlings obtained = No. of seedlings obtained / No. of ovules inoculated.
c): 500 mg/L CH was added to the basal media.
d): mMS2 medium with reduced ammonium concentration by half (825 mg/L NH_4NO_3), increased potassium level (adding 350 mg/L KCl) and doubled calcium chloride concentration (880 mg/L $CaCl_2 \cdot 2H_2O$).

Table 2. Relative efficiency of ovule culture in *Lycoris* using mMS2 + CH medium

Source of ovules		No. of ovules cultured [a)]	% of ovules germinated [b)]	% of seedlings obtained [c)]
L. albiflora × *L. sanguinea*		26	23.1	15.4
L. radiata × *L. sanguinea*		37	54.1	43.2
L. albiflora [d)]	selfed	10	30.0	20.0
L. radiata [d)]	selfed	26	15.4	11.5
L. sanguinea [e)]	selfed	18	88.9	83.3

a): The ovules were isolated 30 days after self- or cross-pollination.
b): % of ovules germinated = No. of ovules germinated / No. of ovules inoculated.
c): % of seedlings obtained = No. of seedlings obtained / No. of ovules inoculated.
d): *L. albiflora* and *L. radiata* are "sterile" species with irregular meiotic division.
e): *L. sanguinea* is a "fertile" species with normal meiotic division.

and seedling formation rates. The addition of 500 mg·L^{-1} CH to mMS2 medium (mMS2 + CH) promoted the germination and seedling formation rates (Table 1)[27]. The seedling formation rates in *L. sanguinea* were satisfactorily high compared with the current record of 0.12 selfed plants per floret[35]. These results indicate that higher levels of potassium and calcium and lower level of ammonium are more suitable for ovule culture, and CH would contribute to the promotion of *in vitro* growth of immature embryos as well as to the inhibition of abnormal embryo development as reported by Ziebur et al.[43].

L. sanguinea (diploid, 2n = 22) is a "fertile" species defined by the normal meiotic division[32], whereas *L. albiflora* (diploid, 2n = 17) and *L. radiata* (triploid, 2n = 33) are "sterile" ones defined by the irregular meiotic division[8,9] and had no record of selfed plants[9,10]. It has been difficult to obtain selfed progenies as well as hybrid plants in "sterile" species even through the Mizusashi method[15,18] because of nutrient shortage to developing embryos[15] and/or chromosomal unbalance in recombination[8,9]. The mMS2 + CH medium proved satisfactory for producing them in "sterile" *Lycoris* species (Table 2)[27]. The seedling formation rates of selfed *L. albiflora* and *L. radiata* were 20.0% and 11.5%, and the rates of hybrids were 15.4% in *L. albiflora* × *L. sanguinea* and 43.2% in *L. radiata* × *L. sanguinea*. The hybridity was proved by their chromosome numbers (2n = 20–21 in *L. albiflora* × *L. sanguinea* and 2n = 24–28 in *L. radiata* × *L. sanguinea*)[26]. These satisfactory results are considered to be attributed to the adequate nutrient supply to the immature embryo from mMS2 + CH medium.

2. Interspecific hybrids and selfed plants via ovule culture

A wide range of interspecific crosses by using "fertile" and "sterile" species was carried out using our ovule culture method, in order to prove the applicability for producing interspecific hybrids in *Lycoris*. The S_1 plants were obtained in 5 fertile species and 2 sterile species, and F_1 plants were obtained in 17 out of 21 combinations carried out (Table 3)[25]. Even through ovule culture, the combinations of sterile × sterile did not produce hybrid plants (Table 3)[25]. The hybridity of F_1 plants was confirmed by cytological studies[25,26], and in the case that both parents have identical karyotype, isozyme analysis was done to confirm the hybridity[25]. In the results, all the F_1 plants produced were proved to be true hybrids, and all the hybrids of sterile × fertile were aneuploids, for example 2n = 23 to 31 in *L. radiata* × *L. sanguinea* and 2n = 23 to 28 in *L. radiata* × *L. sprengeri*, providing new breeding sources like the promising aneuploid cultivars bred in *Nerine*[11] and *Narcissus*[4,40].

3. Interspecific hybrids between *Lycoris incarnata* and other species through embryo culture

Several traits of *L. incarnata* (2n = 3χ = 30, 3M + 5T + 20A + 1M' + 1m)[7,23] such as funnel flower form, light rose color, and strong scapes are desirable for developing varieties with these characters. However, it has been difficult to produce interspecific hybrids using *L. incarnata* because of its sterility with irregular meiotic division. We made interspecific crosses between sterile species (*L. incarnata*) and four fertile species (*L. sanguinea*, *L. sprengeri* and *L. radiata* var. *pumila* = 2χ = 22A; *L. aurea* = 2χ = 8M + 6T). The embryos, excised from ovules grown 20–30 days in the Mizusashi method after pollination, were cultured on the mMS + CH medium[27]. Interspecific crosses using *L. incarnata* as a female parent yielded 26 hybrid plants, whereas that of *L. sanguinea* × *L. incarnata* produced none (Table 4)[28]. Cytological studies revealed that all hybrids were tetraploid (Table 5)[28], which would have originated from the fertilization of an unreduced 2n – egg of the female parent (*L. incarnata*) and a normal haploid gamete of the male parent. The hybridity was also confirmed by isozyme analysis in 26 hybrid plants (Fig. 1)[28]. This is the first record in producing true interspecific hybrid plants using the sterile species, *L. incarnata*.

Speciation in genus *Lycoris*

Genetic relationship is useful for evolutionary studies and selecting of suitable parents in a breeding

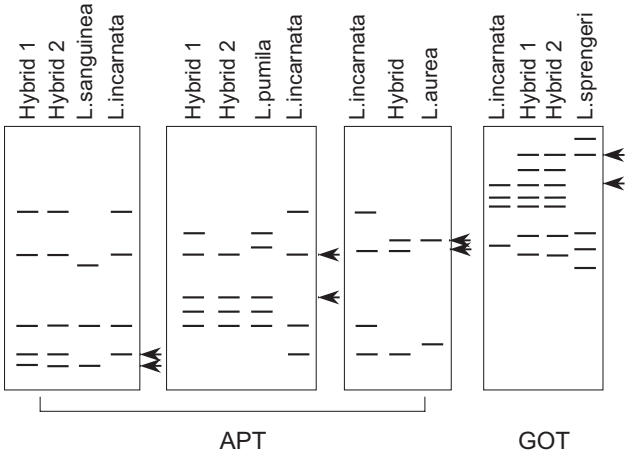

Fig. 1. Zymograms for APT and GOT in interspecific hybrids and their parents

Table 3. Interspecific hybrids and selfed plants produced through ovule culture in *Lycoris*

Cross combination		No. of florets pollinated	% of fruit set	No. of ovules cultured [a)]	Medium	% of germination	% of seedling formation
(♀)	(♂)						
L. sanguinea	self	143	66.4	124	1/2 MS	69.4	56.5
				18	mMS2 + CH	88.9	83.3
	× *L. pumila* [b)]	18	50.0	16	mMS2 + CH	50.0	50.0
	× *L. squamigera*	101	56.4	59	1/2 MS	16.9	15.3
L. sprengeri	self	52	71.2	39	1/2 MS	48.7	43.6
				65	mMS2 + CH	53.8	49.2
	× *L. sanguinea*	12	91.7	34	1/2 MS	38.2	38.2
	× *L. albiflora*	9	77.8	23	1/2 MS	65.2	56.5
L. aurea	self	30	83.3	49	1/2 MS	85.7	65.3
				46	mMS2 + CH	95.7	93.5
	× *L. sanguinea*	7	85.7	12	1/2 MS	83.3	66.7
	× *L. sprengeri*	10	90.0	20	1/2 MS	60.0	60.0
	× *L. albiflora*	8	75.0	11	1/2 MS	90.9	50.0
L. albiflora	self	56	62.5	9	1/2 MS	11.1	11.1
				10	mMS2 + CH	30.0	20.0
	× *L. sanguinea*	45	44.4	17	1/2 MS	5.9	5.9
				26	mMS2 + CH	23.1	15.4
	× *L. sprengeri*	35	57.1	25	1/2 MS	40.0	28.0
	× *L. aurea*	14	42.9	6	1/2 MS	50.0	33.3
	× *L. squamigera*	18	27.8	0	–	–	–
	× *L. radiata*	44	40.9	12	1/2 MS	0	–
				8	mMS2 + CH	0	–
L.radiata	self	35	68.6	39	1/2 MS	12.8	5.1
				26	mMS2 + CH	15.4	11.5
	× *L. sanguinea*	96	79.2	102	1/2 MS	41.2	28.4
				37	mMS2 + CH	54.1	43.2
	× *L. sprengeri*	41	68.3	46	1/2 MS	28.3	21.7
	× *L. aurea*	54	48.1	27	1/2 MS	18.5	14.8
	× *L. pumila*	10	30.0	6	mMS2 + CH	16.7	16.7
	× *L. squamigera*	33	30.3	14	1/2 MS	0	–
	× *L. albiflora*	35	20.0	3	1/2 MS	0	–
				4	mMS2 + CH	0	–
L. squamigera	self	59	61.0	51	1/2 MS	0	–
	× *L. sanguinea*	40	60.0	41	1/2 MS	17.1	12.3
	× *L. sprengeri*	24	41.7	20	mMS2 + CH	10.0	5.0
	× *L. aurea*	25	40.0	13	mMS2 + CH	23.1	23.1
L. pumila	self	32	75.0	60	mMS2 + CH	58.3	51.7
L. traubii	self	11	81.8	40	mMS2 + CH	90.0	82.5

a): The ovules were isolated 30–35 days after self- or cross-pollinations.
b): *L. radiata* var. *pumila*.

Table 4. Results of pollination and *in vitro* culture in interspecific hybridization between *L. incarnata* and four *Lycoris* species

Cross combinations		Male	Female		No. of embryos or ovules		No. of seedlings obtained [b)]
(♀)	(♂)	Pollen germination rate (%)	No. of florets pollinated	Capsule formation (%) [a)]	Cultured	Germinated	
L. incarnata	× *L. sprengeri*	52.0	35	94.3	4 (embryo)	4	4
	× *L. pumila*	61.8	56	60.7	4 (embryo)	4	3
	× *L. aurea*	50.5	40	80.0	2 (embryo)	1	1
	× *L. sanguinea*	56.6	177	75.7	20 (embryo)	20	16
L. sanguinea	× *L. incarnata*	0.5	16	50.0	80 (ovule)	0	0
L. incarnata	self	2.0	42	88.1	90 (ovule)	0	0
L. sanguinea	self	46.6	10	90.0	27 (ovule)	19	18

a): (No. of capsules developed / No. of florets pollinated) × 100, 20–30 days after pollination.
b): After 8–12 weeks of culture.

Table 5. Chromosome number and karyotype of interspecific hybrids and their parental plants

Chromosome no. and karyotype of parental plants		Hybrids		No. of plants observed
(♀)	(♂)	Chromosome no.	Karyotype	
L. incarnata 2n = 30 (3M+ 5T+20A+1M'+1m)	× *L. sanguinea* 2n = 22 (22A)	2n = 41	3M+5T+31A+1M'+1m	16
	× *L. sprengeri* 2n = 22 (22A)	2n = 41	3M+5T+31A+1M'+1m	4
	× *L. pumila* 2n = 22 (22A)	2n = 41	3M+5T+31A+1M'+1m	5
	× *L. aurea* 2n = 14 (8M+6T)	2n = 37	7M+8T+20A+1M'+1m	1

M: Metacentrics, T: Telocentrics, A: Acrocentrics, M': Small metacentrics, m: Very small metacentrics.

program[3,11,12]. These studies have been performed in many plant species including some ornamental bulbs such as *Tulipa*[1] and *Iris*[5]. In *Lycoris*, there is almost no report of genetic relationships except for some cytological studies focusing on karyotype[20–23]. The few genetic studies in *Lycoris* are probably attributed to its asexual reproduction, difficulties in producing sufficient progenies as genetic materials and long juvenile period of progenies. As mentioned in the previous section, selfed plants and interspecific hybrids were produced through ovule and embryo cultures in order to overcome these limitations. Meanwhile, in recent decades, the development of molecular marker systems (isozyme and DNA-based markers) allows genetic analyses of progenies in the seedling stage[1,3]. Thus, we conducted the cytological, allozyme and genomic *in situ* hybridization (GISH) studies in selfed plants and interspecific hybrids for revealing specific relationships in *Lycoris*[29,30,33].

1. Speciation of polyploid species estimated from cytological studies in selfed progeny

Root tips that were excised from seedlings of selfed progenies in Table 3 and their parental plants were used for cytological study for clarifying the speciation processes of polyploid *Lycoris* species. For diploid species, almost all S_1 progenies observed were diploid with the same karyotype as the corresponding parental species (Table 6)[29]. However, S_1 progenies from *L. sprengeri* (2n = 22A) included two triploids with 2n = 33A and one aneuploid with 2n = 32 (31A + 1M), and one S_1 from *L. aurea* (2n = 14, 8A + 6T) was tetraploid with 2n = 28 (16M + 12T). The triploid of *L. sprengeri* was discovered in China by Zhang et al.[42], and this result demonstrated that triploid and triploid-neared aneuploid plants were induced through self-pollination of *L. sprengeri*. Since there was no report[7,23] of wild tetraploid in *L. aurea*, the tetraploid plant induced in Ma & Tarumoto[29] is the first one in *L. aurea*. For triploid species, *L. radiata*

Table 6. Chromosome number and karyotype of selfed plants

Parent		S_1	
Species	2n	2n	No. of S_1 observed
L. sanguinea	22A (2χ)	22A	84
L. sprengeri	22A (2χ)	22A	45
		33A	2
		32(31A + 1M)	1
L. aurea	14 = 8M + 6T (2χ)	14(8M + 6T)	63
		28(16M + 12T)	1
L. pumila [a)]	22A (2χ)	22A	21
L. traubii	12 = 10M + 2T (2χ)	12(10M + 2T)	33
L. radiata [b)]	33A (3χ)	24A	1
		25A	2

a): *L. radiata* var. *pumila*.
b): *L. radiata* var. *radiata*.

Table 7. Polyploidy found in natural population of *Lycoris* species

Taxon	2n	Reference
L. sprengeri	22 (2χ)	Inariyama (1951a)[8]
	33 (3χ)	Zhang et al. (1999)[42]
L. radiata var. *radiata*	33 (3χ)	Inariyama (1951a)[8]
L. radiata var. *pumila*	22 (2χ)	Kurita (1988)[21]
L. sanguinea var. *sanguinea*	22 (2χ)	Inariyama (1951a)[8]
	32 (3χ)	Kurita (1989)[22]
L. sanguinea var. *kiushiana*	22 (2χ)	Kurita (1988)[21]
	33 (3χ)	Kurita (1988)[21]
	44 (4χ)	Kurita (1988)[21]

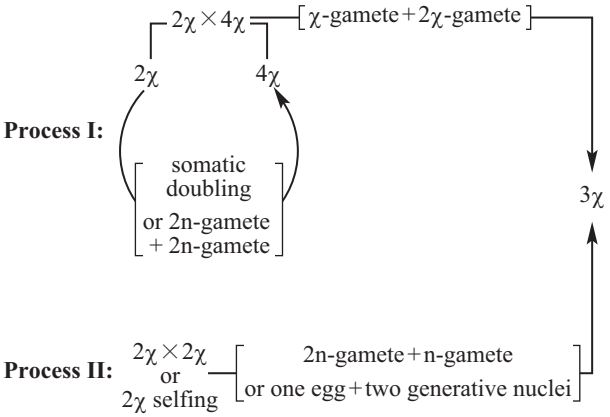

Fig. 2. Hypothesis for karyotype evolution in *Lycoris* species

(2n = 33A), all the S_1 plants observed were aneuploid with chromosome number near to its diploid taxon, *L. radiata* var. *pumila* (2n = 22A). From the results, the evolution routes of natural polyploids shown in Table 7[25] are hypothesized in Fig. 2[25]. For *L. sprengeri* and *L. radiata*, because there is no tetraploid found in nature[7,23], those triploids would have originated by 'Process II', suggesting that unreduced gametes occasionally formed through self-pollination in 'fertile' species would be a factor inducing polyploid species in genus *Lycoris*.

2. Genetic segregation of allozymes in selfed plants of diploid species

Seven diploid species with normal meiotic division, *L. aurea, L. traubii, L. sanguinea, L. sprengeri, L. radiata* var. *pumila (L. pumila*, hereafter*), L. longituba*, and *L. chinensis*, were considered to be progenitors of the other species mostly on the basis of cytological studies[7,23]. For

Table 8. Allozyme variation in selfed progenies of several *Lycoris* species

Locus	Band pattern	*L. aurea*	*L. traubii*	*L. sprengeri*	*L. sanguinea*	*L. pumila* [a)]
Apt-1	P	47	25	30	0	21
	A	0	4	0	64	0
Apt-2	P	47	24	18	33	17
	A	0	5	12	31	4
Got-2	F	35	0	0	64	0
	FS	12	29	0	0	0
	S	0	0	30	0	21
Got-3	F	–	–	0	–	6
	FS	–	–	0	–	10
	S	–	–	30	–	5
Got-4	F	0	0	0	2	–
	FS	0	0	0	42	–
	S	47	29	30	20	–
Est-1	F	–	–	12	13	–
	FS	–	–	10	34	–
	S	–	–	8	17	–
Est-3	P	0	0	0	24	15
	A	47	29	30	40	6
No. of S_1		47	29	30	64	21
No. of parental plants		6	3	8	16	5

P: Present, A: Absent, F: Fast migration, S: Slow migration.
– : No band. a): *L. radiata* var. *pumila.*

these diploid progenitor species, from the characteristic and karyotype analyses in interspecific hybrids, Takemura[36,37] assumed that *L. aurea, L. sanguinea, L. sprengeri*, and *L. pumila* would be homozygous, while Caldwell[2] postulated that *L. sprengeri* and *L. pumila* would be partly heterozygous in their genetic background. It is important to know the zygotic site of the diploid species in evolutional study as well as breeding. For this aim, seedlings of selfed progenies that showed the same chromosome complements as that of their corresponding parents (Table 6), five diploid progenitor species, except *L. longituba* and *L. chinensis*, were used and the allozyme variations were investigated in them. Among 14 allozyme loci of ATP, GOT and EST, seven polymorphic loci were detected (Table 8)[30]. Allozyme segregations were observed in all five species[30], suggesting that each of the five diploid species, *L. aurea, L. traubii, L. sanguinea, L. sprengeri*, and *L. pumila*, is heterozygous. This result proved that the five diploid progenitor species originated from hybrids, and it supported that hybridization was one of the important modes of speciation[21,23].

3. Genome differentiation in *Lycoris* species identified by genomic *in situ* hybridization

Kurita[20,21] reported the major types of chromosomes [M (metacentrics), T (telocentrics), A (acrocentrics), and m (very small metacentrics)] for classifying karyotypes in the genus *Lycoris*. Kurita[21] also suggested that genomes composed of M and T type chromosomes like *L. longituba* (6M + 10T) were ancestral and that genomes composed entirely of A type chromosomes like *L. sprengeri* (22A) were derivatives. However, detailed genome analysis in *Lycoris* had not been performed. Thus, the chromosomes of six *Lycoris* species and three interspecific hybrids were investigated using genomic *in situ* hybridization (GISH) to clarify the chromosome constitution in this genus. Total DNA from *L. aurea* (2n = 13, 9M + 4T) and *L. sprengeri* (2n = 22, 22A) was used as probe and blocking DNA, respectively. All the chromosomes of *L. aurea* (9M + 4T) and *L. longituba* (6M + 10T) were hybridized with probe DNA and were visualized as yellow labeled chromosomes, while those of *L. sprengeri* (22A), *L. pumila* (22A) and *L. sanguinea* (22A) remained unlabeled and appeared as red chromosomes

Table 9. Origins, cytological characteristics, and results of GISH analyses of 6 *Lycoris* species and 3 interspecific hybrids in which total DNA from *L. aurea* and *L. sprengeri* were used as probe and blocking DNA, respectively

Species (origin) and interspecific hybrids		Chromosome no.	Karyotype	No. of chromosomes detected by GISH			
				L. aurea type		*L. sprengeri* type	
L. aurea	(China)	2*n* = 13	9M+4T	13	(9M, 4T)	0	
L. longituba	(China)	2*n* = 16	6M+10T	16	(6M, 10T)	0	
L. sprengeri	(China)	2*n* = 22	22A	0		22	(22A)
L. pumila [a)]	(China)	2*n* = 22	22A	0		22	(22A)
L. sanguinea	(Japan)	2*n* = 22	22A	0		22	(22A)
L. incarnata	(China)	2*n* = 30	3M+5T+20A+1M'+1m	8	(3M, 5T)	22	(20A, 1M', 1m)
L. incarnata × *L. sprengeri*		2*n* = 41	3M+5T+31A+1M'+1m	8	(3M, 5T)	33	(31A, 1M', 1m)
L. incarnata × *L. pumila*		2*n* = 41	3M+5T+31A+1M'+1m	8	(3M, 5T)	33	(31A, 1M', 1m)
L. incarnata × *L. sanguinea*		2*n* = 41	3M+5T+31A+1M'+1m	8	(3M, 5T)	33	(31A, 1M', 1m)

M: Metacentrics, T: Telocentrics, A: Acrocentrics, M': Small metacentrics, m: Very small metacentrics.
a): *L. radiata* var. *pumila*.

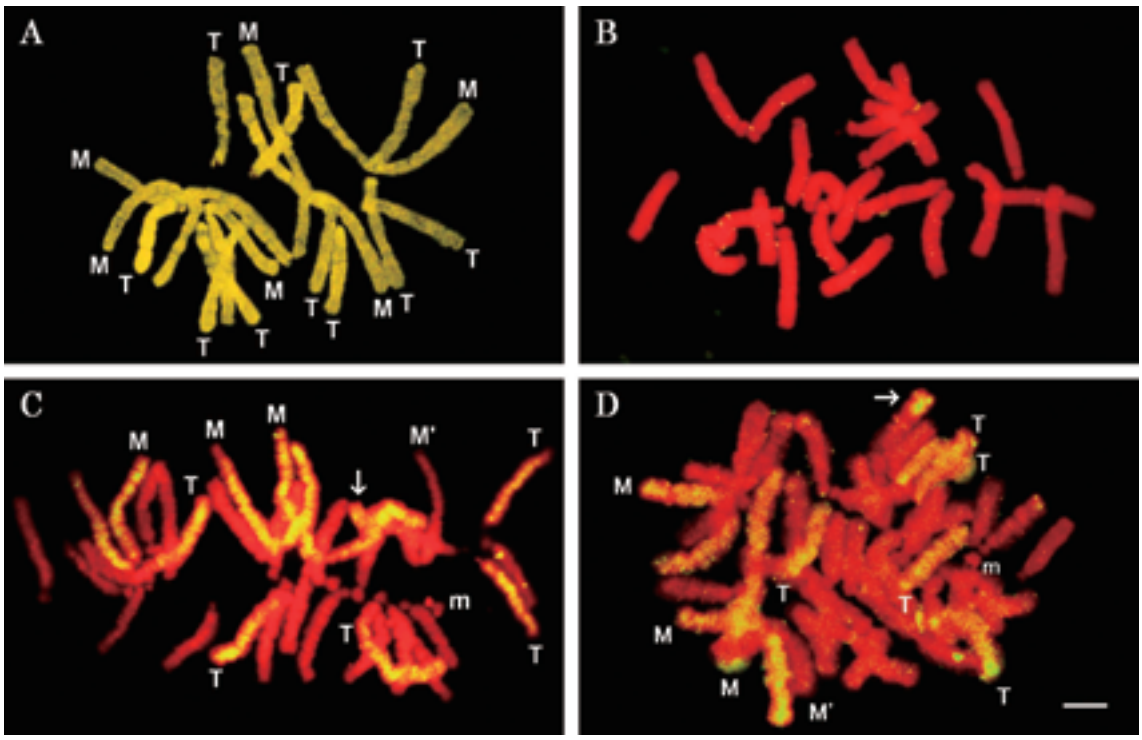

Fig. 3. Genomic *in situ* hybridization on the chromosomes of *L. longituba* (A), *L. pumila* (B), *L. incarnata* (C), and *L. incarnata* × *L. pumila* (D)

Total DNA of *L. aurea* and *L. sprengeri* were used as probe and blocking DNA, respectively. Hybridization signals are visualized in yellow. Each chromosome type except A is displayed in the figure. Arrows indicate the translocation on A type chromosomes. Scale bar = 10 μm.

(Table 9 & Fig. 3)[33]. Eight labeled (3M + 5T) and 22 unlabeled chromosomes (20A + 1M' + 1m) in *L. incarnata*, and eight labeled (3M + 5T) and 33 unlabeled chromosomes (31A + 1M' + 1m) in three interspecific hybrids between *L. incarnata* and 3 diploid species were detected in GISH (Table 9 & Fig. 3)[33]. The distinction between M + T and A type chromosomes at the DNA sequence level by GISH demonstrated that genome differentiation had occurred in the genus *Lycoris*. *L. incarnata* is confirmed to be an allotriploid species derived from the fusion of normal reduced gametes of a species with 6M + 10T and unreduced gametes of a species with 22A or 20A + 1M' + 1m, which was assumed by Kurita[21]. Intergenomic translocation was considered to be one of the factors in speciation in *Lycoris*[21] as well as in *Avena*[6], *Triticum*[41] and others[13,14]. Translocation from M or T type chromosomes to A type chromosomes was observed in *L. incarnata*, suggesting that intergenomic translocation affected the speciation of *L. incarnata*.

Conclusion

This report revealed some progress in overcoming problems which impede genetic and breeding studies in genus *Lycoris*. An improved ovule culture method was developed to rescue abortive embryos, and a large number of selfed plants and true interspecific hybrids in several *Lycoris* species was produced by using it. Hybrids of 3χ (female) × 2χ (male) were all aneuploids, suggesting that interspecific hybridization of $3\chi \times 2\chi$ would become an effective breeding method for increasing the character variation in *Lycoris*. Moreover, interspecific hybrids between *L. incarnata* (sterile) and four fertile species were successfully obtained for the first time and would be promising breeding materials. However, in order to produce hybrid plants more efficiently, the mechanism of embryo abortion and methods of embryo rescue need to be studied further.

In cytological studies of the S_1 obtained through ovule culture, polyploidization and aneuploid reduction caused by self-pollination were shown to be among the major factors affecting karyotype evolution in some *Lycoris* species. The allozyme segregation in S_1 proved the heterozygous nature of the fertile *Lycoris* species, suggesting that varietal and specific differentiation would be easily caused by self- and cross-pollination in *Lycoris*. Not only the genomic differentiation but also intergenomic translocation from M or T type chromosomes to A type chromosomes were revealed by genomic *in situ* hybridization. These results will become useful information for making progress in genetic and breeding studies in genus *Lycoris*.

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