Genes Genet. Syst. (2006) **81**, p. 243–253

Genetic variations in the chloroplast genome and phylogenetic clustering of *Lycoris* species

Tada-aki Hori[1*], Akiko Hayashi[1], Tsuneo Sasanuma[2] and Siro Kurita[3]

[1]*Transcriptome Profiling Group, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan*
[2]*Kihara Institute for Biological Research, Yokohama City University, Maioka-cho 641-12, Totsuka-ku, Yokohama 244-0813, Japan*
[3]*Department of Biology, Faculty of Science, Chiba University. 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*

(Received 5 January 2006, accepted 10 July 2006)

The genus *Lycoris* of *Amaryllidaceae* comprises approximately 20 species that are distributed only in the moist warm temperate woodlands of eastern Asia. The objectives of this study were: (1) to clarify the phylogeny of the *Lycoris* species by using the definitive DNA sequencing method and (2) to examine the possible maternal donor of the hybrid origin *Lycoris* species and the Japanese triploid strains of *Lycoris radiata* var. *radiata*. The nucleotide sequence of the maturase K (*matK*) gene and the noncoding intergenic spacer (IGS) between the *atpB* and *rbcL* genes in the chloroplast genome were determined in a total of 27 strains of 11 species of the genus *Lycoris*. Variation among taxa was mainly due to nucleotide substitution, although deletions and an insertion were found in the IGS. For two chloroplast regions, the phylogenetic trees showed essentially similar topology, indicating the existence of four clades, I, II, III, and IV. For all the species except *L. radiata*, intraspecific variation was smaller than interspecific variation. For *L. radiata*, triploid strains were divided into clades I and II, and diploid strains were divided into clades I and IV. This implies that the diploid species of *L. radiata* var. *pumila* is a probable ancestral species. The clustering indicated that the chloroplast genome has not evolved in parallel with the karyotype in genus *Lycoris*. Regarding the hybrid origin species, the maternal parents of *L. squamigara*, *L. albiflora* and *L. rosea* were revealed to be *L. longituba*, *L. radiata* and *L. radiata* var *pumila*, respectively. We also suggest that a diploid strain of *L. radiata* var. *pumila* in clade I might be a candidate of the maternal donor of the Japanese triploid strains. A possible model of the maternal donor of *Lycoris* species is proposed.

Key words: nucleotide variation, phylogenetic tree, *matK* gene, *atpB-rbcL* intergenic spacer, chloroplast genome, *Lycoris*

INTRODUCTION

The genus *Lycoris* of *Amaryllidaceae* consists of approximately 20 species. It is distributed only in the warm temperate and subtropical zones of East Asia from southwestern China to southern Korea and Japan, with a few species extending to northern Indochina and Nepal (Hsu et al., 1994). *Lycoris radiata* var. *radiata*, a variety of the species of *L. radiata* (L'Heit.) Herb, is widely distributed throughout Japan except on the island of Hokkaido and is well known by the Japanese vernacular name 'HIGAN-BANA'. This species has been shown to be completely sterile due to the triploid nature of its genomic constitution of 2n = 3x = 33 (Nishiyama, 1928; Inariyama, 1931). Inariyama (1944) further reported that the triploid sterile species was generated from *L. radiata* var. *pumila,* which is diploid, with 2n = 2x = 22. Since the diploid variety of *L. radiata* var. *pumila* is endemic to China, but not distributed in Japan (Maekawa, 1954; Kurita, 1987b), it has been suggested that in prehistoric times, when the practice of rice cultivation from China was introduced in Japan, the triploid sterile species was introduced into the southern part of Japan as a companion plant. Subsequently it spread throughout Japan, accompanying the rapid expansion of rice cultivation to

Edited by Toru Terachi
* Corresponding author. E-mail: horitadaaki810@aol.com

the northern parts of the country.

In our previous study (Hayashi et al., 2005), we investigated the nucleotide sequence variation of the lectin gene in the nuclear genome and the maturase K (*matK*) gene in the chloroplast genome to identify the maternal donor of Japanese triploid *L. radiata* var. *radiata*. In that study, we demonstrated that the two diploid Chinese strains of *L. radiata* var. *pumila* examined were not the probable maternal ancestor of the triploid strains of *L. radiata* var. *radiata* that are currently found in Japan.

Some other *Lycoris* species than *L. radiata* are also popular as gardening plants. Regarding the classification of the genus *Lycoris*, however, the phylogenetic relationships among the species are still unknown because of the continuous variation of morphological and physiological characters within and between species. Karyotype is also complicated in this genus, for example, even in diploids, 2n = 12, 16, and 22 species are present, which can be explained by the theory of chromosome fusion or fission (Hsu et al., 1994). In addition, it has been difficult to identify newly acquired *Lycoris* accessions due to natural hybridization. Among the many diploid fertile species, hybrids with diverse morphological characteristics occur in nature and in cultivated areas. Karyotypical and phenological studies reported by Kurita (1987a), Hsu et al. (1994), and Lee and Kim (1987) have suggested that a revision of the work by Traub and Moldenke (1949), which has long been accepted in the *Lycoris* taxonomy, may be necessary.

As to the molecular studies in this genus, Roh et al. (2002) have recently conducted a large scale population study on 80 samples of *Lycoris* species and unidentified accessions collected from various sources in different countries for the identification and classification of these samples by the random amplified polymorphic (RAPD) method. They reported that the clustering of *Lycoris* species based on RAPD polymorphic bands generally agrees with taxonomic treatments that are based on morphological, karyotypical and phenological observations. In our previous study (Hayashi et al., 2005), completely identical nucleotide sequences were detected in 11 Japanese and four Chinese triploid strains and also between two Chinese diploid strains. This clear genetic constancy in their nucleotide sequences was consistent with the findings obtained from previous chromosome karyotype analysis (Kurita, 1987b) and allozyme analysis (Chung et al., 1999). However, in spite of such genetic uniformity in each geographical area, the triploid strains of *L. radiata* were clearly genetically diverged between Japan and China.

The objectives of the present study were (1) to clarify the phylogeny of the *Lycoris* species by using the definitive DNA sequencing method and (2) to examine the possible maternal donor of the hybrid origin *Lycoris* species and the Japanese triploid strains of *Lycoris radiata* var. *radiata*. New findings made in this study were the identification of four major phylogentic groups in *Lycoris* species, and clarification of the maternal donors of hybrid origin species as suggested by the early cytogenetical studies.

MATERIALS AND METHODS

Plant materials: *Lycoris* species A total of 27 strains representing 11 species of the genus *Lycoris* were examined in this study. The species name, strain number, locality, seed fertility, chromosome karyotype and references are shown in Table 1. These materials were collected as bulbs by us, and several bulbs were obtained from Shiroshita Farm (Nagasaki) and cultivated in our experimental gardens. Photographs of flowers and karyotypes are stored as vouchers. The leaf and/or DNA samples are available for reference upon request.

Extraction of genomic DNA and PCR The total genomic DNA was extracted from plant leaves according to the procedures described in our previous report (Hayashi et al., 2005). Two genomic regions in the chloroplast of genus *Lycoris* were examined, namely, the maturase K (*mat K*) gene and the noncoding intergenic spacer (IGS) between *atpB* and *rbcL*.

A major part of the *matK* gene was amplified by PCR employing Taq DNA polymerase : the PCR involved 35 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The following primers were employed: 5'-CTATATCCACTTATCTTTCAGGAGT-3' and 5'-AAAGTTCTAGCACAAGAAAGTCGA-3', which were designed by using the nucleotide sequence of the partial coding region of the *Lycoris traubii matK* gene (accession No AB017290) reported by Ito et al. (1999)

The noncoding intergenic spacer (IGS) between the *atpB* and *rbcL* genes was amplified by PCR with LA Taq (TAKARA BIO INC.) and 35 cycles of denaturation at 95°C for 20 sec, annealing and extension at 68°C for 2 min. The primers used were 5'-CGAAAATAAATGTCCGATAGCAAGT-3' and 5'-AACATAGGCATAATTTCATCCATGA-3', which correspond in position to the sequence that is common to 23 *Lycoris* species and subspecies (accession Nos AB090927 and DQ020498 to DQ020518)

DNA sequencing of the *matK* gene and the IGS between the *atpB* and *rbcL* genes in the chloroplast genome Unlike in the case of the nuclear genomic sequence, these chloroplast genomic sequences were obtained as homogeneous material. The PCR products were separated by agarose gel electrophoresis and confirmed to exhibit a single band signal for both genomic regions (data not shown). To avoid artificial sequence alterations caused by PCR, the amplified products were

Table 1. Source of the materials for the taxa in the genus *Lycoris* examined in this study

No. Species Name	Locality	Fertility*	Karyotype**	(References***)
1. *Lycoris radiata* (L'Herit.) Herb. var. *radiata*				
		Sterile	2n = 3x = 33A	(Refs. 2, 5)
(1) radiata-1	Miyagi, Japan			(Strain No.11 in Japan, Ref.1)
(2) radiata-2	Chiba, Japan			(Strain No.10 in Japan, Ref.1)
(3) radiata-3	Kyoto, Japan			(Strain No. 7 in Japan, Ref.1)
(4) radiata-4	Nagasaki, Japan			(Strain No. 2 in Japan, Ref.1)
1. *Lycoris radiata* (L'Herit.) Herb. var. *radiata*				
		Sterile	2n = 3x = 33A	(Refs. 2, 5)
(5) radiata-5	Fujian, China			(Strain No. 1 in China, Ref.1)
(6) radiata-6	Jiangxi, China			(Strain No. 2 in China, Ref.1)
(7) radiata-7	Zhejiang, China			(Strain No. 3 in China, Ref.1)
(8) radiata-8	Guangxi, China			(Strain No. 4 in China, Ref.1)
2. *Lycoris radiata* (L'Hérit.) Herb. var. *pumila* Grey				
		Fertile	2n = 2x = 22A	(Refs. 2, 5)
(9) pumila-1	Zhejiang, China			(Strain No. 1 in China, Ref.1)
(10) pumila-2	Zhejiang, China			(Strain No. 2 in China, Ref.1)
(11) pumila-3	China			
(12) pumila-4	China			
Other related species in *Lycoris*				
3. *Lycoris sanguinea* Maxim.				
		Fertile	2n = 2x = 22A	(Refs. 2, 4, 5)
(13) sanguinea-1	Japan			
4. *Lycoris sprengeri* Comes ex Baker				
		Fertile	2n = 2x = 22A	(Refs. 2, 5
(14) sprengeri-1	Jiangsu, China			(Refs. 2, 5)
(15) sprengeri-2	China			
5. *Lycoris haywardii* Traub (sensu Hsu et al. 1994)				
		Fertile	2n = 2x = 22A	(Ref. 5)
(16) haywardii-1	Zhejiang, China			(Ref. 5)
6. *Lycoris rosea* Traub & Moldenke (sensu Hsu et al. 1994)				
		Fertile	2n = 2x = 22A	(Ref. 5)
(17) rosea-1	Zhejiang, China			
7. *Lycoris traubiii* Hayward				
		Fertile	2n = 12 = 10M+2T	(Refs. 2, 5)
(18) traubii-1	Kagoshima, Japan			(Ref. 2)
(19) traubii-2	Japan			
8. *Lycoris chinensis* Traub				
		Fertile	2n = 16 = 6M+10T	(Ref. 5)
(20) chinensis-1	Zhejiang, China			(Ref. 5)
9. *Lycoris longituba* Y.Hsu & G.J.Fan				
		Fertile	2n = 16 = 6M+10T	(Ref. 5)
(21) longituba-1	Jiangsu, China			(Ref. 5)
10. *Lycoris albiflora* Koidzumi				
		Sterile	2n = 17= 5M+1T+11A	(Refs. 2, 5)
(22) albiflora-1	Kagoshima, Japan			(Refs.2, 5)
(23) albiflora-2	Japan			
(24) albiflora-3	Japan			
11. *Lycoris squamigera* Maxim.				
		Sterile	2n = 27= 6M+10T+11A	(Refs. 2, 5)
(25) squamigera-1	Aomori, Japan			
(26) squamigera-2	Aomori, Japan			
(27) squamigera-3	Iwate, Japan			

Notes:
* Seed fertility: Fertile species can propagate by seeds, while sterile species propagate exclusively by division of bulbs.
** Karyotype: A, acrocentric chromosome; M, metacentric chromosome; T, telocentric chromosome.
*** References: (1), Hayashi et al., 2005; (2), Kurita, 1987a ; (3) Kurita, 1987 b; (4) Kurita, 1989; (5) Hsu et al., 1994.

No.	Species	Variant site (base No.) of nucleotide variations detected in 1136 bases for *matK* gene										
		49	274	341	529	581	643	801	825	967	1019	1054
13	*sanguinea*	TACTC	AATTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
18	*traubii*	TACTC	AATTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
14	*sprengeri-1*	TACTC	AATTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
15	*sprengeri-2*	TACTC	AATTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
16	*haywardii*	TACTC	AATTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
9	*pumila-1*	TACTC	AACTT	CCCAT	TGTGT	TTACG	TCCGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
20	*chinensis*	TACTC	AACTT	CCCAT	TGTGT	TTACG	TCTGC	TTTTC	TTTAC	TACCG	TCCTT	CCATC
1	*radiata-1*	TATTC	AATTT	CCTAT	TGCGT	TTGCG	TCTGC	TTGTC	TTTAC	TACCG	TCTTT	CCGTC
5	*radiata-5*	TATTC	AATTT	CCTAT	TGTGT	TTGCG	TCTGC	TTGTC	TTGAC	TACCG	TCTTT	CCGTC
11	*pumila-3*	TATTC	AATTT	CCTAT	TGCGT	TTGCG	TCTGC	TTGTC	TTTAC	TACCG	TCTTT	CCGTC
12	*pumila-4*	TATTC	AATTT	CCTAT	TGTGT	TTGCG	TCTGC	TTGTC	TTTAC	TACCG	TCTTT	CCGTC
22	*albiflora*	TATTC	AATTT	CCTAT	TGCGT	TTGCG	TCTGC	TTGTC	TTTAC	TACCG	TCTTT	CCGTC
17	*rosea*	TATTC	AATTT	CCTAT	TGTGT	TTGCG	TCTGC	TTGTC	TTGAC	TACCG	TCTTT	CCGTC
21	*longituba*	TATTC	AATTT	CCCAT	TGTGT	TTGCG	TCTGC	TTGTC	TTCAC	TACCG	TCTTT	CCATC
25	*squamigera*	TATTC	AATTT	CCCAT	TGTGT	TTGCG	TCTGC	TTGTC	TTCAC	TAGCG	TCTTT	CCATC

Fig. 1. Multiple sequence alignment for the *matK* gene in 15 representative strains of 11 species of genus *Lycoris*. Among the 1,136 bases examined, 11 base substitutions (0.96%) were detected. Variant nucleotides are indicated by a colored letter in the middle of five bases.

No.	Species	Variant site (base No.) of nucleotide variations detected in 389 bases for IGS region							
		176	194	250	327	350	373		384
15	*sprengeri-2*	CACCT	TCTGT	TGCTC	TATATATATATATAAGTTATATATATAAAAACTAACTATATA				ATGGT
14	*sprengeri-1*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
18	*traubii*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
9	*pumila-1*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
13	*sanguinea*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
16	*haywardii*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
20	*chinensis*	CACCT	TCTGT	TGCTC	TATATATATATA—AGTTATATATATAAAAACTA----TATA				ATGGT
25	*squamigera*	CATCT	TCCGT	TGCTC	TATATATA-------AGTTATATATATAAAAACTA----TATA				ATGGT
21	*longituba*	CATCT	TCCGT	TGCTC	TATATATA-------AGTTATATATATAAAAACTA----TATA				ATGGT
12	*pumila-4*	CATCT	TCCGT	TGTTC	TATATATA-------AGTTATATATAGAAAAACTA----TATA				ATGGT
5	*radiata-5*	CATCT	TCCGT	TGCTC	TATATATA-------AGTTATATATAGAAAAACTA----TATA				ATAGT
1	*radiata-1*	CATCT	TCCGT	TGCTC	TATA----------AGTTATATATAGAAAAACTA----TATA				ATGGT
11	*pumila-3*	CATCT	TCCGT	TGCTC	TATA----------AGTTATATATAGAAAAACTA----TATA				ATGGT
22	*albiflora*	CATCT	TCCGT	TGCTC	TATA----------AGTTATATATAGAAAAACTA----TATA				ATGGT
17	*rosea*	CATCT	TCCGT	TGCTC	TATATATA-------AGTTATATATAGAAAAACTA----TATA				ATGGT

Fig. 2. Multiple sequence alignment for the *atpB-rbcL* IGS region in 15 representative strains of 11 species of genus *Lycoris*. Among the 389 bases examined, five base substitutions (1.29%) were detected. In addition, deletions with different numbers of TA in the TA repeat region and an insertion of ACTA were detected. Variant nucleotides are indicated by colored letters.

directly subjected to nucleotide sequencing with the Applied Biosystems 3730 DNA sequencer and the BigDye Terminator ver.3.1 Cycle Sequencing Kit (Applied Biosystems), according to the procedures that we described previously (Saito et al., 1998; Hayashi et al., 2005).

Analysis of DNA sequences (CLUSTALW) The sequences that were obtained were aligned using CLUSTALW (Thompson et al., 1997:http://hypernigg.nig.ac.jp/homology/clustalw.shtml) and the selected discordant nucleotides among the specimens are shown in Figs 1 and 2 for better comprehension.

Phylogenetic analysis The phylogenetic trees for each of the two chloroplast genomic regions were constructed using the neighbor-joining (NJ) and parsimony methods with the software PHYLIP 3.65 (Felsenstein, 2004) and PAUP 4.0b (Swofford, 2005). For NJ trees, genetic distance was estimated with the Jukes-Cantor method (Jukes and Cantor, 1969) and bootstrap values for 1000 replicates were calculated. For parsimonious trees, semistrict consensus trees were reconstructed based on the parsimonious trees found with a heuristic search. To root the trees, the nucleotide sequences of *Chlidanthus fragrans*, a species belongs to the family *Amaryllidaceae*, were used for both regions. This species is considered to be a suitable outgroup because its genetic distance from *Lycoris* species is more than twice that within the *Lycoris* genus (see Results) and also it is not so different from *Lycoris* species, as mentioned above. The DDBJ/EMBL/GenBank accession numbers for the two regions of the outgroup species were AB017277 for *matK* and AY460390 for the *atpB-rbcL* IGS.

RESULTS

DNA sequence analysis-multiple sequence alignments The distinct nucleotide discordance observed in

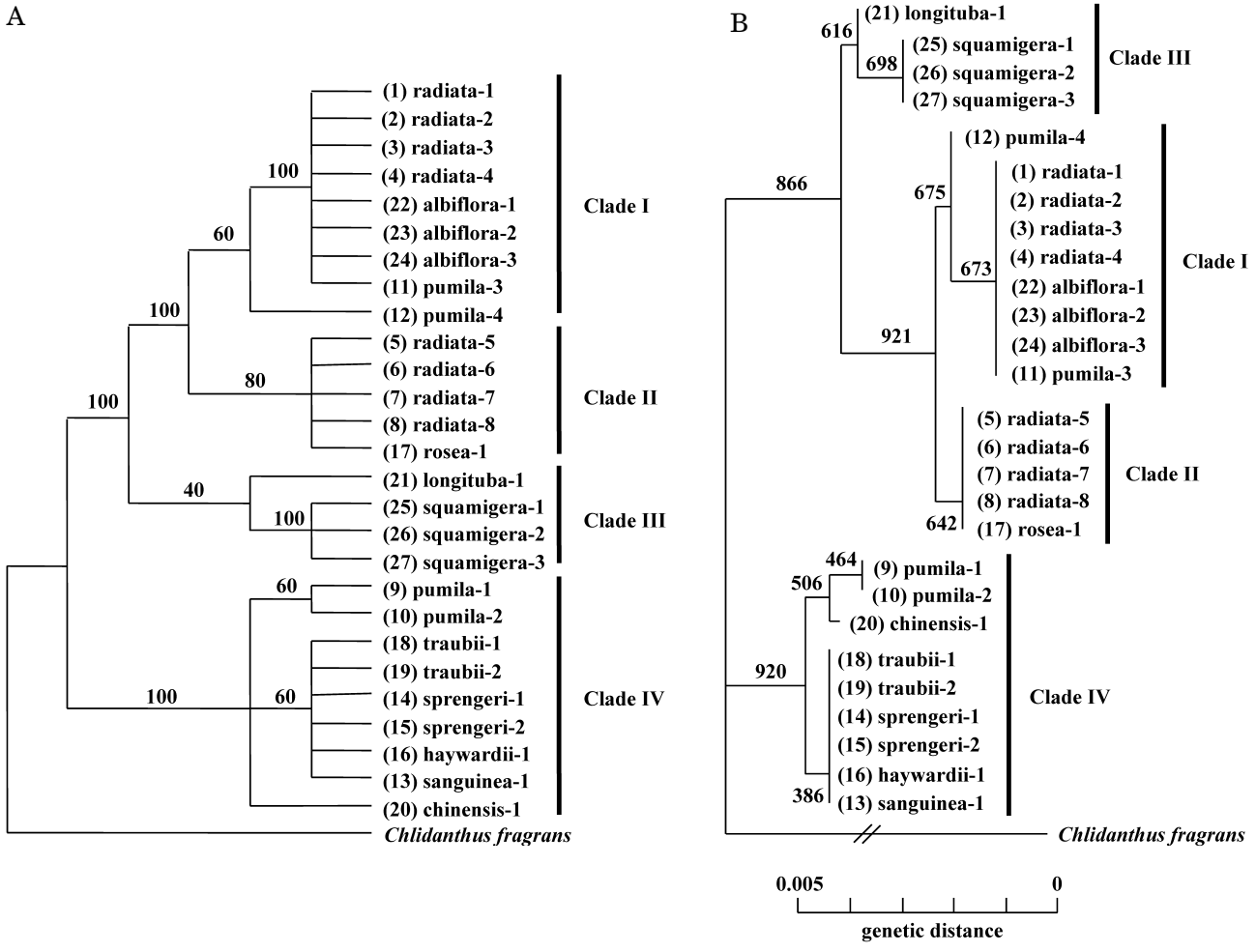

Fig. 3. Phylogenetic tree for *matK*. (A) Semistrict consensus parsimonious tree and (B) Neighbor-Joining (NJ) tree are shown. The numbers beside the branches indicate the consensus indices in the parsimonious tree and bootstrap values for 1000 replicates in the NJ tree. For the NJ tree, genetic distance is shown below the tree.

the two genomic regions of the chloroplast are summarized non-redundantly in Figs.1 and 2. Polymorphic nucleotides were identified at 11 positions in the examined region of the *matK* gene and at seven positions in the IGS between the *atpB* and *rbcL* genes. The sequence alignment revealed that the IGS also contains a variable number of TA dinucleotide repeats. These nucleotide sequences for the chloroplast *matK* gene and the IGS between the *atpB* and *rbcL* genes in *Lycoris* have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB243640 - AB243668.

Construction of phylogenetic trees The phylogenetic trees were constructed using the neighbor-joining (NJ) and the parsimony methods, based on multiple sequence alignment analysis. For the *matK* gene, as shown in Fig. 3, four distinct clades were observed in both trees. Clade I comprises nine strains including four Japanese strains of *L. radiata* var. *radiata* (triploid sterile taxa), two Chinese strains of *L. radiata* var. *pumila* (diploid fertile taxa) and three strains of *L. albiflora*; clade II four Chinese strains of *L. radiata* var. *radiata* and *L. rosea*; clade III, one strain of *L. longituba* and three strains of *L. squamigera;* clade IV, five diploid species, namely, the two remaining strains of *L. radiata* var. *pumila* and all the strains of *L. traubii, L. sprengeri, L. haywardii* and *L. sanguinea*. For all the species except for *L. radiata*, the strains from the same species were included in the same clade, i.e., the intraspecific variation was smaller than

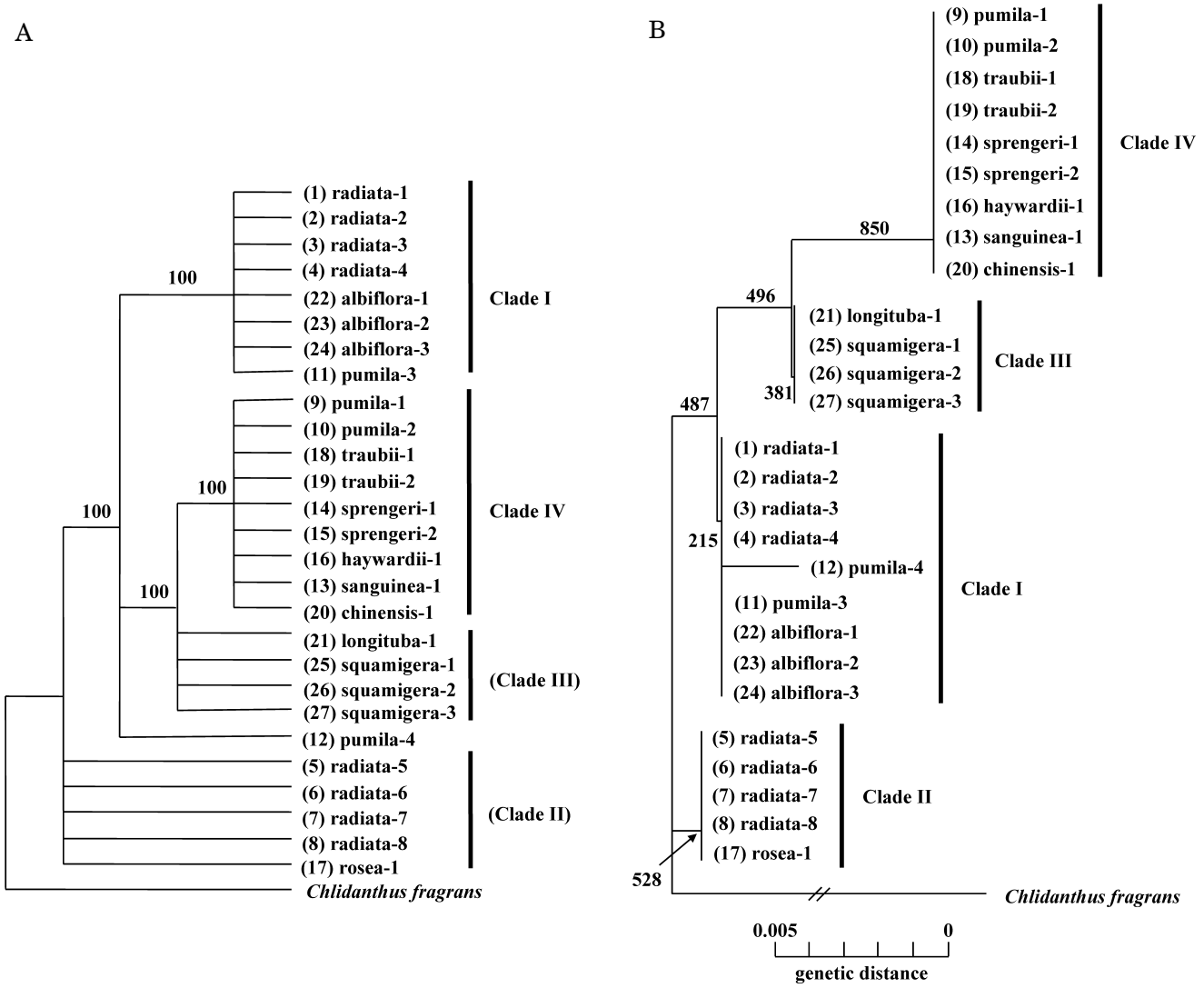

Fig. 4. Phylogenetic tree for *atpB-rbcL* IGS. (A) Semistrict consensus parsimonious tree and (B) Neighbor-Joining (NJ) tree are shown. The numbers beside the branches indicate the consensus indices in the parsimonious tree and bootstrap values for 1000 replicates in the NJ tree. For the NJ tree, genetic distance is shown below the tree. Clades II and III for the parsimonious tree were not a really clade because they did not form a cluster, but to show the correspondence with the other trees they were indicated as clades with parentheses for convenience.

the interspecific variation. In the case of *L. radiata*, triploid strains of var. *radiata* were divided into clades I and II, and diploid strains of var. *pumila* were divided into clades I and IV, indicating that the variation within *L. radiata* covered all the variations of the genus *Lycoris*. The rooting point determined using the outgroup species, *Chlidanthus fragrans*, indicated that the divergence between clade IV and the other clades first occurred in the genus *Lycoris*. This result implies that the intraspecific divergence of *L. radiata* might have preceded the speciation of the other *Lycoris* species, because four diploid strains of *L. radiata* var. *pumila* that were included in clusters I and IV were divided into two superclades. When we used another *Amaryllidacae* species, *Crinum moorei* (DDBJ/EMBL/GenBank accession number AB017279), as an outgroup species, the same topology and rooting point was obtained for both trees. (data not shown).

For the *atpB-rbcL* IGS region, as shown in Fig. 4, four clades were observed in the NJ tree, as well as the trees constructed based on the *matK* gene. The members of each clade were consistent with the *matK* gene trees, but the bootstrap values for each clade were much lower than that for the *matK* gene. For the parsimonious tree, only two clades, which corresponded to I and IV in the other trees, were observed. In addition, pumila-4, which was included in clade I for the other trees, was separated from clade I. These results indicated that the tree for the *atpB-rbcL* IGS region was relatively less informative than that for the *matK* region.

To confirm the clustering, phylogenetic trees based on the hypothetical sequence made by joining the two different chloroplast regions were reconstructed (Fig. 5). The clustering pattern based on the hypothetical joining sequence was largely consistent with the tree for matK, but the bootstrap values for each clade increased

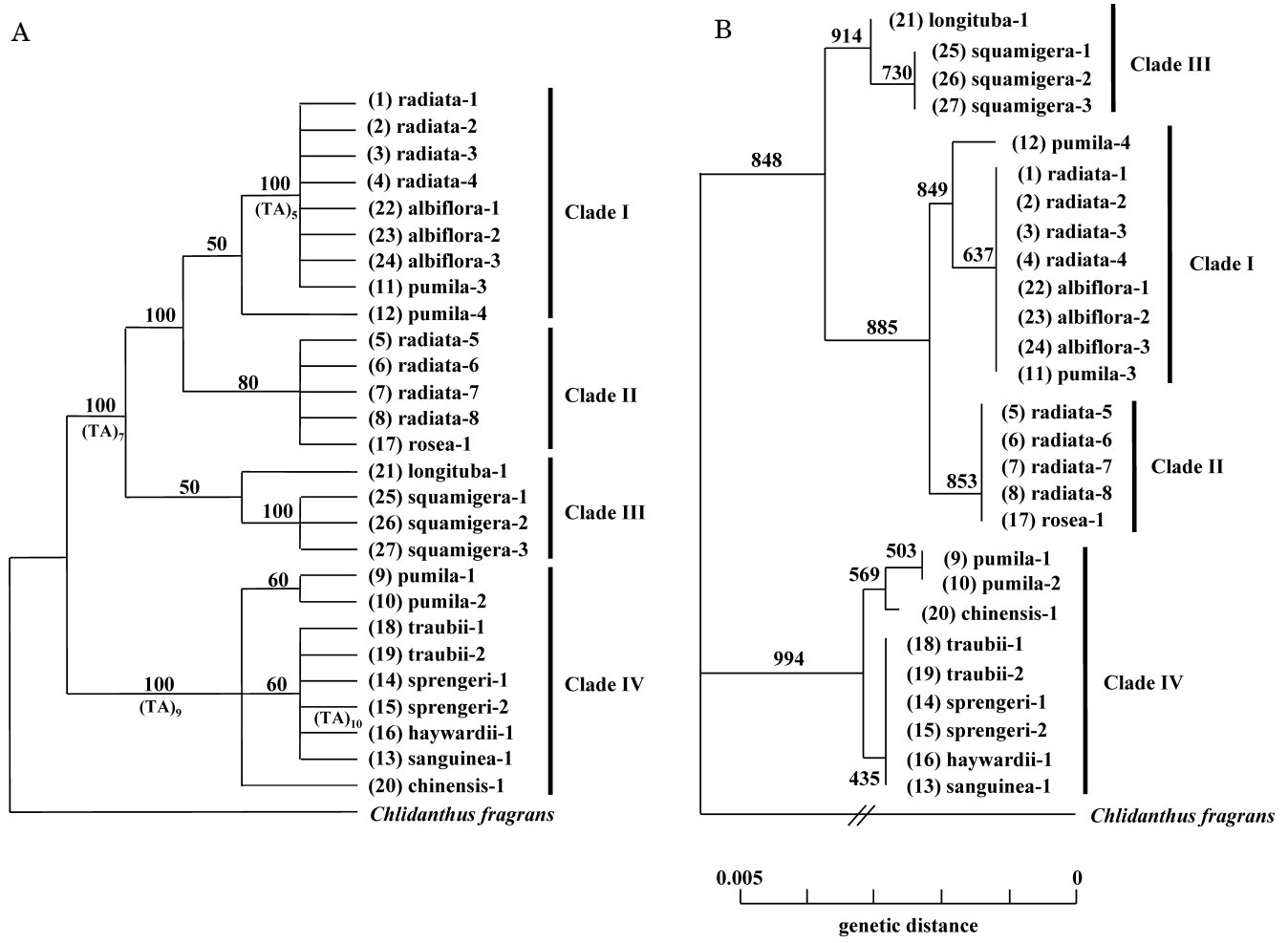

Fig. 5. Phylogenetic tree based on the hypothetical sequence constructed by joining the sequences of *matK* and *atpB-rbcL* IGS regions. (A) Semistrict consensus parsimonious tree and (B) Neighbor-Joining (NJ) tree are shown. The numbers beside the branches indicate the consensus indices in the parsimonious tree and bootstrap values for 1000 replicates in the NJ tree. The parsimoniously deduced changing point of the number of TA repeats located in the middle of *atpB-rbcL* IGS region are indicated below the parsimonious tree. For the NJ tree, genetic distance is shown below the tree.

to more than 80% for the NJ tree. The variation of the number of TA repeats positiond in the middle of the *atpB-rbcL* IGS region was well consistent with the evolutionary distance estimated by the parsimonious tree (Fig. 5A).

DISCUSSION

Phylogenetic relationship of species in the genus *Lycoris* based on two chloroplast genomic regions Based on the sequences of the two chloroplast regions, the *Lycoris* species can be classified into four major clades, namely, I, II, III and IV. The results for the two regions were mainly but not completely consistent with each other. Clades II and III for the *atpB-rbcL* IGS were not clearly formed. This is because of the small number of informative nucleotide changes in the *atpB-rbcL* IGS region. To solve the obscure clustering for each region, we created the hypothetical sequence joining the two different chloroplast regions. The clustering based on the hypothetical joining sequence gave us more reliable information, since it has more informative sites. Thus, this discussion is mainly based on the results for the hypothetical joining sequence. As shown in Fig. 5, the triploid strains of *L. radiata* var. *radiata* form two separate clades, namely, clade I containing Japanese triploid strains and clade II containing Chinese triploid strains. This differentiation between Japanese and Chinese triploid strains is consistent with our previous report in which the Japanese and Chinese triploid strains were considerably different in the patterns of nucleotide variation in two genomic regions, i.e., the nuclear lectin and chloroplast maturase genes, although identical nucleotide sequences were detected in 11 Japanese triploid strains and in four Chinese triploid strains (Hayashi et al., 2005). In the case of the diploid strains of *L. radiata* var. *pumila*, the four strains used in this study were split into two divergent clades, namely, I and IV. This spread of *L. radiata* at both ploidy levels indicates a high level of intraspecific chloroplast variation of *L. radiata*. No other *Lycoris* species showed such a high level of intraspecific variation in this study. This implies that the intraspecific divergence of *L. radiata* occurred coincidently with or preceding the speciation of the other *Lycoris* species, i.e., *L. radiata* is possibly an ancestral species of the genus *Lycoris* and the other species were derived from this species relatively recently.

The classification of *Lycoris* species had been conducted based on the morphological and phenological characters (Traub and Moldenke, 1949), but it is still difficult and complicated due to the gradual changes of morphological and physiological characters within and between species and frequent production of hybrids in nature and in cultivation. Furthermore, recent cytological analyses revealed karyotypical and phenological variation within a species, indicating the necessity of reclassification of *Lycoris* species (Hsu et al., 1994). In our study, the whole variation among *Lycoris* species was included in the intraspecific variation of *L. radiata*. This may indicate that the genetic divergence between each of the present *Lycoris* "species" is not at a species level, but an infra-species category level (subspecies, variety or even ecotype). Another possibility is that the chloroplast phylogeny does not represent the evolutionary process of the genus, which was caused by nuclear introgression throughout interspecific hybridization. In that case, the divergent groups of *L. radiata* based on the chloroplast genome would possess a similar nuclear genome to each other. This seems possible since interspecific hybridization is frequently observed in this genus and even sterile hybrids can survive in nature because of their perenniality and vegetative reproducibility. Since our present study is only based on the chloroplast genome we can not determine which possibility is better able to explain the whole phylogeny of the genus *Lycoris*. Additional information about nuclear genetic variation will be necessary to obtain a conclusive classification.

Evolutionary relationship between chloroplast phylogeny and karyotype variation in the genus *Lycoris* One of the important features of the genus *Lycoris* is the arrangement of chromosomes. In the karyotypes of the genus *Lycoris*, three major types of chromosomes are identified: A (acrocentric), M (metacentric), and T (telocentric). It is likely that the basic chromosome number of 11 is the primitive type, and the total arm number of any species is always a multiple of 11 (Inariyama, 1931; 1951; Jones, 1978). However, it is still unsolved whether a successive decrease in the chromosome number via centric fusion or increase by centric fission has been the essential mechanism for karyotype evolution and speciation in the genus *Lycoris* (Hsu et al., 1994). One of the authors (Kurita, 1988) reported that the M chromosome is not a simple fusion product of two A chromosomes and is thought to have changed into two T chromosomes by direct centric fission and then into two A chromosomes by pericentric inversion. He is inclined to the fission theory, based on the karyological and karyogeographical evidence, together with C-banding patterns and DNA contents of certain species. The result of this study shed new light on the karyotype evolution in the genus *Lycoris*. The phylogenetic analysis suggested that *L. radiata* is the ancestral species of this genus. The diploid strain *L. radiata* var. *pumila* has 2n = 22A chromosomes, so that this karyotype should be original in *Lycoris*, i.e., centric fusionis a more likely process than centric fission. The observation of the production of telocentric chromosomes by centric fission of metacentric chromosomes (Kurita, 1988) may indicate that the change of chromosome numbers is not a unidirectional but a

reversible evolutionary process.

The phylogenetic clustering in the present study is not always consistent with previous studies on karyotype evolution. In the present study, four clear clusters were formed, but each cluster contains several different karyotype species, For example, 2n = 22A species were found in clades I and IV and 2n = 6M + 10T species were found in clades III and IV. This indicates that the divergence of the chloroplast genome is not parallel to the chromosome rearrangement. Two species possessing the same karyotype, 2n = 6M + 10T, *L. chinensis* and *L. longituba*, were spread into different clusters, IV and III, respectively. Based on our conclusion that the 2n = 22A species was ancestral, two explanations are possible. One is that the two species were generated polyphyletically by independent chromosome fusion events. The other is that one species formed at first and the other species was produced by introgression of the nuclear genome into a cytoplasmically divergent species. Based on our present information, we could not determine which process has occurred. Future analysis of nuclear genome variation in *Lycoris* species will help to clarify this.

Clarification of maternal donor of hybrid *Lycoris* species In this study, we used three hybrid origin species, *L. squamigera*, *L. albiflora*, and one diploid *L. rosea*. The origin of these species has been studied cytogenetically. *L. squamigera* (2n = 27 = 6M + 10T + 11A) is a hybrid between *L. sprengeri* (2n = 22 = 22A) and *L. longituba* (2n = 16 = 6M + 10T) (Inariyama, 1953; Takemura, 1961; Kurita, 1987a), and *L. albiflora* (2n = 17 = 10M + 2T) is a hybrid between *L. traubii* (2n = 12 = 10M + 2T) and *L. radiata* var. *pumila* (2n = 2x = 22 = 22A) (Inariyama, 1944) or *L. radaiata* var. *radiata* (2n = 3x = 33 = 33A) (Makino, 1943; Kurita, 1987b), and *L. rosea* (2n = 22 = 22A) is considered to be a natural hybrid between two diploid species carrying 2n = 22 = 22A, *L. sprengeri* and *L. radiata* var. *pumila* (Hsu et al., 1994). However the maternal origin of these hybrid species has been unknown to date. The present study gave an important clue to clarify the maternal lineage of these hybrid species. In the phylogenetic trees, *L. squamigera* and *L. longituba* formed a single cluster (clade III), indicating that *L. longituba* is the maternal donor of *L. squamigera*. For both analysed chloroplast regions, the three strains of *L. albiflora* have nucleotide sequences completely identical to four Japanese triploid *L. radiata* and one diploid *L. radiata* but different from *L. traubii*, and therefore it is concluded that the mother of *L. albiflora* is *L. radiata* although it cannot be determined whether it was diploid or triploid.

As for *L. rosea*, the maternal origin was not clearly indicated because in the trees *L. rosea* did not form a clear cluster with either parental diploid species. However, we suppose that *L. radiata* var. *pumila* is a likely candidate for the cytoplasmic donor of *L. rosea*, because *L. rosea* formed a cluster with Chinese strains of *L. radiata* var. *radiata,* which is the autotriploid derived from *L. radiata* var. *pumila,* and the other parental species, *L. sprengeri,* was located in a different cluster from *L. rosea*. Probably unidentified strain(s) of *L. radiata* var. *pumila*, which should be included in clade II, contributed the cytoplasm to *L. rosea* and the Chinese strains of *L. radiata* var. *radiata*.

In Fig. 6. we suggest a possible model of the maternal donor of hybrid origin species in the genus *Lycoris*, based on the molecular phylogenetic analyses in the present study. It must be noted that the figure is one of the possible models which is based on the assumption that the nuclear introgression has not occurred and the chloroplast phylogeny obtained in this study was parallel to the speciation process.

In a natural habitat in Jiangsu Province, China, Kurita has observed that two populations of diploid fertile species, i.e., *L. chinensis* (2n = 16) and *L. sprengeri* (2n = 22), were separated approximately 100 m, and possible interspecific sterile hybrids having the same chromosome number of 2n =19, showing identical karyotype of 3M + 5T + 11A in each population, were identified, but no cytological and morphological evidence of successive backcrosses (introgressive hybridization) between the hybrids and parental species could be demonstrated.

Possible origin of Japanese triploid, sterile strains of *L.radiata* var. *radiata* Although cytogenetic studies on the genus *Lycoris* have been conducted by several authors, the origin of the Japanese triploid strains of *L. radiata* var. *radiata* has not been identified. Early studies concluded that *L. radiata* var. *radiata* is an autotriploid of diploid strains *L. radiata* var. *pumila* based on the cytological observations (Nishiyama, 1928; Inariyama, 1931, 1944). In our previous study (Hayashi et al, 2005), we clearly demonstrated that the triploid strains of *L. radiata* in Japan are genetically quite uniform at the nucleotide level in both the nuclear and chloroplast genomes. This implies that after their introduction into Japan from China they rapidly propagated vegetatively via bulbs from only one strain having a unique DNA sequence and karyotype. Since the diploid *L. radiata* var. *pumila* is distributed only in China and not found in Japan, the diploid maternal donor of Japanese triploid *L. radiata* must be in China. To date the mother and birthplace of Japanese triploid *L. radiata* in China are unknown. In a previous study, we investigated two Chinese diploids *L. radiata* var. *pumila* (strain Nos. 9 and 10 in the present study), but the nucleotide sequences of their lectin and *matK* genes were not identical to those of Japanese triploid *L. radiata*, indicating that they were not probable candidates for the mother of the Japanese triploid *L. radiata*.

In the present study, we added two other diploid

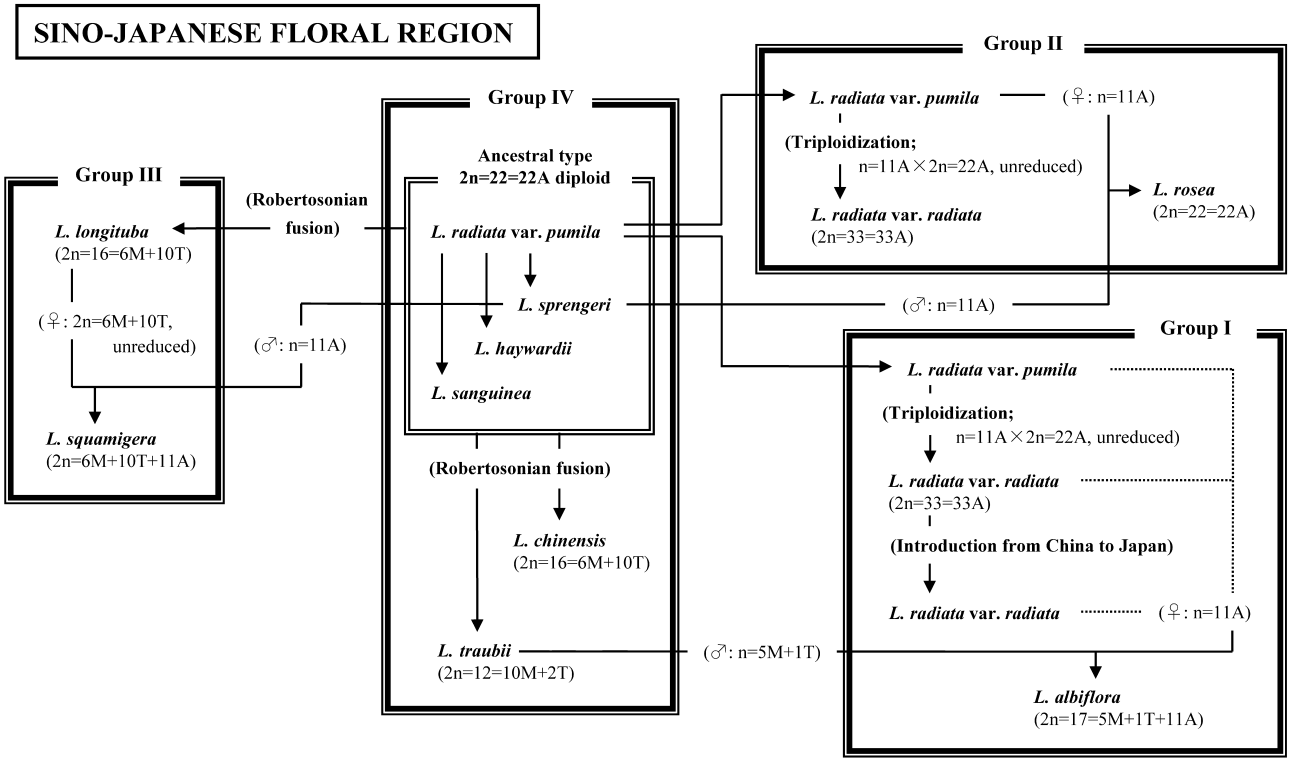

Fig. 6. The schematized evolutionary process in the genus *Lycoris*. Based on the phylogenetic clustering of *Lycoris* species, a possible evolutionary process is shown schematically. Groups I, II, III and IV correspond to clades I, II, III and IV in Fig. 3 and 4, respectively.

strains of *L. radiata* var. *pumila* (strain Nos. 11 and 12). These newly used *L. radiata* var. *pumila* were clustered with Japanese triploid *L. radiata* (clade I in Figs. 3, 4 and 5). Especially, of these two, one strain (strain no. 11) had nucleotide sequences completely identical to Japanese triploid *L. radiata* for both *matK* and *atpB-rbcL* IGS regions. Therefore, this strain is a strong candidate for the maternal parent of Japanese triploid *L. radiata* or its very close relative. Unfortunately, the collection site of this strain was not recorded, and thus we have not yet achieved our final goal of clarifying the origin of Japanese *L. radiata*. In the present study, we demonstrated a high level of intraspecific variation in *L. radiata* var. *pumila*. Although we examined only four strains of the variety in the present study, the high level of variation detected indicates the necessity of examining more strains of *L. radiata* var. *pumila* in order to identify the mother of Japanese *L. radiata* more precisely.

The authors appreciate two anonymous reviewers concerning improvements to this paper. We are very grateful to Dr. Toshiyuki Saito for his continuous support of our studies, critical reading of the manuscript and valuable comments. We also thank Mr. Shigeharu Shiroshita (Shiroshita Farm) and Mrs. Reiko Katakura for supplying some of the materials.

REFERENCES

Chung, M. G. (1999) Notes on allozyme variation in *Lycoris radiata* (*Amaryllidaceae*) from Korea. Bot. Bull. Acad. Sin. **40**: 227–230.

Felsenstein, J. (2004) PHYLIP version 3.65. Distributed by the author. Department of Genetics, University of Washington, Seatttle, WA 98195–7730, USA.

Hayashi, A., Saito, T., Mukai, Y., Kurita, S., and Hori, T. (2005) Genetic variations in *Lycoris radiata* var. *radiata* in Japan. Genes Genet. Syst. **80**: 199–212.

Hsu, P.-S., Kurita, S., Yu, Z.-Z., and Lin, J.-Z. (1994) Synopsis of the genus *Lycoris* (*Amaryllidaceae*). Sida **16**: 301–331.

Inariyama, S. (1931) Cytological studies in the genus *Lycoris* (Preliminary notes). Bot. Mag. Tokyo 45:11–24.

Inariyama, S. (1944) Origin of *Lycoris radiata* and *L. albiflora*. Jap. J. Genet. **23**: 15–16.

Inariyama, S. (1951) Cytological studies in the genus *Lycoris* (II). Sci. Rep. Tokyo Bunrika Digaku Sect. **B7**: 103–156.

Inariyama, S. (1953) Cytological studies in *Lycoris*. Rep. Kihara Inst. Biol. Res. **6**: 5–10.

Ito, M., Kawamoto, A., Kita, Y., Yukawa, T., and Kurita, S. (1999) Phylogenetic relationships of *Amaryllidaceae* based on *matK* sequence data. J. Plant Res. **112**: 207–216.

Jones, K. (1978) Aspects of chromosome evotution in higher plants. In *Recent Advances in Botanical Research* (ed. H.W. Woolhous) **6**: 119–194. Acedemic Press.

Jukes T. H., and Cantor, C. R. (1969) Evolution of protein molecules. In *Mammalian Protein Metabolism* (ed. H. N. Munro), pp.21–132. Academic Press.

Kurita, S. (1987a) Variation and evolution in the karyotype of *Lycoris*, *Amaryllidaceae* II. Karyotype analysis of ten taxa among which seven are native to China. Cytologia **52**: 19–40.

Kurita, S. (1987b) Variation and evolution in the karyotype of *Lycoris*, *Amaryllidaceae* IV. Interspecific variation in the

karyotype of *L. radiata* (L'Herit.) Herb. and origin of this triploid species. Cytologia **52**: 137–149.

Kurita, S. (1988) Variation and evolution in the karyotype of *Lycoris*, *Amaryllidaceae* VII. Mode of karyotype evolution within species and probable trend of karyotype evolution in the genus. Cytologia **53**: 323–325.

Kurita, S. (1989) Variation and evolution in the karyotype of *Lycoris*, *Amaryllidaceae* V. Chromosome variation in the karyotype of *L. sanguinea* Maxim. Plant Species Biol **4**: 47–60.

Lee, S., and Kim, M. (1987) Palynological study of some *Lycoris* species. Kor. J. Plant Taxon **17**: 147–154.

Maekawa, F. (1954) Prehistoric naturalized plants to Japan proper. Acta Phytotax. Geobot. **27**: 274–279.

Makino, T. (1943) On *Lycoris albiflora*. A cta. Phytotax. Geobot. **13**: 17–19.

Nishiyama, I. (1928) Reduction division in *Lycoris*. Bot. Mag. Tokyo **42**: 509–513.

Roh, M. K., Kurita, S., Zhau, X. Y., and Suh, J. K. (2002) Identification and classification of the genus *Lycoris* using molecular markers. J. Kor. Soc. Hort. Sci. **43**: 120–132.

Saito, T., Matsuda, Y., Ishii, H., Watanabe, F., Mori, M., Hayashi, A., Araki, R., Fujimori, A., Fukumura, R., Morimyo, M., Tatsumi, K., and Hori, T. (1998) Mouse Cdc-21 only 0.5 k upstream from Dna-pkcs in a head-to-head organization: an implication of co-evolution of ATM family members and cell cycle regulation genes. Mammal. Genome **9**: 769–772.

Swofford, D. L. (2005) PAUP. Phylogenetic Analysis Using Parsimony Version 4 Beta. Sinauer Associates, Sunderland, Massachusetts.

Takemura, E. (1961) Morphological and cytological studies on artificial hybrids in the genus *Lycoris*. I. On the hybrid *L. sprengeri* Comes. and *L. straminea* Lindal. Bot. Mag. Tokyo **74**: 524–531.

Thompson, J. D., Gibbon, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by analysis tools. Nucleic Acids Res. **25**: 4876–4882.

Traub, H. P., and Moldenke, H. P. (1949) *Amaryllidaceae*: Tribe *Amallese*. Amer. Pl. Life Soc. Stanford, CA.