Genetic variations in *Lycoris radiata* var. *radiata* in Japan

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The genetic variations of *Lycoris radiata* var. *radiata*, a completely sterile triploid from Japan, were examined by comparing the nucleotide sequences of genomic DNA regions in 11 triploid strains sampled from Japan and four triploid strains sampled from China, and in two diploid strains of *Lycoris radiata* var. *pumila*, which is endemic to China and fertile. For this purpose, two genes were analyzed, the lectin gene in the nuclear genome and the maturase gene in the chloroplast genome. A clear genetic constancy was observed in their DNA nucleotide sequences. For both genes, completely identical nucleotide sequences were detected in the 11 Japanese and four Chinese triploid strains and also between the two Chinese diploid strains. However, some genetic variations were observed between the Japanese and Chinese triploid strains, and between the triploid and diploid strains. These results are consistent with the findings obtained from previous chromosome karyotype analyses and allozyme analyses. In addition, in our preliminary FISH analysis of the physical mapping of the rRNA gene family, the 18S-5.8S-26S rRNA and 5S rRNA loci were localized on six and four chromosomes, respectively. Regarding the 18S-5.8S-26S rRNA loci, two were associated with two SAT chromosomes. The remaining four were distinguished by having no secondary constriction. Localization of 5S rRNA loci to chromosome spreads revealed three sites on the proximal part of the long arm of three acrocentric chromosomes and one site on the distal part of the long arm of the SAT chromosome; the latter site was juxtaposed to the 18S-5.8S-26S rRNA loci. These findings indicate that *L. radiata* var. *radiata* is not a typical autotriploid. The present paper discusses the possible origin of *L. radiata* var. *radiata* from a diploid variety of *L. radiata* var. *pumila*, based on the molecular cytogenetic analysis and DNA sequence analysis.

Key words: Chromosome, FISH, nucleotide variation, nuclear lectin gene, chloroplast maturase gene, *Lycoris*

INTRODUCTION

The genus *Lycoris*, a small group of *Amaryllidaceae* comprises 20 species (Hsu et al, 1994); it is distributed only in the moist warm temperate woodlands of eastern Asia, from China to Japan and Korea, with a few species extending to northern Indochina and Nepal.

*Lycoris radiata* var. *radiata* is a variety of the species of *L. radiata* (L’Herit.) Herb and is completely sterile due to its triploid genomic constitution. This triploid species is very common and widely distributed throughout Japan except Hokkaido and its habitat is always restricted to the edge of paddy fields, the margins of plantations and graveyards.

It has been suggested that during prehistoric times, approximately 3000 years ago, when the practice of rice cultivation was brought into Japan from China, this triploid sterile species was introduced into Japan from China, this triploid sterile species was introduced into the southern part of Japan as a companion plant. Then it spread throughout Japan, accompanying the rapid expansion of rice cultivation to the northern parts of the country.

Cytogenetic studies on the species *L. radiata* var. *radi-
ata have been carried out by several authors. Nishiyama (1928) was the first cytogeneticist to clarify that the complete sterility of this species was due to its triploid nature (2n=3x=33). In addition, he found 11 trivalent configurations of chromosomes pairing at the first meiotic metaphase and irregular chromosome segregation at meiotic anaphase. Therefore, he attributed the sterility of this species to autotriploidy. Inariyama (1931) also observed 33 rod-shaped chromosomes that were divided into 3 identical sets consisting of 11 chromosomes each; this supported Nishiyama’s view. In addition, Inariyama (1944) also suggested that this autotriploid variety was generated from L. radiata var. pumila which is diploid with a chromosome constitution of 2n=2x=22, by the fusion of a haploid gamete with 11 acrocentric chromosomes and a nonreduced gamete with 22 chromosomes from a diploid species.

Kurita (1987) conducted an extensive study of the population cytogenetics of triploid strains in Japan by analyzing 519 bulbs from 58 different localities and reported genetic constancy at the level of their chromosome constitution, by providing evidence that the standard karyotype was found in 97.7% of the plants examined. Chung (1999) carried out allozyme analysis in eight Korean populations of L. radiata var. radiata as well as in one Korean population of L. chinesis Traub. (2n =16) to estimate levels of allozyme diversity and reported that Lycoris radiata var. radiata was monomorphic at all 24 allozyme loci surveyed, whereas L. chinesis was polymorphic at 19% of the 21 loci analyzed, with 0.06 gene diversity. These results strongly indicated that only one or a few bulbs of L. radiata var. radiata were introduced from China or, secondarily, from Japan to a temple in Korea and were then naturalized in the southern Korean peninsula via a strong vegetative reproduction by the rapid formation of new bulbs.

In the present study, in order to further examine the genetic constancy in the triploid sterile strains of L. radiata var. radiata revealed by karyotype analysis of Japanese populations and allozyme analysis of Korean populations, we have investigated genetic variations at the level of genomic DNA in the nucleotide sequences of the nuclear lectin gene and the chloroplast maturase gene in triploid strains from different populations in Japan and China.

**MATERIALS AND METHODS**

1. **Lycoris radiata** (L'Herit.) Herb. This species is a bulbous perennial herb and the following two natural varieties are known (Hsu et al, 1994).

(1) **L. radiata** (L'Herit.) Herb. var. radiata: 1) Botanical characteristics: Leaves narrow, strap-shaped, up to 50 cm long, 7–15 mm wide, deep green with a whitish stripe in the center. Scape 30–55 cm long. Pedicel, 12–15 mm long, tepaltube 5–8 mm long; tepals 3.5–4.5 cm long, 6–8 mm wide, clearly crisped margin and recurved. Stamens 80–85 mm long. Style 90 mm long.


3) Distribution: Japan except Hokkaido, southern part of Korea, and south-western China.

In Japan, this species blooms during the autumnal equinoxial week in September, and Japanese Buddhists regard this plant as a celestial flower and refer to it as HIGAN-BANA (Flower of Nirvana) or MANJYUSHAGE (in Sanskrit, Manjyuusaka flower)

(2) **L. radiata** (L'Herit.) Herb. var. pumila: This species resembles var. radiata in external morphology. Leaves narrow strap-shaped, up to 40 cm long, 10–15 mm broad, deep green with a whitish stripe in the center. Scape 30–55 cm long. Tepaltube 4–6 mm long; tepal 3.5–4.5 cm long, 5–6 mm broad, finely crisped margin and recurved. Stamens 5.5–6.5 cm long. Style 6.0–7.5 cm long. Leaves appear in autumn; scape produced in August. Endemic to China. This species is called KOHIGAN-BANA (small HIGAN-BANA) in Japan, because it is smaller than HIGAN-BANA.

In the present study, a total of 17 strains were examined, including 11 Japanese var. radiata strains, four Chinese var. radiata strains and two Chinese var. pumila strains. The list of materials and their localities are shown in Table 1 and Fig. 1. Representative flowers of both L. radiata (L’Herit.) Herb. var. radiata and L. radi-
The genetic variations in *Lycoris* var. *pumila*, and their somatic chromosomes are shown in Fig. 2 (see Results).

2. Chromosome analysis and fluorescence in situ hybridization (FISH) techniques

1) Karyotype analysis

The somatic chromosomes in the root meristems were examined. Root tips were placed in 0.1% colchicine solution at room temperature for 3 hrs, and then fixed in an alcohol-acetic acid mixture (3:1, v/v) for 24 hrs at 4°C. After the tips were washed, they were macerated in 1N HCl at 60°C for 3 min, and then stained with acetocarmine or aceto-orcein solution for 12–24 hrs. The well-stained root tips were squashed by tapping with a toothpick or by pressing with a thumb or a hand roller (Hori and Tsunewaki, 1969; Kurita, 1987, Mukai et al. 1990).

2) Fluorescence in situ hybridization (FISH)

In order to determine the karyotype of the genus *Lycoris*, physical mapping of the 18S-5.8S-26S and 5S ribosomal RNA gene families on the chromosomes was carried out by using the methods described by Mukai et al. (1990, 1991). FISH analysis with biotin-labeled 18S-5.8S-26S rDNA and digoxigenin-labeled 5S rDNA sequence repeats was carried out as described by Raina et al. (2001). For the detection of fluorescent signals on the DAPI-counterstained chromosomes, digital image analysis was performed as described by Rahman et al. (1997).

3. Extraction of genomic DNA, PCR and DNA sequencing

The total genomic DNA was extracted from plant leaves (100 mg wet weight/leaf) by using a Master Pure Leaf Purification Kit (EPICENTRE, USA).

Two genes were analyzed. The lectin gene in the nuclear genome of *Lycoris* was amplified using the polymerase chain reaction (PCR) with LA-Taq polymerase (TAKARA BIO), using 35 cycles of denaturation at 95°C for 20 sec, annealing and extension at 68°C for 2 min. Based on the nucleotide sequence of the full-length cDNA of the *L. radiata* lectin gene reported by Zhao et al. (2003) and registered in GenBank (accession no AY191306), the following primer set was designed: F(5’-AAAAACCCAAAACAAGCAAAATCAACA-3’) and R(5’-GGCAGCAGAACC-...
CATTTACATCCA-3').

As a chloroplast gene, the maturase gene was amplified using PCR with Taq polymerase, using 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.

By using the nucleotide sequence of the partial coding region of the \textit{L. traubii} chloroplast gene for maturase, reported by Ito et al. (1999) and registered in GenBank (accession no AB017290), the following primer set was designed: F(5'-CTATATCCACTTATCTTTCAGGAGT-3') and R(5'-AAAGTTCTAGACAAGAAGAAGTCGA -3').

Since DNA gel electrophoresis analyses of the PCR products for both genes exhibited a single unique band (data not shown), nucleotide sequencing of the genes was carried out with an Applied Biosystems 3730 DNA analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the procedures reported by Saito et al. (1998).

1) Flowering of \textit{Lycoris radiata} var. \textit{radiata} from Japan: somatic chromosomes, 2n=3x=33A

2) Flowering of \textit{Lycoris radiata} var. \textit{radiata} from China: somatic chromosomes, 2n=3x=33A

3) Flowering of \textit{Lycoris radiata} var. \textit{pumila} from China: somatic chromosomes, 2n=2x=22A

Fig. 2. Flowers and somatic chromosomes in \textit{Lycoris}.
RESULTS

1. Karyotype analysis and FISH analysis of the somatic cells of *Lycoris*  
   First, to examine the ploidy of the materials used, the karyotype of the somatic chromosomes was determined. As shown in Fig. 2, the triploid strains of *L. radiata* var. *radiata* collected in Japan and China were typical triploids with chromosome constitution of 2n=3x=33A (A implies acrocentric chromosomes) and the diploid strains of *L. radiata* var. *pumila* exhibited the karyotype of 2n=2x=22A.

   In our preliminary FISH analysis of the physical mapping of the rRNA gene family, the 18S-5.8S-26S rRNA and 5S rRNA loci were localized on six and four chromosomes, respectively, as shown in Fig. 3. Regarding the 18S-5.8S-26S rRNA loci, two were associated with two SAT chromosomes. The hybridization signals extended over the whole short arm, a secondary constriction and a satellite. The remaining four were distinguished by having no secondary constriction. Their signals labeled only the short arm of four A chromosomes.

   Two SAT chromosomes were observed in karyotype analysis of mitotic metaphase. The possibility of more than two SAT chromosomes is undeniable because the satellite often fuses to the short arm. The number of nucleoli in the interphase cells was examined on the FISH samples, as shown in Fig. 3. In a total of 172 cells examined, the distribution of the numbers of cells containing one nucleolus, two nucleoli and three nucleoli was 84 (48.8%), 79 (45.9%), and 9 (5.2%), respectively. The variations in the number of active nucleoli may be due to the sensitivity of the FISH method applied. However, it seems likely that the triploid strain contains three chromosomes that carry the active rRNA genes.

   Localization of 5S rRNA loci to chromosome spreads revealed three sites on the proximal part of the long arm of three A chromosomes and one site on the distal part of the long arm of the SAT chromosome; the latter site was juxtaposed to the 18S-5.8S-26S rRNA loci.

2. Genetic variations in the nuclear lectin gene of *Lycoris*  
   We then examined genetic variations at the DNA nucleotide sequence level of the lectin gene in the nuclear genome in 11 triploid strains from Japan and four triploid strains from China, as well as two diploid strains from China.

   A nucleotide sequence of 503 bp in the genomic DNA including a 477-bp open reading frame of the lectin gene, was aligned with the reference sequence using CLUSTALW (Thompson et al., 1997). The essential parts of the multiple alignment data are shown in Fig. 4; only variant bases are summarized. In a total of 20 bases (4.0%) out of 503 bp examined, 18 base substitutions, one deletion and one insertion were detected, compared with the reference sequences deposited in GenBank.

   It is noteworthy that no genetic variations were detected among the 11 Japanese triploid strains, four Chinese triploid strains and two Chinese diploid strains, indicating completely identical nucleotide sequences of the region examined. However, the Japanese and Chinese triploid strains differ by 3 base changes. The Japanese and Chinese triploid strains differ from the Chinese diploid strains by one and two base changes, respectively.

   In Appendix-1, the full 503 nucleotide sequences of three representative strains from different localities in Japan and China are compared with the reference

![Fig. 3. FISH analysis of somatic chromosomes in *Lycoris*. A) Metaphase plate of somatic chromosomes of *Lycoris radiata* var. *radiata* in Japan (stained with acetocarmine), and B) Localization of signals from 18S-5.8S-26S rRNA genes (green fluorescence) and 5S rRNA gene (red fluorescence) on the same metaphase chromosomes as in A). Large and small arrows indicate 18S-5.8S-26S rRNA and 5S rRNA loci, respectively. Black arrows indicate the satellite. “n” indicates the nucleolus.](image-url)
sequence. These nucleotide sequences for the nuclear lectin gene in Lycoris have been deposited in the DNA Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank nucleotide sequence databases with the accession numbers AB214117-AB214119.

It is clear from Fig. 4 and Appendix-1 that in the nuclear lectin gene, significant base changes were found between our nucleotide sequence data and that reported by Zhao et al. (2003) and deposited in the GenBank. These differences could be attributed to the materials examined and might reflect intraspecific variations of L. radiata var. radiata in different populations.

3. The double peaks detected in the nucleotide sequences of the nuclear lectin gene To avoid possible artifacts from the cloning process, a mixture of the PCR products was directly subjected to DNA sequencing. As shown in Fig. 5, double peaks of the nucleotide sequence were clearly identified, particularly in the case of the Japanese triploid strains. Since this may imply a heterozygous condition of the alleles, we isolated several clones and determined the nucleotide sequences of the individual clones. The results obtained for the two kinds of clones shown in Table 2 clearly indicated that the original PCR products were mixtures of the products of two different alleles.

4. Genetic variations in the chloroplast maturase gene of Lycoris The nucleotide sequence of 1,136 bp of the genomic DNA including a partial coding frame of the maturase gene was aligned with the reference sequence
The genetic variations in Lycoris

The essential parts of the multiple alignment data are shown in Fig. 6, where only variant bases are summarized. Base substitutions were detected in a total of only 10 bases (0.9%) out of the 1,136 bp examined as compared with the reference sequences reported for another species of L. traubii and deposited in GenBank. This appears to be due to interspecific variations.

It is clear from Fig. 6 that no genetic variations were detected among the 11 Japanese triploid strains, four Chinese triploid strains and two Chinese diploid strains, indicating completely identical nucleotide sequences. However, Japanese and Chinese triploid strains differ by only two base changes, and both the Japanese and Chinese triploid strains differ from the Chinese diploid strains by nine base changes.

In Appendix-2, the full 1,136 nucleotide sequences of three representative stains are compared with the reference sequence. These nucleotide sequences for the chloroplast maturation gene in Lycoris have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB214120-AB214122.

**DISCUSSION**

1. The triploid nature of L. radiata var. radiata in Japan and its genetic constancy at the level of chromosome constitution. Cytogenetic studies of the species of L. radiata var. radiata have been made by several authors (Nishiyama, 1928; Inariyama 1931, 1944; Kurita, 1987). In the present study, we determined that the triploid strains of L. radiata var. radiata collected in Japan and China were typical triploids with chromosome constitution of 2n=3x=33A and the diploid strains of L. radiata var. pumila had the karyotype of 2n=2x=22A, confirming the previous findings.

Nishiyama (1928) found 11 trivalent configurations of chromosomes pairing at the first meiotic metaphase and irregular chromosome segregation at meiotic anaphase. Therefore, he attributed the sterility of this species to autotriploidy.

Inariyama (1931) also observed 33 rod-shaped chromosomes that were divided into three identical sets, consist-
ing of 11 chromosomes each; this supported Nishiyama’s view. Inariyama (1944) further suggested that this autotriploid variety was generated from \textit{L. radiata} var. \textit{pumila} which is diploid, having a 2n=2x=22 chromosome constitution.

Kurita (1987) conducted an extensive study on the population cytogenetics of the Japanese triploid strains and reported genetic constancy of their karyotypes.

2. The triploid sterile species of \textit{L. radiata} var. \textit{radiata} is not a simple autotriploid. Kurita (1987) reported that the genetic constancy at the level of chromosome constitution with the standard karyotype includes two A type satellite chromosomes heterozygously, i.e., the size of the short arm of one satellite chromosome is smaller than that of the other. In addition, he observed that the number of nucleoli in an interphase nucleus ranges from one to three, and two-thirds of the nuclei have two nucleoli, one large and one small. Three nucleoli were observed in approximately one-tenth of the cells. This suggests that there are three nucleolar organizer regions (NORs) in a chromosome complement of \textit{L. radiata} although only two satellite chromosomes are detectable. These observations suggest that the triploid species is not a simple autotriploid.

In our FISH analysis shown in Fig. 3, localization of 5S rRNA loci to chromosome spreads revealed three sites on the proximal part of the long arm of three A chromosomes and one site on the distal part of the long arm of the SAT chromosome; the latter site was juxtaposed to the 18S-5.8S-26S rRNA loci. These findings indicate that \textit{L. radiata} var. \textit{radiata} is not a typical autotriploid, supporting Kurita’s notion (1978).

In order to clarify the complex nature of the triploidy, additional molecular cytogenetic studies of the genome structure will be needed to apply more chromosome-specific DNA marker probes.
3. The genetic constancy at the level of DNA nucleotide sequence in *L. radiata* var. *radiata* and possible origin of the Japanese triploid species

As shown in Fig. 4 and 6, for both the nuclear lectin gene and chloroplast maturase gene, completely identical nucleotide sequences were detected in the 11 Japanese and four Chinese triploid strains and also between the two Chinese diploid strains. However, some genetic variations were observed between the Japanese and Chinese triploid strains, and between the triploid and diploid strains. These results are consistent with the findings obtained from previous chromosome karyotype analysis (Kurita, 1978) and allozyme analysis (Chung, 1999).

The heterozygosity detected in the nucleotide sequence shown in Fig. 5 might be related to the heterozygous conditions of the satellite chromosomes reported by Kurita (1987). Based on these circumstantial findings, it seems likely that the original triploid strains were generated from a hypothetical unidentified hybrid between two diploid strains which had different genotypes and a different morphology of satellite chromosomes.

The extent of nucleotide differences observed among the samples tested for maturase in the chloroplast genome was rather low compared with that of the lectin gene in the nuclear genome. This appears to be due to the low nucleotide substitution rate of chloroplast genes compared with that of the nuclear-encoded genes (Wolfe et al. 1987).

According to the cytogenetic karyotype analysis, the triploid sterile species of *Lycoris radiata* var. *radiata* originated in China from the diploid fertile species of *Lycoris radiata* var. *pumila*. This hypothesis was supported by karyotype analysis and meiotic behavior resulting in the formation of trivalent chromosomes (Nishiyama, 1928; Inariyama, 1931, 1944), and the fact that no *Lycoris radiata* var. *pumila* has been found so far in Japan.

The present studies clearly indicate that the triploid species of *Lycoris* in both Japan and China are genetically quite uniform. The genetic constancy of Japanese triploid strains at the nucleotide level in both the nuclear and chloroplast genomes may imply that after their introduction into Japan from China, they rapidly propagated vegetatively by bulbs from only one strain having a unique standard DNA sequence in the standard karyotypes.

In the case of the nuclear lectin gene, it seems likely that the DNA nucleotide sequences of the diploid strains in China are more similar to those of the Japanese triploid strains than to those of the Chinese diploid strains (Fig. 4 and Appendix-1). They differ by only one base substitution (G→T) at the 488th position near the end of the 503 bp analyzed.

On the other hand, in the case of the maturase gene in the chloroplast genome (Fig. 6 and Appendix-2), the triploid strains from Japan and China are very similar, differing by only two base changes. However, they differ from the diploid strains of *Lycoris radiata* var. *pumila* in China by nine base changes.

Owing to cytoplasmic inheritance and the haploid nature of the chloroplast gene, the two diploid strains examined are not probable candidates for the mother of the triploid strains of *Lycoris radiata* var. *radiata* that are found today in Japan.

Although it is difficult to suggest the possible origin of the triploid strains from the diploid strains in China, it is conceivable that an unidentified triploid other than the four Chinese strains examined in the present study could be the mother clone of all the strains in Japan. More population survey studies of triploid and diploid strains in China would help to clarify this issue.

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Appendix-1. Multiple Sequence Alignment of the Nuclear Lectin Gene in Lycoris

The 503 base pairs of the genomic DNA region including a 477-bp open reading frame of the lectin gene were aligned using CLUSTALW with the reference sequence registered in the GenBank. Variant nucleotides are indicated by colored letters (red or blue). The R and K represent a double peak of A/G and G/T, respectively. Each small bar (– and –) indicates a deletion.

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<th>Location</th>
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Appendix-2. Multiple Sequence Alignment of the Chloroplast Maturase Gene in *Lycoris*
The genetic variations in *Lycoris*
Notes:
Chiba represents strains of *Lycoris radiata* var. *radiata* in Japan.
AY191306 indicates DNA sequence of the lectin gene registered in GenBank.
Guangxi represents strains of *Lycoris radiata* var. *radiata* in China.
Mogan Shan represents strains of *Lycoris radiata* var. *pumila* in China.