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*Full Length Research Paper*

# rDNA internal transcribed spacer sequence analysis of *Lycoris* Hert.

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**The interspecific relationships of *Lycoris* species were studied by internal transcribed spacer (ITS) sequences. ITS fragments of 14 species were amplified, sequenced and analysed. The results showed that ITS sequences of 14 species were different from each other and the ITS lengths of 14 species were about 652 bp. The GC content of ITS2 sequences was bigger than that of ITS1. Clustering results based on ITS sequences showed that *Lycoris* species could be divided into three clades. The classification was basically consistent with those of karyotype and morphology. This paper suggested that the likelihood of hybrid origin of *Lycoris* species was supported and ITS could be used as a good molecular marker to identify plants of *Lycoris*.**

**Key words:** *Lycoris* Hert., internal transcribed spacer (ITS), molecular taxonomy, interspecific relationship.

## INTRODUCTION

The genus *Lycoris* Herb. belongs to the family Amaryllidaceae, and it is mainly distributed in China and Japan. It has about 20 species in the world and about 15 species and two varieties in China, which are mainly distributed in the south of the Yangtze River, especially in warm regions (Xu et al., 1985; Yuan et al., 2008). *Lycoris* spp. show that its bulb has important medicinal value and exploitation-utilization prospects with rich galantamine, lycorine and other alkaloids (Xie et al., 2007). In *Lycoris*, hybridization has been proved to be one of the important modes of speciation (Kurita and Hsu, 1996). And development of leaves of *Lycoris* does not coincide with its flowering during its growth and development. It is difficult to identify the species with only morphological features. Therefore, it is very important to identify accurately *Lycoris* species by means of alternative method, such as using the techniques of karyology and the molecular taxonomy. The interspecific relationships and identification of *Lycoris* species were performed by example cytology (Zhou et al., 2005), random amplified polymorphic DNA (RAPD) (Zhang et al., 2002) and inter simple sequence repeat (ISSR) makers (Yuan et al., 2007). The karyotype studies on *Lycoris* showed that chromosomes of *Lycoris* were classified into three types: 1) chromosomes with constrictions in median region (M); 2) chromosomes with constrictions in terminal region (T); and 3) chromosomes with constrictions in subterminal region (ST) (Zhao et al., 2008). Some information on classification of *Lycoris* was provided, but the origin and relationship of *Lycoris* species are not clear (Ma et al., 2004). The internal transcribed spacer (ITS) region of the 18S-5.8S-26S nuclear ribosomal DNA (nrDNA) has been commonly used for phylogenetic inference in plants. Two ITS regions, ITS1 and ITS2, generally evolve more rapidly than coding regions and have been shown to be equally informative, and able to differentiate between closely related species (Baldwin, 1992; Christopher et al., 2009; Vijaykumar et al., 2010; Yan et al., 2010). In this study, the genetic polymorphisms and relationships of 14 species in *Lycoris* were evaluated by using ITS fragment in order to provide theoretical support for the phylogenetic relationship and identification of *Lycoris* resources.

## MATERIALS AND METHODS

A total of 14 species of *Lycoris*, three individuals per species, from

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**Table 1.** Source of *Lycoris*.

| S/N | Species | Chromosome number* |
|---|---|---|
| 1 | *Lycoris straminea* Lindl. | 3M+5T+11ST |
| 2 | *Lycoris anhuiensis* Y. Hsu & Q. J. Fan | 6M+10T |
| 3 | *Lycoris aurea* (L' Her.) Herb. | 6M+10T |
| 4 | *Lycoris caldwellii* Traub. | 6M+10T+11ST |
| 5 | *Lycoris chinensis* Traub. | 6M+10T |
| 6 | *Lycoris haywardii* Traub. | 22ST |
| 7 | *Lycoris houdyshelii* Traub. | 3M+5T+22ST |
| 8 | *Lycoris incarnata* Comes ex C. Sprenger | 4M+3T+22ST+1m |
| 9 | *Lycoris longituba* Y. Hsu & Q. J. Fan | 6M+10T |
| 10 | *Lycoris longituba* var. *flava* Y. Hsu & X. L. Huang | 6M+10T |
| 11 | *Lycoris radiata* (L' Her.) Herb. | 22ST |
| 12 | *Lycoris rosea* Traub & Moldenke | 22ST |
| 13 | *Lycoris sprengeri* Comes ex Baker | 22ST |
| 14 | *Lycoris squamigera* Maxim | 6M+10T+11ST |

*Chromosome number (Wu et al., 2007; Zhao et al., 2008).

Yuanling, Zhongfang, Hangzhou botanical garden and Nanjing botanical garden Mem Sun Yat-sen (Table 1) were used. Each species were planted in 3 plots with size of 2 × 5 m and grown at normal fertilization and watering condition. Total genomic DNA was extracted from young leaves according to a modified DNA extraction procedure reported in Sharpe et al. (1989).

ITS primers were chosen according to White (1990): 5'-TCCTCCGCTT ATTGA TAT GC -3' and 5'-GGAAGGTAAAAGTC AAGG-3'. PCR were performed in 50 µl reaction system containing 5.0 $mm^3$ 10 × PCR buffer, 1.0 $mm^3$ 10 $mmol.L^{-1}$ dNTPs, 1.0 $mm^3$ 50$\mu mol.L^{-1}$ primer, 1.5 U Taq enzyme and 40 ng template under the following conditions: 95°C denaturation for 5 min, followed by 35 cycles of 94°C denaturation for 45 s, 56°C annealing for 45 s and 72°C extension for 45 s and a final extension at 72°C for 10 min (Wu et al., 2007; Yuan et al., 2008). The PCR products were fractionated on 1% agarose gel, and the gel images were obtained with the GelLogic 100 image system. The target fragments were isolated from the agarose gel under UV radiation, reclaimed and purified with the reagent kit (Tiangel midi purification kit), and then directly sequenced. Sequence analysis was performed with Dnaman, Garli and MEGA 5.05. Unweighted pair group method with arithmetic mean (UPGMA) was used to make cluster analysis by soft MEGA 5.05 (Felsenstein, 1989; Tamura et al., 2011).

## RESULTS

### Length and GC content of ITS sequences

The ITS lengths of 14 species were about 652 bp. The internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) sequences were about 235 and 254 bp, respectively. GC contents of *Lycoris* species changed slightly, of which ITS1 and ITS2 were 65.7 to 69.9% and 70.8 to 73.6%, respectively. The GC content of ITS2 sequences was larger than that of ITS1 sequences (Table 2).

### Genetic distance of *Liquors*

The genetic distances of 14 species in *Lycoris* were relatively small, from 0.001 to 0.066, their average was 0.04154. *Lycoris haywardii* and *Lycoris caldwellii*, *Lycoris sprengeri* and *Lycoris caldwellii* had the largest genetic distances with 0.066. *Lycoris longituba* and *Lycoris longituba* var. *flava* had the shortest genetic distance with 0.001(Table 3).

### Phylogenetic tree with bootstrap method

The phylogenetic tree constructed on the basis of the ITS sequences showed that 14 species were divided into three clades: clade I with *Lycoris longituba*, *Lycoris longituba* var. *flava*, *Lycoris anhuiensis, Lycoris aurea* and *Lycoris chinensis*; clade II with *Lycoris radiata*, *Lycoris haywardii*, *Lycoris rosea* and *Lycoris sprengeri;* clade III with *Lycoris caldwellii*, *Lycoris straminea*, *Lycoris houdyshelii*, *Lycoris incarnata* and *Lycoris squamigera* (Figure 1).

## DISCUSSION

ITS sequence had more informative sites and it was widely applied to the fields of intraspecific variation and interspecific relationships of plants in recent years. This study showed that the ITS sequences of 14 species of *Lycoris* were different from each other and they were divided into three branches (Figure 1), which were consistent with the analysis of chromosome number and karyotype. *L. chinensis*, *L. aurea*, *L. anhuiensis, L.*

**Table 2.** Length and GC content of ITS sequences.

| species | ITS | ITS1 | | ITS2 | |
|---|---|---|---|---|---|
| | Length | Length | GC content (%) | Length | GC content (%) |
| *Lycoris straminea* Lindl. | 652 | 235 | 66.8 | 254 | 72.0 |
| *Lycoris anhuiensis* Y. Hsu & Q. J. Fan | 653 | 235 | 68.7 | 254 | 72.4 |
| *Lycoris aurea* (L' Her.) Herb. | 653 | 235 | 68.5 | 255 | 73.0 |
| *Lycoris caldwellii* Traub. | 653 | 235 | 67.2 | 255 | 71.0 |
| *Lycoris chinensis* Traub. | 652 | 235 | 66.8 | 254 | 72.0 |
| *Lycoris haywardii* Traub. | 653 | 236 | 69.9 | 254 | 70.8 |
| *Lycoris houdyshelii* Traub. | 652 | 236 | 67.4 | 253 | 73.6 |
| *Lycoris incarnata* Comes ex C. Sprenger | 652 | 236 | 65.7 | 253 | 72.8 |
| *Lycoris longituba* Y. Hsu & Q. J. Fan | 653 | 235 | 68.1 | 255 | 72.2 |
| *Lycoris longituba* var. *flava* Y. Hsu & X. L. Huang | 653 | 236 | 68.3 | 254 | 72.0 |
| *Lycoris radiata* (L' Her.) Herb. | 653 | 235 | 68.9 | 255 | 72.2 |
| *Lycoris rosea* Traub & Moldenke | 653 | 236 | 69.9 | 254 | 70.8 |
| *Lycoris sprengeri* Comes ex Baker | 653 | 235 | 69.8 | 255 | 71.0 |
| *Lycoris squamigera* Maxim | 652 | 236 | 66.1 | 253 | 72.8 |

**Table 3.** The pairwise distance of *Lycoris.*

| Parameter | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. straminea* | 1.000 | | | | | | | | | | | | | |
| *L. anhuiensis* | 0.049 | 1.000 | | | | | | | | | | | | |
| *L. aurea* | 0.053 | 0.030 | 1.000 | | | | | | | | | | | |
| *L. caldwellii* | 0.019 | 0.056 | 0.057 | 1.000 | | | | | | | | | | |
| *L. chinensis* | 0.054 | 0.030 | 0.006 | 0.059 | 1.000 | | | | | | | | | |
| *L. haywardii* | 0.061 | 0.051 | 0.044 | 0.066 | 0.048 | 1.000 | | | | | | | | |
| *L. houdyshelii* | 0.015 | 0.051 | 0.049 | 0.019 | 0.051 | 0.056 | 1.000 | | | | | | | |
| *L. incarnata* | 0.017 | 0.052 | 0.054 | 0.022 | 0.056 | 0.063 | 0.017 | 1.000 | | | | | | |
| *L. longituba* | 0.049 | 0.006 | 0.023 | 0.056 | 0.023 | 0.048 | 0.051 | 0.052 | 1.000 | | | | | |
| *L.longituba* var. *flava* | 0.049 | 0.006 | 0.023 | 0.056 | 0.023 | 0.048 | 0.051 | 0.052 | 0.001 | 1.000 | | | | |
| *L. radiata* | 0.056 | 0.051 | 0.041 | 0.061 | 0.043 | 0.015 | 0.048 | 0.057 | 0.048 | 0.048 | 1.000 | | | |
| *L. rosea* | 0.056 | 0.049 | 0.043 | 0.064 | 0.044 | 0.005 | 0.054 | 0.058 | 0.046 | 0.046 | 0.017 | 1.000 | | |
| *L. sprengeri* | 0.058 | 0.048 | 0.043 | 0.066 | 0.044 | 0.006 | 0.056 | 0.059 | 0.044 | 0.044 | 0.019 | 0.002 | 1.000 | |
| *L. squamigera* | 0.014 | 0.052 | 0.054 | 0.022 | 0.056 | 0.059 | 0.014 | 0.003 | 0.052 | 0.052 | 0.054 | 0.054 | 0.056 | 1.000 |

*longituba* and *L. longituba* var. *flava* were classified into clade I with the same chromosome numbers and karyotype (6M+10T). They had leaves in spring except *L. aurea* which had leaves in autumn. The results were also consistent with the analysis of cytology (Deng and Zhou, 2005) and comparative anatomy (Zhou et al., 2006). *L. anhuiensis* and *L. longituba* had close genetic relationship, the evidences of morphology (Zhou et al., 2005) and RAPD markers (Zhang et al., 2002) supported it. *L. sprengeri*, *L. rosea*, *L. haywardii* and *L. radiata* were classified into clade II with the same chromosome number and karyotype (22ST), and had leaves in autumn. The flower color of *L. radiata* and *L. rosea* was red, that of *L. sprengeri* and *L. haywardii* was red and blue, and these were consistent with the analysis of morphology, cytology and molecular markers (Yuan et al., 2008; Kurita, 1987; Zhou et al., 2005).

*L. squamigera* (6M+10T+11ST), *L. incarnate* (4M+3T+22ST+1m), *L. houdyshelii* (3M+5T+22ST), *L. straminea* (3M+5T+11ST) and *L. caldwellii* (6M+10T+11ST) were classified into clade III, their karyotype was a mix of chromosomes with constrictions in median, terminal and subterminal region (M+T+ST), but their chromosome number had big variation. Hybridization in *Lycoris* is a very common phenomenon. Hybrid played a key role in the formation of *Lycoris* species (Liu and Hsu, 1990). Although, the origin and relationship of *Lycoris* species are not clear, seven diploid species among them were considered to be progenitors of the other species on the basis of

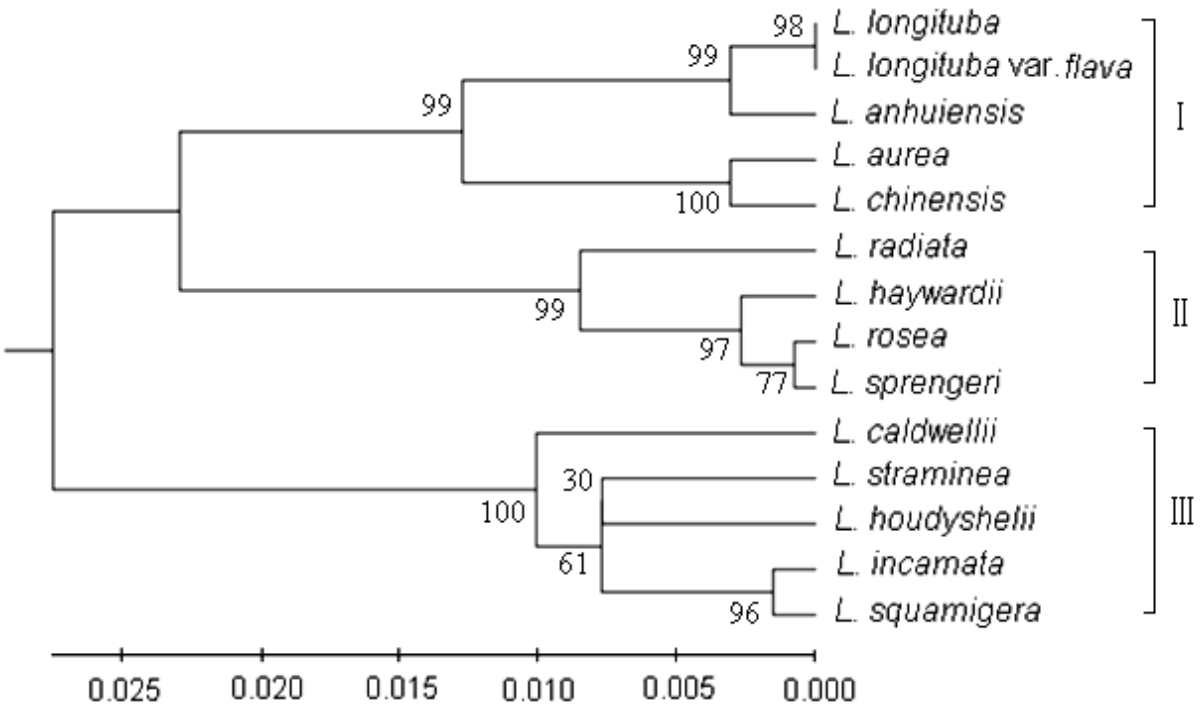


**Figure 1:** Phylogenetic tree of Lycoris based on ITS sequences with bootstrap method

cytological studies and hybridization results (Hsu et al., 1994; Ma et al., 2004). Bose (1963) and Kurita (1987) showed that the 2n=19 (such as *L. straminea* and *L. albiflora*) in *Lycoris* was diploid hybrids of 2n=16 and 2n=22, 2n=27 (such as *L. caldwellii* and *L. squamigera*) was hybrids of gametes not subtrahend of 2n=16 and normal gametes of 2n=22. From the results of this study, it is thus supposed that the species of clade III could be hybrids of diploid species of the other clades. The likelihood of hybrid origin of *Lycoris* species was supported.

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