Phylogenetic Relationships among *Lilium auratum* Lindley, *L. auratum* var. *platyphyllum* Baker and *L. rubellum* Baker Based on Three Spacer Regions in Chloroplast DNA

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We postulate the phylogenetic relationships of section *Archelirion* (genus *Lilium*) based on the nucleotide sequence variations of three spacer regions in chloroplast DNA, *trnT-trnL*, *trnL-trnF* and *atpB-rbcL*. The sequences aligned provided a matrix of 1,627 bp, and were analyzed phylogenetically by the maximum parsimony method. Section *Archelirion* was divided into two major clades, one consisting of *L. auratum* var. *auratum* and *L. rubellum*, and the other consisting of the rest of taxa in *Archelirion*. The cytoplasm of the former clade was regarded as the rubellum type (eastern type), and that of the latter clade as the japonicum type (western type). Each taxon was monophyletic, but two varieties of *L. auratum* were belonged to a different clade. The phylogeny of *L. auratum* var. *auratum* is different from that of *L. auratum* var. *platyphyllum*, and it has a sister-relationship with *L. rubellum*. These results provide a more convincing phylogenetic relationship within section *Archelirion*.

Key Words: Liliaceae, *Lilium*, *Archelirion*, *auratum*, *platyphyllum*, *rubellum*, phylogeny, chloroplast DNA.

Introduction

Lilium auratum Lindley is native to Japan, and it is an important genetic resource for Oriental hybrids, a major lily cultivar group. This lily has white out-facing flowers with large spots and a yellow central stripe, and it is an ornamental plant that is valuable itself. *L. auratum* has two major varieties, *auratum* and *platyphyllum*. The former is indigenous wild populations in northern to southwestern part of Honshu, Japan, and the latter is indigenous to Izu islands, stretching away in a southerly direction from the entrance to Tokyo Bay. Variety *platyphyllum* has larger leaves and flowers than variety *auratum*, and its flowers have modest spots. Variety *platyphyllum* was differentiated as a different

species, *L. platyphyllum* Makino, but it is generally classified as a variety of *L. auratum* at present.

L. rubellum Baker is indigenous in the northeastern part of Honshu. The flower with funnel shaped and pink petals is valuable itself as an ornamental plant, and it is also an important genetic resource for Oriental hybrids because it produces early-flowering offsprings when crossed with other Oriental lily species and hybrids (McRae 1998).

According to the classification by Comber (1949), who established the most authoritative classification, genus *Lilium* has seven sections; *Martagon*, *Pseudolirium*, *Liriotypus*, *Archelirion*, *Sinomartagon*, *Leucolirion* and *Dauroilirion*. *L. auratum* and *L. rubellum* were classified into section *Archelirion*, and this section included five other species, *L. speciosum*, *L. alexandrae*, *L. japonicum*, *L. nobilissimum* and *L. brownii*. However, *L. brownii* was polyphyletic to other *Archelirion* and had a sister relationship to subsection 6b (*Leucolirion*) in the phylogenetic trees based on sequences of the internal transcribed spacer region of nuclear ribosomal DNA (Nishikawa *et al.* 2001) and *matK* gene of chloroplast DNA (Nishikawa *et al.* 2000). Therefore, *L. brownii* should be classified into subsection 6b, and the other six species into the true section *Archelirion*.

This section *Archelirion* was monophyletic in trees based on both morphological characters (Lighty 1968, Asano 1986) and molecular biological analyses (Haruki *et al.* 1997, Nishikawa *et al.* 1999, 2000, Dubouzet and Shinoda 1999). However, the relationships within this section were different in each tree, and they were not even monophyletic in the tree based on the sequence data of *rbcL* and *matK* gene (Hayashi and Kawano 2000). Therefore, the relationships within section *Archelirion* are not clear in spite of their importance as genetic resources.

There are many different intergenic regions in the chloroplast genome that could potentially be used for phylogenetic inference at lower taxonomic levels (Soltis and Soltis 1998). In the phylogenetic analysis of *Lilium*, coding regions of chloroplast genome were appropriate to the intragenus level, but not to the intrasection level (Hayashi and Kawano 2000, Nishikawa *et al.* 2000). Therefore, intergenic spacer regions of chloroplast genome were expected to be appropriate for intrasection analysis of *Archelirion*. We analyzed three spacer regions, *trnT-trnL*, *trnL-trnF* and *atpB-rbcL*, in order to increase the number of informative mutations for

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raising the reliability of phylogenetic tree.

In the present study we investigated the relationships within section *Archelirion*, particularly two varieties of *L. auratum* and *L. rubellum* by phylogenetic analysis based on the sequence data of three intergenic spacer regions in chloroplast DNA, *trnT-trnL*, *trnL-trnF* and *atpB-rbcL*.

Materials and Methods

Plant Materials

Nine taxa of the section *Archelirion* were used (Table 1). *L. henryi* was used as an outgroup because it is classified into section *Leucolirion* (subsection 6a), the nearest neighbor of section *Archelirion* (Nishikawa *et al.* 1999). Four taxa were used plural individuals, and the 29 individuals were totally analyzed. Samples were collected from individuals growing in the wild except for commercial ones, which were sampled from the field of the National Institute of Agrobiological Sciences. Leaves were stored at -80°C until DNA extraction.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted by the same method as that used by Nishikawa *et al.* (1999), and used as template DNA for PCR. Three spacer regions of chloroplast DNA were selected for PCR as shown in Fig. 1. Every PCR was performed on a thermal cycler (MJ Research PTC-100) using the KOD Dash DNA polymerase (Toyobo, Japan) and following primer pairs: 5'-CAAATGCGATGCTCTAACCT-3' and 5'-AGTCCGTAGCGTCTACCGAT-3' for *trnT-trnL*, 5'-TCCGTCGACTTTATAAGTCGTG-3' and 5'-TGCCAGG AACCAGATTTGAACT-3' for *trnL-trnF*, and 5'-GAAGT AGTAGGATTGGTTCTCA-3' and 5'-CCAACACTTGCT TTAGTTTC-3' for *atpB-rbcL*. The primer pairs were designed on the basis of the sequence data of chloroplast DNA from rice (Hiratsuka *et al.* 1989). Each reaction mixture was 25 μl , containing 10 ng of genomic DNA, 2.5 μl of 10x buffer, 2.5 μl of dNTP mixture (a 2 mM concentration of each dNTP), 10 pmol of both primers, and 1.25 U of KOD Dash. The thermal cycler program was run as follows: Initial denaturation was for 2 min. at 94°C , followed by 30 cycles of 30 sec. at 94°C , 2 sec. at 50°C , and 90 sec.

Table 1. Taxonomic position and source of plant materials

No.	Taxa	Origin	DDBJ ¹⁾ accession number		
			<i>trnT-trnL</i>	<i>trnL-trnF</i>	<i>atpB-rbcL</i>
1	<i>Lilium alexandrae</i>	Kagoshima	AB064557	AB064567	AB064577
2	<i>L. auratum</i> var. <i>auratum</i>	Iwate (Shizukuishi)	AB064558	AB064568	AB064578
3		Ibaraki (Kanasago)	ditto	ditto	ditto
4		Yamanashi (Uenohara)	ditto	ditto	ditto
5		Niigata (Shitada)	ditto	ditto	ditto
6		Nagano (Miyota)	ditto	ditto	ditto
7		Aichi (Tsukude)	ditto	ditto	ditto
8		Mie (Iitaka)	ditto	ditto	ditto
9		Nara (Higashi-Yoshino)	#9 ²⁾	ditto	ditto
10	<i>L. auratum</i> var. <i>platyphyllum</i>	Tokyo (Hachijo)	AB064559	AB064569	AB064579
11		Tokyo (Hachijo)	ditto	ditto	ditto
12		Tokyo (Hachijo)	ditto	ditto	ditto
13		Tokyo (Aogashima)	ditto	ditto	ditto
14		Tokyo (Aogashima)	ditto	ditto	ditto
15		Tokyo (Aogashima)	ditto	ditto	ditto
16		Commercial	ditto	ditto	ditto
17	<i>L. japonicum</i> var. <i>japonicum</i>	Toyama	AB064560	AB064570	AB064580
18		Toyama	ditto	ditto	ditto
19	<i>L. japonicum</i> var. <i>abeanum</i>	Commercial	AB064561	AB064571	AB064581
20	<i>L. nobilissimum</i>	Commercial	AB064562	AB064572	AB064582
21	<i>L. rubellum</i>	Yamagata (Oguni)	AB064563	AB064573	AB064583
22		Fukushima (Atsushio-kano)	ditto	ditto	ditto
23		Fukushima (Fukushima)	#18, #32 ³⁾	ditto	ditto
24		Fukushima (Nango)	#29, #32 ³⁾	ditto	ditto
25		Niigata (Shibata)	AB064563	ditto	ditto
26		Niigata (Shitada)	#32 ³⁾	ditto	ditto
27	<i>L. speciosum</i> var. <i>speciosum</i>	Kagoshima	AB064564	AB064574	AB064584
28	<i>L. speciosum</i> var. <i>clivorum</i>	Commercial	AB064565	AB064575	AB064585
	<i>L. henryi</i> (Outgroup)	Commercial	AB064566	AB064576	AB064586

¹⁾ DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>)

²⁾ The number shows mutation site in Table 2 against AB064558.

³⁾ The numbers show mutation sites in Table 2 against AB064563.

Fig. 1. Schematic representation of three spacer regions of chloroplast DNA sequenced in this study. IR shows inverted repeat.

at 74°C. The reaction mixture was purified with Suprec-02 (Takara, Japan), and resuspended in 50 µl of TE [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA].

DNA Sequencing

The purified DNA solution was cycle sequenced in both directions with the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, USA) on the Model 373S automated sequencer (Applied Biosystems, USA). The reaction mixture contained 50 ng of template DNA, 4.5 µl of sterile water, 4 µl of Terminator Premix, 0.5 µl of primers (3.2 µM). Primers for cycle sequencing were the same as described above. The thermal cycler program was run for 25 cycles (96°C, 10 sec.; 50°C, 5 sec.; 60°C, 4 min.).

Phylogenetic Analysis

Three kinds of sequences of all samples were aligned using Clustal X version 1.81 (Thompson *et al.* 1997). The total of 1,627 bp, which consisted of 816 bp nucleotides in *trnT-trnL*, 176 bp in *trnL-trnF*, 635 bp in *atpB-rbcL* were used for the following analysis. Phylogenetic analysis was performed by the maximum parsimony method using PAUP* version 4.0b8 (Swofford 1998). The most parsimonious tree was obtained using the heuristic search option involving 100 replications of random addition sequence and tree-bisection-reconnection (TBR) branch-swapping. All characters were specified as unweighted. Bootstrap analysis (Felsenstein 1985) was conducted to evaluate branching orders. One thousand replications were calculated using the heuristic search option with TBR branch-swapping and simple sequence addition sequence algorithms.

Results

Sequences of three spacer regions of chloroplast DNA for 10 taxa were obtained and registered in the DDBJ (DNA Data Bank of Japan) DNA database (Table 1). Sequences were identical among the same taxon except for *trnT-trnL* of *L. auratum* var. *auratum* and *L. rubellum*.

Table 2 shows the mutations in the three spacer regions of chloroplast DNA in section *Archelirion*. The alignment of the total sequences in the three regions provided a matrix of 1,627 bp, which consisted of 816 bp in *trnT-trnL*, 176 bp in *trnL-trnF* and 635 bp in *atpB-rbcL*. *TrnT-trnL* was the most variable among three spacer regions (36 sites), but eight sites, #21, #22 and #30 to #35 in *trnT-trnL*, were excluded from the following analyses because of their equivocal alignment. Therefore, 44 mutations were analyzed in total, and 32 were phylogenetically informative.

In section *Archelirion*, the most parsimonious tree was divided into two major clades (Fig. 2), designated as follows: Group I comprised *L. auratum* var. *auratum* and *L. rubellum*; Group II comprised other 7 taxa. All taxa were clearly monophyletic, but two varieties of *L. auratum* clearly separated into different clades. Var. *auratum* was more closely related to *L. rubellum* than var. *platyphyllum*. In 23 mutation sites used for phylogenetic analysis, var. *auratum* corresponded to *L. rubellum* at 17 sites, but to var. *platyphyllum* at only 3 sites (Table 3).

Discussion

In this study, the most interesting point is the distance between the two varieties of *L. auratum*. Surprisingly, the two varieties were clearly separated in section *Archelirion* (Fig. 2), and *L. auratum* var. *auratum* was closely related to *L. rubellum* rather than *L. auratum* var. *platyphyllum* (Table 3). *L. auratum* var. *platyphyllum* is classified into a variety of *L. auratum* even though it was formerly regarded as a distinct species, *L. platyphyllum* Makino (Makino 1914). *L. auratum* var. *platyphyllum* and *L. auratum* var. *auratum* have many morphological characters in common such as flower shape, and the two are distinguished in several morphological characters such as leaf shape and modest spots on flowers. Then, is var. *platyphyllum* closely related to the type, var. *auratum*?

In other phylogenetic studies based on DNA variation, the relationship of the two varieties was unclear because of lack of var. *auratum* (Hayashi and Kawano 2000) or var. *platyphyllum* (Dubouzet and Shinoda 1999), and low reliable divergence within the clade of *Archelirion* (Nishikawa *et al.* 1999, 2000). Only two studies using *rbcL* (Haruki *et al.* 1997) and *matK* (Nishikawa *et al.* (not published)) showed the relationship of the two varieties, and suggested a polyphyletic relationship between the two varieties of *L. auratum*. However, they did not consider the diversity of each taxon. We analyzed eight individuals of var. *auratum*, seven var. *platyphyllum* and six *L. rubellum* for estimation of their

Table 2. Mutations in the three spacer regions of chloroplast DNA in the section *Archelirion*

Regions	Aligned length [sequence range] (bp)	GC content [range] (%)	Mutation			
			Code	Position	Type ¹⁾	Accession ²⁾
<i>trnT-trnL</i>	816 [756-798]	22.45 [21.71-22.88]	#1	11 bp downstream <i>trnT</i>	Transversion	1
			#2	17 bp downstream <i>trnT</i>	Transversion	17, 18
			#3	97 bp downstream <i>trnT</i>	Deletion	2-9
			#4	130 bp downstream <i>trnT</i>	Insertion	2-9, 21-26
			#5	137 bp downstream <i>trnT</i>	Transversion	10-16
			#6	141 bp downstream <i>trnT</i>	Transversion	1
			#7	169 bp downstream <i>trnT</i>	Transversion	2-9, 21-26
			#8	203 bp downstream <i>trnT</i>	Transversion	28
			#9	215 bp downstream <i>trnT</i>	Transversion	9, 17, 18, 20
			#10	246 bp downstream <i>trnT</i>	Insertion	1
			#11	249 bp downstream <i>trnT</i>	Insertion	19
			#12	258 bp downstream <i>trnT</i>	Deletion	2-9, 21-26
			#13	305 bp downstream <i>trnT</i>	Deletion	1
			#14	313 bp downstream <i>trnT</i>	Transversion	2-9, 21-26
			#15	319 bp downstream <i>trnT</i>	Transversion	2-9, 21-26
			#16	320 bp downstream <i>trnT</i>	Transversion	1-16, 21-26, 28
			#17	337 bp downstream <i>trnT</i>	Insertion	21-26
			#18	341 bp downstream <i>trnT</i>	Transversion	23
			#19	384 bp downstream <i>trnT</i>	Insertion	21-26, 28
			#20	391 bp downstream <i>trnT</i>	Insertion	19
			#21	406 bp downstream <i>trnT</i>	Insertion	21-26
			#22	410 bp upstream <i>trnL</i>	Deletion	2-9
			#23	392 bp upstream <i>trnL</i>	Transversion	1-18, 20-26, 28
			#24	385 bp upstream <i>trnL</i>	Transversion	17, 18
			#25	371 bp upstream <i>trnL</i>	Transition	1-9, 21-26
			#26	363 bp upstream <i>trnL</i>	Transversion	1-16, 21-26, 28
			#27	353 bp upstream <i>trnL</i>	Insertion	10-16
			#28	347 bp upstream <i>trnL</i>	Insertion	2-9, 21-26
			#29	341 bp upstream <i>trnL</i>	Transition	24
			#30	332 bp upstream <i>trnL</i>	Insertion	1-9, 21-26
			#31	330 bp upstream <i>trnL</i>	Insertion	1, 21-26
			#32	329 bp upstream <i>trnL</i>	Insertion	1, 21, 22, 25
			#33	328 bp upstream <i>trnL</i>	Insertion	1
			#34	135 bp upstream <i>trnL</i>	Deletion	2-9, 20-26
			#35	134 bp upstream <i>trnL</i>	Insertion	1
			#36	110 bp upstream <i>trnL</i>	Insertion	17, 18
<i>trnL-trnF</i>	176 [175-176]	27.33 [25.71-27.43]	#1	80 bp downstream <i>trnL</i>	Transition	2-9, 21-26
			#2	46 bp upstream <i>trnF</i>	Insertion	2-9, 21-26
			#3	40 bp upstream <i>trnF</i>	Transition	2-9
<i>atpB-rbcL</i>	635 [625-628]	25.71 [25.60-25.84]	#1	34 bp downstream <i>atpB</i>	Transversion	17-19
			#2	42 bp downstream <i>atpB</i>	Deletion	1-9, 21-26
			#3	101 bp downstream <i>atpB</i>	Transversion	2-9, 21-26
			#4	145 bp downstream <i>atpB</i>	Insertion	2-9, 21-26
			#5	175 bp downstream <i>atpB</i>	Transversion	2-9
			#6	205 bp downstream <i>atpB</i>	Deletion	2-9, 21-26
			#7	236 bp downstream <i>atpB</i>	Transition	2-9, 21-26
			#8	308 bp downstream <i>atpB</i>	Transversion	1
			#9	296 bp upstream <i>rbcL</i>	Transversion	2-9, 21-26
			#10	281 bp upstream <i>rbcL</i>	Insertion	20
			#11	174 bp upstream <i>rbcL</i>	Insertion	21-26
			#12	141 bp upstream <i>rbcL</i>	Transversion	1-26, 28
			#13	123 bp upstream <i>rbcL</i>	Transversion	2-9, 21-26

¹⁾ The mutation types are considered by contrast with *L. speciosum* var. *speciosum* in each position.²⁾ The number shows mutating accessions listed in Table 1.

Fig. 2. Phylogenetic tree of section *Archelirion* based on sequence data of three chloroplast DNA regions using the maximum parsimony method. The tree is strict consensus of the eight most parsimonious trees. Tree length=58, Consistency index (CI)=0.8966, Retention index (RI)=0.9791, Rescaled consistency index (RC)=0.8778. The numbers above the nodes represent bootstrap value expressed as percentage of 1,000 bootstrap replications. Each box shows similarity of cytoplasm.

diversity. Although *L. auratum* var. *auratum* has diverse morphological characters regionally and environmentally (e.g. leaf length and leaf width) (Shimizu 1987), it had little diversity of chloroplast DNA. Each taxon was monophyletic, and no individuals of the two varieties of *L. auratum* showed a close relationship to any other variety (Fig. 2). Therefore, their diversity is too small to overlap with that of other taxa.

Noda (1987) reviewed the relationships among genus *Lilium* originated in Japan based on cytological studies. He estimated that three taxa, *L. auratum* var. *auratum*, *L. japonicum* and *L. rubellum*, were closely related because of the presence of common nucleolar chromosomes in the standard karyotype and B-chromosome. *L. rubellum* particularly had all nucleolar chromosomes jointly with *L. auratum* var. *auratum*, although one chromosome was distinctive. On the other hand, *L. auratum* var. *platyphyllum* had two out of four nucleolar chromosomes jointly and no B-chromosome. Therefore, it is appropriate that *L. auratum* var. *auratum* was more distinct to var. *platyphyllum* than to *L. rubellum* based

on cytological studies.

According to the above-mentioned circumstances, it is appropriate that the phylogeny of *L. auratum* var. *auratum* is different from that of *L. auratum* var. *platyphyllum*, and *L. auratum* var. *auratum* has a sister-relationship with *L. rubellum*. Although introgression may have happened between the two varieties of *L. auratum* because of the high cross compatibility shown by Shimizu (1971), most of their morphological similarity owes to inheritance from a common ancestor, and the rest probably owes to homoplasy.

The relation between *L. rubellum* and *L. japonicum* is similar to that between the two varieties of *L. auratum*. *L. rubellum* closely resembles *L. japonicum* in morphological characters (Asano 1986), and Shimizu (1987) estimated that *L. rubellum* was originated from *L. japonicum* adapted to the snowy area at the northeastern end of the distribution. However, *L. rubellum* was more closely related to *L. auratum* var. *auratum* than to *L. japonicum* in the present study (Fig. 2), and the karyotype of *L. rubellum* was similar to that of *L. auratum* var. *auratum* as well as that of *L. japonicum* (Noda

Table 3. Bases at the 28 mutation sites between *L. auratum* and *L. rubellum*

Species	Mutation sites												
	<i>trnT-trnL</i>												
	#3	#4	#5	#7	#12	#14	#15		#17		#19	#27	#28
<i>L. auratum</i> var. <i>auratum</i>	-----	GTAT	T	A	—	T	G		-----		—	—	T
<i>L. auratum</i> var. <i>platyphyllum</i>	ATTTATTATT	---	A	T	A	G	T		-----		—	T	—
<i>L. rubellum</i>	ATTTATTATT	GTAT	T	A	—	T	G	TATAMTATAATGTATATAATA			T	—	T

Species	Mutation sites												
	<i>trnL-trnF</i>			<i>atpB-rbcL</i>									
	#1	#2	#3	#2	#3	#4	#5	#6	#7	#9	#11	#13	
<i>L. auratum</i> var. <i>auratum</i>	T	T	G	—	C	TTCAAT	T	-----	C	G	—	T	
<i>L. auratum</i> var. <i>platyphyllum</i>	C	—	A	G	A	-----	G	CTTTTTT	T	C	—	G	
<i>L. rubellum</i>	T	T	A	—	C	TTCAAT	G	-----	C	G	A	T	

1987). Therefore, it is probable that the nearest neighbor of *L. rubellum* is not *L. japonicum* but *L. auratum* var. *auratum*. Of course more investigation is necessary for the diversity of *L. japonicum* because of their wide distribution and morphological variation. However, the diversity of *L. japonicum* will not greatly exceed that between the two varieties, var. *japonicum* and var. *abeanum* because they showed close relationship (Fig. 2).

Consequently, section *Archelirion* was separated into two major groups phylogenetically as indicated in Fig. 2. We call them here rubellum type and japonicum type. The former consists of *L. auratum* var. *auratum* and *L. rubellum*, and the latter consists of the other members of section *Archelirion*. Based on their distribution, they are also regarded as eastern type (rubellum) and western type (japonicum), while the two types overlap in central Honshu (Fig. 3). Three stories can be hypothesized in the phylogeny of section *Archelirion*: (1) the two types were divergent from proto-*Archelirion*, (2) the rubellum type was divergent from the japonicum type, (3) the japonicum type was divergent from the rubellum type. Under the hypothesis of Noda (1987), hypothesis (2) is probable because he estimated that section *Archelirion* was developed from *L. speciosum*. However, (1) is actually probable because his "*L. speciosum*" might mean proto-*speciosum*, and current *L. speciosum* is too divergent to be regarded as an ancestor of section *Archelirion*. The rubellum type has a large difference from the japonicum type in chloroplast DNA, so that this type might be divergent in early times after the proto-*Archelirion* was formed. Or the difference may be due to the bottleneck effect caused by the glacial period, i.e., relict *Archelirion* distributed in eastern area throughout glacial have spread over northeastern Japan in postglacial like beech (Tsukada 1982).

The nucleotide sequence variations of three spacer regions in chloroplast DNA enabled to determine more convincingly the phylogenetic relationships in section *Archelirion*, particularly in three taxa, *L. auratum* var. *auratum*, *L. auratum* var. *platyphyllum* and *L. rubellum*. It will provide breeders with useful information for using genus *Lilium* as a genetic resource. Since our hypothesis also re-

Fig. 3. Distribution of two cytoplasm types in section *Archelirion*. This is a modification of the map of Shimizu (1987).

flects the evolutionary process, it is likely to contribute to the improvement of the *Lilium* phylogeny.

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