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Phylogenetic analysis of the internal transcribed spacer region of Japanese *Lilium* species

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Abstract The DNA from 16 *Lilium* species and one variety endemic to or naturalized in Japan were obtained and their internal transcribed spacer regions of nuclear ribosomal DNA (nrDNA) were amplified by PCR and sequenced by cycle sequencing. Phylogenetic analysis of the ITS sequences supported the validity of Comber's classification system. It has also provided molecular evidence for the transfer of *Lilium dauricum* to sect. *Sinomartagon*. The phylogenetic relationships revealed by ITS DNA analysis were supported by previously published crossability data. The molecular phylogeny of Japanese *Lilium* species was discussed with reference to the putative migration routes of these species.

Key words *Lilium* · Molecular systematics · ITS DNA sequences · Phylogeny

Introduction

There are more than 90 *Lilium* species distributed all over the northern hemisphere and 13–15 species occur in Japan (Asano 1986). Only six species are unique to Japan, while the rest of the endemic species are also found in neighboring China, Taiwan, Korea or Russia (Okazaki 1996).

Lilies have long been treasured in Japan for their ornamental and food values (Shimizu 1969). Hence, selection work in lilies was started in Japan several centuries ago. Japanese lilies have subsequently played a major role in the development of important cultivars classified under Easter lilies, Asiatic hybrids and Oriental hybrids (Okazaki 1996).

Comber (1949) used classical taxonomic methods based on 15 morphological characters to establish the most widely accepted classification of the genus *Lilium*. Recent research used more detailed floral or vegetative characteristics (Baranova 1988), numerical analysis of morphological characters (Asano 1986), or C-banding of chromosomes (Smyth et al. 1989) to provide a greater resolution of some heterogeneous groups in Comber's classification.

In *Allium* subg. *Melanocrommyum*, Dubouzet and Shinoda (1998 b) demonstrated the general concordance between a phylogeny derived from ITS sequence analysis and a classification system based on morphological, chemical, serological and cytological data. In the present report, we discuss the relationships among Japanese *Lilium* species according to a phylogenetic analysis of the DNA sequences found in the internal transcribed spacer (ITS) region of the nuclear genome.

Materials and methods

DNA was extracted from the species listed in Table 1. DNA purification, PCR amplification of the ITS region, cycle sequencing and phylogenetic analysis followed the procedures described by Dubouzet and Shinoda (1998 b). Forward and reverse sequencing reactions were performed at least twice for each taxon using the primers ITSL (Hsiao et al. 1994) and ITS4, respectively. The internal primers ITS2 and ITS3 were used to improve resolution near the priming sites for ITSL and ITS4. The sequences for ITS2, 3 and 4 were obtained from T. Brun's homepage (plantbio. berkeley. edu/~bruns/primers.html). The sequences generated in the present study were submitted to GenBank (NCBI, USA) under GenBank accession numbers AF074466–AF074479, AF088193, AF088200 and AF088204.

SEQPUP v 0.6 (D. Gilbert of Indiana University, USA) was used to generate the reverse complement sequence of the reverse-primed

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Table 1 Taxonomic classification and natural distribution of some *Lilium* species endemic to or naturalized in Japan. Entries are currently maintained at the Lily Park (Sapporo, Japan)

Species names[c]	Classification system				Distribution
	Wilson (1925)	Comber (1949)	Haw (1986)	Baranova (1988)	
alexandrae hort. Wallace	*Leucolirion*		*Regalia*	*Archelirion*	Kagoshima (southern Kyushu)[c]
auratum Lindl.	*Archelirion*	*Archelirion*	*Archelirion*	*Archelirion*	Central to northern Honshu[c]
callosum Sieb. & Zucc.	*Martagon*	*Sinomartagon*	*Sinomartagon*	*Nepalensia*	Fukuoka (northern Kyushu) to Okinawa[3]; China, Korea, Taiwan, Russia[a]
concolor Salisb.	*Pseudolirium*	*Sinomartagon*	*Asteridium*	*Sinolirium*	Aomori (northern Honshu) to Miyazaki (central Kyushu)[c]; NE Asia[a]
dauricum Ker-Gawl.	*Pseudolirium*	*Daurolirion*	*Pseudolirium*	*Pseudolirium*	Hokkaido to Aomori[c]; NE Asia[a]
formosanum Wallace	*Leucolirion*	*Leucolirion*	*Regalia*	*Regalia*	Taiwan
hansonii Leichtl.	*Martagon*	*Martagon*	*Martagon*	*Martagon*	E Russia, Korea and Japan[a]; naturalized in Hokkaido[d].
japonicum Thunb.	*Leucolirion*	*Archelirion*	*Regalia*	*Archelirion*	Niigata (northern Honshu) to Miyazaki[c]
lancifolium Thunb.	*Martagon*	*Sinomartagon*	*Sinomartagon*	*Sinomartagon*	Nagasaki (northwestern Kyushu)[c], Cheju Island (Korea)[b]; E China[a]
lancifolium Thunb. var. *flore-pleno* Regel	*Martagon*				Japan
leichtlinni Hook. f. var. *maximowiczii* (Regel) Baker	*Martagon*	*Sinomartagon*	*Sinomartagon*	*Sinomartagon*	Aomori to Kagoshima[c]; Central Korea[a]
longiflorum Thunb.	*Leucolirion*	*Leucolirion*	*Regalia*	*Regalia*	Kagoshima to Okinawa[c]; Taiwan[b]
maculatum Thunb.	*Pseudolirium*		*Pseudolirium*	*Pseudolirium*	Aomori to Shizuoka (central Honshu)[c]
medeoloides A. Gray	*Martagon*	*Martagon*	*Martagon*	*Martagon*	Hokkaido to Shikoku[3]; China, S Korea, Russia[a]
nobilissimum Mak.	*Leucolirion*	*Archelirion*	*Regalia*	*Archelirion*	Kagoshima[c]
rubellum Baker	*Leucolirion*	*Archelirion*	*Regalia*	*Archelirion*	Niigata to Miyagi (northern Honshu)[c]
speciosum Thunb.	*Martagon*	*Archelirion*	*Archelirion*	*Archelirion*	Shikoku to Kagoshima[c]; China and Taiwan[b]

[a] Bryan and Griffiths (1995)
[b] Okazaki (1996)
[c] Shimizu (1987)
[d] Wilson (1925)

reactions and to align the sequences of each treatment replication both within species and among species. Neighbor-Joining analysis and bootstrapping of the aligned sequences were performed using CLUSTAL W (Thompson et al. 1994). The genetic distances in Table 2 were calculated by the DNADIST program in PHYLIP v 3.5 using the Kimura 2-parameter model of base substitution (Felsenstein 1993). UPGM analysis of the genetic distances was also performed using the NEIGHBOR program in PHYLIP. The dendrograms in Figs. 1 A and B were illustrated by NJPLOT (M. Gouy, University of Lyon, France). The sequence lengths, % GC content and the number of base differences were calculated by SITES v 1 (Hey and Wakeley 1997). These software programs are freely available in the Internet.

Results

PCR amplification of the ITS region generated an approximately 650-bp band whose intensity varied among species. DNA sequencing of purified PCR products was easy, except for *Lillium longiflorum* and the species in the section *Archelirion* which generated electropherograms muddled by overlapping peaks. In most of these problem cases, a large portion of the reverse sequencing reaction was unreadable, indicating: (1) the existence of ITS4 priming sites in non-homologous sequences, and/or (2) non-specific priming by ITS4 due to poor homology. The ITSL-primed forward reactions of *L. longiflorum* were also cluttered with multiple peak overlaps. Peak overlaps were resolved by (1) multiple replication, (2) visual inspection of the electropherograms, (3) sequencing using the internal primers ITS2 and ITS3, and/or (4) excision from agarose gels and subsequent purification of the amplification product.

The ITS sequences were 626-bp long, except for species classified in sect. *Archelirion* which were 632-bp long. After the introduction of six indels, the aligned ITS sequences were 632-bp long, and the length of the ITS1, 5.8S and ITS2 regions were 230, 164 and 238 bases, respectively. There were 70 ($11.1\% = 100 \times 70/632$) informative sites in the ITS region. The transition/transversion ratio was 1.83 (66/36). The mean GC content was 61.4 ± 0.96. One indel in ITS1 and five in ITS2 were inferred to obtain full alignment.

Discussion

Non-homologous ITS paralogues

ITS sequences generated from half of the species were generally clutter-free and easily aligned and resolved. As mentioned above, resolving the ITS sequences of species classified under sect. *Archelirion* was very difficult due to a high frequency of peak overlaps in the reverse sequencing reaction with ITS4. This phenomenon can be due to the presence of non-homologous sequences that also contain regions complementary to ITS4. We had more serious problems with *L. longiflorum* since both ITSL- and ITS4-primed reactions generated cluttered sequences. These non-homologous sequences are probably divergent paralogues that have mutated rapidly after de-activation and release from selection pressure (Buckler et al. 1997).

The almost complete peak overlap shown by the ITSL-primed reactions (*L. longiflorum*) and the ITS4-primed reactions (*L. longiflorum* and sect. *Archelirion*) indicate extensive mutation of the paralogues rather than incomplete homogenization by concerted evolution. Incomplete homogenization as a probable cause of these heterogeneous sequences can be ruled out since it is expected to show only a few overlapping peaks at the relatively few polymorphic sites in the *Lilium* ITS region, instead of almost completely muddled sequences. Multiple random deletions in (at least two) different paralogues in a single sequencing reaction can lead to the production of confused sequences.

Sequences from each species in sect. *Archelirion* were only clarified by aligning several sequences generated by the internal primer ITS3 along with those generated by ITSL and ITS4. Gel excision followed by purification of the ITS band of *L. longiflorum* enabled the generation of readable sequences from ITSL-primed forward reactions. However, only ITS3-primed reactions produced intelligible sequences from the 3′ portion of the ITS region.

ITS phylogeny and the classification of Japanese *Lilium* species

Of the 17 entries in Table 1, seven are found only in Japan, viz. *L. alexandrae*, *L. auratum*, *L. lancifolium* var. *flore-pleno*, *L. japonicum*, *L. maculatum*, *L. nobilissimum* and *L. rubellum*. In this report, sect. *Archelirion* consists exclusively of Japanese species. The rest of the species are also found in neighboring China, Korea and/or Russia. *L. callosum*, *L. dauricum* and *L. medeloides* are the most widely distributed of these species. *L. formosanum*, a species from Taiwan, was introduced into Japan in the 1920s; it has escaped from cultivation and is currently distributed all over Japan.

Wilson (1925) classified the lilies of eastern Asia (including the Japanese species) into four main sections, viz. *Leucolirion*, *Archelirion*, *Martagon* and *Pseudolirium* (Table 1). Subsequently, Comber (1949) redistributed the Japanese lilies into five sections, eliminating the Asian species from sect. *Pseudolirium*, and transferring *L. dauricum* to sect. *Daurolirium* and *L. concolor* to sect. *Sinomartagon*. He also transferred *L. japonicum*, *L. nobilissimum*, *L. rubellum* and *L. speciosum* to sect. *Archelirion*. Although recent researchers proposed more sections (Haw 1986), and even subsections (Baranova 1988), based on more detailed morphological analysis, Comber's classification is still the most widely accepted.

In Fig. 1 A and B, the *Lilium* species are grouped in clusters corresponding to the sections *Martagon*,

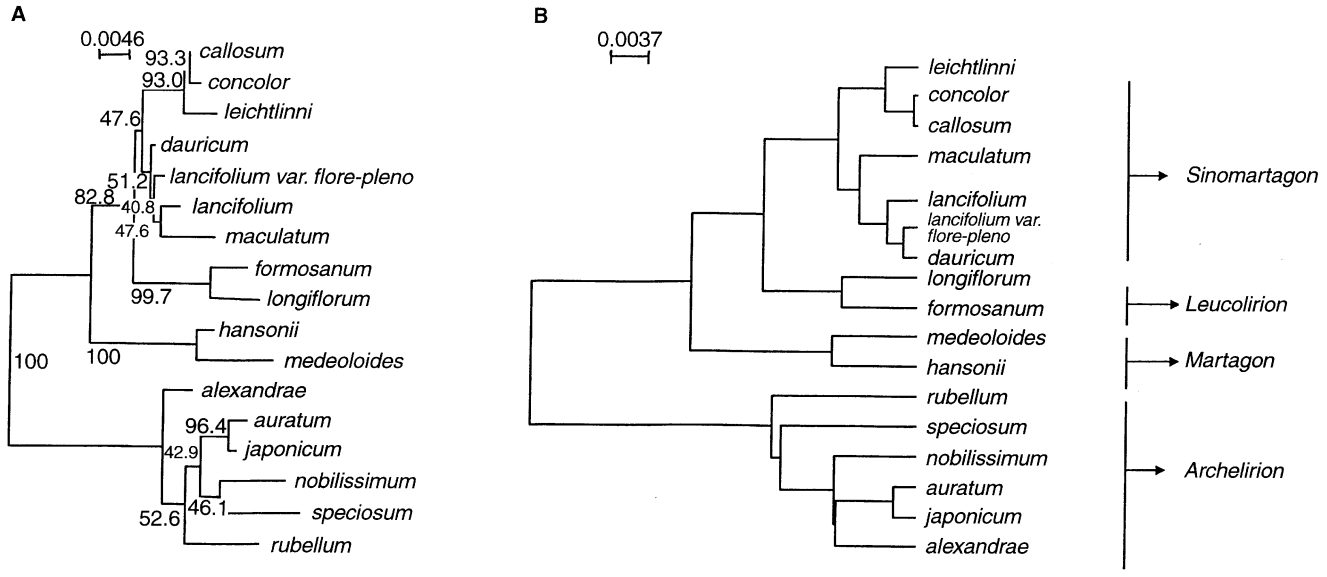

Fig. 1A, B Phylogeny of Japanese *Lilium* species according to **A** Neighbor-Joining analysis and bootstrapping of ITS DNA sequences generated by CLUSTAL W after gap exclusion and correction for multiple substitutions, and **B** NEIGHBOR's implementation of Unweighted pair group mean analysis of the Kimura 2-parameter genetic distances calculated by DNADIST (PHYLIP 3.5). Treefiles are illustrated by NJPLOT. Whole numbers represent bootstrap values in percent

Leucolirion, *Sinomartagon* and *Archelirion* as defined by Comber (1949). The consonance between Figs. 1 A and B as well as the bootstrap values indicate the reliability of the overall topology. The concordance between our results and Comber's system is quite remarkable in view of the fact that Comber analysed only 15 qualitative morpho-physiological characters. Asano (1986) used numerical analysis to evaluate 53 multi-state morpho-physiological and chemical characters and came up with a topology similar to Fig. 1 B.

The major discordant species is *L. dauricum*, which Comber separated in the monotypic sect. *Daurolirion* because it had a set of taxonomically important traits that were found in three disparate sections (*Martagon*, *Pseudolirium* and *Sinomartagon*). He did point out that the majority of taxonomic traits shown by *L. dauricum* are also found in sect. *Sinomartagon*. In 1960, Lighty (as cited by De Jong 1974) rejected sect. *Daurolirion* and returned *L. dauricum* to sect. *Sinomartagon*. Smyth et al. (1989) also commented that *L. dauricum* fits readily into Comber's *Sinomartagon* if less importance is attached to their hypogeal germination (epigeal in *Sinomartagon*) and the jointed bulb scales of *L. dauricum* (entire in *Sinomartagon*).

Asano (1986) showed a dendrogram that lumped *L. dauricum* with the Japanese species classified in sect. *Sinomartagon*. He further stated that *L. dauricum* is highly crossable with the *L. tigrinum* (syn. *L. lancifolium*) group (a) of Comber's *Sinomartagon*. Figure 1 A also shows the close relationship between *L. dauricum*, *L. maculatum* and *L. lancifolium*. The number of base differences and the corresponding genetic-distance values between *L. dauricum* and the other members of sect. *Sinomartagon* are also low (Table 2).

L. maculatum is unique to Japan and is found just outside the natural range of *L. dauricum* in Japan. These two species are believed to be the parents of the natural hybrid, *L.* × *elegans* (Shimizu 1969). As discussed above, *L. dauricum*, and consequently *L. maculatum*, belongs to the sect. *Sinomartagon*.

L. lancifolium var. *flore-pleno* has the most divergent flower shape among the entries. This 'monstrosity', according to Wilson (1925), was obtained from a shipment originating from Japan. As far as we know, there are no records of its natural distribution in Japan. Hence this triploid (Shimizu 1969) is a possible spontaneous mutant that is clearly related to *L. lancifolium* (Fig. 1).

Haw (1986) used Baranova's original definition of sect. *Regalia* which included the Japanese species Comber classified in section *Archelirion*. Subsequently, Baranova (1988) re-defined sect. *Regalia* and transferred these Japanese species back to sect. *Archelirion*. This section is clearly distinct from sect. *Leucolirion* (see Fig. 1). Comber did not include *L. alexandrae* in his classification of the genus (Table 1). This species is clustered with other species in sect. *Archelirion*.

Comparison of the more recent classifications by Haw and Baranova (Table 1) with the distribution of species in Fig. 1 indicates that the establishment of the sections *Asteridium* (Haw 1986), *Nepalensia* and *Pseudolirium* (Baranova 1988) unnecessarily broke up the natural (genetically based) grouping of these species in Comber's sect. *Sinomartagon*.

Table 2 Relationships among *Lilium* species according to the genetic distances calculated using the Kimura two-parameter model of base substitution (decimal fractions above the diagonal) and the number of base differences in the ITS region (below the diagonal)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *alexandrae*		0.02	0.05	0.05	0.05	0.06	0.07	0.06	0.06	0.06	0.07	0.01	0.07	0.07	0.02	0.03	0.02
2 *auratum*	10		0.06	0.06	0.06	0.07	0.08	0.07	0.07	0.07	0.08	0.00	0.07	0.08	0.02	0.02	0.03
3 *callosum*	32	38		0.00	0.01	0.04	0.03	0.01	0.01	0.01	0.03	0.06	0.02	0.05	0.07	0.07	0.07
4 *concolor*	32	38	1		0.01	0.04	0.03	0.01	0.01	0.01	0.03	0.06	0.02	0.05	0.07	0.07	0.07
5 *dauricum*	32	38	6	7		0.03	0.02	0.01	0.02	0.00	0.02	0.06	0.01	0.04	0.07	0.07	0.07
6 *hansonii*	38	42	23	24	18		0.04	0.03	0.04	0.03	0.05	0.07	0.03	0.02	0.08	0.07	0.08
7 *formosanum*	40	48	19	20	13	27		0.03	0.03	0.02	0.01	0.08	0.03	0.06	0.08	0.08	0.09
8 *lancifolium*	36	42	7	8	4	20	16		0.02	0.01	0.03	0.07	0.01	0.04	0.08	0.07	0.08
9 *leichtlinni*	36	42	4	5	10	25	19	10		0.02	0.03	0.07	0.03	0.05	0.08	0.07	0.08
10 *lancifolium* var. *flore-pleno*	34	39	7	8	2	19	15	4	11		0.02	0.06	0.01	0.04	0.07	0.07	0.08
11 *longiflorum*	44	49	20	21	14	28	9	17	20	15		0.08	0.03	0.06	0.09	0.09	0.09
12 *japonicum*	9	3	37	37	37	41	47	41	41	38	48		0.07	0.08	0.01	0.02	0.03
13 *maculatum*	39	43	12	13	7	21	20	7	16	7	21	42		0.05	0.08	0.08	0.08
14 *medeoloides*	44	50	30	31	24	10	33	25	30	26	34	49	28		0.09	0.09	0.09
15 *nobilissimum*	10	10	42	42	42	48	50	46	46	43	53	9	49	54		0.03	0.02
16 *rubellum*	17	14	40	40	40	44	50	44	44	41	51	13	45	51	20		0.03
17 *speciosum*	13	18	44	44	44	50	52	46	48	45	52	17	49	53	15	20	

Relationships among Japanese *Lilium* species based on ITS analysis

The distribution of species in Fig. 1 B basically resembles that reported by Asano (1986). A major difference between the two dendrograms lies in the position of *L. medeoloides* which was the outspecies in Asano's dendrogram. Among the Japanese *Lilium* species, Asano (1986) considered *L. medeoloides* as the most ancient because it is the most widely distributed and possesses the greatest number of 'putatively primitive' morphological structures. This concept of 'ancestry' was also adopted by Comber (1949). *L. hansonii*, which is also classified in sect. *Martagon*, is closely associated with *L. medeoloides*. Lighty (1968) hypothesized that this might be the oldest extant *Lilium*.

If sect. *Archelirion* is excluded, the supposition of the 'ancestry' of *L. medeoloides* is supported by its genetic distance and its number of base differences (Table 2). However, the genetic distances in Table 2 clearly indicate that sect. *Archelirion* is of a distinctly different and equally ancient derivation from the rest of the species.

Branch lengths after a molecular 'fork' can be used to estimate the amount of time that transpired after divergence. Figure 1 A was created by Neighbor-Joining analysis without the assumption of a 'molecular clock' whereas Fig. 1 B was generated using UPGMA, which assumes a 'molecular clock'. Both Fig. 1 A and B agree on the basic clustering of species vis-a-vis Comber's taxonomic classification. Both also coincide in the amount of divergence (branch lengths) between the four main clusters (sections).

The differences between the two figures lies in the relative positions of *L. maculatum* and *L. alexandrae*. Hybridization data cited by Asano (1987) and Shimizu (1969) support the position of *L. maculatum* in Fig. 1 A. We do not know of any previous report that can help resolve the conflicting positions of *L. alexandrae*.

The dendrogram reported by Asano (1986) placed *L. longiflorum* (sect. *Leucolirion*) in the cluster containing sect. *Archelirion*. In contrast, *L. longiflorum* and *L. formosanum* are clustered much closer to sect. *Sinomartagon* than to sect. *Archelirion* in Fig. 1 A. This corroborates the results of Haruki et al. (1997) who reported that PCR-RFLP patterns of the chloroplast and nuclear rDNA genes of *L.* × *formolongi* (*L. formosanum* × *L. longiflorum*) revealed that it was more closely related to sect. *Sinomartagon* than to sect. *Archelirion*.

Asano (1987) reported that the numerically weighted percent-crossability between *L. longiflorum* and Japanese species belonging to the sections *Archelirion*, *Martagon*, and *Sinomartagon* were 18%, 25% and 28%, respectively. The corresponding average genetic distances (extracted from Table 2) between *L. longiflorum* and the species in these three sections are 0.08, 0.05, 0.04, respectively. These values are illustrated in the phylogenetic relationships shown in Fig. 1 and contradict

Asano's 1986) dendrogram showing a close relationship between sections *Archelirion* and *Leucolirion*. However, recent improvements in in vitro culture and breeding techniques have resulted in some successful crosses between members of these two sections (Okazaki 1996).

Based on the analysis of presumably 'primitive' morphological characters, Lighty (1968) suggested that the Japanese species classified under sect. *Archelirion* gave rise to *L. longiflorum* and its relatives. Figure 1 shows that sect. *Archelirion* diverged from the other Japanese species way back in evolutionary time; i.e. it is practically a different lineage.

Molecular phylogeny and evolutionary migration

Asano (1986) speculated that *L. medeoloides* and the Japanese species classified under sect. *Sinomartagon* were distributed in the Makinoesia region (eastern Asian continent and Japan) during the Oligocene epoch (approximately, 23.5–34 mya), before Japan was isolated from mainland Asia. He postulated that species classified under the sections *Leucolirion* (6b) and *Archelirion* migrated into Japan later, after the waters rose and formed the Japanese archipelago. This migration theory implies that the latter two sections are of a provenance different from that of *L. medeoloides* and the Japanese species classified under sect. *Sinomartagon*.

Noda (1987) postulated that *L. speciosum* came to Japan from South China and gave rise to other members of sect. *Archelirion*. The section *Archelirion* is unquestionably different from the rest of the Japanese species and its isolated position in Fig. 1 supports Noda's 'South China origin' theory.

Apart from *L. formosanum*, the closest relative of *L. longiflorum* (sect. *Leucolirion*) is sect. *Sinomartagon* (Fig. 1). Noda (1987) noted the karyotypic similarity of *L. longiflorum*, *L. formosanum* and *L. philippinense* and he proposed that the Japanese branch of sect. *Leucolirion* originated from the south (Philippines) and then migrated northward to Japan through Taiwan. However, he did not offer any clue as to the origin of *L. philippinense* and its relationship to the other *Liliums* in mainland Asia.

Wild *L. longiflorum* grows on coral rocks by the sea (Wilson 1925; Haw 1986). It is the only member of Comber's section 6b that is considered to be adapted or tolerant to alkaline soil (Woodcock and Stearn 1950; Bryan and Griffiths 1995). According to Wilson (1925), it appears to be a maritime species and, unlike most lilies, a limestone plant. In fact, it grows poorly on acidic soils. If Noda's 'northern migration' theory is correct, then this species arose as an alkali-tolerant variant of its southern relative, *L. formosanum*. *L. formosanum*'s short life cycle, prolific seed production and wide adaptation (Wilson 1925) make it a plausible ancestral species of *L. longiflorum*. Their close relationship is attested to by the existence of their hybrid progeny, *L.* × *formolongi*. The adaptation of *L. longiflorum* to limestone soils may have allowed it to colonize part of the coral-based Ryukyu archipelago, but it may also have been an important factor that hindered its northward spread to nearby Kagoshima (southern Kyushu) which has poor, acidic soils.

In contrast, Lighty (1968) and Haw (1986) suggested that *L. longiflorum* originated in Japan. Lighty (1968) was of the opinion that *L. longiflorum* and its relatives (Comber's subsection 6b) evolved from sect. *Archelirion* and then spread southwards to the Philippines and westwards to India. As mentioned above, this is not supported by our data.

Comber (1949) grouped the Taiwanese, Philippine and Himalayan trumpet lilies along with the Japanese trumpet lily. Noda's 'northern migration' theory would be more credible if the connection between the Himalayan, Philippine, Taiwanese (or mainland Chinese) and Japanese trumpet lilies were to be clarified. Molecular data may be useful in establishing the linkages among these species in relation to their center of diversity in the Sino-Himalayan region.

Conclusions

The almost exact correspondence between Comber's classification with the grouping of *Lilium* species according to a phylogenetic analysis of ITS DNA sequences justifies the general acceptance of Comber's system for the classification of *Lilium*. Conversely, the congruence between (Comber's) morphological classification and the groups established by molecular (ITS DNA) sequence analysis validates the future use of the latter method for determining genetic relationships among a wider set of *Lilium* species. It is also useful in supporting the reclassification of species (e.g. *L. dauricum*) that possess morphological characters that typify different sections.

Our results also show that phylogenetic analysis of ITS DNA sequences is useful in verifying the phylogenetic relationships obtained from the traditional morphological analysis of Japanese *Lilium* species. Relationships identified by ITS sequence analysis are also corroborated by the results of hybridization between species belonging to different sections. Lastly, it may also be a useful tool in tracing the evolutionary migration of *Lilium* species.

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