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ITS DNA sequence relationships between *Lilium concolor* Salisb., *L. dauricum* Ker-Gawl. and their putative hybrid, *L. maculatum* Thunb.

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Abstract Cycle sequencing of the ITS region of nuclear ribosomal DNA of *L. concolor*, *L. dauricum* and *L. maculatum* generated surprisingly homogenous sequences from these three species. Analysis of the few (13 out of 639) polymorphic nucleotide sites in the ITS region produced results that do not support the belief that *L. maculatum* is a hybrid of the two other species. Neighbor-Joining analysis of the genetic distances calculated using the Kimura 2-parameter model of base substitution confirmed the close relationship between *L. dauricum* and *L. maculatum*. The phylogenetic tree, in conjunction with the distribution pattern and morphological similarities of the two species, suggest that *L. maculatum* is derived from the more widely distributed *L. dauricum*. The results also revealed that there is sufficient molecular divergence between *L. maculatum* and *L. dauricum* to support their status as separate species.

Key words ITS sequence analysis · Cycle sequencing · *Lilium* · Hybrid verification

Introduction

L. maculatum Thunb. refers to a group of Japanese lilies which have generated a lot of confusion among *Lilium* taxonomists (Shimizu 1987). There are many forms, mostly dwarf and well-fitted for pot culture and the rock garden (Woodcock and Stearn 1950). According to Jellito and Schacht (1990), the various clones of *L. maculatum* in the trade are often wrongly listed as *L. elegans* and that these are actually hybrids (of *L. maculatum* and *L. dauricum*) developed by Japanese gardeners 200 years ago. In contrast, Shimizu (1969) reported that *L.* × *elegans* is a hybrid between *L. maculatum* and *L. dauricum*. One apparent source of confusion is that *L. elegans* is considered by some, e.g. Woodcock and Stearn (1950), to be a synonym of *L. maculatum*.

Berckmüller (1927) obtained several *L. concolor* Salisb. × *L. dauricum* Ker-Gawl. hybrids whose morphological characters were well within the range of *L. maculatum*. Hence, some *Lilium* authorities use *L.* × *maculatum* to acknowledge its hybrid nature (Woodcock and Stearn 1950; Leslie 1982; Bryan 1989).

Much of the contemporary taxonomic work done on *L. maculatum* has been conducted by Japanese researchers relying on classical morphological differences (Kitamura et al. 1977; Ohwi 1972; Satake 1982). *L.* × *maculatum* is not used in Japan because no Japanese *Lilium* specialist believes that *L. concolor* had anything to do with the evolution of *L. maculatum*. Okazaki (1996) reported that Japanese researchers are more concerned with the exact relationship between *L. maculatum* and *L. dauricum*, as each has been considered to be a subspecies of the other. Karyotype analysis of *L. maculatum*, *L. maculatum* ssp. *dauricum* (syn. *L. dauricum* Ker-Gawl.) and *L. concolor* belied the hybrid origin of *L. maculatum* (Noda 1987).

The 18S-5.8S-28S section of nuclear ribosomal DNA is known as the internal transcribed spacer region (ITS). ITS DNA sequences are widely used in plant molecular systematics to infer phylogenetic relationships. In addition, interspecific hybrids show additivity of nucleotide states at each site where the putative parental species differ (Campbell et al. 1993). Subsequently, Wendel et al. (1995) reported that additivity of nucleotide states applies to first-generation hybrids

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but, due to biased gene conversion and concerted evolution leading to homogenization, ITS sequences eventually resemble only one parent.

The study presented here describes a protocol, from DNA extraction to cycle sequencing, which is useful for the characterization of DNA sequences in the internal transcribed spacer region of *Lilium*. It also demonstrates the utility of ITS sequence analysis in determining the relationships between *L. concolor*, *L. dauricum* and their putative hybrid, *L. maculatum*.

Materials and methods

The genetic materials were obtained from the collection maintained at the Lily Park in Sapporo. The DNA extraction procedure described by Dubouzet et al. (1997) for *Alstroemeria* was modified as follows: minute (20–50 mg) samples from young leaves of *L. concolor* (acc. 9452130) and *L. dauricum* (acc. 9742949) and from pre-soaked seeds of *L. maculatum* (acc. 8030397) were homogenized in 300 µl extraction buffer (4 *M* guanidine thiocyanate, 0.1 *M* EDTA-2Na, 1% cetyltrimethyl-ammonium bromide and 1% polyvinylpyrrolidone in 0.1 *M* TRIS-HCl, pH 8) in 0.6-ml centrifuge tubes. After the addition and thorough mixing of 300 µl (24 : 1) 1-bromo-3-chloropropane: isoamyl alcohol, the suspensions were centrifuged and the aqueous phase (approx. 250 µl) transferred to another centrifuge tube. The nucleic acids were precipitated by adding 350 µl 2-propanol. The precipitates were washed with 70% ethanol, decanted, air-dried and then resuspended in 0.1 *M* TRIS-HCl buffer. Aliquots were adjusted to 2 ng/µl using the Hoefer DyNaQUANT fluorometer.

The ITS amplification reaction consisted of 12.5 µl DNA extract, 4 µl 25 mM $MgCl_2$, 3.4 µl ddi water, 2.5 µl 10 × buffer, 2 µl dNTP mix, 4.3 pM ITSL (Hsiao et al. 1994), 4.1 pM ITS4 (White et al. 1990) primers and 0.125 µl AmpliTaq DNA polymerase (Perkin Elmer, USA). The DNA aliquots were distributed in 0.2-ml thin-walled polymerase chain reaction (PCR) tubes and cooled in ice water, and then the rest of the components were added. The Perkin Elmer 9600 thermal cycler was preheated to 90°C before placing the sample tubes in the heating block. Thermal cycling parameters reported by Dubouzet et al. (1998) for *Allium* ITS were followed.

PCR product purification, cycle sequencing with AmpliTaq FS dye terminators, sequence alignment, and phylogenetic analysis were performed as in our previous report (Dubouzet and Shinoda 1998). Sequencing reactions using either forward (ITSL) or reverse (ITS4) primers were performed at least twice.

SEQPUP V 0.6 (D. Gilbert of Indiana University, USA) was used to format the data and generate the reverse complement sequence of the reverse-primed reactions. Using CLUSTAL W (Thomson et al. 1994) we aligned the sequences with those of *Allium senescens* (Dubouzet and Shinoda 1998) to locate the boundaries of ITS1, 5.8S and ITS2. Identification of polymorphic sites was performed using SITES v 1 (Hey and Wakeley 1997). Genetic distance was calculated using DNADIST with the Kimura 2-parameter model of base substitution, and a treefile was generated using the NJ option of the NEIGHBOR program in PHYLIP (Felsenstein 1993). The treefile was illustrated using NJPLOT (M. Gouy, University of Lyon, France). These software programs are available by anonymous ftp on the Internet.

Results

Extraction of genomic DNA from young leaves was easily accomplished using the above-mentioned protocol. However, in the case of the seed sample, a lot of non-nucleic acid components (probably polysaccharides) co-precipitated with the nucleic acids after the addition of 2-propanol and centrifugation. Our subsequent trials revealed that it is much easier to excise out the embryos from the presoaked seeds followed by DNA extraction.

Amplification of the ITS region by PCR generated a single, approximately 650-bp band. Electropherograms generated by the ABI Prism DNA sequencing software showed a relatively homogenous signal from each species. The overlapping peaks were resolved by replication and combined analysis of the forward and reverse reactions.

The sequence of the ITS regions of the species is shown in Fig. 1. All species had 639-bp ITS sequences with no apparent indels. In all species the ITS1, 5.8S and ITS 2 regions were 229, 164 and 244 bp long, respectively. There were 13 polymorphic sites among the three species (Fig. 1). Of these, *L. maculatum* had 6 nucleotides identical to that of *L. dauricum*. The remaining 7 polymorphic nucleotides in *L. maculatum* could not be attributed to either putative parent. Conversely, *L. concolor* and *L. dauricum* had similar nucleotides in 6 of the 13 polymorphic sites. *L. concolor* and *L. maculatum* differed in 13 polymorphic sites.

Discussion

Taxonomy of *L. dauricum* and *L. maculatum*

Wilson (1925) distinguished between *L. dauricum* and *L. maculatum* because the latter has a compact, non-jointed bulb with a directly growing flowering stem. According to Kitamura et al. (1977), however, these traits are invalid because they can also be found among the many forms of *L. dauricum*. In fact, there are two consistent distinguishing morphological characters that Japanese taxonomists can mention: (1) *L. maculatum* Thunberg has less pubescence on the pedicel and outer surface of the perianth segments than *L. maculatum* ssp. *dauricum*, and (2) the basal portion of the flowering stem of *L. maculatum* is more papillose than that of *L. maculatum* ssp. *dauricum*. Pubescence on the perianth is a probable survival mechanism that partly determines the success of sexual reproduction in temperate climates.

Japanese taxonomists aver that the aforementioned morphological differences between *L. maculatum* and *L. dauricum* are too slight to confer to them the status of separate species. Hence, in adherence to norms of taxonomy, *L. maculatum*, which was coined by Thunberg in 1794, takes precedence over *L. dauricum*, which was proposed by Ker-Gawler in 1809. The conundrum is that these taxonomists agree that the taxon other

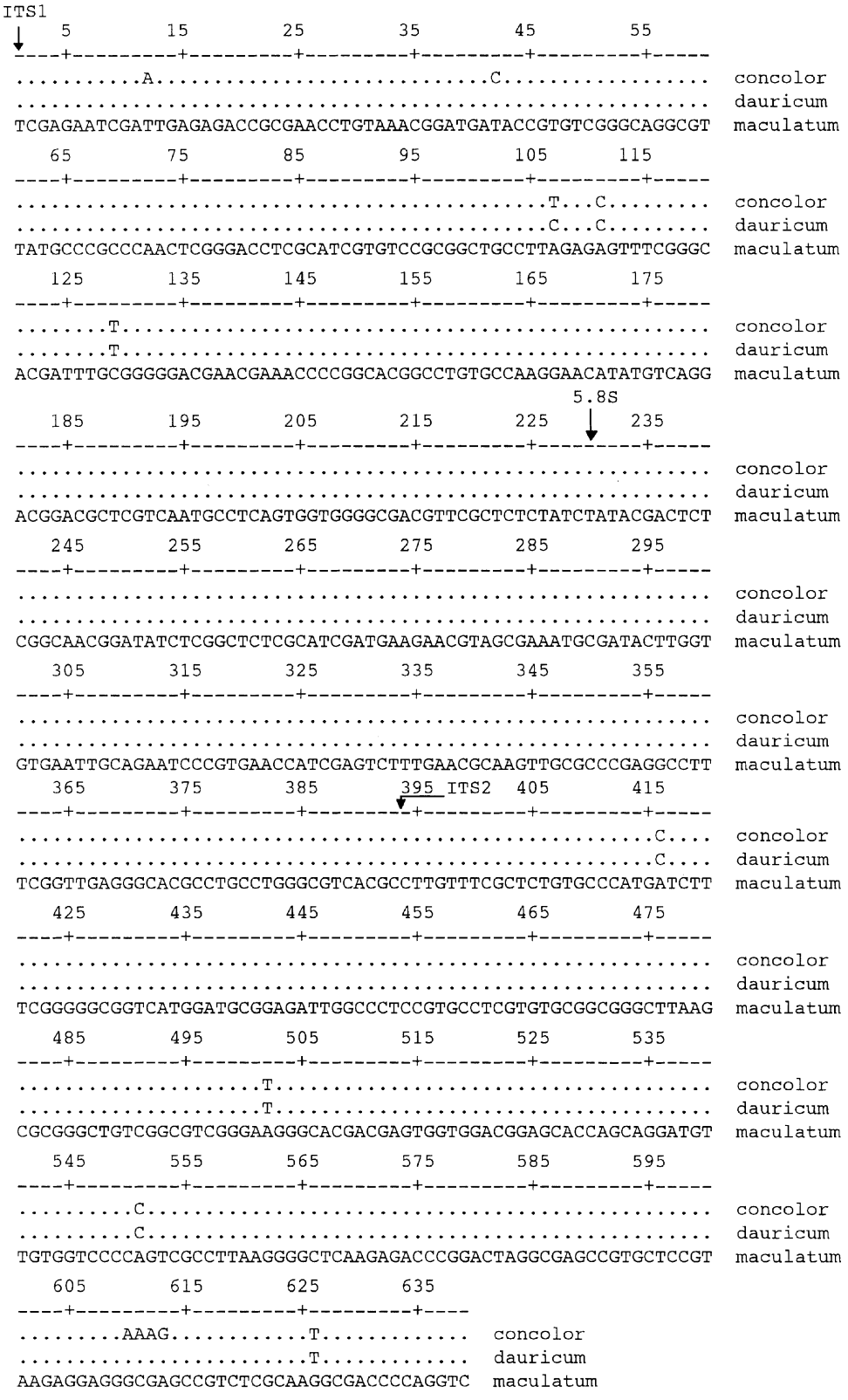

Fig. 1 DNA sequence of the ITS region of three *Lilium* species. Polymorphic sites are denoted by *capital letters* in *L. concolor* and *L. dauricum*. Modified from a print file made by SEQPUP

people know as *L. maculatum* is derived from the taxon also known as *L. dauricum*.

Ohwi (1972) was of the opinion that the morphological differences between these two taxons were of minor importance and hence referred to *L. dauricum* as *L. maculatum* var *dauricum* (Ker-Gawl.) Ohwi. Kitamura et al. (1977) and Satake et al. (1982) agreed that the differences between the two taxons are substantial

enough to confer subspecies status so they adapted the nomenifer *L. maculatum* subsp. *dauricum* (Baker) Hara to refer to *L. dauricum*. More recently, Hayashi (1990) referred to these two taxons as *L. maculatum* ssp. *dauricum* and *L. maculatum* ssp. *maculatum*. Hence, no contemporary Japanese taxonomist of sufficient repute uses the nominifer *L. dauricum* Ker-Gawl.

The existence of *L.* × *elegans*, which is believed to be a natural hybrid between *L. maculatum* and *L. dauricum* (Shimizu 1969), supports the notion that these two are just variants of one species, since barriers to interspecific hybridization are usually (but not always) a characteristic of a distinct species. Noda (1987) presented the karyotypes of all known Japanese lilies and showed that the karyotypes of *L. dauricum* (syn. *L. maculatum* ssp. *dauricum*) and *L. maculatum* are essentially the same. Therefore, the dearth of significant and consistent distinguishing morphological characteristics, the absence of a reproductive barrier and the karyotypic similarity of these two taxons favor the viewpoint of the Japanese taxonomists.

The other group consists of *Lilium* horticulturists in Japan who believe that these two taxons are different species. Asano (1989) judged that the morphological differences are significant enough to justify the separation of these two taxons into distinct species. Shimizu (1987), though aware of the karyotype data (from Noda 1987), still concluded that the abovementioned morphological characteristics, in addition to their different seed germination patterns (*L. dauricum* is hypogeal whereas *L. maculatum* is epigeal), justify their establishment as separate species.

The mode of germination is a critical evolutionary character, as it determines whether the sexual progeny of a species can compete and survive in a given temperate environment. According to Bryan and Griffiths (1995), hypogeal germination is a genetic delay mechanism that inhibits growth aboveground until a cool period, representing winter, has passed. The distinct delineation in their natural distributions, i.e. adaptation, also supports the notion that they are separate species.

Taxonomy of *L. concolor*

L. concolor has been placed in several different sections, including *Pseudolirium* by Wilson, *Sinomartagon* by Comber and *Lophophorum* by Wang and Tang (Haw 1986). Baranova (1988) classified *L. concolor* under sect. *Sinolirium* Vrischz, whereas Haw (1986) proposed the monotypic sect. *Asteridium* Haw to accommodate this species. In short, results from morphological evaluation by these *Lilium* taxonomists suggest that *L. concolor* and the other two species belong to two different sections.

Noda (1987) showed that the karyotypes of *L. maculatum* ssp. *dauricum* (syn. *L. dauricum* Ker-Gawl.) and *L. maculatum* differ from *L. concolor* by the presence of an additional constriction in chromosome k (no. 11) in the latter species.

On the putative hybridity of *L. maculatum*

The widely accepted notion that *L. maculatum* is a hybrid is based on the claims of Berckmüller (1927) who fertilized *L. concolor* with pollen from *L. dauricum* and obtained a segregating F_1 population whose characters were well within the range of *L. thunbergianum* Schultes (syn. *L. maculatum* Thunb.). In conformation to breeding conventions, Berckmüller (1927) specifically wrote that he obtained *L. concolor* × *L. dauricum* hybrids, where the second species was the pollen donor. Berckmüller recommended that other gardeners repeat his feat, but he was confident that they would get the same results. Woodcock and Stearn (1950) believed his claims and, unfortunately, wrote that the hybrid was *L. dauricum* × *L. concolor*. Hence, some contemporary *Lilium* students think that *L.* × *maculatum* (*L. dauricum* × *L. concolor*) is the correct epithet.

Berckmüller also added that the *L. thunbergianum* he was referring to did not set seed but apparently had pollen potent enough for interspecific hybridization. This is a rather strange observation since Kitamura et al. (1977) reported that *L. maculatum* sets seeds freely in the wild. Interspecific *Lilium* hybrids often (but not always) produce nonviable pollen (Asano 1982). Considering the taxonomic confusion regarding this species in Europe (e.g. Jellito and Schact 1990), it is quite possible that Berckmüller was talking of a taxon different from the Japanese *L. maculatum*. In addition, Noda (1987) showed that there was no karyological characteristic in *L. maculatum* that can be attributed to its putative maternal parent, *L. concolor*. Hence, no Japanese specialist on *Lilium* has ever accepted the veracity of Berckmüller's claims.

Breeders at CPRO-DLO (Netherlands) crossed *L. dauricum* and *L. concolor* and, as expected, the hybrids showed phenotypes intermediate between the two parents and were not similar to *L. maculatum* (Jaap van Tuyl, personal communication).

Role of species distribution

L. maculatum occurs naturally only in the northern half of Honshu, Japan (Shimizu 1969). In Japan, *L. dauricum* is limited to the northern tip of Honshu, (Aomori Prefecture) and Hokkaido, whereas *L. concolor* is widely distributed from Kyushu to Honshu (Shimizu 1969, 1987); both species can also be found in Korea and China. The current distribution of *L. dauricum* indicates that it is adapted to the colder regions of the aforementioned countries. The delimitation of *L. maculatum* to the area between the natural

boundaries of *L. dauricum* and *L. concolor* in Japan may bolster certain suppositions in the hybrid nature of *L. maculatum.*

ITS sequence relationships among the three species

The DNA sequences of the ITS regions of these three species are shown in Fig. 1. Only 13 out of the 626 nucleotide sites turned out to be polymorphic. None of the polymorphic sites in *L. maculatum* could be attributed to the putative maternal parent, *L. concolor.* The lack of additivity in these polymorphic sites can not be explained away by concerted evolution since the remainder ($98\% = 100 \times 1 - 13/639$) of the sequences of these three species are homogenous. Hence, the only conclusion that can be drawn is that *L. concolor* does not have any parental relationship with *L. maculatum.* This corroborates the results of karyotype analysis presented by Noda (1987).

Six of the polymorphic sites in *L. maculatum* were identical to those of *L. dauricum.* This identity can be interpreted as being due to similar heritage (kinship) rather than as evidence of a parent-offspring relationship. The most important proof of the absence of a parent-offspring relationship among the three species is the occurrence of 7 polymorphic sites, with no indication of mixed signal, unique only to *L. maculatum.*

Figure 2 shows the close relationship between *L. dauricum* and *L. maculatum.* It graphically explains the ease by which these two species can be hybridized, also evidenced by the existence of several registered varieties (Leslie 1982). *L. concolor* is the outspecies in Fig. 2, which also shows that *L. maculatum* could have evolved from *L. dauricum.* Analysis of the polymorphic sites (above) also leads to this conclusion. The restricted distribution of *L. maculatum* is another major indication of its derived nature.

The presence of 7 polymorphic nucleotide sites between *L. dauricum* and *L. maculatum* raises the following dilemma – just how many nucleotide differences are required to confirm the distinction between section/species/subspecies in *Lilium*? The number of base differences is directly proportional to the amount of genetic divergence. There are only 7 polymorphic sites between *L. concolor* and *L. dauricum*, and *Lilium* taxonomists agree that these two belong to different sections. Logically, a subspecies relationship should be characterized by much fewer (than 7) polymorphisms. Hence, our results indicate that *L. maculatum* shows sufficient molecular divergence in its ITS DNA sequence to qualify as an independent species, derived from but not a subspecies of *L. dauricum.*

It is quite plausible that forms of *L. dauricum* spread south towards central Honshu after the last Ice Age and that most were eliminated in a subsequent period of global warming. The remnant populations, geographically isolated from the main *L. dauricum* population, developed mechanisms (e.g. immediate epigeal germination) that allowed them to compete and survive in the milder climate. Hence, recent speciation can explain the close similarity between *L. dauricum* and *L. maculatum.*

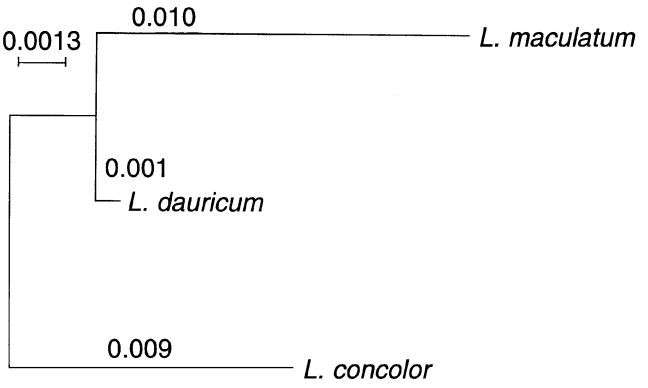

Fig. 2 Phylogenetic relationships among the three *Lilium* species according to Neighbor Joining analysis of their Kimura-2 parameter distance values. Treefile illustrated by NJPLOT

Conclusions

Sequence analysis of the ITS region of *L. concolor*, *L. dauricum* and *L. maculatum* has belied the hybrid origin of *L. maculatum.* Hence the use of *L.* × *maculatum* to refer to this species should be discontinued. Our results also indicate that there is sufficient molecular divergence between *L. maculatum* and *L. dauricum* to support their establishment as separate species. In other words, the present molecular evidence does not support the use of *L. maculatum* var *dauricum* or *L. maculatum* ssp. *dauricum* and *L. maculatum* ssp. *maculatum* to replace *L. dauricum* Ker-Gawl. and *L. maculatum* Thunb.

Our results indicate the validity of cycle sequence analysis to provide molecular fingerprints of *Lilium* species. These ITS sequences can also be useful, as we have shown here, in verifying the pedigree of a putative hybrid. Hence, the technique can be used in providing definitive proof of parent-offspring relationships.

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