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Genetic Similarity of *Lilium brownii* var. *colchesteri* in Japan and Korea

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Genetic similarity of *Lilium brownii* var. *colchesteri* cultivated in Japan and Korea was investigated with 302 AFLP markers. Average, maximum and minimum genetic similarity value within the whole accessions of Japan and Korea was 0.9788, whereas those between them and one Chinese accession was 0.9477. The threshold similarity value for identification of clones was estimated to be 0.97 by the same method with 10 clonally propagated individuals of *L. longiflorum* 'Hinomoto' and referring previous reports. 90.2% of pairs within the whole accessions of Japan and Korea represented higher the values than the threshold. Excluding five individuals, 98.9% of pairs showed higher the values. The individuals of *L. brownii* var. *colchesteri* in Japan and Korea, therefore, are considered to be almost one clone, and the introduction of the lily into Japan seems to have been through Korean Peninsula.

INTRODUCTION

In the genus *Lilium* that includes approximately 100 species (McRae, 1998), *L. brownii* F. E. Brown var. *colchesteri* Wilson is classified into *Leucolirion* Section out of four sections based on flower shapes (Wilson, 1925) or *Archelirion* Section out of seven sections based on many other characters such as seed germination manner, leaf arrangement, bulb scale and seed characters, bulb shape and habit, etc. as well as flower shapes (Comber, 1949). The recent molecular study reveled that it is closely related to *L. formosanum* Wallece and *L. longiflorum* Thunb. of *Leucolirion* Section (Nishikawa *et al.*, 2001).

Lilium brownii var. *colchesteri* is native to central China at elevations up to 1500 meters above sea level (McRae, 1998). Its bulbs have been used as a vegetable and traditional medicine in China (Chen *et al.*, 1980), but not cultivated for ornamental purposes there. The species have been cultivated as an ornamental plant widely in Japan for about 400 years. It is considered that it was introduced from China or via Korean Peninsula, since there remain some literature, paintings, poems and silk fabrics in those days (Shimizu, 1971; Okubo, 2006). It remains, however, only in a few regions in Japan in present days (Shimizu, 1971), whereas it is widely cultivated mostly in kitchen gardens of farmhouses in South Korea.

The lily has unique and ornamental floral characteristics such as light and elegant fragrance and perianth color rapidly changing from yellowish cream to white during anthesis (Okubo, 2006). It was introduced into England in 1835 and then spread to Belgium and Holland (Willson, 1925). There are a few reports available on the crossing of the species (MacRae, 1998). Cultivars and hybrids of the lily have not yet been bred. In order to promote sustainable maintenance of the species as a genetic resource for developing lily breeding, genetic variation of *L. brownii* var. *colchesteri* cultivated in Japan and Korea was compared by AFLPs.

MATERIALS AND METHODS

Plant materials

The source of the 41 accessions used in this study is presented in Table 1. Sixteen Japanese accessions were obtained from three sites of Fukuoka, Gifu and Nagasaki Prefectures. Korean accessions were collected from nine and ten sites of Chungcheongnam–do and Kangwon–do Provinces, respectively. The distance of the collection sites in two provinces is about 200 km. One Chinese accession introduced a long time ago into Japan and maintained in Gifu Prefecture. Three samples maintained in a herbal garden of Takeda Pharmaceutical Company Ltd. in Kyoto Prefecture were also used. One accession of *L. formosanum* from Kaohsiung, Taiwan and of *L. longiflorum* from Lanyu Island, Taiwan were used as out–group species.

Most of *Lilium* cultivars are clonally propagated (McRae, 1998). Ten plants of *L. longiflorum* 'Hinomoto' were supplied to estimate a threshold value of the genetic similarity for identifying clones of *L. brownii* var. *colchesteri*.

One or two fresh leaves for each accession were sampled and stored at −80 ˚C until DNA extraction.

AFLP fingerprinting

Total DNA was extracted by the modified CTAB method (Kobayashi *et al.*, 1998).

AFLP fingerprinting was conducted by the following procedure. Approximately 2 μg of total DNA was digested with 2.5 U of *Eco*RI (Toyobo Co., Ltd., Osaka, Japan)

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Table 1. Geographic location and origin of *Lilium brownii* var. *colchesteri*, *L. formosanum* and *L. longiflorum* accessions used in the present study

Species	Accession group	No. of accession sites	No. of tested samples	Origin
L. brownii var. *colchesteri*	Fukuoka (Japan)	1	1	Privately cultivated in Fukuoka Prefecture, Japan
	Gifu (Japan)	1	1	Privately cultivated in Gifu Prefecture, Japan
		1	3	Reported as vegetatively propagated from G1
	Iki (Japan)	1	11	Privately cultivated in Iki Island, Nagasaki Prefecture, Japan
	Takeda (Japan)	1	3	Cultivated in a herbal garden of Takeda Pharmaceutical Company Ltd. in Kyoto Prefecture, Japan
	Choongchungnam (Korea)	9	9	Privately cultivated in Choongchungnam–do Province, Korea
	Kangwon (Korea)	10	10	Privately cultivated in Kangwon–do Province, Korea
	China	1	1	Reported as introduced from China more than two decades ago and privately cultivated in Gifu Prefecture, Japan
L. formosanum	Kaohsiung (*L. formosanum*)	1	1	Introduced from Kaohsiung County, Taiwan and cultivated in Kyushu University, Fukuoka Prefecture, Japan
L. longiflorum	Lanyu (*L. longiflorum*)	1	1	Introduced from Lanyu Island, Taitung County, Taiwan and cultivated in Kyushu University, Fukuoka Prefecture, Japan

and 0.5 U of *Mse*I (New England BioLabs Inc., MA, USA) and ligated to 1 U of *Eco*RI and *Mse*I adaptors {Applied Biosystems Inc. (ABI), CA., USA} with 5 U *T4* DNA ligase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a total volume of 2.5 μL containing 10x *T4* ligase buffer with ATP (Wako), 25 pg L^{-1} bovine serum albumin (BSA, New England BioLabs) and 0.5 M NaCl (Nacalai Tesque Inc., Kyoto, Japan) for 16 h at 22 ˚C.

Then, the reaction was stopped for 20 min at 65 ˚C and stored at 4 ˚C until the amplification reactions. Digested–ligated DNA fragments were diluted 1:10 with TE buffer and used as templates for the first amplification reaction. The preselective PCR was performed in a 10 μL solution containing 2.0 μL of the diluted digested–ligated DNA product, 1.1 mM $MgSO_4$, 2 mM of dNTPs, 0.2 U KOD–Plus–DNA polymerase, 10x KOD–Plus buffer (Toyobo), and each 5 μM of preselective primers (Genenet Co., Ltd., Fukuoka, Japan). The primer pair used in the preselective amplification was 5'– GAC TGC GTA CCA ATT CA – 3' (E) for *Eco*RI primer and 5'– GAT GAG TCC TGA GTA ACA G– 3' (M) for *Mse*I primer. PCR was performed using a thermocycler (PC–701, Astec) under the temperature program of 1 cycle for 2 min at 94 ˚C, 25 cycles for 15 s at 94 ˚C, for 30 s at 56 ˚C and for 90 s at 68 ˚C, and then 1 cycle for 30 min at 60 ˚C. The pre–amplification products were then diluted to 200 μL with TE. The selective PCR was performed in a 5 μL solution containing 0.75 μL of the diluted primary amplification product, 1.1 mM $MgSO_4$, 2 mM dNTP, 0.1 U KOD–Plus–DNA polymerase, 10x KOD–Plus buffer (Toyobo), and each 5 μM selective primers. We pre–screened 64 selective primer pairs and finally chose the three most informative ones for this study; a pair of E+CT (ABI)/ M+TT (Genenet), E+CA (ABI)/ M+CC (Genenet), E+AG (ABI)/ M+CA (Genenet). The selective PCR was carried out using a thermocycler (PC–808–02, Astec) under the temperature program of 1 cycle for 2 min at 94 ˚C, 10 cycles for 15 s at 94 ˚C, 30 s at 66 ˚C (annealing temperature was reduced by 1.0 ˚C at each cycle), 1 min at 68 ˚C, 20 cycles for 15 s at 94 ˚C, 30 s at 56 ˚C and 1 min at 68 ˚C, and then 1 cycle for 30 min at 60 ˚C.

Fingerprint data were obtained by running the amplified samples on an ABI Prism 310 DNA Sequencing System using P/N AFLP Plant Mapping Protocol (P/N 4303146). Band scoring was completed with the Genescan software (ABI).

Statistical analysis

Visually distinguishable AFLP markers were transformed into a binary matrix, with '1' for the presence and '0' for the absence of a band. Genetic distance between individuals was calculated based on pair wise comparisons according to Nei and Li (1978) by RESTDIST, a program of PHYLIP version 3.6 (Felsentein, 2004). Genetic similarity was calculated based on the results of genetic distance.

Genetic similarity = 1 – (genetic distance)

RESULTS AND DISCUSSION

Variation within clones of *L. longiflorum* 'Hinomoto' and determining the threshold value

A total of 184 AFLP fragments was identified

between 50 bp and 500 bp in size from the tested 10 clonally propagated *L. longiflorum* 'Hinomoto'. One hundred fifty–three out of the 184 AFLP makers were monomorphic but no samples that all the fragments perfectly corresponded to each other were found. Average, maximum and minimum values of genetic similarity were 0.9854, 0.9952 and 0.9756, respectively. The frequency histogram of pair wise genetic similarity for 10 individuals is shown in Fig. 1A.

Many threshold values for identification of clones have been proposed in AFLP fingerprinting. Anens *et al*. (1998) set the threshold similarity at 0.98 in poplar (*Populus nigra*), whereas Winfield *et al*. (1998) found the similarities from 0.96 to 1.00 in *Populus nigra* subsp. *butulifolia*. Douhovnikoff and Dodd (2003) established 0.983 in *Salix exigua* and Douhovnikoff *et al*. (2004) determined the value of 0.974 in *Sequoia sempervirens*. These reports suggest that the similarity threshold value exists between 0.96 and 0.98. Considering these proposed values with the results with *L. longiflorum*, the threshold value of around 0.97 was adopted in this study for estimation of clones in *L. brownii* var. *colchesteri*.

Genetic similarity of *L. brownii* var. *colchesteri*

A total of 302 AFLP fragments among which seven markers were monomorphic was identified between 50 bp and 500 bp in size from the 41 tested accessions.

Average values of the similarity within Japanese and

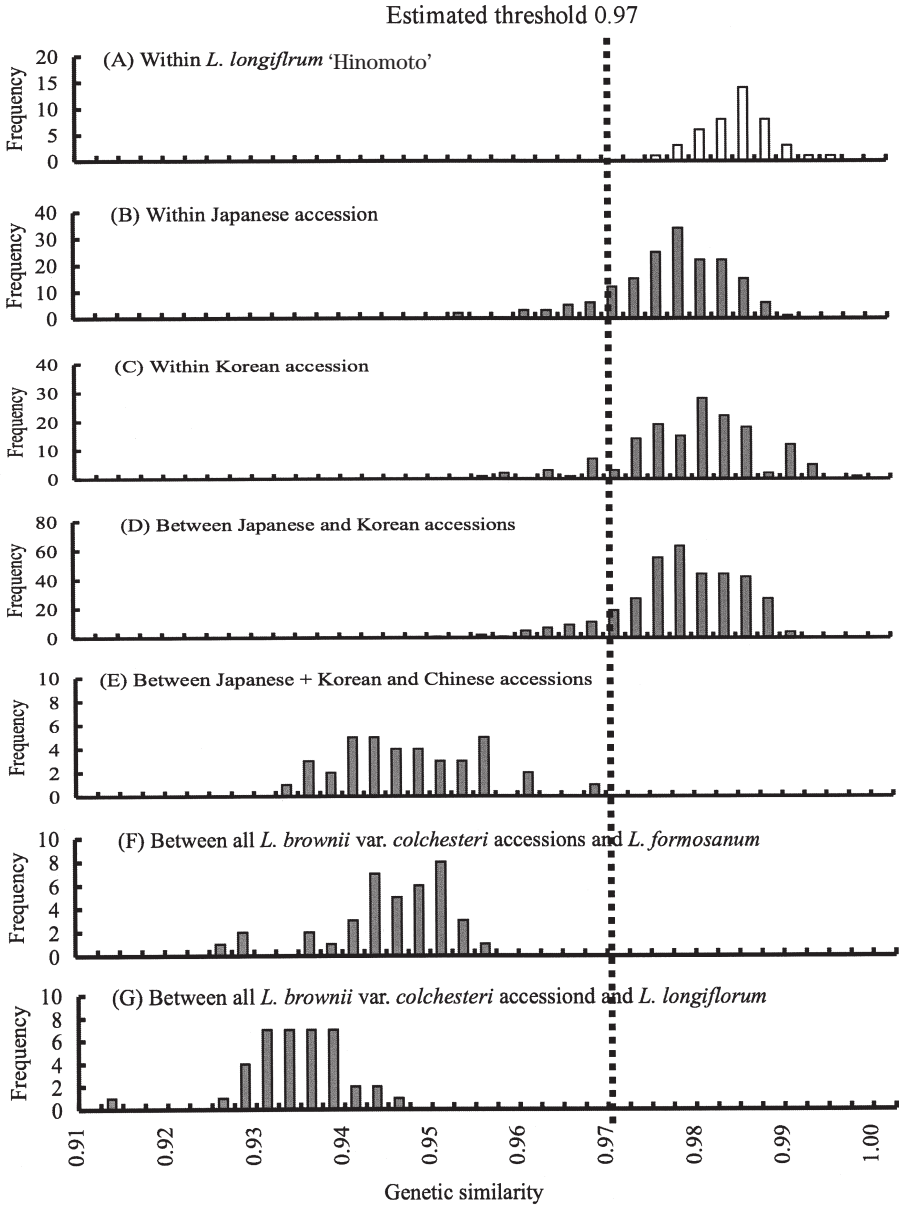

Fig. 1. Frequency histograms of pair wise genetic similarity for *L. longiflorum* 'Hinomoto', *L. brownii* var. *colchesteri*, *L. formosanum* and *L. longiflorum* used in this study. (A) within *L. longiflorum* 'Hinomoto', (B) within Japanese accession, (C) within Korean accession, (D) between Japanese and Korean accessions, (E) between Japanese+Korean and Chinese accessions, (F) between all *L. brownii* var. *colchesteri* accessions and *L. formosanum* and (G) between all *L. brownii* var. *colchesteri* accessions and *L. longiflorum*.

within Korean accessions were respectively 0.9779 and 0.9813, while the value between Japanese and Korean accessions was 0.9788, which was higher than the value within Japanese accessions (Table 2 and Figs. 1B–D). These values were higher than the threshold value of 0.97. When one Chinese accession was included in the analysis, the values lowered to 0.9477, lower than 0.97.

The frequency histograms of pair wise genetic similarity within Japanese (Fig. 1B) and Korean (Fig. 1C) accessions and between Japanese and Korean accessions (Fig. 1D) were similar. These histograms were not resembled to those between Japanese＋Korean and Chinese accessions (Fig. 1E), between *L. formosanum* and all the *L. brownii* var. *colchesteri* accessions (Fig. 1F) and between *L. longiflorum* and all the *L. brownii* var. *colchesteri* accessions (Fig. 1G). These results indicate that Japanese and Korean accessions used in the present study are considered to be almost clones.

Whithin Japanese accessions, within Korean accessions, and between the accessins, 88.9%, 92.4% and 87.5% of pairs showed higher value of the similarity threshold of 0.97. When one individual of Gifu, three of Takeda and one of Choongchungnam accessions were excluded, the percentages risen rose to 100%, 99.3% and 98.5%, respectively (Fig. 2). Excluding these five individuals, it is highly possible that the other accessions in Japan and Korea are genetically almost identical, they are clones.

Fruit set was not observed in some Japanese and Korean *L. brownii* var. *colchesteri* accessions used in this experiment when they were self– and cross–pollinated (by H. Saruwatari, data not shown). It is reported that the species showed strong self–incompatibility (Shimizu, 1971). The results support our suggestion.

The results also support the idea of Okubo (2006) that *L. brownii* var. *colchesteri* in Japan was introduced through the Korean Peninsula in old days, but not directly from China.

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Table 2. Average genetic similarity within and between accession groups with different locality and origin. Accession groups are the same as Table 1

Accession group		Accession group			
		Japan	Korea	China	*L. formosanum*
Japan	(N＝19)	0.9779			
Korea	(N＝19)	0.9788	0.9813		
China	(N＝ 1)	0.9490	0.9463	–	
L. formosanum	(N＝ 1)	0.9468	0.9452	0.9259	–
L. longiflorum	(N＝ 1)	0.9364	0.9345	0.9128	0.9687

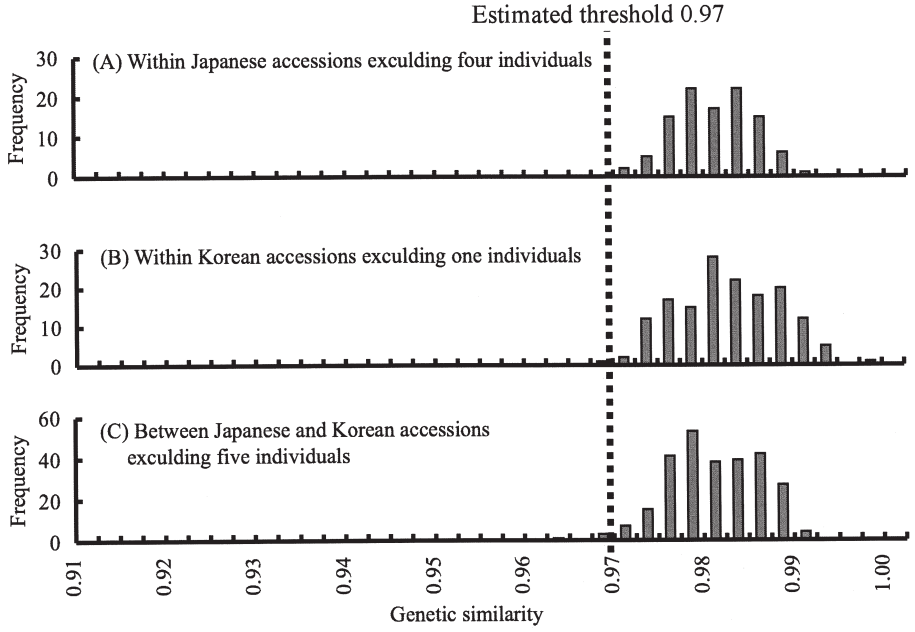

Fig. 2. Frequency histograms of pair wise genetic similarity for *L. brownii* var. *colchesteri* used in this study. (A) within Japanese accessions excluding one individual of Gifu and three of Takeda accessions, (B) within Korean accessions excluding one individual of Choongchungnam accession and (C) between Japanese and Korean accessions excluding one individual of Gifu, three of Takeda and one of Choongchungnam accessions.

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