

Genetic variation of maiden lily (*Lilium rubellum* Baker) within and among three natural populations in Fukushima Prefecture in Japan

Variación Genética del Lirio Rosa (*Lilium rubellum* Baker) dentro y entre tres poblaciones naturales de la Prefectura de Fukushima en Japón

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Abstract

Lily (genus *Lilium*) is one of the most important flowers for bulb and cut flower production in the world. *Lilium rubellum* Baker is a native species of Japan that exhibits early flowering, is pink coloured, and possesses a pleasant fragrance, which makes it an important genetic resource for breeding lilies. In this work, the genetic diversity of *L. rubellum* was studied among three natural populations located in Fukushima Prefecture, Japan, which have been designated as the Mt. Azuma, Nango and Atsushio-kano populations. A total of 31 accessions collected in 1997 were analysed using Random Amplified Polymorphic DNA (RAPD) markers. Eighteen decamer primers produced 98% polymorphic RAPD bands; furthermore, 11 of the 18 primers produced 10 or more polymorphic bands with a mean per primer Polymorphic Information Content of 0.382. Among the three populations, the mean Shannon index, Nei's gene diversity and percentage of polymorphic loci were 0.2749, 0.4099 and 76.7%, respectively. These data revealed that there is a high genetic diversity within all the populations. Analysis of the three genetic diversity indices within populations showed that the Nango population had the highest genetic diversity, whereas the Atsushio-kano population had the lowest. An unweighted pair group method with arithmetic mean dendrogram based on Jaccard's similarity coefficient was constructed, and the three populations of *L. rubellum* collected in the Fukushima prefecture were clearly differentiated at 0.61 similarity index. The Nango and Mt. Azuma populations were genetically closer than the Atsushio-kano population. The analysis of molecular variance showed a 29.53% variance among the populations. This study revealed that there is high genetic diversity within populations and moderate genetic diversity among the three natural populations of *L. rubellum* Baker in the Fukushima Prefecture of Japan.

Keywords: genetic diversity, RAPD marker, *Lilium rubellum*

Resumen

El Lirio (género *Lilium*) es una de las flores bulbosas de corte más importantes en el mundo. *Lilium rubellum* Baker es una especie nativa de Japón, produce una floración muy temprana de color rosado, y una agradable fragancia, por lo que esta especie es un importante recurso genético para el mejoramiento genético de los lirios. En este trabajo se estudió la diversidad genética de *L. rubellum* entre tres poblaciones naturales de la prefectura de Fukushima en Japón: Mt. Azuma, Nango y Atsushio-Kano. Se analizaron un total de 31 accesiones mediante la prueba de ADN polimórfico amplificado aleatorio (RAPD). 18 decámeros cebadores produjeron 98% de bandas polimórficas, 11 de estos 18 cebadores producen 10 o más bandas polimórficas con una media por cebador de Contenido de Información Polimórfica (PIC) = 0.382. La media del índice de Shannon Ho = 0.2749, de la diversidad genética de Nei He = 0.4099 y el porcentaje de loci Polimórficos = 76.7 revelaron que existe una alta diversidad genética dentro de todas estas poblaciones. Los análisis de los tres índices de diversidad genética intrapoblacional, mostraron que la población de Nango tenía la mayor diversidad genética y la población de Atsushio-kano la más baja. Se construyó un dendrograma UPGMA basado en el coeficiente de similitud de Jaccard y con un índice de similitud de 0.61, las tres poblaciones de *L. rubellum* recolectadas en la prefectura de Fukushima fueron claramente diferenciadas. Las poblaciones de Nango y de Monte Azuma estaban más cercanamente relacionadas genéticamente que con la población de Atsushio-kano. Con el análisis de la varianza molecular (AMOVA) se observó que existe un 29,53% variación interpoblacional. Este estudio reveló que existe una alta diversidad genética dentro de las poblaciones y una moderada diversidad genética entre las tres poblaciones naturales de *L. rubellum* Baker en la prefectura de Fukushima en Japón.

Palabras clave: Diversidad genética, marcador RAPD, *Lilium rubellum*

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Introduction

Ornamental plants are used by humans because of their beauty, symbolic significance (Mendonça de Carvalho, 2011), colour (Kaufman & Lohr, 2008), fragrance and therapeutic (Matsuo, Takaesu, & Asano, 2008) and emotional value. The lily has become one of the most important ornamental plants grown for its flower bulbs and cut flowers worldwide over the last 50 years (Van Tuyl & Arens, 2011). Furthermore, *Lilium* species can have pharmaceutical applications since they contain steroidal saponins, glycoalkaloids and phenolic glycerides as well as antioxidants (Mammadov, Deniz, Rakhimzhanova, Kılınçarslan, & Mammadov, 2017).

The centre of origin of the *Lilium* genus is the Himalayan region in Asia (De Jong, 1974). This genus has about 100 species grouped into seven sections distributed across the Northern hemisphere extending as far south as the Southern Asia (Van Tuyl et al., 2011). Japan is known to be rich in *Lilium* species. Fifteen species are distributed across all prefectures of Japan, and Japanese *Lilium* species have played an important role in the development of major important cultivars of Eastern Lily, Asiatic Hybrids and Oriental Hybrids (Okasaki, Asano, & Oosawa, 1994). However, *Lilium* species are also under threat of genetic erosion, requiring increased attention and efforts to conserve *Lilium* germplasm (Yi, Lee, Chung, Lee, & Lim, 2013).

Lilium rubellum Baker grows wild in the mountainous region of Northeastern Honshu, Japan (McRae, 1998; Shimizu, 1971). It flowers early and has an attractive pink colour and pleasant fragrance (Lim & Van Tuyl, 2007). It is easily propagated by seed and is cultivated in the Fukushima Prefecture (Okasaki et al., 1994). The desirable quality of this delicate, pink-coloured lily is that it can hybridise with other lilies of the section *Archelirion*, and their offspring are referred to as Oriental Hybrids, one of the popular variety groups of lilies (Okasaki et al., 1994). Understandably, *L. rubellum* is an important germplasm resource for breeding lilies, and evaluating its genetic diversity will provide valuable understanding of distinct populations.

Only seven *Lilium* species have had their genetic diversity assessed using molecular techniques (Arzate-Fernández, Miwa, Shimada, Yonekura, & Ogawa, 2005; Huang et al., 2009; İkinci & Oberprieler, 2010; Persson, Lundquist, & Nybom, 1998; Wen & Hsiao, 2001; Wu et al., 2015). One of the first techniques used in order to assess genetic diversity was Random Amplified Polymorphic DNA (RAPD) markers (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990). This technique is based on the amplification of discrete DNA segments by polymerase chain reaction (PCR) using small size oligonucleotide primers of random design (Halward, Stalker, LaRue, & Kochert, 1992; Sharma, Dawson, & Waugh, 1995)

Currently, there are many other, more modern molecular techniques, but RAPD markers are still useful in large-scale genetic studies of diverse populations (Velasco-Ramírez, Torres-Morán, Molina-Moret, De Jesús Sánchez-González, & Santacruz-Ruvalcaba, 2014). Despite its low resolving power, the simplicity and low cost of agarose gel electrophoresis has made the use of RAPD markers prevalent. The markers are quick, easily generated by PCR, and do not require prior sequence information (Idrees & Irshad, 2015). Furthermore, their effectiveness in *Lilium* was established by Yamagishi (1995), who developed RAPD markers for 13 species. In this study, RAPD markers were used (i) to assess the genetic differentiation among populations of *L. rubellum* in the prefecture of Fukushima, Japan, and (ii) to estimate the levels of genetic diversity within *L. rubellum* populations. This information will be useful in planning conservation and breeding programmes of *L. rubellum*.

Materials and Methods

Plant materials

Leaf samples of 31 individuals of *L. rubellum* were collected from three different sites in Fukushima Prefecture (Figure 1) located in the north central region of Honshu Island, Japan: 1) Mt. Azuma-Kofuji, Fukushima City (altitude 1,300 MASL; Figure 2); 2) Atsushio-kano Village (currently Kitakata City; altitude, 500 MASL; Figure 3); and 3) Nango Village, figure 4, which consisted of Aburakubo (altitude, 700 MASL), Shimoyama (altitude, 600 MASL), Misawa (altitude, 600 MASL) and Nagachisawaguchi (currently Minamiaizu Town; altitude, 700 MASL). The collections were made in June and July of 1997 (Table 1). The leaves were stored at -80°C until DNA extraction.

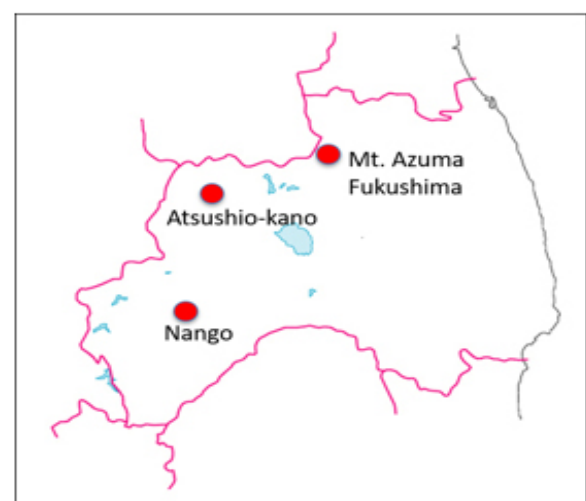


Figure 1. Collection Cities in Fuskushima Prefecture



Figure 2. Rose Lily in Mt. Azuma



Figure 3. Rose Lily in Atsushio-kano



Figure 4. Rose Lily in Nango

DNA extraction

Total DNA was extracted by the CTAB method (Murray & Thompson, 1980). Approximately 1 g of frozen leaves was

Table 1. Sample Name Collected in Fukushima Prefecture

Population	Accession Number	Sample Name	
1. Mt Azuma	1	Azuma A 8	
	2	Azuma A 16	
	3	Azuma B 14	
	4	Azuma B 11	
	5	Azuma B 5	
	6	Azuma A 18	
	7	Azuma C 13	
	8	Azuma C 17	
	9	Azuma C11	
	17	Azuma A 7	
	18	Azuma A 6	
	19	Azuma B 4	
	20	Azuma B 20	
	21	Azuma C 16	
	22	Azuma C 19	
	2. Atsushio-kano	27	Atsushio-kano Village1
		28	Atsushio-kano Village 3
		29	Atsushio-kano Village 6
		30	Atsushio-kano Village 10
		31	Atsushio-kano Village 12
	3. Nango	10	Nango Misawa 5
		11	Nango Misawa 1
12		Nango Nagachisawaguchi 2	
13		Nango Nagachisawaguchi 13	
14		Nango Aburakubo B1	
15		Nango Aburakubo 18	
16		Nango Shimoyama 1	
23		Nango Misawa 6	
24		Nango Nagachisawaguchi 18	
25		Nango Aburakubo 9	
26	Nango Aburakubo B8		

ground to a fine powder with liquid nitrogen in a mortar with a pestle. The powder was transferred to a beaker containing 5 mL of 2× CTAB buffer (2% CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA and 1.4 M NaCl) and immediately mixed. The mixture was transferred to a centrifuge tube and incubated for 10 min at 65 °C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently for 30 min at room temperature. Five millilitres of 1× CTAB buffer (1% CTAB, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA and 0.7 M NaCl) was added to the mixture. After centrifugation at 4,000 rpm for 15 min, the supernatant was transferred to a new tube by decanting. An equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently for 10 min, then centrifuge again at 4,000 rpm for 15 min. The supernatant

was transferred to a new tube and 0.1 vol of 10% CTAB (10% CTAB and 0.7 M NaCl) was added to obtain a CTAB–nucleic acid precipitate. After gentle inversion, the mixture was left for 30 min at room temperature. The precipitate was recovered by centrifuging at 4,000 rpm for 15 min and removing the supernatant. The precipitate was dissolved in 5 mL of HS-TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA and 1 M NaCl) containing 5 μ L of RNase and incubated at 55 °C until completely dissolved. Five millilitres of isopropanol was added to the solution to remove the CTAB. After gentle inversion, the solution was centrifuge at 4,000 rpm for 10 min and the supernatant was removed. The precipitate was washed with 5 mL of 70% ethanol and dissolved in 3 mL of TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA).

Amplification by RAPD procedures

A total of 31 accessions from three natural populations of this prefecture were analysed using 18 random decamer primers. For DNA amplification, 10 μ L of the reaction mixture was prepared, containing 10 ng of DNA, 1 μ L PCR 10 \times buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 1 μ L of dNTPs (2.0 mM each), 1.2 μ L of MgCl₂ (25 mM), 0.5 units of DNA polymerase (AmpliTaq Gold), 0.1 μ L of a decamer primer (20 pmol/ μ L) and sterilised, distilled water to a final volume of 10 μ L. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) as follows: first cycle at 95 °C for 10 min; followed by 50 cycles at 94 °C for 1 min,

35 °C for 1 min and at 72 °C for 2 min; and 1 cycle at 94 °C for 1 min; 35 °C for 1 min at 72 °C for 5 min; and a final hold at 4 °C. For each RAPD assay, primers of the PC, RA, OPA, OPB and OPC series were randomly selected (Table 2). The amplified DNA segments were separated in 1.5% agarose gels (20 \times 15 cm) prepared in TBE buffer (Sambrook, Fritsch, & Maniatis, 1989). Electrophoresis was performed at 80 V for 4 h. Then the gel was stained with ethidium bromide (0.5 μ g/mL) and the bands were visualised using ultraviolet light.

Data analysis

Scoring of polymorphic DNA fragments was done using 1 or 0 representing the presence or absence of a polymorphic band, respectively. From these data, the Polymorphic Information Content (PIC) per primer and the genetic similarity between genotypes of the three sampled populations were estimated using the Jaccard similarity coefficient, which was calculated using NTSYS-pc software version 2.2 (Rohlf, 1992; Van Tuyl & Arens, 2011; Van Tuyl et al., 2011). Based on the resulting similarity matrix, a grouping analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA). For assessing genetic variation within populations, the programme GenAlEx 6.5 (Peakall & Smouse, 2012) was used. Nei's gene diversity (1973) (H_g) and Shannon index (H_o) were also estimated. Analyses of molecular variance (AMOVA) between and within populations were calculated using the ARLEQUIN software version 3.5 (Excoffier & Lischer, 2010)

Table 2. Primers and Polymorphic Information Content

Primer Code	Sequence	Number of Amplified Bands	Number of Polymorphic Bands	% GC	PIC
OPA-08	5'-GTGACGTAGG-3'	13	13	60	0.344
OPA-09	5'-GGGTAACGCC-3'	14	13	70	0.414
OPA-10	5'-GTGATCGCAG-3'	9	9	60	0.413
OPB-01	5'-GTTTCGCTCC-3'	14	14	60	0.457
OPB-08	5'-GTCCACACGG-3'	11	11	70	0.380
OPB-17	5'-AGGGAACGAG-3'	14	12	60	0.283
OPC-01	5'-TTCGAGCCAG-3'	9	8	60	0.377
OPC-08	5'-TGGACCGGTG-3'	12	12	70	0.364
OPC-09	5'-CTCACCGTCC-3'	8	8	70	0.391
OPC-15	5'-GACGGATCAG-3'	7	7	60	0.358
OPC-04	5'-CCGCATCTAC-3'	16	16	60	0.428
Pc 3	5'-AACCGCGCTC-3'	14	14	70	0.382
Pc 7	5'-CGCGGACGAT-3'	11	11	70	0.432
RA 3	5'-CGATCGAGGA-3'	9	9	60	0.431
RA5	5'-AAGCAGCAAG-3'	10	10	50	0.341
RA35	5'-AAGCTCCCCG-3'	9	9	70	0.456
RA37	5'-TGTGGCCGGT-3'	11	11	70	0.378
RA41	5'-GAGTGCGCAG-3'	9	9	70	0.317
Total Number of Bands		200	196		

Results

Degree of polymorphism

A total of 200 bands were amplified using 18 primers, and 196 were polymorphic (98%; Table 2). They ranged in size from 0.20 to 2.24 kb. The number of bands per primer varied from 7 to 16 with a mean of 11.1. In this study, 100% of the primers used produced polymorphic bands.

Table 3. Genetic diversity indices of three populations of *Lilium rubellum*

Populations	H_e	H_o	% polymorphic loci
Mt. Azuma	0.284103443	0.421575859	78.5
Atsushio-kano	0.24402529	0.365970296	68.5
Nango	0.296599687	0.442330341	83
Mean	0.274909473	0.409958832	76.67

H_e , genetic diversity of Nei; H_o , Shannon index.

Table 4. Summary of analysis of molecular variance for the three populations of *Lilium rubellum*

Source of Variation	Sum of squares	Variance Components	Percentage Variation
Among populations	314.52	13.21	29.52
Within populations	883.03	31.54	70.46
Total	1197.55	44.75	

$F_{st} = 0.2952$

Genetic diversity

In this study, the Nei's gene diversity or expected heterozygosity H_e within populations ranged from 0.244 (Atsushio-kano population) to 0.296 (Nango population) as shown in Table 3. The Shannon index H_o ranged from 0.365 (Atsushio-kano population) to 0.442 (Nango population). It is important to note that the Shannon index ranges from 0 to 1, and the closer the value is to one, the higher the diversity. The percentage of polymorphic loci ranged from 68.5% (Atsushio-kano population) to 83% (Nango population). The Nei's gene diversity H_e , the Shannon index H_o , and the percentage of polymorphic loci were 0.275, 0.409 and 76.7%, respectively; this showed a relatively high genetic diversity within the populations of *L. rubellum*.

Genetic structure

The similarity matrix calculated using the Jaccard coefficient between genotypes based on RAPD markers exhibited coefficients of similarity between pairs of genotypes that ranged from 0.54 to 0.85 (Figure 5). The greatest similarity was found between the individuals from C 13 and C 17 from Mt. Azuma (0.85). In the dendrogram at 0.61 similarity index, the three populations of *L. rubellum* collected in Fukushima Prefecture were clearly differentiated. Population 1, made up of 15 samples taken in Mt. Azuma-Fukushima City, had a percentage of similarity with a range of 0.66%–0.85%; Population 2, made up of five samples taken in Atsushio-kano Village, had a percentage of similarity with a range of 0.62%–0.74%; and Population 3, made up of 11 samples taken in

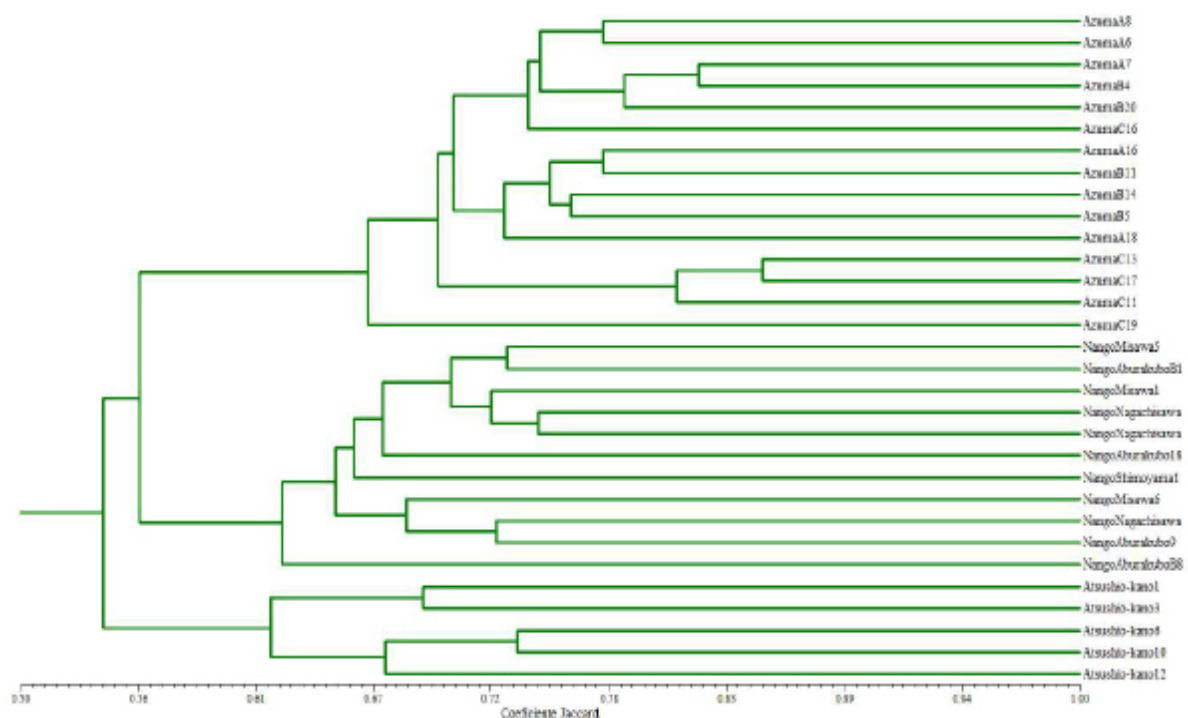


Figure 5. UPGMA dendrogram based on Jaccard's similarity coefficients

the Nango villages, had a percentage of similarity with a range of 0.63%–0.76% (Table 1; Figure 5). The Nango and Mt. Azuma populations were closer genetically than the Atsushio-kano population. As shown in Table 4, AMOVA analysis revealed that of the total variation, 29.52% occurred among the populations and the remainder within the populations.

Discussion

Three natural populations of *L. rubellum* in Fukushima Prefecture, Japan, were analysed by RAPD marker analysis with 18 primers to produce bands, 98% of which were polymorphic. Eleven of these primers showed ≥ 10 polymorphic bands (OPA-08, OPA-09, OPB-01, OPB-08, OPB-17, OPC-08, OPC-04, Pc 3, Pc 7, RA5 and RA37) with a PIC mean of 76.7%. In future analyses, primers that produce ≥ 10 polymorphic bands would be useful in evaluating the genetic variability in *L. rubellum*.

The genetic diversity mean of *L. rubellum* ($H_o = 0.2749$, $H_e = 0.4099$, and percentage of polymorphic loci = 76.7%) compared with other narrowly distributed species of the genus *Lilium*, was moderately high (Guo et al., 2011) and very similar to the genetic diversity of *L. regale* ($H_o = 0.2750$). Of the three populations analysed, the Nango population showed the highest genetic diversity and the Atsushio-kano population showed the lowest genetic diversity within the respective populations. The Mt. Azuma population had intermediate values of the three indices analysed. Since these samples were collected near the alpine belt (approximately 1,300 MASL), the population was expected to have greater variation because subalpine species have been shown to harbour greater variation in the highlands of central Japan (Ohsawa & Ide, 2011). The relatively high genetic diversity of *L. rubellum* may be attributable to its potential mixed mating system and long-range pollen and seed dispersal, which is also common in other *Lilium* species like *L. regale* (Wu et al., 2015)

In the UPGMA dendrogram at 0.61 similarity index, the three populations of *L. rubellum* collected in Fukushima Prefecture were clearly differentiated. The analysis of interpopulation variation through AMOVA showed that there was 29.52% of variation among populations. Moreover, Nango is located at the southern edge of the distribution of *L. rubellum*, and it is closer geographically to the Atsushio-kano Village (outside Kitakata Town) than to Mt. Azuma, Fukushima City (Figure 1). Despite geographic distances, as can be seen in the dendrogram (Figure 5), the populations of Mt. Azuma and Nango were in the same cluster (0.55), indicating that they are closely related compared to the Atsushio-kano population. However, the number of samples collected representing the Atsushio-kano population was quite small. In future analysis, a higher number of samples collected in this village should be taken in order to validate the accuracy of

population clustering.

Conclusions

The assessment of genetic diversity is of great importance to the sustainability of plant populations (Wang et al., 2007). The evolutionary potential of a species largely depends on its level of genetic variability, and, for plants, the overall level of genetic variability in a population is often correlated with the geographical distribution range of the species (Hamrick & Godt, 1996). The wide separation between populations found based on results of the AMOVA analysis may result in increased genetic diversity due to the fact that there may be little cross pollination among the three populations included in this study. The populations sampled in this study were separated by many kilometres, and distance could be a significant factor accounting for the relatively high genetic variation among populations.

We hope that the genetic diversity evaluated in germplasm collected over 20 years ago will serve to compare with the genetic diversity that can be found using other molecular markers. The *L. rubellum* populations reported here offer important information on the conservation strategies for this endangered species and may be of value to breeding programmes.

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