Genetic Analysis of *Lilium speciosum* Thynb. (Liliaceae) in the Koshiki Islands and the Southwestern Kyushu Populations, in Japan

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Abstract

We studied the phylogeny and genetic structure of *Lilium speciosum* Thynb. (Liliaceae), collected from natural populations in the Koshiki Islands and southwestern Kyushu Island, using the Multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) method. A total of thirty-three samples were collected from nine sites in Kami-koshiki-shima, Naka-koshiki-shima, and Shimo-koshiki-shima of the Koshiki Islands in Satsuma-sendai-City, and Hashima of Ichiki-kushikino City southwestern Kyushu Island (all belonging to Kagoshima Prefecture, Japan). All plants were genetically divergent from each other. Three groups were recognized in a phylogenetic tree. Ten plants in Kyushu Island and all plants in Shimo-koshiki-shima consisted of a derived monophyletic clade. Another derived clade consisted of all plants in Naka-koshiki-shima, three in Kyushu Island, and one in Shimo-koshiki-shima. Genetic structure analysis results showed that plants in the Koshiki Islands and Kyushu Island tend to be genetically different from each other. The plants of Naka-koshiki-shima and Kyushu Island were polymorphic.

Key words: Genetic Structure, Intraspecific Phylogeny, MIG-seq, Natural Populations, Speciosum Lily

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Introduction

Lilium speciosum Thunb., Liliaceae, is a perennial monocotyledon and distributed on the Kyushu and Shikoku areas of southwestern Japan, and in parts of mainland China and Taiwan. Over 100 wild species are included in the genus *Lilium* Tourn. ex L. Their molecular phylogeny or genetic relationship has been studied over the past 25 years (Nishikawa *et al*. 1999, Hayashi and Kawano 2000, Du *et al*. 2014, Huang *et al*. 2018). According to the newest classification by Watanabe *et al*. (2021), *L. speciosum* belongs to a new section, *Japonica* (Baranova) S. T. Watan. et M. N. Tamura, with other Japanese wild lilies: *Lilium japonicum* Thunb. ex Houtt., *L. nobilissimum* (Makino) Makino and *L. ukeyuri* Veitch ex R. Hogg.

Flowers of *L. speciosum* are reddish pink or light pink with dark red spots or rarely whole white. There are three wild varieties, var. *speciosum*, var. *clivorum* S. Abe & Tamura, and var. *gloriosoides* Baker, and several horticultural races. Natural habitats of *L. speciosum* var. *speciosum* occur in the Koshiki Islands (Satsuma-sendai City), Nagashima Island (Nagashima Town), Takaonomachi-euchi (Izumi City), Hashima and Nishi-ichiki (Ichiki-kushikino City), and Nishi-minami-kata (Minami-satsuma City) of Kagoshima Prefecture, Amakusa Islands in Kumamoto Prefecture and Munakata (Munakata City) in Fukuoka Prefecture. *Lilium speciosum* var. *clivorum* grows in Kochi and Tokushima Prefectures in Shikoku, and in the Nishi-sonogi Peninsula and the Kuju-ku-shima Islands in Nagasaki Prefecture (Hatushima 1986, Tamura and Takahashi 2015, Hayashi 2016, Suzuki *et al.* 2022). *Lilium speciosum* var. *gloriosoides* is distributed in the mainland China and Taiwan (Zhang 2016, Zhong & Xu 2016).

Japanese wild lilies have been used as garden plants and for breeding horticultural races in Japan and overseas, but their natural distributions have recently become limited. *Lilium speciosum* var. *speciosum* was categorized into Endangered II (Vulnerable: VU) on the Red List 2025 (https://www.env.go.jp/press/press_04578.html) by the Ministry of the Environment of Japan as few natural populations remain in Kyushu Island and other outlying small islands (Kagoshima Prefectural Office 2016). Moreover, its habitats are partly protected. For example, a large area of the Koshiki Islands was designated a Kagoshima Prefectural Park in 1981, and then upgraded to a Quasi National Park in 2015. *Lilium speciosum* var. *speciosum* usually grows in the coastal grassland and forest margins on three inhabited islands, including Kami-koshiki-shima (44.1 km^2), Naka-koshiki-shima (7.3 km^2), and Shimo-koshiki-shima (66.1 km^2), as well as on some small, uninhabited islands near these three main islands (Ogawa 2001). In contrast, several small populations living outside protected areas in southwestern Kyushu Island are seriously endangered or almost extinct.

In this research, we analyzed the genetic structure of 33 plants of *Lilium speciosum* var. *speciosum*, collected from nine natural populations in the Koshiki Islands and southwestern Kyushu Island, Japan. We used the Multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) method (Suyama *et al*. 2021) to clarify their phylogenetic relationship and genetic structure. This method is PCR-based single nucleotide

polymorphism genotyping using the next-generation sequencer (Suyama and Matsui 2015). We expected to detect genome-wide genetic variations despite the large size of the *Lilium* species genome.

Materials and Methods

Plant samples were collected with official permissions from two populations in Kami-koshiki-shima, two populations in Naka-koshiki-shima, and four populations in Shimo-koshiki-shima in the Koshiki Islands (Satsuma-sendai City) in 2023. The samples from Kuyshu Island were collected from one population in Hashima (Ichiki-kushikino City) in 2004. Distances between the growing positions of the two optional sampled plants were over 1 m in each plant. Information on the plant samples is shown in Table 1, and a map of the collection sites is shown in Fig. 1. Young leaves of each plant were collected and rapidly dried using silica gel desiccant sachets. Dried voucher specimens of whole plants without bulbs were stored in the laboratories of the Faculty of Agriculture or Faculty of Science of Kagoshima University.

Total genomic DNA was extracted from 50 mg dried leaf tissue of each material using a DNA extraction kit (DNeasy Plant Kit, QIAGEN, Germantown, MD, USA). The DNA solution was concentrated to approximately 50 ng/µl by the ethanol precipitation method. The MIG-seq analysis was conducted by Bioengineering Lab. Co. Ltd (Sagamihara, Japan) used the protocols of Suyama *et al*. (2021) and Suyama and Matsui (2022). The genome

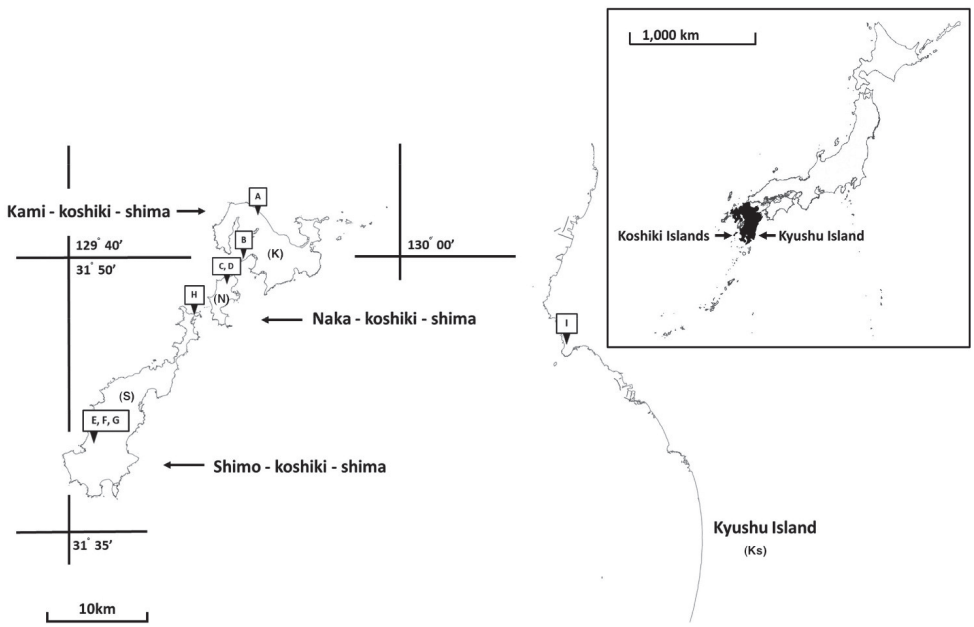

Fig. 1 A right map showing the location of Koshiki Islands and Kyushu Island in Japan. A left map showing the location of the collecting sites in Kami-koshiki-shima (K), Naka-koshiki-shima (N), Shimo-koshiki-shima (S) and Kyushu Island (Ks). Abbreviations A-I represent the site names in Table 1.

library was constructed by using the two-step tailed PCR method. The first PCR products were amplified by using tailed inter-simple sequence repeats (ISSRs) primers and used as templates for the second-tailed PCR. Reaction mixtures were treated at 94℃ for 1 min. 25 cycles of 94℃ for 30 sec, 38℃ (1st PCR) or 60℃ (2nd PCR) for 1 min and 72℃ for 1min, then 72℃ for 10 min (1st PCR) or 5 min (2nd PCR) on a thermal cycler. The primer sequences for the first and the second PCR are shown in Table 2. The PCR products were purified,

Table 1. Sampling information of 33 plants of 9 collecting sites (A-I).

Site name	Plant No.	Latitude	Longitude	Altitude (m)	Island name (Abbreviation)	Geographical information	Vegetation environment	Sampling date (dd-mm-yy)
A	1. 2. 3	31° 52' 15.42" N	129° 52' 02.00" E	9	Kami-koshiki-shima (K)	Segami, Kami-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	19-Jul-23
B	4, 5, 6, 7	31° 49' 56.24" N	129° 50' 51.59" E	7	Kami-koshiki-shima (K)	Naka-koshiki, Kami-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	19-Jul-23
C	13, 14, 15, 16, 17	31° 48' 59.37" N	129° 50' 10.27" E	109	Naka-koshiki-shima (N)	Taira, Kami-koshiki-cho, Satsuma-sendai, City, Kagoshima Pref., Japan	grassland	21-Jul-23
D	18, 19, 20, 21, 22	31° 48' 52.67" N	129° 50' 19.86" E	72	Naka-koshiki-shima (N)	Taira, Kami-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	21-Jul-23
E	8, 9	31° 40' 02.39" N	129° 40' 48.60" E	85	Shimo-koshiki-shima (S)	Katanoura, Shimo-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	20-Jul-23
F	10, 11	31° 39' 59.82" N	129° 40' 50.22" E	120	Shimo-koshiki-shima (S)	Katanoura, Shimo-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	20-Jul-23
G	12	31° 39' 59.77" N	129° 40' 54.42" E	13	Shimo-koshiki-shima (S)	Katanoura, Shimo-koshiki-cho, Satsuma-sendai City, Kagoshima pref., Japan	grassland	20-Jul-23
H	23	31° 47' 03.42" N	129° 48' 02.96" E	64	Shimo-koshiki-shima (S)	Imuta, Shimo-koshiki-cho, Satsuma-sendai City, Kagoshima Pref., Japan	grassland	21-Jul-23
I	24, 25, 26, 27, 28, 29, 30, 31, 32, 33	31° 46' 09.63" N	130° 11' 28.31" E	131	Kyushu Island (Ks)	Hashima, Ichiki-kushikino-City, Kagoshima Pref., Japan	roadside grassy belt	4-Apr-04

Table 2. Sequences of primers for the first and second PCR.

	Primer name	Sequence (5'—3')
Forward primers for the first PCR	(ACT)4 TG-f	GAACGACATGGCTACGATCCGACTTNNNCTGACTACTACTACTTG
	(CTA)4 TG-f	GAACGACATGGCTACGATCCGACTTCTGCTACTACTACTATG
	(TTG)4 AC-f	GAACGACATGGCTACGATCCGACTTNCTGTTGTTGTTGTTGAC
	(GTT)4 CC-f	GAACGACATGGCTACGATCCGACTTNNCTGGTTGTTGTTGTTCC
	(GTT)4 TC-f	GAACGACATGGCTACGATCCGACTTNCTGGTTGTTGTTGTTTC
	(GTG)4 AC-f	GAACGACATGGCTACGATCCGACTTNNNNCTGGTGGTGGTGGTGAC
	(GT)6 TC-f	GAACGACATGGCTACGATCCGACTTCTGGTGTGTGTGTGTTC
	(TG)6 AC-f	GAACGACATGGCTACGATCCGACTTNNCTGTGTGTGTGTGTGAC
Reverse primers for the first PCR	(ACT)4 TG-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNNNGACACTACTACTACTTG
	(CTA)4 TG-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTGACCTACTACTACTATG
	(TTG)4 AC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNNNNGACTTGTTGTTGTTGAC
	(GTT)4 CC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNNGACGTTGTTGTTGTTCC
	(GTT)4 TC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNGACGTTGTTGTTGTTTC
	(GTG)4 AC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNNNGACGTGGTGGTGGTGAC
	(GT)6 TC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTNNGACGTGTGTGTGTGTTC
	(TG)6 AC-r	GTCTTCCTAAGACCGCTTGGCCTCCGACTTGACTGTGTGTGTGTGAC
Forword primer for the second PCR	2ndF	GAACGACATGGCTACGATCCGAC
Reverse primer for the second PCR	2ndR	TGTGAGCCAAGGAGTTG-Index1- TTGTCTTCCTAAGACCGCTTGGCCTC

and approximately 500-900 bp fragments were selected using DNA Clean Beads (MGI Tech, Shenzhen, China). The concentration of the library was measured with a dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) on a Qubit 3.0 Fluorometer. Circular DNAs and the DNA nano-ball (DNB) were made by using the MGI Easy Circularization Kit and DNB Rapid Make Reagent Kit (MGI Tech). Fragment sequences were detected on a sequencer, DNBSEQ-T7 (MGI Tech), using a High-throughput Sequencing Kit and High-throughput Pair-End Sequencing Primer Kit (MGI Tech). The primer and adaptor regions were trimmed from each sequence using fastx_trimmer (ver. 0.0.14) and cutadapt (ver. 4.0) software, and the paired-reads with over 75 nucleotide sequences and over 30 quality scores were selected using sickle (ver. 1.33). The data of paired-reads with 75 nucleotide sequences were used for genotyping SNPs. A phylogenetic tree was constructed by the maximum likelihood method by using the GTR+gamma model of RAxML (ver. 8.2.9) and the pgsumtree of Phylogears2 (ver. 2.0.2015.11.30). The genetic structure was analyzed using the 'structure' program of Stacks (K=1-8, R=0.8), and the delta K was calculated by Structure Harvester (ver. 0.6.93).

Table 3. Numbers of pair-reads, length of segments (bp) and numbers of selected pair-reads of 33 samples.

Plant No.	Numbers of pair–reads	Length of segments (bp)	Numbers of selected pair–reads
1	1,038,333	311,499,900	866,707
2	1,152,201	345,660,300	964,202
3	1,134,113	340,233,900	928,814
4	1,054,444	316,333,200	893,782
5	1,110,450	333,135,000	926,831
6	1,047,544	314,263,200	889,180
7	1,080,300	324,090,000	919,676
8	847,783	254,334,900	711,909
9	1,061,824	318,547,200	893,470
10	847,759	254,327,700	712,504
11	995,614	298,684,200	824,463
12	594,594	178,378,200	503,718
13	884,816	265,444,800	746,803
14	643,427	193,028,100	543,038
15	1,146,564	343,969,200	951,567
16	1,114,358	334,307,400	938,552
17	1,026,059	307,817,700	866,407
18	937,801	281,340,300	798,099
19	1,068,863	320,658,900	871,875
20	964,292	289,287,600	795,803
21	1,150,973	345,291,900	934,089
22	1,016,873	305,061,900	857,846
23	1,103,982	331,194,600	911,414
24	1,025,174	307,552,200	845,505
25	878,774	263,632,200	741,351
26	829,361	248,808,300	687,762
27	878,133	263,439,900	651,568
28	904,838	271,451,400	682,623
29	915,624	274,687,200	708,242
30	873,568	262,070,400	720,665
31	918,929	275,678,700	755,280
32	970,772	291,231,600	842,611
33	813,475	244,042,500	693,489
Total	32.031,615	9,609,484,500	26,579,845

Results and Discussion

The concentration of the genome library was 5.6 ng/μl. The number of paired-reads, length of segments (bp), and the number of paired-reads, selected by sickle are shown in Table 3. In total, sequences of 9,609,484,500 base pairs of 32,031,615 paired-reads were clarified, and 26,579,845 paired-reads were selected. The range of selected paired-reads numbers per sample varied from 503,718 (Plant No. 12) to 951,567 (Plant No. 15). No identical sequences were found among the 33 samples, although *L. speciosum* and many other lilies were able to asexually reproduce by dividing bulbs. Therefore, all material plants in this research seemed to be reproduced sexually by seeds or clones of seedlings. These sequence data of the

selected paired-reads were used for estimating the phylogeny and analyzing the genetic structure.

A neighbor-joining phylogenetic tree constructed by the maximum likelihood method is shown in Fig. 2. Three groups, I, II and III, were recognized on the tree. Group I included plants No. 1, 2, 3 and 7, collected from Kami-koshiki-shima. Group II was a clade consisting of No. 24-33 from Kyushu Island and No. 8-12 from Shimo-koshiki-shima. Group III included No. 4-6 of Kami-koshiki-shima, No. 13-22 of Naka-koshiki-shima and No. 23 of Shimo-koshiki-shima.

The delta K value at K=2 to K=8 was shown in Fig. 3. The value at K=3 was the highest. The genetic structure analysis at K=2 to K=8 is shown in Fig. 4. Numbers 1-33, letters A-I and K, S, N, and Ks at the bottom of Fig. 4 are the plant number, the site name, and the abbreviations of islands in Table 1, respectively. The Kami-koshiki-shima population (No. 1-7) belonged to a same group at K=2 to K=6, and the populations from Site A and Site B were slightly different at K=7 to K=8. The Shimo-koshiki-shima population (No. 8-13) belonged to a same group at K=2 to K=8. One plant (No. 23 of Site H) from Shimo-koshiki-shima was genetically similar to No. 21 of Site D in Naka-koshiki-shima. Naka-koshiki-shima population (No. 13-22) was polymorphic at K=3 to K=8. Plants No. 16 and 17 of Site C belong to the same group but different from all other plants. These two plants showed genetically very close to each other in the phylogeny (Fig. 2). The Kyushu Island population (No. 24-33) was polymorphic from K=2 to K=8. Plant No. 33 included similar parts of some major elements of plants from the Koshiki Islands, such as, No. 1-3 of Kami-koshiki-shima, No. 8-12 of Shimo-koshiki-shima, and No. 14, 15, 18, 19, 20 and 22 of Naka-koshiki-shima. Results of the genetic structure analysis showed that plants of the Koshiki Islands and Kyushu Island tended to be genetically different, and that the plants of Kyushu Island and Naka-koshiki-shima were polymorphic.

Three individuals (No. 1-3) remained at Site A at the highest latitude in the northern part of Kami-koshiki-shima. In contrast, populations in Naka-koshiki-shima, Shimo-koshiki-shima, and Kyushu were derived relatively. The phylogenetic Group II consisted of plants of the Kyushu and Shimo-koshiki-shima populations. A bootstrap value of the inner node at the common ancestor of these plants was 0.53. This value was not high, but the genetic structures between Kyushu and Shimo-koshiki-shima populations were largely different, even at K=2 (Fig. 3). The genetic structure of plants of sites, E, F, and G in Katanoura in the southwestern part of Shimo-koshiki-shima were tended to be unique. The phylogenetic Group III included plants of Sites B, C, D, and H. These four sites were geographically close despite Site B being in Kami-koshiki-shima, C and D in Naka-koshiki-shima, and H in the northern peninsula of Shimo-koshiki-shima. A small island, Naka-jima, is located between Kami-koshiki-shima and Naka-koshiki-shima, with two narrow channels between the islands. Imuta-seto Strait is between Naka-koshiki-shima and Shimo-koshiki-shima, and the narrowest distance between the islands is approximately 1 km. Therefore, the pollination by insects and/or the seed dispersal by strong wind are possible across the channels or Imuta-seto Strait.

The geographical and lithological conditions of the research areas are as follows: The

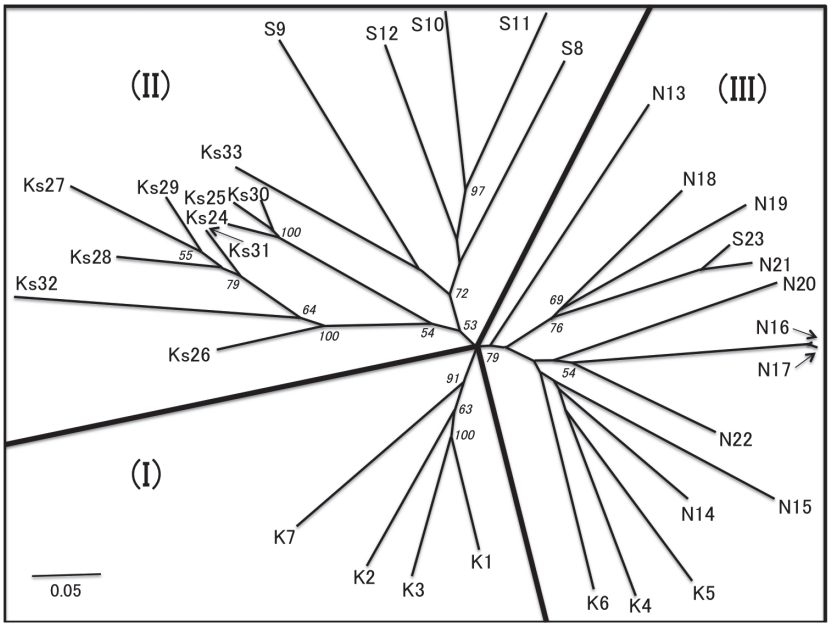

Fig. 2 A phylogenetic tree of 33 plants of Lilium speciosum var. speciosum constructed by maximum likelihood method. The three separated areas show phylogenetic groups, I, II and III. Letters, K, N, S and Ks, and numbers 1-33 at the end of branches are the abbreviations of island names and sample numbers of Table 1, respectively. Bootstrap values over 50 were shown as italic numbers at internal nodes. The bar (0.05) at the bottom is a scale for the genetic distance.

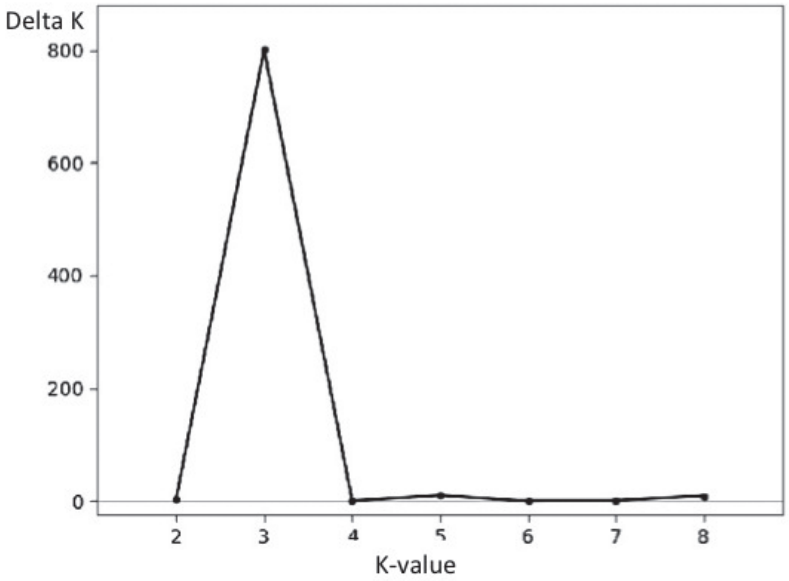

Fig. 3 The delta K value at K = 2 to K = 8.

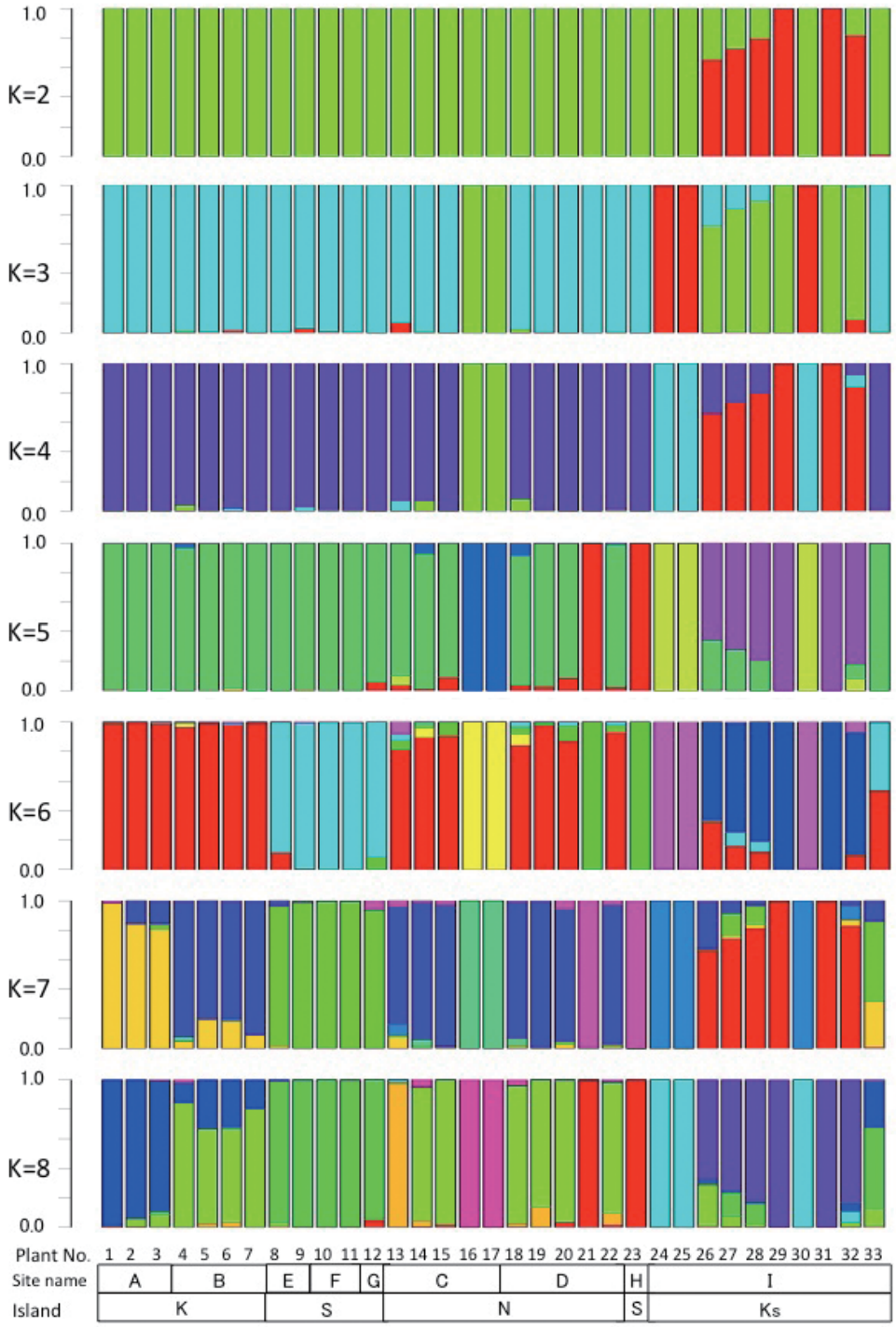

Fig. 4 The genetic structure of 33 plants. Numbers 1-33 at the bottom are the plant numbers. Letters A-I represent collection sites. Letters K, S, N, and Ks are abbreviations of islands.

Koshiki Islands are located 45 km west of Kyushu Island and spread approximately 38 km from the northeast in a southwest direction. Hashima of Kyushu Island (Site I) has igneous rocks, whereas the Koshiki Islands have other rock types, such as sandstone, mudstone, and granodiorite, formed during the eras of Cretaceous of the Mesozoic to Quaternary of the Cenozoic (Inoue *et al*. 1982, Rehman *et al*. 2024). The distribution of these rocks in the Koshiki Islands is as complicated as a mosaic. The interaction between the geographical lithological condition and the intraspecific genetic variation of this plant has not been recognized. The collection sites used for this research are located in the grasslands of the Koshiki Islands (Sites A-H) and in the roadside grassy belt surrounded by secondary forests in Hashima of Kyushu (Site I). All of these areas are in the warm temperate zone and are expected by broad-leaved evergreen forests such as *Symploco glaucae-Castanopsietum sieboldii* community as phytosociological vegetation classification. The present vegetation of the Koshiki Islands is mainly secondary forest following logging and sprouting and wind-exposed grasslands and semi-natural grasslands maintained by fire burning are in the islands (Terada and Kawanishi 2021, Kawanishi *et al*. 2024).

We need to pay attention to the artificial disturbance of the natural habitats of *L. speciosum* caused by humans during the past several centuries. *Lilium speciosum* has been used outside its naturally distributed area as a garden plant at least since the 17th Century, and was introduced to Europa in the 18th Century. Indeed, many *L. speciosum* bulbs were exported worldwide in the 19th and 20th Centuries (Miyamoto *et al*. 2024). The bulbs or plants were transplanted by humans when the plants carried attractive flowers and/or other proper characteristics for horticulture. We collected plant samples from their natural habitats, but the possibility of prior human disturbance of the *Lilium* populations cannot be excluded.

It is difficult to fully understand the relationship between genetic variations and the growing environment of the plants. However, Sites E, F, and G in the Katanoura of southwestern Shimo-koshiki-shima are worth mentioning. The genetic structure of the plants from these three sites tended to be monophyletic. The plants grow in wind-exposed grassland on the seaside slope that faces the East China Sea. The slope is surrounded by 350-450 m hill-ridges covered by thick broad-leaved evergreen forest. This grassland on the slope is isolated from other population of *L. speciosum.* This isolation possibly caused the genetically originality in this area.

In conclusion, a part of the genetic variation and relationship was clarified in natural populations of *L. speciosum* var. *supeciosum* in the Koshiki Islands and southwestern Kyushu Island. Further investigation is necessary to fully understand the phylogeny and genetic structure of this species, including all varieties in other natural habitats and related horticulture races.

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