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A new record of *Ardisia* × *walkeri*, a hybrid of *A. japonica* and *A. pusilla*, (Primulaceae) from Jeju Island, Korea

Goro Kokubugata[1,*], Satoshi Kakishima[2,*], Chan-ho Park[3,*], Takuro Ito[4], Atsushi Abe[5], Chikako Ishii[1] and Gwan-Pil Song[6]

[1]*Department of Botany, National Museum of Nature and Science, Tsukuba, Ibaraki 305-0005, Japan*
[2]*Center for Molecular Biodiversity Research, National Museum of Nature and Science, Tsukuba, Ibaraki 305-0005, Japan*
[3]*National Institute of Biological Resources, Seo-gu, Incheon 22689, Republic of Korea*
[4]*The Center for Academic Resources and Archives/Botanical Gardens, Tohoku University, Sendai, Miyagi 980-0862, Japan*
[5]*Okinawa Churashima Research Center, Okinawa Churashima Foundation, Ishikawa, Motobu, Okinawa 905-0206, Japan*
[6]*Jeju Environment Research Institute, Jeju city, Jeju 63040, Republic of Korea*

**Correspondent: gkokubu@kahaku.go.jp; kakishima@kahaku.go.jp; ddony@korea.kr*

We conducted phylogenetic analyses using multiplexed inter-simple sequence repeat genotyping by sequencing and compared chloroplast DNA sequences among *Ardisia japonica*, *A. pusilla*, and morphologically intermediate plants found on Jeju Island, Korea. Our network analysis demonstrated that the intermediate plants were genetically positioned between *A. japonica* and *A. pusilla*. Our comparison of the intergenic spacer between the *psb*A and *trn*H genes in chloroplast DNA indicated that four nucleotide substitutions separate *A. japonica* and *A. pusilla*, whereas the intermediate plants exhibited the *A. japonica* haplotype. Our results suggest that the intermediate plants on Jeju Island represent a natural hybrid of *A. japonica*, as the maternal species, and *A. pusilla*, and that they are attributable to *Ardisia* × *walkeri*. This record constitutes the first documented occurrence of the hybrid taxon in Korea.

Keywords: cpDNA, hybridization, Jeju Island, Korea, MIGseq

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Introduction

Ardisia Swartz comprises 400–500 species of trees, shrubs, subshrubs and (rarely) herbs that are distributed mainly in East and Southeast Asia (Jie and Pipoly III, 1996). Four species have been documented in Korea, including *A. crenata* Sims, *A. crispa* (Thunb.) A.DC., *A. japonica* (Hornsted) Blume (Fig. 1A), and *A. pusilla* de Candolle (Fig. 1B) (Chang *et al*., 2017). These species are distributed throughout China, Japan, Korea, and Taiwan (Yamazaki, 1993; Chang *et al*., 2017). In the four species, both *A. japonica* and *A. pusilla* belong to *Ardisia* sect. *Bladhia* (Thunberg) Mez ex Walker, and are typically small shrubs characterized by their leaf texture and the presence or absence of long hairs on young stems and petioles. The former has subcoriaceous leaves (Fig. 1A) and lacks long hairs on the young stems and petioles (Fig. 2A), whereas the latter has chartaceous leaves (Fig. 1B) and long hairs on the young stems and petioles (Fig. 2C) (Yamazaki, 1993). Previously, a natural hybrid of *A. japonica* and *A. pusilla*, *A.* × *walkeri* Yuen P. Yang, studied herein, has been documented in Japan.

Ardisia × *walkeri* was formally described based on a type specimen collected from Shizuoka Prefecture, Japan (Yang and Dwyer, 1989). It was previously treated as *A. japonica* var. *montana* Miq. or *A. montana* (Miq.) Siebold ex Franch. et Sav., but Yang and Dwyer (1989) noted that the epithet *montana* has already been applied to other *Ardisia* species. *Ardisia* × *walkeri* has been found in the Kanto and Kyushu Districts of Japan, as well as the Izu Islands and Tokunoshima Island in the Ryukyu Archipelago east of the Japanese mainland (Koyama and Kokubugata, 1998), and recent studies consider it to be endemic to Japan (Takahashi, 2017). Previous studies based on morphological data have revealed that *A.* × *walkeri* has subcoriaceous leaves, similar to *A. japonica* (Fig. 1C), and long hairs on young stems and petioles, similar to *A. pusilla* (Fig. 2C), and thus, it has been thought to be a natural hybrid of the two species (Walker, 1954; Yang and Dwyer, 1989; Yamazaki, 1993; Koyama and Kokubugata, 1998). Based

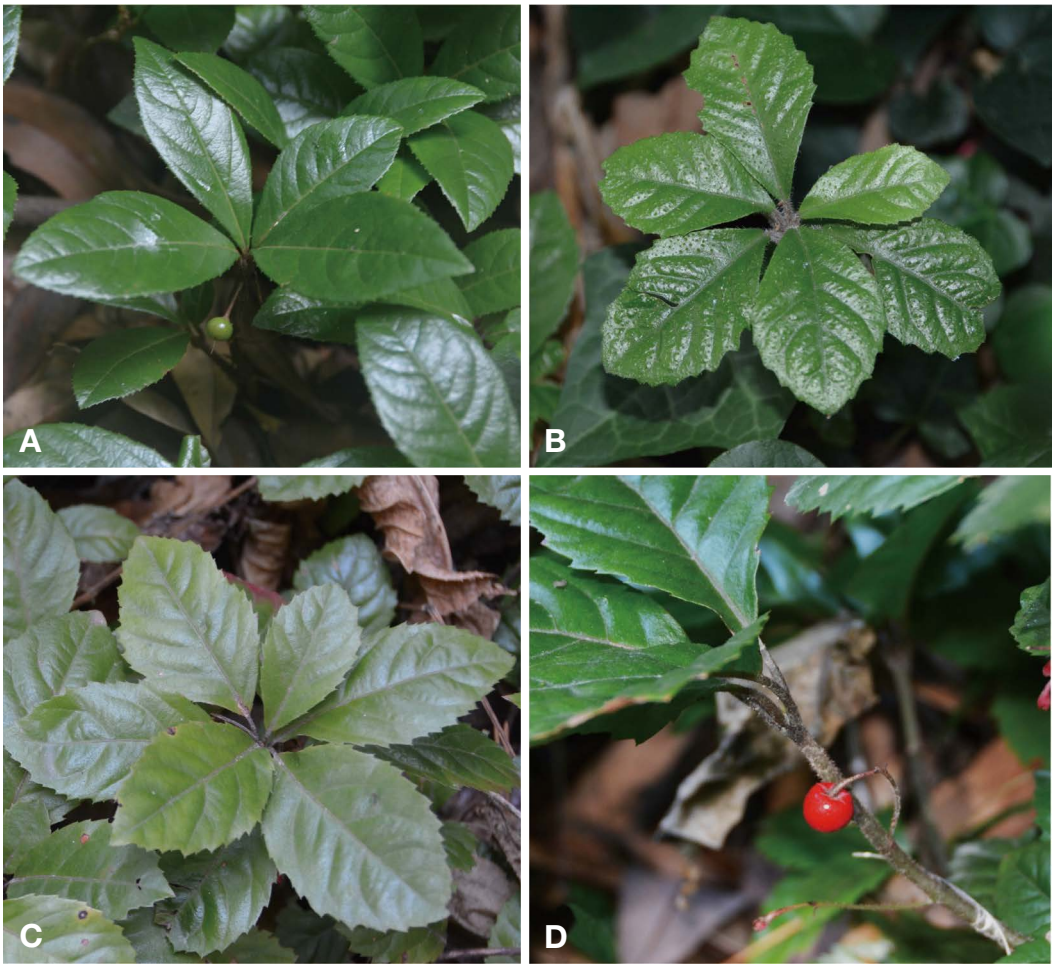

Fig. 1. Plants of the two *Ardisia* species and the putative hybrid from Jeju Island. A. *A. japonica* from Japan (*G. Kokubugata9130*). B. *A. pusilla* from Japan (*G. Kokubugata21622*). C. *Ardisia* spp. from Jeju Island (*G. Kokubugata21540*). D. Fruit of *Ardisia* spp. from Jeju Island (*G. Kokubugata21540*). Refer to Table 1 for voucher specimen numbers.

on molecular comparisons using the internal transcribed spacer sequence (ITS) of nuclear DNA, Kokubugata *et al*. (2019) confirmed that plants from the Kanto District (*J. Hagiwara38574* and *H. Koyama10231*, both included in our study) morphologically identified as *A.* × *walkeri* originated from a hybrid of *A. japonica* and *A. pusilla*. Furthermore, Kokubugata *et al*. (2019) confirmed *A. japonica* as the maternal parent based on the intergenic spacer sequence between the *psb*A and *trn*H genes (*psb*A-*trn*H) of chloroplast DNA (cpDNA).

Jeju Island is a volcanic island located off the southern coast of the Korean Peninsula. The island is 73 km east to west and 31 km north to south, with an area of 1,847 km^2. Mount Halla is the highest mountain on the island (Woo *et al*., 2013). Jeju Island is believed to have received floristic elements from mainland Korea and China, as well as from Kyushu in southern Japan. Tropical species occur in the lowlands (e.g., Chung *et al*., 2013), whereas temperate species are found at higher elevations (e.g., Kong, 2000; Dolezal *et al*., 2012). By contrast, a few species, such as *Sedum tosaense* Makino (Crassulaceae), have a disjunct distribution and occur on both Jeju Island and Shikoku, situated on the Pacific Ocean side of Japan (Ito *et al*., 2014). In 2019, we conducted field surveys on Jeju Island and found *Ardisia* plants with subcoriaceous leaves (Fig. 1C) and long hairs on the young branchlets and petioles (Fig. 2B), and were identifiable as *A.* × *walkeri*, which hitherto has not been reported in Korea.

Our objectives were to conduct molecular phylogenetic analyses on the putative *A.* × *walkeri* plants from Korea, specifically multiplexed inter simple sequence repeat genotyping by sequencing (MIG-seq), to confirm their origin from both *A. japonica* and *A. pusilla*. In addition, we compare the *psb*A-*trn*H intergenic spacer region of cpDNA to identify the maternal parent of the hybrid.

Fig. 2. Young stems and petioles of *Ardisia* plants from Jeju Island. A. *A. japonica* (*G. Kokubugata21530*). B. *Ardisia* spp. (*G. Kokubugata 21540*). C. *A. pusilla* (*G. Kokubugata21533*). The bar indicates 3 mm. Refer to Table 1 for voucher specimen numbers.

Materials and Methods

Specimen collection and DNA extraction

Ardisia japonica and *A. pusilla* were collected from Japan and Jeju Island, and three individuals morphologically similar to *A.* × *walkeri* were collected from a population on Jeju Island (Table 1). Voucher specimens were deposited in the herbaria of the National Museum of Nature and Science, Japan (TNS) and the National Institute of Biological Resources, Korea (KB).

DNA extraction, PCR, and sequencing

DNA was extracted from fresh leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. DNA samples from *A. japonica*, *A. pusilla*, and *A.* × *walkeri* obtained from previously collected Japanese plants (Kokubugata *et al*., 2019) were used for the present MIG-seq analyses.

We used sequences from *psb*A-*trn*H in cpDNA for maternal species identification following the same protocols used in an earlier study of *A.* × *walkeri* from Japan (Kokubugata *et al*., 2019). The *psb*A-*trn*H sequence was amplified using psbAF (5′-GTT ATG CAT GAA CGT AAT GCT C-3′) as the forward primer and trnHR (5′-CGC GCA TGG ATT CAC AAA TC-3′) as the reverse primer (Sang *et al*., 1997). The PCR temperature cycling program comprised 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a hold at 4°C to terminate the reaction. PCR was performed on the GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) using EmeraldAmp PCR Master Mix (TaKaRa, Otsu, Japan). The PCR products were examined by electrophoresis prior to purification using the Illustra^{TM} ExoProStar (GE Healthcare UK Ltd., Chalfont St. Giles, UK). Cycle sequencing was performed with the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) using the PCR primers. The cycle sequencing products were purified by ethanol precipitation. Automated sequencing was performed on the 3130xl Genetic Analyzer (Applied Biosystems). The electropherograms were assembled using ATGC ver. 4.01 (Genetyx Co., Tokyo, Japan). We compared the *psb*A-*trn*H sequences among the three *Ardisia* species and deposited the sequencing data in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The *psb*A-*trn*H sequences of *Ardisia* species obtained for a different study (Kokubugata *et al*., 2019) were used in the cpDNA haplotype comparison (Table 1).

Network analysis using MIG-seq data for single-nucleotide polymorphisms

We used MIG-seq, which can detect single-nucleotide polymorphisms (SNPs) (Suyama and Matsuki, 2015), to clarify the genetic differences among *Ardisia* species (Table 1, BioProject accession number PRJNA1000073). Preparation of the MIG-seq library followed Suyama and Matsuki (2015). In the first PCR, Primer Set 1 was used (Suyama and Matsuki, 2015), and the effect of different cycle numbers (25, 30, or 35 cycles) was investigated. All products were used in the subsequent analyses. We low-

Table 1. Plant materials investigated, and their GenBank accession numbers and variable sites in *psb*A-*trn*H sequences.

Taxon	Collection locality	Collection number (herbarium)	Abbreviation*	GenBank accession no.**	*psb*A-*trn*H sequence position			
					165	187	321	322
A. japonica	JAPAN, Tohoku, Akita, Senhoku City	*G. Kokubugata21787* (TNS)	JJ-GK21787	LC773641[a]	T	T	A	T
	JAPAN, Tohoku, Miyagi, Sendai City	*G. Kokubugata21872* (TNS)	JJ-GK21872	LC773642[a]	T	T	A	T
	JAPAN, Hokuriku, Toyama, Toyama City	*G. Kokubugata19452* (TNS)	JJ-GK19452	LC773643[a]	T	T	A	T
	JAPAN, Kanto, Ibaraki, Ishioka City	*G. Kokubugata19525* (TNS)	JJ-GK19525	LC773644[a]	T	T	A	T
	JAPAN, Kanto, Ibaraki, Hitachi-ota City	*G. Kokubugata21762* (TNS)	JJ-GK21762	LC773645[a]	T	T	A	T
	JAPAN, Izu Islands, Tokyo, Kozu Is., Kozu-cho	*A. Ebihara & Y. Saito 2834* (TNS)	JJ-ES2834	LC437979[b]	T	T	A	T
	JAPAN, Tokai, Gifu, Ibikawa Town	*G. Kokubugata8724* (TNS)	JJ-GK8724	LC437978[b]	T	T	A	T
	JAPAN, Kyushu, Kumamoto, Itsuki Village	*G. Kokubugata9130* (TNS)	JJ-GK9130	LC437981[b]	T	T	A	T
	JAPAN, Kyushu, Kagoshima, Isa City	*G. Kokubugata9088* (TNS)	JJ-GK9088	LC437982[b]	T	T	A	T
	JAPAN, Kyushu, Kagoshima, Kagoshima City	*G. Kokubugata19868* (TNS)	JJ-GK9868	LC773646[a]	T	T	A	T
	JAPAN, Ryukyus, Kagoshima, Toshima Village, Nakano-shima Is.	*G. Kokubugata11320* (TNS)	JJ-GK11320	LC437983[b]	T	T	A	T
	KOREA, Jeju, Jeju Is. Jeju City	*G. Kokubugata21525* (TNS)	JK-GK21525	LC773647[a]	T	T	A	T
	KOREA, Jeju, Jeju Is. Jeju City	*G. Kokubugata21526* (TNS)	JK-GK21526	LC773648[a]	T	T	A	T
	KOREA, Jeju, Jeju Is. Jeju City	*G. Kokubugata21530* (TNS)	JK-GK21530	LC773649[a]	T	T	A	T
	KOREA, Jeju, Jeju Is. Seogwipo City	*G. Kokubugata21531* (TNS)	JK-GK21531	LC773650[a]	T	T	A	T
	KOREA, Jeju, Jeju Is. Seogwipo City	*G. Kokubugata21532* (TNS)	JK-GK21532	LC773651[a]	T	T	A	T
	KOREA, Jeju, Jeju Is. Seogwipo City	*G. Kokubugata21538* (TNS)	JK-GK21538	LC773652[a]	T	T	A	T
A. × *walkeri*	JAPAN, Kanto, Chiba, Isumi City	*J. Hagiwara38574* (TNS)	WJ-JH38574	LC437985[b]	T	T	A	T
	JAPAN, Kanto, Chiba, Kominato Town	*H. Koyama10231* (TNS)	WJ-HK10231	LC437986[b]	T	T	A	T
Ardisia sp.	KOREA, Jeju, Seogwipo City	*G. Kokubugata21540* (TNS)	K-GK21540	LC773653[a]	T	T	A	T
	KOREA, Jeju, Seogwipo City	*G. Kokubugata21541* (TNS)	K-GK21541	LC773654[a]	T	T	A	T
	KOREA, Jeju, Seogwipo City	*G. Kokubugata21542* (TNS)	K-GK21542	LC773655[a]	T	T	A	T
A. pusilla	JAPAN, Tokai, Shizuoka, Shizuoka City	*G. Kokubugata21622* (TNS)	PJ-GK21622	LC773656[a]	G	G	C	G
	JAPAN, Tokai, Shizuoka, Shizuoka City	*G. Kokubugata21627* (TNS)	PJ-GK21627	LC773657[a]	G	G	C	G
	JAPAN, Shikoku, Kochi, Yasuda Town	*G. Kokubugata21588* (TNS)	PJ-GK21588	LC773658[a]	G	G	C	G
	JAPAN, Shikoku, Kochi, Toyo Town	*G. Kokubugata10166* (TNS)	PJ-GK10166	LC773659[a]	G	G	C	G
	JAPAN, Kyushu, Oita, Usuki City, Kuroshima Is.	*G. Kokubugata21648* (TNS)	PJ-GK21648	LC773660[a]	G	G	C	G
	JAPAN, Kyushu, Kumamoto, Amakusa City	*G. Kokubugata21636* (TNS)	PJ-GK21636	LC773661[a]	G	G	C	G
	JAPAN, Kyushu, Kumamoto, Ashikita City	*G. Kokubugata21633* (TNS)	PJ-GK21633	LC773662[a]	G	G	C	G

Table 1. Continued.

Taxon	Collection locality	Collection number (herbarium)	Abbreviation*	GenBank accession no.**	*psbA-trn*H sequence position			
					165	187	321	322
A. pusilla	JAPAN, Kyushu, Kagoshima, Ibusuki City	*G. Kokubugata19219* (TNS)	PJ-GK19219	LC773663[a]	G	G	C	G
	JAPAN, Kyushu, Kagoshima, Ibusuki City	*G. Kokubugata19220* (TNS)	PJ-GK19220	LC773664[a]	G	G	C	G
	JAPAN, Ryukyus, Tanega-shima Is., Nishinoura City	*G. Kokubugata19214* (TNS)	PJ-GK19214	LC773665[a]	G	G	C	G
	JAPAN, Ryukyus, Tanega-shima Is., Nishinoura City	*G. Kokubugata18030* (TNS)	PJ-GK18030	LC773666[a]	G	G	C	G
	JAPAN, Ryukyus, Okinawa Is., Kunigami Village	*G. Kokubugata8980* (TNS)	PJ-GK8980	LC437989[b]	G	G	C	G
	JAPAN, Ryukyus, Okinawa Is., Higashi Village	*G. Kokubugata9030* (TNS)	PJ-GK9030	LC437990[b]	G	G	C	G
	JAPAN, Ryukyus, Okinawa Is., Nago City	*A. Abe1059-1* (TNS)	PJ-AA1059-1	LC773667[a]	G	G	C	G
	JAPAN, Ryukyus, Taketomi Town, Iriomote Is.	*A. Abe1042-1* (TNS)	PJ-AA1042-1	LC773668[a]	G	G	C	G
	JAPAN, Ryukyus, Taketomi Town, Iriomote Is.	*G. Kokubugata21384* (TNS)	PJ-GK21384	LC773669[a]	G	G	C	G
	KOREA, Jeju, Jeju City	*G. Kokubugata21533* (TNS)	PK-GK21533	LC773670[a]	G	G	C	G
	KOREA, Jeju, Seogwipo City	*G. Kokubugata21534* (TNS)	PK-GK21534	LC773671[a]	G	G	C	G

*Abbreviations refer to the network Fig. 3.
**The *psbA-trn*H sequences registered in the DNA Data Bank of Japan (DDBJ): [a]The present study; [b]Kokubugata *et al.* (2019).

ered the annealing temperature from the original protocol to 38°C to obtain more loci. The products from the second PCR, obtained using the first PCR products as templates, were purified, and fragments of 300−800 bp were isolated using BluePippin (Sage Science, Beverley, MA, USA). The final concentrations were measured using the Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA) and 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

The multiplexed library was sequenced using the Illumina MiSeq Sequencer and MiSeq Reagent Kit ver. 3 (150 cycles; Illumina, Inc., San Diego, CA, USA). The obtained reads (80 bp each) were trimmed to remove the adapter sequences (GTCAGATCGGAAGAGCACACGTCTGAA CTCCAGTCAC and CAGAGATCGGAAGAGCGTCGT GTAGGG AAAGA), the first five bases, and the last base, whereas low-quality regions (quality value <15 within a 4-bp sliding window) and short reads (<74 bp) were removed using Trimmomatic ver. 0.39 (Bolger *et al.*, 2014). Ipyrad (ver. 0.9.20) was used to assemble the reads and obtain SNP markers (Eaton and Overcast, 2020). The minimum sample number was set to 4, the depth of coverage to 3, and the clustering threshold to 0.85. Other parameters were set to the default values.

We used SplitsTree4 ver. 4.18.2 (Huson and Bryant, 2006) to construct a Neighbor-Net network (Bryant and Moulton, 2004) for all samples using the uncorrelated P distance matrix calculated from the SNP matrix. The individual-based genetic structure was estimated using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) in the ipyrad-analysis toolkit. The sample coverage with the minimum number of SNPs was set at 0.8. Ten independent Markov Chain Monte Carlo runs with 100,000 iterations were performed for K (number of clusters) values of 2−5, following a burn-in period of 100,000 steps. The optimum K was estimated based on delta K values, and the mean log-likelihood probabilities were calculated using the ipyrad-analysis toolkit.

Results

Habitat of putative *A.* × *walkeri* plants on Jeju Island

Putative *A.* × *walkeri* plants occurred in a dark, moderately moist environment under a broadleaf canopy at a park with trails in Seogwipo City, Jeju Island. *Ardisia japonica* and *A. pusilla* were not detected in forested habitats.

Network analysis and genetic structure analysis using MIG-seq SNP data

The Neighbor-Net network divided *Ardisia* from Japan and Jeju Island into two terminal clades following estab-

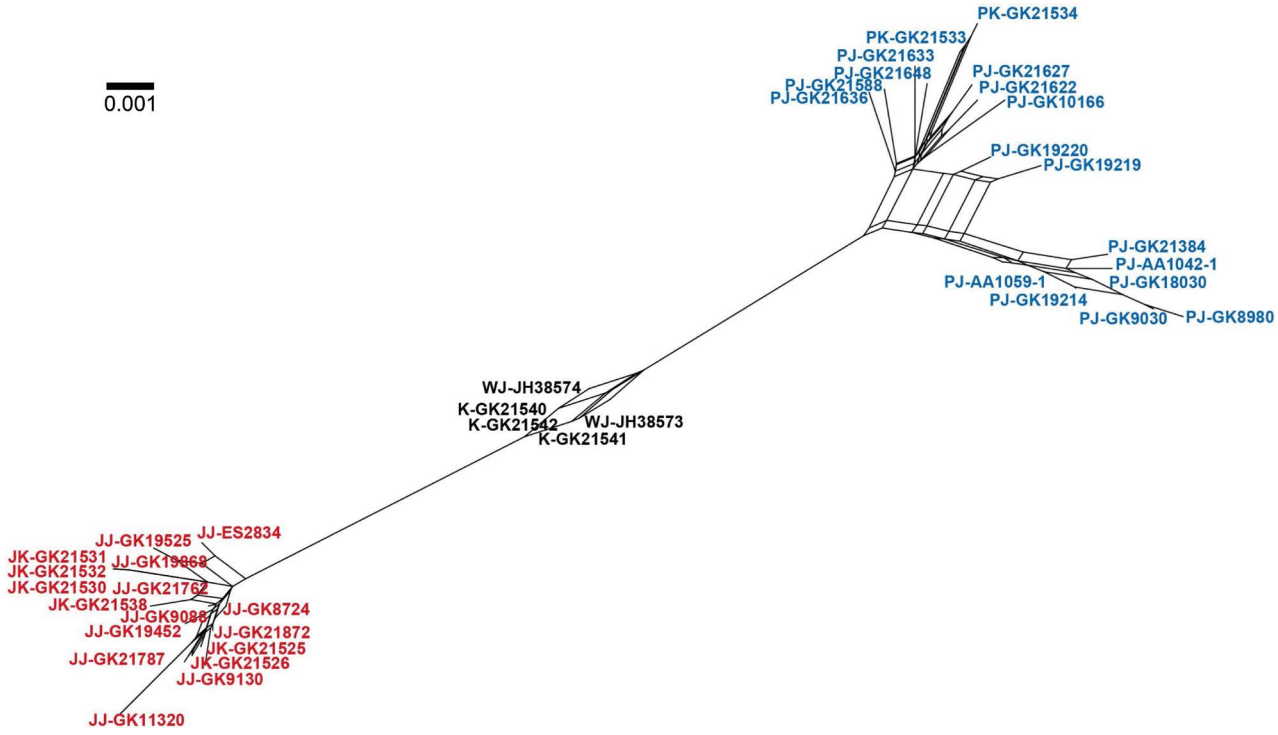

Fig. 3. Neighbor-Net network (lnL = −756467.33) of *Ardisia* plants based on MIG-seq SNP data from the first PCR after 35 cycles (472,598 bp from 3,724 loci).

lished species delimitations: one clade comprised *A. japonica* (red in Fig. 3) and the other *A. pusilla* (red in Fig. 3). Three putative *A.* × *walkeri* specimens from Jeju Island, as well as two *A.* × *walkeri* specimens from Japan, were placed in a separate clade between these two clades (Fig. 3).

The population analysis based on SNP data indicated that K = 2 was the optimal number of clusters for the datasets (Evanno *et al*., 2005) (Fig. 4). Using K = 2, the data supported the two clusters comprising *A. japonica* (red bars in Fig. 3B) and *A. pusilla* (blue bars in Fig. 4). The two *A.* × *walkeri* specimens from Japan and the putative *A.* × *walkeri* specimens from Jeju Island were genetically intermediate to *A. japonica* and *A. pusilla* (red and blue bars in Fig. 4).

Determination of the maternal parent using cpDNA sequences

Interspecific comparison of *psb*A-*trn*H in the cpDNA indicated that seven variable nucleotide positions occur in this sequence, of which four (positions 181, 203, 337, and 338, 5′−3′) were useful for separating *A. japonica* from *A. pusilla* (Table 1). The four nucleotide positions of the putative *A.* × *walkeri* specimens from Jeju Island were identical to those of *A. japonica*.

Discussion

Taxonomic treatment of the Jeju Island plants

The network and population analyses using the MIG-seq SNP data clearly indicate that the putative *A.* × *walkeri* plants from Jeju Island originate from a hybrid of *A. japonica* and *A. pusilla*, similar to the Japanese plants reported by Kokubugata *et al*. (2019). The leaf texture and indumentum on the young branchlets and petioles of the Jeju Island plants correspond to descriptions of *A.* × *walkeri* from Japan (Walker, 1954; Yang and Dwyer, 1989; Yamazaki, 1993; Koyama and Kokubugata, 1998). These results suggest that the Jeju Island plants are attributable to *A.* × *walkeri* and originate from a hybrid of *A. japonica* (maternal parent) and *A. pusilla*. This record constitutes the first documented occurrence of the hybrid taxon in Korea.

Phylogenetic background of *A.* × *walkeri* on Jeju Island

A single branch between *A. japonica* and *A.* × *walkeri* and between *A. pusilla* and *A.* × *walkeri* shown in our network analysis suggests that the Japanese and Korean *A.* × *walkeri* specimens might have originated from a common ancestor. With respect to seed dispersal, *A. japonica* can colonize via long-distance dispersal of fruits by

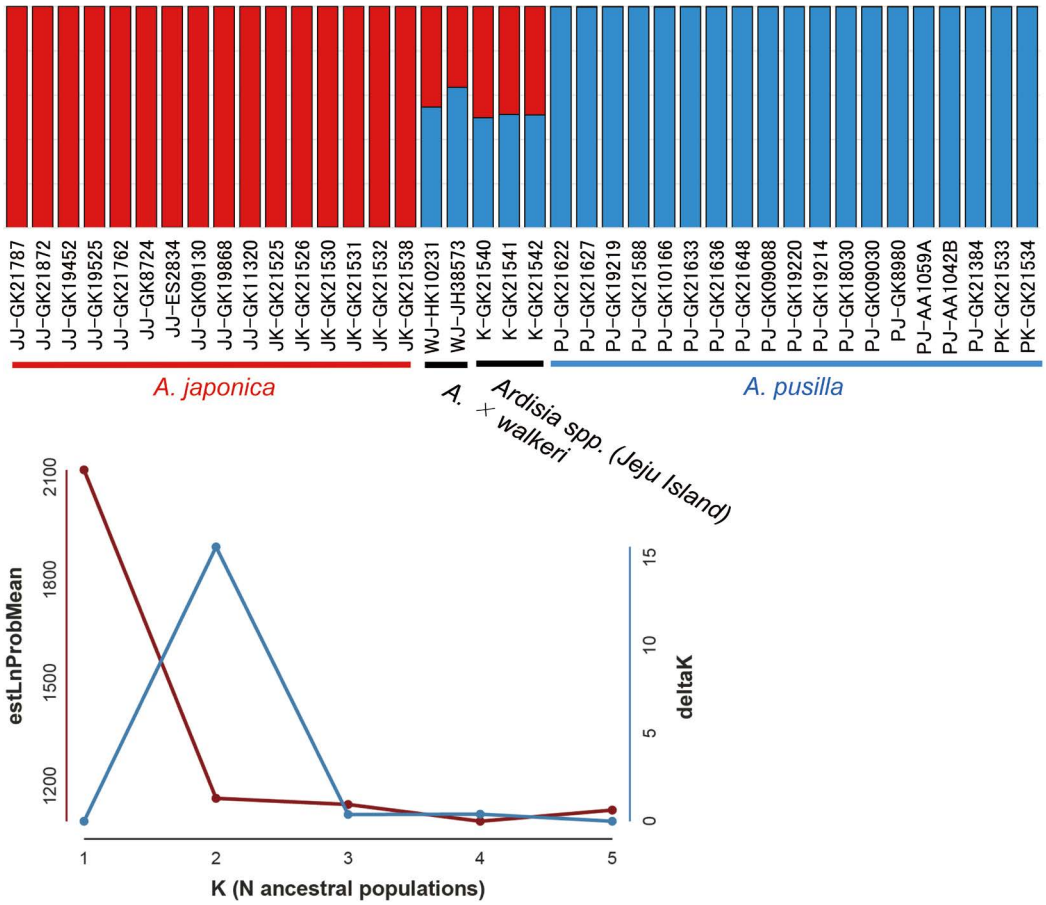

Fig. 4. Results of the STRUCTURE analysis using 934 MIG-seq SNPs. A. Individual-based genetic structure based on K = 2. B. Delta K and mean log probabilities for each K.

birds (Cheon *et al*., 2002). Indeed, the semi-migratory *Hypsipetes amaurotis* Temminck eats the fruits of *A. japonica* on Jeju Island (Kim *et al*., 2015). Fruits of *A.* × *walkeri* are round and reddish, with a diameter of 5−7 mm (Fig. 2D), and are similar to those of *A. japonica* (Yang and Dwyer, 1989). It is therefore possible that fruits of *A.* × *walkeri* dispersed from Japan to Jeju Island or vice versa following the hybridization event. Our cpDNA analysis, which indicated that the maternal parent of *A.* × *walkeri* is *A. japonica* in both the Japanese and Jeju Island plants, is consistent with our network analysis results, which suggest a single hybridization event. Similar to its parent species, *Ardisia* × *walkeri* has long creeping rhizomes and propagates quickly via vegetative expansion (Yang and Dwyer, 1989). In addition, *A. japonica* (Cheon *et al*., 2002) and most other *Ardisia* species (Bawa *et al*., 2002) are reported to have high self-compatibility. These reproductive characteristics may have contributed to the expansion of the distribution in Japan and Korea following the dispersal of one or a few plants. Further studies, including assessments of pollen and seed fertility, floral and cytological comparisons, and phylogenetic analyses using other sequencing data, could clarify the processes underlying the hybridization event and dispersal between Japan and Korea.

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