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NOTE

First record of *Goodyera* × *tamnaensis* (Orchidaceae) from Boso Peninsula, Chiba Prefecture, Japan, based on morphological and molecular data

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ABSTRACT: Several individuals of an unknown taxon of *Goodyera* were discovered in Boso Peninsula, Katsuura City, Chiba Prefecture, Japan. Detailed morphological investigation suggests that this taxon is a presumably natural hybrid between *G. schlechtendaliana* and *G. velutina* based on traits such as leaf venation pattern, the color of bract, ovary and inflorescence, the shape of the lip, lateral petal and sepal and column, hair length on inflorescence and ovary. Molecular data based on genome-wide markers using the next-generation sequencing platform (i.e., MIG-seq data) provide further support of the hybrid status of the plants from Boso Peninsula, Chiba Prefecture, Japan. The natural hybrid between *G. schlechtendaliana* and *G. velutina* was recently described as *G. ×tamnaensis* in Jeju Island, South Korea. Therefore, based on our findings, we reported the first occurrence of *G. ×tamnaensis* in Japan.

KEY WORDS: DNA barcoding, *Goodyera schlechtendaliana*, *Goodyera velutina*, MIG-seq, hybridization.

INTRODUCTION

The genus *Goodyera* R. Br. (Orchidaceae) includes ca. 60 species distributed in southern Africa, Asia, northeastern Australia, Europe, Madagascar, North America, Mesoamerica, and the southwestern Pacific islands (Chen *et al.,* 2009; Guan *et al.,* 2014). Species of *Goodyera* are terrestrial, lithophytic or epiphytic and typically grow in the shade, on mossy rocks or along moist tracks of perennial mountain streams (Hu *et al.,* 2016).

The genus is characterized by creeping rhizomes, leaves that often have white or golden venation on the upper surface, saccate lip, two sectile pollinia attached to a viscidium and a single stigmatic lobe (Hu *et al.*, 2016). Furthermore, the flowers of *Goodyera* usually have dissimilar sepals, a concave dorsal sepal connivent with the petals to form a hood over the column. The lateral sepals are usually connivent with a lip formed from the concave-saccate hypochile and sessile epichile (Guan *et al.*, 2014; Suetsugu and Hayakawa, 2019). Although they are well-known in horticulture as jewel orchids due to the beautiful coloration and venations of their leaves, the species taxonomy within the genus remains to be revised, owing to ambiguous diagnostic characters and similar floral features (Guan *et al.,* 2014; Hu *et al.,* 2016; Shin *et al.,* 2002; So and Lee, 2017; Suetsugu *et al.,* 2019).

During the recent botanical survey, the unknown taxon of *Goodyera* was collected in Boso Peninsula, Katsuura City, Chiba Prefecture, Japan. Intriguingly, these plants exhibited intermediate morphological characters between *G. schlechtendaliana* Rchb.f. and *G. velutina* Maxim. ex Regel (Fig. 1). Therefore, this unknown taxon might be a natural hybrid between *G. schlechtendaliana* and *G. velutina*. The natural hybrid of them was described as *G.* × *tamnaensis* in Jeju Island, South Korea (Lee *et al.,* 2010, 2012). Recently, molecular studies of the genus *Goodyera* have succeeded in elucidating phylogenetic relationships within the genus by using multiple gene markers (Hu *et al.,* 2016; Lee *et al.,* 2012), although the resolution based on a single gene is sometimes too low to be useful for species identification (Suetsugu *et al.*, 2019). Therefore, we investigated the identity of the unknown taxon, based on not only morphological traits but also the genome-wide sequences amplified with multiplexed inter-simple sequence repeat (ISSR) primers.

MATERIALS AND METHODS

Field sampling

Three individuals of the putative *Goodyera* × *tamnaensis* were collected in Boso Peninsula, Katsuura City, Chiba Prefecture, Japan, situated in a warm temperate area of Eastern Japan. The study site containing ca. 10 flowering individuals is a coniferous forest dominated by *Pinus densiflora*. In addition, 16

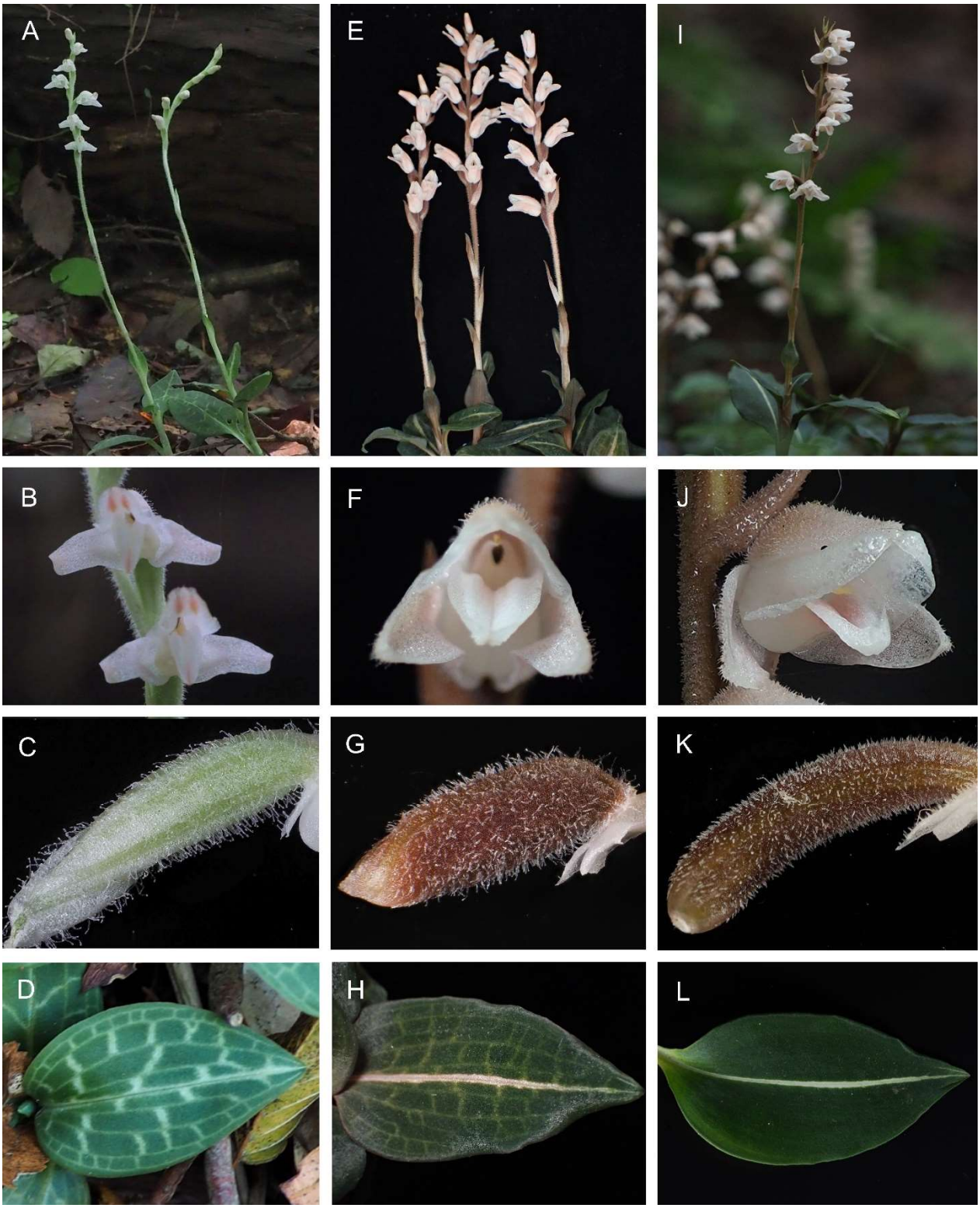

Fig. 1. Comparison among ***Goodyera schlechtendaliana*** (**A**–**D**), ***G.* ×*tamnaensis*** (**E**–**H**), and ***G. velutina*** (**I**–**L**). **A**, **E**, **I**. Habit. **B**, **F**, **J**. Flower. **D**, **H**, **L**. Leaf.

individuals of *G. velutina* and 10 individuals of *G.schlechtendaliana* (including four individuals of *G. schlechtendaliana* var. *yakushimensis*), were collected throughout Japan for comparative study (Table S1). For molecular analysis, the leaves were dried with silica gel until DNA extraction.

Morphological observation

We compared the morphological characters of the putative *Goodyera* × *tamnaensis* from Boso Peninsula, Katsuura City, Chiba Prefecture, Japan, *G. schlechtendaliana* and *G. velutina* using the samples mentioned above (Table S1). The morphological variation of *G.* × *tamnaensis*, *G. schlechtendaliana* and *G. velutina* was also investigated by reviewing the literature and herbarium specimens collected in other localities and deposited in KYO, TI and TNS. Morphological characters were observed visually and under a stereomicroscope and measured using a digital caliper.

Next-generation DNA sequencing - MIG-seq

Genomic DNA was extracted from approximately 10 mg (dry weight) of leaf material using cetyl trimethyl ammonium bromide (CTAB) method. We amplified thousands of short sequences by using the primers of MIG-seq for 29 *Goodyera* samples, following the protocol of Suyama and Matsuki (2015). Our protocol followed standard conditions and described primer sequences, except for a decrease in annealing temperature (38°C) and an increase in cycle number (30 cycles) in the first PCR step. Two PCR steps were performed; for the 1st PCR step, we amplified ISSR regions from genomic DNA with MIG-seq tailed ISSR primer set-1 and each purified 1st PCR product was used for 2nd PCR (Suyama & Matsuki 2015). The 2nd PCR step was conducted with common and indexed primers. The 2nd PCR products were then pooled as a single mixture library. Fragments of size range 350–800 bp were isolated from the mixture of 2nd PCR products by AMpure XP (Beckman Coulter, Brea, CA, USA). The concentration was measured by quantitative PCR (Library Quantification Kit; Clontech Laboratories, Mountain View, CA, USA) and then sequenced by Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA) with a MiSeq Reagent Kit v3 (150 cycle, Illumina) (Suyama & Matsuki 2015). All raw MIG-seq data were deposited at the DDBJ Sequence Read Archive (DRA) with accession number DRA011506. The sequencing of the first 17 bases of read 1 and 2 (SSR primer regions and anchors) was skipped using 'DarkCycle'.

Low-quality reads and extremely short reads were removed using Trimmomatic 0.39 (Bolger *et al.*, 2014). After quality control, 2936279 reads (101251 ± 5147 reads per sample) were obtained. Stacks 2.41 pipeline was used for *de novo* SNP discovery (Catchen *et al.*, 2013; Rochette *et al.*, 2019) with the following parameters: minimum depth of coverage required to create a stack (m) = 3, maximum distance allowed between stacks (M) = 2, number of mismatches allowed between sample loci when building the catalog (n) = 2. Only SNPs retained by 14 or more samples were extracted, and loci containing SNPs with high heterozygosity (Ho ≥ 0.6) were removed. Moreover, the SNP site with a minor allele frequency of less than 5% was filtered out. We only used the first SNP from each locus to avoid the inclusion of linked SNPs. Finally, 895 SNPs were used for the subsequent analyses.

Population structure was examined by STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). We performed 50 independent runs with a burn-in of 100,000 steps and additional 100,000 steps with the admixture model and estimated log-likelihoods for each number of clusters (K = 1–10). Optimal K values were determined using the Delta K method (Evanno *et al.*, 2005) in Structure Harvester (Earl and vonHoldt, 2012). Graphical results were obtained using CLUMPAK (Cluster Markov Packager Across K (Kopelman *et al.*, 2015) and R package Pophelper 2.2.9 (Francis, 2017). A Neighbor-Net network was constructed by SplitsTree4 4.14 (Huson and Bryant, 2006) using the uncorrelated P distance matrix calculated from the SNPs matrix.

RESULTS AND DISCUSSION

Detailed morphological examination revealed that the morphological characters of an unknown taxon of *Goodyera,* including leaf venation pattern, the color of bract, ovary and inflorescence, the shape of the lip, lateral petal and sepal and column, hair length on inflorescence and ovary, are intermediate between *G. schlechtendaliana* and *G. velutina* (Figs. 1–4). Molecular data based on genome-wide markers using the MIG-seq data provide conclusive evidence for the hybrid status of the plants from Boso Peninsula, Katsuura City, Chiba Prefecture, Japan (Figs. 5–6). Consequently, the taxon is a presumably natural hybrid between *G. schlechtendaliana* and *G. velutina.* Since the natural hybrid between *G. schlechtendaliana* and *G. velutina* was described as *G.* × *tamnaensis* in Jeju Island, South Korea (Lee *et al.*, 2010, 2012), we hereby report its first occurrence in Japan.

Notably, *G.* × *tamnaensis* from Boso Peninsula, Katsuura City, has a distinctly patterned venation, which it has probably inherited from the bold central vein of *G. velutina* and the reticulate venation of *G. schlechtendaliana* (Fig. 1; Lee *et al.*, 2010, 2012). In general, *G.* × *tamnaensis* has velutinous dark green leaves with a white central vein and reticulate venation (Fig. 1). While *G. schlechtendaliana* has non-velutinous green leaves with reticulate venation (Suetsugu and Hayakawa, 2019), *G. velutina* has velutinous dark purplish-green leaves with one white mid-vein and no

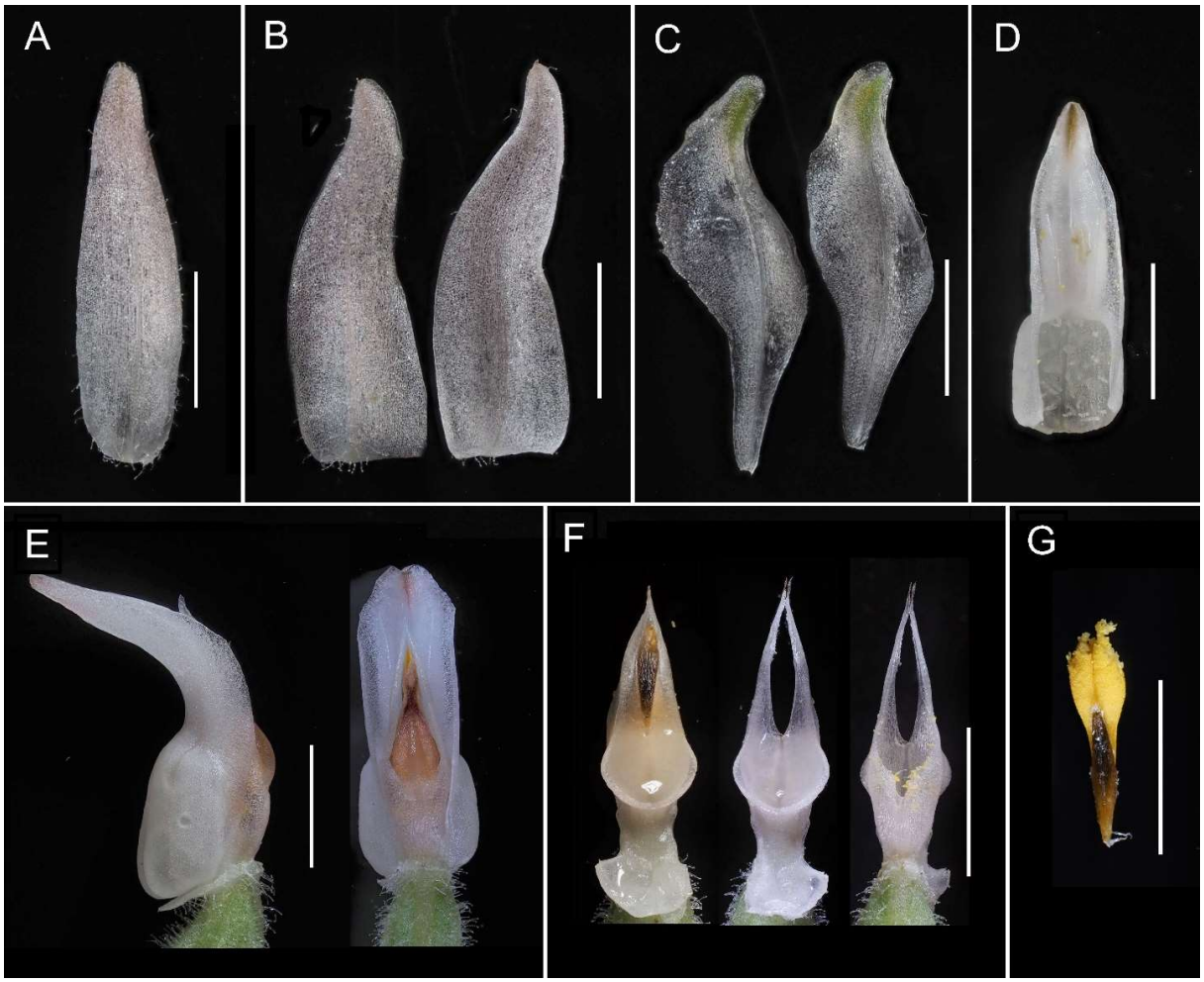

Fig. 2. ***Goodyera schlechtendaliana*** (*Masayuki Ishibashi KS812*, TNS). **A**. Dorsal sepal, outside view. **B**. Lateral sepals, outside view (left) and inside view (right). **C**. Lateral petals, outside view. **D**. Lip. **E**. Lip and column, lateral view (left) and dorsal view (right). **F**. Column, ventral view (left), ventral view, pollinaria removed (middle) and dorsal view, pollinaria removed (right). **G**. Pollinarium. All scale bars = 3 mm.

reticulate venation (Lee *et al.*, 2010; Suetsugu *et al.*, 2019). In addition, *G. schlechtendaliana* can be distinguished from *G. velutina* by the shape of the lateral sepal. Therefore, it is noteworthy that that of *G.* × *tamnaensis* (ovate-lanceolate, recurved and twisted, Fig. 3B) is intermediate between *G. schlechtendaliana* (lanceolate, strongly recurved and twisted, Fig. 2B) and *G. velutina* (ovate, not recurved and twisted, Fig. 4B) (Suetsugu & Hayakawa 2019; Suetsugu *et al.* 2019). Furthermore, while the aspect ratio of the lateral petal in *G.* × *tamnaensis* is ca. 2.8, that of *G. schlechtendaliana* and *G. velutina* is ca. 3.0 and ca. 2.1, respectively. Overall, *G.* × *tamnaensis* has the intermediate characteristics or the characteristics of either one of its putative parents. Therefore, morphological data support the hybrid origin of *G.* × *tamnaensis*. For a detailed comparison of morphological characters among *G.* ×*tamnaensis*, *G. schlechtendaliana*, and *G. velutina*, see Table 1.

Molecular data provide further support of hybrid status in *G.* × *tamnaensis* from Boso Peninsula, Katsuura City. The STRUCTURE analysis showed that *G. schlechtendaliana* (including its intraspecific variant *G. schlechtendaliana* var. *yakushimensis*) and *G. velutina* were differentiated into two clusters, and *G.* × *tamnaensis* had genetic components of both *G. schlechtendaliana* and *G. velutina* (Fig. 5). The Neighbor-Net (SplitsTree) phylogenetic analysis also indicated that *G. schlechtendaliana* and *G. velutina* represented two distinct genetic clusters, and *G.* × *tamnaensis* occupied an intermediate position between *G. schlechtendaliana* and *G. velutina* (Fig. 6). Therefore, both the STRUCTURE and Neighbor-Net network analyses based on MIG-seq data indicated a hybrid origin of *G.* × *tamnaensis* investigated here.

Hybridization in plants has played a crucial role in plant evolution (Pace *et al.*, 2019). Several studies have shown that hybridization can provide significant evolutionary consequences such as increased genetic diversity, the origin of new species or ecotypes, and

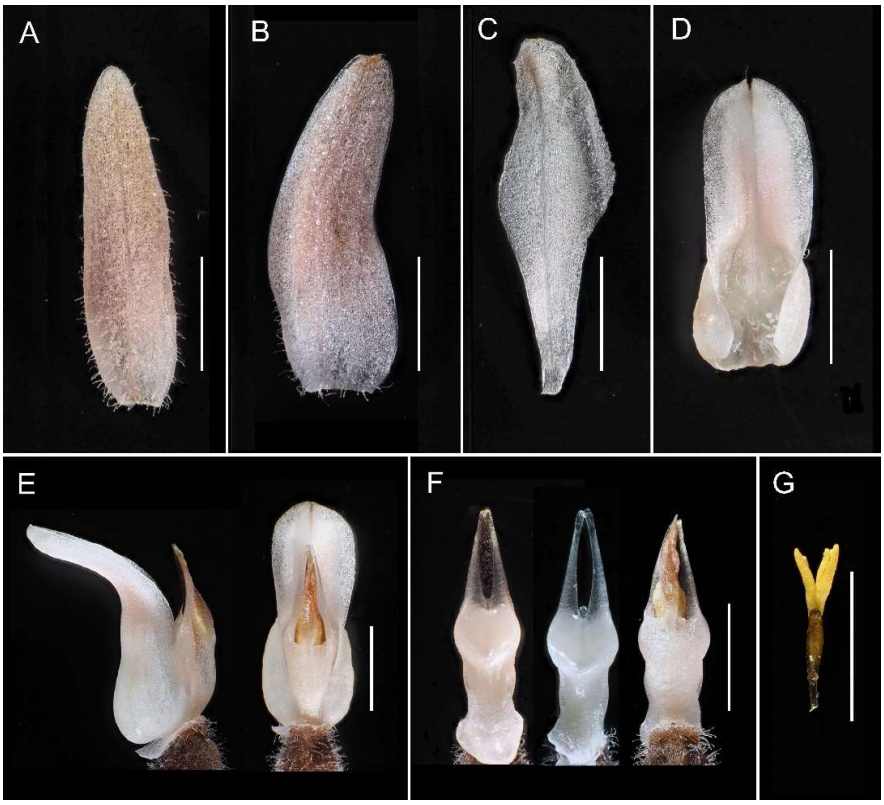

Fig. 3. *Goodyera* × *tamnaensis* (*Akiko Sakakibara KS556*, TNS). **A**. Dorsal sepal, outside view. **B**. Lateral sepals, outside view. **C**. Lateral petals, outside view. **D**. Lip. **E**. Lip and column, lateral view (left) and dorsal view (right). **F**. Column, ventral view (left), ventral view, pollinaria removed (middle) and dorsal view (right). **G**. Pollinarium. All scale bars = 3 mm.

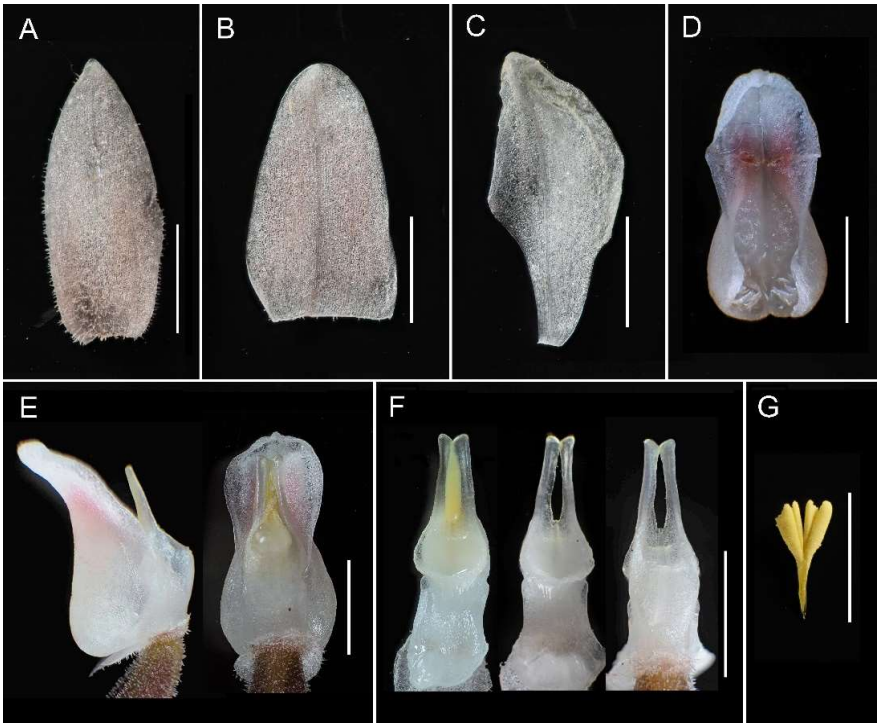

Fig. 4. *Goodyera velutina* (*Masayuki Ishibashi KS811*, TNS). **A**. Dorsal sepal, outside view. **B**. Lateral sepals, inside view. **C**. Lateral petals, inside view. **D**. Lip. **E**. Lip and column, lateral view (left) and dorsal view (right). **F**. Column, ventral view (left), ventral view, pollinaria removed (middle) and dorsal view, pollinaria removed (right). **G**. Pollinarium. All scale bars = 3 mm.

Table 1. Morphological comparison among ***Goodyera schlechtendaliana***, ***G. ×tamnaensis*** and ***G. velutina*** in Boso Peninsula, Katsuura City, Chiba Prefecture, Japan.

characters	***G. schlechtendaliana***	***G. × tamnaensis***	***G. velutina***
leaf color	green	velutinous dark green	velutinous dark green
leaf shape	elliptic-ovate	lanceolate-ovate	ovate
central vein	faint	prominent	prominent
lateral vein	prominent	intermediate	hidden
reticulate pattern	prominent	faint	none
inflorescence length	ca. 15 cm	10 – 15 cm	6 – 10 cm
hair status and length on peduncle and ovary	0.3 – 0.4 mm, clavate	0.3 – 0.4 mm, clavate	0.1 mm, subulate
color of bract, ovary and inflorescence	pale green	reddish-brown	reddish-brown
flower color	white	light reddish pink	light reddish pink
shape of lip apex	strongly recurved	recurved	slightly recurved
shape of lateral sepal	lanceolate, strongly recurved and twisted	ovate-lanceolate, recurved and twisted	ovate, slightly recurved and twisted
shape of lateral petal	rhombic-oblanceolate to oblong-oblanceolate, apex of hood strongly recurved	rhombic-oblanceolate to oblong-oblanceolate, apex of hood recurved	rhombic-oblanceolate, apex of hood slightly recurved
aspect ratio of lateral petal	ca. 3.0	ca. 2.8	ca. 2.1
rostellum shape	narrowly triangular, 1/2 as long as column, apex acuminate	narrowly triangular, 1/2 as long as column, apex flattened and wedge-shaped	oblong to rectangular, 2/5 as long as column, apex flattened and wedge-shaped

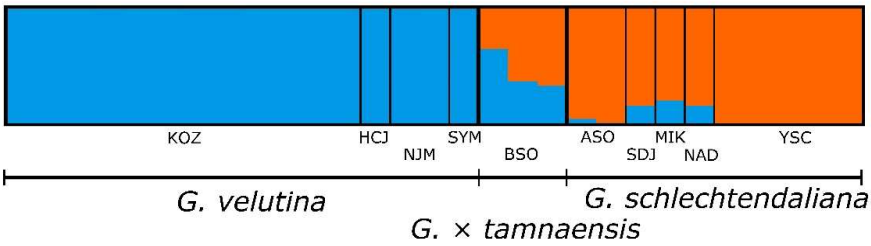

Fig. 5. Population structure derived from 29 *Goodyera* samples inferred by the STRUCTURE algorithm. *K* = 2 has the largest delta *K* for our data, indicating that *K* = 2 was optimal. Species and populations are separated by broad and narrow vertical black lines, respectively.

breakdown or reinforcement of isolating barriers (Lee *et al.*, 2012; Nakahama *et al.*, 2019; Pace *et al.*, 2019; So and Lee, 2017). Although gene flow may proceed only to the formation of F1 hybrids due to hybrid sterility, the STRUCTURE analysis suggested a somewhat differential level of hybridization among *G.* × *tamnaensis* individuals (Fig. 5), suggesting the existence of some putative backcrossed hybrids with one of the parental species. Taken together with its wide distribution (Lee *et al.*, 2012), *G.* × *tamnaensis* could a stabilize hybrid. Since both species are neither autogamous nor apogamous but are pollinator-dependent, natural hybridization must have occurred via shared pollinators of both *Goodyera* species. Given that *G. maximowicziana* and *G. velutina* often have overlapped distribution in other locations in Japan, further surveys may reveal a broader distribution of *G.* × *tamnaensis*. While little is known about the role of breeding systems and hybridization in the evolution of the genus *Goodyera*, it is suspected that *G. tesselata* Loddiges is a hybrid origin between *G. oblongifolia* Raf. and *G. repens* (L.) R. Brown var. *ophioides* Fern. (Kallunki, 1976). *Goodyera maximo-velutina* is also considered as a natural hybrid between *G. maximowicziana* and *G. velutina* (So and Lee, 2017; Suetsugu *et al.*, 2019). Further studies are needed to assess the role of hybridization in the evolution of the genus.

Goodyera* × *tamnaensis N.S. Lee, K.S. Lee, S.H. Yeau & C.S. Lee Korean J. Pl. Taxon. 40: 252 (2010)

Specimen examined: JAPAN. Chiba Prefecture, Boso Peninsula, Katsuura City, 16 September 2018, *Akiko Sakakibara KS556* (TNS).

Distribution: Japan (Boso Peninsula, new record) and South Korea (Jeju Island). In Japan, *Goodyera* × *tamnaensis* is currently only known from a single population of ca. 10 flowering individuals in a coniferous forest dominated by *Pinus densiflora* in Boso Peninsula, Katsuura City, Chiba Prefecture. Two parental species (i.e., *G. schlechtendaliana* and *G. velutina*) bloom sympatrically in the same season in the population.

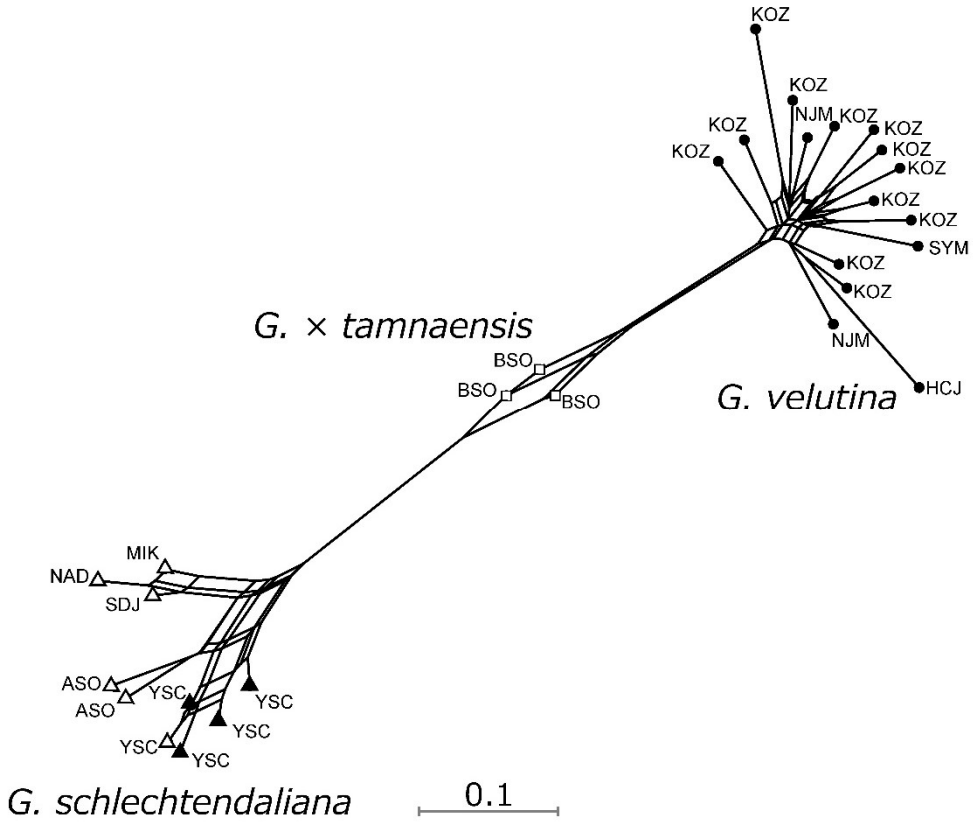

Fig. 6. Neighbor-Net network derived from 29 *Goodyera* samples based on uncorrected P distance calculated by 895 SNPs. Filled circle: *G. velutina*. Open square: *G. × tamnaensis*. Open triangle: *G. schlechtendaliana*. Filled triangle: *G. schlechtendaliana* var. *yakushimensis*.

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Supplementary materials are available from Journal Website.