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A new natural hybrid, *Goodyera ×tanakae* (Orchidaceae) from Japan with a discussion on the taxonomic identities of *G. foliosa*, *G. sonoharae*, *G. velutina*, *G. ×maximo-velutina* and *G. henryi*, based on morphological and molecular data

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ABSTRACT: *Goodyera ×tanakae* is described as a new taxon based on molecular and morphological comparison with its closely related species *G. foliosa*, *G. velutina*, *G. henryi* and *G. maximo-velutina*. Detailed morphological examination has revealed that morphological characters of *G. ×tanakae*, such as leaf coloration and venation and the shape of lip and column, are intermediate between *G. foliosa* and *G. velutina*. Molecular data based on genome-wide markers using the next-generation sequencing platform (MIG-seq data) have also supported that *G. ×tanakae* is a natural hybrid between *G. foliosa* and *G. velutina.* Both maximum likelihood and Neighbor-Net phylogenetic analysis indicated that *G. ×tanakae* occupies an intermediate position between *G. foliosa* and *G. velutina.* The STRUCTURE analysis also showed that *G. ×tanakae* has genetic components of both *G. foliosa* and *G. velutina*. Therefore, we concluded that this taxon is a natural hybrid between *G. foliosa* and *G. velutina* based on morphological and molecular data. The taxonomic identity of its closely related species *G. foliosa*, *G. velutina*, *G. henryi* and *G. maximo-velutina* has also been discussed.

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

INTRODUCTION

The genus *Goodyera* R. Brown includes ca. 70 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). The genus is characterized by evergreen plant, 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).

Explicit species delimitation is fundamental for testing evolutionary theory and implementing conservation strategies (Botes *et al.*, 2020; Pace *et al.*, 2019). However, species identification of the genus *Goodyera* is sometimes difficult, especially for closely related species. Although they are well-known as jewel orchids due to the beautiful coloration and venations of their leaves, previous studies pointed out the difficulty in its species delimitation, owing to convergent morphological features (Guan *et al.*, 2014; Hu *et al.*, 2016; Shin *et al.*, 2002; So and Lee, 2017; Suetsugu *et al.*, 2019). In this respect, it is noteworthy that molecular techniques have emerged as valuable tools to understand the phylogenetic relationships (Botes *et al.*, 2020; Pace *et al.*, 2019). In particular, the internal transcribed spacer (ITS) region of nrDNA with moderate interspecific variation has been the primary source of phylogenetic analysis at lower taxonomic levels in plants (Baldwin *et al.*, 1995; Guan *et al.*, 2014). However, the resolution based on Sanger sequencing, such as ITS region, might sometimes be too low to be useful for species identification in *Goodyera.* The ITS sequences of morphologically distinct species *G. velutina* Maximowicz ex Regel and *G. repens* (L.) R. Brown were identical to each another (Shin *et al.*, 2002). Moreover, although a more comprehensive phylogenetic study of *Goodyera* has been conducted based on not only ITS but also two plastid regions (*trnL-F* and *matK*), discordances with the morphological characteristics have remained (Hu *et al.*, 2016). Therefore, a higher resolution genetic marker is probably required to elucidate the complex evolutionary history of closely related *Goodyera* species.

The advent of high-throughput sequencing technology has rendered a large number of loci accessible even in non-model organisms. Therefore, studies using such technology would help reveal species boundaries and evolutionary history of closely-related species (Tamaki *et al.*, 2017; Yoichi *et al.*, 2018; Hirano *et al.*, 2019). One available method for this purpose is the MIG-seq [multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing], which is a recently developed

genome-wide genotyping method using a high-throughput sequencing platform (Suyama and Matsuki, 2015). MIG-seq is a microsatellite-associated DNA sequencing technique - a type of reduced representation sequencing that includes restriction site-associated DNA sequencing (RAD-seq) (Suyama and Matsuki, 2015). The genome-wide SNP data obtained by MIG-seq are helpful for detecting reproductive isolation and hybridization of taxa even for recently divergent species (Hirano *et al.*, 2019; Tamaki *et al.*, 2017; Yoichi *et al.*, 2018).

Here we focused on an unknown taxon of *Goodyera* similar to *G. foliosa* (Lindl.) Benth. ex C.B. Clarke and *G. velutina* discovered in Fukuoka Prefecture, Japan, during recent botanical surveys (Figs. 1–3). A previous molecular analysis based on the nuclear ribosomal ITS and plastid (*matK* and *trnL-F*) regions suggested that *G. foliosa* is a polyphyletic taxon forming two strongly supported clades with *G. velutina* and *G. henryi* Rolfe (Hu *et al.*, 2016). However, *G. foliosa* and *G. velutina* can be distinctly distinguished by morphological features such as leaf venation pattern and hair status and length on peduncle and ovary (Hu *et al.*, 2016). Consequently, species delimitation between *G. foliosa* and *G. velutina* has never been questioned. Therefore, discordances with the morphological characteristics indicate the necessity of higher resolution genetic information (Hu *et al.*, 2016).

In this study, we aimed to elucidate the identity of the unknown *Goodyera* taxon and its closely related species *G. foliosa* and *G. velutina* based on MIG-seq data and evidence from plant morphology. From the combined morphological and molecular results, we concluded that the unknown taxon is a natural hybrid between *G. foliosa* and *G. velutina*. We herein describe the taxon as *G. ×tanakae* named after Koji Tanaka, who collected the type specimens.

MATERIALS AND METHODS

Field sampling

Two individuals of *Goodyera ×tanakae* were collected in Kitakyushu City, Fukuoka Prefecture Japan. In addition, 16 individuals of *G. velutina* and 11 individuals of *G. foliosa* (including seven individuals previously recognized as *G. sonoharae* Fukuyama, a synonym of *G. foliosa*) were collected throughout Japan. Furthermore, despite distinct morphological traits, *G. henryi* (syn. *G. maximowicziana* Makino) is sometimes treated as the intraspecific taxon of *G. foliosa* such as *G. foliosa* var. *maximowicziana* (Makino) S.S.Ying. Therefore, 12 individuals of *G. henryi* were also collected. Three individuals of *G. maximo-velutina*, a probable natural hybrid between *G. henryi* and *G. velutina* (So and Lee, 2017; Suetsugu *et al.*, 2019) were also collected for comparative study (Table S1). Leaves for DNA analysis were immediately dried using silica gel and stored at room temperature until DNA extraction. At least one voucher specimen from each population was deposited in KYO, SPMN and TNS (Table S1). The herbarium acronyms follow Index Herbariorum (Thiers, 2021).

Morphological observation

We compared the morphological characters of *Goodyera ×tanakae*, *G. foliosa*, *G. velutina*, *G. henryi* and *G. maximo-velutina* using the samples mentioned above. The morphological variation of *G. ×tanakae*, *G. foliosa* 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.

High-throughput genomic analysis - MIG-seq

Genomic DNA was extracted from silica-dried leaves by CTAB method. A MIG-seq library for 44 *Goodyera* samples was prepared according to the protocol outlined in Suyama and Matsuki (2015) with a slight modification (Suetsugu *et al.*, 2021). The library was sequenced using an Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA) with a MiSeq Reagent Kit v3 (150 cycle, Illumina). It should be noted that the raw MIG-seq data of 16 *G. velutina* samples had already been deposited at the DDBJ Sequence Read Archive (DRA) with accession number DRA011506 for Suetsugu *et al.* (2021). The remaining raw MIG-seq data were deposited at the DDBJ Sequence Read Archive (DRA) with accession number DRA011700.

After removing primer regions and sequencing reads with low quality (Suetsugu *et al.*, 2021), 4,507,773 reads (102,449 ± 4407 reads per sample) were obtained from 4,654,922 raw reads (105,794 ± 4519 per sample). 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 22 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 counts of less than four was filtered out. We only used the first SNP from each locus to avoid linked SNPs, providing 430 SNPs for the subsequent analyses.

Maximum likelihood (ML) phylogeny based on SNPs was inferred using RAxML 8.2.10 (Stamatakis, 2014) with GTR model of substitution. We performed 1,000 replicates of parallelized tree search bootstrapping. To examine interspecific hybridization, 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. Population structure was also examined by STRUCTURE 2.3.4 (Pritchard *et*

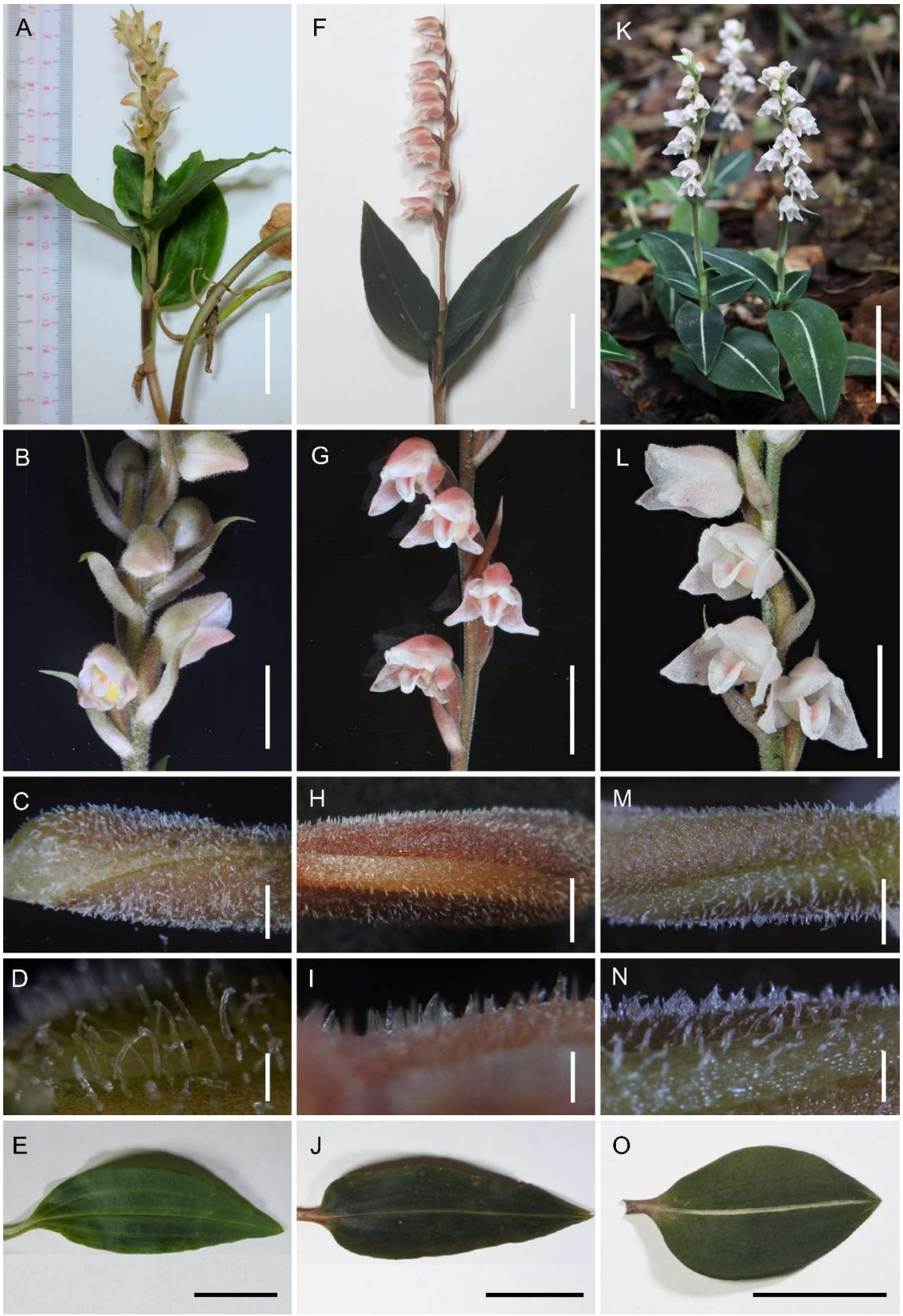

Fig. 1. Comparison among *Goodyera foliosa*, *G. ×tanakae*, and *G. velutina*. **A–E**. *Goodyera foliosa*. **F–J**. *G. ×tanakae*. **K–O**. *G. velutina*. **A**, **F**, **K**. Habit. Scale bars = 3 cm. **B**, **G**, **L**. Flowers. Scale bars = 1 cm. **C**, **H**, **M**. Ovary. Scale bars = 1 mm. **D**, **I**, **N**. Hairs on the ovary Scale bars = 0.3 mm. **E**, **J**, **O**. Leaf. Scale bars = 1 cm.

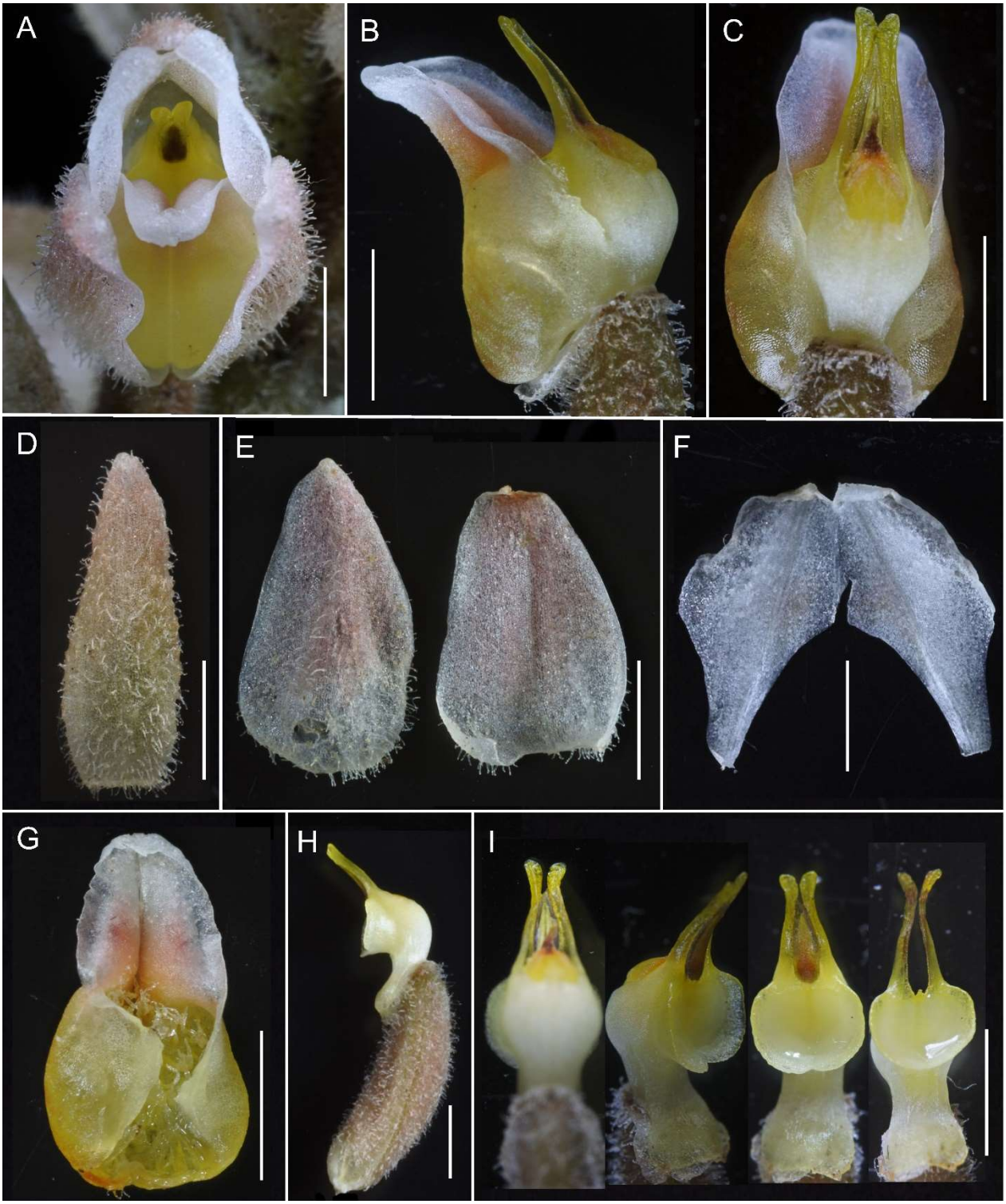

Fig. 2. *Goodyera foliosa* at Munakata City, Fukuoka Prefecture (*Koji Tanaka KS817*, KYO). **A**. Flower, front view. **B**. Lip and column, lateral view. **C**. Lip and column, dorsal view. **D**. Dorsal sepal, outside view. **E**. Lateral sepals, outside view. **F**. Lateral petals, outside view. **G**. Lip. **H**. Column and ovary. **I**. Column, dorsal view, lateral view, ventral view, and ventral view (pollinaria removed). All scale bars = 3 mm.

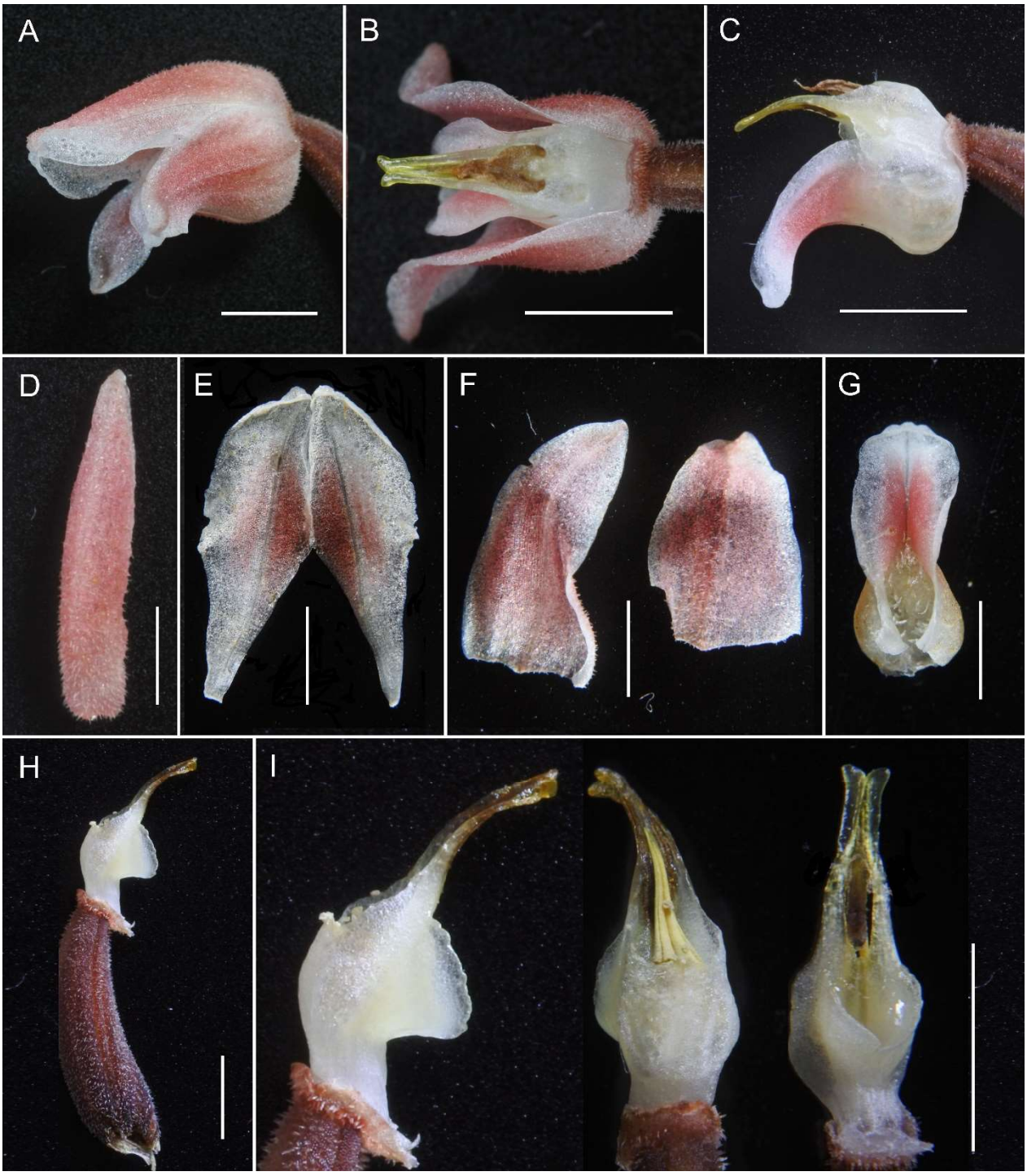

Fig. 3. *Goodyera ×tanakae* (*Koji Tanaka KS818*, KYO). **A**. Flower, lateral view. **B**. Lateral sepals, lip and column, dorsal view. **C**. Lip and column, lateral view. **D**. Dorsal sepal, outside view. **E**. Lateral sepals, outside view. **F**. Lateral petals, outside view. **G**. Lip. **H**. Column and ovary. **I**. Column, lateral view (anther cap removed), dorsal view (anther cap removed), and ventral view. All scale bars = 3 mm.

al., 2000) for estimating genetic admixture. We performed 20 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 of Evanno *et al.* (2005) in Structure Harvester (Earl and von Holdt, 2012). Graphical results were obtained using CLUMPAK (Kopelman *et al.*, 2015) and R package Pophelper 2.2.9 (Francis, 2017).

Table 1. Morphological comparison among *G.* ×*tanakae* and its closely related species.

characters	*G.* ×*tanakae*	*G. foliosa*	*G. velutina*	*G.* ×*maximo-velutina*	*G. henryi*
leaf color	velutinous dark green	green	velutinous dark green	dark green	green
leaf shape	lanceolate-ovate	lanceolate-ovate	ovate	lanceolate-ovate	lanceolate-ovate
length/width ratio of leaf lamina	2.0 – 2.9	2.0 – 2.9	1.3 – 1.9	2.0 – 2.9	2.0 – 2.9
central vein	faint	faint	prominent	faint	faint
lateral vein	hidden	faint	hidden	faint	faint
peduncle length	2.5 – 5 cm	2.5 – 5 cm	3 – 6 cm	1 – 2.5 cm	less than 1 cm
rachis length	1/2-2/3 of inflorescence	ca. 2/3 of inflorescence	ca. 1/2 of inflorescence	ca. 3/4 of inflorescence	nearly all the inflorescence
hair status and length on peduncle and ovary	0.1 mm, subulate	0.3 – 0.5 mm, clavate	0.1 mm, subulate	0.05 mm, subulate	none
color of bract, ovary and peduncle	reddish brown	pale green tinged with reddish brown	reddish brown	reddish brown	pale green
flower color	light reddish pink	light reddish pink	white or light reddish pink	light reddish pink	light reddish pink
length/width ratio of lateral petal	ca. 2.4	ca. 2.1	ca. 2.2	ca. 2.6	ca. 3.0
lip color	hypochile yellow, epichile pale red	hypochile yellow, epichile pale red	hypochile white, epichile pale red	hypochile white, epichile pale red	hypochile white, epichile yellow
epichile/hypochile length ratio	ca. 1.0	less than 1	more than 1	ca. 1.0	ca. 1.0
color and shape of stigma	surrounded by a hood-like rim, rim prominent at the base, translucent yellow	surrounded by a hood-like rim, rim entirely prominent, translucent yellow	surrounded by a hood-like rim, rim prominent at the base, translucent white	surrounded by a hood-like rim, rim prominent at the base, translucent white	surrounded by a hood-like rim, rim entirely prominent, translucent pale yellow

RESULTS AND DISCUSSION

Morphological character of *G.* ×*tanakae* and its closely-related species

Detailed morphological examination has revealed that the morphological characters of *G.* ×*tanakae,* such as leaf coloration and venation and the shape of column, are intermediate between *G. foliosa* and *G. velutina* (Figs. 1–3). For example, leaf texture, coloration and venation of *G.* ×*tanakae* is intermediate between that of *G. foliosa* and *G. velutina*. *Goodyera* ×*tanakae* has velutinous dark green leaves with a faint central vein (Fig. 1). While *G. foliosa* has non-velutinous green leaves with a faint central vein (Suetsugu and Hayakawa, 2019), *G. velutina* has velutinous dark purplish-green leaves with prominent white central vein (Lee *et al.*, 2010; Suetsugu *et al.*, 2019). In addition, the color and shape of stigma in *G.* ×*tanakae* is intermediate between that of *G. foliosa* and *G. velutina* (Figs. 2–3). Furthermore, *G.* ×*tanakae* and *G, foliosa* can be distinguished from *G. velutina* by leaf shape (lanceolate-ovate, 2.0–2.9 in length/width ratio vs. ovate, 1.3–1.8 in length/width ratio). In contrast, *G.* ×*tanakae* and *G. velutina* are also distinguishable from *G. foliosa* by hair status and length on peduncle and ovary (0.1 mm, subulate v.s. 0.3–0.5 mm, clavate). Overall, *G.* ×*tanakae* has the intermediate characteristics or the characteristics of either one of its putative parents. Therefore, morphological data supported the hybrid origin of *G.* ×*tanakae*. It is also noteworthy that *G. henryi* and *G.* × *maximo-velutina* is easily distinguishable from *G. foliosa*, *G.* ×*tanakae* and *G. velutina* by shorter peduncle and elongated lateral petal. For a detailed comparison of morphological characters among *G.* ×*tanakae*, *G. foliosa*, *G. velutina*, *G. henryi* and *G.* ×*maximo-velutina*, see Table 1. Additional descriptions and illustrations of *G. velutina*, *G. henryi* (as *G. maximowicziana*) and *G.* ×*maximo-velutina* are also available in Lee *et al.* (2010), So and Lee (2017) and Suetsugu *et al.* (2021).

Molecular phylogeny of *G.* ×*tanakae* and its closely-related species

Although *G. foliosa* and *G. velutina* are morphologically distinct, previous phylogenetic studies based on nrITS and plastid (*matK* and *trnL-F*) regions did not indicate the monophyly of each taxon (Hu *et al.* 2016). However, our ML phylogenetic tree based on MIG-seq analysis showed that *G. foliosa* (including individuals previously recognized as *G. sonoharae*: KGM population), *G. velutina* and *G. henryi* are divided into three clades: the monophyly of each species was supported by 90%, 93%, and 100% bootstrap values, respectively (Fig. 4). The Neighbor-Net (SplitsTree) phylogenetic analysis also indicated that *G. foliosa*, *G. henryi* and *G. velutina* represent three distinct genetic clusters (Fig. 5). Although the STRUCTURE analysis at $K = 2$ (the largest delta K for our data; Fig. S1) showed that *G. foliosa* and *G. henryi*

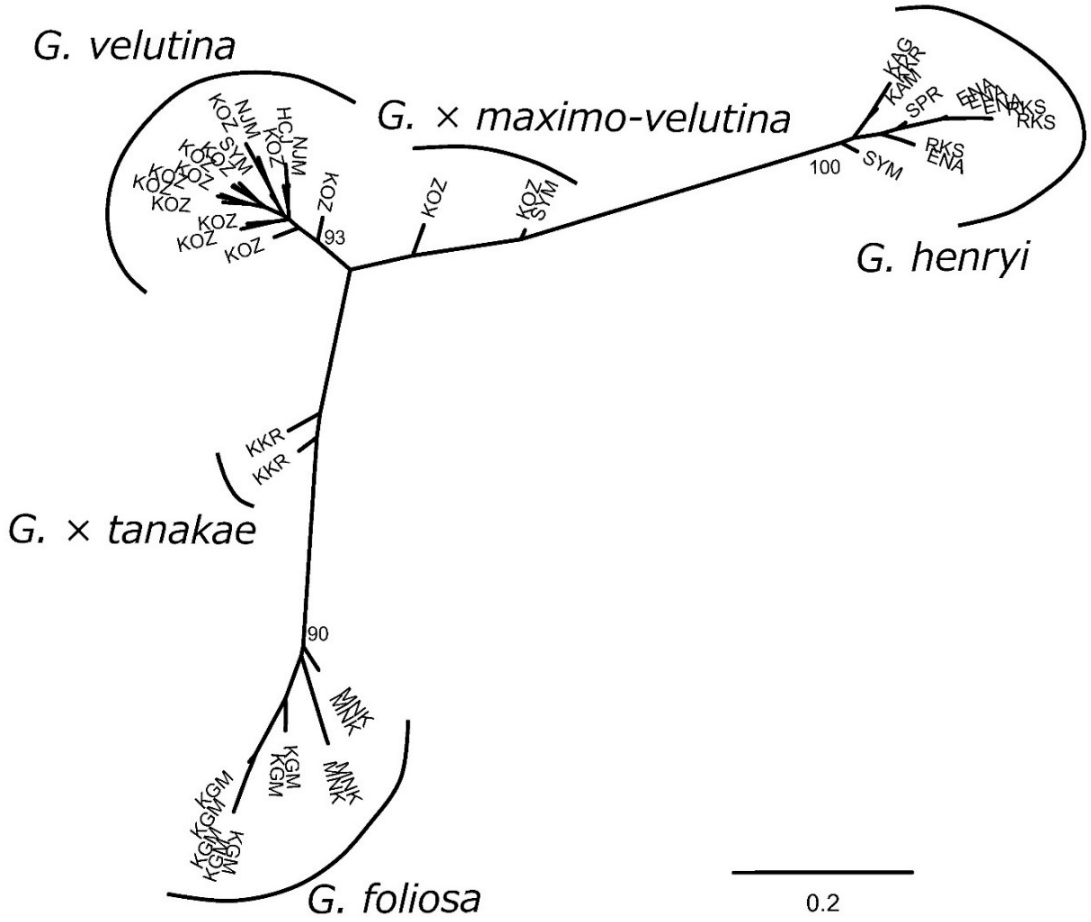

Fig. 4. Phylogenetic tree of 44 *Goodyera* samples, reconstructed using MIG-seq data. Bootstrap values within species and those less than 50% are not shown. Branch length represents the average number of substitutions per SNP site.

were classified into the same clusters, the analysis at K = 3 (the second-largest delta K) showed that *G. foliosa*, *G. henryi* and *G. velutina* could be classified into three groups (Fig. 6). Taken together with the fact that *G. henryi* is also distinguished from *G. foliosa* by many morphological traits (Chen *et al.* 2009; Hu *et al.* 2016; Suetsugu *et al.* 2019), we also consider that *G. henryi* is not so much an intraspecific variant of *G. foliosa* as an independent species, based on the phylogenetic distinctness at least in the ML and SplitsTree phylogenetic analysis. Since *G. foliosa* and *G. velutina* can be distinguished by leaf venation pattern and many other morphological features (Table 1), Hu *et al.* (2016) concluded that further studies are needed to solve the complex relationships among *G. foliosa*, *G. henryi* and *G. velutina*. The present study demonstrated the effectiveness and robustness of MIG-seq data for defining the recently divergent *Goodyera* species that were otherwise indistinguishable using genetic markers based on Sanger sequencing. In addition, MIG-seq data confirmed the previous notions that it is appropriate to synonymize *G. sonoharae* under *G. foliosa* (Chen *et al*., 2009).

Molecular data have also supported that *G.* ×*tanakae* is a natural hybrid between *G. foliosa* and *G. velutina.* The Neighbor-Net (SplitsTree) phylogenetic analysis indicated that *G.* ×*tanakae* occupies an intermediate position between *G. foliosa* and *G. velutina* (Fig. 5). The STRUCTURE analysis also showed that *G.* ×*tanakae* has genetic components of both *G. foliosa* and *G. velutina* (Fig. 6). Therefore, our genetic analysis confirmed that *G.* ×*tanakae* has a different evolutionary origin from *G. maximo-velutina*, a natural hybrid between *G. henryi* and *G. velutina* (So and Lee, 2017; Suetsugu *et al.*, 2019): *G. maximo-velutina* occupies an intermediate position between *G. henryi* and *G. velutina* in the phylogenetic tree and the Neighbor-Net (Figs. 4, 5) and has genetic components of both *G. henryi* and *G. velutina* (Fig. 6). Since these *Goodyera* species are neither autogamous nor apogamous but are pollinator-dependent (Sugiura and Yamaguchi, 1997), natural hybridization must have occurred via their shared pollinators. Although little is known about the pollinator assembles of *Goodyera* species, information on pollination biology in these *Goodyera* species could improve our understanding of the origins of *G.* ×*tanakae* and *G. maximo-velutina*.

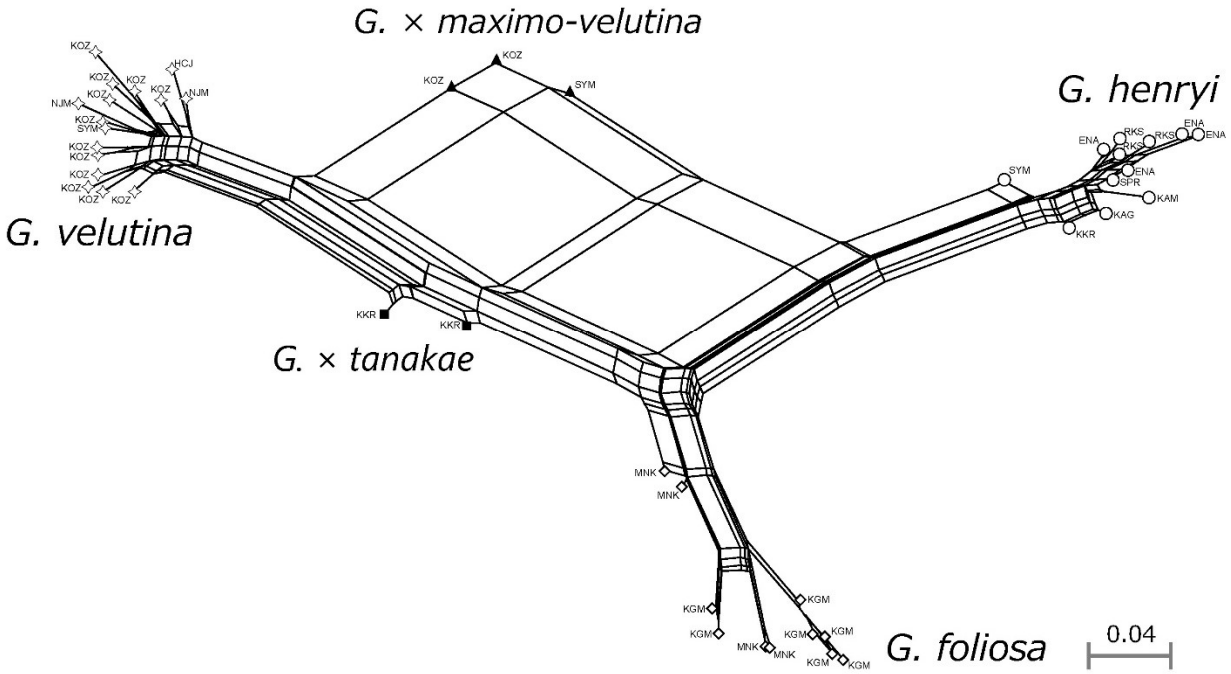

Fig. 5. Neighbor-Net network derived from 44 *Goodyera* samples based on uncorrected P distance calculated from 430 SNPs. Open star: *G. velutina*. Filled square: *G. ×tanakae*. Open lozenge: *G. foliosa*. Open circle: *G. henryi*, Filled triangle: *G. maximo-velutina*.

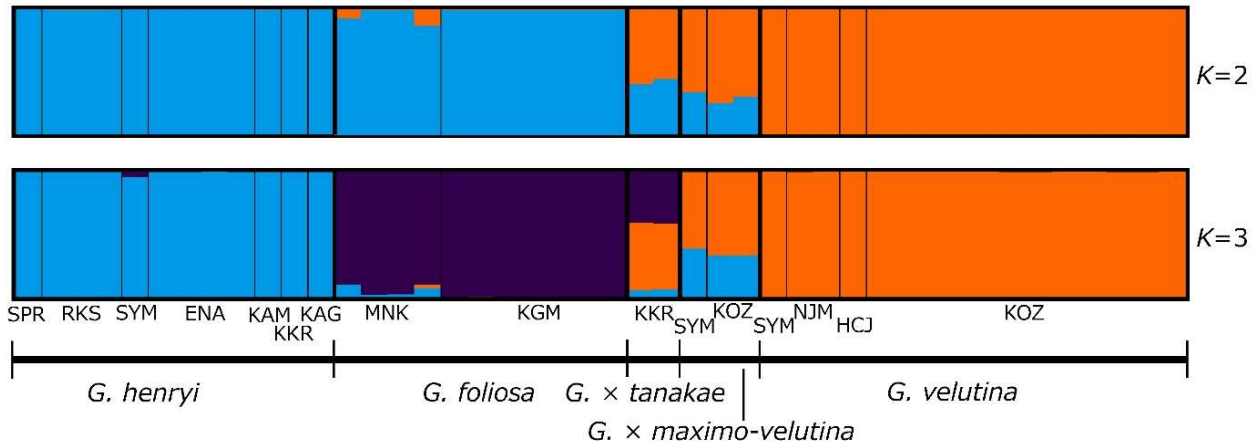

Fig. 6. Population structure derived from 44 *Goodyera* samples inferred by STRUCTURE. *K* = 2 and 3 have the largest and second-largest delta *K* for our data, indicating that *K* = 2 and 3 were the most and second most optimal. Species and populations are separated by broad and narrow vertical black lines, respectively.

TAXONOMIC TREATMENT

Goodyera* ×*tanakae Suetsugu, ***sp. nov.***

Figs. 1F–J & 3

Type: JAPAN. Fukuoka Prefecture, Kitakyushu City, Kokura-minami-ku, Dobaru, 22 September 2016, *Koji Tanaka KS818* (holotype: KYO, herbarium specimen and liquid-preserved material, labelled as the same specimen).

***Diagnostic characters*:** *Goodyera* ×*tanakae* is similar to *G. velutina* but differs in its lanceolate-ovate leaves with a faint central vein and more densely arranged flowers.

Terrestrial herbs, 10–20 cm tall. Rhizome slender, few noded. Roots yellowish brown, with minute root hairs. Stems erect, 5.5–11.0 cm long, 4–6-leaved. Leaves alternate, widely spaced or somewhat clustered toward apex; lamina velutinous dark green without distinct white central vein, lanceolate–ovate, 3.5–5.9 × 1.6–2.5 cm, length/width ratio 2.0–2.9, base rounded, margin entire, apex acute; petiole-like base and tubular sheath 0.8–1.3 cm long. Peduncle green, 2.5–5.0 cm, pubescent, with 1

or 2 sterile bracts; rachis 3–6 cm, sublaxly 6–15-flowered, secund; floral bracts reddish-brown, lanceolate, 10.0–15.0 × 2.5–3.0 mm, pubescent. Flowers opening weakly, white tinged pink; ovary reddish brown, cylindric-fusiform, 8–10 mm long, pubescent. Sepals pubescent on outer surface; dorsal sepal oblong, 8.5–11.5 × 2.2–3.0 mm, concave, forming hood with petals, apex obtuse; lateral sepals obliquely ovate-elliptic, ovate, 8.0–10.5 × 3.5–5.0 mm, apex obtuse. Petals obliquely rhombic, 8.0–11.0 × 3.3–4.5 mm, 1-veined, narrowly contracted at base, apex obtuse; lip ovate, 7.5–8.5 mm long; hypochile concave-saccate, inside papillose; epichile ligulate, 3.7–4.2 mm, apex recurved. Column ca. 5.5 mm long, clawed at base; rostellum slender, yellowish, deeply bifid, apex flattened and wedge-shaped; stigma orbicular, surrounded by a hood-like rim, rim prominent at the base; anther cap ovate, much shorter than rostellar arm; pollinia 2, oblanceolate attached to a narrow translucent ellipsoid viscidium. Fruit reddish brown, capsule, 9–11 mm long, erect, pubescent. Seeds fusiform.

***Etymology*:** *Goodyera ×tanakae* is named after Koji Tanaka, who collected the type specimens.

Distribution: *Goodyera ×tanakae* is currently known only from the type locality. The population harbors dozens of flowering plants under humid evergreen broadleaf forest dominated by *Aucuba japonica* Thunb. The new hybrid is not sympatric with parental taxa *G. foliosa* and *G. velutina*. In particular, the nearest population of *G. foliosa* is more than 10 km away, while *G. velutina* can be observed within a few kilometers square. Therefore, *Goodyera ×tanakae* may have different niche preferences from *G. foliosa* and *G. velutina*, although the allopatric distributional pattern does not necessarily indicate niche partitioning, given that orchid seeds are typically dispersed over long distances by the wind.

It is known that interspecific hybridization in plants has played a crucial role in plant evolution (Soltis and Soltis, 2009) including adaptation to novel environments and generation of phenotypic diversity (Goulet *et al.*, 2017; Nakahama *et al.*, 2019; Pace *et al.*, 2019). Because several studies have also suggested that hybridization is an essential mechanism in the evolution of the genus *Goodyera* (Kallunki, 1976; So and Lee, 2017), further studies are needed to assess the evolutionary role of hybridization in genus *Goodyera.*

Additional specimens examined: JAPAN. Fukuoka Prefecture, Kitakyushu City, Kokura-minami-ku, Dobaru, 27 September 2016, *Koji Tanaka KS819* (KYO).

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