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First record of *Goodyera* ×*maximo-velutina* (Orchidaceae) from Kozu Island, Japan

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ABSTRACT: Several individuals of an unknown taxon of *Goodyera* were discovered on Kozu Island, Japan. Detailed morphological investigation suggests that this taxon is considered as a natural hybrid between *G. maximowicziana* and *G. velutina* based on morphological characteristics, such as length of peduncle. Since the natural hybrid between *G. maximowicziana* and *G. velutina* was recently reported in Jeju Island, South Korea and described as *G. ×maximo-velutina,* here we recorded the first occurrence of *G. ×maximo-velutina* from Japan. Investigation on chromosome number among *G. maximowicziana, G. velutina*, and *G. ×maximo-velutina* provides the further support of hybrid status in *G. ×maximo-velutina* (Orchidaceae) from Kozu Island, Japan. We also discussed the utility of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA for detecting the hybrid between *G. maximowicziana* and *G. velutina.*

KEY WORDS: Chromosome number, *Goodyera maximowicziana*, *Goodyera velutina*, hybridization, Izu Islands, Japan.

INTRODUCTION

The genus *Goodyera* R. Br. (Orchidaceae) includes ca. 100 species that are 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 and leaves that often have white or golden venation on the upper surface (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). The flowers also have a single anther with sectile pollinia. The hairs within the concave-saccate hypochile of the lip clearly distinguish *Goodyera* from related genera (Chen *et al.* 2009). Within the genus, however, species are somewhat difficult to distinguish, owing to ambiguous diagnostic characters and similar floral features (Shin *et al.* 2002; Guan *et al.* 2014; Hu *et al.* 2016; So & Lee 2017; Suetsugu & Hayakawa 2019).

During recent botanical survey in Kozu Island, Japan, we collected an unknown taxon of *Goodyera*. There are three *Goodyera* species (i.e., *G. maximowicziana* Makino, *G. velutina* Maxim. ex Regel, and *G. hachijoensis* Yatabe) that bloom in same season in the investigated population. It seems that this plant exhibited intermediate morphological characters between *G. maximowicziana* and *G. velutina*. Therefore, this unknown taxon can be considered as a natural hybrid of them. The natural hybrid between *G. maximowicziana* and *G. velutina* was recently reported in Jeju Island, South Korea and described as *G. ×maximo-velutina,* which is named after two parental taxa *G. maximowicziana* and *G. velutina* (So & Lee 2017). Detailed morphological investigation reported here actually showed that this taxon is considered as a natural hybrid between *G. maximowicziana* and *G. velutina*. Therefore, here we recorded the first occurrence of *G. ×maximo-velutina* from Japan. In addition, we discussed the utility of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA for detecting the hybridity between *G. maximowicziana* and *G. velutina*. Cytological investigations among *G. maximowicziana, G. velutina*, and *G. ×maximo-velutina* were also carried out.

MATERIALS AND METHODS

Field sampling

Living materials were collected from 2017 and 2018 in Kozu Island, Tokyo Metropolis, Japan, which is situated in a warm temperate area of Eastern Japan. Kozu Island is one of the seven main islands of the Izu Islands group, which stretches south from the entrance of the Bay of Tokyo. In total, fifteen individuals of *G. velutina*, seven individuals of *G. maximowicziana*, and three individuals of *G. ×maximo-velutina* were collected in

Kozu Island, Tokyo Metropolis. Voucher specimens of these 25 individuals were deposited in the herbariums of National Museum of Nature and Science (TNS) and Museum of Natural and Environmental History, Shizuoka (SPMN) after collection on the data on morphological and cytological characters.

Morphological observation

We compared the morphological characters of the putative *Goodyera* ×*maximo-velutina* from Kozu Island, Japan with the sympatric *G. maximowicziana* and *G. velutina* using aforementioned 25 individuals. The morphological variation of *G. maximowicziana* and *G. velutina* was also investigated by reviewing the literature and herbarium specimens collected in other localities and deposited in TNS. Morphological characters were observed visually and under a stereomicroscope and also measured using a digital caliper.

Molecular analysis

We analyzed ITS sequences of three individuals of *G. maximowicziana* and three individuals of *G. velutina* collected in the Honshu, Shikoku, and Kyushu districts, Japan to determine the utility of the ITS regions for detecting the hybridity between *G. maximowicziana* and *G. velutina.* For our molecular analyses, we isolated total DNA from leaves using a DNeasy® Plant Mini Kit (Qiagen KK, Tokyo, Japan) following the manufacturer's instructions. We amplified the ITS regions using the universal primers "ITS5W" and "ITS4" (White et al. 1990). The isolated DNA was amplified by PCR in 16 μL reaction solutions each containing 0.5 μM of each forward and reverse primer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, and 0.05 units of TaKaRa Ex Taq® DNA polymerase (Takara Bio Inc. Shiga, Japan) in a 1 × concentration buffer supplied by the manufacturer. We used the following thermal cycle profile for amplification: 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C for 35 cycles, followed by a 5 min final extension at 72°C. To check amplification, 5 μL of each product was loaded onto 1.0% agarose gels for electrophoresis. The PCR products were purified with Exo-SAP-IT® (USB Corporation, Cleveland, OH, USA) and directly sequenced in both directions using a BigDye® Terminator kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. The primers used for amplification were also used for sequencing. The resulting sequences were submitted to GenBank (accession numbers LC464141, LC485469 and LC485470 for *G. maximowicziana* and LC464142, LC464143 and LC485472 for *G. velutina*) (Table 2). To reveal genetic polymorphisms among the two species, we obtained sequences of the ITS regions of *G. maximowicziana* and *G. velutina* from the DDBJ database (Table 2).

Cytological observation

Root tips were collected from fifteen individuals of *G. velutina*, seven individuals of *G. maximowicziana*, and three individuals of *G.* ×*maximo-velutina* in Kozu Island, Tokyo Metropolis. They were used for mitotic chromosome counts. In summary, they were pretreated with 2 mM 8-hydroxyquinoline solution for 4–5 h, fixed in Carnoy's solution for 1–24 h, macerated in 1 N HCl at 60°C for 1 min, and then squashed in aceto-orcein. The sample was observed under a light microscope. The photo image of the orcein-stained chromosomes was taken using a digital camera connected to a light microscope.

RESULTS AND DISCUSSION

Detailed morphological examination revealed that the morphological characters of an unknown taxon of *Goodyera*, such as leaf coloration and veins, length of peduncle and aspect ratio of the lateral petal, are intermediate between *G. maximowicziana* and *G. velutina* (Figs. 1, 2). In particular, we showed that the length of peduncle greatly differs among *G. maximowicziana*, *G. velutina*, and *G.* ×*maximo-velutina*. Consequently, the taxon should be considered as the natural hybrid, *G.* ×*maximo-velutina*.

The leaf morphology of *G.* ×*maximo-velutina* from Kozu Island (hereafter, *G.* ×*maximo-velutina*) is more similar to the *G. maximowicziana*, since the leaf is green, midvein is not clear, and with 1 or 2 pairs of lateral veins (So & Lee 2017). However, that of *G.* ×*maximo-velutina* is still somewhat similar to *G. velutina* since the coloration of *G.* ×*maximo-velutina* is darker than *G. maximowicziana* (Fig. 1C). In addition, *G. maximowicziana* can be distinguished from *G. velutina* by shorter peduncle. Therefore, it is noteworthy that the length of *G.* ×*maximo-velutina* peduncle (1.5–3 cm) is intermediate between *G. maximowicziana* (usually less than 1 cm) and *G. velutina* (usually more than 3 cm; Fig. 1A). While So and Lee (2017) did not considered the peduncle length as the diagnostic character, the photos of *G.* ×*maximo-velutina* from Jeju Island, South Korea is also intermediate between these two parental species. Furthermore, *G.* ×*maximo-velutina* is intermediate between its putative parents in some floral characters. While aspect ratio of the lateral petal in *G. maximowicziana* is ca. 2.9, that in *G. velutina* is ca. 2.1. In addition, that of *G.* ×*maximo-velutina* is ca. 2.6 (Fig. 2C, I, O). For a detailed comparison of morphological characters among *G.* ×*maximo-velutina*, *G. maximowicziana*, and *G. velutina*, see Table 1.

So and Lee (2017) conducted morphological and molecular analyses to prove hybridity of *G.* ×*maximo-velutina* and suggested utility of the ITS regions. So and Lee (2017) noted that *G.* ×*maximo-velutina* shared 3

Table 1. Morphological comparison among ***Goodyera maximowicziana***, ***G.* ×*maximo-velutina*** and ***G. velutina*** in Kozu Island, Tokyo, Japan.

	G. maximowicziana	***G.* ×*maximo-velutina***	***G. velutina***
leaf color	green	dark green	dark purplish green
central vein	faint	intermediate	distinct
lateral vein	1–2 pairs	1–2 pairs	none
peduncle length	less than 1 cm	1.5–3 cm	more than 3 cm
pedicel hairiness	puberulent	puberulent	densely puberulent
pedicel color	pale green to reddish brown	pale green to reddish brown	reddish brown
ovary hairiness	puberulent	puberulent	densely puberulent
ovary color	pale green to reddish brown	pale green to reddish brown	reddish brown
bract color	pale green	pale green to reddish brown	reddish brown
aspect ratio of the lateral petal (mean)	(2.4–)2.9(–3.4)	(2.5–)2.6(–2.7)	(1.9–)2.1(–2.3)

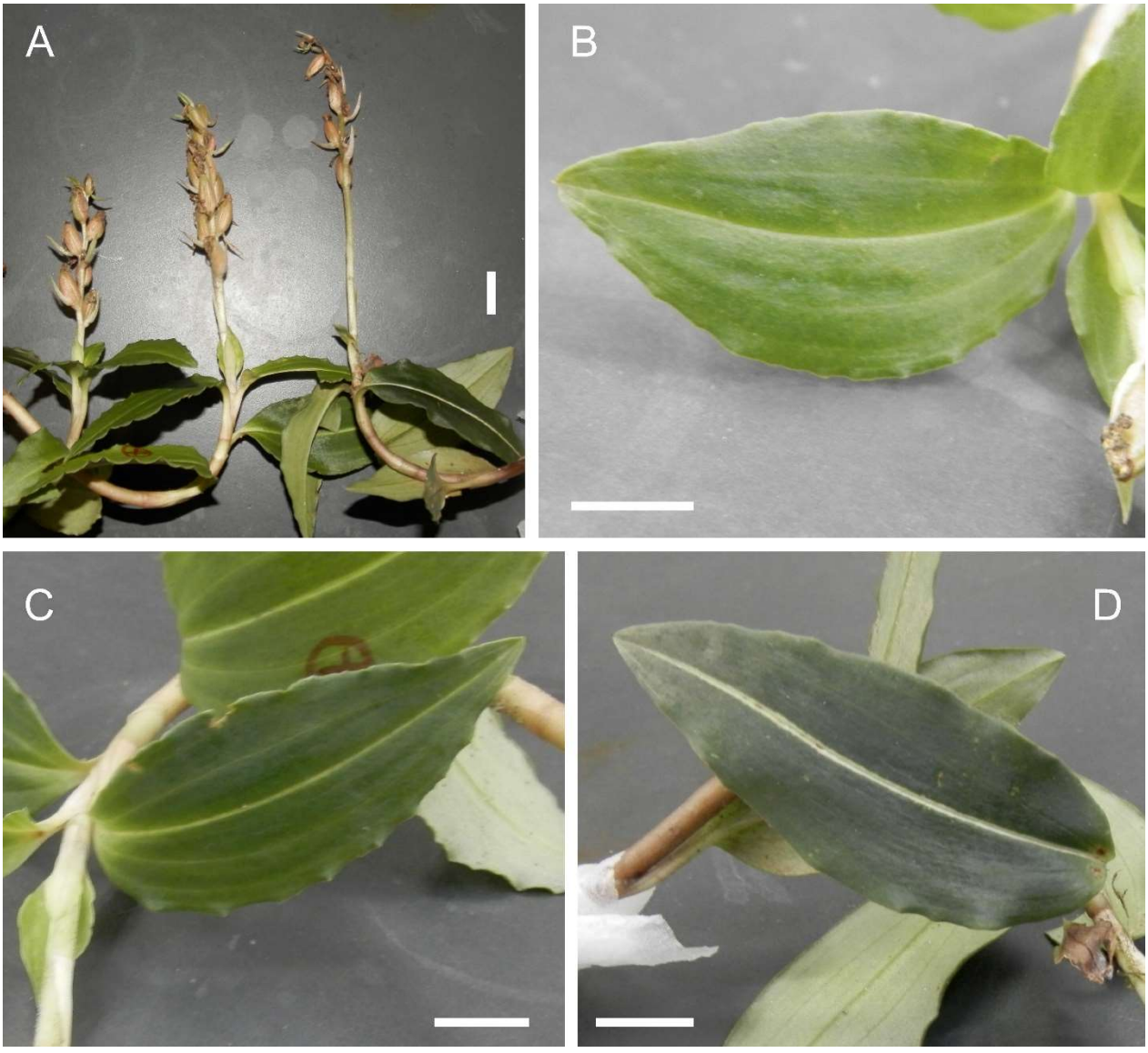

Fig. 1. Comparison among ***Goodyera maximowicziana***, ***G.* ×*maximo-velutina***, and ***G. velutina***. **A.** Habit (left ***G. maximowicziana***, middle ***G.* ×*maximo-velutina***, right ***G. velutina***). **B.** ***G. maximowicziana*** leaf. **C.** ***G.* ×*maximo-velutina*** leaf. **D.** ***G. velutina*** leaf. All scale bars = 1 cm.

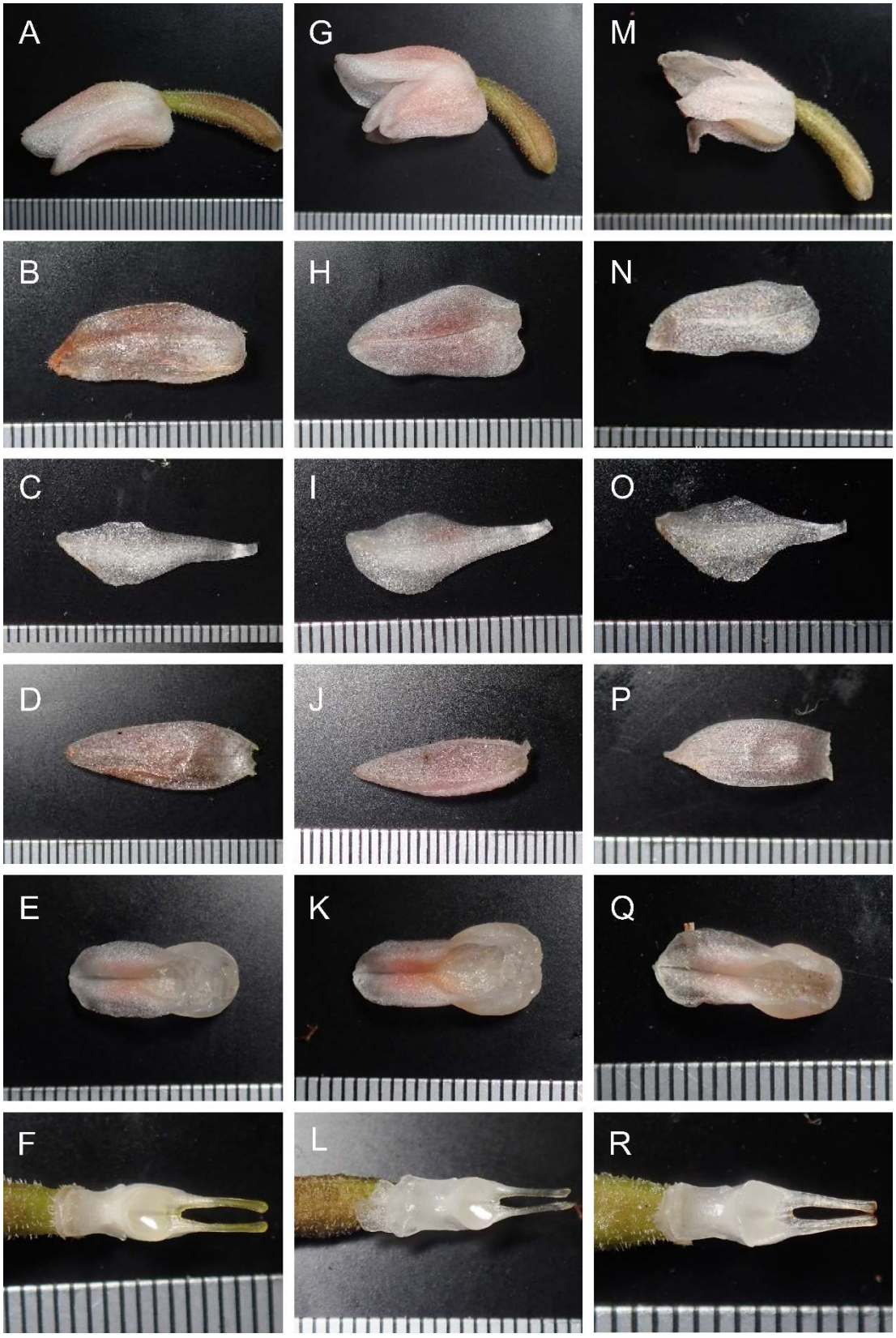

Fig. 2. Comparison of floral parts among ***Goodyera maximowicziana*** (**A**–**F**), ***G. ×maximo-velutina*** (**G**–**L**), and ***G. velutina*** (**M**–**R**). **A.** Flower. **B.** Lateral sepal. **C.** Lateral petal. **D.** Dorsal sepal. **E.** Lip. **F.** Column. **G.** Flower. **H.** Lateral sepal. **I.** Lateral petal. **J**. Dorsal sepal. **K**. Lip. **L**. Column. **M.** Flower. **N.** Lateral sepal. **O.** Lateral petal. **P.** Dorsal sepal. **Q.** Lip. **R.** Column. All scale bars = 0.5 mm.

Table 2. Comparison of informative nucleotide sites in the ITS regions to infer the parental species of ***Goodyera* ×*maximo-velutina*** described by So and Lee (2017).

Species	Site* 112	512	652	Accession	Reference
G. maximowicziana**					
Japan: Fukuoka	C	G	T	LC464141	this study
Japan: Gifu, Ena	A	G	T	LC485469	this study
Japan: Kochi, Kami	C	G	T	LC485470	this study
Japan	C	G	?	HM140998.1	Shefferson *et al.* (2010)
G. velutina					
Japan: Fukuoka, Kitakyushu	C	G	A	LC464142	this study
Japan: Fukuoka, Kitakyushu	C	G	N	LC464143	this study
Japan: Kochi, Kami	C	G	A	LC485471	this study
China	A	T	A	KT344077.1	Hu *et al.* (2016)
China	A	K	A	KT344075.1	Hu *et al.* (2016)
China	C	G	A	KT344073.1	Hu *et al.* (2016)
China	C	G	T	KT344071.1	Hu *et al.* (2016)

*Putative sites are shown because we could not find the sequences descried by So and Lee (2017).

**According to the policy in the taxonomy database, we deposited in the DDBJ database as *G. foliosa* var. *leavis*, a synonym of *G. maximowicziana*.

specific character states in the ITS regions, with *G. maximowicziana* and *G. velutina* at 3 positions: 112 (A/C), 512 (G/T), 652 (A/T) using samples within Jeju Island, South Korea. Unfortunately, we could not find the sequences and accession numbers in the DDBJ database used So and Lee (2017). However, the *G. maximowicziana* and *G. velutina* sequences of samples deposited in the DDBJ database and our samples collected in the Honshu, Shikoku, and Kyushu districts, Japan showed that the 3 characters are not autapomorphy but synapomorphy (Table 2). These results indicate that ITS sequences are not so conclusive to determine the hybridity of *G.* ×*maximo-velutina*. Therefore, other methods such as cytological analysis will be required to determine the origin of *G.* ×*maximo-velutina.*

Investigation on chromosome number among *G. maximowicziana, G. velutina*, and *G.* ×*maximo-velutina* provides the further support of hybrid status in *G.* ×*maximo-velutina* from Kozu Island, Japan. While the chromosome number of $2n = 28$ was counted for most (14/15) *G. velutina* individuals ($2n = 56$ for 1/15), the chromosome number of $2n = 56$ was counted for all (7/7) *G. maximowicziana* individuals. The number is reasonable since the chromosome number $2n = 28$ for *G. velutina* and $2n = 56$ for *G. maximowicziana* is reported as common type in warm temperate region of Japan (Tanaka 1965a, b). In contrast, the chromosome number of all (3/3) *G.* ×*maximo-velutina* individuals was counted as $2n = 42$ (Fig. 3), supporting that hybrid statu in *G.* ×*maximo-velutina* from Kozu Island, Japan.

Interestingly, *G. maximowicziana* from Kozu Island is slightly different from that from Jeju Island and other areas in that the pedicel and ovary are at least somewhat puberulent (So & Lee 2017). Therefore, it may be even possible that introgressive hybridization between *G. maximowicziana* and *G. velutina* widely occur in Kozu Island, and the introgressive hybridization may be the cause for puberulent status of *G. maximowicziana* from Kozu Island. Actually, investigation on chromosome number will not identify the hybridization between *G. maximowicziana* with $2n = 56$ and *G. velutina* with $2n = 56$, while hybridization with *G. velutina* is less conclusive than the plants we recorded here as *G.* ×*maximo-velutina* in most *G. maximowicziana* from Kozu Island, judging from other morphological characters (Table 1). In addition, we could not completely exclude the possibility that a closely related species *Goodyera foliosa* (Lindl.) Benth. ex C.B.Clarke is one of candidate parents in *G.* ×*maximo-velutina* while the possibility is low since *G. foliosa* does not distribute sympatrically not only Kozu Island but also Jeju Island. Further detailed molecular investigation such as microsatellite markers will reveal the pattern and degree of hybridization of *G.* ×*maximo-velutina* in Kozu Island and Jeju Island.

Goodyera* ×*maximo-velutina N.S. Lee & J.H. So Phytotaxa 317: 67 (2017)

Terrestrial herb, 5–15 cm tall. Rhizome pale green to brownish green, rooting at nodes. Roots fleshy, yellowish brown, with minute root hairs. Stem erect, 3–13 cm long, terete, pale green to reddish green. Leaves dark green, ca. 2–6 × 1.5–3 cm, 1 midvein and 1–2 pairs of lateral veins, leaf margin undulated. Inflorescence with (1–)4–11 flowers. Peduncle 1.5–3 cm, pale green to pale reddish brown, puberulent. Rachis 2–5 cm, subdensely-flowered. Floral bracts lanceolate, 8–10 mm long, slightly pubescent on outer surface, apex acuminate, pale green to reddish brown. Flower resupinate, pale pink to reddish pink. Ovary pedicellate, cylindric-fusiform, 8–10 mm long, pale green to pale reddish brown, puberulent. Sepals 3, free, sub-similar, pubescent on outer surface, 1-veined; dorsal sepal narrowly elliptic-lanceolate, cymbiform, 8.5–9.5 × 3.8–4.4 mm, sub-acute at apex, forming hood with petals; lateral sepals ovate-lanceolate to triangular, 8.3–9.3 × 3.3–3.6 mm, apex obtuse. Petals obliquely rhombic-oblanceolate to oblong-oblanceolate, 8.3–9.3 × 3.4–3.8 mm, apex of hood recurved, 1-veined. Lip 7.0–7.8 × 3.6–3.8 mm; hypochile concave-saccate, inside base papillose; epichile ligulate, apex subacute. Column ca. 5.5 mm long; stigma orbicular, slightly protruding.

Distribution: Japan (Tokyo Metropolis, Kozu Island, new record) and South Korea (Jeju Island). In Japan, *Goodyera* ×*maximo-velutina* is currently only known from a single population of ca. 10 flowering individuals

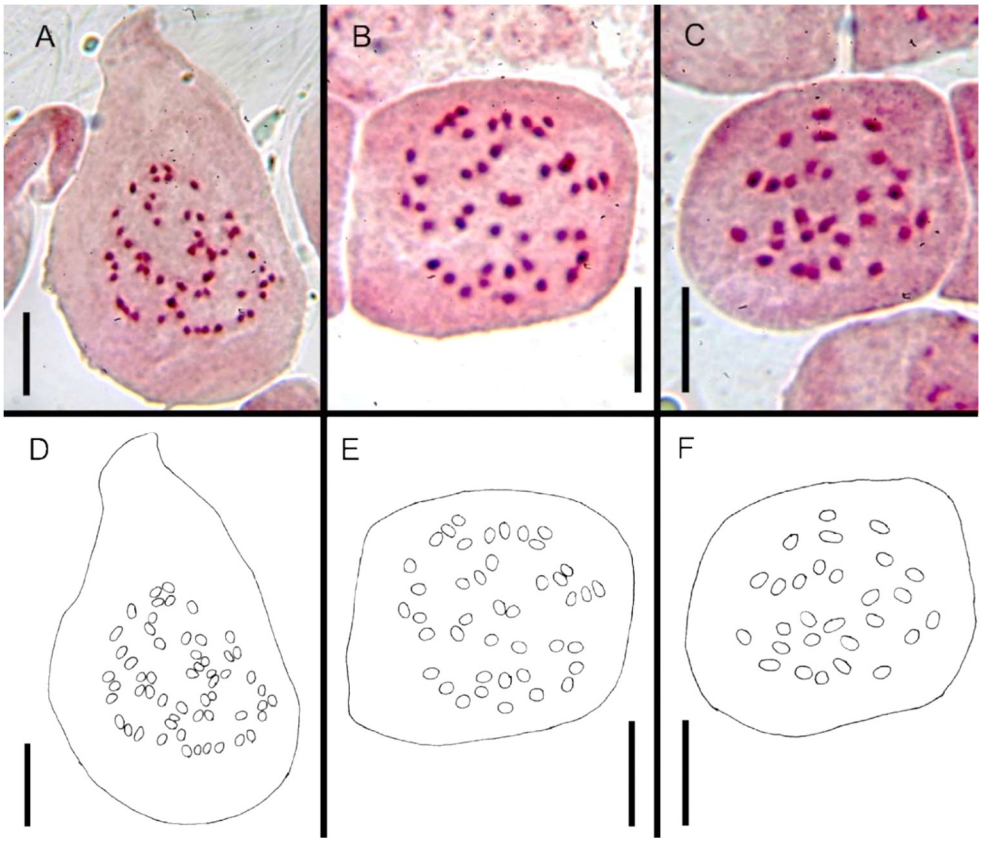

Fig. 3. Somatic chromosomes (**A**–**C**) and explanatory drawings (**D**–**F**) of ***Goodyera***. **A** & **D**. ***Goodyera maximowicziana*** (2*n* = 56). **B** & **E**. ***G. ×maximo-velutina*** (2*n* = 42). **C** & **F**. ***G. velutina*** (2*n* = 28). All scale bars = 10 μm.

in an evergreen broadleaved forest dominated by *Castanopsis sieboldii* (Makino) Hatus. ex T.Yamaz. et Mashiba in Kozu Island, Tokyo Metropolis.

Specimen examined: JAPAN. Tokyo Metropolis, Kozu Island, 16 September 2018, *K. Ishida HT12* (TNS), Tokyo Metropolis, Kozu Island, 1 October 2017, *K. Ishida HH219* (SPMN), Tokyo Metropolis, Kozu Island, 1 October 2017, *K. Ishida HH220* (SPMN).

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LITERATURE CITED

Chen, X., K.Y. Lang, S.W. Gale, P.J. Cribb and P. Ormerod. 2009. *Goodyera. In*: Wu, Z. Y., P. H. Raven and D. Y. Hong (eds.), Flora of China, vol. 25, pp. 45-54. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Guan, Q.X., G.Z. Chen, M.H. Li and S.P. Chen. 2014. *Goodyera malipoensis* (Cranichideae, Orchidaceae), a new species from China: Evidence from morphological and molecular analyses. Phytotaxa **186(1)**: 51-60.

Hu, C., H. Tian, H. Li, A. Hu, F. Xing, A. Bhattacharjee, T. Hsu, P. Kumar and S. Chung. 2016. Phylogenetic analysis of a 'jewel orchid' genus *Goodyera* (Orchidaceae) based on DNA sequence data from nuclear and plastid regions. PloS One **11(2)**: e0150366.

Shefferson, R.P., C.C. Cowden, M.K. McCormick, T. Yukawa, Y. Ogura-Tsujita and T. Hashimoto. 2010. Evolution of host breadth in broad interactions: mycorrhizal specificity in East Asian and North American rattlesnake plantains (*Goodyera* spp.) and their fungal hosts. Mol. Ecol. **19(14)**: 3008-3017.

Shin, K., Y.K. Shin, J. Kim and K. Tae. 2002. Phylogeny of the genus *Goodyera* (Orchidaceae; Cranichideae) in Korea based on nuclear ribosomal DNA ITS region sequences. Journal of Plant Biology **45(3)**: 182-187.

Suetsugu, K. and H. Hayakawa. 2019. A new variety of *Goodyera schlechtendaliana* (Orchidaceae) from Yakushima and Okinawa, Japan. Acta Phytotax. Geobot. **70:** 49-55.

So, J. and N. Lee. 2017. The Origin of New Natural hybrid, *Goodyera* × *maximo-velutina* (Orchidaceae) from Jeju Island, Korea. Phytotaxa **317(1)**: 61-68.

Tanaka, R. 1965a. Chromosome numbers of some species of Orchidaceae from Japan and its neighbouring areas. J. Jpn. Bot. **40**: 54-77.

Tanaka, R. 1965b. Intraspecific polyploidy in *Goodyera maximowicziana* Makino. La Kromosomo **60**: 1945-1950.

White, T., J.T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* Innis, M., D. Gelfand, J. Sninsky and T. J. White. (eds.), PCR protocols: a guide to methods and application, pp. 315-322. Academic Press, San Diego.