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Phylogeny of the Genus *Goodyera* (Orchidaceae; Cranichideae) in Korea Based on Nuclear Ribosomal DNA ITS Region Sequences

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We sequenced the nuclear ribosomal internal transcribed spacer (ITS) regions to determined the phylogenetic relationships of the several *Goodyera* species in Korea and to measure the extent of their differentiation region. ITS 1 was 238 to 239 bp long while ITS 2 was 258 to 259 bp. The 5.8S coding region was 156 bp long. Sequence divergences among species, calculated by Kimura's two- parameter method, ranged from 0.0 to 5.4%. The most parsimonious tree, with a consistency index of 0.935 and a retention index of 0.937, was produced with 337 steps. Our ITS sequence results demonstrate the monophyly of Korean *Goodyera* and support previous morphological, geographical, and RAPD data analyses.

Keywords: *Goodyera*, ITS region, nrDNA ITS sequences, phylogenetic relationships

The genus *Goodyera* R. Br. (Orchidaceae), comprising 40 species, is distributed in all the temperate and tropical regions of the northern hemisphere, except Africa (Li, 1978; Satake et al., 1982). Although most of the species are restricted to far eastern Asia, including China, Taiwan, Korea, and Japan, *Goodyera repens* is more widely scattered. This genus is characterized by creeping rhizomes, simple stamens, and hairs at the bases of the lips, all of which distinguish it from related genera (Ohwi, 1984). However, no overall generic review or no discussions on the infrageneric classification system had been conducted until now.

For some species, investigations have been made of their morphology and phytogeography (Kitagawa, 1939; Fernald, 1950; Kitamura et al., 1980), cytology (Richardson, 1935; Eftimiu-Heim, 1941; Love, 1954; Mutsuura and Nakahira, 1960; Vij and Gupta, 1975; Love and Love, 1981), reproductive processes (Ackerman, 1975), population dynamics (Kallunki, 1976), and palynology (Ikuse, 1956; Ueno, 1976; Konta and Tsuji, 1982). Five *Goodyera* species have been recorded in Korea (Tae et al., 1997). Studies of the relationships, speciation process, and phylogeny among those the Korean species have been based on karyotype analysis (Tae et al., 1997); cluster analysis with morphological, anatomical, cytological, and geographical data (Tae and Ko, 1999); as well as, and RAPD analysis (Tae et al., 1999). Based on those examinations, researchers have suggested that the Korean *Goodyera* species be divided into two groups.

Use of nuclear ribosomal DNA (nr DNA) internal transcribed spacer (ITS) regions has become popular because of the relatively high rate of nucleotide substitutions. That has permitted systematic comparisons of relatively recently diverged taxa and, especially, the low level taxa such as the infrageneric species (Kim and Jansen, 1994; Baldwin et al., 1995; Choi and Kim, 1997; Choi et al., 2000; Erdogan and Mehlenbacher, 2000).

The objective of this study was to analyze the phylogenetic relationships among Korean *Goodyera* species based on their nr DNA ITS region sequences. We also wanted, to compare the results our sequencing with those from previous morphological, cytological, anatomical, and RAPD studies (Vij and Gupta, 1975; Tae et al., 1997, 1999; Tae and Ko, 1999).

MATERIALS AND METHOD

Plant Material and DNA Extraction

Plant materials and collection data are listed in

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Table 1. Leaves, used as DNA sources, were gathered from natural populations. All plant samples were kept in vinyl zipper bags with silicagel until they were stored at −70°C in the laboratory. After pulverizing the leaf tissues in liquid nitrogen, we extracted total genomic DNA using 2X CTAB buffer (Doyle and Doyle, 1987). The DNA samples were then stored at −20° C.

Amplification and Sequencing of ITS Regions

We amplified double-stranded DNA of the complete ITS region (including the 5.8 S coding region) in each genomic DNA. The primers included ITS 4R (5'-TCCT-CCGCTTATTGATATGC-3') and ITS 5F (5'-GGAAG-TAAAAGTCGTAACAAGG-3') (White et al., 1990). Amplifications were performed in 50 μL reactions that contained 10 to 50 ng DNA, 200 μM deoxyribonucleotide triphosphates (equimolar), 1 unit AmpliTaq DNA polymerase (Perkin Elmer, Cetus), 10 mM Tris-HCl, (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and primers at 0.5 to 1.0 μM. Before the symmetric cycles bagan, the mixture was predenatured at 92 °C for 3 min. Each PCR cycle consisted of 30 s at 95 °C for denaturation, 30 s at 50°C for annealing, and 1 min at 72°C for extension. After 30 cycles, the reactions were incubated at 72°C for 7 min to complete the final extension. The ITS PCR products were sequenced completely in both directions using primers ITS 5F and ITS 4R, according to the manufacturer's procedure for an ABI PRISM Dye Dideoxy Chain Terminator Cycle Sequencing Kit (Perkin Elmer; Norwalk, CT, USA). Sequenced fragments were resolved on an ABI 310 automated sequencer (Perkin Elmer). Two sequences from each PCR product were assembled, checked, and corrected where necessary using Sequencer version 3.1.1 (Gene Codes Corp.).

Sequence Alignment and Phylogenetic Analysis

The boundaries of the two ITS regions and three coding regions (18 S, 5.8 S, and 26 S) of nrDNA were determined by comparing them with published sequences (Cho et al., 1996; Choi et al., 1996; Sun et al., 2000). Sequences were aligned, with gap adjustments, using the Clustal X program of Thompson et al. (1997) and, ultimately, were visually determined. For our outgroups, we selected three related orchid genera (*Habenaria*, *Cynorkis*, and *Bonatea*) from the NCBI GenBank. These were used to help interpret the results and to confirm the phylogenetic relationships among our *Goodyera* species (Douzery et al., 1999; GenBank, 2002). Parsimony analyses were performed with unordered parsimony using PAUP* for Macintosh (Swofford, 2001). Gaps were not included in the analyses. The shortest tree was found via the heuristic search method (Option = TBR). Character-state optimization, ACCTRAN, and MULPARS option were used in the tree description. We also used an heuristic search algorithm to carry out bootstrap analysis (Felsenstein, 1985) with 1000 replicates in order to confirm the internal support and tree strength. Sequence divergence values between species were calculated according to the two-parameter method of Kimura (1980) for 462 aligned sites of the ITS 1 and ITS 2 regions. The Kimura's two-parameter method with the transition-transversion ratio set at 1:2 was selected for our corrections. In addition, we produced a neighbor-joining (NJ) tree (Saitou and Nei, 1987) based on the results of Kimura's two-parameter- method analysis.

RESULTS

ITS sequence, Size, Variation, and Base Composition

Complete ITS sequences (including the 5.8 S coding region) were analyzsed for eight *Goodyera* and outgroup species (Table 1). Sequences characteristics are summarized in Tables 2 and 3. Lengths of the ITS 1, 5.8

Table 1. Species of *Goodyera* and outgroup used for nrDNA ITS sequence analyses. The voucher specimens were deposited at HNU (Herbarium of Hannam University).

Species	Voucher specimens	Collection sites	GenBank Accession number
G. macrantha Maxim.	Tae, K.H. (HNU 017561)	Wando, 20 July 1996	AF366894
G. maximowicziana Makino	Tae, K.H. (HNU 022787)	Hallasan, 9 Sep. 1997	AF366895
G. repens (L.) R. Br.	Tae, K.H. (HNU 022788)	Hallasan, 10 July 1997	AF366896
G. schlechtendaliana Rchb. f.	Tae, K.H. (HNU 017564)	Kyeryongsan, 29 Aug. 1995	AF366897
G. velutina Maxim. ex Regel.	Tae, K.H. (HNU 017563)	Hallasan, 5 Oct. 1995	AF366898
Cynorkis galeata			AJ000135
Bonatea speciosa			AJ000121
Habenaria arenaria			AJ000139

Table 2. Sequence characteristics of nrDNA ITS region from *Goodyera* species.

	ITS1	5.8S	ITS2
Length range (bp)	238 - 239	156	258 - 259
Number of indels	3	0	1
G + C content	45.4 - 46.0%	53.9%	49.2 - 50.2%
Transitions (Ts)	3	0	5
Transversions (Tv)	19	0	10
Ts / Tv	0.16	0.00	0.50

Table 3. Size and G+C contents of ITS 1, ITS 2, and 5.8S coding regions of nrDNA from *Goodyera* species.

Species	Length, bp (G + C %)		
	ITS1	5.8S	ITS2
G. schlechtendaliana	239(46.0)	156(53.9)	258(49.6)
G. repens	239(46.0)	156(53.9)	258(49.6)
G. maximowicziana	239(45.6)	156(53.9)	259(50.2)
G. velutina	238(45.4)	156(53.9)	259(49.8)
G. macrantha	239(45.6)	156(53.9)	258(50.0)

S, and ITS 2 regions were within the range recorded for other flowering plants (Baldwin et al., 1995). The lengths of the ITS regions (including the 5.8 S coding region) varied from 653 to 654 bp. For all species, ITS 2 (258 to 259 bp) was longer than ITS 1 (238 to 239 bp). The 5.8 S coding region was 156 bp long in all species.

Sequence alignments for the *Goodyera* species required three and one independent insertion deletion mutations in ITS 1 and ITS 2, respectively. The G + C content was near or below 50%, with a narrow range of variation in ITS 1 (45.4 to 46.0%), ITS 2 (49.2 to 50.2%), and the 5.8 S coding region (53.9%). Eights transitions were made, with three in ITS 1 and five in ITS 2. Transversions totaled 29, with 19 in ITS 1 and 10 for ITS 2 (Table 2). The ratio of transition / transversion in ITS 2 (0.50) was approximately three times higher than for ITS 1 (0.16).

Sequence Divergence and Phylogenetic Analyses

The aligned sequences between the two amplifying primers for our *Goodyara* species included 656 positions, with 204 informative sites among them. No sequence variation was observed for the first 40 bp of ITS 2. Sequence divergences from ITS 1, ITS 2, and the 5.8 S cording region are presented in Table 4.

Between-pair divergences for the *Goodyera* ITS regions ranged from 0.00 to 5.4%, whereas the values between ingroup and outgroup varied from 43.9 to 48.6%. No differences were found between *G. schlechtendaliana* and *G. repens* (Table 4). Using the aligned sequences, the total number of variable sites among the *Goodyera* species was 38 (5.79%), with 23 (9.50%) in ITS 1 and 15 (5.79%) in ITS 2. Of the variable sites, the cladistically informative ones numbered 15 (6.1%) in ITS 1 and 10 (3.9%) in ITS 2.

Based on the analysis of the ITS region, we obtained a single, most parsimonious phylogenetic tree with a consistency index of 0.935 (excluding the uninformative sites, index of 0.918) and a retention index of 0.937. Tree length was 337. The bootstrap value and the number of informative sites for each branch are given in Figure 1. We also constructed an NJ tree to determine the genetic dissimilarities between pairs of taxa (Fig. 2).

DISCUSSION

Among various angiosperms, the ITS regions of nrDNA are very heterogeneous both in size and nucleotide sequences (Baldwin et al., 1995). Although in most

Table 4. Sequence divergences of nrDNA ITS region from species of *Goodyera* and outgroups, as derived by Kimura's two-parameter method.

	GSH	GRE	GAX	GVE	GAC	CGA	BSP	HAR
GSH	–	0	26	27	36	219	212	214
GRE	0.000	–	26	27	36	219	212	214
GAX	0.038	0.038	–	3	22	217	209	210
GVE	0.040	0.040	0.004	–	22	220	212	212
GAC	0.054	0.054	0.032	0.032	–	222	218	213
CGA	0.474	0.474	0.470	0.477	0.486	–	86	94
BSP	0.447	0.447	0.439	0.446	0.466	0.147	–	69
HAR	0.455	0.455	0.444	0.448	0.452	0.164	0.116	–

Calculated sequence divergences are given below the diagonal and observed numbers of nucleotide differences above the diagonal.
*GSH, *G. schlechtendaliana*; GRE, *G. repens*; GAX, *G. maximowicziana*; GVE, *G. velutina*; GAC, *G. macrantha*; CGA, *C. galeata*; BSP, *B. speciosa*; HAR, *H. arenaria*.

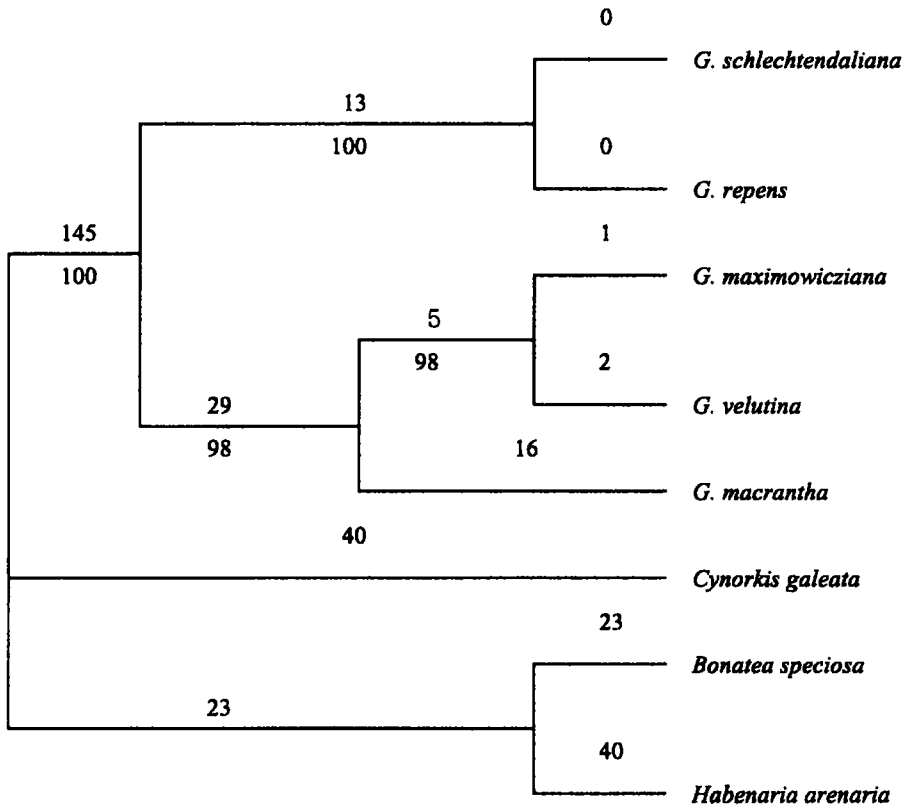

Figure 1. The most parsimonious tree of Korean *Goodyera* species based on the nrDNA ITS sequences. Numbers above the line indicate the nucleotide substitutions and those below the line give bootstrap percentages from 1000 replications. Consistency Index (CI) = 0.935 and 0.918 excluding uninformative characters, and Retention Index (RI) = 0.937.

flowering species, ITS 1 is longer than ITS 2 (Baldwin, 1992, 1993; Kim and Jansen, 1994; Choi and Kim, 1997), in this study, we found the opposite to be true, with ITS 1 (lengths ranging from 238 to 239 bp), and the longer ITS 2 (varying from 258 to 259 bp). The G + C contents for ITS 1 (45.4 to 46.0%) and ITS 2 (49.2 to 50.2%) are lower than those reported for most other angiosperms (approximately 50 to 70%; see Baldwin et al., 1995). The 5.8S coding region (156 bp) in this study was slightly shorter than that in other angiosperms (161 to 164 bp), but the G + C content for that region (53.9%) was similar to the approximately 50 to 60% found in in others species. Evolutionary rates differed between ITS 1 and ITS 2, with the former, changing about one and half times faster than the latter. The sequence divergence values among our ingroup taxa (0.0 to 5.4%) were very low compared with those between the ingroup and outgroup taxa (avg. 44.6%).

The most parsimonious tree from the ITS sequence showed that the *Goodyera* species (Tribe Cranichideae, Subtribe Goodyerinae) can be distinctly grouped as monophyletic, with 100% bootstrap values (Fig. 1). Among the Korean species, the two clades were derived from the node of a hypothetical ancestor. In the first clade, *G. schlechtendaliana* and *G. repens* were grouped together without any base substitutions; these, species are commonly found in the island and inland areas, including the Korean peninsula. In contrast, *G. maximowicziana*, *G. velutina*, and *G. macrantha*, whose ranges are restricted to the island (i.e., Jeju-do, Wan-do, and Ulleung-do areas in Korea), were grouped as a second clade. This finding also supports those of previous morphological and RAPD studies (Tae and Ko, 1999; Tae et al., 1999). Tae et al. (1997) have suggested that distributional center for *Goodyera* could originate from the Far East.

No sequence differences were found between *G. schlechtendaliana* and *G. repens*, as had already been indicated by Tae et al. (1997, 1999) and Tae and Ko (1999). Although these two species share very similar external morphology (Li, 1978; Kitamura et al., 1980; Lee, 1980; Satake et al., 1982), each is distinct in its size,

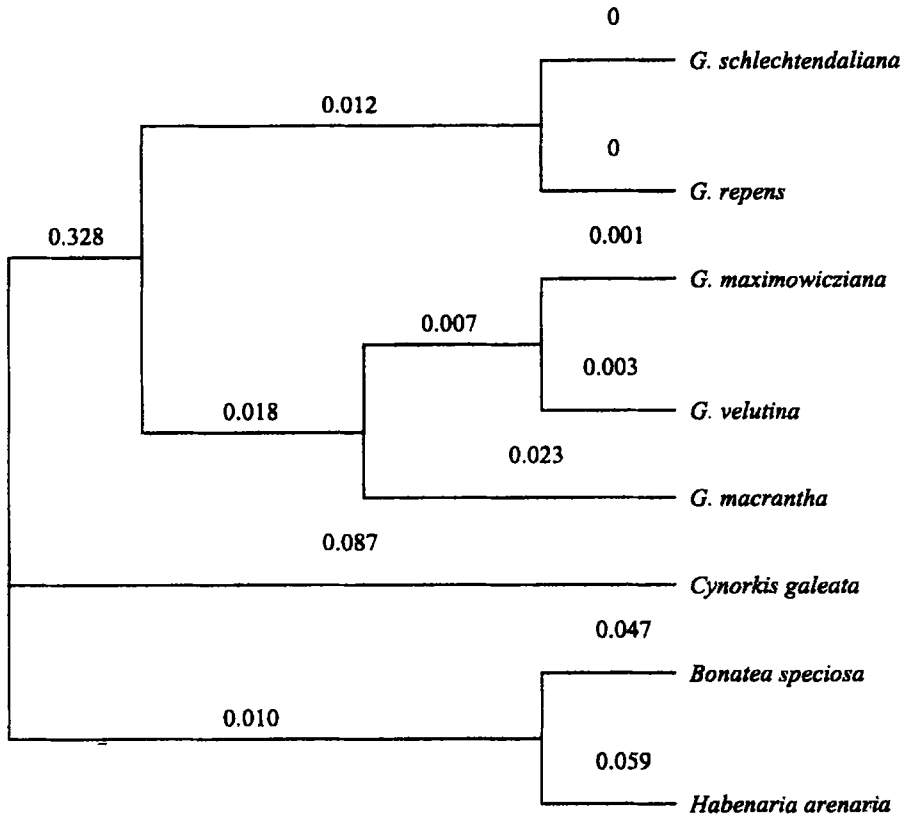

Figure 2. The Neighbor-Joining tree of Korean *Goodyera* species based on the nrDNA ITS sequences. Numbers above the line indicate the genetic distances calculated from the Kimura's two parameter method.

habitat, and the presence of lip hairs (Tae et al., 1997, 1999; Tae and Ko, 1999). However, in this study, we could not utilize the ITS sequence results to confirm any difference between *G. schlechtendaliana* and *G. repens*.

In the second clade, *G. maximowicziana* and *G. velutina* were more closely related to each other than to *G. macrantha*, av observation that agrees with the results of a morphomatric analysis by Tae and Ko (1999). *G. macrantha* is distinguished from the other two by its deep green leaves, small numbers of flowers (one to three), large flower size (2 to 3 cm), and long petals. Based on those morphological characters, Tae et al. (1997) have proposed that *G. macrantha* is a primitive species, as defined by the evolutionary hypothesis of Goldberg (1986).

In general, the results of our nrDNA ITS sequences support those conclusions made from previous morphological, cytological, and RAPD data analyses. Therefore, we suggest that these tools would be useful in defining the phylogenetic relationships among the Korean *Goodyera* species.

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