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Genetic Analyses of Genus *Cypripedium* Found in Northern Japanese Islands and Related Species Endemic to Northeast China

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Summary

Several species and varieties of genus *Cypripedium* grown in Japanese Islands and northeast China were investigated using genetic analysis. *C. macranthos* var. *hotei-atsmorianum* and *C. macranthos* var. *rebunense* were collected in northern Japan. They are regarded as varieties or forms of *C. macranthos* of Eurasia. Although the large subunit of ribulose-1,5-diphosphate decarboxylase/oxydase (*rbc*L) of chloroplast did not show any difference among the species, a difference between *C. macranthos* var. *hotei-atsmorianum* and the other two was found in the sequence of nuclear DNA encoding the internal transcribed spacer (ITS) region of ribosomal RNA. Random amplified polymorphic DNA method (RAPD) revealed a difference between *C. macranthos* var. *rebunense* from others. That *C. calceolus* and *C. macranthos* which were both collected in the Eurasia differed was demonstrated by their *rbc*L analyses and ITS sequences. The genetic analyses confirmed that *C.* × *ventricosum* was a hybrid of *C. calceolus* × *C. macranthos*. Thus, analyses using *rbc*L and ITS region are useful to distinguish species and varieties of *Cypripedium*.

Key Words: *Cypripedium*, DNA sequence analysis, ITS, Japanese Islands, *rbc*L.

Introduction

More than 40 species of genus *Cypripedium* range widely in the temperate zone of the northern hemisphere (Cribb, 1997). *C. macranthos* var. *hotei-atsmorianum* and *C. macranthos* var. *rebunense* are the well-known *Cypripedium* in Japan. They have been regarded as varieties or forms of *C. macranthos* grown in Eurasia (Cribb, 1997; Miyabe and Kudo, 1930; Ohwi, 1965). To classify their relationship, *C. macranthos* and the above two varieties, and *C. calceolus* were investigated by genetic analysis.

C. × *ventricosum*, which grows in northeast China, has been described as a hybrid of sympatric *C. calceolus* and *C. macranthos* (Rolfe, 1910). However, its identification by morphological appearance remains uncertain. Hence, it was subjected to genetic analysis.

The following procedures were used in our experiments. (1) The base sequence of a large subunit of ribulose-1,5-biphosphate decarboxylase/oxydase gene (*rbc*L) was determined to distinguish the species of *Cypripedium*. (2) The base sequence of ITS of nuclear ribosomal gene was determined with a special attention to the difference among the varieties of *C. macranthos*. (3) Electrophoresis by RAPD was used to distinguish *C. macranthos* var. *rebunense* from other species and varieties of *Cypripedium*. The DNA sequences of *rbc*L and ITS were used to classify the genus *Cypripedium*.

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Materials and Methods

Plant

Plants of *C. macranthos*, *C.* × *ventricosum* and *C. calceolus* were collected in Jilin province of northeast China. Plants of *C. macranthos* var. *rebunense* and *C. macranthos* var. *hotei-atsmorianum* were collected in northern Japan. Each plant of *Cypripedium* was classified by its morphological appearance according to the key to the species developed by Cribb (1997). The morphological features that are intermediate between those of *C. macranthos* and *C. calceolus* were assigned to *C.* × *ventricosum* for its classification. The yellow flower of C. macranthos var. rebunense distinguishes it from *C. macranthos* var. *hotei-atsmorianum* with its reddish violet flower. Kamanashi type of *C. macranthos* var. *hotei-atsmorianum* was distinguished from other plants of *C. macranthos* var. *hotei-atsmorianum* by its large pouch and a 2 week delay in flowering. The plants were found in Kamanashi Valley of Nagano Prefecture.

P. haynaldianum of genus *Paphiopedilum* which is endemic to southeast Asia was used as an out group in our experiment.

Sample code, DNA used for sequence analysis, and sites of collections are listed in Table 1.

Table 1. *Cypripedium* and *Paphiopedilum* plants used in this study.

DNA sample No.	Herbarium[z]	Voucher	Geographical distribution
C. macranthos SW.			
M302	KH	CR9631	Jilin, CHINA
M304	KH	CR9503	Jilin, CHINA
M309	KH	CW9621	Jilin, CHINA
M311	KH	CW9622	Jilin, CHINA
M312	KH	CW9623	Jilin, CHINA
M314	KH	CR9632	Jilin, CHINA
M315	KH	CR9633	Jilin, CHINA
M316	KH	CP9642	Jilin, CHINA
M317	KH	CR9634	Jilin, CHINA
M318	KH	CP9643	Jilin, CHINA
C. macranthos var. *hotei-atsmorianum* (Ohwi) Sadovsky			
H201	KH	H9502	Kamanashi, Nagano pref. JAPAN
H202	KH	H9426	Jouzankei, Sapporo, Hokkaido, JAPAN
H203	KH	H9403	Tougeshita, Rumoi, Hokkaido, JAPAN
H209	KH	H9409	Otaru, Hokkaido, JAPAN
H216	KH	H9416	Kamanashi, Nagano pref. JAPAN
H217	KH	H9601	Mt.Butoku, Shibetu, Hokkaido, JAPAN
H224	KH	H9405	Tougeshita, Rumoi, Hokkaido, JAPAN
H230	KH	H9402	Jouzankei, Sapporo, Hokkaido, JAPAN
C. macranthos var. *rebunense* Miyabe et Kudo			
R105	RI	no voucher	Rebun Island, Hokkaido, JAPAN
R110	RI	no voucher	Rebun Island, Hokkaido, JAPAN
R112	RI	no voucher	Rebun Island, Hokkaido, JAPAN
R113	RI	no voucher	Rebun Island, Hokkaido, JAPAN
R202	KH	R9402	Rebun Island, Hokkaido, JAPAN
R205	KH	no voucher	Rebun Island, Hokkaido, JAPAN
R220	KH	R9701	Rebun Island, Hokkaido, JAPAN
R221	KH	R9801	Rebun Island, Hokkaido, JAPAN
C. calceolus L.			
C305	KH	CL9661	Jilin, CHINA
C306	KH	CL9662	Jilin, CHINA
C308	KH	CL9751	Jilin, CHINA
C309	KH	CL9752	Jilin, CHINA
C. × *ventricosum* SW.			
V301	KH	V9703	Jilin, CHINA
V307	KH	V9702	Jilin, CHINA
V321	KH	V9721	Jilin, CHINA
V323	KH	V9501	Jilin, CHINA
V382	OT	V0082	Jilin, CHINA
V383	OT	V0083	Jilin, CHINA
V385	OT	V0085	Jilin, CHINA
V387	OT	V0087	Jilin, CHINA
V388	OT	V0088	Jilin, CHINA
V389	OT	V0089	Jilin, CHINA
V392	OT	V0092	Jilin, CHINA
V393	OT	V0093	Jilin, CHINA
V397	OT	V0097	Jilin, CHINA
P. haynaldianum Stein			
Pah1	KH	no voucher	PHILIPINE

[z] KH, Kita-hiroshima (Hokkaido); RI, Teppu, Rebun (Hokkaido); OT, Ootaki, (Hokkaido).

Preparation of DNA

Leaves from 10 plants of *C. macranthos*, 8 plants of *C. macranthos* var. *rebunense*, 8 plants of *C. macranthos* var. *hotei-atsmorianum*, 4 plants of *C. calceolus*, 13 plants of *C.* × *ventricosum* of genus *Cypripedium* and 1 plant of *P. haynaldianum* of genus *Paphiopedilum* were frozen, pulverized in liquid nitrogen, and the samples stored at −83℃ . Genomic DNA was extracted from the powder by using cetyltrimethylammonium bromide (CTAB) (Sugiura, 1989). When the amount of plant material was too low to extract DNA, a commercial ISOPLANT kit for plant DNA extraction (Nippon Gene) was used. Genomic DNA from each plant was prepared and its sequence determined.

DNA fragments of rbcL and ITS region

DNA fragments of *rbc*L and ITS region were prepared by PCR amplification of each genome of *Cypripedium* and *Paphiopedilum*. Primers for the preparation of *rbc*L DNA were those designed by Kores et al. (1997): rbcL-1F (5'-ATGTCACCACAAACAGAAAC-3') of forward strand and rbcL-1360R (5'-CTTCACAAGCAGCTAGTTC-3') of reverse strand. Primers of ITS region cited by Hsiao et al. (1994) were ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3') for forward strand and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for reverse strand. The amplification protocol was as follows: premelt 95℃ 3 min, followed by 40 cycles (94℃ 1 min denaturation, 55℃ 2 min annealing, 72℃ 3 min extension). After gel electrophoresis of the PCR products, target DNAs were purified by QIAGEN QIA quick gel extraction Kit. The volume was reduced to 20 μL in SPEED VAC concentrator (Savant, USA).

RFLP for analysis of rbcL fragments

The restriction site variation in *rbc*L DNA was examined by using restriction enzymes, such as *Hha*I, *Taq*I, *Msp*I, *Hae*III, *Eco*R I, and *Bgl*II. *Hha*I+*Taq*I, *Eco*RI+*Hae*III and *Msp*I+*Bgl*II were combined and the PCR products were applied to the second incubation with restriction enzymes in a suitable buffer. After incubation at 37℃ for 150-200 min, products were analyzed by gel electrophoresis.

PCR-RAPD procedure for DNA sequence analysis

The RAPD experiment was carried out with genomes isolated from *Cypripedium*. The search for reproducible bands was carried out with a random primer OPA16 of Operon 10 mer KIT-A (5'-AGCCAGCGAA-3'). PCR was carried out in 25 μL reaction mixture of PCR buffer containing 1.5 mM $MgCl_2$, 0.1 mM each of dNTP, 0.5 U Taq polymerase, 0.2 μM of OPA16, and 25 ng genomic DNA. The amplification protocol was as follows: premelt 90℃ 1 min, followed by 45 cycles (94℃ 1 min denaturation; 39℃ 1 min annealing; 72℃ 2 min extension), followed by a 5 min final extension at 72℃. The products were separated by gel electrophoresis.

Sequence determination of DNA

Four primers for sequence analysis of the *rbc*L DNA (408R: 5'-TTCCAGACGTAGAGCTCGCA-3', 634F: 5'-ATGCGTTGGAGAGATCGT-3', 741R: 5'-ACATGTACCCGCAGTTGCA-3' and 907F: 5'-AGGCAGAAGAATCATGGT-3') were newly designed. Samples for Dye terminater cycle sequencing of the DNA fragments of *rbc*L and ITS region were prepared with Thermo Sequence premix Kit ver. 2.0 (Amersham Bioscience, Uppsala, Sweden) according to the manufacture's instruction. The preparations were subjected to an automatic DNA sequencer (PE Applied Biosystem, model 377, USA).

Results

RFLP analyses of rbcL fragments

The *rbc*L fragments of *C. macranthos*, *C. macranthos* var. *rebunense*, and *C. macranthos* var. *hotei-atsmorianum* were digested by using either one restriction enzyme of *Hha*I, *Eco*RI or *Msp*I, or by using either one combination of two restriction enzymes, *Hha*I+*Taq*I, *Eco*RI+*Hae*III, or *Msp*I+*Bgl*II. The *rbc*L digests were analyzed by gel electrophoresis. An identical electrophoretic pattern was obtained with *C. macranthos* and the two varieties when the same restriction enzyme or the same combinations of the enzymes were used for the digestion.

Sequence determination of rbcL

The sequences of *rbc*L fragments of *C. macranthos*, *C. macranthos* var. *rebunense*, *C. macranthos* var. *hotei-atsmorianum*, *C. calceolus* and *C.* × *ventricosum* were determined. Our results indicated that an identical base sequence was obtained from different plants of the same species or variety except *C.* × *ventricosum*. The *rbc*L sequences of *C. macranthos*, *C. macranthos* var. *rebunense*, and *C. macranthos* var. *hotei-atsmorianum* were identical; the sequence of *C.* × *ventricosum* (V301) was identical with the one of *C. macranthos*. A few exceptions were observed with M318 of *C. macranthos* and H230 of *C. macranthos* var. *hotei-atsmorianum*. Each of them showed an extra base substitution at one position from the common *rbc*L sequence (Table 2). The same size of 1378 bp was obtained as the DNA fragments of *rbc*L from the plants described above. The nucleotide sequences of *C. macranthos* (M302) and *C. calceolus* (C305) are available from DDBJ/EMBL/GeneBank accession number AB176548 and AB176549, respectively.

Of the *rbc*L sequences of C305, C306, C308, and C309 of *C. calceolus*, C305, C306 and C308 had an identical base sequence and size was 1378 bp. Thus, it was assigned to the common *rbc*L sequence of *C. calceolus*. C309 of *C. calceolus* contained two

additional substitutions to *rbc*L of *C. calceolus* (Table 2). When the sequence of C305, C306, and C308 of *C. calceolus* was compared with that of *C. macranthos*, that of *C. calceolus* revealed 6 base substitutions from the sequence of *C. macranthos* at positions 363, 390, 688, 753, 982, and 1250 (Table 2). The *rbc*L sequence of *C. macranthos* showed GCGA from the position 390, whereas it was replaced by TCGA in *C. calceolus*. This substitution was confirmed by the digestion of *rbc*L with *Taq*I and the gel electrophoresis of reaction products.

The base substitution of *rbc*L sequence of *P. haynaldianum* from the one of *C. macranthos* was

Table. 2 Matrix of variable nucleotide positions in *rbc*L of the *Cypripedium*.

Species	DNA sample No	Nucleotide position									
		363	384	390	687	688	703	753	982	1250	1262
C. macranthos SW.	M302	A	A	G	A	G	A	A	T	G	G
	M304	·	·	·	·	·	·	·	·	·	·
	M309	·	·	·	·	·	·	·	·	·	·
	M311	·	·	·	·	·	·	·	·	·	·
	M312	·	·	·	·	·	·	·	·	·	·
	M314	·	·	·	·	·	·	·	·	·	·
	M315	·	·	·	·	·	·	·	·	·	·
	M316	·	·	·	·	·	·	·	·	·	·
	M317	·	·	·	·	·	·	·	·	·	·
	M318	·	·	·	·	·	·	·	·	·	C
C. macranthos var. *hotei-atsmorianum* (Ohwi) Sadovsky	H201	·	·	·	·	·	·	·	·	·	·
	H202	·	·	·	·	·	·	·	·	·	·
	H203	·	·	·	·	·	·	·	·	·	·
	H209	·	·	·	·	·	·	·	·	·	·
	H216	·	·	·	·	·	·	·	·	·	·
	H217	·	·	·	·	·	·	·	·	·	·
	H224	·	·	·	·	·	·	·	·	·	·
	H230	·	·	·	·	·	T	·	·	·	·
C. macranthos var. *rebunense* Miyabe et Kudo	R201	·	·	·	·	·	·	·	·	·	·
	R202	·	·	·	·	·	·	·	·	·	·
	R205	·	·	·	·	·	·	·	·	·	·
	R220	·	·	·	·	·	·	·	·	·	·
	R221	·	·	·	·	·	·	·	·	·	·
C. calceolus L.	C305	G	·	T	·	A	·	G	G	C	·
	C306	G	·	T	·	A	·	G	G	C	·
	C308	G	·	T	·	A	·	G	G	C	·
	C309	G	T	T	G	A	·	G	G	C	·
C. × *ventricosum* SW.	V301	·	·	·	·	·	·	·	·	·	·

Dots indicate nucleotides identical with those of *C. macranthos* (M302).

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C. macranthos      1 CATTGTTGAGACAGCAGAATATATGATCGAGTGAATCCGGTGGACTTGTGGTTACTCAGCTCGACATAGGC--TTTGCTTTTGCGGTGACCCTAATTTGT   98
P. haynaldianum    1 CATTGTTGAGACA-CATAATAATTGATCGAGTTAATCTGGAGGATCAGT--TTACTTTAGTCACCCATGGGCATCTGCTCTTGCAGTGACCTGGATTTGC   97

C. macranthos     99 CATTGGGCCTCCTCCAAAGCTTTCCTTGTGGGTTTGAACCTCTAGCACGGTGC-------AGTAT-GCGCCAAGTCATATGAAGCATCACTGATGAATGA  190
P. haynaldianum   98 CATCGAGCCTCCTTGGGAGCTTTC-TTGCTGG---CAA--TCTAAATCGTTGCCCGGCGCAGTCTTGCGCCAAGTCATATCA----------------CA  174

C. macranthos    191 CATTATTGTCAAAAAGTTGGAGTGGAAGCGTGCTATTGCATGCATGCAAATGAATTTTTTATGACTCTCGACAACGGATATCTTGGCTCTTGCATCGATG  290
P. haynaldianum  175 CATAATTG----GAAG-GGGGGCGGCATGCTG-TCTAGACCCTCCCCAAATTATTTTTTGATAACTCTCAGCAACGGATATCTCGGCTCTTGCATCGATG  269

C. macranthos    291 AAGAACGCAGCGAAATGCGATAAGTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGATGCCATCAGGCTAAGGGC  390
P. haynaldianum  270 AAGAACGCAGCGAAATGCGATAAATGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCATCAGGCCAAGGGC  369

C. macranthos    391 ACGCCTGCCTGGGCGTCGTGTGCTGCGTCTCTCCTGTCAATGCTTTCGTATCATATAGACAGGTTTGCATTGCGTGGATGTGAAAGATTGGCCCCTTGTG  490
P. haynaldianum  370 ACGCCTGCCTGGGCATTGCGAGTCATATCTCTCC-CTTAATG-AGGCTGTCCATACATACTGTTCAGC-CACAGCGGATGTG--AGTTTGGCCCCTTGTT  464

C. macranthos    491 CCTAGGTGCGGTGGGTCTAAG----GACTAGTGTTTTGATGGTTCGAAACCTGGCAGGAGGTGGAGGATGTTGGCAGCTATAAGGCTATCATTTGAATCC  586
P. haynaldianum  465 CTTTGGTACGGGGGGTCTAAGAGCTGCATGGGCTTTTGATGGTCCTAAATACGGCAAGAGGTGGACGAAGTATGCTACAACAAAATTGTAGTGCGAAT-G  563

C. macranthos    587 CCCAATATTGTCGTGTTTG-TCGGA---CCTAGAGAAGAACATGTTTGAATCCCAATG-GGGGCAAACAACCCTCGGGCGGTTGATTGCCATTCATATGC  681
P. haynaldianum  564 CCCAGGGTTGTCGTATTAGATGGGCCAGCATAATCTAAAGACCCTTTGAACCCCATTAGAGGCCCATCAACCCATGATCAGTTGATGGCCATTTGGTTGC  663

C. macranthos    682 GACCCCAGGTCAGGC                                                                                  696
P. haynaldianum  664 GACCCCAAGTCAGGT                                                                                  678
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Fig. 1. Alignment of the DNA sequence encoding ITS region of *C. macranthos* and *P. haynaldianum*. Hyphens indicate a gap. Bold letters indicate nucleotides of 5.8S in the ITS region.

observed at 19 positions among 1378 bp of *rbc*L. The nucleotide sequence of *P. haynaldianum* is available from DDBJ/EMBL/GeneBank accession number AB176547.

Sequence determination of ITS region

Analyses of the sequences of ITS region of *C. macranthos*, *C. macranthos* var. *rebunense*, *C. macranthos* var. *hotei-atsmorianum*, *C. calceolus* and *C.* × *ventricosum* demonstrated that the total size of ITS obtained from these plants had an identical size (696 bp). ITS region that consists of three regions of ITS1, 5.8s, and ITS2 formed a line from 5'end to 3'end (Baldwin, 1992). When the boundaries of the three regions of ITS of *Cypripedium* were compared with the ITS sequences of *Daucus carota* and *Vicia faba* (Yokota et al., 1989), the sizes of ITS1, 5.8s, and ITS2 were: 247 bp for ITS1, 164 bp from thymine248 to thymine411 for 5.8s, and 285 bp for ITS2.

The ITS sequence of *C. macranthos* (Fig. 1) is identical with the sequence of *C. macranthos* var. *rebunense*, but a difference exists in one of *C. macranthos* var. *hotei-atsmorianum*. Cytosine was found at position 450 of ITS2 region of *C. macranthos* and *C. macranthos* var.

Table. 3 Matrix of variable nucleotide positions in ITS region of the *Cypripedium* and *Paphiopedilum*.

Species	DNA sample No	Nucleotide position[z]										
		20	98	149	181	302	438	440	450	620	642	653
C. macranthos SW.	M302	T	T	T	T	G	G	A	C	G	G	C
	M304	·	·	·	·	·	·	·	·	·	·	·
	M309	·	·	·	·	·	·	·	·	·	·	·
	M311	·	·	·	·	·	·	·	·	·	·	·
	M312	·	·	·	·	·	·	·	·	·	·	·
	M314	·	·	·	·	·	·	·	·	·	·	·
	M315	·	·	·	·	·	·	·	·	·	·	·
	M316	·	·	·	·	·	·	·	·	·	·	·
	M317	·	·	·	·	·	·	·	·	·	·	·
	M318	·	·	·	·	·	·	·	·	·	·	·
C. macranthos var. *hotei-atsmorianum* (Ohwi) Sadovsky	H201	·	·	·	·	·	·	·	T	·	·	·
	H202	·	·	·	·	·	·	·	T	·	·	·
	H203	·	·	·	·	·	·	·	T	·	·	·
	H209	·	·	·	·	·	·	·	T	·	·	·
	H216	·	·	·	·	·	·	·	·	·	·	·
	H217	·	·	·	·	·	·	·	T	·	·	·
	H224	·	·	·	·	·	·	·	T	·	·	·
	H230	·	·	·	·	·	·	·	T	·	·	·
C. macranthos var. *rebunense* Miyabe et Kudo	R202	·	·	·	·	·	·	·	·	·	·	·
	R205	·	·	·	·	·	·	·	·	·	·	·
	R220	·	·	·	·	·	·	·	·	·	·	·
	R221	·	·	·	·	·	·	·	·	·	·	·
C. calceolus L.	C305	·	C	·	C	A	A	G	T	A	A	T
	C306	·	C	·	C	A	A	G	T	A	A	T
	C308	·	C	·	C	A	A	G	T	A	A	T
C. × *ventricosum* SW.	V301	·	C	·	C	A	A	G	T	A	A	T
	V307	C	C	·	C	A	A	G	T	A	A	T
	V321	·	C	·	C	·	·	G	T	·	·	·
	V323	·	C	·	C	A	A	G	T	A	A	T
	V382	·	C	·	C	R	R	G	Y	R	A	Y
	V383	·	C	·	C	R	R	G	Y	R	A	Y
	V385	·	C	·	C	R	R	G	Y	R	A	Y
	V387	·	C	Y	C	A	A	G	T	A	A	T
	V388	·	C	·	C	R	R	G	Y	R	A	Y
	V389	·	C	·	C	A	A	G	T	A	A	T
	V392	·	C	·	C	A	A	G	T	A	A	T
	V393	·	C	·	C	A	A	G	T	A	A	T
	V397	·	C	Y	C	A	A	G	T	A	A	T
P. haynaldianum Stein	PaH1	·	C	·	A	·	T	T	·	A	·	·

Dots indicate nucleotides identical with those of *C. macranthos* (M302).

[z] R, adenine or guanine; Y, thymine or cytosine.

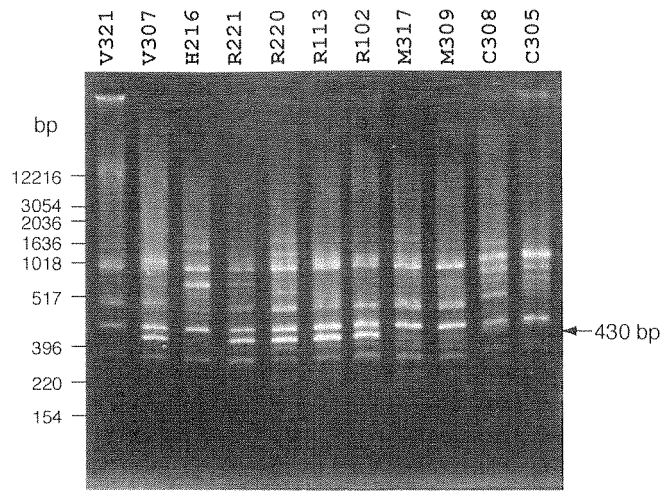

Fig. 2. Gel plate showing the 430 bp band of *C. macranthos* var. *rebunense* by RAPD electrophoresis in the presence of a random primer OPA16. DNA size makers are given in base pair on the left of the panel.

rebunense, but cytosine changed to thymine at the same position 450 of the sequence of *C. macranthos* var. *hotei-atsmorianum* (Table 3). The nucleotide sequences of *P. haynaldianum*, *C. macranthos* (M302) and *C. calceolus* (C305) are available from DDBJ/EMBL/GeneBank accession number AB176592, AB176593 and AB176594, respectively.

The ITS sequences of C305, C306 and C308 of *C. calceolus* were identical so that this sequence was assigned as the sequence of *C. calceolus*. The ITS sequence of *C. calceolus* differed from that of *C. macranthos* which had 9 base substitutions. Among the 9 substitutions,1 substitution was observed in 164 bp of 5.8s region at position 302.

ITS sequences of 13 plants of *C.* × *ventricosum* revealed that five possessed the identical sequence to that of *C. calceolus*. These five had adenine at positions 302, 438, and 620 and thymine at positions 450 and 653. The sequences of V382, V383, V385, and V388 showed that positions 302, 438, and 620 were not always occupied by adenine, but by either one of adenine or guanine. In addition, positions 450 and 653 were not fixed to thymine, but assigned to either one of thymine or cytosine. Furthermore, V387 and V397 showed thymine or cytosine at position 149, although only thymine occupied this position among other plants of *C.* × *ventricosum*. Although V321 was classified as *C.* × *ventricosum* from its morphological appearance (Table 3), its ITS sequence was a mixture of *C. macranthos*- and *C. calceolus*-types; the ITS sequences of other *C.* × *ventricosum* accessions were strictly *C. calceolus*-type.

Comparison of ITS of *P. haynaldianum*, an out group in this study, with that of members of *Cypripedium*, revealed that of the ITS of the former was 671 bp, whereas that of *C. macranthos* was 696 bp; many deletions, additions, and substitutions were observed at 205 positions (Fig. 1). The size of 5.8s coding region of *P. haynaldianum* was 164 bp, the same as that of *C. macranthos*, though 12 substitutions from the sequence of *C. macranthos* were observed.

RAPD gel-electrophoresis

RAPD electrophoresis with a random primer OPA16 distinguished the genome of *C. macranthos* var. *rebunense* from genomes of *C. macranthos*, *C. macranthos* var. *hotei-atsmorianum*, and *C. calceolus* (Fig. 2). The RAPD electrophoresis of *C. macranthos* var. *rebunense* gave a reproducible and distinct band at 430 bp. When the genome of *C. macranthos*, *C. macranthos* var. *hotei-atsmorianum*, or *C. calceolus* was substituted for the genome of *C. macranthos* var. *rebunense*, the band at 430 bp was absent.

RAPD electrophoresis of V307 of *C.* × *ventricosum* with OPA16 gave an exceptionally distinct band of 430 bp, compared with other members of *C.* × *ventricosum*.

Discussions

Genetic analyses of leaf *rbc*L and ITS using RFLP and RAPD gel electrophoreses to species and varieties of genus *Cypripedium* revealed that *C. macranthos* var. *hotei-atsmorianum* could be distinguished from *C. macranthos*, *C. macranthos* var. *rebunense* and *C.* × *ventricosum* by base substitution at one position of ITS sequence. The RAPD analysis in the presence of primer OPA16 also distinguished the genome of *C. macranthos* var. *rebunense* from others. The similarities in *rbc*L sequence of *C. macranthos* and the ITS sequence of *C. calceolus* with those in *C.* × *ventricosum* suggest that *C.* × *ventricosum* is a hybrid between *C. macranthos* and *C. calceolus*. This view coincides of

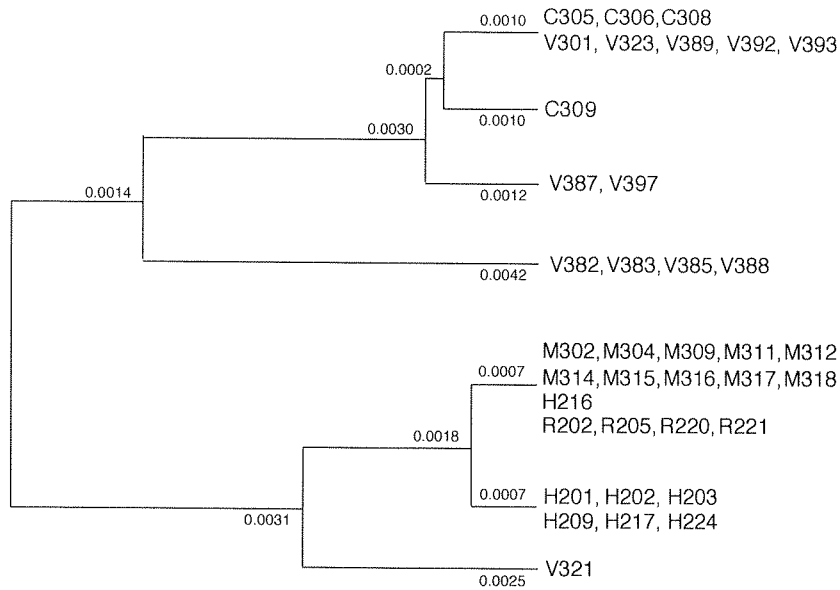

Fig. 3. A dendogram showing the phylogenic relationships among *Cypripedium* species based on the sequences of ITS region. This phylogenic tree was depicted using neighbour-joining method with a program GENETYX-MAC (ver. 10.1.2; Software Development Co., Tokyo). Numbers on nodes represent branch length in terms of percentage divergences.

the morphological analysis (Cribb, 1997; Rolfe, 1904) and the morphologic and allozyme analyses (Averyanov, 2000; Knyasev et al., 2000). Thus, the genetic analysis that was useful for classifying different species and varieties of genus *Cypripedium* should be beneficial for horticulturist.

The 430 bp band obtained in the RAPD with OPA16 in *C. macranthos* var. *rebunense* also occurred in V307 of *C.* × *ventricosum*. The existence of the 430 bp band in plants of *C.* × *ventricosum* collected at Jillin of northeast China and in those of *C. macranthos* var. *rebunense* that is endemic to Rebun Island near Hokkaido (Fig. 2) is inexplicable.

H216 of *C. macranthos* var. *hotei-atsmorianum* that was collected in Kamanashi Valley in Nagano prefecture has been classified as a Kamanashi-type by its morphological appearance. All plants of *C. macranthos* var. *hotei-atsmorianum* have thymine at position 450 of ITS sequence, whereas H216 has cytosine, which corresponds to the sequence of *C. macranthos*. On the other hand, H201 that was collected also in Kamanashi Valleyhas not been classified as a Kamanashi-type because of its morphological appearance, but it has thymine at position 450. These results raise questions as to the classification of kamanashi-type of *C. macranthos* var. *hotei-atsmorianum*: more plants need to be analyzed to obtain an answer.

Six base differences that were observed in the *rbc*L sequences of 1378 bp between *C. macranthos* and *C. calceolus* yield a divergence rate of 0.44%. The divergence rate of *rbc*L gene was estimated as 0.05-0.07% in one million years (Wendel and Albert, 1992). Thus, an estimated value of 6-9 million years could be calculated from a 0.44% divergence between *C. calceolus* and *C. macranthos*. Since paleomagnetic evidence show that the Japanese Islands were separated from Eurasia 15-20 million years ago (Niitsuma, 1985), the divergence of *C. calceolus* and *C. macranthos* is predicted to have occurred after the separation of Japanese Islands. This hypothesis explains why it is difficult to find *C. calceolus* in the Japanese Islands. However, *C. calceolus* was found in Rebun isle, which is close to Sakhalin, Russia. Taniguchi et al. (2001) showed that *C. calceolus* found in Rebun isle differed from that of China or Europe; it could be of Russian origin. The *C. calceolus* found on Rebun isle may have, somehow, migrated from Sakhalin.

A dendrogram (Fig. 3) was obtained from ITS sequences of *C. macranthos, C. macranthos* var. *rebunense*, *C. macranthos* var. *hotei-atsmorianum, C. calceolus* and *C.* × *ventricosum*. The distribution of *C.* × *ventricosum* suggests the heterogeneous processes of its hybridization.

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日本列島の北部と中国の東北地方に生息する *Cypripedium* 属の遺伝子分析による比較研究

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摘　要

日本海を囲む日本列島の北部と中国の東北地方に生育している *Cypripedium* 属植物について，遺伝子分析によって比較研究を行った．ホテイアツモリソウとレブンアツモリソウは日本列島で採取したもので，中国の東北地方で採取した *C. macranthos* の変種または品種と考えられている．葉緑体にある ribulose－1，5－diphosphate decarboxylase/oxygenase の大サブユニット (*rbc*L) の DNA を比較すると，3種はまったく区別できなかった．ところが，核 DNA 上にあるリボソーム RNA の ITS 部分を比較すると，ホテイアツモリソウとレブンアツモリソウ・中国産 *C. macranthos* との間で，1塩基の置換が認められた．また以上3種のゲノムを鋳型にランダムプライマー OPA16 を用いた RAPD 電気泳動を行った結果，レブンアツモリソウにのみ 430 bp の DNA フラグメントが認められた．中国の東北地方で採取した *C. calceolus* の *rbc*L と ITS の塩基配列はいずれも同じ地域で採集した *C. macranthos* と異なっていた．*C.* × *ventricosum* は *rbc*L と ITS の遺伝子分析から，*C. macranthos* と *C. calceolus* の雑種であると確認された．これらのことより *rbc*L と ITS 遺伝子の分析は，*Cypripedium* 属の種や変種の分類に有用であることが示された．