A comparative population study of genetic diversity in *Goodyera foliosa* var. *laevis* (Orchidaceae) native to Hiroshima Prefecture, Japan*

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Abstruct

Molecular analysis was carried out using random primers to infer the characteristics of RAPD generated DNA fragments and to investigate genetic diversity of six populations of *Goodyera foliosa* var. *laevis* in Hiroshima Prefecture, Japan. Using two decamer primers of OPA-01 and OPB-05, 157 reproducible DNA fragments were obtained from 30 individuals of this variety. In cluster analysis, four main clades were found in the tree. One of these clades consisted of all five individuals of Mt. Tateeboshiyama population.

Introduction

Goodyera R. Br. is a member of the Orchidaceae and comprises 40 species widely distributed from the subarctic regions of the Northern Hemisphere to tropical regions in Asia (Satomi 1982). All species in this genus are terrestrial, with one exception. *Goodyera* species mainly grow in primary forests with high humidity and are often associated with dominant forest-trees. Thus, it is suggested that speciation of this group is correlated with certain associated forest-trees (Tanaka 1965).

Taxonomic treatments of *Goodyera*, however, are still imperfect, because the genus contains many rare species with not enough complete herbarium specimen masses (Schlechter 1926, Holttum 1964, Briger 1974-1975, Dressler 1981). Therefore, more examinations must be necessary to clarify these taxonomic treatments, as pointed out by some previous works (Dressler and Dodson 1960, Maekawa 1971, 1978, Seidenfaden 1978). Additionally, as same as species classification, population study to understand genetic diversity in *Goodyera* is quite important for the species conservation and its sustainable management of gene resource, since many of the species are on the decrease in the population number and size even now.

In Japan, more than ten species of *Goodyera* have been recorded (Sera 1990). Of these, *Goodyera foliosa* (Lindley) Bentham ex C. B. Clarke shows intraspecific morphological variation, and has two varieties in Japan. *Goodyera foliosa* (Lindley) Benth. var. *laevis* Finet is common type of the Japanese species, and is distributed from the north main island (Hokkaido) to the south main island (Kyushu) in the Japanese archipelago, whereas another variety, *Goodyera foliosa* (Lindley) Benth. var. *commelinoides* (Fukuyama) F. Maekawa, is native to only Kyushu island and further down south small islands belonging to Kagoshima, Okinawa Prefectures and the Tokyo metropolitan area, Japan. Moreover, in the previously investigated individuals reported by Tanaka (1965) and Sera (1990), *G. foliosa* var. *laevis* has an intraspecific polyploidy, which is generally considered to play a role to promote the geographic segregation explained by their adaptive selection to environments (Ohi *et al.* 2003). The cytotypes with different ploidy levels of *G. foliosa* var. *laevis* are commonly found in south part of the largest main island (Honshu) in Japan, especially in Hiroshima Prefecture (Sera 1990). However, even though cytological studies have been carried out to see chromosome differentiation among related taxa, there is no report of molecular investigation for estimating genetic diversity in the populations with different cytotypes of this Japanese variety.

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Random amplified polymorphic DNA (RAPD) method for DNA amplification (Williams *et al.* 1990) has been widely used as a means of generating genetic markers in many organisms. RAPD technique could identify genotypes directly and help to mitigate complications arising from earlier cytological and morphological studies (Das 2008). Moreover, RAPD method is also used to collect information on the levels and patterns of population genetic diversity in wild plants, because knowledge of genetic diversity is the baseline for conservation (Geburek 1997).

In this study, molecular analysis was carried out using random primers to infer the characteristics of RAPD generated DNA fragments and to investigate genetic diversity of six populations of *G. foliosa* var. *laevis* in Hiroshima Prefecture, Japan.

Materials and Methods

Plant materials

The plant materials used are listed in Table 1. To extract total genomic DNA, leaf materials were obtained from 30 plant individuals collected. All of the plants used here were cultivated in the Laboratory of Plant Environment Science, Department of Plant Science, School of Agriculture, Tokai University.

Table 1. Collection data for *Goodyera foliosa* var. *laevis* distributed in Hiroshima Prefecture

Accession number for collection date and site	Individual serial numbers in the accession	Abbreviation of population	Locality	Geographical ordinates	Altitude (m)
TK03533C01	03, 04	HID	Saeki-ku, Hiroshima City	34°23'54"N, 132°20'12"E	150
TK03533C02	I01, I03, II02, II03, A01	TSK	Taisyakukyo, Jinsekikougen-tyo	34°51'31"N, 133°12'51"E	500
TK03533C03	II01, II03, III04, IV02, IV03, V01, A01	KMN	Kumano, Saijo-tyo, Syobara City	35°01'31"N, 133°05'01"E	600
TK03533C04	01, 03, 05, 07, 08	EBS	Mt. Tateeboshiyama, Saijo-tyo, Syobara City	35°02'56"N, 133°04'03"E	790
TK03544C05	I02, I03, I04, II03, II04	GRY	Mt. Garyuzan, Geihoku, Kitahiroshima-tyo	34°41'13"N, 132°11'58"E	1000
TK03557A01	I01, I02, I03, II01, II03, II05	ETJ	Okimi-tyo, Etajima City	34°13'32"N, 132°11'58"E	160

DNA extraction

Total genomic DNA extraction was followed mainly Hoshi *et al.* (1994). The samples were ground into powder with liquid nitrogen and homogenized in the buffer containing 1 M Tris (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammonium bromide and 0.5% mercaptoethanol. The homogenates were extracted three times with an equal volume of chloroform-isoamyl alcohol (24: 1) for 15 min each and the DNAs were precipitated with an equal volume of isopropyl alcohol at room temperature.

RAPD amplification

RAPD amplification was followed the instructions of RAPD decamer Kit (Operon technologies, Alamenda, CA, USA). To optimize the polymerase chain reaction (PCR) amplification conditions, experiments were carried out with varying concentrations of DNA template, primers, and Taq DNA polymerase. According to previous RAPD work (Tae et al. 1999), two primers of OPA-01 and OPB-05 were selected from the series of Operon technologies (Alamenda, CA, USA).

Amplification was performed on a PCR thermalcycler, Program Temp Control System (Astec, PC-708) with 20 μl reaction mixtures containing 10 ng of template DNA, 0.2 mM of each dNTPs (dATP, dTTP, dCTP and dGTP), 0.5 units of Taq DNA polymerase (Toyobo, TAP-211), 10 pmol of primer, 10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl2. The amplification regime was performed with the following programmes: 94°C for 5 min followed by 45 cycles with 94°C for 30 sec, 42°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified PCR products were performed onto an agarose (1.0% w/v) gel (Nacalai) electrophoresis (iMyrun IMR-201) in 0.5×TAE buffer at 75 V for 50 min. The gel was visualized by ethidium bromide staining and photographed under UV illuminater (Funakoshi, NTM-20).

Data analysis

All amplifications were repeated thrice in order to confirm the reproducible amplification of scored fragments. Second and third reproducible bands were scored for the construction of the data matrix. The marked changes observed in RAPD profiles (disappearance and/or appearance of bands in comparison with untreated control treatments) were evaluated. Each gel of RAPD was analyzed by scoring present (1) or absent (0) bands. The pooled data matrices were entered into the StatPartner version 2.0 package. A dendrogram was constructed by employing UPGMA (Unweighted Pair Group Method with Arithmetic Average) (Sokal and Sneath 1963) to group individual into discrete clusters.

Results

The potential of RAPD fingerprinting for genetic differentiation within *Goodyea foliosa* var. *laevis* was well demonstrated with amplification of purified genomic DNA of 30 individuals of collected from six populations in Hiroshima Prefecture (Fig. 1).

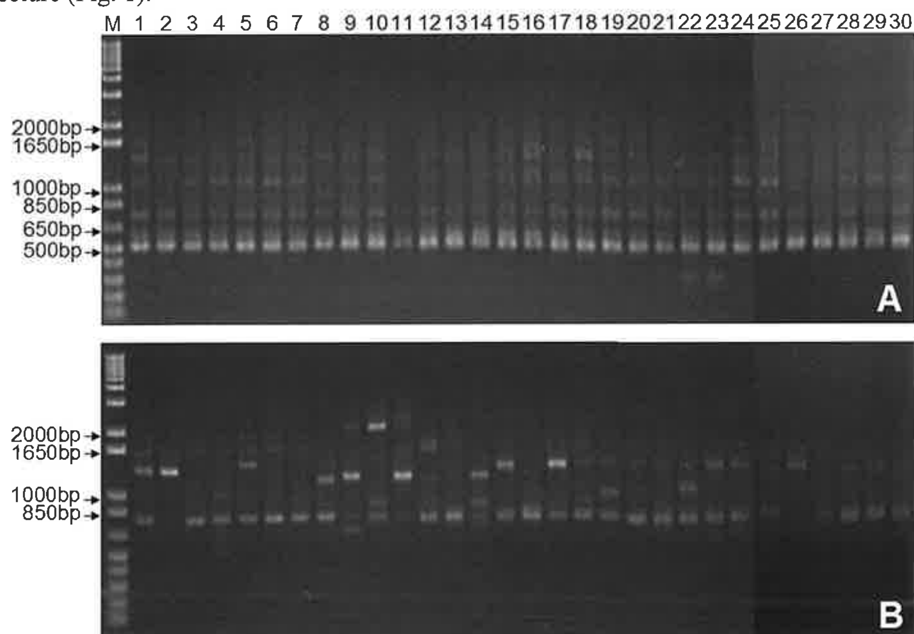

Fig. 1. Random amplified polymorphic DNA of *Goodyera foliosa* var. *laevis* in Hiroshima prefecture, Japan. A, Primer OPA-01. B, Primer OPB-05. Lane M, Marker 1 kb DNA Ladder. 1-2, HID. 3-7, TSK. 8-14, KMN. 15-19, EBS. 20-24, GRY. 25-30, ETJ. Population abbreviations are defined in Table 1 and strain order is the same as that of Table 2.

Using two decamer random primers of OPA-01 and OPB-05, more than six RAPD bands were not obtained in this study. In all, 163 reproducible DNA fragments were amplified, and each primer amplified varying numbers of the fragments. OPA-01 primer generated 98 reproducible bands in the range of 500-1650 bp, while OPB-05 primer generated 65 reproducible bands in the range of 650-2000 bp.

In each population, several bands amplified by OPA-01 primer were shown in the range of 500-1400 bp in KMN and TSK, and 500-1650 bp in EBS, ETJ, GRY and HID. In contrast, one to four bands amplified by OPB-05 primer were shown in the range of 650-1400 bp in KMN, 800-1400 bp in ETJ and HID, 800-1650 bp in GRY, 800-2000 bp in TSK, and 850-1650 bp in EBS.

Table 2. Data matrix of RAPD data for Hiroshima Prefecture, Japan (0, absent; 1, present. Population abbreviations are defined in Table 1)

Primer	bp	Population and individual number																													
		HID		TSK					KMN							EBS					GRY					ETJ					
		03	04	I01	I03	II02	II03	A01	II01	II03	III04	IV02	IV03	V01	A01	01	03	05	07	08	I02	I03	I04	II03	II04	I01	I02	I03	II01	II03	II05
OPA-01	1650	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	1	0	0
	1400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1
	1000	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	700	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0
	550	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	500	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
OPB-05	2000	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1650	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0
	1550	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1400	1	1	0	0	1	0	0	1	1	0	1	1	0	1	0	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1
	1100	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
	1000	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	800	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
	650	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The major amplicon numbers of OPA-01 and OPB-05 were six (DNA fragments with 500, 550, 700, 1000, 1400 and 1650 bp) and nine (DNA fragments with 650, 800, 850, 1000, 1100, 1400, 1550, 1650 and 2000 bp), respectively (Table 2).

In OPA-01 primer, all individuals of *G. foliosa* var. *laevis* studied here had a distinct band at 500 bp. OPA-01 primer also made clear bands of 700 bp and 1400 bp in most individuals. But DNA bands appeared at 550 bp, 1000 bp and 1650 bp were only observed in a few individuals. In contrast to OPA-01, primer of OPB-05 exhibited a characteristic 850 bp band in EBS population. Five amplicons were shown in KMN and TSK, while only two amplicons were shown in ETJ and HID. RAPD bands generated by OPA-01 primer were more commonly found in all individuals and populations than those of OPB-05.

Pairwise genetic distances among whole individuals of six populations, as revealed by RAPD analysis, are shown in Table 3. The highest value of 2.828 with 7 character differences was found between TSK-I01 and EBS-05. The genetic dissimilarity matrix had many high values of 2.646 between EBS and the other four populations. In particular, the majority of these second large values in the other populations were mainly found in ETJ and GRY populations. Zero-values, which indicated exactly same banding pattern in RAPD analysis, were seen not only within the populations, but also among some populations.

Cluster analysis of the genetic distance values was performed to generate a dendrogram showing overall genetic relatedness among *G. foliosa* var. *laevis* individuals (Fig. 2). Using 30 individuals obtained from the six populations, four main clades were found in the cluster tree. One of these clades consisted of all five individuals collected from EBS population, but others were not.

Table 3. Genetic dissimilarity matrix calculated by Nei (1972)'s genetic coefficient based on RAPD data

		Population and individual number																													
		HID		TSK					KMN							EBS					GRY					ETJ					
		03	04	I01	I03	II02	II03	A01	II01	II03	III04	IV02	IV03	V01	A01	01	03	05	07	08	I02	I03	I04	II03	II04	I01	I02	I03	II01	II03	II05
HID	03																														
	04	1.000																													
TSK	I01	2.449	2.236																												
	I03	2.000	1.732	1.414																											
	II02	1.732	1.414	2.236	1.732																										
	II03	2.236	2.000	2.236	1.732	1.414																									
	A01	2.000	1.732	1.414	0.000	1.732	1.732																								
KMN	II01	1.732	1.414	2.236	1.732	1.414	2.000	1.732																							
	II03	2.000	1.732	2.449	2.000	1.732	2.236	2.000	1.000																						
	III04	2.000	1.732	2.000	1.414	1.732	1.732	1.414	1.732	2.000																					
	IV02	1.414	1.000	2.000	1.414	1.000	1.732	1.414	1.000	1.414	1.414																				
	IV03	1.414	1.000	2.000	1.414	1.000	1.732	1.414	1.000	1.414	1.414	0.000																			
	V01	2.000	1.732	1.414	0.000	1.732	1.732	0.000	1.732	2.000	1.414	1.414	1.414																		
	A01	1.732	1.414	2.236	1.732	1.414	2.000	1.732	1.414	1.732	1.000	1.000	1.000	1.732																	
EBS	01	2.000	1.732	1.414	0.000	1.732	1.732	0.000	1.732	2.000	1.414	1.414	1.414	0.000	1.732																
	03	1.000	1.414	2.236	1.732	1.414	2.000	1.732	1.414	1.732	1.732	1.000	1.000	1.732	1.414	1.732															
	05	2.000	1.732	2.449	2.000	1.000	1.732	2.000	1.000	1.414	2.000	1.414	1.414	2.000	1.732	2.000	1.732														
	07	1.732	1.414	1.732	1.000	1.414	1.414	1.000	1.414	1.732	1.000	1.000	1.000	1.000	1.414	1.000	1.414	1.732													
	08	1.732	1.414	1.732	1.000	1.414	1.414	1.000	1.414	1.732	1.000	1.000	1.000	1.000	1.414	1.000	1.414	1.732	0.000												
GRY	I02	1.414	1.732	2.449	2.000	1.000	1.732	2.000	1.732	2.000	2.000	1.414	1.414	2.000	1.732	2.000	1.000	1.414	1.732	1.732											
	I03	2.000	1.732	2.000	1.414	1.000	1.000	1.414	1.732	2.000	1.414	1.414	1.414	1.414	1.732	1.414	1.732	1.414	1.000	1.000	1.414										
	I04	2.000	1.732	2.000	1.414	1.732	1.732	1.414	1.732	2.000	1.414	1.414	1.414	1.414	1.732	1.414	1.732	2.000	1.000	1.000	2.000	1.414									
	II03	1.732	1.414	2.236	1.732	1.414	2.000	1.732	1.414	1.732	1.732	1.000	1.000	1.732	1.414	1.732	1.414	1.732	1.414	1.414	1.732	1.732	1.000								
	II04	1.414	1.000	2.000	1.414	1.000	1.732	1.414	1.000	1.414	1.414	0.000	0.000	1.414	1.000	1.414	1.000	1.414	1.000	1.000	1.414	1.414	1.414	1.000							
ETJ	I01	1.414	1.732	2.449	2.000	1.732	2.236	2.000	1.732	2.000	2.000	1.414	1.414	2.000	1.732	2.000	1.000	2.000	1.732	1.732	1.414	2.000	2.000	1.732	1.414						
	I02	1.732	2.000	2.236	1.732	2.000	2.449	1.732	2.000	2.236	2.236	1.732	1.732	1.732	2.000	1.732	1.414	2.236	2.000	2.000	1.732	2.236	1.732	1.414	1.732	1.732					
	I03	1.414	1.732	2.000	1.414	1.732	1.732	1.414	1.732	2.000	1.414	1.414	1.414	1.414	1.732	1.414	1.000	2.000	1.000	1.000	1.414	1.414	1.414	1.732	1.414	1.414	1.732				
	II01	1.414	1.732	2.449	2.000	1.732	2.236	2.000	1.732	2.000	2.000	1.414	1.414	2.000	1.732	2.000	1.000	2.000	1.732	1.732	1.414	2.000	2.000	1.732	1.414	0.000	1.732	1.414			
	II03	1.732	1.414	2.236	1.732	1.414	2.000	1.732	1.414	1.732	1.732	1.000	1.000	1.732	1.414	1.732	1.414	1.732	1.414	1.414	1.732	1.732	1.732	1.414	1.000	1.000	2.000	1.732	1.000		
	II05	1.732	1.414	2.236	1.732	1.414	2.000	1.732	1.414	1.732	1.732	1.000	1.000	1.732	1.414	1.732	1.414	1.732	1.414	1.414	1.732	1.732	1.732	1.414	1.000	1.000	2.000	1.732	1.000	0.000	

Discussion

A relationship between genetic difference and plant population in this genus has been noticed by a few researchers (Tanaka 1965, Kallunnki 1976, Wong and Sun 1999). Kallunnki (1976) investigated population diversity of *G. tesselata* in North America, and suggested that high morphological variation in this species was due to hybrid origin with polyploidization event. Wong and Sun (1999) reported that Hong-Kong's populations of *G. procera* showed a wide genetic range without any correlation between genetic diversity and geographic distance. In our study, population clustering based on Nei's genetic identities (Nei 1972) generated from RAPD data was similar to the previous results of Wong and Sun (1999). Except for EBS population (Mt. Tateeboshiyama, Shyobara City), a significant relationship was not found between genetic difference and population (Fig. 2). The clade consisting only of EBS individuals was located at basal position in RAPD tree, and each of the individuals had a long genetic distance. Thus, it can be speculated that population of Mt. Tateeboshiyama kept high and unique genetic diversity.

Our RAPD result showed that most individuals in each population were placed in different main clades with long terminal branches, strongly indicating all populations had high genetic diversity. As researched areas in our study, previous cytogenetic work has interesting information about interspecific polyploidy (Sera 1990). Two cytotypes with different chromosome numbers with 2n=28 and 2n=56 were found in *Goodyera foliosa* var. *laevis* in Hiroshima Prefecture. Moreover, the populations distributed more west gave chromosome number of 2n=56. Thus, these results indicated that polyploidal chromosome number of *G. foliosa* var. *laevis* derived from more east population of same varieties with a lower chromosome number and high genetic diversity.

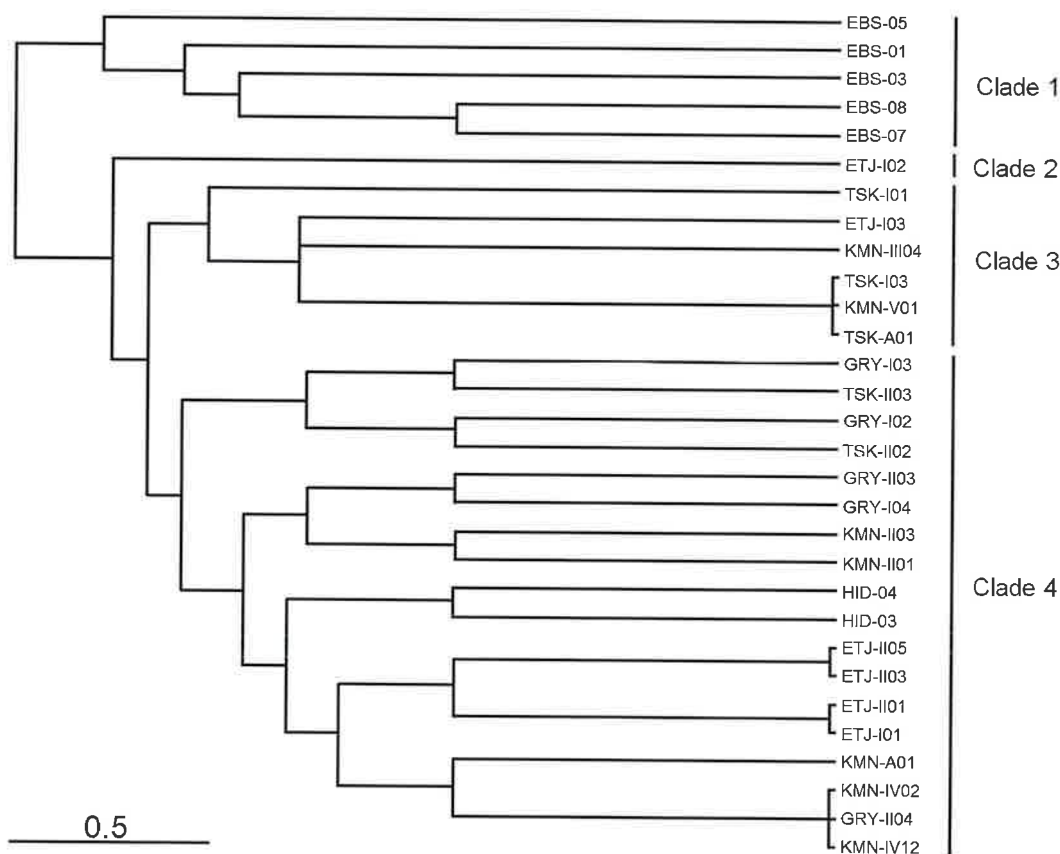

Fig. 2. UPGMA cluster tree of 30 individuals of *Goodyera foliosa* var. *laevis* from six populations in Hiroshima Prefecture, Japan. The scale bar indicates a genetic distance. Population and individual abbreviations are defined in Table 1.

Getting a better understanding of genetic diversity is very important for formulating comprehensive conservation plans (Hamrick 1983, Falk and Holsinger 1991, Loescheke et al. 1994, Geburek 1997). In spite of the threat of extinction, there is little information about genetic diversity of native orchid plant species. The levels and patterns of genetic variation using allozyme and RAPD analyses were provided for wild orchid conservation strategy in Hong-Kong's populations (Wong and Sun 1999). In the comparison with allozyme diversity, higher levels of genetic variation were detected at the RAPD. Therefore, our comparative population study using RAPD method can help us figure out baseline for conservation of the genus *Goodyera* in Hiroshima Prefecture.

Further examinations with other PCR-based molecular markers in many more populations were necessary to clarify the population relationship in this species.

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広島県に自生するアケボノシュスランを用いたRAPD解析

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要　約

広島県に自生するアケボノシュスラン (*Goodyera foliosa* (Lindley) Benth. var. *laevis* Finet) の遺伝的多様性を調査するため，県内6箇所から採取した30個体のRAPD (Random Amplified Polymorphic DNA) 解析を行った．PCR増幅に用いた2種類のランダムプライマーで個体間多型を示すDNA断片が増幅された．得られた多型DNAをもとに遺伝的距離を求めた結果，帝釈峡と立烏帽子山の個体間で見られた値がもっとも高い数値を示した．クラスター解析の結果から，立烏帽子山の個体群は独自かつ高い遺伝的多様性を保っていることが推察された．

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