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Genetic Diversity and Phylogenetic Relationship of Genus *Phyllostachys* by RAPD Markers

Song Jin Lee*, Man Kyu Huh[1], Hyun Cheol Shin[2] and Hong Wook Huh[3]

Damyang-gun Office, Damyang 517-802, Korea
[1]*Department of Molecular Biology, Dongeui University 995 Eomgwangno, Busan 614-714, Korea*
[2]*Foest Research Institute, Southern Forest Center*
[3]*Department of Biology, Pusan National University, 30 Jangjeon-dong, Geumjeong-gu, Busan 609-735, Korea*

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Genus *Phyllostachys* is a long-lived woody species primarily distributed throughout South East Asia. Many species of this genus has been regarded as medically and ecologically important in the world. We evaluated representative samples of the four taxa with RAPD to estimate genetic relationships within the genus *Phyllostachys*. The percentages of polymorphic loci were 8.9-33.3% at the species level. *P. bambusoides* was found to show lower genetic diversity (H=0.018) than other species. Total genetic diversity (H_T) was 0.315, genetic diversity within populations (H_S) was 0.043, the proportion of total genetic diversity partitioned among populations (G_{ST}) was 0.659 and the gene flow (Nm) was 0.0263. As some Korean populations were isolated and patchily distributed, they exhibited low levels of genetic diversity. The four taxa of the genus *Phyllostachys* analyzed were distinctly related to a monophyletic. *P. nigra* var. *henonis*. Stapf was found to be more closely related to *P. pubescens* than to *P. nigra*. *P. bambusoides* was quite distinct from the remaining species.

Key words : Genetic diversity, genus *Phyllostachys*, phylogenetic relationships, RAPD

Introduction

Increased effort to broaden our understanding of molecular variation in different plant species from South East Asia is needed. One important species of liliopsida is bamboo. Bamboos have been useful plants for thousands of years. Bamboo is much easier to produce and equally versatile. Producing four to five times more biomass than the trees felled for wood production, bamboo grows almost everywhere in the world and in enormous variety. In both the tropics and sub-tropics, it achieves great height and thickness in a very short time. Economically the bamboo is one of the most important plants to humans. Bamboos are a cheap and sustainable source of building materials, food, and of a source of decorative ornamental garden plants. The woody bamboos are used for matting, thatch, baskets, bamboo wires, bamboo boxes and scaffolding [11].

The genus *Phyllostachys* is comprised of about 4~5 species in Korea [14]. The taxonomy of *Phyllostachys* has been described mainly through morphological characteristics. However morphological characteristics are restricted in their resolving power mainly because of the small number of variables available. Efficient methods to clarify the taxonomic status of several species are much needed. Until recently, much of the Korean forest has been disturbed by the cutting of trees and shrubs for medicine in rural areas [9].

Typical populations of many *Phyllostachys* species except *P. bambusoides* are small and distributed in patches. The main concern relative to persistence of *Phyllostachys* is continued habitat destruction and fragmentation. Consequently, wild *Phyllostachys* populations have suffered loss individuals, loss of and reduction of populations and fragmentation of remaining populations by human activities such as over-gathering medicinal plants. Thus insights into the relative gene diversity among and within wild populations of *Phyllostachys* would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources [1].

RAPD assay has been useful in determining genetic relationships among closely related species [2,4]. RAPD analysis is quick, robust, and requires minimal preliminary work [13]. We expected that the RAPD analyses assess the amount and structure of genetic diversity within and between natural populations and more finely discriminate all the tested genotypes than the allozymes [16]. Hence, we successfully

***Corresponding author**
Tel : +82-61-380-3473, Fax : +82-61-380-3371
E-mail : ililgu47@daum.net

assess the genetic relationships among the local populations of *Phyllostachys* in Korea.

Molecular markers, like RAPD, AFLP, and ISSR are examples of polymerase chain reaction (PCR)-based genetic markers for rapid screening of genetic diversity. These markers have been found to be effective in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation of subpopulations [18].

The genus *Phyllostachys* (Bambusaceae) has been an abundant plant over its range in Korea. But, reduction of populations is a serious problem. Many manufacturers substitute bamboos for plastic or iron goods. Korea is a developing country. So fields of bamboo are used for the purposing agricultural land to raise products for industry. Population are reproductively isolated may gradually exhibit genetic differentiation [8]. The rapid loss of new plants results in the permanent loss of gene pools with potential for species conservation. The purposes of this paper are: 1) to estimate how much total genetic diversity is maintained in the species, and 2) to describe how genetic variation is distributed within and among populations [3].

Materials and Methods

Plant materials

All of the 12 populations were collected from four species in Korea (Table 1). The plant materials consisted of various populations belonging to four species of the genus *Phyllostachys*. One young leaf per mature tree ($\geq$5 yr) was sampled. Fifteen plants were randomly collected from each population. In addition, one species of the same family, *Sasa japonica* was used as standard to compare the phylogenetic relationships.

DNA extraction

The genomic DNA of the 90 samples including standard was extracted from fresh leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol.

RAPD analysis

Twenty arbitrarily chosen 10-mer primers, the kit D (OPD-01 to 20) of Operon Technologies (Alameda, Colo.) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses. To analyze the DNA of individuals, we selected nine decamer primers that produced RAPD bands in four species in a preliminary test (Table 2).

Amplification reactions were performed in 0.6 ml tubes containing 25 μl of the reaction buffer; 10 mM Tris-HCl, pH 8.8, 50 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP, dTTP, 0.2 mM primer, 2.1 units Taq DNA polymerase, and 25 ng of genomic DNA. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light using Polaroid 667 film. A 100 bp ladder DNA marker (Pharmacia) was used in an adjacent lane for the estimation of fragment size.

Table 1. Code and locations of the genus *Phyllostachys* and the standards in this study

Species	Code	Localities
Phyllostachys bambusoides Sieb. et Zucc.	BAM 1	Gurye-gun, Masan-myeon, Jeonlanam-do
	BAM 2	Ungsang-eup, Deokgye-ri, Gyeongsangnam-do
	BAM 3	Namwon ci, Damyang-eup, Jeonlabuk-do
P. nigra Munro	NIG 1	Ungsang-eup, Deokgye-ri, Gyeongsangnam-do
	NIG 2	Jukheon-dong, Gangneung-si, Gamgwon-do
	NIG 3	Guseo-dong, Geumjeong-gu, Busan
P. pubescens Mazel	PUB 1	Namwon-ci, Damyang-eup, Jeonlabuk-do
	PUB 2	Gwangsan-dong, Gwangsan-gu, Gwangju
	PUB 3	Gajwa-dong Jinju-si, Gyeongsangnam-do
P. nigra var. *henonis* Stapf.	HEN 1	Namwon ci, Damyang-eup, Jeonlabuk-do
	HEN 2	Gajwa-dong Jinju-si, Gyeongsangnam-do
	HEN 3	Geoje-myeon, Geoje-si, Gyeongsangnam-do
Sasa japonica Makino	SAS	Sicheon-myeon, Sanxheong-gun, Gyeongsangnam-do

Table 2. List of decamer oligonucleotide utilized as RAPD primers, their sequences, and associated polymorphic fragments amplified in the genus *Phyllostachys*

Primer	Sequence (5′ to 3′)	No. of fragments detected	Fragment size range (bp)
OPD01	ACCGCGAACG	17	470 - 2540
OPD02	CGACCCAACC	12	350 - 1890
OPD05	GTCGCCGTCA	12	450 - 2380
OPD08	TCTGGTGAGG	6	550 - 1120
OPD10	TGAGCGGACA	11	650 - 1500
OPD12	ACCTGAACGG	6	660 - 1400
OPD15	TTGGCACGGG	8	720 - 1450
OPD18	CTCTGGAGAC	8	520 - 1340
OPD19	GGTCTACACC	10	450 - 1460

Statistical analyses

All RAPD bands were scored by eye and only unambiguously scored bands were used in the analyses. Because RAPDs are dominant markers, it was assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band.

The following genetic parameters were calculated using a POPGENE computer program ver. 1.31 [21]. The percentage of polymorphic loci (P_P); mean numbers of alleles per locus (A); effective number of alleles per locus (A_e); and gene diversity (H) [17]. The degree of polymorphism was quantified using Shannon's diversity index (I) [20].

To analyse the organization of variability within the genus *Phyllostachys*, we examined the genetic variation by partitioning the total genetic diversity (H_T) to within species components (H_S), and the differentiation among species (G_{ST}). Furthermore, gene flow (Nm) between the pairs of populations was calculated from G_{ST} values by $N\text{m} = 0.5(1/G_{ST}\text{-}1)$ [15].

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity [3].

$H = -\sum p_i \log p_i$

where p_i is the frequency of a particular phenotype i [12]. H_o can be calculated and compared for different populations [18].

$H_{POP} = 1/n \sum H_O$

be the average diversity over the n different species and let

$H_{SP} = -\sum p \log p$

be the diversity of species calculated from the phenotypic frequencies p in all the species considered together [18]. Then the proportion of diversity presented within species, H_{POP}/H_{SP}, can compared with that of between species ($H_{SP} - H_{POP}$)/ H_{SP}.

To elucidate the extent of genetic departure of populations from each other, Nei's genetic identity (GI) and genetic distance (GD) were calculated for each pairwise combination of populations [17]. Homogeneity of variance among species was tested by Bartlett's statistics.

A phylogenetic tree was constructed by the neighbor-joining (NJ) method [19] using the NEIGHBOR program in PHYLIP version 3.57 [5].

Results

From the 20-decamer primers used for a preliminary RAPD analysis, nine primers produced good amplification products both in quality and variability (Table 3). Overall, 90 fragments were generated among the tested *Phyllostachys* array. Invariant fragments ranged from 6-17 per primer. In a simple measure of intraspecies variability by the percentage of polymorphic bands, the *P. bambusoides* and *P. nigra* var. *henonis* exhibited the lowest variation (8.9%). The *P. ni-*

Table 3. Measures of genetic variation for RAPD generated among taxa

Species	N_P	P_P	A	Ae	H	I
P. bambusoides	8	8.9	1.044	1.031	0.018	0.026
P. nigra	30	33.3	1.167	1.118	0.074	0.098
P. pubescens	26	28.9	1.144	1.120	0.064	0.092
P. nigra var. *henonis*	8	8.9	1.044	1.037	0.020	0.028
Mean	18	20.0	1.100	1.077	0.044	0.061

The number of polymorphic loci (Np), percentage of polymorphism (Pp), mean number of alleles per locus (A), effective number of alleles per locus (Ae), gene diversity (H), and Shannon's information index (I)

gra showed the highest (33.3%).

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*I*) within populations. The mean *I* of *P. nigra* (0.098) was highest of all species and was significantly differen (paired *t* test) from the others. As the typical populations of *Phyllostachys* were small, isolated, and patchily distributed for natural populations, they maintained a low level of genetic diversity (mean H_T=0.315) (Table 4).

An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 13.8% the total genetic diversity was among species (Table 5). Thus, the majority of genetic variation (86.2%) resided within species. The average number of individuals exchanged between populations per generation (*N*m) was estimated to be very low (0.263).

Clustering of three populations per species, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). The four taxa of genus *Phyllostachys* analyzed were distinctly related to a monophyletic. *P. nigra* var. *henonis* Stapf was found to be more closely related to *P. pubescens* than to *P. nigra*. *P. bambusoides* was quite distinct from remaining species.

Discussion

Genetic diversity and population structure

Genetic diversity of genus *Phyllostachys* can be comparable with that of allozyme analysis and other species, although the use of different methods (e.g., the number of loci, populations sampled, and the enzyme systems studied) may preclude meaningful direct comparisons. For example, its genetic diversity of 0.044 is lower than that for temperate-zone species (0.146), monocots (0.181), species with a sexual and asexual reproduction mode (0.120), and long-lived woody habit (0.177) [6].

In this study, RAPDs were used to determine the genetic relationships among 12 populations and the results were compared to pedigree relationships where they were available.

The ultimate result of this study is lacking of intra-population variation. 34.1% variation was found among species and about 65.9% within species. The genus *Phyllostachys* in Korea is moderate or more differentiated than the other vegetative and predominant asexual-reproductive mode species [7].

If an N_m value (0.263) can be considered lower than 1,

Table 4. Estimates of genetic diversity statistics and polymorphic loci in *Phyllostachys* species by RAPD

Locus	H_T	H_S	G_{ST}	*N*m
OPD01	0.401	0.067	0.831	0.161
OPD02	0.303	0.057	0.601	0.140
OPD05	0.196	0.039	0.318	0.495
OPD08	0.247	0.053	0.496	0.301
OPD10	0.326	0.036	0.678	0.311
OPD12	0.333	0	0.833	0
OPD15	0.358	0.049	0.737	0.123
OPD18	0.372	0.055	0.787	0.484
OPD19	0.294	0.027	0.650	0.354
Mean	0.315	0.043	0.659	0.263

Total genetic diversity (H_T), genetic diversity within species (H_S), proportion of total genetic diversity partitioned among population (G_{ST}) and gene flow (*N*m) between the pairs of populations.

Table 5. Partitioning of the genetic diversity into within and among *Phyllostachys* species by RAPD

Species	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$(H_{sp}-H_{pop})/H_{sp}$
P. bambusoides	1.865	2.119	0.880	0.120
P. nigra	1.812	2.120	0.855	0.145
P. pubescens	1.762	2.059	0.856	0.144
P. nigra var. *henonis*	1.847	2.149	0.859	0.141
Mean	1.822	2.112	0.862	0.138

Compared for different populations (H_{POP}), the average diversity over the n different species (H_{SP}), the proportion of diversity presented within species (H_{POP}/H_{SP}), compared with that of between species ($H_{SP} - H_{POP}$)/ H_{SP}.

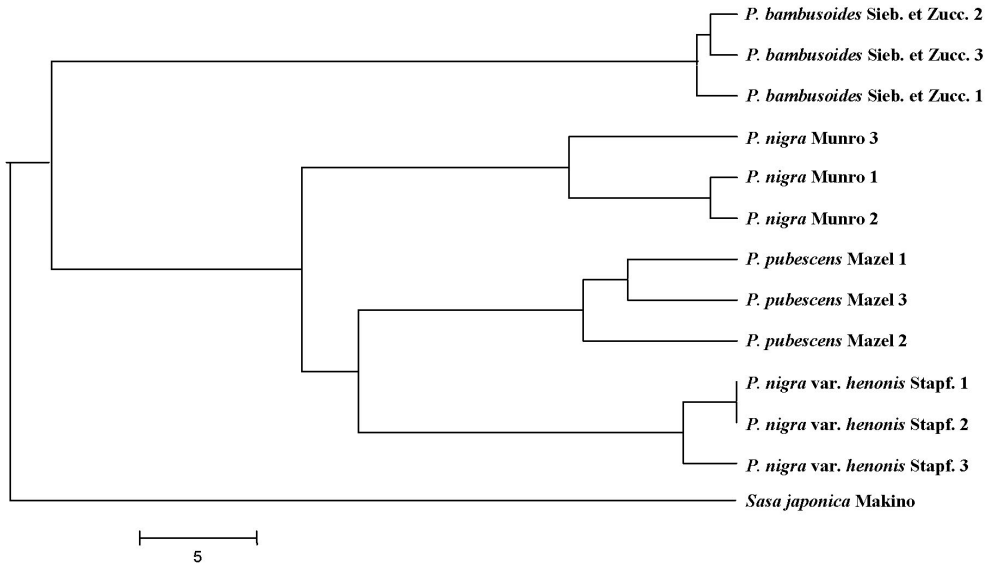

Fig. 1. A phylogenetic tree for *Phyllostachys* based on RAPD analysis.

and as a result, genetic drift should be a factor in genus *Phyllostachys*. Thus, the levels of gene flow we have calculated are not of sufficient magnitude to counterbalance genetic drift and may play a major role in shaping the genetic structure of the populations among species. Fruiting of the genus *Phyllostachys* is exceptionally infrequent event. Depending on the species of bamboo, flowering cycles vary from 10 to over 100 years and it has been observed that numbers of one bamboo species flower at the same time in large geographical areas. Most of the Korean populations are regenerated artificially. Populations are reproductively isolated. Therefore, most of the populations have small population sizes and are isolated from each other.

Phylogenetic relationships within *Phyllostachys*

This position also varied in phylogenetic trees constructed by molecular markers (Fig. 1). In the phylogeny generated, the four taxa of the genus *Phyllostachys* analyzed were distinctly related to a monophyletic. Since species of *Phyllostachys* show a wide range of morphological and geographical variation, it is difficult to elucidate phylogenetic relationships with morphological characteristics [10]. *P. bambusoides* was quite distinct from remaining species. This is almost agreement concerning the morphological characters.

P. nigra var. *henonis* Stapf was found to be more closely related to *P. pubescens* than to *P. nigra*. However *P. nigra* and *P. pubescens* are closely related, previous morphological taxonomic work was not proposed as the closest relative. Stem and branches of *P. nigra* were black, whereas them of *P. nigra* var. *henonis* are not black, but only dark blue and originated from China. At present, the phylogenetic position of this species shown in (Fig. 1). It is seem to be the best, judging from morphological, genetic, and distribution data. This issue will be clarified in future studies. In addition, *P. nigra* var. *henonis* was strongly differentiated in *P. nigra*, and thus deserve taxonomic treatment at subspecies or even at species level. Although the size of sampling was not large enough for the genus *Phyllostachys*, the analyses of RAPDs will certainly provide an enhanced view on the phylogeny of this genus. In addition, It is necessary to identify taxa that additional molecular experiments such as AFLP, SSR, and ITS

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초록 : RAPD분자 마커를 이용한 왕대속 대나무의 유전적 다양성 및 계통 관계

이송진* · 허만규[1] · 신현철[2] · 허홍욱
(부산대학교 생물학과, [1]동의대학교 분자생물학과, [2]국립산림과학원 남부산림연구원)

왕대속 대나무들은 대부분 동남아시아에 분포한다. 전세계적으로 왕대속에 속하는 4종은 의학적, 생태학적으로 중요시 되어 왔다. 이번 연구에서 우리나라에 자생하고 있는 왕대속 4종을 RAPD마커를 이용하여 유전적 관계 분석하였다. RAPD분석결과 왕대속에 속하는 4종의 대나무는 명확하게 분류가 되었고 8.9~33.3%로 다형현상이 나타났다. 특히 왕대는 다른 종들 보다 유전적 다양성이 0.018로 가장 낮게 나왔다. 그리고 집단 내 유전적 다양성(*Hs*)은 0.315, 집단간 다양성(*Gst*)은 0.659 그리고 유전자 유동(*Nm*)은 0.0263로 나타났다. 이는 한국의 왕대속 집단은 지리적 및 환경적 요인을 받아 유전적 다양성이 낮게 나타났으며 본 연구는 대나무 유전적 다양성 연구에 중요한 기초자료가 될 것으로 사료된다.