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

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Four taxa of the genus *Phyllostachys* were analysed with PCR-based molecular inter-simple sequence repeat (ISSR) determine markers to determine their phylogenetic relationships. Many species of this genus are regarded as economical and ecologically important in the world. With the nine primers screened, ISSR assay generated a total of 64 reproducible bands. Analysis of ISSR from individual plants of genus *Phyllostachys* resulted in 43 polymorphic bands with 70.49%. When species were grouped by four taxa, within group diversity was 0.092 (*Hs*), while among group diversity was 0.499 (*Gst*) on a per locus basis. The estimated gene flow (*Nm*) between the pairs of species was 0.559. Hence, we can expect weak or low gene flow among species. Total mean genetic diversity (H_T) for four taxa was 0.291. The ISSR data allowed us to resolve well-supported clades in the genus *Phyllostachys*. The four taxa of the genus *Phyllostachys* analyzed were distinctly related to a monophyletic.

Key words : Genus *Phyllostachys*, ISSR, phylogenetic relationships

Introduction

While a great deal of phylogenetic information on molecular marker of magnoliopsida (dicots) has been accumulated over the last 2 decades, information of liliopsida (monocots) is rudimentary. Especially, almost no information is available from a southeast Asia. We need an understanding of molecular variation in different plant species from these regions. One important species of liliopsida is bamboo. The bamboo is produced easily and is utilized to the multipurpose. 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. Bamboos are a cheap and sustainable source of building materials, food, and of source decorative ornament garden plants. The woody bamboos are used for matting, thatch, baskets, bamboo wires, bamboo boxes and scaffolding [11].

The genus *Phyllostachys* (Bambusaceae) have been distributed mostly in korea. But, reduce of populations is serious. Many manufactures substitute bamboos for plastic or iron goods. Korea is developing country. A bamboo wood was utilized for an agriculture and industry development. Isolated populations may be gradually exhibit genetic differentiation. The rapid loss of new plants results in the permanent loss of gene pools with potential for species conservation.

The genus *Phyllostachys* is comprised of 4 species in Korea [12]. The taxonomy of *Phyllostachys* has processed mainly through morphological characteristics. *P. bambusoides* Sieb. et Zucc is sericeous that get into twig and right angle. *P. nigra* var. *henonis* Stapf accomplishes twig and acute angle. *P. pubescens* Mazel is bamboo joint by one. *P. nigra* Munro is a black very in stem.

However morphological characteristics are restricted 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 [9,10].

Typical populations of many *Phyllostachys* species except *P. bambusoides* are small and distributed in patches. The main concern of persistence of *Phyllostachys* is continued habitat destruction and fragmentation. Consequently, wild *Phyllostachys* populations have suffered loss individuals, loss 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

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for the development of strategies for *ex situ* conservation of plant genetic resources [1].

In recent tears, a number of PCR-based DNA markers, such as RAPD (random amplified polymorphism DNA), SSR (simple sequence repeats), AFLP (amplified fragment length polymorphism), and ISSR (inter simple sequence repeats) has been widely used to investigate systematics and population genetic structure because they overcome the limitations of allozyme markers [13]. Of these, the most popular is RAPD, which has been successfully used in a wide variety of fields [20]. As a less widely used PCR-based marker has a few advantages over other markers. ISSR primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSRs [6]. Also, the sequences that ISSRs target are abundant throughout the eukaryotic genome and evolve rapidly; consequently ISSRs may reveal a much higher number of polymorphic fragments per primer than RAPDs [3,4]. In addition, studies have indicated that ISSRs produce more reliable and reproducible bands compared with RAPDs because of the higher annealing temperature and longer sequence of ISSR primers [17,19]. Therefore, ISSRs have proved to be useful in population genetic studies, especially in decting genetic diversity and fingerprinting closely related individuals [20].

Therefore, the objectives of this study were to estimate how much genetic diversity is maintained in genus *Phyllostachys* and to describe germ-plasm classification within genus.

Materials and methods

Plant Materials

All of the 12 accessions of four taxa were collected from populations in Korea (Table 1). One young leaf per mature tree ($\geq$ 5 yr) was collected each in May. Fifteen plants were randomly collected from each population. In addition, the species of same family Gramineae, *Sasa borealis* was provided for the outgroup and used to compare the phylogenic relationship (Fig. 3).

DNA Extraction and ISSR Analysis

The genomic DNA of the 195 samples including outgroup was extracted from fresh leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, USA.) according to the manufacturer's protocol.

Nine 10-mer primers of Bioneer Technologies (Korea) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses (Table 1).

Amplification reactions were performed in 0.6 ml tubes containing 2.5 μl of the reaction buffer, 10 mM Tris-HCl (pH 8.8), 1.25 mM each of dATP, dCTP, dGTP, dTTP, 5.0 pM primer, 2.5 units Taq DNA polymerase, and 25 ng of genomic DNA. A 100 bp ladder DNA marker (Pharmacia) was used in the end of for the estimation of fragment size. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., USA).

Statistical Analyses

All ISSR bands were scored by eye and only unambiguously scored bands were used in the analyses. Because ISSRs 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) developed by [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) [14].

To elucidate the organization of variability within the genus *Phyllostachys,* we examined the genetic variation by the differentiation among species and the number of migrants per generation (N_m) using the Nei's genetic diversity statistics [15].

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity [2]:

$H_O = - \sum p_i \log p_i$

where p_i is the frequency of a particular phenotype i. H_O can be calculated and compared for different populations [16]. Let

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

be the average diversity over the different populations and let

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

be the diversity calculated from the phenotypic frequencies p in all populations considered together. Then the proportion of diversity present within populations, H_{POP}/H_{SP}, can compared with that of between populations (G_{ST}), ($H_{SP} - H_{POP}$)/H_{SP}.

The estimation of genetic similarity (GS) between geno-

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

Species	Code	Localities
Phyllostachys bambusoides Sieb. et Zucc.	BAM 101	Gurye-gun, Masan-myeon, Jeonlanam-do, Korea
	BAM 103	Ungsang-eup, Deokgye-ri, Gyeongsangnam-do, Korea
	BAM 104	Namwon ci, Damyang-eup, Jeonlabuk-do, Korea
P. nigra Munro	NIG 201	Ungsang-eup, Deokgye-ri, Gyeongsangnam-do, Korea
	NIG 202	Jukheon-dong, Gangneung-si, Gamgwon-do, Korea
	NIG 205	Guseo-dong, Geumjeong-gu, Busan, Korea
P. pubescens Mazel	PUB 303	Namwon-ci, Damyang-eup, Jeonlabuk-do, Korea
	PUB 304	Gwangsan-dong, Gwangsan-gu, Gwangju, Korea
	PUB 305	Gajwa-dong Jinju-si, Gyeongsangnam-do, Korea
P. nigra var. *henonis* Stapf.	HEN 401	Namwon ci, Damyang-eup, Jeonlabuk-do, Korea
	HEN 402	Gajwa-dong Jinju-si, Gyeongsangnam-do, Korea
	HEN 405	Geoje-myeon, Geoje-si, Gyeongsangnam-do, Korea
Sasa borealis Makino	SAS101	Mandeok-dong, Buk-gu, Busan, Korea

types was based on the probability that an amplified fragment from one individual will also be present in another [14]. GS was converted to genetic distance (1-GS). Homogeneity of variance among populations was tested by Bartlett's statistics.

A phylogenetic tree was constructed by the neighbor-joining (NJ) method [18] using the NEIGHBOR program in PHYLIP version 3.57 [5].

Results

From the 11 primers used for a preliminary ISSR analysis, nine primers produced good amplification products both in quality and variability (Table 2). Overall, 64 fragments were generated among the tested genus *Phyllostachys* array. The fragments ranged from 3 to 21 per primer. In a simple measure of interspecies variability by the percentage of polymorphic bands, the *P. bambusoides* exhibited the lowest variation (32.8%) (Table 3). The *P. nigra* showed the highest (49.2%). Mean number of alleles per locus (*A*) and effective number of alleles per locus (Ae) for species were 1.201 and 1.161, respectively. As the typical populations of genus *Phyllostachys* were small, isolated, and patchily distributed for natural populations, they maintained a low level of genetic diversity for ten polymorphic primers. The

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

No. of Primer	Sequence (5′ to 3′)	No. of fragments detected
ISSR-01	5′-AGAGAGAGAGAGAGAGG-3′	7
ISSR-02	5′-CTCTCTCTCTCTCTCT-3′	4
ISSR-03	5′-CACACACACACACACAG-3′	3
ISSR-04	5′-TCTCTCTCTCTCTCT-3′	4
ISSR-05	5′-GGAGAGGAGAGGAGA-3′	8
ISSR-06	5′-GAGAGAGAGAGAGAGAGT-3′	10
ISSR-07	5′-GAGAGAGAGAGAGAGACG-3′	10
ISSR-08	5′-GAGAGAGAGAGAGAGATC-3′	7
ISSR-10	5′-GCGACACACACACACACACA-3′	11

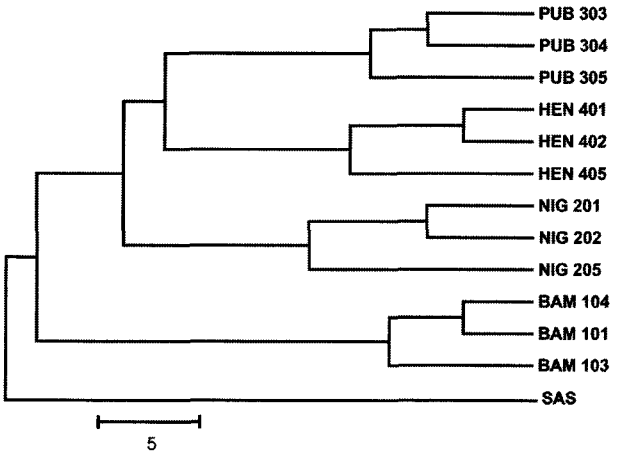

Fig. 1. A phylogenetic tree for *Phyllostachys* based on ISSR analysis

Table 3. Summary of genetic diversity for all loci among four *Phyllostachys* species by ISSR markers

Species	N_P	P_P	*A*	Ae	*H*	I
P. bambusoides	20	32.78	1.1639	1.1311	0.0729	0.1043
P. nigra	30	49.18	1.2459	1.1967	0.1093	0.1565
P. pubescens	24	39.34	1.1967	1.1574	0.0874	0.1252
P. nigra var. henonis	24	39.34	1.1967	1.1574	0.0874	0.1252
Mean	24.5	40.16	1.2008	1.1607	0.0893	0.1278

ns: Not significant; * = $p < 0.05$; *** = $p < 0.001$.

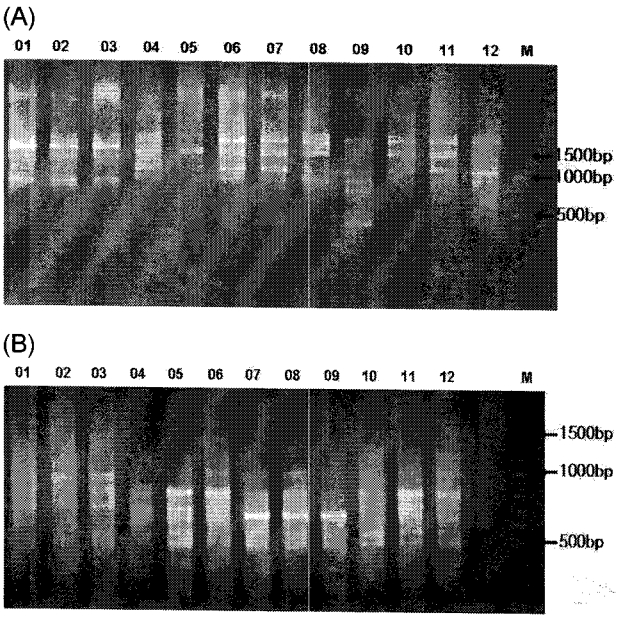

Fig. 2. Amplification progiles of *Phyllostachys* varieties by A. ISSR-01 and B. ISSR-09. Lane number 1-12 represent the varieties as: BAM 101, BAM 103, BAM 104, NIG 201, NIG 202, NIG 205, PUB 303, PUB 304, PUB 305, HEN 401, HEN 402, HEN 405. M is for morlecular weight marker

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

Locus	H_T	H_S	G_{ST}	*Nm*
ISSR-1	0.359	0.109	0.593	0.223
ISSR-2	0.313	0.000	0.750	0.000
ISSR-3	0.301	0.197	0.219	2.411
ISSR-4	0.265	0.122	0.374	0.456
ISSR-5	0.205	0.086	0.280	0.297
ISSR-6	0.286	0.098	0.479	1.027
ISSR-7	0.328	0.059	0.870	0.141
ISSR-8	0.284	0.056	0.565	0.287
ISSR-10	0.291	0.102	0.359	0.186
Mean	0.291	0.092	0.499	0.559

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

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

Species	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$(H_{sp}-H_{pop})/H_{sp}$
P. bambusoides	1.526	2.121	0.720	0.280
P. nigra	1.558	2.102	0.741	0.259
P. pubescens	1.494	2.095	0.713	0.287
P. nigra var. *henonis*	1.518	2.061	0.736	0.264
Mean	1.524	2.095	0.728	0.273

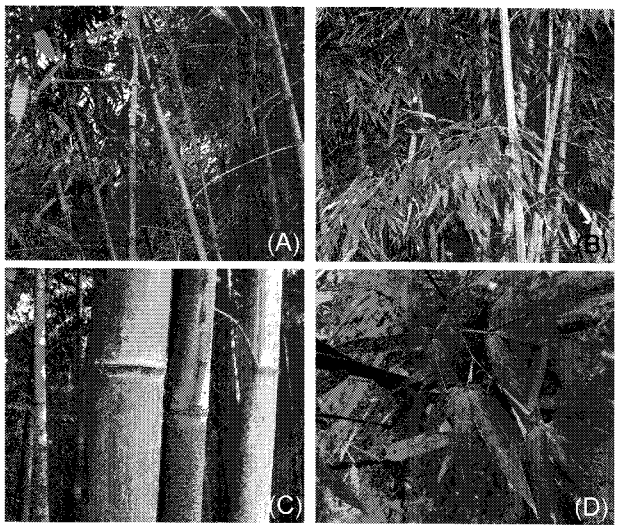

Fig. 3. Amplification progiles of *Phyllostachys* varieties by as: (A) *P. bambusoides* Sieb. et Zucc (B) *P. nigra* var. *henonis* Stapf (C) *P. pubescens* Mazel (D) *P. nigra* Munro

mean *H* was 0.089 across species, varying from 0.073 to 0.109. In particular, *P. nigra* had the highest expected diversity (0.109); *P. bambusoides*, the lowest (0.073) (Fig. 2).

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*I*) within populations. The mean *I* of *P. nigra* (0.157) was highest of all species and showed significant difference (paired *t* test). 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 = 0.291) (Table 4). The average number of individuals exchanged between populations per generation (*Nm*) was estimated to be very low (0.559).

An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 27.3% the total genetic diversity was among species (Table 5). Thus, the majority of genetic variation (72.8%) resided within species.

Clustering of four 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* 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 com-

parable 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 0.089 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) [7]. The percentage of polymorphic loci at the species level was 40.2%. This value is also lower than the average for species with temperature-zone distributions (48.5%), monocots (59.2%), and species with a sexual and asexual reproduction mode (43.8%), but lower than those with a long-lived woody (64.7%) [7].

In case of RAPD, the result was same trend. For example, this level of polymorphism is lower than frequencies reported in sunflower (48%) and genus *Chaenomeles* (93.7%) [1].

Given the proliferation of genetic markers, comparisons between techniques are inevitable. However, there is a need technique is best suited the issues being examined. In this study, ISSRs were used to determine the genetic relationships among 12 populations and the results compared to pedigree relationships where there were available.

A striking feature of this study is the lacking of intra-populations variation. 27.3% variation was found among species and about 72.8% within species. The genus *Phyllostachys* in Korea is moderate or more differentiated than the other vegetative and predominant asexual-reproductive mode species [8]. The population structure of a species is affected by a number of evolutionary factors including mating system, gene flow, seed dispersal, and mode of reproduction as well as natural selection [7]. Most species of genus *Phyllostachys* possess a certain capacity foe sexual reproduction by low-rate selfings [10]. It is not unexpected, therefore, that a much larger proportion of genetic diversity resides among populations of these species; although some selfing species often maintain as much diversity as outcrossing species.

If an N_m value (0.559) can be considered lower than 1, 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 genus *Phyllostachys* is exceptionally low 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. Now most of Korean populations are regenerated artificially. Populations are reproductively isolated Therefore, most populations have small population sizes and isolated each other.

Phylogenetic relationships within Phyllostachys

This position also varied in phylogenetic trees constructed by molecular markers (Fig. 1). In generated phylogeny, the four taxa of 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 and *P. nigra* var. *henonis* are separated from each other and *P. nigra* var. *henonis* 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 : ISSR 분자 마커를 이용한 왕대속 대나무의 유전적 다양성 및 계통 관계

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ISSR분자 마커를 이용하여 왕대속 대나무의 유연관계를 분석 실시하였다. 전세계적으로 왕대속에 속하는 4종은 경제학적, 생태학적으로 중요한 식물 자원중 하나이다. 총 11개의 ISSR 프라이머 중 9개의 프라이머에서 증폭이 일어 났으며 총 64개의 밴드를 확인 할 수 있었다. ISSR분석결과 왕대속 4종의 대나무에서 70.49%인 43개의 다형현상이 나타났다. 4개의 분류군으로 분류된 왕대속 대나무의 집단내 다양성(*Hs*)은 0.092, 집단간 다양성(Gst)은 0.499로 나타났다. 각 종에 대한 유전자 유동(*Nm*)은 0.559로 나타났다. 따라서 왕대속에 속하는 4종의 유전자 유동(*Nm*)은 아주 낮음을 알 수 있었다. 4종의 분류군에 대한 유전자 다양성(*Ht*)은 0.291로 낮게 나타났으며 ISSR 마커로 명확하게 그들은 분류가 되었다. 또한 왕대속의 4종은 단일계통임을 확인할 수 있었다.