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Diversity of I-SSR Variants in *Gingko biloba* L. Planted in 6 Regions of Korea[1]

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國內 6個 銀杏나무 植栽地에 있어서 I-SSR 變異體의 多樣性[1]

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ABSTRACT

Genomic DNAs were extracted from the leaves of 182 ginkgo trees (*Ginkgo biloba* L.) planted in 6 regions and subjected to the analysis of both I-SSR and RAPD markers. A total of 227 amplicon variants were generated by PCR using 15 I-SSR primers and 67 amplicons by PCR with 5 RAPD primers. Levels of genetic diversity within 6 populations were turned out to be similar (Shannon's Index, I-SSR : 0.35～0.40; mean of 0.38, RAPD : 0.31～0.38; mean of 0.35, combined : 0.35～0.40; mean of 0.37). Ranks of the level of genetic diversity estimated from I-SSR, RAPD, and combined data were not coincided each other. Majority of genetic diversity was allocated among individuals within populations (I-SSR : 94.31%, RAPD : 93.62%, combined : 93.57%), which resulted in pretty low level of population differentiation. Genetic differentiation between male and female groups was turned out to be quite low (I-SSR : 0.03, RAPD : 0.091, combined : 0.043), which slightly fluctuated when analysis was restricted to the data obtained from 3 regions where both male and female trees were sampled (I-SSR : 0.038, RAPD : 0.084, combined : 0.047). Genetic relationships among the populations, reconstructed by UPGMA, were not coincided with geographic affinity, which might be resulted from sharing of seed sources in some regions. Whereas independent cluster analyses with I-SSR data and RAPD data, respectively, reclassified by sexes revealed two sexual groups in which all the male and the female populations were clustered together, cluster analysis with combined data did not show clear sexual grouping.

Key words : *I-SSR, RAPD,* Gingko biloba, *genetic diversity degree of population differentiation, cluster analysis*

要 約

6개 지역에 식재된 은행나무 182개체의 잎으로부터 DNA를 추출하여 I-SSR과 RAPD 표지자를 분석하였다. 15개 I-SSR primer를 사용하여 PCR을 수행해서 227개의 증폭산물 변이체를 확인하였고, 5개 RAPD primer를 사용하여 67개의 증폭산물 변이체를 확인했다. 6개 집단에 있어서 집단내의 유전적 다양성 정도는 유사한 것으로 나타났다 (Shannon's Index, I-SSR : 0.35～0.40; 평균 0.38, RAPD : 0.31～0.38; 평균 0.35. 통합 : 0.35～0.40; 평균 0.37). 3개의 자료 (I-SSR 표지자, RAPD 표지자 및 통합 자료)로부터 평가된 유전적 다양성 정도의 등급은 상호간에 일치하지 않았다. 유전적

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다양성의 대부분이 집단내 개체간에 존재하는 것으로 나타났으며 (I-SSR; 94.31%, RAPD : 93.62%, 통합 : 93.57%), 결과로써 집단간 분화정도는 상당히 낮게 나타났다. 수나무와 암나무 그룹간의 유전적 분화정도는 매우 낮았으며 (I-SSR : 0.03, RAPD : 0.091, 통합 : 0.043), 수나무와 암나무를 동시에 채집한 3개 지역만을 대상으로 분석했을 경우에는 약간의 변동이 있었다 (I-SSR : 0.038, RAPD : 0.084, 통합 : 0.047). UPGMA법을 이용해서 분석한 집단간의 유전적 유연관계는 지리적 거리와 일치하지 않았는데, 이는 몇몇 지역들이 종자 공급원을 공유하고 있기 때문일 것으로 추정된다. I-SSR과 RAPD data를 성별로 재편성해서 유집분석을 수행한 결과, 모든 수나무 집단들과 암나무 집단들이 각각 별개의 성별 분지군을 형성하는 양상을 보였으나, 2개의 data를 통합해서 유집분석을 수행했을 경우에는 성에 따른 명백한 분지군이 형성되지 않았다.

INTRODUCTION

Ginkgo tree (*Ginkgo biloba* L.) is the only species of the Ginkgoaceae survived at present. Unlike other gymnosperms, ginkgo tree is dioecious and has motile sperm-cells (Li, 1956). Arboricultural use of ginkgo tree as a road tree or a garden tree generally prefers male individuals to avoid stenchy of the ripen fruits of female trees. Pomocultural demand of seeds, which are edible, prefers female individuals for seed production. As a being of living fossil, ginkgo tree has existed several millions of years on the Earth. Although economical value of ginkgo tree is getting raised as food, medical resources, and timber, yet no serious consideration to estimate its genetic background has been taken. Estimating the genetic diversity of a species is the fundamental step to understand the genetic architecture of the species of interest. Ginkgo trees in Korea were introduced from China over a thousand years ago and planted mainly for roadside tree and scenic tree, which gave rise to inevitable prediction of narrow genetic base of them in Korea compared to other endemic tree species. The economic importance considered, proper silvicultural practice should be considered to keep broadening genetic base and to prevent genetic resources from being lost although ginkgo tree is not an endemic tree species in Korea.

In present study, ginkgo trees of 93 males and 89 females were sampled from 6 regions and subjected to the analysis of both I-SSR and RAPD markers. On the basis of the observed DNA variants, population genetic parameters were estimated to monitor the genetic resource of ginkgo trees in Korea.

MATERIALS AND METHODS

1. DNA extraction and PCR amplification

Foliages of Gingko trees were collected from 182 individual trees (93 male and 89 female trees, respectively) in 6 populations (Table 1) in 1999 and stored at -20℃ until DNA extraction. Total genomic DNAs were extracted from foliage tissues by a modified CTAB method (Hong *et al.*, 1993). Twenty-five microliter of reaction mixture was prepared for PCR amplification, which contained 5ng of template DNA, 0.2mM each of dNTPs, 0.025% of BSA (Boeringer Mannheim, Germany), 5$\mu\ell$ of 1.5μM primers, 1.2$\mu\ell$ of 25mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Advanced Biotechnique, UK). The lid-heated DNA thermal cycler PTC-200 (MJ Research, USA) was used for PCR with the following 2 temperature profiles : 1) for amplification of I-SSR marker, 5 min at 94℃ for 1 cycle for initial denaturation; 45 cycles of 30 sec. at 94℃ for denaturation, 30 sec. at 52℃ for annealing and 1 min. at 72℃ for extension. After 45 cycles, 10 min. of final extension was performed at 72℃. 2) for amplification of RAPD marker, 5 min at 94℃ for 1 cycle for initial denaturation; 45 cycles of 30 sec. at 94℃ for denaturation, 30 sec. at 36℃ for annealing and 1 min. at 72℃ for extension. After 45 cycles, 10 min. of final extension was performed at 72℃. Amplification products were fractionated on 2% agarose gel containing EtBr in 1X TBE

Table 1. Estimates of phenotypic diversity within populations on the basis of observed I-SSR and RAPD amplicon variants.

Populations	No.	I-SSR		RAPD		Combined	
		S.I.[a]	R[b]	S.I.	R	S.I.	R
Taejeon	21	0.3476	6	0.3516	3	0.3485	6
Suncheon	34	0.4067	1	0.3787	1	0.4003	1
Jeonjoo	58	0.3716	5	0.3621	2	0.3694	4
Jinjoo	35	0.3736	4	0.3514	4	0.3686	5
Keochang	18	0.3890	2	0.3086	6	0.3707	3
Masan	16	0.3877	3	0.3352	5	0.3757	2

a : Shannon's index b : ranks

buffer and photographed over UV trans-illuminator. Sizes of amplification products were calculated by comparing with 100 base-pair DNA ladder (MBI, Lithuania) which was loaded in two separate lanes on the same gel for electrophoresis. Fifteen I-SSR primers, which showed clear amplification products from the preliminary primer screening, were chosen and used for PCR with 182 diploid DNAs from 6 populations.

2. Data analysis

Gel photos were scanned with biological imaging system (pdi, USA). After computer scanning of the photographs, the presence of the amplicons of the same size was verified via computer image analysis (Diversity One, pdi). Variants of the amplification products were recorded as the presence (1) versus the absence (0) of the amplicon of the same size. Shannon's index of phenotypic diversity (*Ho* : Shannon, 1948) for both I-SSR and RAPD amplicons (i.e., distribution of I-SSR and RAPD amplicon variants among the categories of presence or absence within population) was estimated as $-\sum p_i \log_2 p_i$ by POPGENE v.1.21 (Yeh et. al., 1997), where *pi* is the frequency of presence or absence of the PCR amplicon of the same size. Genetic distance was calculated by Euclidean metric of Excoffier et al. (1992) and population genetic parameters were estimated by AMOVA v1.55 (Excoffier *et al.*, 1992). AMOVA was performed at either 2 or 3 hierarchical levels according to data reprepared to test the distribution patterns of genetic diversity among plantations and between sexes. In comparison with the result of AMOVA, degree of population differentiation, θ(theta : Weir and Cockerham, 1984), an analogue of F_{ST} and Φ_{ST} in AMOVA, was computed by TFPGA v1.3 (Miller, 1997) on the basis of 2 different estimators of allele frequencies from the phenotypically dominant markers of I-SSR and RAPD : First, the frequency of the recessive allele was simply defined as the square root of the recessive homozygote frequency at a locus (Weir, 1990). Second, an alternative and apparently less biased estimator of the frequency of the recessive allele was calculated on the basis of a Taylor expansion (Lynch and Milligan, 1994). Genetic relationships among populations were reconstructed by UPGMA (phylip v3.5c; Felsenstein, 1993) on the basis of pairwise Manhattan distance (Wright, 1978) between populations computed by RAPDDIST v1.0 (Black, 1996). Statistical test of the topology on the UPGMA tree was performed with 100 bootstrapped samples prepared by RAPDDIST v1.0 (Black, 1996).

RESULTS AND DISCUSSION

For I-SSR markers, a total of 227 I-SSR variants, amplified with 15 I-SSR primers [UBC #810(14 variants), 813(6), 814(7), 817(26), 827(25), 834(20), 835(13), 836(20), 843(6), 859(6), 864(10), 866(15), 873(34), 874(11), 880(14)], were observed in 182 Gingko trees (15.1 variants/primer)(Figure 1A). DNA fingerprint-like amplicon profiles of all the analyzed Gingko trees were obtained by pooling phenotypic data of the observed 227 amplicon variants. Estimates of Shannon's index revealed similar level of genetic diversity within populations, ranged from 0.35 (Taejeon) to 0.40 (Suncheon) with the mean of 0.38 (Table 1), which was similar to that observed from I-SSR analyses in 5 populations of *Torreya nucifera* (mean of 0.353; Hong et al., 2000). Majority of genetic diversity was allocated within populations

Table 2. Analysis of molecular variance for I-SSR and RAPD amplicon variants.

Source of variance	d.f.	Variance Component		
		I-SSR	RAPD	Combined
Among populations[a]	5	5.69%	6.38%	6.43%
Within populations[a]	176	94.31%	93.62%	93.57%
Among sexes[b]	1	3.80%	8.38%	4.71%
Among populations within sexes[b]	4	5.22%	6.02%	5.70%
Within populations[b]	121	90.98%	85.61%	89.58%

a : AMOVA was performed with data from whole samples.
b : AMOVA was performed with data from 127 samples from
3 populations where both male and female trees were sampled.

(94.31%), which resulted in low degree of population differentiation (Φ_{ST}=0.0569) (Table 2). This level of population differentiation was somewhat lower than that observed from I-SSR analyses in 5 populations of *Torreya nucifera* (Φ_{ST}=9.35%; Hong et al., 2000) and in 6 populations of *Koelreuteria paniculata* (Φ_{ST}=14.3; Son et al., 2000) which are both endemic and distributed in the restricted regions of Korea. This observation suggests that the introduced tree species of *Ginkgo biloba* has narrow genetic base compared to the endemic tree species of being distributed in the restricted regions. When AMOVA was performed with 3 populations where both male and female trees were sampled, low degree of genetic differentiation (Φ_{CT}=0.038) was observed between male and female groups. This observation suggested that I-SSR variants, observed in this study, as a whole, were evenly distributed in both sexes, which was also confirmed by common principal component analysis (data not shown). Genetic relationships among the populations, reconstructed by UPGMA, were not coincided with geographic affinity, which might be resulted from sharing of seed sources in some regions (Figure 2A). For examples, populations of Keochang and Masan might be originated from the same seed source (C.I.= 100%). Populations of Taejeon, Jeonjoo, Suncheon,

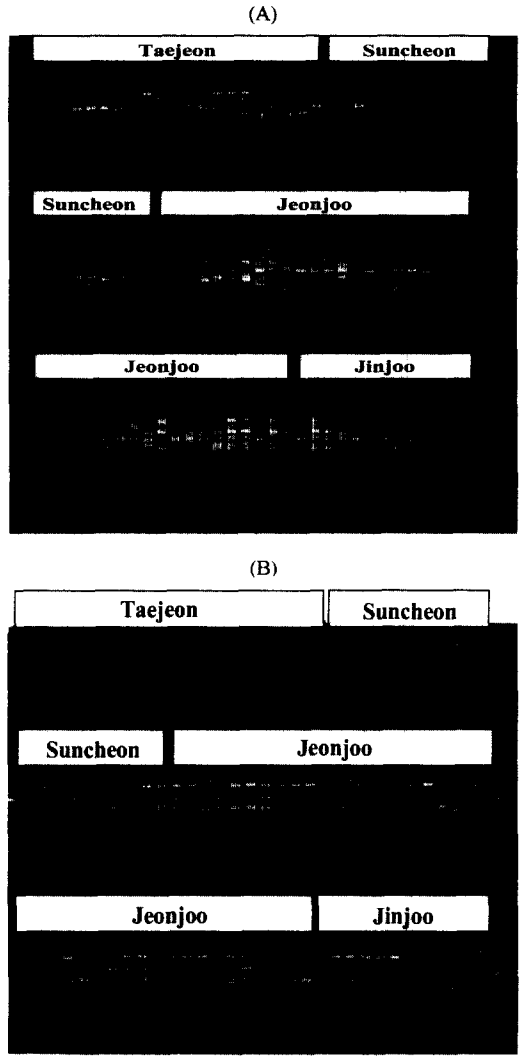

Figure 1. Example of I-SSR (A) and RAPD (B) profiles of ginkgo trees. I-SSR PCR was performed with UBC primer #859. RAPD PCR was performed with OPA-07.

and Jinjoo might be shared the same seed source (C.I.=100%) with an distinct subgroup of Jeonjoo, Suncheon, and Jinjoo (C.I.=100%). Although all subgroups of the same sex were clustered together when male and female trees were treated as subgroups in each sampled population, statistical confidence was not significant (C.I.=73%) (Figure not shown).

For RAPD markers, a total of 67 RAPD variants, amplified with 4 RAPD primers [Operon : A01(18

variants), A07(16), B02(13), B12(20)], were observed in 182 Gingko trees (16.75 variants/primer) (Figure 1B). DNA fingerprint-like amplicon profiles of all the analyzed Gingko trees were also obtained by pooling phenotypic data of the observed 67 amplicon variants. Estimates of Shannon's index revealed similar level of genetic diversity within populations, ranged from 0.31 (Keochang) to 0.38 (Suncheon) with the mean of 0.35, which was relatively lower than those estimated from I-SSR marker (mean of 0.38) (Table 1). Ranks of the level of genetic diversity were not coincided with those for I-SSR markers except for Suncheon (first) and Jinjoo (fourth). On the basis of estimation by AMOVA, distribution of genetic diversity revealed that most of the genetic diversity was allocated within population (93.62%), which was somewhat lower than that of I-SSR marker (94.31%) (Table 2). Level of population differentiation was also turned out to be low (Φ_{ST}=6.38%), which was much lower than that observed from RAPD analyses in the populations of *Pinus densiflora* (Φ_{ST}=29%; Kang 1997) and of *Abies holophylla* (Φ_{ST}=19.81%; Kim, 1998) distributed in large area of Korea. This observation suggests that the introduced tree species of *Ginkgo biloba* has much narrow genetic base compared to the endemic tree species of being widely spreaded in Korea. Additional evidence for the narrow genetic base of *Ginkgo biloba* planted in Korea could be provided by the study on 4 newly established seedling stands of *Populus tremuloides* in burned areas inside Yellowstone National Park, where high level of population differentiation (Φ_{ST}=31%) was observed (Tuskan et al. 1996).

When AMOVA was performed with 3 populations where both male and female trees were sampled, 8.38% of the genetic diversity was allocated between male and female groups. This observation suggested that RAPD variants might be relatively more differentiated or differently distributed by chance between 2 sexes than I-SSR variants (Φ_{CT}=0.038). This difference also resulted in relatively high confidence interval (89%) for grouping of the male subgroups on the dendrogram reconstructed by UPGMA (Figure not shown). On the basis of cluster analysis, although the overall pattern of grouping among 6 populations was similar to that based on I-SSR marker, confidence interval for each node was critically lower than that based on I-SSR marker (Figure 2B). This might be partially due to small number of RAPD variants (67) compared to that of I-SSR variants (227) or different nature of RAPD and I-SSR variants (i.e., different compositions of DNA sequences for primers : arbitrary 10 nucleotides for RAPD vs. a few repeats of the simple sequences with 1 to 3 additional arbitrary nucleotides for I-SSR analyses). Such difference in the sequences of primers (i.e., representing different priming sites) raises a conjecture that the data sets obtained by RAPD and I-SSR analyses may be collected from different parts of the genome which might be undergone different evolutionary processes (ex. different evolutionary rate).

For combined data of 227 I-SSR and 67 RAPD amplicons, overall trend of allocation of the genetic diversity was maintained to be same as those estimated with either I-SSR or RAPD amplicons, respectively. Estimates of Shannon's index revealed similar level of genetic diversity within populations, ranged from 0.35 (Taejeon) to 0.40 (Suncheon) with the mean of 0.37, which was closer to that estimated from I-SSR marker (mean of 0.38) than RAPD marker (mean of 0.35) (Table 1). Overall trend of ranks of the level of genetic diversity was not coincided with those for both I-SSR and RAPD markers except for Suncheon which showed the highest level of genetic diversity for both markers. Distribution of genetic diversity revealed that most of the genetic diversity was allocated within population (93.57%), which was close to that of RAPD marker (93.62%) and somewhat lower than that of I-SSR marker (94.31%) (Table 2). Level of population differentiation was also turned out to be low (Φ_{ST}= 0.0643). When AMOVA was performed with 3 populations where both male and female trees were sampled, 4.71% of the genetic diversity was allocated

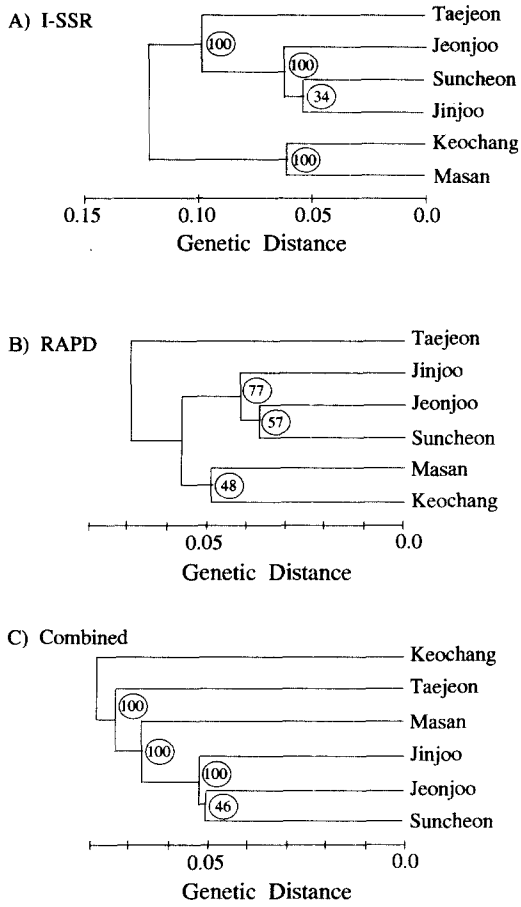

Figure 2. Phenogram constructed by the UPGMA analysis on the basis of A) I-SSR, B) RAPD, and C) combined data. The numbers in the circles to the right of relevant nodes represent the confidence intervals calculated from 100 bootstrap replicates.

between male and female groups (Table 2). Level of sexual differentiation estimated from the combined data (i.e., 294 DNA markers) was much lowered compared to that of RAPD variants (8.38%) but somewhat increased compared to that of I-SSR variants (3.8%). Different degree of fluctuation of sexual differentiation by combining I-SSR and RAPD variants might be due to buffering effect resulted from relatively large number of I-SSR markers (227) compared to RAPD markers (67) analyzed in this study. On the basis of cluster analysis, the overall patterns of grouping among 6 populations were not coincided to those based on I-SSR and RAPD variants (Figure 2C). This might be induced by different nature of RAPD and I-SSR variants (i.e., different compositions of DNA sequences for primers : arbitrary 10 nucleotides for RAPD vs. a few repeats of the simple sequences with 1 to 3 additional arbitrary nucleotides for I-SSR analyses). Such difference in the sequences of primers (i.e., representing different priming sites) raises a conjecture that the data sets obtained by RAPD and I-SSR analyses may be collected from different parts of the genome which might be undergone different evolutionary processes (ex. different evolutionary rate). This discrepancy might result in failure to satisfy the most critical assumption of constant evolutionary rate among compared units for UPGMA. This inference could be reinforced by observation that subgroups of the same sex were not clustered together (Figure not shown).

In conclusion, estimation of genetic diversity and genetic differentiation might be improved by combining data of I-SSR and RAPD variants which might cover the genome more evenly. However, scrupulous caution should be taken an account on reconstruction of genetic relationships on the basis of combined data of I-SSR and RAPD variants, which might result in incorrect topology on dendrogram on account of probable irregularity in the evolutionary rates of different DNA markers. In considering that the introduced tree species of *Ginkgo biloba* has narrow genetic base compared to the endemic tree species and that almost all of ginkgo trees in Korea were planted, sustaining efforts should be made to maintain genetic diversity and to blend genetic make-up of the local seed sources. To attain this object, local afforestation should be practiced to broaden seed sources by gathering seedlings from several suppliers in different locations.

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