Korean J. Plant Res. 25(3):308-316(2012)
http://dx.doi.org/10.7732/kjpr.2012.25.3.308

Original Research Article

Variant Identification in *Platanus occidentalis* L. Using SNP and ISSR Markers

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Abstract - The purpose of this study was to identify the variant of *Platanus occidentalis,* whose bark looks white, also can be classified as *P. occidentalis* and to examine its genetic difference from the general *P. occidentalis*. For the variant identification of *P. occidentalis,* SNP and ISSR analysis were used in this study. Thirteen samples of *P. occidentalis* white variant were collected in Cheongju and 24 samples of normal *P. occidentalis* obtained in Cheongju, Pyongtaek, Ansan, Suwon, Osan and Jincheon area. ITS 1 and ITS 2 sequences of white variants were identical with those of *P. occidentalis*. We could not find any sequence difference between normal and white *P. occidentalis*. So we concluded that the white variant belongs to normal *P. occidentalis* even their bark is white and peeled easily. By ISSR test, 98 amplicons were acquired using 10 primers. *P. occidentalis* and white *P. occidentalis* showed different band patterns from the UBC #834. According to the result of Nei (1979)'s genetic distance analysis, the members of white *P. occidentalis* were grouped more tightly than the members of normal *P. occidentalis.* The UPGMA dendrogram shows that the variant and *P. occidentalis* divided widely into two groups. These results show that the phenotype of *P. occidentalis* white variant is caused by genetic factors rather than by environmental factors.

Key words - Primer, Genetic distance, Dendrogram, Amplicon, White Platanus

Introduction

In Korea, there are 3 types of tree species that are of *Platanaceae* and, of these, *P. occidentalis* is a deciduous broadleaf tree that belongs to *Rasales*, *Platanaceae*. It is native to North America and is commonly called *Platanus* and it is often referred to as a "bell tree" for its small bell-like fruits. Because the *P. orientalis* spp. has excellent transplanting capability and greenery, it is a species that is planted often as roadside trees and, in particular, *P. orientalis* spp. is prevalently planted as roadside trees and trees for greeneries.

Trees that are planted as ornamental trees for their high horticultural value are in lot of cases planted for the aesthetic beauty of the flowers; however, they are also often used as ornamental trees for the beauty of the bark or the overall shape of the tree. Since the bark sometimes cracks or splits away as the tree grows, it can be considered to be undergoing continuous changes during the lifetime of the tree. For *P. occidentalis* whereas its cracked outer layers are relatively loosely attached to the bark and therefore are easily eliminated, the unaesthetic outer layers that are not loosely attached to the bark can degrade the aesthetics of the tree and also can serve as a haven for the sycamore lace bugs (*Corythucha ciliata*). As shown, the outer barks of most of *P. occidentalis* are not easily eliminated and stay intact, however, a *P. orientalis* spp. was discovered where - while the brown colored outer bark are all eliminated and only the white colored inner bark remains - the entire tree trunk appears as if it has been painted white, whereby attracting visual attention with a high aesthetic value (Fig. 1). For the *P. orientalis* spp. that has white bark provides a neat groomed image with its smooth tree trunk and is also easily eye-catching, it was deemed suitable to be used as a landscaping material for the locations that are for attracting traffic flow.

The sycamore lace bugs that live on *P. orientalis* spp. suck juices from the leaves and cause damages and turn leaves into dark yellow in color whereby harming the aesthetics of urban landscape (Park *et al.*, 2007). In addition, sycamore lace bugs

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Fig. 1. *P. occidentalis* with general bark (left) and White bark (right).

spend winter as adult insects and use rough tree bark as a hiding place and in April come out from the cracks in the bark and move towards the top areas, and if the bark, which act as a hiding place for them, can be eliminated, then it could be expected that the damages caused by sycamore lace bugs would be reduced as well. In order to utilize this variant species that is deemed to have horticultural values and is also resistance to bugs as a genetic resource, it would be necessary, first, to examine whether the differences are attributable to organic differences or environmental conditional differences.

SNP refers to the variations of DNA sequencing caused by the change in the single nucleotides of the two alleles, and it is useful for analyzing the relationship between the alleles and phenotypes (Jorde, 2000). Although initially it was used in the higher plants including crops and a few species, nowadays it is used in animals as well and has been garnering attention as the next generation marker (Gupta *et al.*, 2001).

The ISSR marker was first developed by Zietkewicz *et al.* (1994) during their effort to create genome map through the proliferation of DNA, and this is a way to amplify the sequencing between two SSRs. This method can be used even in cases where genetic information is deficient, as in *P. occidentalis*, and, since it uses only 1 primer, the manipulation of experiments is rather simple.

Due to the fact that it was difficult to conduct morphological identification of the variant individual bodies, which were white in external appearance as a result of the brown colored bark being stripped off, because of the variations in the leaf shapes and in the number of bell-shaped fruits, analyses of genetic information in order to accurately ascertain the species were necessary. As such, this study attempted to test - among the *P. orientalis* spp. planted as roadside trees - whether the individual bodies that had all of the brown colored outer bark eliminated and their entire tree trunks were appearing white in color were of the similar species as *P. occidentalis* by using the SNP (Single Nucleotide Polymorphism) and ISSR (Inter-Simple Sequence Repeat) molecular markers, and our study also attempted to investigate the differences of genetic characteristics between *P. occidentalis* and *P. orientalis* spp. that has white bark.

Materials and Methods

Materials

P. orientalis spp. (hereinafter referred to as "white platanus"), which appear to be variant individual bodies with white bark, and typical *P. occidentalis* were used in the testing (Fig. 1). As shown in Table 1, 13 test samples of white platanus with all of their brown colored outer layers eliminated were collected in the City of Cheongju and a total of 24 test samples of typical *P. occidentalis* were collected in the Cities of Cheongju, Pyeongtaek, Ansan, Suwon, Osan and Jincheon. After collection, the test samples were packaged to prevent any mix-ups and were kept in a freezer at -20℃.

Extraction of Genomic DNA

In order to extract the DNA of white platanus and *P. occidentalis*, test samples were acquired from the 37 individual bodies and the leaves of about 2-3 g that were stored in a freezer were triturated by using liquid nitrogen, and then through the CTAB method, the DNA was isolated.

Selection of Primer

SNP PCR

Because the genetic information of *P. occidentalis* was deficient, the primers that were used for other species were applied. The primer for the division of *P. occidentalis* (Cemmamo and Cafasso, 2002) and the primer sets for amplifying non-coding portions were used (Table 2).

Table 1. Locality of *P. occidentalis*

Symbol	Locality	Symbol	Locality
W1	112 Saun-ro Heungdeok-gu Cheongju	P1	15 Hyanggun-ro Sangdang-gu Cheongju
W2	120 Saun-ro Heungdeok-gu Cheongju	P2	163 Sangdang-ro Sangdang-gu Cheongju
W3	32 Daewon-ro Heungdeok-gu Cheongju	P3	155 Sangdang-ro Sangdang-gu Cheongju
W4	45 Daewon-ro Heungdeok-gu Cheongju	P4	32 Daewon-ro Heungdeok-gu Cheongju
W5	50 Daewon-ro Heungdeok-gu Cheongju	P5	79 Sajikdae-ro Heungdeok-gu Cheongju
W6	37 Daewon-ro Heungdeok-gu Cheongju	P6	393-1 Seongbong-ro Heungdeok-gu Cheongju
W7	47 Daewon-ro Heungdeok-gu Cheongju	P7,8,9	43 Seonghodae-ro Ohsan-si
W8	19 Daewon-ro Heungdeok-gu Cheongju	P10,11,12	13 Geomeundeul 1-ro Seobuk-gu Cheonan-si
W9	17 Daewon-ro Heungdeok-gu Cheongju	P13,14,15	2 Songwha 2-ro Paengseong-eup Pyengtaek-si
W10,11	13 Daewon-ro Heungdeok-gu Cheongju	P16,17,18	1232 Nongdari-ro Jincheon-gun
W12	79 Sajikdae-ro Heungdeok-gu Cheongju	P19	14 Cheonnam-ro Danwon-gu Ansan-si
W13	293-1 Seongbong-ro Heungdeok-gu Cheongju	P20	8 Jeongjo 776-ro Paldal-gu Suwon-si
		P21,22,23,24.	52 Naesudong-ro Heungdeok-gu Cheongju

W: White bark, P: General bark.

ISSR PCR

Among the extracted DNA samples, 4 samples were randomly selected and conducted PCR analyses against the UBC (University of British Columbia) 800 quadrant 100 primers. After electrophoresis, by comparing the amplification patterns of amplified fragments, 10 samples that had excellent reproducibility and displayed polymorphism and, of which the amplification products are clearly distinguishable, were selected, and PCR analyses were conducted against the entire 37 individual bodies (Table 3).

PCR Conditions and Electrophoresis

The PCR reaction mixture was comprised - by using AccuPower® PCR PreMix from Bioneer - of 10 ng template DNA, 0.6 μM ISSR Primer, 1.5 mM $MgCl_2$, 250 μM dNTP Mix, 10 mM Tris-HCl (PH 9.0), 1 U Tag DNA Polymerase and 30 mM KCl for every 20 μl of reaction mixture, and, regarding the SNP PCR conditions, after a 5 minute pretreatment at 94℃ and after repeating 30 times of 30 seconds at 94℃ 1 minute at 55℃ and 45 seconds at 72℃, amplification reaction was amplified for the final time for 7 minutes at 72℃.

Regarding the ISSR PCR conditions, after a 5 minute pretreatment at 94℃ and after repeating 45 times of 30 seconds at 94℃ 30 seconds at 50℃ or 30 seconds at 55℃ and 60 seconds at 72℃, amplification reaction was amplified for the final time for 10 minutes at 72℃. By using a 1% agarose gel and by performing electrophoresis for 90 minutes at 60 V, the band patterns were analyzed at the UV trance illuminator level and photographed.

Sequence Analysis

SNP Analysis

For an analysis of base sequencing 10 μl of the leftover after the electrophoresis - among the amplified fragments obtained after PCR - was put through a purification processing by using the GeneAll Expin PCR SV protocol. The purified PCR amplified fragments were diluted into a third level purified water and were analyzed using an ABI RPISM® 3730 XL analyzer. The base sequencing analyzed through a sequencing analysis software was obtained in text documents and *.abl format documents. The obtained base sequencing was classified by primers, and the *.abl files were loaded using the Clustal X 1.83 software and by arranging in an array the SNP was confirmed.

ISSR Analysis

Regarding the amplified fragments, the data for two types of phenotypes (Appearance: 1, No appearance: 0) was processed, based on the presence of bands, via a binary scale. By

Table 2. Primer for SNP markers and sequences

No.	primer sequence	Reference
1	F: CAT TAC AAA TGC GAT GCT CT R: TCT ACC GAT TTC GCC ATA TC	Taverlet *et al*.,1991.
2	F: CAT TAC AAA TGC GAT GCT CT R: GGG GAT AGA GGG ACT TGA AC	
3	F: CAT TAC AAA TGC GAT GCT CT R: ATT TGA ACT GGT GAC ACG AC	
4	F: CGA AAT CGG TAG ACG CTA CG R: TCT ACC GAT TTC GCC ATA TC	
5	F: CGA AAT CGG TAG ACG CTA CG R: GGG GAT AGA GGG ACT TGA AC	
6	F: CGA AAT CGG TAG ACG CTA CG R: ATT TGA ACT GGT GAC ACG AC	
7	F: ATT TGA ACT GGT GAC ACG AC R: GGG GAT AGA GGG ACT TGA AC	
8	F: GGT TCA AGT CCC TCT ATC CC R: ATT TGA ACT GGT GAC ACG AC	
9	F: AAG CGG AAT TTG TGC TTG T R: TAG ACA TCG GTA CTC CAG TGC	Lee *et al*., 1994.
10	F: AAC ATT CCC ACC AAG CCT AAT C R: ATG AGA ACG ACA CAA CTG GCA A	
11	F: TCC GTA GGT GAA CCT GCG C R: TCC TCC GCT TAT TGA TAT GC	Kaundun and Matsumoto, 2003
12	F: CCT TGG GGT TAT CCT GCA CT R: ACT GCA ATT TTA GAG AGA CGC G	White *et al*.,1990.
13	F: GGA GAA GTC GTA ACA AGG TTT CCG R: ATC CTG CAA TTC ACA CCA AGT ATC G	Cemmamo and Cafasso, 2002.
14	F: ATC CTT TAC TCA GTG AAT GAG R: GCT TTA GTC TCT GTT TGT GG	Shinozaki *et al*.,1986.

Table 3. List of ISSR primer codes and sequence employed for this analysis

Primer	Sequence (5'→3')
UBC 811	GAG AGA GAG AGA GAG AC
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 835	AGA GAG AGA GAG AGA GTC
UBC 844	CTC TCT CTC TCT CTC TRC
UBC 846	CAC ACA CAC ACA CAC ART
UBC 855	ACA CAC ACA CAC ACA CYT
UBC 856	ACA CAC ACA CAC ACA CYA
UBC 879	CTT CAC TTC ACT TCA
UBC 881	GGG TGG GGT GGG GTG
UBC 897	CCG ACT CGA GNN NNN NAT GTG G

using the POPGENE program, the genetic distance of each individual body was calculated through the calculation method of Nei & Li (1979), and the phylogenetic relationships among the populations were prepared in a dendrogram by using the UPGMA (Unweighted Pair-Group Method using Arithmetic Average) method, and the genetic similarities of each of the individual bodies were examined.

Results and Discussion

In order to ascertain through molecular markers what species the variant individual body that appears white in color with the outer bark peeled off was and in order to investigate the differences of genetic characteristics between typical *P. occidentalis* and this variant individual body, 24 individual bodies of typical *P. occidentalis* and 13 individual bodies of white platanus that had all of their brown colored outer bark eliminated were used as test samples.

SNP Analysis

In order to verify the species of the variant individual bodies in which the outer bark layers were all eliminated, SNS analysis was conducted. Among the 14 primer sets (Table 2) used in SNP analysis, bands were ascertainable in the primers developed by Cemmamo and Cafasso (2002) for the amplification of the ITS region, and it was possible to obtain sequencing by purifying the area.

As a result of analysis, as shown in Table 4, both the *P. occidentalis* and white platanus were seen as being consistent in terms of Adenine, A, in the ITS 1 region, and in the ITS 2 region as well both the *P. occidentalis* and white platanus were seen as being consistent in terms of Cytosine, C, whereby making it possible to ascertain that the variant individual bodies in which the outer bark layers are all eliminated and are white in color were also *P. occidentalis*.

In addition, although sequencing was obtained through the amplification of the non-coding areas of chloroplast DNA of *P. occidentalis*, it was not possible to find the markers that are tied together in common in accordance with the tree bark (Fig. 2).

ISSR Analysis

In order to test the genetic differences between *P. occidentalis* and the variant individual bodies, an ISSR analysis was carried out. As shown in Table 5, as a result of ISSR analysis by using the UBC 800 quadrant primers, it was possible to verify the presence of bands where polymorphism appears clearly in 10 primers, including UBC 811, 834, 835, 844, 846, 856, 779, 881, 897, etc., and in 500~2,100 bp, a total of 98 amplification products were obtained. Of these 98 amplification products, 7 bands that accounted for about 7.1% were all seen in common in all individual bodies, and the total number of bands that were polymorphistic and showed differences in terms of DNA arrays between individual bodies was seen to be 91.

In particular, in the UBC 834 primer, a tendency of separation between the population of typical *P. occidentalis* and the population of white platanus that had outer layers eliminated was seen. In the case of UBC 834 primer, there were a total of 10 bands that appeared as a result of amplification in 550,

Table 4. ITS 1 and 2 sequence of analyzed *Platanus* species

ITS 1 sequence	Result of Cemmamo & Cafasso	*P. orientalis*	GGTCG**T**GTGGGCTGTCG
		P. occidentalis	GGTCG**A**GTGGGCTGTCG
		P. acerifolia	GGTCG**G**GTGGGCTGTCG
	Result of Experiment	common platanus	GGTCG**A**GTGGGCTGTCG
		white platanus	GGTCG**A**GTGGGCTGTCG
ITS 2 sequence	Result of Cemmamo & Cafasso	*P. orientalis*	GGCGGCAGC**A**TCGCGAC
		P. occidentalis	GGCGGCAGC**C**TCGCGAC
		P. acerifolia	GGCGGCAGC**M**TCGCGAC
	Result of Experiment	common platanus	GGCGGCAGC**C**TCGCGAC
		white platanus	GGCGGCAGC**C**TCGCGAC

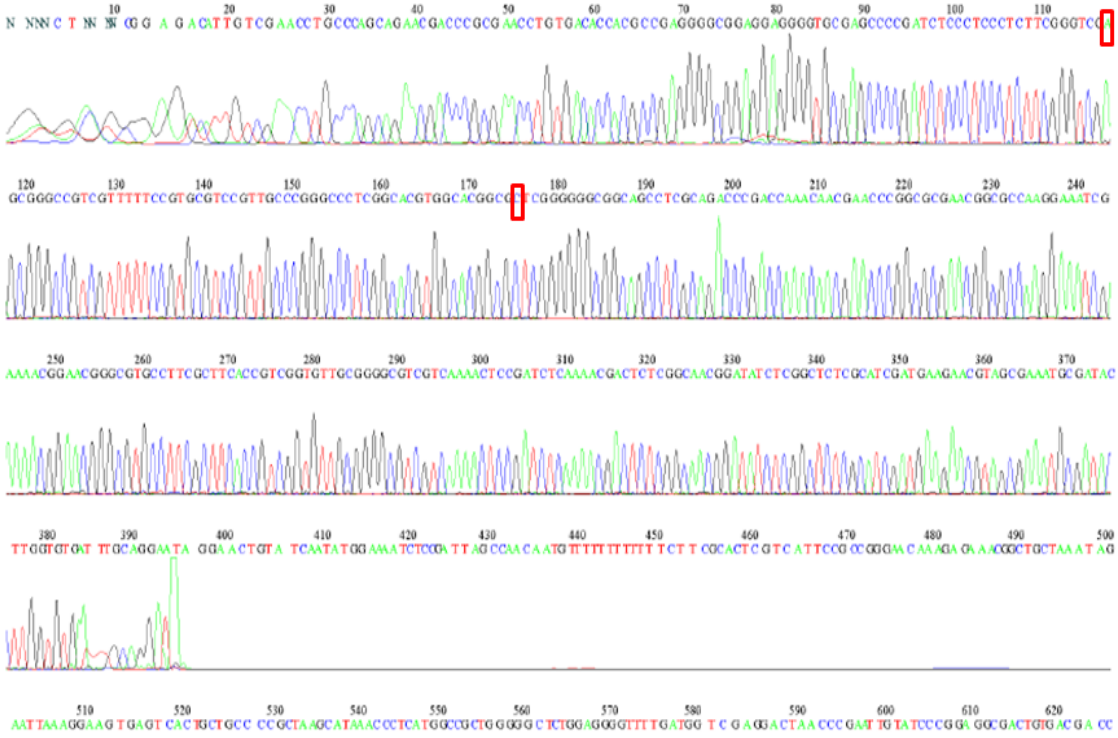

Fig. 2. Automatic sequencing profile of the *P. occidentalis* Variant (W5).

Table 5. List of 10 ISSR primers and the result for *P. occidentalis*

Primer	Total no. of bands	No. of polymorphic bands	Fragment size range (bp)
UBC 811	8	6	500-1200
UBC 834	10	9	500-1500
UBC 835	10	10	500-1500
UBC 844	10	9	500-1300
UBC 846	9	9	500-1200
UBC 855	10	8	500-1300
UBC 856	10	10	500-1400
UBC 879	9	9	500-2100
UBC 881	13	12	500-2000
UBC 897	9	9	500-1200
Total	98	91	500-2100

700, 750, 800, 850, 900, 1000, 1100, 1300 and 1500 bp, and, of these, common bands were seen in 550 bp, and there were 9 bands that were polymorphistic. Although there might be differences in terms of resolution, as compared to the typical *P. occidentalis*, the white individual bodies did not show bands in 850, 900, 1000, 1100, 1300 and 1500 bp, whereby displaying a tendency in which the typical *P. occidentalis* and white platanus separated (Fig. 3).

As a result of obtaining -based on the total of 98 amplified fragments obtained from 10 primers- a dissimilarity index matrix through the genetic distance of Nei (1972), the individual bodies of the white platanus population were seen to be genetically closer than the individual bodies of the *P. occidentalis* population.

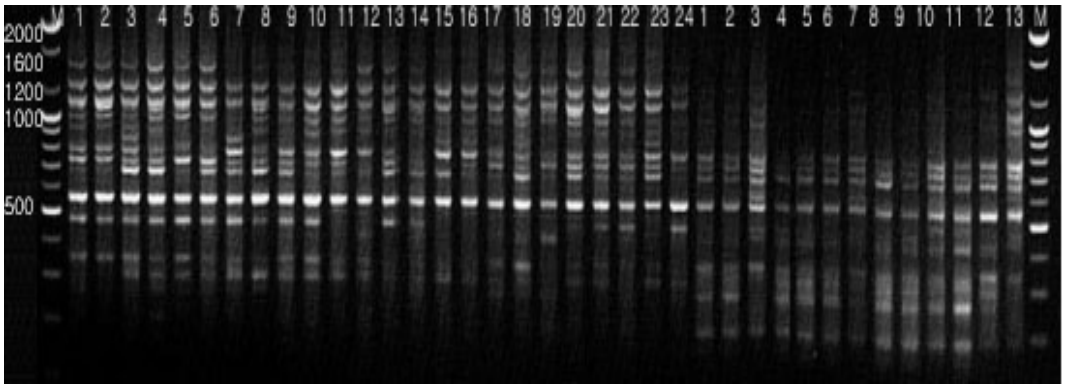

Fig. 3. ISSR bands amplified by the primer UBC 834.

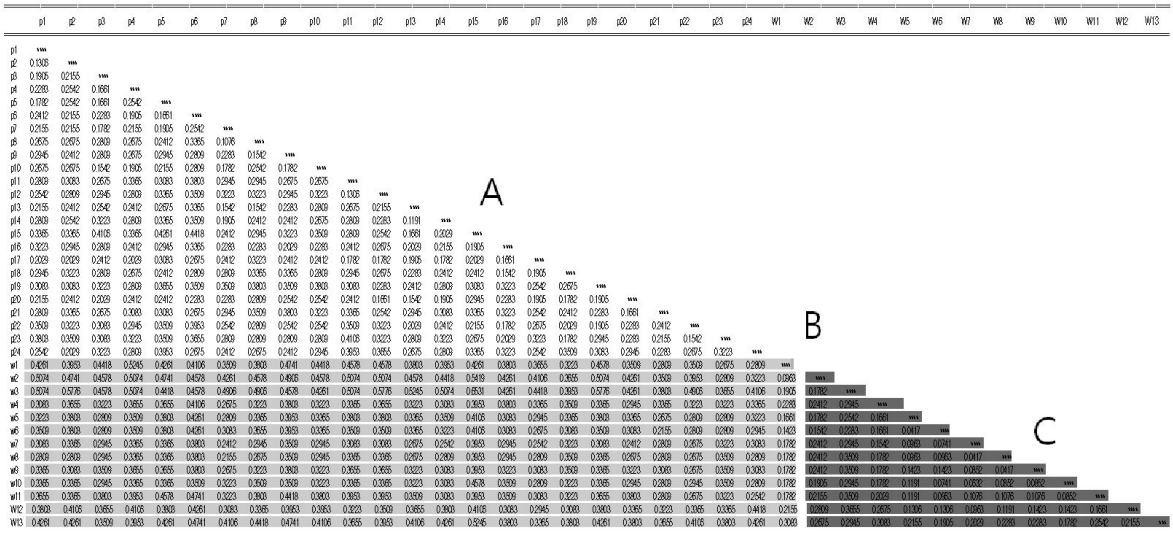

Fig. 4. UPGMA dendrogram showing the relationships among 37 population, based on data of genetic distance obtained by ISSR.

In regard to the genetic distance, the distance between white individual bodies W7 and W8 showed the closest genetic relationship with 0.041, and the P19 (collected in Ansan) of the typical *P. occidentalis* and white individual body W3 were seen to be the farthest with 0.577. In a comparison in terms of inter populations, the average genetic distance of the white platanus individual body population was seen to be 0.181 (Part C of Fig. 4), the typical *P. occidentalis* individual body population was 0.259 on average (Part A of Fig. 4), and the average genetic distance between the individual bodies of the white platanus and typical *P. occidentalis* was seen to be 0.364 (Part B of Fig. 4). As such, the individual bodies of the white platanus population were genetically closer than the individual bodies of the typical *P. occidentalis* population, and the genetic distance between the typical *P. occidentalis* and the white platanus was found to be relatively distant.

In addition, as a result of conducting a cluster analysis using the genetic distance in accordance with the UPGMA method as shown in Fig. 5, the white platanus individual bodies were largely clustered together, whereby becoming fractionized as an independent binary population. As such, the white platanus was deemed to have a genetic uniqueness, and it was deemed to be determined by genetic factors rather than the environment, regardless of the collection areas.

In conclusion, because variations were seen in the leaf shapes and in the number of bell-shaped fruits of the white platanus, which seems to be a variant of *P. occidentalis*, assessments based on the types were difficult to be carried out; therefore, white platanus was identified as *P. occidentalis* through the results of conducting SNP analyses.

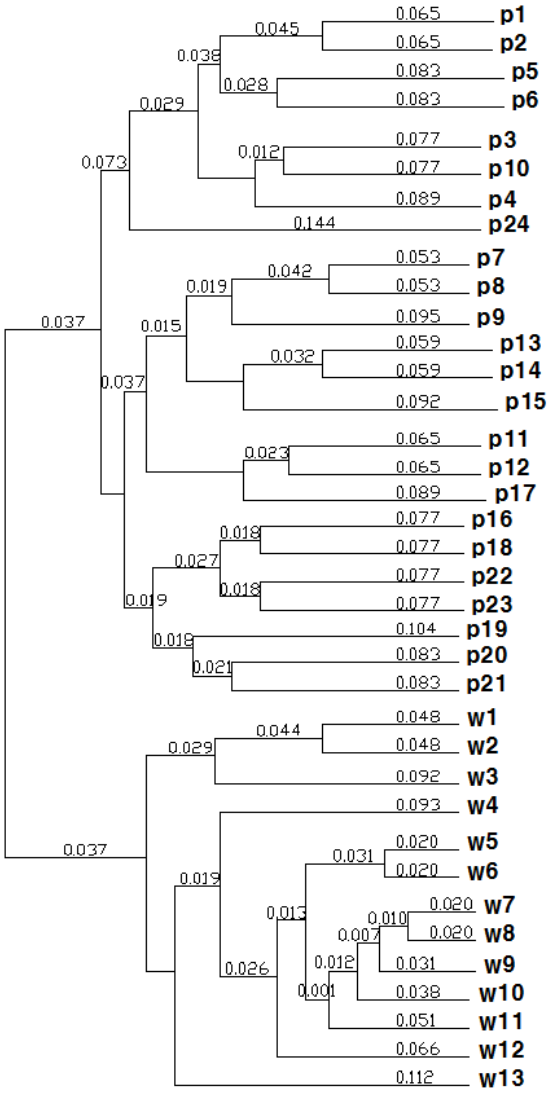

Fig. 5. UPGMA dendrogram showing the relationships among 37 population, based on data of genetic distance obtained by ISSR.

White platanus, by displaying a tendency of separating from the typical *P. occidentalis* in the UBC 834 through ISSR analysis, was found to show differences in terms of genetics as compared to the typical *P. occidentalis*. In addition, as a result of a cluster analysis using the genetic distance, based on the fact that the clustering of white individual bodies was observed, *P. occidentalis* that has white bark has a genetic uniqueness and this was confirmed as being attributable to the genetic factors rather than the environment.

"Gene center theory" of Vavilov (1926) is that there are more recessive individual bodies in occur places farther away. Thus, white platanus is the same species with *P. occidentalis* but it is estimated to be propagated that individual bodies expressed recessive gene are propagated by unsexual propagation.

Acknowledgements

This work was supported by the research grant of the Chungbuk National University in 2010.

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(Received 23 May 2012 ; Revised 21 June 2012 ; Accepted 24 June 2012)