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Random Amplified Polymorphic DNA Analysis of Genetic Relationships Among *Acanthopanax* Species

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Random amplified polymorphic DNA (RAPD) analysis was used to determine the genetic relationships among seventeen species of the *Acanthopanax* species. The DNA isolated from the leaves of the samples was used as template in polymerase chain reaction (PCR) with twenty random decamer primers in order to distinguish plant subspecies at the level of their genomes. The RAPD patterns were compared by calculating pairwise distances using Dice similarity index, and produced to the genetic similarity dendrogram by unweighted pair-group method arithmetic averaged (UPGMA) analysis, showing three groups; a major cluster(twelve species), minor cluster (4 species) and single-clustering species. The results of RAPD were compatible with the morphological classification, as well as the chemotaxonomic classification of the *Acanthopanax* species. The *Acanthopanax* species containing 3,4-*seco*-lupane type triterpene compounds in their leaves corresponded to the major cluster, another species having oleanane or normal lupane type constituents to minor clusters, and one species not containing triterpenoidal compound to single-cluster.

Key words: *Acanthopanax* species, RAPD, UPGMA, Genetic similarity, Morphological classification, Chemotaxonomy

INTRODUCTION

The genus *Acanthopanax* belonging to the family Araliaceae consists of about 30 species in the world, distributing in East and South Asia (Ohwi, 1972). The root and stem barks of these species have been employed as a tonic and prophylactic in oriental herbal medication as well as Ginseng, a root of *Panax ginseng* (Araliaceae).

Various kinds of the *Acanthopanax* species have been used as the herbal drugs of same purpose. Especially, two *Acanthopanax* species, *A. gracilistylus* and *A. senticosus*, have been mainly used as medicinal plants in China (Namba, 1994). In Korea, *A. sessiliflorus* and its relative species have been used as herbal drugs for a long time, and *A. senticosus* forma *inermis* and *A. divaricatus* var. *albeofructus* have been cultivated recently for medicinal use (Yook, 1997).

In recent years, DNA marker techniques based on PCR amplification have become increasingly important at the study of genetic relationships among plants. For the taxonomic studies of the inter- and intra-specific level, the amplification of anonymous fragments from genomic DNA templates using arbitrary primers (random amplification polymorphic DNA analysis, RAPD) has become a popular method (Dettori *et al.*, 2000; Paul *et al.*, 1997) And RAPD techniques provide a fast and easy approach to the problem of plant genetic variation.

Thus, the RAPD analysis can be a useful tool to identify pharmaceutical drug plants as well as for morphological and microscopic analysis (Shaw *et al.*, 1995; Hosokawa *et al.*, 2000).

Though many kinds of health food made by *Acanthopanax* species are taken popularly in Korea, there are few products conformed their origin of the crude material. And, *Acanthopanax* species, included many varieties and forma, would be classified into deferent groups based on only their phenotypic characteristics. Such kind of classification is common in the botanical literature despite the lack of a scientific basis.

We, therefore, evaluated the use of RAPD analysis

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for the identification of *Acanthopanax* plants, and their genetic relationships. It is able to provide a convenient and rapid assessment of differences in the genetic composition of related individuals. Furthermore, our previous phytochemical studies on the *Acanthopanax* species were compared with the results of the RAPD analysis.

MATERIALS AND METHODS

Plant materials

Seventeen species of the genus *Acanthopanax,* including 10 typical species, four forma and three varieties, were collected from several habitats in Korea and the Medicinal Plant Garden, Faculty of Pharmaceutical Sciences, in Kumamoto University, Japan, and were identified by Prof. C.S. Yook, College of Pharmacy in Kyung-Hee University, Korea. The plant materials were used for the analysis and the rest of the voucher specimens have been deposited at the Herbarium of the Collage of Pharmacy in Kyung-Hee University (Table I).

DNA Isolation

Genomic plant DNA was isolated from the leaves using the Dneasy Plant Mini Kit (QIAGEN Ltd.) according to the provided manual. The prepared DNA was dissolved in an appropriate volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration and purity were determined spectrophotometrically, and adjusted to approx. 20 ng/μL.

Amplification of fragments

The polymerase chain reaction (PCR) was performed in 25 μL reaction mixture which is contained 10 mM Tris-HCl (pH 8.5), 200 μM dNTPs, 1.5 μM $MgCl_2$, two kinds of 1.5 μM primers, 1.25U AmpliTaq DNA polymerase and 20 ng of template DNA. The nucleotide sequences of 10 decamer primers (Operon technologies, Alameda CA) are shown in Table II. Amplification was performed in a Santhermo PCR, Model WS-145 (SankoJunyaku Co., Ltd.). The PCR protocol was as follows: initial denature at 94°C for 2 min, 40 cycles consisting of 40°C for 30 sec, 72°C for 60 sec, and 94°C for 30 sec, and final extension at 72°C for 5 min. Amplification products were separated in 1.0% agarose/TBE gels in the presence of ethidium bromide, and photographed under UV light. 100-bp DNA ladder marker and λ/*Hind III* were used as molecular weight size marker.

Analysis of RAPD

Twenty random primers of the OPA-series were used to amplify some target DNA in preliminary trials. Only ten primers showing clear polymorphic patterns in these trials were selected to amplify the DNA from the plant materials (Table II). In this PCR analysis, random primers were employed as pairs, that is, OPA2+7, OPA3+9, OPA8+11, OPA13+14, and OPA10+18. Polymorphic bands on agarose gels were scored as present (1) or absent (0) and the fragment readings were entered in a computer file as a binary matrix.

A pairwise similarity matrix, as recoded in Table III, was constructed by using the Dice similarity index $SD=2N_{ab}/$

Table I. The species and source of plant materials used for the analysis

No	Plants species	Source
1	*Acanthopanax japonicus* Franch. et Savat	Medicinal Plant Garden, Kumamoto University
2	*Acanthopanax sieboldianus* Makino	Gyengsangnam-do (Mt. Chiisan), Korea
3	*Acanthopanax koreanum* Nakai	Jezu-do (Mt. Hanrasan), Korea
4	*Acanthopanax senticosus* Harms	DMZ (Mt. Dae-Am san), Korea
5	Acanthopanax sciadophyloides Franch. et Savat	Oita (Mt. Kuzyu), Japan
6	*Acanthopanax chiisanensis* Nakai	Gyengsangnam-do (Mt. Chiisan), Korea.
7	*Acanthopanax divaricatus* var. *albeofructus* Yook	Kyungki-do (Kwangrung), Korea
8	*Acanthopanax senticosus* forma *inermis* Harms	Kyungki-do (Kwangrung), Korea
9	*Acanthopanax sessiliflorus* forma *chungbuensis* Yook	Kyungki-do (Mt. Chunmasan), Korea
10	*Acanthopanax divaricatus* forma *nambonenesis* Yook	Gyengsangnam-do (Mt. Chiisan), Korea
11	*Acanthopanax divaricatus* var. *distigamtus* Yook	Kyungki-do (Mt. Papyungsan), Korea
12	Acanthopanax sessiliflorus Seemenn	Kyungki-do (Mt. Kwangduksan), Korea
13	*Acanthopanax sessiliflorus* var. *tristigmatus* Yook	Kyungki-do (Mt. Papyungsan), Korea
14	*Acanthopanax divaricatus* forma *flavi-flos* Yook	Chungchungbuk-do (Mt. Susinsan), Korea
15	*Acanthopanax divaricatus* Seemenn	Medicnal Plant Garden, Kumamoto University
16	*Acanthopanax pedunculus* D.Han et C.S. Yook	Gyengsangnam-do (Mt. Chiisan), Korea
17	*Acanthpanax seoulense* Nakai	Kyungki-do (Mt. Kwangduksan), Korea

Table II. List of decamer oligonucleotide primers employed for the RAPD analysis

Primer Code	Sequence (5' to 3')	Primer Code	Sequence (5' to 3')
OPA-2	TGCCGAGCTG	OPA-10	GTGATCGCAG
OPA-3	AGTCAGCCAC	OPA-11	CAATCGCCGT
OPA-7	GAAACGGGTG	OPA-13	CAGCACCCAC
OPA-8	GTGACGTAGG	OPA-14	TCTGTGCTGG
OPA-9	GGGTAACGCC	OPA-18	AGGTGACCGT

(N_a+N_b), where N_{ab} is the number of shared bands between a pair of genotypes 'a' and 'b;, N_a the number of scored bands in genotype 'a' and N_b the number of scored bands in genotype 'b' (Sneath *et al.*, 1973). Similarity estimates were analyzed by the unweighted pair-group method arithmetic averaged (UPGMA), expressing the genetic similarity dendrogram.

RESULTS AND DISCUSSION

To evaluate the genetic relationships, ten primers producing polymorphic bands strong and clear enough for the analysis were selected from 20 random primers for fingerprinting the samples of the genus *Acanthopanax*. A total of 21 informative polymorphic fragments were obtained from 17 DNA samples of the 10 primers (Fig. 1). The relationships of the samples were analyzed using Dice similarity index finally to produce the genetic similarity dendrogram of the 17 species through the cluster analysis (UPGMA), separating into three groups; major cluster, a minor cluster, and a single cluster in the dendrogram (Fig. 2).

The major cluster consisted of 12 accessions (No. 9, 17, 8, 6, 15, 10, 7, 11, 12, 13, 16, and 14), showing the close genetic relationships in this cluster (high similarity values, at least 0.706 in the Dice index). The species in this group is quite different from the other species on the RAPD analysis results. (Low similarity values at most 0.365) Especially, the similarity values calculated between the ten accessions (No. 9, 17, 8, 6, 15, 10, 7, 11, 12, and 13) in the major cluster were particularly high, ranging from 0.842 to 1.000. *A. divaricatus* forma *flavi-flos* and *A. pedunclus* were divided at early phase relatively in the major cluster, the clustering levels 78.9% and 81.3% on the dendrogram, respectively, implying that they are somewhat related to but reasonably classified from the other ten accessions in the major cluster.

The minor cluster (No. 2, 4, 1, and 3) is composed of two sub-clusters divided by 43.4% of the clustering level on the genetic dendrogram. The similarity index between *A. japonicus* and *A. koreanum* was 0.800 in the first sub-cluster, indicating they are a relatively close. The similarity index between *A. senticosus* and *A. sieboldianus* was 0.600 in the second sub-cluster.

All the similarity values of *Acanthopanax scidophyloides* with the other species were very low, ranging from 0.125 to 0.400, thereby forming a single cluster in the genetic

Table III. Similarity indices for the pairwise comparison among the *Acanthopanax* species for the ten primers

No[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	-																
2	0.769	-															
3	0.333	0.462	-														
4	0.167	0.500	0.462	-													
5	0.429	0.286	0.267	0.143	-												
6	0.235	0.471	0.111	0.471	0.421	-											
7	0.333	0.333	0.211	0.444	0.400	0.870	-										
8	0.235	0.353	0.111	0.471	0.421	0.909	0.957	-									
9	0.333	0.444	0.211	0.444	0.400	0.870	0.917	0.870	-								
10	0.316	0.421	0.200	0.421	0.381	0.917	0.960	0.917	0.960	-							
11	0.316	0.421	0.200	0.421	0.381	0.917	0.960	0.917	0.960	1.000	-						
12	0.353	0.353	0.222	0.471	0.316	0.818	0.870	0.818	0.870	0.917	0.917	-					
13	0.333	0.444	0.211	0.444	0.300	0.870	0.917	0.870	0.917	0.960	0.960	0.957	-				
14	0.143	0.286	0.133	0.571	0.250	0.737	0.800	0.842	0.800	0.762	0.762	0.632	0.700	-	-		
15	0.25	0.375	0.118	0.500	0.444	0.857	0.909	0.952	0.909	0.870	0.870	0.762	0.818	0.889	-		
16	0.286	0.143	0.267	0.571	0.125	0.632	0.800	0.737	0.700	0.762	0.762	0.842	0.800	0.750	0.667	-	
17	0.333	0.444	0.211	0.444	0.400	0.870	0.917	0.870	1.000	0.960	0.960	0.870	0.917	0.800	0.909	0.700	-

[a] The numbers are the *Acanthopanax* species No. listed in Table I.

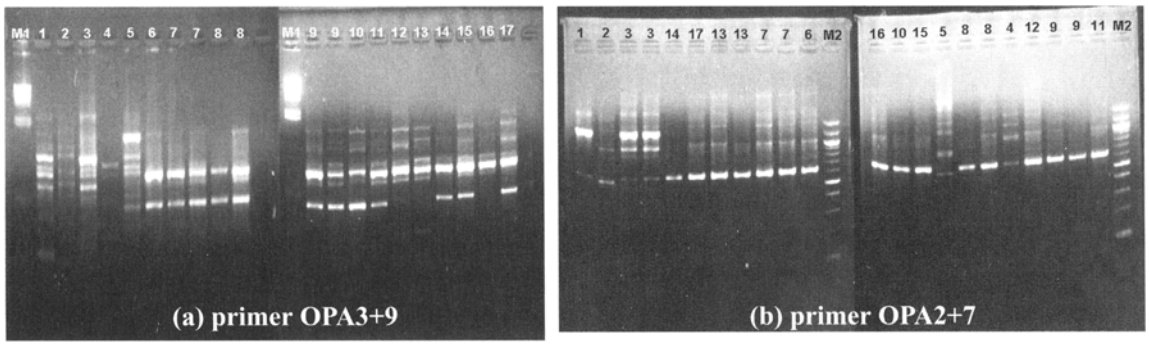

Fig. 1. RAPD fragments obtained by the primers OPA3+OPA9 (a) and OPA2+OPA7 (b). Lane number 1~18 corresponds to the code number in Table I. Lines M1 and M2, show the marker fragments, *λ/hind III* and 100-bp DNA ladder marker, respectively.

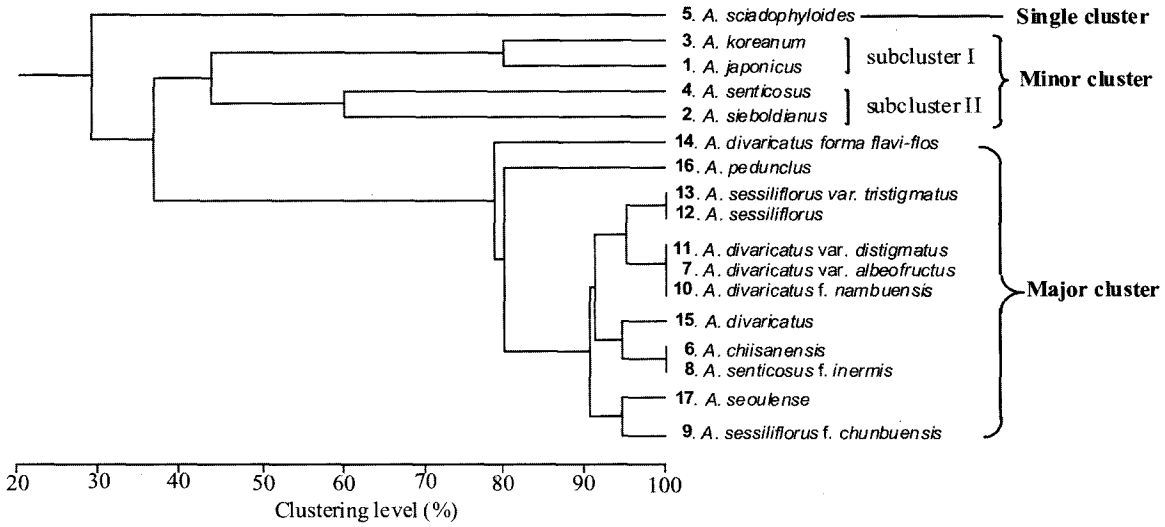

Fig. 2. Unweighted pair-group arithmetic average clustering (UPGMA) phenogram generated for the Acanthopanax species using the Dice similarity metrics.

dendrogram. Its low similarity index was consistent with the morphological difference with the other accessions (Ohwi, 1972).

From the previous studies on the phytochemical constituents of *Acanthopanax* genus, this genus was classified into four groups according to the type of triterpene containing in their leaves, this is 3,4-*seco*-lupane, oleanane, normal lupane triterpenoid and no triterpene components (Miyakoshi *et al.*, 1999; Park *et al.*, 2000; Kasei *et al.*, 1986; Shirasuna *et al.*, 1997; Oh *et al.*, 2000; Sawada *et al.*, 1993; Shao *et al.*, 1988; Shao *et al.*, 1989; Chang *et al.*, 1998; Yook *et al.*, 1998; Chang *et al.*, 1999; Kitajima *et al*, 1989).

This chemotaxomomic classification is compatible with the results of the PCR analysis. The first group corresponds to the major cluster, the second and third groups to the minor cluster, and the fourth group to the single-cluster species on the genetic dendrogram.

Consequently, the distributions of the 17 species in the dendrogram were almost consistent with the classical classification, especially with our morphological observation. High similarity was designated within the major cluster of 12 accessions for the DNA banding patterns, as well as morphology. Especially, the ten accessions of them might be reclassified as one species because of their high genetic similarity. The chemotaxonomic classification had also shown good agreement with the results of the RAPD analysis, as well as the morphological classification. Additionally, *A. senticosus* forma *inermis* seems to be reclassified as the forma or variety of *A. divaricarus* or *A. chiisanensis,* but not *A. senticosus* in view of the genetic, morphological and phytochemical similarity.

The results obtained in this study allow us to verify and complement the classification of the *Acanthopanax* species, although the relevance of this result has to be investigated further.

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