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Genetic Relationships and Phylogeny of the *Asplenium antiquum* Makino (Aspleniaceae) and its relative species based on RAPD Analysis

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ABSTRACT

This study characterized the genetic variations of 13 populations of *Asplenium antiquum* and its relative species using randomly amplified polymorphic DNA (RAPD) markers. A total 88 scorable RAPD bands were generated by the 12 random oligo primers and were analyzed by Nei and Li's genetic distance. High genetic variability was detected between *A. antiquum* and *A. nidus*, with the range from 0.568 to 0.682. And slightly low genetic variations showed within the populations of same species. Seven populations of *A. antiquum* showed slight differences (0.000-0.216), and five populations of *A. nidus* showed similar low genetic variations (0.114 to 0.171). Two individuals from Sup-seom Island which are growing in might be the regenerated one from abroad. *A. antiquum* were clustered as two groups (Group I, Group II) by UPGMA phenogram. And five populations of *A. nidus* were clustered as two groups correlated with geographical distribution. The RAPD data was very useful to define the genetic variations and to discuss the phylogenetic relationships among *A. antiquum* and the related species..

Key Words : *Asplenium antiquum*, RAPD, UPGMA, NJ, relationships, phylogeny

INTRODUCTION

The genus *Asplenium* (Aspleniaceae) comprises approximately 700 species ferns distributed widely in the nothern hemisphere. Three genera, *Asplenium*, *Ceterach*, and *Camptosorus*, have been recognized within the Aspleniaceae (Melchior, 1964). The nine sections, *Euasplenium, Sarelii, Oligophlebium, Hymenasplenium, Phyllites, Boniniella, Neottopteris, Tarachia*, and *Platyneuron*, of the largest genus *Asplenium* have been recorded by morphological characters (Momose, 1962). *A. antiquum, A. nidus* and some related species which have been included in *Neottopteris* are distributed in the northwestern Pacific areas including Far Eastern Asia (Momose, 1962). The genus *Asplenium* had recorded and examined by Linne (1735, 1753), Makino (1969), Hayata (1927), Nakai (1933), and etc. In the present, Mitui *et al.* (1989) discussed the phylogeny on the Japanese 8 species with cytological data. Also Kato *et al.* (1990) tried to define the systematic status of sect. *Hymenasplenium* based on the vascular system, raphides, and chromosomal numbers. And Murakami and Hatanaka (1988)

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Table 1. Materials and collection data used in this study.

Taxa	Localities	Collection date
Asplenium antiquum	456 Bomok-dong, Seogwipo-si, Jeju-do (1)	July 26, 1997
Makino	557-8 Bomok-dong, Seogwipo-si, Jeju-do (2)	July 26, 1997
	*clone 1, Seopseom Isl., Seogwipo-si, Jeju-do (3)	July 26, 1997
	*clone 2, Seopseom Isl., Seogwipo-si, Jeju-do (4)	July 26, 1997
	Jeju Orchid garden, Seogwipo-si, Jeju-do (5)	July 25, 1997
	Wulai, Taiwan (6)	Aug. 15, 1997
	Yakushima, Kagoshima, Japan (7)	Sept. 9, 1997
Asplenium nidus	Yeomiji Botanic Garden, Jungmoon, Jeju-do (1)	July 25, 1997
Linne	Wulai, Taiwan (2)	Aug. 15, 1997
	Taipei University, Taipei, Taiwan (3)	Aug. 16, 1997
	Yakushima, Kagoshima, Japan (4)	Sept. 9, 1997
	Shinjuku-gyoen Botanical Garden (5)	Dec. 2 1997
Asplenium australasicum	*Okinawa, Japan	Dec. 8, 1997
(J. Sm) Hooker		

* Natural Monument of Korea and Japan

performed the chemotaxonomic study on Aspleniaceae, and Watano and Iwatsuki (1988) classified Japanese *Asplenium* species into four biotypes by reproductive types and genetic variations based on isozyme analysis.

A. antiquum had been recorded firstly as a new species with Japanese material (Makino, 1929), even Mori (1921) recorded the Korean one as *A. nidus* before. And Chung (1955), Lee (1979), and Lee(1995) had recorded its distribution in Korea. Especially *A. antiquum* in Sup-seom Island, the sole habitat in Korea, had been determined as a National Monument No. 18 in 1962 because that this area was confirmed as the northest natural distribution and rarity. But, after that time, the natural habitat had been destroyed on and on by artificial interference and most of the individuals were disappeared, and nowadays there are very little individuals in Sup-seom Island. Also there are numerous transplanting efforts and records of *A. antiquum* to recover its habitat from 1966 to 1983.

Molecular genetic markers have become popular to resolve the traces and its availability has been confirmed by many studies (Williams *et al.*, 1990; Baldwin *et al.*, 1995; Erdogan and Mehlenbacher, 2000, etc). Especially RAPD and AFLP are very useful to trace the origin on especially low level taxa (Tae *et al.*, 1999; Sim and Kim, 2002; Kim *et al.*, 2002). Also RAPD method overcomes many of the technical limitations of RFLP and has been used in many genetic analyses, including population genetics studies (Hadrys *et al.*, 1992; Chalmers *et al.*, 1992; Ashburner *et al.*, 1997; Gallois *et al.*, 1998; Gauer and Cavalli-Molina, 2000).

The objective of this study was to investigate the genetic variations and to apply the phylogenetic relationships among *A. antiquum* and related species in Far East including Korea, Japan, and Taiwan. Also we discussed the identity of *A. antiquum* individuals in Sup-sepm Island. which are transplanted ones from unknown ancestors, and we tried to discriminate their status.

MATERIALS AND METHOD

Plant Materials and DNA Extraction

Table 2. Code and sequences of primer analysed, total number of bands analysed and fragment size.

Primer	Sequence (5′ →3′)	Total no. of bands	Fragment size range
NAPS-01	CCT GGG CTT C	9	200-1500bp
NAPS-06	CCT GGG CCT A	4	300-1500bp
NAPS-43	AAA ACC GGG C	13	200-2000bp
NAPS-44	TTA CCC CGG C	6	300-1500bp
NAPS-72	GAG CAC GGG A	8	300-1500bp
NAPS-75	GAG GTC CAG A	9	200-2000bp
NAPS-77	GAG CAC CAG G	9	300-2000bp
NAPS-78	GAG CAC TAG C	2	300-1500bp
NAPS-81	GAG CAC GGG G	6	300-1500bp
NAPS-82	GGG CCC GAG G	14	300-1500bp
NAPS-83	GGG CTC GTG G	3	500-1500bp
NAPS-84	GGG CGC GAG T	5	200-1500bp
Total	-	88	-
Mean/primer		7.33	

The plant materials used in this study and their collection data are given in Table 1. Leaves used as sources of DNA were collected from natural populations. All plant materials were kept in vinyl zipper bag with silica-gel until returned to the laboratory and stored at -70℃ in the laboratory until use. Total genomic DNA was extracted from fresh leaf tissue pulverized in liquid nitrogen using the 2X CTAB buffer (Doyle and Doyle, 1987). DNA samples were stored at -20℃ until use.

RAPD Reaction

Randomly amplified polymorphic DNA in each genomic DNA was amplified by 35 cycles with No. 1-100 oligo primers by NAPS (Univ. of British Columbia). Amplifications were perfomed in 25 μl reactions containing 10-50ng DNA, 200 M dNTP (equimolar), 0.5 units Ampli*Taq* DNA polymerase (Perkin & Elmer, Cetus), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and primers at 1.0 M. Before the PCR cycles, PCR mixture was predenatured at 92℃ for 3 minutes. The PCR cycle consisted of 30 seconds at 95℃ for denaturation, 30 seconds at 50℃ for annealing, and 1 minutes at 72℃ for extension. After 35 cycles the PCR reactions were incubated at 72℃ for 7 minutes to complete final extension. After the useful primers were screened, we conducted 3 times repetitive reactions with same primers to confirm their availability. Agarose gel electrophoresis were employed to check the amplified DNA products by 1.5% agarose gel with 10-4% EtBr. And the correct band positions were determined with 1D-PCR program by Universal Software (AAB, 2000).

Data Analysis

Each RAPD band was assigned a number and treated as a unit character coded as 0 (absent) or 1 (present) by the operational taxonomic units (OTUs), and the data matrix was conducted. The genetic variations were calculated by Nei and Li' s genetic distance (1979) with the distance program of PAUP* (Swofford, 2001, ver. 4.08b). The UPGMA phenogram were produced by the RAPD results, and the Nei and Li' s distance was to used to construct a Neighbor-Joining tree (NJ tree, Saitou and Nei, 1987).

Table 3. Genetic dissimilarity matrix of *Asplenium antiquum* and the related species caculated by Nei and Li's genetic distance based on the RAPDs data.

	A.antiquum1	A.antiquum2	A.antiquum3	A.antiquum4	A.antiquum5	A.antiquum6	A.antiquum7	A.nidus1	A.nidus2	A.nidus3	A.nidus4	A.nidus5	A.australasicum
A.antiquum1	0												
A.antiquum2	0.0227	0											
A.antiquum3	0.1250	0.1477	0										
A.antiquum4	0.1250	0.1477	0.0000	0									
A.antiquum5	0.1932	0.2159	0.0682	0.0682	0								
A.antiquum6	0.1705	0.1477	0.1136	0.1136	0.1136	0							
A.antiquum7	0.1591	0.1364	0.1250	0.1250	0.1250	0.0114	0						
A.nidus1	0.5682	0.5682	0.6250	0.6250	0.6477	0.6250	0.6136	0					
A.nidus2	0.6023	0.6023	0.6364	0.6364	0.6591	0.6591	0.6477	0.1477	0				
A.nidus3	0.5795	0.5795	0.6364	0.6364	0.6818	0.6591	0.6477	0.1477	0.1136	0			
A.nidus4	0.5909	0.5909	0.6250	0.6250	0.6705	0.6023	0.5909	0.1136	0.1477	0.1705	0		
A.nidus5	0.6023	0.6023	0.6364	0.6364	0.6818	0.6136	0.6023	0.1023	0.1591	0.1818	0.0568	0	
A.australasicum	0.5682	0.5682	0.6250	0.6250	0.6250	0.6023	0.5909	0.4545	0.4432	0.4886	0.4091	0.3977	0

* Find the Abbreviation in Table 1

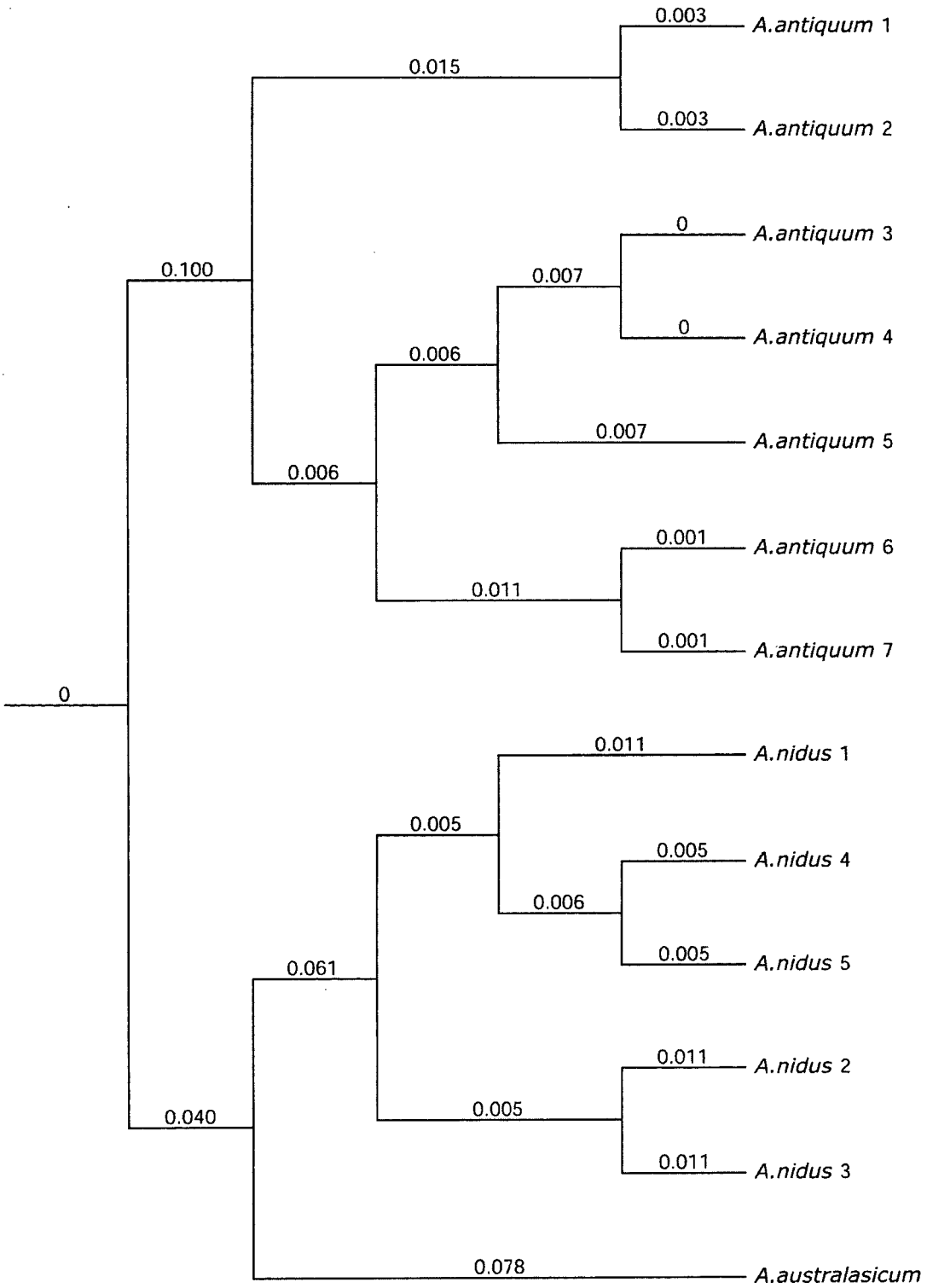

Fig. 1. The UPGMA phenogram of *Asplenium antiquum* and its related species based on the results of RAPD analysis

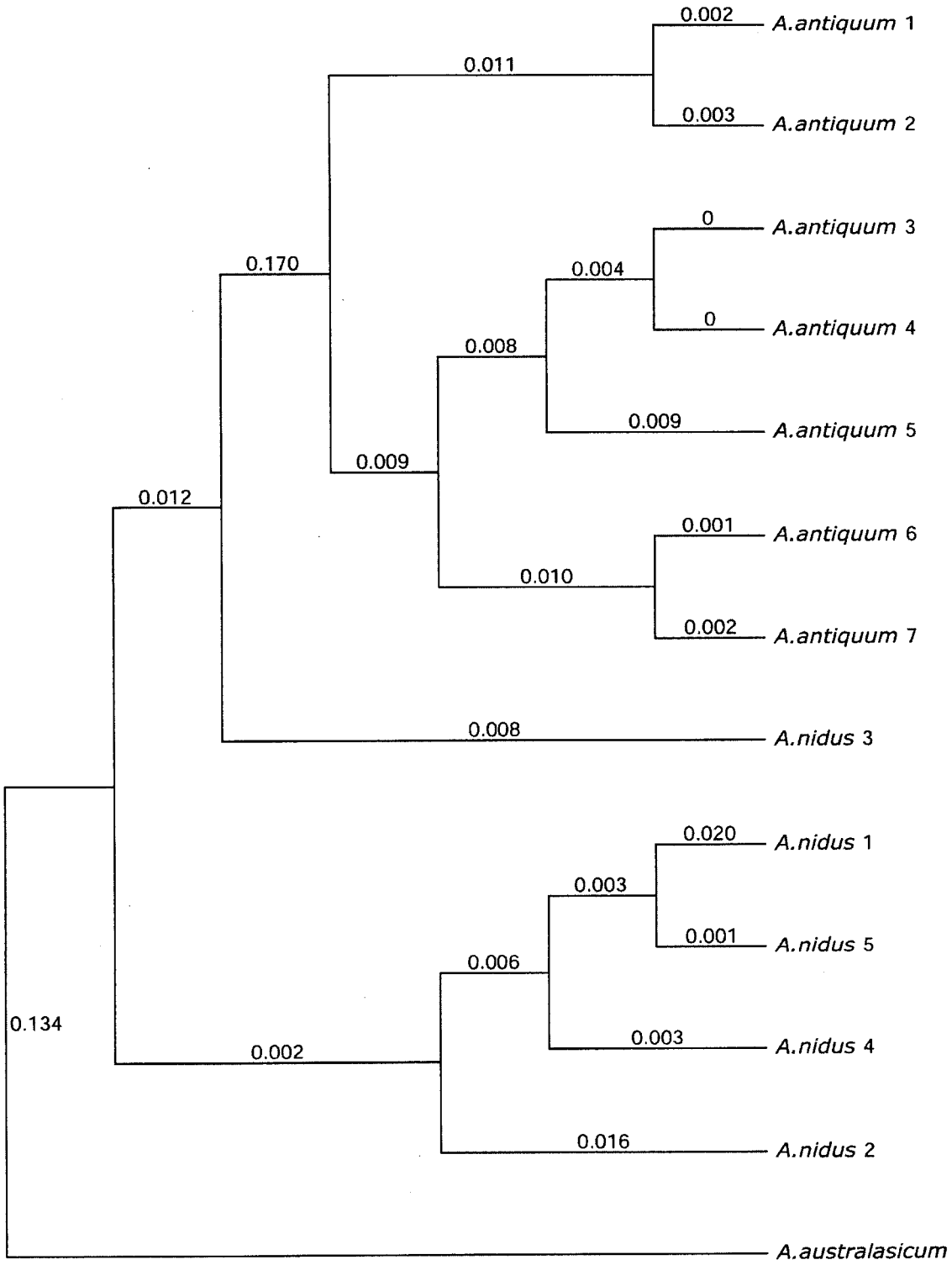

Fig. 2. The Neighbor-Joining tree of *Asplenium antiquum* and its related species based on the results of RAPD analysis.

RESULTS AND DISCUSSION

Thirty-two primers were screened from 100 primers which were used in this study. Most of the reacted 32 primers showed the relatively high G+C contents (60-80%), and this results supported the previous study on RAPD (William *et al.*, 1990; Fritsch *et al.*, 1993). And 12 primers of them presented the same results from all the taxa after three times repetitive reaction (Table 2). The considered RAPD markers which showed consistent amplification were found in the ranges between 200bp and 2000bp, and 2 to 14 bands per primer were amplified according to the taxa (Table 2). Nearly same RAPD band patterns from same species were detected and those from other taxa were different. And the RAPD bands from regional populations within the same taxa were same almost. Eighty-eight scorable markers from 12 primers were applied to generate the genetic dissimilarity matrix by Nei and Li's distance (Table 3).

Seven populations of *A. antiquum* showed slight differences comparing with other taxa in the levels of genetic distance, ranging from 0.000 to 0.216 (Table 3). Two individuals from Sup-seom Island which are growing in presented no genetic differences, and it might be regenerated from same plant. And two Bomok-dong ones which were regarded as the descendent of the original individual in Sup-seom Is. (Han, HJ., per. comm) showed very low level of variations (0.023). Also, present two transplanted clones at Sup-seom Is. were recognized as the closest ones (0.068) with the one from Jeju Orchid Garden which was immigrated in 1974 from Hachijodo, Japan (Kang, BJ., pers. comm.). Five populations of *A. nidus* showed only little slight genetic differences in the levels of genetic distance, ranging from 0.114 to 0.171 (Table 3). But, distinct genetic discontinuity (0.568-0.682) presented between *A. antiquum* and *A. nidus*.

Figure 1 shows a UPGMA phenogram for 13 populations of *A. antiquum* and its related species analysed. Based on the UPGMA tree, grouping of same species was showed. In *A. antiquum*, two individuals from Sup-seom Island and the one from Jeju Orchid Garden were clustered, and these were formed a group (Group I) with those from Taiwan and Yakushima (Japan). On the other hand, two Bomok-dong ones were clustered as another group (Group II) independently. This result indicate that present transplanted individuals in Sup-seom Is. might be the regenerated ones from abroad, even though seven populations of *A. antiquum* clustered as a same species. Also, five populations of *A. nidus* were clustered as two groups correlated with geographical distribution (Japan and Taiwan). And *A. autralasicum* was clustered with *A. nidus* together (Fig. 1). The NJ tree which applied *A. autralasicum* as an outgroup indicated that *A. antiquum* and *A. nidus* were derived from the common ancestor with independent evoultionary route (Fig. 2). Also, it showed the similar grouping patterns likewise UPGMA phenogram. With the NJ tree, even if we would believe or not its artificial immigration, we could guess an speciation-adaptation process among *A. antiquum* and its related species as follows : firstly, two ancestral type of *A. antiquum* and *A. nidus* were diverged from hypothetical ancestor long times ago. And they had performed the successive differentiations and speciations to adapt themselves into the diverse habitats. And two species got to distinguished distinctly, and finally some individuals of *A. antiquum* were isolated in Sup-seom Island. Also, those were attacked their habitat and destroyed by some mankind several decades ago, and the other people came to transplant the regenerated ones from abroad.

From above, the RAPD data was useful to define the genetic relationships and to convince the identity of *A. antiquum* and the related species.

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