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Characterization of Chromosomal DNA and DNA Polymorphism in Korean Cultivars of *Allium sativum* L.

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The genetic background of the garlic (*Allium sativum* L.) is not well understood, since it is cultivated exclusively by vegetative propagation. To understand its genetic background, a local cultivar, Danyang, was chosen, and several basic characteristics of its chromosomal DNA were examined. Its G + C content was 40.6%, and the relative proportion of fast reassociated sequences, intermediate reassociated sequences, and slow reassociated sequences were 12%, 40%, and 48%, respectively. The genome size, calculated based on reassociation kinetic experiments, was 1.11×10^{10} bp or 12.16 pg per haploid genome. To compare the genetic variation among four local cultivars, Munkyung, Seosan, Euiseong, and Danyang, random amplified polymorphic DNA (RAPD) analysis was performed. By using slightly longer primers, 18-24 nucleotides in size, than the traditional primers used for such analysis, more reliable RAPD results were obtained. 15 primers gave rise to amplified bands, and the results could be grouped into two categories. The patterns of amplified products produced by 12 primers, group A, were polymorphic. These results were analyzed using a NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), and a dendrogram grouping the four local cultivars was produced. The three primers of group B gave rise to a monomorphic band pattern from four local garlic clutivars, indicating that these primers possibly recognize garlic specific sequences. These primers were useful in identifying genetic variations among the *Allium* species.

Keywords: DNA polymorphism, DNA sequence organization, garlic, RAPD, reassociation kinetics

Garlic (*Allium sativum* L.) is a perennial plant that belongs to *Allium* genus, and its product ranks in importance next to common onions as a food additive. Most garlic is sterile and is vegetative propagated by cloves, except several cultivars collected in Soviet Central Asia (Etoh and Ogura, 1978; Etoh, 1985, 1986). Cytogenetic analysis has revealed that garlic is a diploid (2n = 16), with two pairs of satellite chromosomes (Kim and Seo, 1991), and that its nuclear DNA amount to be estimated at 35.7 pg per cell by microspectrophotometric measurements using Feulgen densitometry (Bennett and Smith, 1991). However, despite its importance and use as a food additive and a medicinal ingredient, its genetic background is poorly understood.

To understand the global genetic backgrounds of certain organisms, analysis of genome organization is very useful. Experimental evidence suggests that repetitive sequences are interspersed in single copy DNA, and eukaryotic genome composition, including higher plants, bears remarkable similarity to each other (Zimmerman and Goldberg, 1977). Reassociation kinetic analysis of chromosomal DNA is used to measure kinetic complexity, genome size, and nucleotide composition of higher eukaryotes (Gupta et al., 1981; Leutiwiler et al., 1984). This kind of study may contribute substantially to our understanding of the chromosome structure and genome organization of the garlic.

It might be expected that garlic would show little intraspecific variations because of its vegetative reproduction. However, there are many distinct local cultivars in regions where garlic has long been grown. Traditionally, different garlic cultivars have been distinguished according to the following criteria; morphological characteristics such as the bolting type, the number, color and size of the primary cloves and the bulb weight, and physiological and ecological characteristics such as maturity, long-day requirements for bulb formation, winter hardness, and bulb dormancy (Johns and Mann, 1963). Recently, attempts have been made to differentiate garlic cultivars on the basis of DNA-related traits. Since Maaβ and Klaas (1995) separated garlic into four major groups based on isozyme and random amplified polymorphic DNA (RAPD) analysis, other similar approaches using DNA fingerprinting methods have been accomplished, involving RAPD analysis (Bradley et al., 1996; Eom and Lee, 1996; Lee et al., 1996; Kim et al., 1997).

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The DNA fingerprinting method has been shown to be very useful to identify subtle genetic differences among closely related organisms, especially when the typical genetic approach could not be applicable, like as with garlic produced, which can only reproduce via vegetative propagation. RAPD markers have been adopted as a convenient and powerful means for DNA fingerprinting (Welsh and McClelland, 1990; Williams et al., 1990). RAPD markers are generated by PCR of chromosomal DNA using primers with arbitrary random sequences. It is apparent that this technique offers a fast and efficient method for detecting DNA polymorphism in a wide range of species. More recently, RAPD analysis has been applied to a wide range of species for many purposes, such as gene mapping, identifying the markers linked to traits (Paran et al., 1991), and analyzing the phylogenetic relationships (Demeke et al., 1992; Cho et al., 1994; Seok and Choi, 1998).

As the primers used in RAPD analysis are generally short arbitrary sequences, the annealing temperature is rather low and the pattern of amplified bands is not always reproducible. To minimize the reproducibility problem, we tried longer primers 18-24 nucleotides in size and more stringent conditions for RAPD analysis. With this new method, the genetic variations of four local cultivated garlics and several *Allium* species were examined. In addition, we investigated the genomic sequence organization by reassociation kinetic study to further expand our understanding of its genetic backgrounds.

MATERIALS AND METHODS

Experimental Plants

Four local garlic cultivars, 'Danyang', 'Munkyung', 'Seosan', 'Euiseong', and a cultivar of onion, 'Haenam', were obtained from local farms or local markets. Sanmanul (*Allium victorialis* var. *platyphyllum*) was collected from natural population of Ulreung Island. Seeds of Welsh onions (*Allium fistulosum* L.) were purchased from Daenong Seed-Company in Seoul.

Chromosomal DNA Extraction

DNA was extracted from the leaves of the four local garlic cultivars, onions, Sanmanul, and Welsh onions as described by Rogers and Bendich (1988) with minor modifications. Frozen leaves were ground to a fine powder with liquid nitrogen and transferred to a centrifuge tube. One volume of hot (65°) 2x cetyltrimethylammonium bromide (CTAB) buffer (2% (w/v) CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone (Mr 40,000), pH 8.0) was added, gently mixed with one volume of chroloform/isoamylalcohol (24:1) for 10 min, and then centrifuged at 3,000*g* for 15 min. The upper phase was transferred to a new tube. After adding 0.1 volume of 10% CTAB solution (10% CTAB, 0.7 M NaCl), another extraction with chroloform/isoamylalcohol was performed. One volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) was added for precipitating nucleic acids, mixed gently, and centrifuged at 3,000*g* for 30 min. After drying, the DNA pellet was dissolved in a high-salt TE buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 8.0) and then precipitated with two volumes of 95% ethanol. The DNA precipitate was pooled out with a hooked glass rod, vacuum-dried after washing with 70% ethanol, and resuspended in TE buffer. Then the DNA was treated with RNase A and was extracted successively with phenol, phenol/chloroform, chloroform, followed by ethanol precipitation (Sambrook et al., 1989). The DNA pellet was resuspended in TE buffer and stored at −20°.

Measurement of G + C Content

Thermal denaturation was performed in order to determine the G + C content of garlic chromosomal DNA. The purified DNA of the Danyang cultivar at a concentration of 25 μg/mL in 0.12 M phosphate buffer (0.12 M sodium phosphate, pH 6.8) was used to obtain a melting curve. The temperature was raised from 60°C to 100°C at the rate of 1°C/min using a thermal-controlled spectrophotometer (Gilford 2600), and a change of absorbance at 260 nm was monitored. The G + C content based on measurement of melting temperature (Tm) was determined using equation of Felsenfeld (1971).

$$\%\,(G + C) = 2.44\,(Tm - 69.35)$$

Reassociation Kinetics

Analysis of repetitive DNA sequences by reassociation was performed as described by Britten et al. (1974) with minor modification. DNA was sheared to an average length of 300~400 base pairs by sonication using an ultrasonicator (Sonic & Materials, Inc., Model VCX200, Danbury, CT.,USA) at 200 W for 3 min and was resuspended in 0.12 M phosphate buffer (pH 6.8) at concentrations ranging from 3 μg/mL to 10

mg/mL. The DNA solutions were denatured by boiling for 5 min and were reassociated in a 60°C water bath to the desired C_0t values. After reassociation to the appropriate C_0t value, the unreassociated and reassociated DNA fractions were separated by hydroxyapatite (Bio-Rad, DNA grade, Bio-Gel HTP, Hercules, USA) chromatography in a 60°C water-jacketed column at 0.12 M and 0.4 M phophate buffer (pH 6.8), respectively, and amounts of DNA were determined by the measurement of absorbance at 260 nm. Results were analyzed by plotting C/C_0 and C_0t values (Britten and Kohne, 1968; Allen et al., 1975; Leutwiler et al., 1984), where C_0 = original total single-stranded DNA concentration (mole/L), C = concentration of single-stranded DNA at time t (mole/L), and t = time after initiation of reassociation (sec).

To find the C/C_0 value for quickly reassociated DNA sequences with C_0t values of approximately 10^{-2}–10^{-1}, a completely denatured DNA solution at 100°C was transferred to a recording spectrophotometer which was kept at 60°C, and changes of its absorbance at 260 nm were monitored. The amounts of reassociated DNA were calculated based on hypochromicity, the fall in UV absorption that occurs as double strands are formed (Britten et al., 1974).

RAPD Analysis

For RAPD analysis, initially 35 different primers, ranging 18 to 24 nucleotides in size, were tested. PCR reactions were performed under the following condition; 40 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension 72°C for 2 min, typically in a 20 µL volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.9 mM $MgCl_2$, 200 µM each dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 pmole primers, 50 ng of chromosomal DNA and 1 unit of *Taq* DNA polymerase (Promega, Medison, USA). The PCR products were analyzed on 1.4% agarose gel and visualized by staining with ethidium bromide. Fifteen primers, shown in Table 1, yielded amplified PCR products and were useful for RAPD analysis.

Band Analysis

The bands in each lane were scored in binary data, 1 indicating band presence and 0 indicating band absence. From this data, a similarity matrix was constructed using Jaccard's coefficient (Sokal and Sneath, 1963). A cluster analysis of data in the similarity matrix was performed with the program NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System, version 1.70, Exeter Software, Rohlf, F. J., New York, USA) using the UPGMA (Unweighted Pair-Group Method Arithmetic average, Sokal and Michener, 1958). The clusters were then shown as a dendrogram indicating the estimated relatedness between the local garlic cultivars.

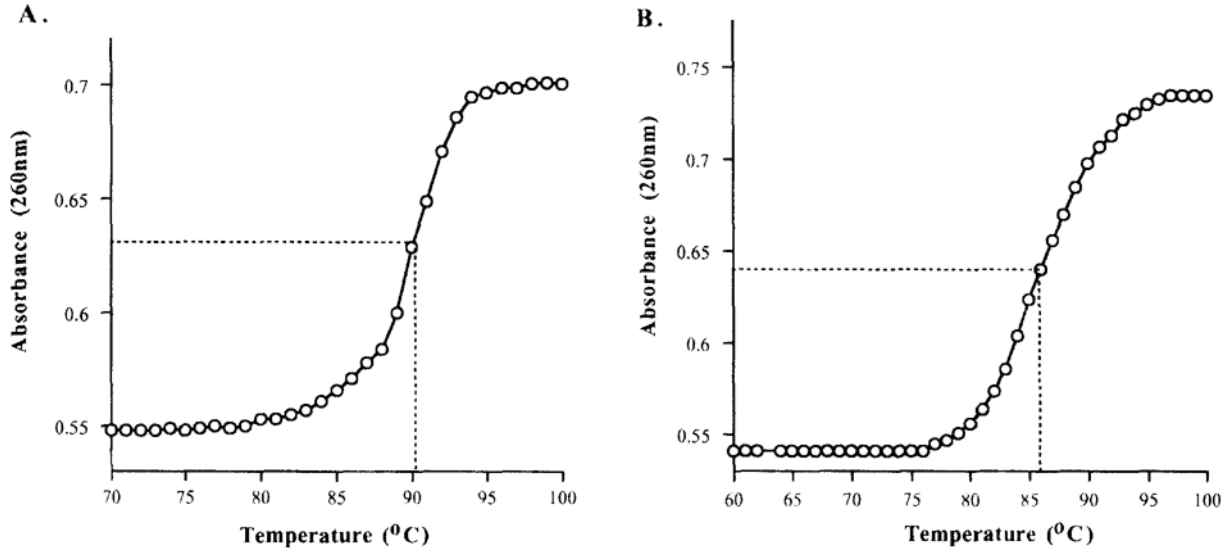

Figure 1. Melting curve of garlic chromosomal DNA. Thermal denaturation profiles measured by absorbance at 260 nm. (A) garlic chromosomal DNA (B) *Escherichia coli* chromosomal DNA. The temperature was increased from 60°C to 100°C at the rate of 1°C/min.

Table 1. Primers used for RAPD analysis.

name of primer	sequence (5' → 3')	origin of sequence
1018	CTGTCTGACAATGCGTCGCGT	*Gonyaulax polyedra*'s LBP* gene (Lee et al., 1993)
1022	ACAGGAAACAGCTATGACCATG	Reverse sequencing primer
Y1	GAACGTCGACACCACGAACGCA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y3	TCGTTCTTGCCGCCAAGGTCGT	*G. polyedras* Luciferase gene (Bae and Hastings, 1994)
Y5	TCGAAGCCTATTCCGCTCCAGA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y7	CGAGGCCAAGAAGATTAT	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y9	AGCTGAAACAGCGCTCCATCGT	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y10	TGAAGATGCTCGGGGAGCACAG	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y11	GAGCCGTCCAACTCTTTGTCAT	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y12	AGGTCTACTCCACTGGGTTTCA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y13	GTCCTCTCCTCAGGCGGTACT	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y14	GCGTCTTGTCGCCGTAGTCGAA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y15	ACGCCAAGCATGTGGAGAAGTA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y17	CCTGCACAAGCCAATGGAGGTT	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)
Y18	CCAGCAGTAATGAAACCCAGTGGA	*G. polyedra*'s Luciferase gene (Bae and Hastings, 1994)

*LBP; luciferase binding protein.

RESULTS AND DISCUSSION

Thermal Denaturation of Chromosomal DNA

The melting curves for garlic chromosomal DNA and *Escherichia coli* chromosomal DNA are shown in Figure 1. The Tm of garlic was estimated as 86 ± 0.7°C and the G + C content was 40.6 ± 1.7% judged from the results of three independent tests. Mawmur and Doty (1962) reported that many higher eukaryotes have G + C content between 35.6 and 49.1%. Therefore the G + C content of garlic was within the range of that of higher eukaryotes. The Tm and the G + C content of *E. coli* used for the control was 90.4 ± 1.0°C and 51.3 ± 2.5%, respectively, which was identical to the known value, 90.5~91.9°C and 51.0% (Brown, 1991).

Reassociation Kinetics

To understand the DNA sequence organization of garlic and its genome size, chromosomal DNA of the Danyang cultivar which was sheared to an average size of 300~400 base pairs was prepared and used for reassociation kinetic experiments. As shown in Figure 2, a typical eukaryotic C_0t curve was obtained. This curve can be divided into three kinetic components (dashed lines): about 12% of genomic DNA was fast reassociated (or highly repetitive) sequences, 40% was intermediate reassociated (or middle repetitive) sequences, and 48% was slow reassociated (or slightly repetitive or unique) sequences.

The genome size of an organism can be estimated from the reassociation kinetic parameters of its genomic sequences and a known kinetic standard

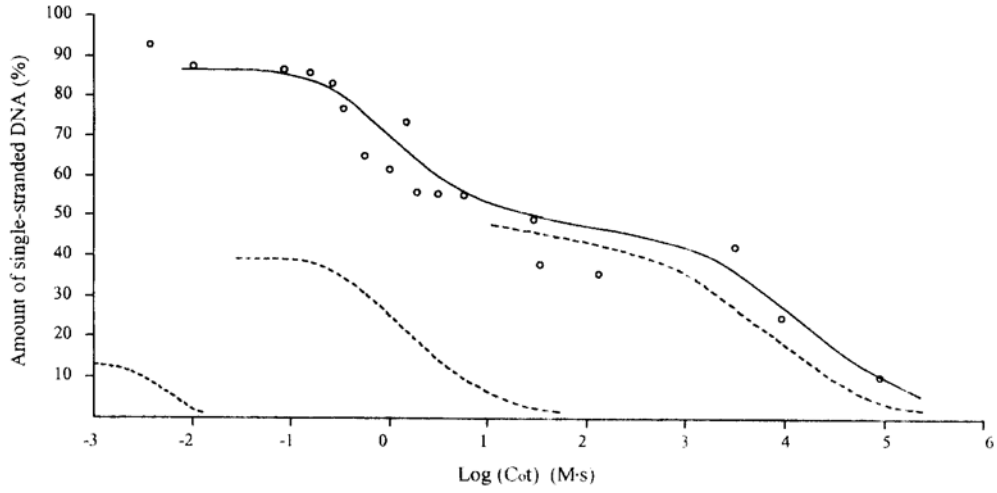

Figure 2. Reassociation kinetics of garlic chromosomal DNA. DNA was sheared to an average length of 300~400 bp by sonication, resuspended in 0.12 M phosphate buffer (pH 6.8), and denatured by boiling for 5 min. Samples were reassociated at 60°C until it reaches to appropriate C_0t value. For the fast reassociated sequences, the spectrophotometric method was used. Otherwise, the reassociated DNA and unreassociated DNA were separated by hydroxyapatite chromatography. The amount of single-stranded and double-stranded DNA was determined by measuring the absorbance at 260 nm (solid line; C_0t curve, dashed line; predicted reassociation kinetics of pure components).

such as *E. coli* (Britten and Kohne, 1968; Zimmerman and Goldberg, 1977; An et al., 1996). The copy number of fast, intermediate, and slow reassociated sequences of the garlic genomic DNA were 1.43×10^6, 3.03×10^3, and 1, respectively, assuming that the copy number of slow reassociated sequence is one. The kinetic complexity of each component was 7.84×10^2 bp, 1.23×10^6 bp, and 6.2×10^9 bp, respectively, when the kinetic complexity of *E.coli* genome (i.e. genome size) is 4.2×10^6 bp, and its $Cot_{1/2}$ is 4.5 (Table 2). As the sum of each repetitive sequences is $(1.43 \times 10^6) \times (7.84 \times 10^2) + (3.03 \times 10^3) \times (1.23 \times 10^6) + 1 \times (6.2 \times 10^9)$, the genome size of garlic can be estimated to be 1.11×10^{10} bp or 12.16 pg per haploid genome. This value is comparable to 17.9 pg per haploid nuclear DNA amounts, the result obtained by microspectrophotometric measurement (Bennet and Smith, 1991).

Analysis of DNA Polymorphism Among Four Local Garlic Cultivars

To examine the genetic variation among local cultivars, RAPD analysis with the four local garlic cultivars, Danyang, Seosan, Munkyung, and Euiseong, was performed. RAPD analysis based on PCR has been regarded as a fast and efficient method for detecting DNA polymorphism. But the pattern of amplified bands is not always reproducible, as the primers for RAPD are generally short and arbitrary sequences. To minimize this problem of reproducibility, PCR for RAPD was carried out with longer primers, 18~24 nucleotides in size, and with more stringent conditions, a higher annealing temperature than that used in general PCR for RAPD analysis. Thirty-five such primers were tested with a 52°C annealing temperature. Twenty primers out of 35 examined, which

Table 2. Kinetic parameters of galic chromosomal DNA.

Component	fraction of genome[a]	$C_0t_{1/2obs}$[b] (M·s)	K_{obs}[c] ($M^{-1}\cdot s^{-1}$)	$C_0t_{1/2pur}$[d] (M·s)	No. of copy[e]	Kinetic[f] Complexity (bp)
Fast	0.12	0.007	142.9	0.00084	1.43×10^6	7.84×10^2
Intermediate	0.4	3.3	0.303	1.32	3.03×10^3	1.23×10^6
Slow	0.48	1.0×10^4	1.0×10^{-4}	4.8×10^{-3}	1	6.2×10^9

[a]Fraction of 300~400 bp DNA fragment containing sequence of each component, [b]Based on reassociation kinetics curve, [c] $1/C_0t_{1/2obs}$, [d] $C_0t_{1/2obs} \times$ fraction of genome, [e]Derived by ratio of (K_{obs} repetitive/K_{obs} single copy), [f]Calculated by comparison of the rate of reassociation of each component to that of *E. coli* DNA ($C_0t_{1/2}$ = 4.5 M·s, kinetic complexity = 4.2×10^6 bp).

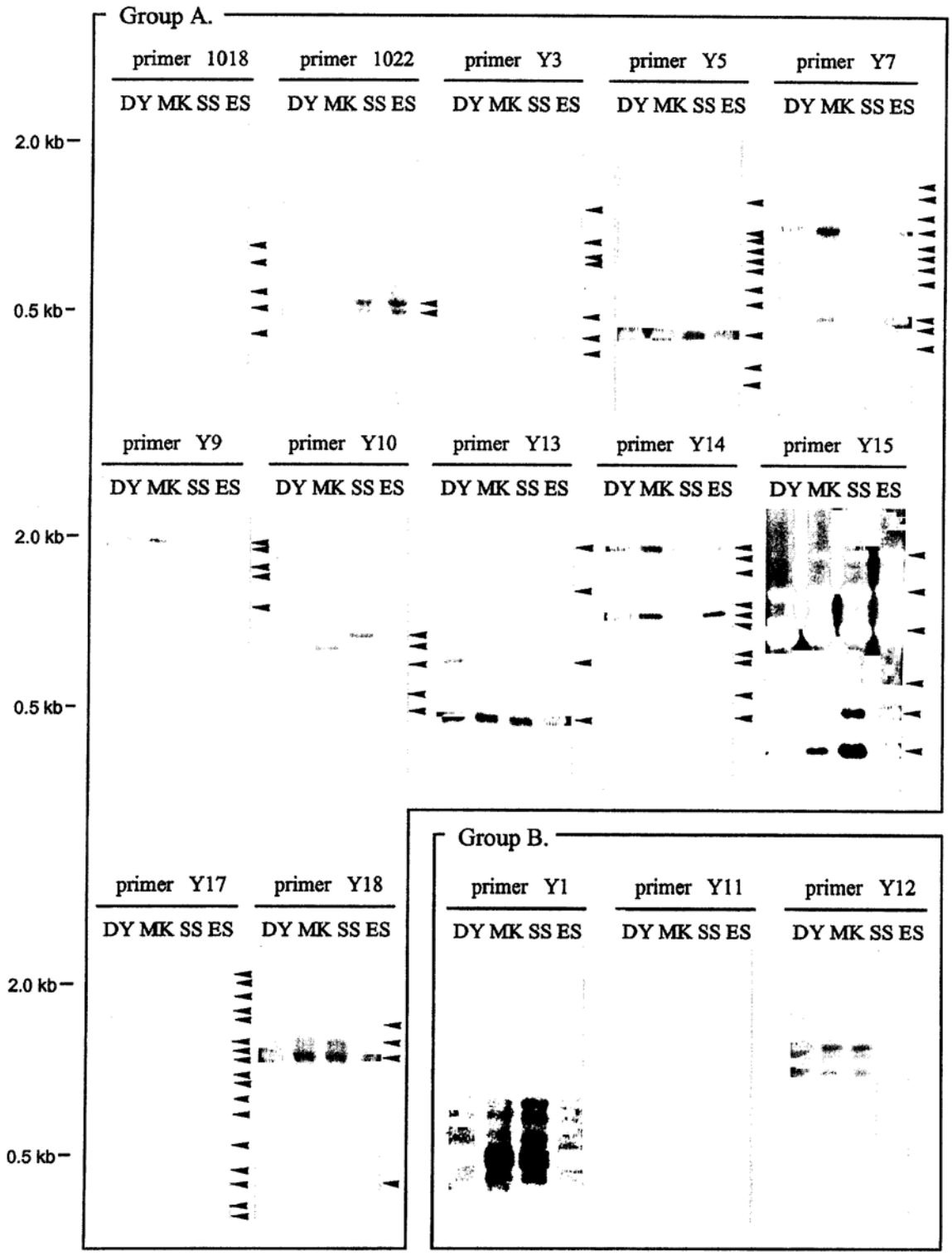

Figure 3. DNA polymorphism of four local garlic cultivars using RAPD analysis. DNA polymorphism among four local garlic cultivars was examined by RAPD analysis: Danyang (DY), Munkyung (MK), Seosan (SS), and Euiseong (ES). PCR reactions were performed under the following conditions; 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min with 10 pmol of primer and 50 ng of chromosomal DNA. PCR products were analyzed on 1.4% agarose gel.

either gave rise to any amplified band or generated a PCR product by itself, without template DNA, were not used further experiments. Fifteen primers, as shown in Table 1, gave rise to amplified bands that fall into a range between 100~2000 base pairs in size as shown in Figure 3. Although such modifications yielded a reduced number of amplified bands per primer, we were able to obtain enough and more reliable PCR products.

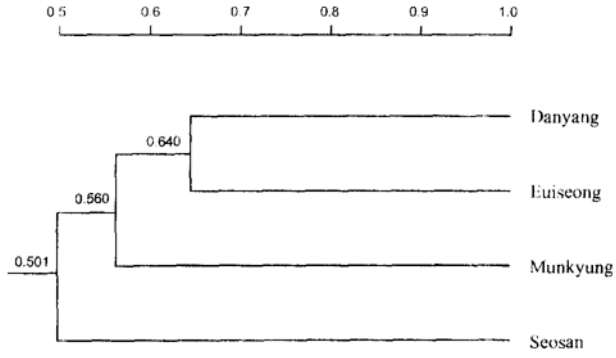

Figure 4. Dendrogram generated by cluster analysis of the similarity matrix for four local garlic cultivars. The analysis was carried out by the program 'NTSYS-PC' (Numerical Taxonomy and Multivariate Analysis System, version 1.70, Exeter Software, Rohlf, F. J., New York, USA) using the unweighted pair-group method arithmetic average. Numbers refer to numerical similarities.

The PCR products produced by each of the 15 independent primers from the four garlic cultivars were compared and classified into two categories, polymorphic and monomorphic. Group A primers including 1018, 1022, Y3, Y5, Y7, Y9, Y10, Y13, Y14, Y15, Y17, and Y18 gave rise to many polymorphic PCR products, while group B primers including Y1, Y11, and Y12 gave rise to monomorphic band patterns in four local garlic cultivars (Fig. 3).

The 12 primers of group A amplified a total of 87 polymorphic bands varying in size from 100 to 2000 base pairs. The band patterns were transformed into a binary format and used to calculate genetic similarities using Jaccard's coefficients. The results from such calculations were then used to construct a neighbor-joining dendrogram to differentiate the local cultivars and to

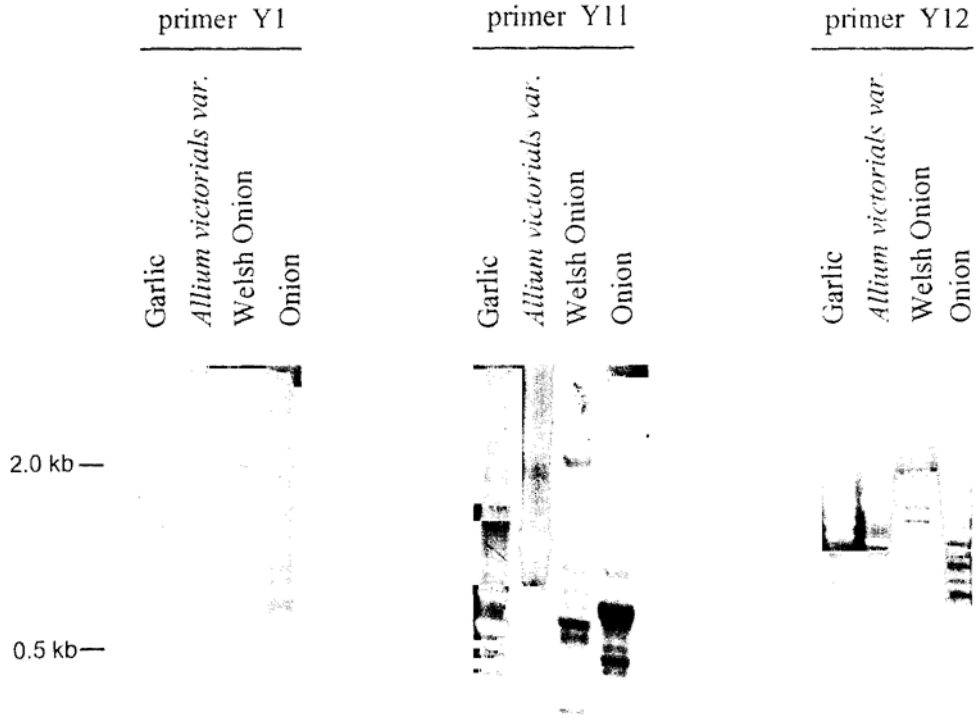

Figure 5. Interspecific DNA polymorphism using RAPD analysis. Group B primers, Y1, Y11, and Y12, shown in Figure 3, were used to RAPD analysis of four species of *Allium* genus. PCR reactions were performed under the following condition; 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min with 10 pmol of primer and 50 ng of chromosomal DNA. PCR products were analyzed on 1.4% agarose gel.

identify the genetic distances among them (Fig. 4). Their relationships were found to be consistent with the results obtained by Hwang (1993) and Lee et al. (1996).

Three primers out of the fifteen produced no distinctive polymorphic bands among the four local cultivars (group B in Fig. 3), indicating that these primers recognize possibly garlic specific sequences.

Interspecific Polymorphism of *Allium* Species

Since group B primers produced no polymorphic bands among the four garlic cultivars, we tested whether these primers can be used to distinguish species in the *Allium* genus. As shown in Figure 5, the amplified band patterns from the Welsh onion (*A. fistulosum* L.), onion (*Allium cepa* L.), Sanmanul (*A. victorials var. platyphyllum*) and Danyang garlic local cultivar were quite different, implying different genetic backgrounds among them. Sanmanul, despite its name, appears to be phylogenetically less related with garlic, since it shared little polymorphic band with any of them. This result is in agreement with the classical classification (Satake et al., 1982).

The primers Y1, Y11, and Y12 have clearly shown to be a useful tool to detect genetic variation among four *Allium* species. To analyze the detailed phylogenetic relationship among *Allium* species, these three primers along with other primers could be used to generate RAPD markers. Recently, RAPD markers have been used to investigate phylogenetic relationships among species, to study interspecific polymorphism, and to trace the putative origin of crop plants (Halward et al., 1992; Kazan et al., 1993; Prince et al., 1995; Ishii et al., 1996).

Marker-assisted selection improves efficiency in breeding by allowing for rapid identification of plants carrying a desirable trait, especially in the early generation of selection. RAPD analysis with group A primers can be a good tool for identifying garlic cultivars based on the genetic background in breeding. We are going to investigate more detailed analysis of garlic cultivars in Korea, based on our results with primers used in this study.

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