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Detection of DNA Sequence Polymorphism by Polymerase Chain Reaction in *Fraxinus mandshurica* Rupr Growing in Korea[1]

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P.C.R 技法을 利用한 들메나무 DNA sequence의 變異調査[1]

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ABSTRACT

It has been reported that there are two distinct phenotypes in *Fraxinus mandshurica* Rupr. growing in Korea. Recently developed polymerase chain reaction(PCR) was used to detect DNA sequence polymorphism in the species. Using a thermostable DNA polymerase and synthetic DNA primers, unknown DNA sequences from the species were randomly amplified. The two types of the species produced different DNA amplification pattern with three different primers tested. Although DNA polymorphism was detected among individuals within types, each type has its own distinct pattern. The two types could be easily differentiated by thier characteristic predominant bands.

Key words : polymerase chain reaction, PCR, *Fraxinus mandshurica*, DNA polymorphism

要　約

들메나무(*Fraxinus mandshurica* Rupr.)는 우리나라에서 두가지의 서로 다른 形態가 自生하고 있는 것으로 알려져 있다. 最近에 開發된 PCR技法을 利用하여 이 두 形態의 들메나무 DNA의 變異를 調査하였다. DNA 合成 酵素와 人工合成된 primer를 利用하여 이 樹種의 DNA를 增幅시켜 比較한 結果 이 두 形態는 DNA 排列에서 서로 다른것으로 나타났다. DNA變異는 같은 形態內의 個體間에도 나타나나 각 形態別로 뚜렷하게 區分되어 形態別로 特徵的인 band들이 觀察되었다. 이러한 特徵的인 band들로 두 形態를 區分할 수 있었다.

INTRODUCTION

Fraxinus mandshurica Rupr. is a native deciduous species widely growing in korea. Previously, we reported that there were at least two different types (Type A and B) in the species(Song et al. 1988). Although they are classified as one species, the two types are different in many morphological and physiological characteristics including leaf shape, terminal winter bud, rachis shape, branch color, and requirements for seed germination.

Isozymes have provided good genetic markers in detecting such variations from wild populations

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(Park 1977, Kim and Hong 1982, Ryu 1982, Son et al. 1989, Chung 1991). However, the technique has some limitations. First, there are only a limited number of isozymes which could be used. Second, isozymes represent only the products of coding region of DNA. Therefore, it may be impossible to detect any change at DNA level unless the DNA codes for the enzyme. Restriction fragment length polymorphism (RFLP) has recently been developed to examine DNA sequence polymorphism (Asins and Carbonell 1988, Lynch 1988, Nybom and Rogstad 1990, Crowhurst et al. 1990). However, it is very expensive and laborious to use for the analysis of plant genomic DNA. The major drawbacks of RFLP are the use of radioactive or other types of markers to prepare a probe and subsequent DNA/DNA hybridization procedure.

Recently, PCR technique has also been developed to amplify any specific DNA segment using thermostable DNA polymerase and artificial primers (Sheffield et al. 1989, White et al. 1989, Mullis 1990). The technique has proven powerful tool for many areas of molecular biology including cDNA library construction, mutation induction, detection of mutation or isolation of specific DNA sequences. One area of research now emerging may be the analysis of DNA variation and DNA finger printing by PCR (Nakamura 1990, Weining and Lanrgidge 1991). The principle of this approach is the association of primer to target sites after denaturing template DNA by heating to over 90℃ and subsequent DNA synthesis by DNA polymerase at lower temperature. Since the enzyme used for this reaction is relatively thermostable, several million copies of DNA could be obtained after 30-40 cycles in 3-4 hrs (Fig. 1). If two individuals have different DNA arrangement within (or downstream of) primer target sequence, the amplification products should be different. It is easily expected that DNA arrangement between related individuals be more similar than that between unrelated ones. By comparing the size and the number of the amplified DNA fragments, it should be possible to detect any deletion, or insertion in specific DNA sequences. The number of the fragments reflects the number of sites which are homologous to primer sequence used.

This technique has recently been used to detect DNA sequence polymorphism and demonstrated as a powerful tool for crop plant breeding (Hu and Quiros 1991, Weining and Langridge 1991, Waugh and Powell 1992).

Here, we report the detection of DNA sequence polymorphism between and within two types of *Fraxinus mandshurica* using PCR techniques.

MATERIALS AND METHODS

DNA isolation

Plant DNA isolation was done according to Junghans and Metzlaff (1990) with a slight modification. A single leaf was taken from grafts of two types of *Fraxinus mandshurica* as well as from those of *F. rynchophylla* growing in the greenhouse. The leaf was ground to fine powder with mortar and pestle in the presence of liquid nitrogen. Four ml of lysis buffer (50mM TrisHCl, pH7.6, 100mM NaCl, 50mM EDTA, 0.5% SDS, and 10mM 2-mercaptoethanol) were added to the powder and the mixture was left in room temperature for 20-30min. It was then transferred to 3ml centrifuge tubes. An equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1) was added and they were gently mixed by inverting and shaking several times. The mixture was then centrifuged at 9,000xg for 3min. The top layer was transferred to new tubes and chloroform/isoamylalcohol extraction was done twice. After adding 30μg/ml RNase, the tubes were incubated for 30-40min at 37℃. Phenol/chloroform and chloroform extractions were done again. DNA was precipitated by adding one nineth volume of 3M sodium acetate and two volumes of ice cold ethanol, and incubating the tubes in -20℃ for 4-6hrs. The precipitated DNA was recovered by centrifugation at 9,000xg for 15min. The pellets were rinsed with 70% ethanol twice and air-dried.

PCR reaction

For PCR, 1-5ng of DNA in 3ul of water were used for a reaction. The deoxynucleotide primers were synthesized in Korea Basic Science Center (Seoul, Korea). The primer 1, 2 and 3 are 15, 18 and 16 bases long, respectively, and have the following

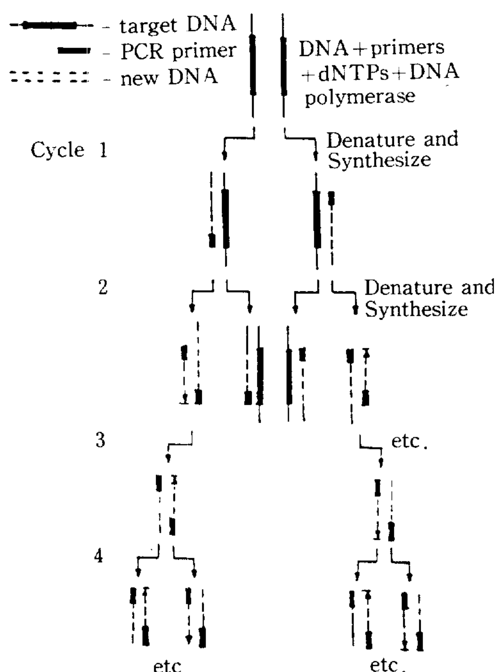

Fig. 1. Schematic diagram for PCR. Millions copies of DNA can be amplified after 30-40 cycles in 4-5 hrs.

sequences.

Primer 1 : 5'-AGC AAG TTC AGC CTG CTT AAG-3'

Primer 2 : 5'-TCC GGA GCT TGC ATG TTT-3'

Primer 3 : 5'-TCA CTA GTT GCA GTA G-3'

Total 30μl reaction mixture contained following gradients ; 0.4μM primer, 1 unit of Taq DNA Polymerase, 200μM dNTPs, 50mM KCl, 10mM TrisHCl, pH8.8, 1.5mM $MgCl_2$, and 0.1% Triton X-100.

All the gradients were purchased from Promega Corp. The reaction mixtures in 0.5ml microfuge tubes were covered with 40μl of mineral oil and placed in an automated thermocycler (Taitec, TR-100). The DNA was heated to 95℃ for 5min before going through the cycle. They were then subjected to 40 cycles (each cycle consisting of 0.5min at 94℃, 0.5min at 37℃, and 2min at 72℃). After amplification, 30μl of the amplification products were mixed with 3μl of loading buffer (20% (w/v) Ficoll 400, 0.1 M Na2-EDTA, 1% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) and loaded in 1.2% (w/v) agarose gel in TBE buffer (0.089M Tris base, 0.089M boric acid, and 0.02mM EDTA, pH8.0). The gel was run at 90 volts (ca. 50mA) for 2hrs. DNA was visualized by staining the gel with (5 μg/ml final conc.) ethidium bromide for 10 min followed by rinsing with running tap water for 30 min.

RESULTS AND DISCUSSION

As shown in Fig. 2, primer 1 generated several bands which appeared in all the samples. However, the two types displayed some bands unique to their type. All the plants produced 2.1 Kb and 570bp fragments. DNA polymorphism was detected within types as well as between types. The plant A-1 (lane 6) did not produce 550bp fragment. The plants A-3 and 4 (lane 8 and 9) did not produce 1.8Kb fragment. The plant A-3 (lane 8) had a unique band of about 1 Kb fragment which appears only in *F. rynchophylla* (lane 1 and 2). The type B (lane 3, 4 and 5) showed similar pattern to the type A. However, the intensity of the bands which reflects the homology of primer to the target sites was very different. In type

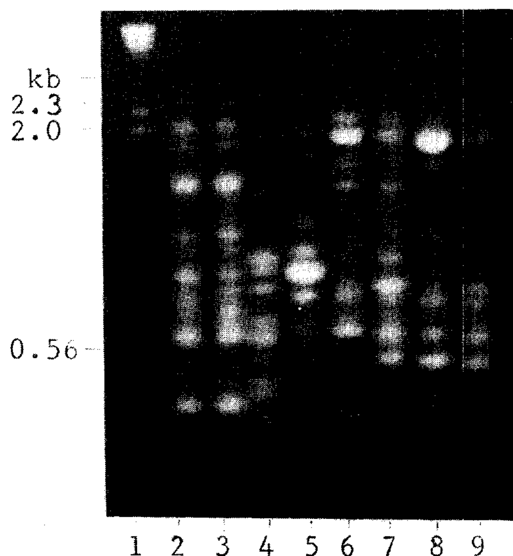

Fig. 2. PCR amplified DNA from *Fraxinus mandshurica* and *F. rhynchophylla*. When primer 1 was used to amplified DNA. Lane 1 : standard DNA marker (Hind III cut lamda DNA), Lanes 2 and 3 : *F. rhynchophylla*, lanes 4 and 5 : Type B plants, and Lanes 6, 7, 8, and 9 : Type A plants.

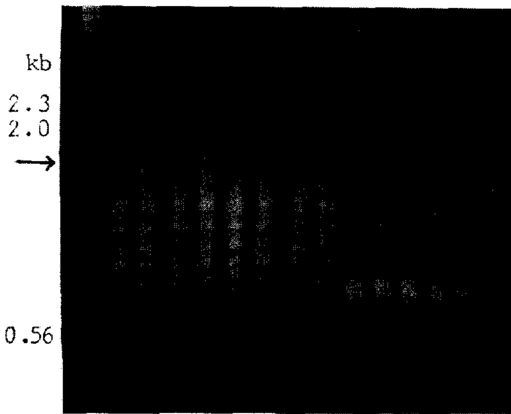

Fig. 3. Amplification of *F. mandsurica* DNA with primer 2. Lane 1 : standard DNA marker (Hind III cut lamda DNA). Lanes 2 to 9 : Type B plants, and Lanes 10 to 15 : Type A plants.

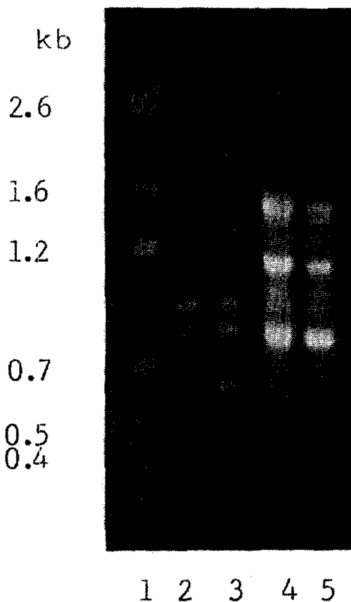

Fig. 4. Amplification of F. mandsurica DNA with primer 3. Lane 1 : pGEM DNA Marker, Lanes 2 and 3 : Type B plants, and Lanes 4 and 5 : Type plants.

B, the two plants compared were slightly different from each other, *F. rhynchophylla* produced somewhat different pattern. However, it also shares many common bands(2 and 0.57kb) with *F. mandsurica*.

The two types could be easily separated by specific profiles resulting from primer 2 amplification(Fig. 3). Whereas a major double bands(about 650 and 680 bp) were detected in all the type A plants(lane 10 to 15), all the type B plants produced 3 bands ranging from 400 to 600bp(lane 2 to 9). Variation was also detected within type A as well as type B plants. The band in size of 1.8Kb(arrow) was polymorphic in type B while a 1.2Kb fragment was missing in some type A plants(lane 10 and 12).

When primer 3 was used, the two types produced very different amplification pattern(Fig. 4). While type A plants displayed very thick 3 predominant bands(lane 4 and 5), type B plants produced thinner ones(lane 2 and 3).

The two types could easily be differentiated with these three primers(especially with primer 2 and 3). We have tested 8 plants per each type collected from all-over the country. Although type A plants grow mainly in KangWon area, they are often found in other areas like ChunBuk. The type A plants growing outside KangWon also showed the same DNA amplification pattern as those in KangWon area, suggesting the two types be genetically distinct. To our knowledge, no attempts have been made to explain why there are two distinct phenotypes in the species. Polyploidy which is common occurrence in *Fraxinus* may have played in the differentiation of the types since no intermediate individuals have been known(Schaeffer and Miksche 1977).

Based on our admittedly small sample, we could demonstrate that the two types might be genetically distinct and PCR could be used to detect such variations. However, a definitive conclusion should be drawn only after more samples including *F. ryncho-phylla* are investigated. Conventional methods such as chromosome counting and hybridization to *F. rynchophylla* may help answer many problems regarding the differentiation.

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