Comparison of Random Amplified Polymorphic DNA Profiles of Crude Extracts of Pollen DNA in Allium, using Thermus aquaticus and Pyricoccus furiosus DNA polymerase combinations

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Comparison of Random Amplified Polymorphic DNA Profiles of Crude Extracts of Pollen DNA in *Allium*, using *Thermus aquaticus* and *Pyricoccus furiosus* DNA polymerase combinations

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Introduction

Sporophytic tissues are the usual sources of DNA for extraction, amplification and analysis. Studies on the use of gametophytic cells for genetic analysis are rare[6]. The DNA content in pooled pollen samples and in vegetative tissues is expected to be similar so a method that allows the genetic characterization of pollen samples will be useful. It will enable the breeder to verify the presence of a particular genetic marker in a pollen sample before hybridization.

Pollen is a convenient source of DNA since it is readily stored for long periods at-20℃[3]. However, standard extraction procedures for plant DNA usually require at least 50 mg sample[10] so these are not suitable for pollen. Although extraction of *Allium* DNA of reasonable purity is tedious and difficult[11], it is possible to amplify DNA from crude extracts of diploid plant tissues[9]. The authors are not aware of any previous report on the generation of random amplified polymorphic DNA (RAPD) markers from crude extracts of pollen DNA.

Taq (*Thermus aquaticus*) DNA polymerase is the most polular enzyme for DNA amplification[4] but it does not have a proof-reading ability[7]. This lack of proof-reading ability may be the reason Taq DNA polymerase can only amplify short strands of DNA[1,2]. Addition of a small amount of Pfu (*Pyricoccus furiosus*) DNA polymerase, which has proof -reading ability and the highest fidelity among current DNA polymerases[7], enabled the Taq DNA polymerase to synthesize longer strands of DNA[2].

The lack of proof-reading ability of Taq DNA polymerase results in relatively lower fidelity in DNA amplification: it can lead to the amplification of artifacts containing recombinations, insertions and point substitutions that can complicate the analysis of DNA sequences for which the extent of polymorphism is not known[5]. Recently, RAPD profiles were used to establish genetic "fingerprints" of several plant species with little or no prior genetic characterization[12]. However, the exclusive use of Taq DNA polymerase to generate RAPDs may result in RAPD profiles containing artifacts. The addition of a small amount of

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Pfu DNA polymerase to Taq DNA polymerase greatly improves the fidelity of DNA amplification[1].

In this study, Taq DNA polymerase and 7:1, 31:1 and 63:1 (v/v) Taq and Pfu DNA polymerase combinations were used to generate RAPD markers from crude extracts of pollen DNA from *Allium cowanii* Lindl., *A. giganteum* Regel, *A. moly* L. and *A. unifolium* Kellogg.

Materials and Methods

Crude extraction of pollen DNA

Pollen of *A. cowanii*, *A. giganteum*, *A. moly* and *A. unifolium* were stored at -20℃ using a procedure described previously[3]. A crude procedure for DNA extraction[9] was modified as follows. A disposable plastic pestle was dipped in four-month-old pollen until the arrowhead tip was well covered with pollen. The pollen was macerated to a paste-like consistency in a matching 1.5 ml centrifuge tube. After the addition of 30 $\mu\ell$ of 0.5N NaOH, maceration was continued for a few more seconds. The tube was briefly centrifuged at 10,000 rpm and then a 10 $\mu\ell$ aliquot of the supernatant was transferred to another tube containing 990 $\mu\ell$ of either TE (0.1 M Tris-HCl and 0.01 M EDTA at pH 8.9) or Tr (0.1 M Tris-HCl at pH 8). The solution was vortexed for 15 sec and adjusted to an optical density at 260 nm (OD_{260}) = 0.5.

For comparison, DNA extracts from leaves of the four *Allium* species were also prepared using a SDS-based extraction procedure[10] that was modified by adding a washing step using a HEPES (0.1 M)-PVP (0.1%)-2 mercaptoethanol (4%) solution to remove phenolic compounds after the initial extraction step. These extracts were adjusted to an OD_{260} = 0.2.

RAPD analysis

On a 25 $\mu\ell$ basis, the basic Taq solution contained 17 $\mu\ell$ distilled water, 2.5 $\mu\ell$ 10× buffer (500 mM KCl, 100 mM Tris-HCl at pH 8 and 1% Triton X-100), 2 mM $MgCl_2$, 10 pM primer (OPD 1-OPD 20, Operon Technologies, USA), 1 $\mu\ell$ DNA extract, 50 mM dNTP and 0.25 $\mu\ell$ (0.625 units) Taq DNA polymerase (Toyobo, Japan). The Pfu supplement consisted of 21.52 $\mu\ell$ distilled water, 3.16 $\mu\ell$ 10 × buffer (200 mM Tris-HCl at pH 8, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1% Triton X-100 and 1 ug nuclease-free bovine serum albumin) and 0.316 $\mu\ell$ (0.79 units) native Pfu DNA polymerase (Stratagene, USA).

For the DNA polymerase combination trials, 2.82, 0.64 or 0.31 $\mu\ell$ of the Pfu solution was added to 25 $\mu\ell$ of the basic Taq solution to obtain the 7:1, 31:1 or 63:1 combinations (v/v, Taq and Pfu DNA polymerases), respectively. The required Pfu solution was pipetted into the inner rim of the lid of a centrifuge tube containing 25 $\mu\ell$ of the Taq solution and then the 25 $\mu\ell$ mineral oil overlay was added.

Mixing the two solutions long before thermal cycling led to highly variable results. This may be partly due to the tendency of the Pfu polymerase to degrade either the primer[1] or the template DNA[8]. Thus, the Taq and Pfu solutions were prepared separately and mixed by centrifugation just before the thermal cycling.

A thermal cycler (Taitec TR-100) was programmed to perform an initial denaturation step at 94℃ for 1 min, followed by 45 cycles of 90℃ for 5 sec, 33℃ for 5 sec and 72℃ for 2 min and a final DNA extension step of 5 min at 72℃. In the initial trials, temperature control was obtained by dipping the thermocouple in a tube containing 25 $\mu\ell$ each of mineral oil and the PCR solution. Since this thermocontrol setup was prone to evaporation losses, the thermocouple was inserted in a tube containing 40 $\mu\ell$ mineral oil. Amplification of the

various treatment combinations was performed at least twice to confirm the repeatability of the RAPD profile.

RAPD fragments were generated using cyclic denaturation steps ranging from 94℃ for 5 sec to 90℃ for 30 sec. However, higher denaturation temperatures resulted in shorter RAPD fragments. This may be due to depurination and nicking of the DNA at high temperatures[1]. The cyclic denaturation step was reduced to 90℃ for 5 sec to obtain longer RAPD fragments.

About 60% of the OPD primers produced RAPD fragments from *A. giganteum* at a 45℃ annealing temperature. All of the OPD primers generated RAPD fragments from *A. giganteum* at 33℃, which is the average melting temperature of these primers.

Amplification of long DNA strands requires a correspondingly long DNA extension step[1, 2]. In this study, RAPD fragments were generated at extension conditions ranging from 72℃ for 5 sec to 68℃ for 10 min. At the less stringent denaturation (90℃ for 5 sec) and annealing (33℃ for 5 sec) conditions, the best RAPD profiles were obtained only with an extension step at 72℃ for 2 min.

Ethidium bromide at 1 mg/ml and 1% agarose S (Nippon Gene, Japan) were dissolved in TAE (40 mM Tris-acetate and 1 mM EDTA) buffer. Aliquots (7 $\mu\ell$) from the amplified reactions and 0.5 μg of Hind Ⅲ-Eco R1 double digest of lambda DNA (Nippon Gene) were electrophoresed in TAE buffer at a constant 3.3 V/cm for 2 hours.

Results and Discussion

Crude extraction of pollen DNA

The amount of pollen that adhered to the tip of the pestle ranged from 0.2 to 0.9 mg in *A. cowanii* and 0.3 to 0.7 mg in *A. giganteum*, depending on the surface properties of both pollen and tip and the amount of pollen available. Maceration in 0.5N NaOH produced a thick and viscous suspension that had to be briefly centrifuged to reduce the proportion of pollen debris in the aliquot. Since *Allium* DNA extracts contain non-nucleic acid contaminants that absorb strongly at 260 nm[11], the samples were adjusted to $OD_{260} = 0.5$ to insure enough DNA in each sample. The average $OD_{260} - OD_{320}$ value (which estimates the amount of DNA in the presence of protein impurities) of these extracts was 0.26.

Fig. 1 shows the RAPD profiles obtaind after amplification using the basic Taq DNA polymerase solution. With OPD 11, almost identical RAPD profiles were obtained from crude extracts of pollen DNA of a given species in TE and Tr storage buffers and the corresponding SDS-based extracts of leaf DNA. Using OPD 12, OPD 13 and OPD 14, distinct RAPD profiles were also generated from Tr extracts of the four species. Thus, Fig. 1 clearly demonstrates the utility of curde extracts of pollen DNA for routine generation of RAPD profiles in *Allium*.

Pollen are desirable sources of DNA because they are easily stored, require very little storage space and, with the current procedure, <1 mg is needed to produce 300 to 700 $\mu\ell$ of crude DNA extract adjusted to $OD_{260} = 0.5$. Hence, with this procedure, it is now possible to detect the presence of specific RAPD markers in pollen before they are used for breeding. Thus, pollen may be the best material for the construction of large libraries of genetic resources. In contrast, leaves, which can be freeze-dried, can not be used for hybridization.

The RAPD profiles in Fig. 1, though repeatable, display a background of nonspecific amplification products between the DNA bands. In addition, the biggest bands are less than

1.58 kilo base pairs (kbp) long.

Optimization of Taq and Pfu DNA polymerase combinations

Fig. 2 shows the RAPD profiles of Taq DNA polymerase solutions when Pfu DNA polymerase solution was added at 7:1, 31:1 and 63:1 ratios. With OPD 12, the RAPD profiles from the four species differed only in the minor (less bright) bands. Comparing the RAPD profiles of the four species obtained with OPD 12, *A. cowanii* produced the most stable RAPD profile with or without the Pfu DNA polymerase supplement (Figs. 1 and 2). With OPD 12, the 7:1 Taq and Pfu DNA polymerase combination produced the lowest total number of amplified bands from the four species (23 bands, Fig. 2) whereas Taq DNA polymerase generated RAPD profiles with the most bands (40 bands, Fig. 1). This may indicate that the Pfu DNA polymerase supplement successfully reduced artifact formation and reduced the production of spurious bands. There was almost no background smearing generated by the 31:1 Taq and Pfu DNA polymerase combination. However, at the highest level of Pfu DNA polymerase supplementation (7:1), background smearing increased in *A. giganteum*, *A. moly* and *A. unifolium*. Background smearing may be due to the degradation of the DNA template by the exonuclease activity of Pfu DNA polymerase[8]. The elongation effect of Pfu DNA polymerase supplementation was not observed in amplification reactions involving OPD 12.

The addition of Pfu DNA polymerase solution to Taq DNA polymerase solutions containing OPD 14 (Fig. 2) effectively eliminated the background smearing that was quite pronounced in the reactions where Taq DNA polymerase was the sole enzyme (Fig. 1). The

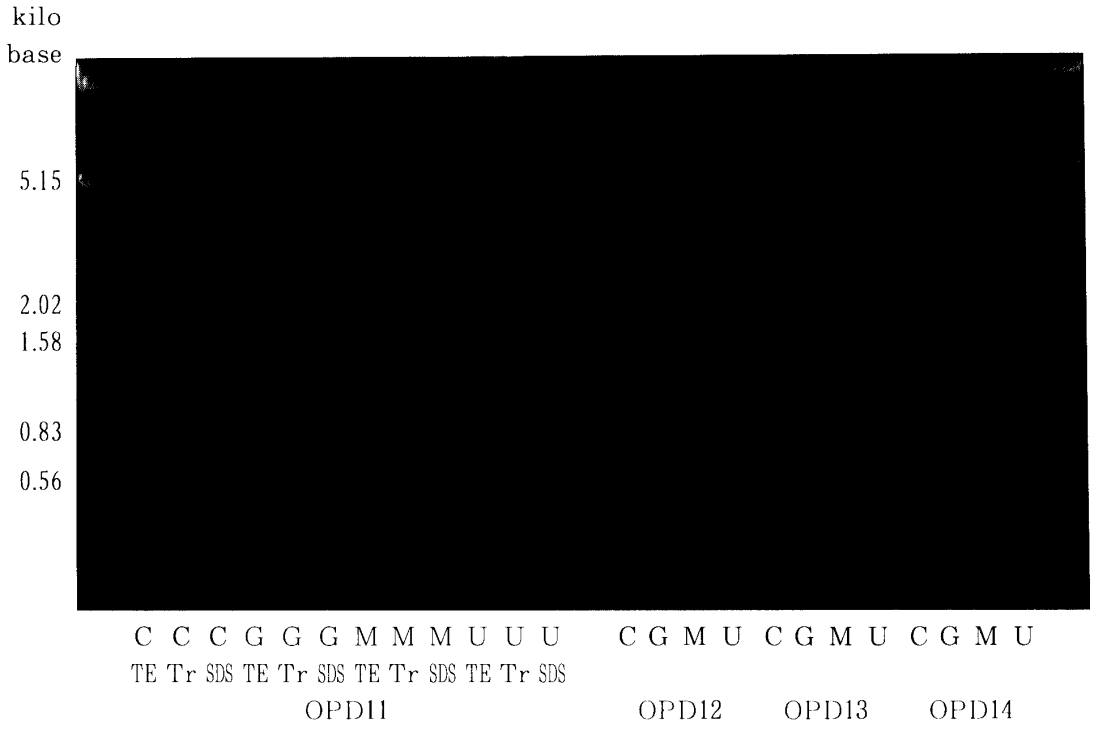

Fig. 1. RAPD profiles obtained from Taq DNA polymerase solutions. C = *A. cowanii*, G = *A. giganteum*, M = *A. moly* and U = *A. unifolium*; TE = pollen macerated in 0.5 N NaOH then suspended in 0.1 M Tris-HCl and 0.01 M EDTA, Tr = pollen macerated in 0.5 N NaOH then suspended in 0.1 M Tris-HCl, SDS = leaf DNA purified using a modified SDS-based DNA extraction procedure.

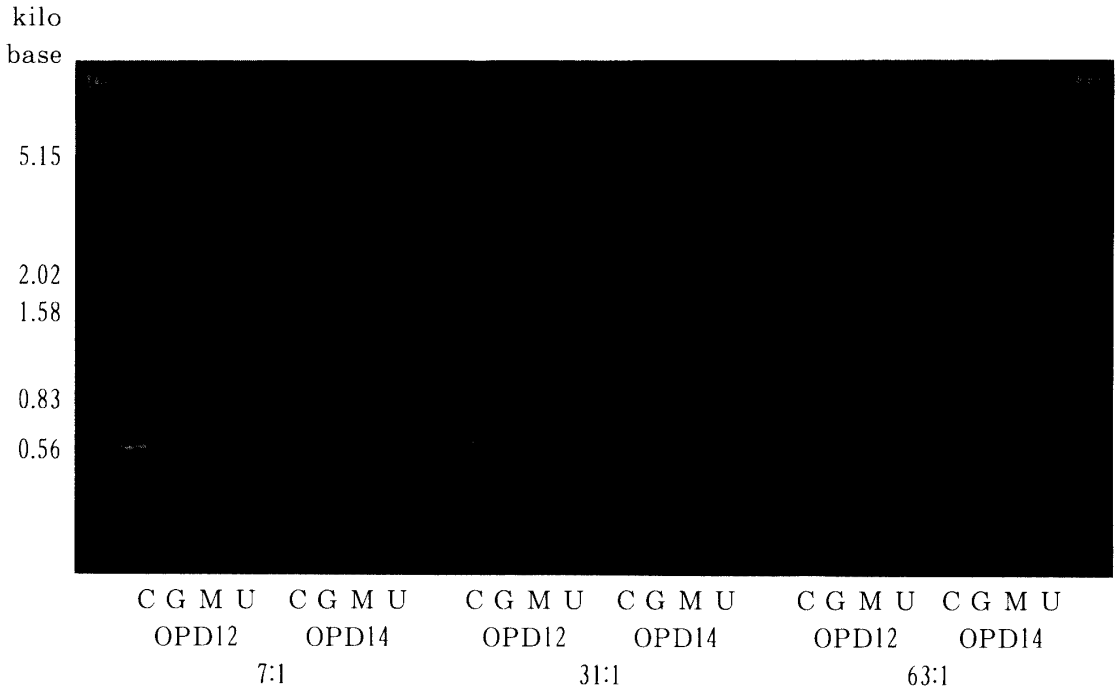

Fig. 2. RAPD profiles obtained from Taq and Pfu DNA polymerase combinations. Ratios refer to the volume of Taq DNA polymerase relative to Pfu DNA polymerase in the reaction. Other abbreviations are as defined in Fig. 1.

longest RAPD band (~5.15 kbp) was obtained from *A. unifolium* at the 63:1 Taq and Pfu combination (Fig. 2). The 7:1, 63:1 and 7:1 Taq and Pfu DNA polymerase combinations generated the longest bands in *A. cowanii*, *A. giganteum* and *A. moly*, respectively. The general increase in the length of amplified bands from the various species in the presence of Pfu DNA polymerase is in accord with previous findings[1,3]. Similar to OPD 12, the Taq DNA polymerase solution generated the greatest total number of RAPD fragments from the four species (44 bands, Fig. 1). The 31:1 Taq and Pfu DNA polymerase combination elicited the lowest total number of RAPD fragments (18 bands, Fig. 2).

RAPD fragments produced by Taq DNA polymerase are generally less than 2 kbp long. Of these species, *A. unifolium* exhibited the best response to the elongation effect of Pfu DNA polymerase supplementation.

The lack of proof-reading ability of Taq DNA polymerase may lead to artifact formation and the proliferation of pseudo-polymorphisms in the RAPD profile. The reduction in the number of RAPD fragments produced by Taq and Pfu DNA polymerase combination may be due to the minimization of such amplification artifacts.

Repeatability is an essential characteristic of a genetic "fingerprint". The foregoing discussion points out that the stability of the RAPD profiles produced by Taq DNA polymerase varies with the DNA template, random primer and the rate of Pfu DNA polymerase supplementation. The response to Pfu DNA polymerase supplementation depended on the species and the random primer. Thus, more studies on the factors that affect the stability of the RAPD profile are needed before using RAPD markers as genetic diversity probes of *Allium* germplasm resources.

Summary

Thermus aquaticus (Taq) DNA polymerase generated distinctive random amplified polymorphic DNA (RAPD) profiles from crude extracts of pollen DNA of *Allium cowanii* Lindl., *A. giganteum* Regel, *A. moly* L. and *A. unifolium* Kellogg. These profiles resembled those of purified leaf DNA of these species obtained through a sodium dodecyl sulfate (SDS) -based DNA extraction procedure. Nonspecific amplification products, however, created a background smear that complicated the classification of the RAPD fragments.

The addition of native *Pyricoccus furiosus* (Pfu) DNA polymerase to the Taq DNA polymerase reactions at ratios of 7:1, 31:1 and 63:1 (Taq:Pfu, v/v) produced RAPD patterns that were mostly different from those obtained with Taq DNA polymerase alone. With OPD 14, the Taq and Pfu DNA polymerase combinations increased the maximum length and decreased the total number of RAPD fragments compared to those obtained from the Taq DNA polymerase reactions. Background smearing was minimized or absent in the reactions containing Taq and Pfu DNA polymerase combinations.

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