

SHORT COMMUNICATION

Single Strand Conformation Polymorphism Method for Initial Detection DNA Sequences Homogeneity

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In molecular phylogenetic study, homogeneity of DNA sequences is a prerequisite before putting it into practice. Internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) has been common in phylogenetic study, but a homogenous sequence is often difficult to obtain. Here we use single-stranded conformation polymorphism (SSCP) method to detect homogeneity for nine pooled amplified products of ITS region. Our results suggested that SSCP method has been applicable in detection homogeneity of ITS region prior to using it in sequencing processes.

Key words: homogenous sequences, internal transcribed spacer region, molecular phylogenetic, SSCP method

INTRODUCTION

Rapid advances of molecular techniques such as polymerase chain reaction (PCR) bring a great impact for the use of DNA sequences in molecular phylogenetic studies. DNA sequences have offered a wealth of potential characters for inferring phylogenetic relationships because the large differences in substitution rates take place in different loci (e.g., Clegg & Durbin 1990; Li 1998). There are numerous DNA regions available, of which Internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) is one of among useful DNA regions that have become increasingly common in phylogeny inference of many groups of organism.

The ITS region includes 5.8S subunit and two spacers (ITS-1 and ITS-2). The nrDNA itself is consisted of three genes that encoded small subunit rRNA (16S to 18S), large subunit rRNA (26S to 28S) and gene 5.8S. An external transcribed spacer (ETS) is located just outside the ITS region. Along with the other components of the nrDNA, the ITS region is highly repeated up to many thousands copies in plant nuclear genome. Non-transcribed spacer (NTS) separates from one to another copy. Several general features of the ITS region promote its use for phylogenetic analyses, i.e., are high copy number (Hillis & Dixon 1991; Playford *et al.* 1992), small size in length (approximately 600 bp in angiospermae), and highly conserved flanks (Baldwin 1992).

However, a major problem when dealing with the ITS region is whether the sequences obtained are

homogenous. Homogeneity of DNA sequences is one of prerequisite prior to putting the sequences into practice (Li & Graur 1991). As mentioned above, ITS region is arranged in several hundreds to thousands tandem repeats copies in which these copies do dependently evolve in concert. However, when concerted evolution fails to homogenize the sequences, possibility of several different sequences, which have different evolutionary histories, will be produced. This failure is primarily caused when the arrays of copies occur at different chromosomal loci (Muster *et al.* 1990; Hillis & Dixon 1991; Sastri *et al.* 1992). If so, this can present opportunities of a real danger to phylogenetic analysis. To overcome this situation, Ritland *et al.* (1993) suggested that cloning and sequencing of individual repeat-types may be necessary.

Still, whether sequencing of individual PCR clones or of pooled PCR product is arguable. For large-scale phylogenetic studies that include the large numbers of pooled PCR product, cloning may be laborious, expensive and time consuming. In this case, sequencing of pooled amplified product is the only way to be chosen instead individual PCR clones. Consequently, screening of pooled amplified product should be first performed before sequencing to ascertain that the sequences obtained are homogenous.

Many methods have been established recently, of which single-strand conformation polymorphism (SSCP) method seems likely to be desired method for screening of pooled amplified product (Sunnucks *et al.* 2000). This method is one of the most widely used methods to identify genetic location encoding mutation (Orita *et al.* 1989). Although this method is still largely empirical due to the

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absence of a robust theory for predicting the dependence of conformation on sequence and of mobility on conformation, SSCP is a convenient mutation screening method.

In view of the tandem repeat copies of the ITS region and sequences difference among individual copies, the SSCP method seems likely to be able to apply in confirming homogeneity of amplified products of ITS region. We thus carried out first SSCP analysis to initial detect homogeneity of pooled amplified product of ITS regions.

MATERIALS AND METHODS

Nine pooled PCR products (amplicons) used in this study were derived from the orchid plant subtribe Aeridinae (Hidayat 2005). Protocol of SSCP method developed by Orita *et al.* (1989) was used. Basically, a small PCR product (amplicon) was taken, denatured and electrophoresed via a non-denaturing polyacrylamide gel. SSCP method consisted of these three following steps: (i) PCR amplification using primers that flank the DNA region of interest; (ii) Denaturation of the double-stranded PCR product, followed by rapid chilling to prevent re-annealing of the strands; and (iii) electrophoretic separation of the single-stranded DNA on a non-denaturing gel such as polyacrylamide.

In the first step, a primer pair, namely, AB101 and 102 (Figure 1; Sun *et al.* 1994), was used to amplify the DNA region of interest with setting-up normal cold PCR reaction. SSCP gel was prepared while PCR reaction was running. To this, several reagents and solutions such as polyacrylamide gel solution (BioWhittaker Molecular Application, USA), glycerol and TBE were required.

The second step was begun with picking up small quantity of PCR product (about 2 µl) and placing it into a new PCR tube, and 18 µl of loading dye is added. A "hot start" denaturation (95 °C for 3 minutes) was performed to denature the double-stranded DNA. After 3 minutes, DNA samples that currently in a single-stranded DNA were removed from thermocycler machine and immediately placed on the ice bath. SSCP electrophoresis was subsequently initiated by loading samples to the polymerized gel and was run overnight (10 hours) at 300 Volt in 20 °C (Orita *et al.* 1989).

The final step was staining the gel through a series of staining solution. The gel was immersed in fixation solution

and was shaken for 30 minutes. The fixation solution was replaced by silver solution and shaken for 20 minutes after then the gel was washed by destiluted water (DW) 3 times; each time was shaken for 2 minutes. After washing the gel with DW, developed solution was subjected to the gel and shaken for a few minutes until the DNA bands appeared. Stop solution was added and shaken for 10 minutes to end the staining step. The gel in preservation solution was then stored for overnight in refrigerator 4 °C. At final stage, the gel was dried and photographed for documentation.

RESULTS

The amplified products presented on agarose gel (Figure 2) sharply delimited single band. In contrast, two bands were showed up on non-denaturing polyacrylamide gels of SSCP analysis (Figure 3). They all were in similar sizes, i.e approximately 1000 bp. However, each band moved in different mobility across to samples used, indicating different conformation due to different mutation process occurred in each samples. This result suggested that homogeneity of all amplified products of ITS region examined were confirmed.

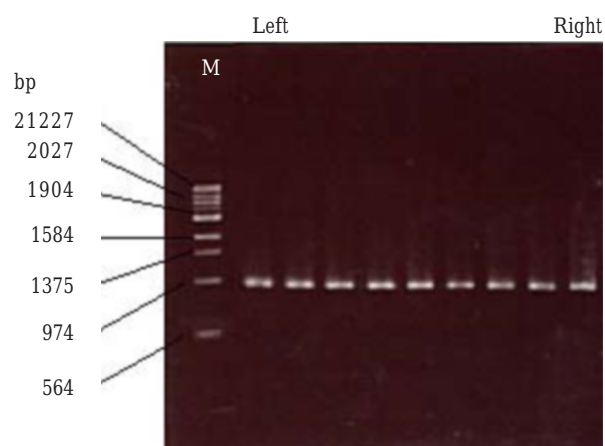
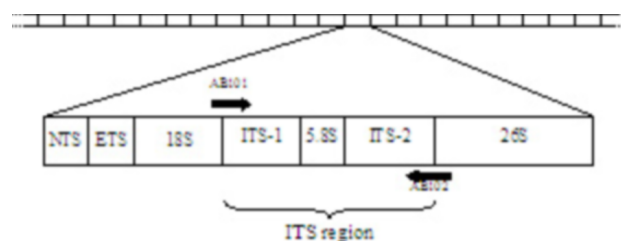


Figure 2. Nine pooled PCR products of ITS region of orchid species used for SSCP analysis. Size of all fragments is around 1000 bp with respect to the DNA size standard (marker/M) used: DNA Lambda cutted by *HindIII/EcoRI*. Plant materials from left to right are *Vanda*, *Phalaenopsis*, *Pomatocalpa*, *Trichoglottis*, *Aeriedes*, *Sarcochillus*, *Luisia*, *Bogoria*, and *Seidenfendenia*, respectively.

Nuclear ribosomal DNA



AB101 = ACGAATTCATGGTCCGGTGAAGTGTTCG
AB102 = GAATTCCTCCGGTTCGCTCGCCGTAC

Figure 1. Organization of ITS region of nrDNA with location of primers used in this study. Information of primers is also provided.

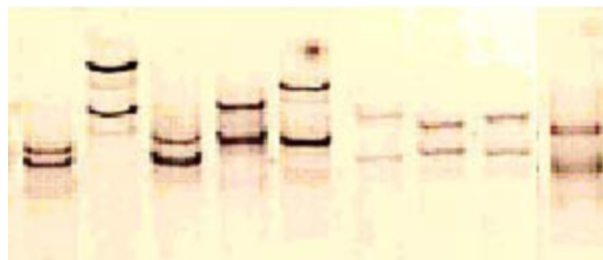


Figure 3. DNA band pattern resulted in SSCP analysis of ITS region on polyacrilamide gel. Sampels from left to right are corresponded to Figure 2. All bands have similar sizes of nucleotides, indicating different mobility due to different mutation in each sample.

DISCUSSION

The principle of SSCP method is based on the fact that the electrophoretic mobility of nucleic acid in a non-denaturing gel is sensitive to both size and shape. Denaturation processes has made double-stranded DNAs (dsDNAs) are changed into single-stranded DNAs (ssDNAs). Unlike dsDNAs, ssDNAs are flexible and will adopt a conformation determined by intra molecular interactions and base stacking that is uniquely dependent on sequence composition. This conformation of the ssDNAs can be affected even an alteration of a single base, that can be detected in different electrophoretic mobility due to substitutional sequences differences such as insertions and deletions.

Accordingly, if the amplified products of ITS do not homogenous, more than two bands are presumably expected being show up in SSCP analysis. This is because individual copy represents different composition of nucleotide due to concerted evolution, through unequal crossing-over and gene conversion (e.g., Zimmer *et al.* 1980; Hillis *et al.* 1991; Baldwin *et al.* 1995).

Some factors such as primer and *Taq* DNA polymerase have been pointed out which is responsible in determining selective amplification of only a single copy. Universal primers for ITS amplification have been created and have been widely used in plant phylogenetic study (Baldwin 1995). However, it is becoming increasingly clear that this advantage of ITS region may also make this region problematic because universal primers facilitated not only PCR amplification of contaminants but also in producing many repeat-type variants.

Taq DNA polymerase catalyzes template-directed synthesis of DNA from nucleotide triphosphates. Concentration of enzyme often leads to erroriness in PCR reaction such as misamplified products and under sampling of any repeat-type variants (Baldwin *et al.* 1995). High concentration of enzyme, possibly because of the high glycerol concentration in the stock solution, often results in an amplification of various copies, whereas too low concentration of enzyme may result in the lack of amplification products. Therefore, optimal enzyme concentration is required in order to determine selective amplification of only a single repeat-type. In addition to these, applying a small quantity of some selective amplification agents such as dimethyl sulfoxide (DMSO) into PCR mixture may increase probability of amplifying only a single copy (Chase *et al.* 2003).

It is apparent that SSCP analysis is useful in initial detection of whether amplified products of ITS region are

homogenous. Although some factors of amplifying only single copy of ITS region have been presented, screening homogeneity of amplified products obtained are still important step prior to applying them in further analysis.

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