

DNA POLYMORPHISMS GENERATED BY SINGLE-STRAND CONFORMATIONAL POLYMORPHISM AND RANDOM AMPLIFIED POLYMORPHIC DNA TECHNIQUE ARE USEFUL AS TOOLS FOR THAI VETIVER GENOME ANALYSIS

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Abstract

The genomic relationships of Thai vetiver and related taxa have been intensively studied by two different techniques: RAPD provided a simple, quick and reliable alternative to identify genetic variation whereas SSCP was used to screen for nucleotide sequence polymorphisms of DNA mutation. Even though the ecotypic and phenotypic characterizations were routinely used to identify vetiver grasses in Thailand, the minor nucleotide variations may trigger morphological, physiological and biological differences. We report here to determine genetic variations of Thai vetiver in the intron region of $\Delta 9$ stearoyl-acyl carrier protein desaturase gene (SAD, EC 1.14.99.6) for oleic fatty acid synthesis by SSCP technique and DNA fingerprinting by RAPD technique. Two degenerate oligonucleotide primers (5' TT/CT GGA CT/AA GGG CT/AT GGA C 3' and 5' ATG TCG/A/T GCG/A TAG/A TCT/CT TG/T GC 3') near consensus sequence flanking around 500 base pairs of SAD gene and two arbitrary primers (J-4 5' CCG AAC ACG G 3' and S-16 5' AGG GGG TTC C 3') are performed to PCR-amplified genomic DNA of 35 ecotypes of Thai vetiver. The results show that both SSCP and RAPD analyses of their DNA polymorphisms are sufficient to distinguish each ecotype of *Vetiveria zizanioides* Nash and *V. nemoralis* A. Camus.

Introduction

His Majesty King Bhumibol Adulyadej of Thailand has long realized that soil degradation and erosion are a serious environmental problem. He has recognized the potential of vetiver grass ('Ya Faek') as a practical and inexpensive yet effective method for soil and water conservation, because of (i) its ability to grow upright while its stiff stem is able to form a dense hedge and (ii) its vigorous deep root system that can penetrate vertically down like a dense subterranean net curtain. It is, as His Majesty has remarked, a "living wall", since its above-ground wall (i.e. hedgerow) caters for erosion control while the underground wall (i.e. roots) enhances slope stability (Hengchaovanich 1998).

RAPD or Random Amplified Polymorphic DNA (William et al. 1990; and Welsh and McClelland 1990) and SSCP or Single-Strand Conformational Polymorphism (Hayashi 1991) are PCR-based techniques. The RAPD technique is based on the amplification of genomic DNA with a single primer of arbitrary nucleotide sequences. This primer detects polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers and can be used to construct genetic maps. RAPD provide a simple, quick and reliable alternative to identify genetic variations, whereas SSCP, which was originally used as a quick technique to screen for nucleotide sequence polymorphisms of the DNA mutation, has been developed to screen for genetic variants, especially in nucleotide substitution at any position along a region of DNA. SSCP is a simple method for detecting sequence variations as small as single base point mutations.

Kresovich et al. (1994) reported that the genome of vetiver within the same clones generated stable RAPD patterns but they were able to distinguish between various vetiver accessions. Pattana et al. (1998) investigated RAPD markers for detection among *Vetiveria nemoralis* A. Camus and *V. zizanioides* Nash in Thailand. Adams and Dafforn (1998) found that 86% of 121 accessions of vetiver

appeared to be from a single clone. Na Nakorn (1993) classified Thai vetiver into two groups including an upland species, 'Ya Faek Don' (*V. nemoralis* A. Camus), and a wetland species, 'Ya Faek Hom' (*V. zizanioides* Nash). The morphological, physiological and biological differences may be based on minor genetic variations. Investigations of DNA sequences can provide information about the general genetic background of organisms and thereby allow conclusions about relatedness. Moreover, since minor nucleotide variations may not be expressed at the protein level and since parts of the DNA consist of non-coding regions, DNA sequence studies are more informative than morphological or biochemical studies. Thus, we report here the use of a PCR-based DNA fingerprinting method using both RAPD and SSCP to detect DNA sequence diversity among vetiver species in Thailand.

Material and Methods

Plant Material

Forty-two ecotypes of 'Ya Faek' provided by the Land Development Study Centre in Thailand were tested for the presence of RAPD and SSCP markers. They include, for 'Ya Faek Hom' (*V. zizanioides*): Chiang Mai, Chiang Rai, Doi Tung, Fiji, India Khao Kho, Kamphaeng Phet 2, Indonesia, Japan, Mae Hae, Mae Hong Son, Mae La Noi, Mae Tae, Monto, North India, Phra Ratchathan, Songkhla 1, Songkhla 2, Songkhla 3, South India, Sri Lanka, Surat Thani, Thai Ping, Trang 1, and Trang 2; and for 'Ya Faek Don' (*V. nemoralis*): Chanthaburi, Chaiyaphum, Huai Kha Khaeng, Kanchanaburi, Kloathoa, Kamphaeng Phet 1, Loei, Nakhon Phanom 1, Nakhon Phanom 2, Nakhon Sawan, Phitsanulok, Prachuap Khirikhan, Ratchaburi, Roi Et, Saraburi 1, Saraburi 2, Udon Thani 1 and Udon Thani 2.

Isolation of Genomic DNA

DNA was extracted from young leaves of each variety of 'Ya Faek'. Approximately 2 g of plant material were extracted with 10 ml of buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM EDTA and 0.1 M β -Mercaptoethanol) at 65°C for 30 min. After centrifugation for five min, isopropanol was added to an equal volume of supernatant and mixed well at room temperature. DNA was concentrated by centrifugation for 15 min. DNA pellets were dried and dissolved in 4 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). 20 μ l of Rnase A (10 mg/ml) was added and incubated at 37°C for 30 min before adding 4 ml of CTAB buffer (0.2 M Tris-HCl, pH 7.5, 2 M NaCl, 0.05 M EDTA DNA and 2% 3-p-cetyltrimethylammonium bromide). The mixture was incubated at 65°C for 15 min and extracted twice with an equal volume of chloroform: isoamyl alcohol and DNA were precipitated and washed with ethanol. The pellet was dissolved in 1 ml of TE buffer.

RAPD Method

Six arbitrary 10-mer primers, J-4 5' CCGAACACGG 3' and S-16 5' AGGGGGTTCC 3' (Srifah et al. 1996) and USDA4 5' GTCCGGATG 3', USDA5 5' CAGAAGCGGA 3', USDA7 5' CTGGACGTCA 3' and USDA8 5'GTGACCGAGT 3' (Kresovich et al. 1994) were selected from previous reports. PCR was performed in a volume of 10 μ l reaction containing 10 mM Tris-HCl (pH 9), 0.2 mM dNTP, 1.5 mM MgCl₂, 20 mg of 'Ya Faek' genomic DNA, 0.2 μ M each of those primers and 0.5 unit Taq polymerase (Perkin Elmer) with the thermal cycle of 35 cycles of 94°C for 1 min, 43°C for 1 min and 72°C for 2 min DNA fragment products were determined on 1% agarose gel.

SSCP Method

Two degenerate primers (5' TT/CT GGA CT/AA GGG CT/AT GGA C 3' and 5' ATG TCG/A/T GCG/A TAG/A TCT/CT TG/T GC 3') near consensus sequence flanking around 500 base pairs of SAD gene were used to perform PCR. 0.15 μ g of DNA was amplified with 0.5 μ M of primer and Taq polymerase (AmpliTaq Gold™, Perkin Elmer) using 1 cycle denaturizing at 94°C for 10 min and 35

cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. PCR products were subjected to SSCP electrophoresis by mixing 2 µl of PCR product with 9 µl of denaturing solution (95% formamide, 0.01 M NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue), heated at 94°C for 2 min, then chilled and subjected to non-denaturing polyacrylamide gel using 0.6x TBE running buffer and 2 watts constant power for 18 hours. DNA polymorphic bands were stained with silver nitrate and scored.

Data Analysis

Each of the amplification products was identified by its size and number of DNA fragments. The presence or absence of individual amplified product DNA bands was scored. A pair-wise difference matrix between ecotypes was determined in each variety of 'Ya Faek' by using the computer program NTSYS-PC version 1.80 (Applied Biostatistics Inc.).

Results and Discussion

RAPD Marker

In our laboratory, we have found that 8 out of 114 different 10-mer oligonucleotide primers from Operon Technology Ltd, J-4, J-14, P-2, Q-2, R-2, R-6, R-12 and S-16, generate the relative intensity bands which showed polymorphism of DNA profiles among the 'Ya Faek Don' and 'Ya Faek Hom' groups (data not shown). Several factors may cause the presence of faint bands, for example single copy of template DNA, tertiary folding of DNA, competitions between fragments for Taq polymerase during amplification. In contrast, two of those primers, J-4 and S-16, always give an intensity band very reproducible. Therefore, primer J-4 and S-16 are used routinely. In this experiment, we found that J-4 and S-16 were more successful than USDA4, USDA5, USDA7 and USDA8 for application of molecular diagnostics of 'Ya Faek Don' and 'Ya Faek Hom' groups in Thailand. Fig. 1 shows DNA polymorphisms generated by J-4 (S-16 and USDA data not shown). There is obviously one different DNA fragment at approximate size 650 base pairs that could be used as potential RAPD marker to identify the vetiver species.

SSCP Marker

Sunflower SAD gene marker has been developed to determine genetic variations of Thai vetiver in the intron region of $\Delta 9$ stearoyl-acyl carrier protein desaturase gene (SAD, EC 1.14.99.6) for oleic fatty acid synthesis by SSCP technique with two degenerate primers near consensus sequence flanking around 500 base pairs of SAD gene. The lengths of PCR products in the intron region of SAD allele between 'Ya Faek Don' and 'Ya Faek Hom' were variable. All 17 ecotypes of 'Ya Faek Don' were generated from two DNA fragments around 750 and 800 base pairs in length whereas 20 ecotypes of 'Ya Faek Hom' were 550 and 750 bp detected on 1% agarose gel. The polymorphic SSCP marker of each group of Ya Faek had three alleles, two of which were different in profiles on SSCP electrophoresis gel (Fig 1).

Relationships among Vetiver Ecotypes

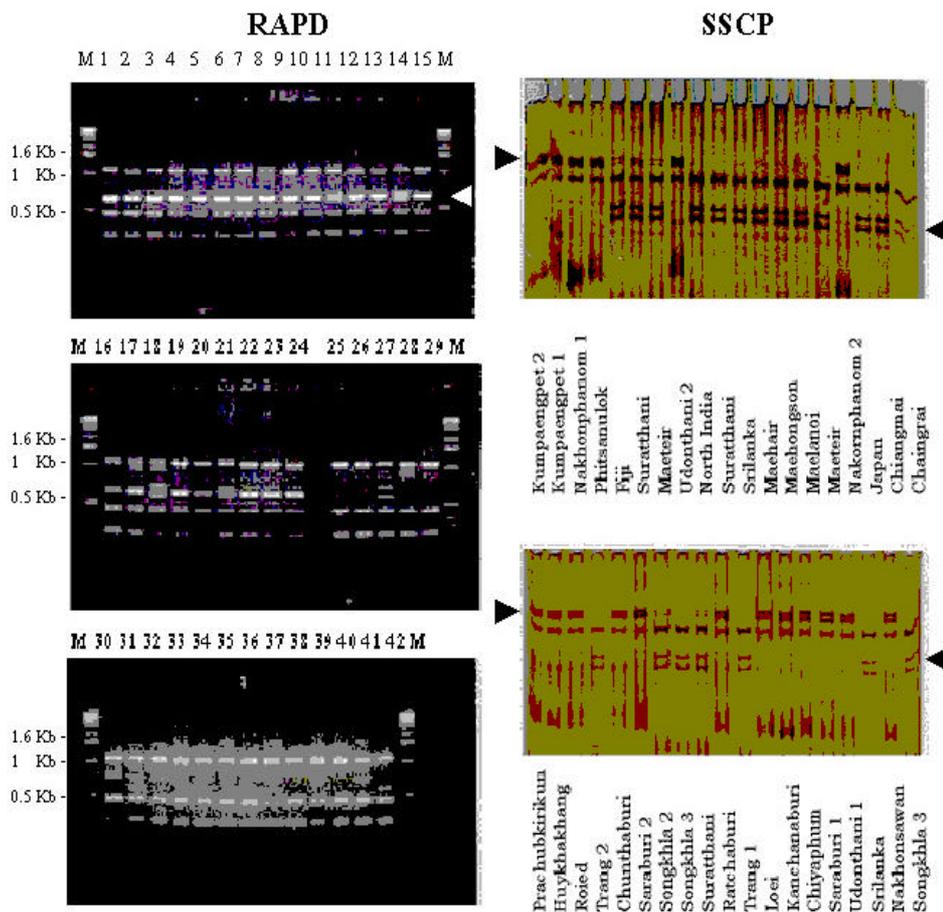
The RAPD amplifications resulted in 66 bands. In general, the size of amplified DNA fragments by 6 primers (J-4, S-16, USDA4, USDA5, USDA7 and USDA8) ranged from 400-2 300 base bp. We obtained an estimate of intragroup diversity for all primers by determining the pairwise distance matrix among pooled data of each DNA polymorphism of ecotypes computed by the NTSYS-PC program. In addition, the same samples have been re-amplified and re-analyzed at least twice. The Phylogenetic relationships were constructed from calculations based on the presence or absence of 1 435 specific RAPD bands.

All 42 'Ya Faek' ecotypes were readily placed into two distinct groups pattern type (Fig. 2). *Group 1*: 24 ecotypes of 'Ya Faek Hom' were divided into two subgroups; each clustered together except India-

Khao Kho was the most distinct one. *Group 2*: ‘Ya Faek Don’ - the population in this group is quite homogeneous. This homogeneity may reflect a geographically gene pool of the same clone of Thai vetiver and the nature of this weed favors in the vegetative propagation. The heterogeneity increase in ‘Ya Faek Hom’ group. One possible reason was that more than half of ‘Ya Faek Hom’ ecotypes in Thailand were imported from other countries as a gift. However, the DNA polymorphism present in the ‘Ya Faek’ ecotypes and the speed of the RAPD and SSCP markers make them feasible as a new and efficient tool for identification.

Polymorphism of the SSCP DNA product profile for SAD primer pairs among 38 ecotypes of Thai vetiver. Variation of intron length and conformation of single DNA in SAD alleles could be observed in lanes Prachuab Khirikhan, Huai Kha Khaeng and Roi Et for ‘Ya Faek Don’, and in lanes Trang 2, Songkhla 2, Songkhla 3, Surat Thani, etc., for ‘Ya Faek Hom’.

Fig. 1. Polymorphism of RAPD DNA product profiles for J-4 primer of 42 ecotypes of Thai vetiver grass ‘Ya Faek Hom’ (*V. zizanioides* Nash) in lanes 1-24 and ‘Ya Faek Don’ (*V. nemoralis* A. Camus) in lanes 25-42. Possible RAPD DNA marker was marked



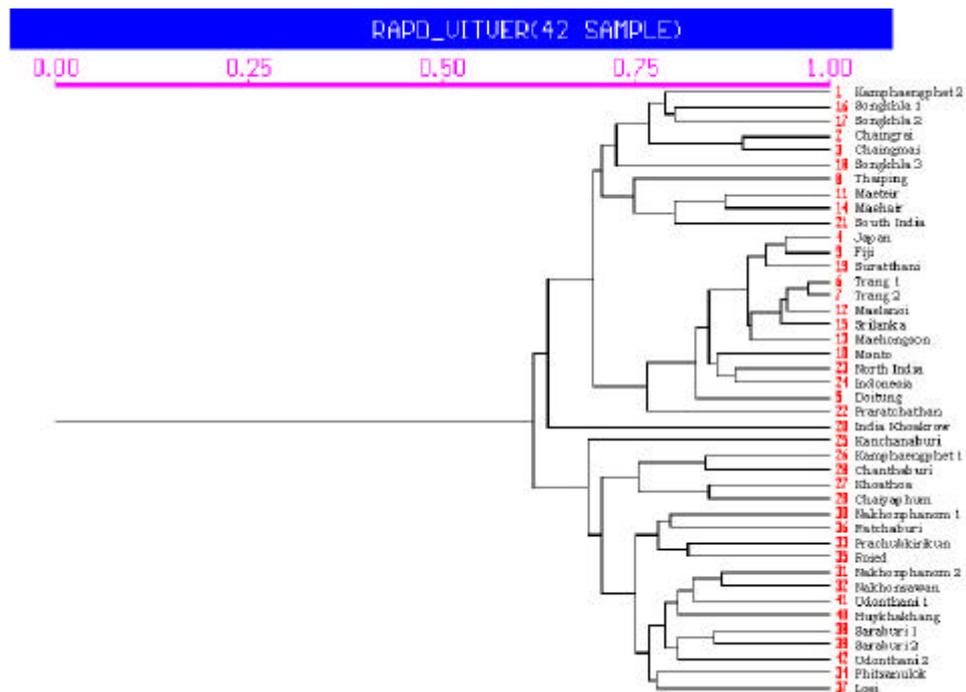


Fig. 2. Phylogenetic tree showing relationships based on the presence and absence of RAPD DNA products among 42 vetiver ecotypes found in Thailand

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