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Phylogenetic Analysis of *Cucumis sativus* Using RAPD Molecular Markers

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Abstract: In this study, the phylogenetic relationships among twelve lines of *Cucumis sativus* were studied using Randomly Amplified Polymorphic DNA (RAPD) markers. A modified CTAB DNA extraction protocol was introduced to obtain genomic DNA from young leaves. Of forty random primers tested, only fifteen primers were able to successfully amplify all genomic DNA samples and the results obtained were interpreted based on total and polymorphic scores. A total of 187 bands were produced in which 144 (~77%) were polymorphic. Additionally, a phylogenetic tree was constructed using the UPGMA analysis. Based on the results, the cucumbers examined could be divided into two major subgroups in which the similarity values were between 0.67-0.93.

Key words: *Cucumis sativus*, cucumber, RAPD, genetic diversity

INTRODUCTION

Identification of plant cultivars is essential not only to trace for their phylogenetic relationship but also to provide useful information for agricultural business. For example, the identified best cultivars giving highest yields or able to tolerate drought can be used to promote agricultural industry and subsequently for breeding programmes. Traditionally, plant diversity has been evaluated using morphological (tree height and canopy shape), chemical (essential oil compositions) and biochemical (isozymes) markers (Hickey, 1973; Ochocka *et al*., 2002; Cogolludo-Agustin *et al*., 2000). These approaches have their limitations and often produce unreliable results for specific differentiation. This is due to various kinds of external factors such as temperature, humidity and light which can alter plant phenotypes investigated by these systems (Bradshaw, 1965). In the past decade, a number of studies have introduced molecular markers to differentiate plant cultivars. These DNA-based techniques such as randomly amplified polymorphic DNA and Amplified Fragment Length Polymorphism (AFLP) are widely used since they are not influenced by environment and thus surpassing the drawbacks generated by phenotypic plasticity (Williams *et al*., 1990; Vos *et al*., 1995). The RAPD technique in particular is a simple and rapid means which can be used to detect polymorphic DNA sequences. The polymorphic DNAs are initially amplified by Polymerase Chain Reaction (PCR) using arbitrary oligonucleotide primers and subsequently separated by gel electrophoresis. The pattern of amplified DNA fragments is then determined for genetic relationship based on appropriate algorithm developed.

Cucumber (*Cucumis sativus* L.) grown in most temperate countries is one of the leading commercial vegetables in the global market ranking fourth after tomato, onion and cabbage (Tatlioglu, 1993). It is estimated that more than a million hectares of cucumbers were planted worldwide, yielding approximately 23 million metric tons of fresh product (Pitrat, 1999). Many countries have the intensive improvement programmes for cucumbers and thus generating several cucumber cultivars in response to specific environment as well as consumer preference. As a result,

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various kinds of hybrid cucumbers are available and although they often differ in morphology, their genetic base particularly to the commercial lines is not that heterogenous (Staub and Meglic, 1993). For cucumbers, genetic markers prove to be useful for cultivar characterisation including the use of isozymes (Staub and Meglic, 1993; Meglic and Staub, 1996), RFLP (Dijkhuizen *et al*., 1996) and RAPD (Horejsi and Staub, 1999). This study was performed as a part of cucumber breeding programme to evaluate the genetic diversity among indigenous cucumber cultivars preserved at Almatha Seeds, Co., Ltd., Thailand by using the RAPD technique.

MATERIALS AND METHODS

Plant Materials

Twelve lines of cucumber obtained as seeds from the Almatha Seeds, Co., Ltd., Thailand were grown during June-October 2005 in the experimental field of Mae Fah Luang University. They were given the codes as follows: ASC-01 (No. 1), ASC-03 (No. 2), ASC-04 (No. 3), ASC-05 (No. 4), ASC-06 (No. 5), ASC-11 (No. 6), ASC-12 (No. 7), ASC-13 (No. 8), ASC-14 (No. 9), ASC-16 (No. 10), ASC-17 (No. 11) and ASC-19 (No. 12).

DNA Extraction

The young leaves of cucumbers were used to extract genomic DNA using the modified method of Agrawal *et al*. (1992). Leaves were initially placed in a mortar and ground into fine powder in liquid nitrogen. The powder was then quickly transferred to the centrifuge containing 500 μL of DNA extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0 and 0.2% mercaptoethanol) and incubated at 65°C for 1 h. The suspension was subsequently extracted with equal volume of chloroform: Isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected to a new microfuge tube and the DNA was precipitated by addition of one volume of cold isopropanol and incubated at -20°C for 30 min. After centrifugation, the DNA pellet was resuspended in 500 μL of RNase buffer (10 mMTris-HCl pH 8.0, 15 mM NaCl). 2 μL of RNase A (10 mg mL^{-1}) was added and the DNA suspension was incubated at 37°C for 1h. The resulting solution was then extracted once with 400 μL of phenol: chloroform: isoamyl alcohol (25:24:1) and once with 500 μL of chloroform: Isoamyl alcohol (24:1) followed by ethanol precipitation and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The DNA samples were then used directly or stored at -20°C until required.

DNA Analysis by RAPD Technique

The RAPD analyses were carried out using forty random primers (Operon Technologies, USA). For PCR, a reaction of 15 μL volume was prepared consisting of 200 μM of dNTPs, 2.5 mM $MgCl_2$, 0.3 U Taq DNA polymerase, 5 pmol of each primer and 50 ng of DNA template. Thermal profile conditions were as follows: initial denaturation 10 min at 95°C, 40 cycles of 1 min at 93°C, 1 min at 37°C, 1.2 min at 72°C, with a final elongation of 5 min at 72°C. Amplification products were subsequently separated on 1.5% agarose gel with ethidium bromide staining.

Data Analysis

The DNA profiles generated were scored as discrete variables using 1 to indicate presence and 0 to indicate absence of a band. The data recorded were then used to calculate using the NTSYS-pc programme version 2.01e (Rohlf, 1990). A phylogenetic tree was then constructed using the unweighted pair-group method with arithmetical averages (UPGMA) as described by Sneath and Sokal (1973).

RESULTS AND DISCUSSION

In this present paper, we used RAPD analysis to investigate the relationship of twelve *C. sativus* cultivars. The genomic DNA extracted from their young leaves was shown to be high quality and thus ready to use as templates. For RAPD, the establishment of PCR conditions is required to ensure reliable and reproducible results. The optimum amplification was determined and presented as follows: 50 ng of template DNA, 2.5 mM of $MgCl_2$, 200 μM of dNTPs, 5 pmol of primers and 0.3 U of Taq enzyme in the 15 μL reaction mixture; such an optimum condition was best in generating the RAPD profiles (data not shown).

For preliminary screening, forty primers were used and it was found that only fifteen primers with a 60-70% GC content could successfully amplify all genomic DNA samples. The cucumber cultivars examined produced a total of 187 bands in which 144 (~77%) were polymorphic suggesting high genetic diversity among these tested cultivars. The amplified products were then categorised based on their size ranging from 438 to 11,600 bp and the numbers of bands produced ranged from 6 using OPC-19 and OPC-20 primers to 18 using OPA-12 primer (Table 1). It should also be noted that of the fifteen primers employed, there were only two primers producing the distinct bands for individual cucumber cultivars: OPA-18 for ASC-16 (No. 10) and OPA-04 for ASC-11 (No. 6) (Fig. 1).

The data obtained were then used to determine the genetic relationship of these cucumbers. Based on Nei's method (Nei and Li, 1979), the similarity values were calculated by scoring the total number and the polymorphic DNA number of bands produced with each primer. Similarity indexes ranged from 0.67 between ASC-04 and ASC-17 to 0.93 between ASC-03 and ASC-11 (Table 2). In addition, the results from the UPGMA cluster analysis were performed and used to construct the phylogenetic tree and showed as a dendogram in Fig. 2. As shown, there were two discernable clusters; the first contained solely the cucumber line ASC-17 whereas the rest formed the second group with higher genetic diversity.

RAPD analysis has proved to be a rapid, inexpensive and useful tool for assessing phylogenetic relationships in plant species. Particularly to *Cucumis* spp., various experiments have showed that the RAPD technique is appropriate for determining intra-genera relationship (Staub *et al*., 1997; Horejsi and Staub, 1999). In addition, it is also used successfully to reveal genetic diversity among African *C. sativus* (Mliki *et al*., 2003). This present study has been performed in which our results confirmed the valuable use of RAPD markers in Thai *C. sativus* cultivars developed by the Almatha Seeds, Co., Ltd. The data obtained are also expected to be important as genetic markers and useful for cucumber improvement programme.

Table 1: Primers used for *Cucumis sativus* identification by RAPD and the number of bands produced. Only fifteen arbitrary primers that successfully amplified all genomic DNA samples are given; they produced 187 bands in total

Primers	Sequences (5' to 3')	GC (%)	No. of polymorphic bands	No. of bands
OPA-04	AATCGGGCTG	60	14	15
OPA-05	AGGGGTCTTG	60	12	13
OPA-11	CAATCGCCGT	60	12	14
OPA-12	TCGGCGATAG	60	16	18
OPA-13	CAGCACCCAC	70	10	17
OPA-18	AGGTGACCGT	60	12	14
OPA-19	CAAACGTCGG	60	7	10
OPC-02	GTGAGGCGTC	70	8	11
OPC-04	CCGCATCTAC	60	13	16
OPC-05	GATGACCGCC	70	12	17
OPC-10	TGTCTGGGTG	60	6	7
OPC-14	TGCGTGCTTG	60	7	12
OPC-18	TGAGTGGGTG	60	10	11
OPC-19	GTTGCCAGCC	70	2	6
OPC-20	ACTTCGCCAC	60	2	6

Table 2: Similarity matrix from twelve lines of *C. sativus* generated from Nei's method (Nei and Li, 1979). The sample numbers from 1 to 12 are given according to the cucumber cultivars (see Materials and Methods)

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.000											
2	0.917	1.000										
3	0.874	0.929	1.000									
4	0.818	0.856	0.820	1.000								
5	0.806	0.833	0.817	0.868	1.000							
6	0.878	0.931	0.897	0.816	0.847	1.000						
7	0.860	0.916	0.890	0.852	0.847	0.901	1.000					
8	0.817	0.867	0.839	0.825	0.821	0.834	0.863	1.000				
9	0.781	0.807	0.773	0.758	0.810	0.814	0.821	0.812	1.000			
10	0.706	0.732	0.698	0.725	0.728	0.742	0.748	0.740	0.793	1.000		
11	0.682	0.707	0.673	0.699	0.712	0.699	0.731	0.690	0.731	0.686	1.000	
12	0.819	0.837	0.812	0.752	0.780	0.826	0.833	0.786	0.779	0.704	0.798	1.000

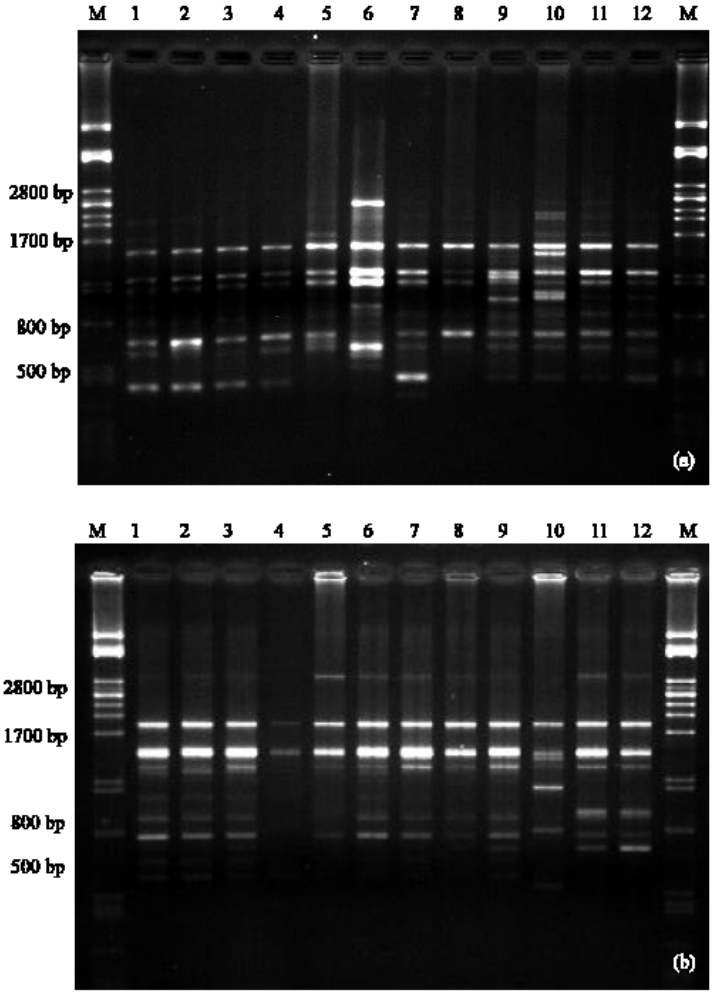

Fig. 1: Representative of RAPD profiles from the genomic DNA of *C. sativus* generated by primers OPA-04 (a) and OPA-18 (b). Lane M, Lambda DNA digested with *Pst*I; lane 1-12, sample numbers of cucumber cultivars

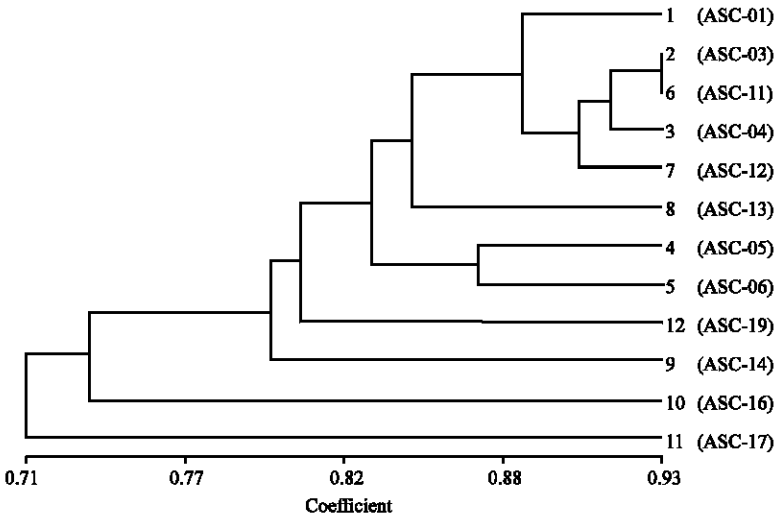

Fig. 2: Dendogram of *C. sativus* cultivars based on UPGMA cluster analysis of RAPD data generated by fifteen random primers

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