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MOLECULAR CHARACTERIZATION OF SOYBEAN CULTIVARS USING MICROSATELLITE AND RAPD MARKERS

El-Shemy, H. A.; M. I. Kobeasy and S. H. Ahmed
Dept. of Biochemistry, Fac. of Agric, Cairo Univ.

ABSTRACT

Microsatellite markers or SSR (Simple Sequence Repeats) and RAPD have proved to be an excellent tool for cultivars identification, pedigree analysis and the evaluation of genetic distance among organisms. Soybean cultivars have been characterized mainly by morphological and biochemical traits. However, these traits have not been sufficient to characterize the large number of cultivars eligible to receive protection. In order to define new soybean cultivars markers the RAPD and SSR markers were used to distinguish the morphologically similar groups. A dendrogram based on the RAPD and SSR profiles showed a good agreement with the cultivars pedigree information.

Keywords: RAPD, SSR (Simple sequence repeat) microsatellites, molecular markers, soybean cultivars.

INTRODUCTION

Plant breeders have traditionally used morphological and biochemical traits to register and protect their varieties. Although these traits remain predominant and important, they present limitations, particularly in closely related cultivars. In plants with a narrow genetic base in their gene pool, such as soybean, they may not be sufficient, taking into account the large number of cultivars eligible to be protected. In such cases, molecular descriptors can provide additional information about the characterization, degree of diversity and genetic constitution of the existing germplasm.

Microsatellites or SSR are sequences of a few repeated and adjacent base pairs, well distributed over the eukaryote genome (Powell *et al.*, 1996). Variations in the number of repeats can be detected by polymerase chain reaction (PCR), with the development of primers (20 to 30 base pairs) specifically built for amplification and complementary to single sequences flanking the microsatellite.

Soybean is an excellent protein source for farm animals and is also an important component of the human diet. Soybean seeds contain cells called protein bodies in which storage proteins are preserved. The protein fraction of soybean seeds has gained importance mainly due to an increase in the standard of living throughout the world and the need for more protein for animals. SDS-PAGE technique was used to construct the phylogenetic tree of some soybean cultivars (El-Shemy *et al*, 2000). Mean-while, the Brazilian authors determined the number of alleles and the gene diversity of trinucleotide loci in a group of soybean cultivars fit to be grown in Brazil (Priolli *et al*, 2002). In this work we described procedures for construction the poputaion genetics of soybean obtained from various parts.

MATERIALS AND METHODS

Soybean plant material and DNA isolation

A group of 11 soybean cultivars, was selected to represent the complete range of cultivars. The cultivars are listed in Table 1. Five plants of each soybean cultivar were grown in a greenhouse for DNA isolation. 4 leaf tissue samples were collected from each cultivar, frozen in liquid nitrogen and lyophilized for 1-2 days. DNA was isolated from the bulked lyophilized leaf tissue (of the plants) of each cultivar by a mini-prep procedure based on Doyle and Doyle (1990). DNA was evaluated by electrophoresis in 0.8% agarose gel stained with ethidium bromide.

Solutions required

1- Extraction buffer: 100 mM Tris-HCl (pH 8), 50 mM EDTA, 1.4 M NaCl, 2% CTAB (Cetyltrimethyl ammonium bromide) .
2- 5M potassium acetate, phenol-chloroform-isoamylalcohol (25:24:1 [v/v/v])
3- TE buffer: 10 mM Tris-HCl (pH 8), 0.1 mM EDTA

DNA isolation

1. Dry leaf tissue (200 mg) was ground to a fine powder in liquid nitrogen.
2. Prewarmed extraction buffer in the amount of 800 uL was added to the sample and ground once more in the buffer.
3. After the samples were taken to the 1.5-mL eppendorf tubes, 4 uL proteinase K (10 mg/mL) were added.
4. The solution was incubated in 37°C for 20 min and then incubated in 65°C for, another 20 min.
5. Following these incubation periods, samples were cooled to room temperature, 250 uL of 5 M potassium acetate were added, and another incubation period on ice was employed for 30 min.
6. The, samples were centrifuged at 17,000 *g* (14,000 rpm) for 15 min, and the supernatant was transferred to a new clean tube.
7. Samples were extracted with 500 uL phenol-chloroform-isoamylalcohol (25:24:1) with gently mixing by inversion 40-50 times. At this stage, it was observed that incubating the samples for at least for 15 min on ice increased the efficiency of DNA yield.
8. After centrifugation for 5 min at 17,000 *g*, supernatant was transferred to a fresh tube, and equal volume of isopropanol was added. Samples were incubated on ice for 15 min. This step is not mandatory, but it can be prolonged until several hours.
9. The samples were then centrifuged for 5 min at 17,000 *g*, and 76% ethanol were added to the pellet for washing them centrifuged once more for 10 min at 17,000 *g*.
10. The pellet was once more washed with 70% ethanol optionally and air-dried until all ethanol was removed.
11. The obtained nucleic acids pellet was dissolved in an appropriate amount of TE buffer (30-60 uL).
12. The nucleic acids dissolved in TE buffer were treated with ribonuclease (RNase, 10 mg/mL) and stored at –20°C until use. DNA was quantified via spectrophotometric measurement of UV absorption at 260 nm

(Shimadzu UV-260). DNA was also quantified by means of agarose gel electrophoresis with ethidium bromide fluorescence and a DNA ladder as the DNA size marker.

SSR loci

Eleven pairs of soybean primers flanking the microsatellite regions, previously developed and published by Cregan *et al.* (1999), were selected. They were coded as Soy 1, Soy 2, Soy 3, Soy 4, Soy 5, Soy 6, Soy 7, Soy 8, Soy 9, Soy 10, and Soy 11. The sequences of the Forward and Reverse primers are available at the soybean Website USDA-ARS Soybean Genome Database. The primers comprise 11 of the 20 soybean linkage groups.

PCR analysis

Different parameters were detected for optimization of PCR reactions. PCR reactions for RAPD and SSR analysis were performed in a 25-uL volume containing 200 ng of genomic DNA, 2.5 uL of 10X reaction buffer, 4 uL of 25 mM $MgCl_2$, 2 uL of 2.5 mM dNTPs, 200 ng primer, and 1 U *Taq* DNA polymerase (Promega).

The reactions were heated in an initial step of 94°C for 2 min and then subjected to 30 cycles of the following program: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified DNA was electrophoresed in a 1% agarose gel containing 0.5 mg/mL ethidium bromide and photographed on a UV transilluminator.

Amplification products also were separated in denaturing gels containing 10% polyacrylamide, 8 M urea and 1 X TBE, during approximately 4 h at 15 mA. The size of each band was estimated by DNA Ladder. Amplified SSR fragments of different sizes were considered as different alleles. The fragments were detected by silver staining, following the Sanguineti *et al.* (1994) protocol.

A genetic dissimilarity coefficient was calculated for each pair of cultivars, according to Diwan and Cregan (1997), to determine the effectiveness of the group of 11 SSR loci in distinguishing each of 11 soybean cultivars.

Table (1) The soybean cultivars used in the present study.

No.	Cultivar	Origin
1	Sanga	USA
2	Tamahomare	Japan
3	Lee	China
4	Athow	USA
5	Macon	USA
6	Gin-Daizu	Japan
7	Fukuyataka	Japan
8	Akishiroma	Japan
9	Crawford	Egypt
10	Cutler	Egypt
11	Clark	Egypt

RESULTS AND DISCUSSION

Figures 1 and 2 show RAPD and SSR differences among soybean cultivars from a narrow genetic base. The primers used differed in size of amplified products (Fig. 1), the highest variation (250-1500 pb) were observed for Tamahomare, Lee and Fukuyataka, cultivars. On the other hand, the smallest one was for Clark line and the cultivars of Athow, Macon and Gin-Daizu did not show any variation (Fig. 1). As new cultivars are developed and released, other comparisons will become important. Phylogenetic trees are genealogical trees that are formed with the data gained from the comparison of PCR products in samples from different cultivars (Fig. 1 and 3) using agarose electrophoresis patterns. The close relationship between some of the soybean cultivars tested (Tamahomare and Lee, Athow and Gin-Daizu, Macon and Akishiroma, Sanga and Cutler) suggests that the cultivars have been produced by one genetic source. Some cultivars, such as Clark and Crawford and Namashixu, were genetically very different (Fig. 1 and 3). Nuswantara *et al.* (1997) investigated the phylogenetic position of rhizobial strains isolated from the root nodules of *A. mangium* at various locations in Indonesia by the analysis of PCR-amplified 16S rRNAgenes. A previous study using AFLP (amplified fragment length polymorphisms) and SSLP (amplified sequence length polymorphisms) on a series of closely related Japanese rice showed that both types of markers were suitable for detecting genetic variation between cultivars, (Mickill *et al*, 1996) with both AFLP and SSLP identifying a high level of polymorphism. Bligh *et al.* (1999), demonstrated that SSLP was a more robust and efficient method than AFLP for the identification of rice cultivars.

Banding profiles obtained in Figure (1) were separately sufficient to identify all materials studied. These results suggested that a small number of RAPD primers are sufficient to prepare genotype specific banding patterns for soybean cultivars identification.

One major application of microsatellite markers in viticulture is the identification and the distinction between cultivars (Thomas *et al.* 1994; Bowers *et al.* 1996; Sefc *et al.* 1998). Therefore, the potential of the markers to yield different genotypes for as many cultivars as possible is of great interest, and selection of the most-informative markers reduces the number of loci to be investigated for reliable cultivar distinction.

All the 11 SSR markers were polymorphic as shown in Figure (2). The results obtained confirm the usefulness and suitability of SSR markers for soybean cultivars identification. The highest variation was observed for Clark line compared with the lowest one such as Pickett, Pickett 71 and Pecking (Fig.2).

The polymorphism of SSR loci detected in this study was consistent with previous studies by Akkaya *et al.*, (1992), Morgante and Olivieri (1994), Maughan *et al.* (1995), Doldi *et al.* (1997) and Narvel *et al.* (2000), but lower than that was obtained by Rongwen *et al.* (1995) and Diwan and Cregan (1997).

Dendrogram of SSR results was construct and the data showed that a close genetic distance relationship between some of the soybean cultivars

such as Tamahomare and Lee, Athow and Macon, Sanga and Cutler (Fig 4). On the other hand, the cultivars of Clark and Crawford and Gin-Daizu, were genetically very different (Fig. 2 and 4)

In comparison between the results of RAPD and SSR markers there are similarity in both dendograms. One possible reason for this difference is that the materials used in the present study were all from breeding programs, thus having a relatively narrow genetic base.

However, Song *et al.* (1999), using this procedure, detected 10 new alleles in 66 soybean cultivars, and Narvel *et al.* (2000) recorded 32 alleles specific for elite cultivars, within a total of 397 alleles that had been detected in 40 lines and in 39 soybean cultivars. Abdelnoor *et al.* (1995) did not find much variation in the genetic distances among 38 Brazilian soybean cultivars, as estimated by RAPD molecular markers. The obtained data suggest that this group of 11 microsatellite loci can be used to distinguish soybean cultivars from each other, in as much as 98.9% of the assayed cultivars could be identified. Furthermore, in referring to some morphological traits, identical cultivars could be distinguished by the same SSR loci.

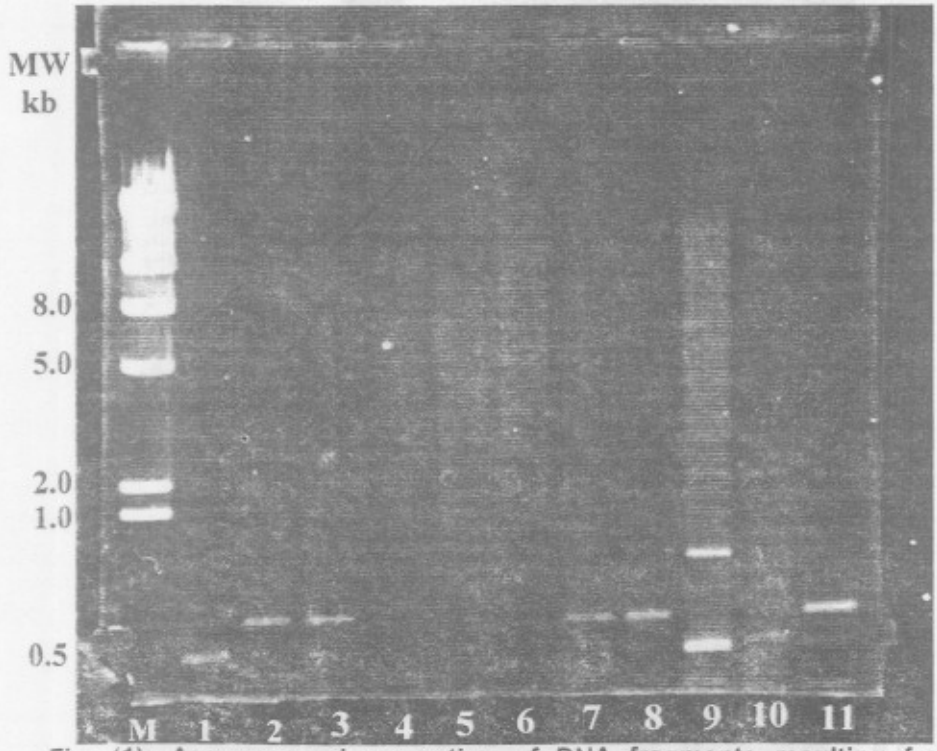

Fig. (1): Aagarose gel separation of DNA fragments resulting from amplification of DNA of various soybeans with RAPD.
The lane M was loaded with DNA standard and lanes from 1 to 11 were loaded of PCR products.

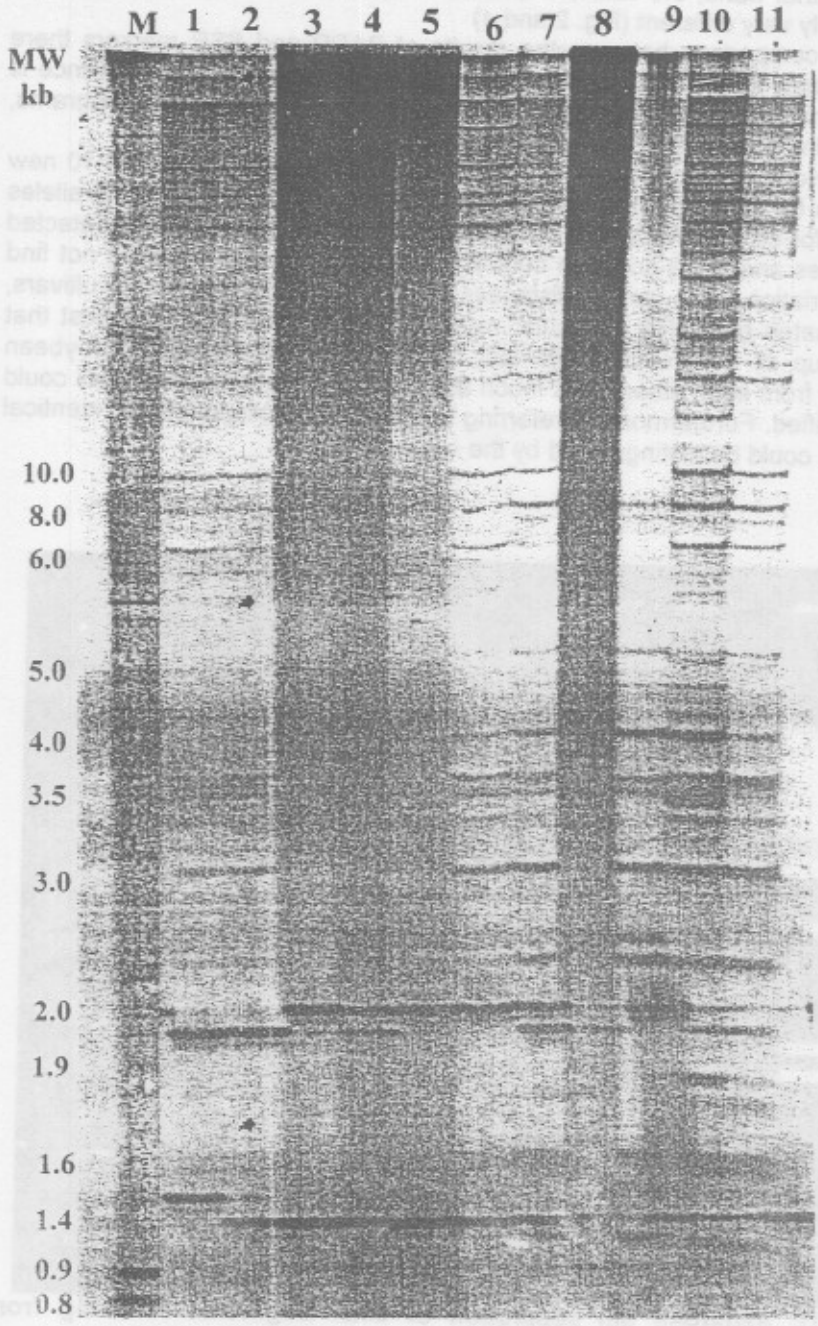

Fig. (2): Polyacrylamide gel showed the banding pattern of SSR for soybean cultivars

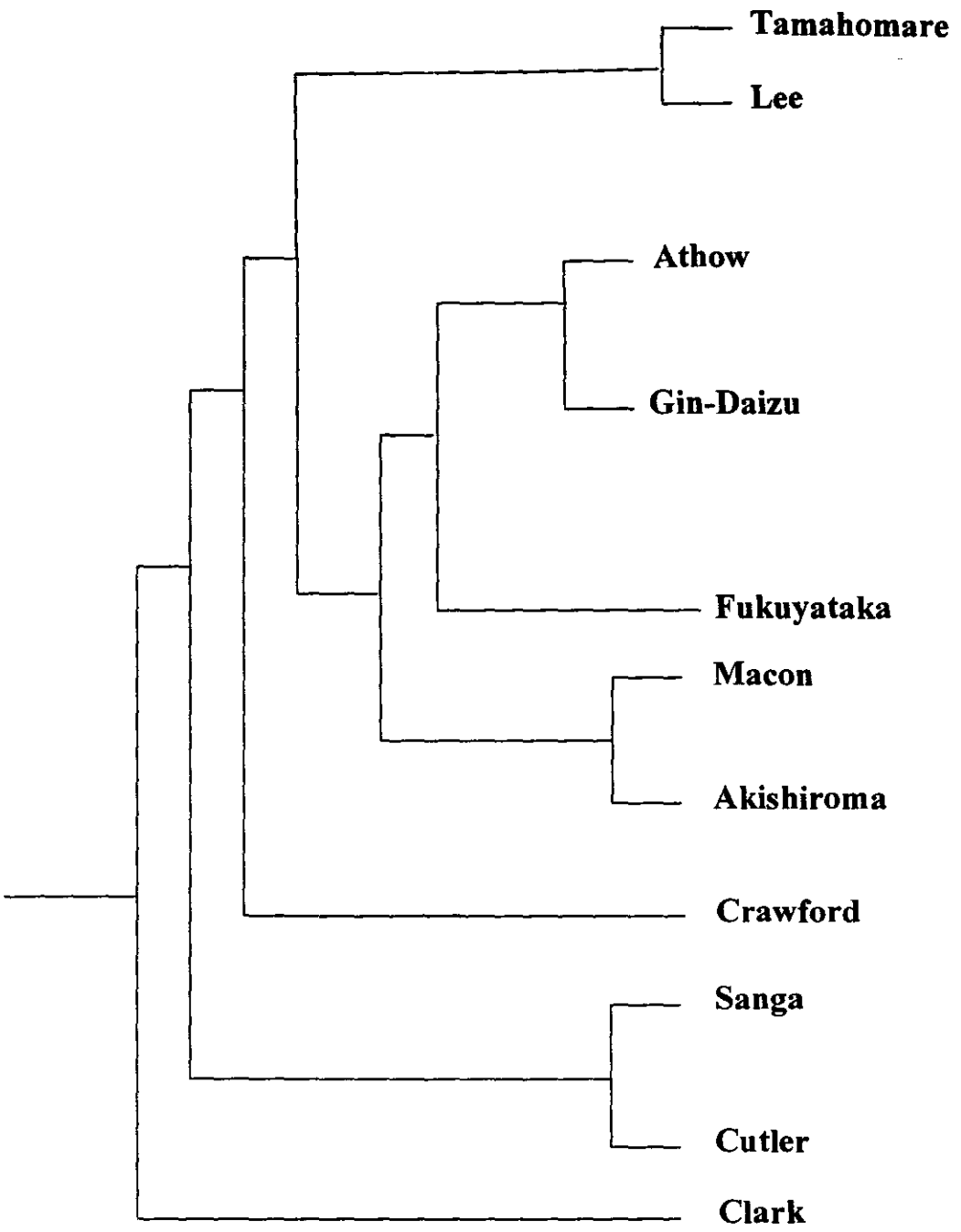

Fig. (3): Dendrogram of 11 soybean cultivars. Genetic distance were based on the information for 11 microsatellite loci.

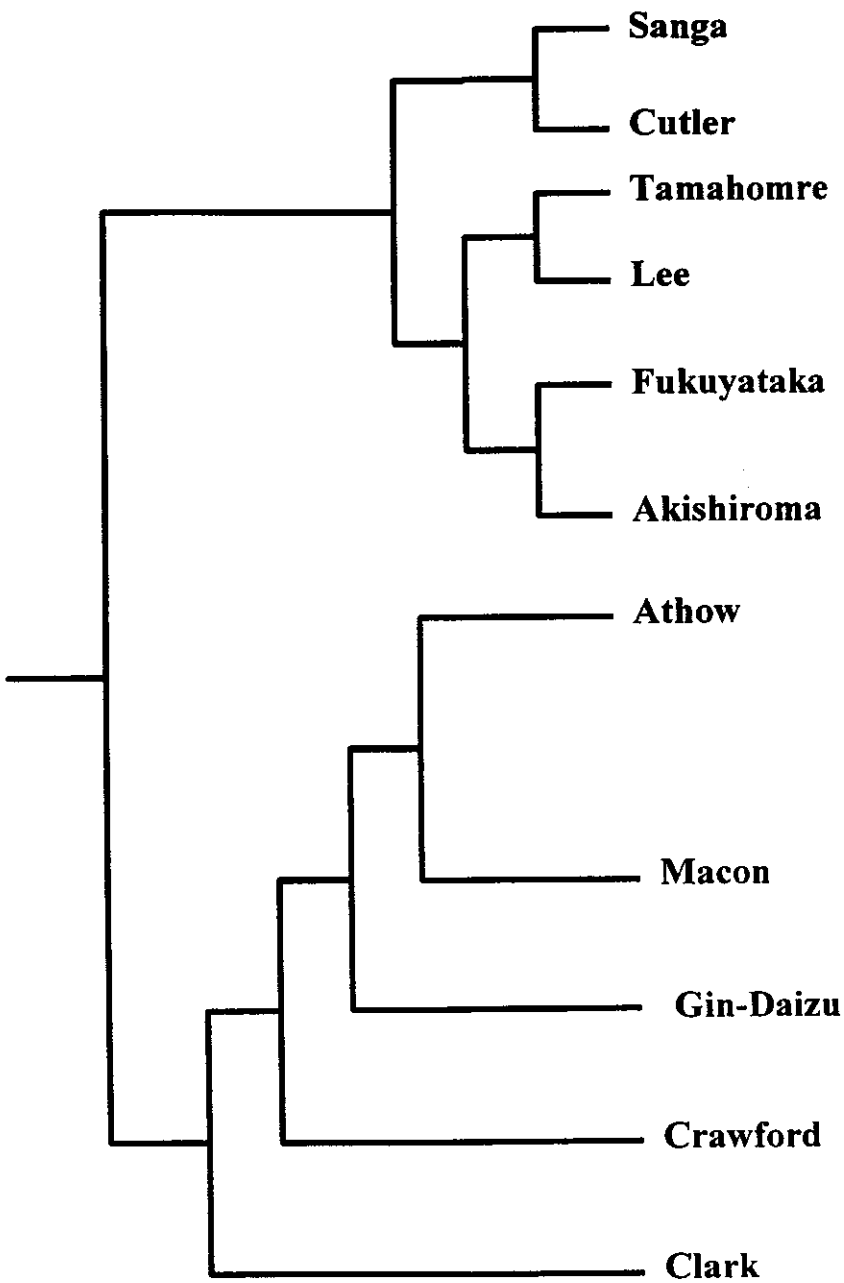

Fig. (4): Dendrogram of 11 soybean cultivars. Genetic distance were based on the information of RAPD gel analysis.

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الصفات الوراثية لأصناف فول الصويا باستخدام تكنيك الميكروستالايت والرابيد

هانى عبد العزيز الشيمى، محمد ابراهيم قبيصى، شريف حلمى أحمد

قسم الكيمياء الحيوية – كلية الزراعة – جامعة القاهرة

نظراً لتعدد أصناف بعض المحاصيل الهامة ومنها فول الصويا كان لابد من إيجاد طريقة لتصنيفها والتعرف عليها بدقة ولذلك يعتبر تكنيك الميكروستالايت والرابيد RAPD من الطرق الناجحة لعمل درجة القرابة الوراثية بين الأصناف المختلفة لهذا المحصول الهام وبذور فول الصويا كان يتم تصنيفها من قبل بالطرق المورفولوجية والطرق الكيمياوية الحيوية ولكن هذه الطرق كانت غير كافيه لتعريف الأصناف الجديدة وحمايتها ولهذا فى هذه الدراسة تم استخدام كل من تكنيك الميكروستالايت والـ RAPD لدراسة درجة القرابة الوراثية بين اصناف فول الصويا المختلفة وأظهرت الدراسة وجود تنوع فى درجة القرابة وأيضاً أشتراك بعض الأصناف فى بعض الصفات المتشابهة.