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Full Length Research Paper

Studies of the genetic diversity of seven sweetsop (*Annona squamosa* L.) cultivars by amplified fragment length polymorphism analysis

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Seven sweetsop germplasm resources were studied, and amplified fragment length polymorphism (AFLP) analysis system was established by detecting the results of several reactions, such as DNA extraction, enzyme restriction, ligation, preamplification and selective amplification in the reaction system of AFLP. The seven germplasm resources of sweetsop were studied by using AFLP technique, and UPGMA clustering results showed genetic distance of 0.572 ~ 0.818. Small genetic distance was 0.572 between 'Yuanhua' sweetsop and other sweetsop cultivars; high genetic distance was 0.818 between 'AP' and 'Fenglishijia' sweetsop. These results suggested that there was relative family relationship between them. This aims to study the family relationship in seven sweetsop germplasms resources, in order to select parents in sweetsop breeding and grafted rootstock.

Key words: Sweetsop, DNA extraction, amplified fragment length polymorphism (AFLP), genetic diversity.

INTRODUCTION

Sweetsop (*Annona squamosa* L.) is a species of *Annona*, native to the tropical Americas, India and Pakistan, and was planted in the mainland of China in the Mid-17th century. Its exact native origin was unknown due to its extensive cultivation, but is thought to be in the Caribbean. The species was described in Jamaica. It was planted more in Central and South America, Philippines, Thailand, Malaysia, India, and Fujian, Hainan, Guangxi, Guangdong, Yunnan Provinces in mainland of China. It is a semi-evergreen shrub or small fruit tree reaching 6 to 8 m tall. The leaves are alternate, simple, oblong-lanceolate, 5 to 17 cm long and 2 to 5 cm broad. The flowers are produced in clusters of 3 to 4; each flower is 1.5 to 3 cm across, with three large petals and three minute ones, having yellow-green spotted purple at the base. The fruits are usually round, slightly pine cone-like, 6 to 10 cm in diameter and weighing 100 to 230 g, with a scaly or lumpy skin. There are variations in shape and size. The fruit flesh is sweet, white to light yellow, and resembles and tastes like custard. It can maintain the charming appearance, strengthen immune system of the body, prevent scurvy, and act as an anticancer; it is known as the upper tonic, high nutritional value in the ancient times of China (Xie et al., 2009). The edible portion coats the seeds generously, a bit like the gooey portion of a tomato seed. Sugar-apple had a very distinct, sweet-smelling fragrance. The texture of the flesh that coats the seeds was a bit like the center of a very ripe guava (excluding the seeds). It was slightly grainy, a bit slippery, very sweet and very soft. In Annona per 100 g flesh fruit, it contained 8.36 g water, 18.0~26% soluble solids, 0.6~0.7% minerals, 0.14~0.3% fat, 265 mg vitamin C, 23.9% carbohydrate, 0.42% organic acids, 15.3~18.3% total sugar, 0.2% calcium, 1.0% iron, 0.04% phosphorus, 1.55% protein (Fruit roll, 1993). The seeds were scattered throughout the fruit flesh; the seed coats

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Abbreviations: PCR, Polymerase chain reaction; **CTAB,** cetyl trimethyl ammonium bromide; **EDTA,** ethylene diamine tetraacetic acid; **AFLP,** amplified fragment length polymorphism; **BSA**, bulked segregant analysis; **RAPD,** random amplified polymorphic DNA.

were blackish-brown, 12–18 mm long, and hard and shiny.

There were also new varieties being developed in Taiwan-pineapple sugar-apple, which was similar in sweetness but had a very different taste. Like the name suggests, it tasted like pineapple. The arrangement of seeds was in spaced rows, with the fruit's flesh filling most of the fruit and making grooves for the seeds, instead of the flesh only occurring around the seeds. Amplified fragment length polymorphism (AFLP) was a technique developed for genomic DNA fingerprinting (Pieter et al., 1995). It combined the techniques of restriction endonuclease digestion and polymerase chain reaction (PCR) amplification of restriction fragments, and thus possesses the advantages of these two techniques. AFLP analysis had a higher resolution and sensitivity than RAPD analysis in revealing allelic polymorphism (Barker et al., 1999), with good stability and repeatability characteristics of RFLP. Hence, it was suggested to be a good technique for studying genetic variation (Mueller et al., 1999). This technique had been widely applied to study genotyping, population differentiation (Bryang et al., 2002) and genetic diversity in a wide variety of organisms (Fang et al., 2001; Van et al., 2002), unknown gene cloning, localization of unknown gene, gene expression and regulation (Ei et al., 2002; Sun et al., 2001; Wang et al., 2001). AFLP analysis technology could also combine with other methods, such as bulked segregant analysis (BSA), low acid gene cloned from apple with both combinations success (Yu et al., 2007). In this study, AFLP technology was used to study genetic polymorphism in seven sweetsop germplasms resources, in order to select parents in sweetsop breeding and grafted rootstock.

MATERIALS AND METHODS

'Fenglishijia' sweetsop (*Annona atemoya*), 'Niuxin' sweetsop (*Annona reticulat)*, 'Milu' sweetsop (*A. reticulat)* 'Yuanhua' sweetsop *(Annona glabra*), 'AP' sweetsop (*A. atemoya)*, 'Ciguo' sweetsop (*Annona muricata)*, 'Shanci' (*Annona Montana)*, were planted in Tropical Germplasm Resourse Research Institute, Hainan China.

DNA extraction

Genomic DNA was extracted from fresh leaves using improved CTAB method (Dellaporta et al., 1983). Leaves (2 to 3 g) from field-grown plants were excised, washed thoroughly and blot dried. The midrib was removed and the leaves were homogenized to a fine powder using liquid nitrogen before adding 10 ml of lysis buffer (freshly prepared 100 mM Tris–HCl, (pH8.0); 20 mM EDTA, (pH8.0); 1.4 M NaCl; 2% CTAB; 2% (w/v) polyvinylpyrrolidone-40,000; 2%β-mercaptoethanol). The homogenate was mixed thoroughly by inverting the tube several times and incubated in a water bath at 65°C for 1 h. Equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly by gently inverting the tubes for 10 min. After centrifugation at 4,000 rpm for 10 min at 4°C the upper aqueous layer was collected in a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently by inverting the tubes for 10 min and centrifuged at 4000 rpm for 10 min at 4°C. Upper aqueous layer was collected and an equal volume of isopropanol was added, mixed gently by inverting the tubes and kept at -20°C for 1 h. DNA precipitate was pelleted by centrifugation at 8000 rpm at 4°C for 10 min and the supernatant was removed. The pellet was washed with 70% ethanol, air-dried and dissolved in 0.5 ml TE (10 mMTris–HCl, pH 8.0; 1mM EDTA, pH 8.0) containing 0.2 mg/ml RNase. The tubes were incubated at 37°C for 1 h and 1/3 volume 3 M sodium acetate (pH5.2) was added, and kept at 4°C for 1 h. DNA precipitate was pelleted by centrifugation at 12,000 rpm for 10 min. Upper aqueous layer was collected, and DNA was precipitated by adding 1/10 volume 3 M sodium acetate (pH 5.2), and 2 volumes of ethanol and kept at -20°C for 1 h - overnight. DNA was pelleted by centrifuging at 10,000 rpm, washed with 70% ethanol, air dried and dissolved in TE (10mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0).

AFLP reactions

DNA templates for AFLP reactions were generated by restriction digestion and ligation. Initially, about 100 ng of total DNA was digested with 5U of *Eco*RI and *Mse*I in 10× buffer 2ul, at 37°C for 12 h. To generate DNA template for subsequent PCR amplification, the digested DNA fragments were ligated with 10 pmol of *Eco*RI and 10 pmol *Mse*I adapters in a reaction mixture containing 10×buffer 2 µl, 0.1 U T_4DNase I at 16°C for 10 h. Preamplification PCR reaction was conducted using a MJ Mini^{TM} Personal Thermal cycler (BIO-RAD) with a pair of primers containing a single selective nucleotide. Amplification was performed at the cycling conditions for 4 min at 94°C, followed by 30 cycles of 94°C for 60 s, 55°C for 30 s, 72°C for 120 s and a final extension of 72°C for 10 min. The 20 µl PCR product mixture was diluted 10-fold with distilled water and used as templates for the subsequent selective PCR amplification. The selective amplification was performed using three pairs of primers, *Eco*RI-CGA/ *Mse*I-CAA, *Eco*RI-AGG/*Mse*I-CTA and *Eco*RI-ACG/*Mse*I-CTG. Fingerprint patterns were visualized on a 6% denaturing polyacrylamide gel using silver staining method.

Gel electrophoresis and silver staining

The PCR products were mixed with 10 ul AFLP loading buffer (99% formamide, 10 mM EDTA, 0.05% bromophenol and 0.05% xylene cyanol). The product mixtures were denatured and concentrated at 90°C for 20 min, and quickly cooled down in an ice bath after denaturation. A 6% denaturing polyacrylamide gel was prerun at 120 W for 30 min. Each well was loaded with 10 µl of sample. The gel was electrophoresed for 2.5 h in a BioRad Sequi-Gen GT DNA sequencing cell (38 × 50 cm) at 110 W and 50°C. Silver staining procedures were derived and modified from Merril et al. (1979). After electrophoresis, the gel was fixed in 1% ethanoic acid for at least 30 min. The gel was rinsed in distilled water and stained with a mixture of 0.2% silver nitrate and 0.007% benzene sulphonic acid for 30 min. The stained gel was rinsed again with distilled water and immersed in a developing solution (2.5% sodium carbonate, 0.037% formaldehyde, 0.002% sodium thiosulphate). The development was subsequently stopped with 1% ethanoic acid when the bands became visualized and reached desirable intensity. Band sizes were estimated by a standard AFLP DNA ladder and analyzed using the Gel Imaging Analyzing System.

Data analysis

AFLP bands were scored manually as (1) for presence or (0) for

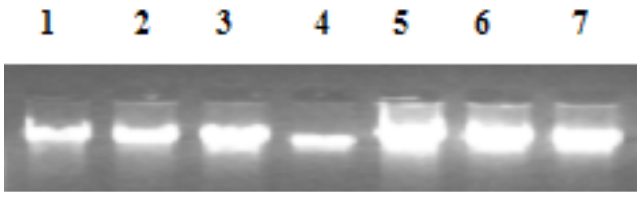

Figure 1. Sweetsop DNA extraction with improved CTAB method. 1, 'Yuanhua' *A. glabra*; 2, 'AP' *A. atemoya*; 3, 'Shanci' *A, Montana*; 4, 'Ciguo' *A. muricata*; 5'Milu' *Annona cherimola*, 6, 'Fenglishijia' *A. atemoya*, 7, 'Niuxin' *A. reticulat*.

absence, and transformed into 0/1 binary character matrix. Fragments that could not be scored unambiguously were not included in the analysis. The total number of individuals analyzed was 30, with 5 to 7 individuals for each species. The data were analyzed using a set of programs NTSYS2.10.

RESULTS

DNA extracted by CTAB method and digested by *Mse*I/*Eco*RI

DNA was extracted from the leaves of varieties sweetsops by improved CTAB method. DNA samples were ivory that easily dissolved in water and TE; value obtained was 1.7-1.9 using spectrophotometer with OD_{260}/OD_{280}. DNA was obviously main belt, relatively complete, having no protein pollution, but with obvious impurity in 1% agarose gel electrophoresis (Figure 1). DNA samples were digested with *Mse*I/*Eco*RI, and products were eletrophoresised in 1% agarose gel (Figure 2A).

Pre-amplification and selective amplification

Product of pre-amplification ranged from 500 to 2000 bp on the 1.2% agarose gel electrophoresis with a pair of primers, containing a single selective nucleotide (Figure 2B). In the reaction process, dNTPS, Mg^{2+} concentration, Taq polymerase concentration, and reaction progress were appropriately optimized. The seven samples were analysed through appropriate reaction conditions, having good results in polyacrylamide gel electrophoresis (Figure 3).

Clustering analysis

The seven DNA templates for AFLP reactions, the three pairs of primers sets yielded a total of 662 scoreable bands (size range: 80-550 bp). The number of bands generated from a single individual ranged from 80 to 122, while a single species produced 90 to 130. The similarity coefficient of seven samples ranged from 0.572 to 0.818. There was minimum similarity coefficient (0.572) between

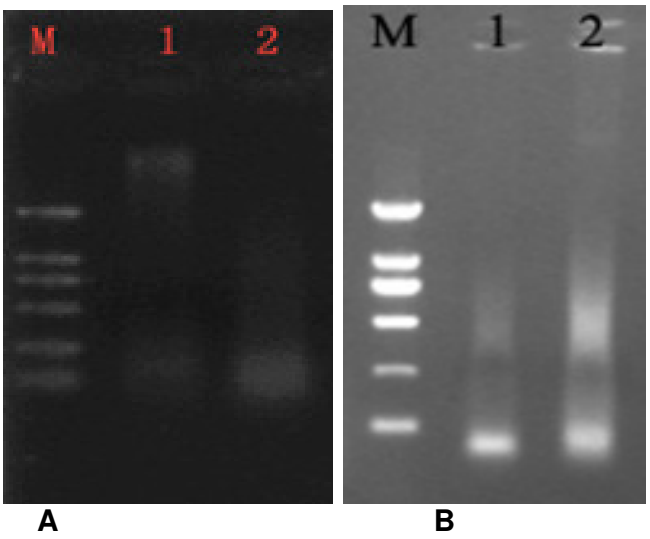

Figure 2. The results of 'AP' sweetsop by enzyme restriction and preamplication. M: DNA marker (DL2000); A1: *Eco*RI restriction; A2: *Mse*I restriction; B1: the result of AP sweetsop by selective amplification, B2: the result of AP sweetsop by preamplification.

'yuanhua' and other varieties, and the highest similarity coefficient (0.818) between 'AP' and 'Feilishijia' (Figure 4). The similarity coefficient was 0.782 between 'Niuxin' and 'Ciguo', and 0.717 between 'Milu' and 'Shanci'. It was shown that the genetic relationship was close between 'AP' and 'Feilishijia', and farther with 'yuanhua'.

DISCUSSION

The fruit and leaves of sweetsops contained more protein, polysaccharide, and polyphenols. DNA of samples was extracted by improved CTAB method. DNA of the samples could digest with *Mse*I/*Eco*RI, and fit for AFLP analysis. The appropriate reaction system and progress were taken in this study. AFLP technology was a highly reproducible DNA fingerprinting technique (Jones et al., 1997). The reproducibility was associated with the consistency of amplification using specific selective primers, with PCR conditions optimized at a high annealing temperature. The use of polyacrylamide gel electrophoresis for separation of PCR products had greatly enhanced the resolution and sensitivity in the identification of alleles (Koeleman et al., 1998). AFLP technology was widely used in studies of population differentiation and genetic diversity in a variety of plants.

In this study, we attempted to use the AFLP fingerprints in phylogenetic analysis of the seven sweetsop species. The result showed that individuals of the same species were grouped into distinct clades. AFLP markers could reveal genetic diversity of the seven varieties and the genetic relationship between each other. The results from this study suggest that distance analysis based on AFLP fingerprints was of good phylogenetic capacity at the

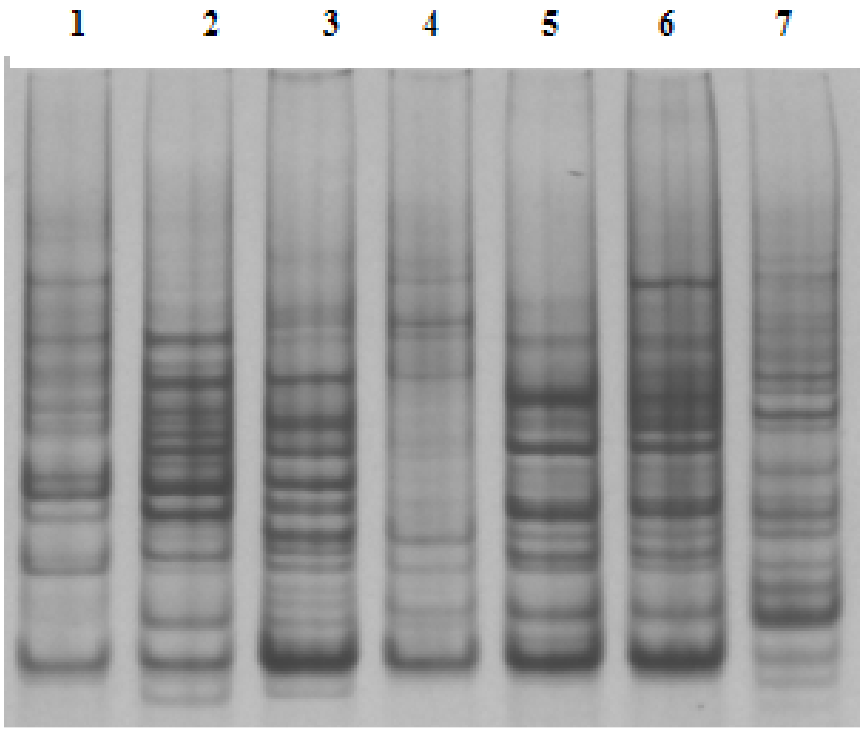

Figure 3. AFLP amplification profile of 7 sweetsop cultivars with primer *Eco*RI-CGA/ *Mse*l-CAA. 1, 'Yuanhua' *A. glabra*; 2, 'AP' *A. atemoya*; 3, 'Shanci' *A. Montana*; 4, 'Ciguo' *A. muricata*; 5, 'Milu' *A. cherimola*, 6, 'Fenglishijia' *A. atemoya*, 7. 'Niuxin' *A. reticulat*.

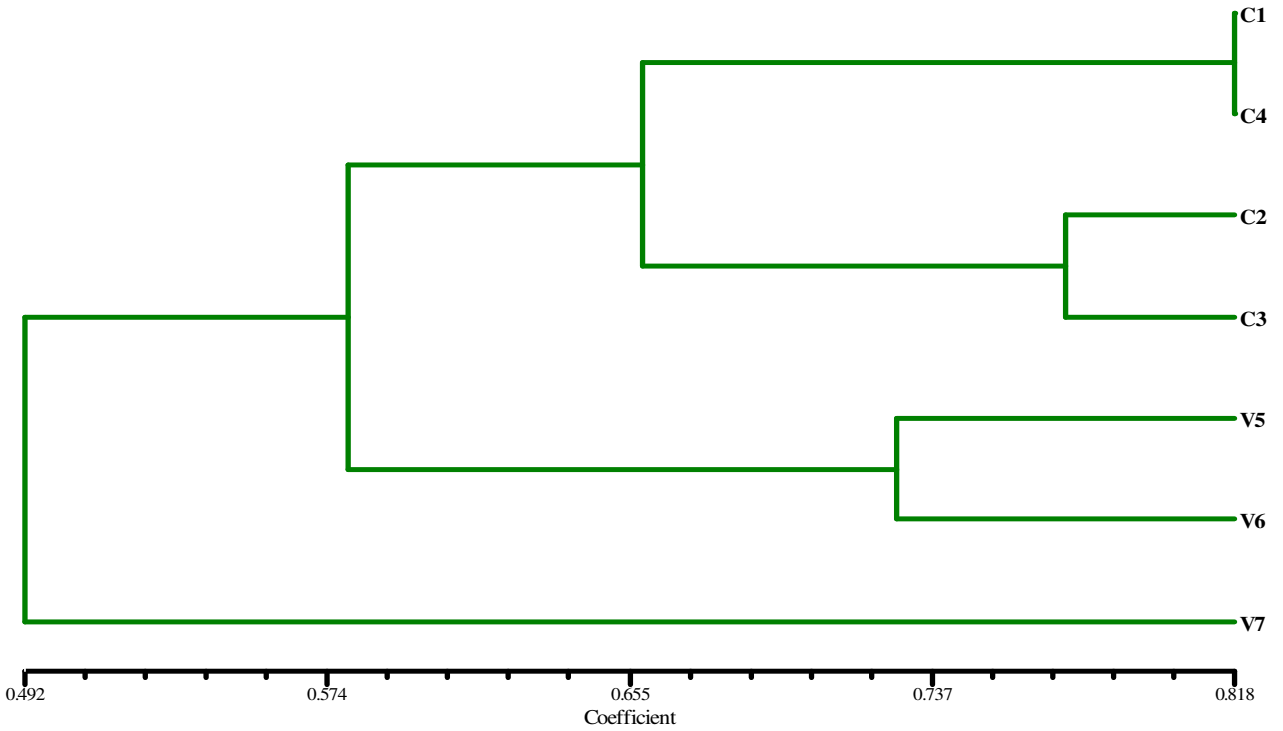

Figure 4. Dendrogram of cluster analysis for sweetsop cultivars based on AFLP markers. C1: 'AP' *A. atemoya*; C2: 'Niuxin' *A. reticulat*; C3: 'Ciguo' *A. muricata*; C4: 'Fenglishijia' *A. atemoya*; V5: 'Milu' *A. cherimola*; V6: 'Shanci' *A. montana*; V7: 'Yuanhua' *A. glabr*.

species level. The genetic relationship of the seven varieties was closely related with the leaf form and smooth, the fruit characters and certain scale. The ‘Niuxin’ and ‘Ciguo’ had good compatibility of grafting, and clustering analysis closely. The genetic relationship could provide the theoretical basis for sweetsop breeding, selection, and grafting. The root disease of sweetsop in South China was the main limiting factors for cultivation and production. The genetic relationship of seven materials was helpful for disease resistance breeding.

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