

Full Length Research Paper

# The earlier identification of the seedless characteristic of the wampee [*Clausena lansium* (Lour.) Skeels] hybrid by a random amplified polymorphic DNA (RAPD) marker

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Wampee as an important fruit is widely grown in South China and conventional breeding methods for the production of new seedless wampee cultivars in recent years were operated. Hybridization was conducted using the seed as female parents and the seedless as male parents. However, the seeds could be gotten from cross combination. An efficient operation was that, non-woven fabrics bag could increase burliness rate in hybridization. In order to earlier identify seedless seedlings in hybrid progeny, the objective of this work is to identify molecular markers linked to seedless trait through bulked segregant analysis. Two bulks, seedless and seed, were prepared by pooling DNA of individual identified plants and used in polymerase chain reaction (PCR) analysis. Out of the 100 primers tested, only S06 primer could get a specific 930 bp restriction random amplified polymorphic DNA (RAPD) amplicon to seedless bulk. As a result, a hybrid was tested with this primer in two cross combinations.

**Key words:** Earlier identification, seedless wampee, bulked segregant analysis, randomly amplified polymorphic DNA.

## INTRODUCTION

Wampee [*Clausena lansium* (Lour.) Skeels] is a tropical, subtropical and very remote citrus fruit tree, belonging to subtribe *Clauseninae* and tribe *Clauceneae* of the orange subfamily *Aurantioideae* (Swingle and Reece, 1967). Wampee has more than 30 species, 11 of which are native to China (Yu, 1982). Among these, only *C. lansium* (Lour.) Skeels and *Clausena indica* (Dalz.) Oliv are edible and commercially cultivated. Chinese wampee (*C. lansium*)

was originally native to the southern part of China and had a long history of cultivation. Wild wampee was found in Guangdong, Guangxi and Hainan Provinces. Recently, its large commercial cultivation areas were the Guangdong, Guangxi, Fujian and Hainan Provinces (Guo and Deng, 1999).

Wampee fruit ripens from May to July in southern China; it tastes similar to grapefruit when ripe, resembles a diminutive lemon and is about 2.0 - 4.0 cm in diameter. It contains 0 - 3 seeds and the pulp is sweet or slightly acidic. The pulp can be added to fruit cups, gelatines and other desserts, or made into pie, jam or jelly. Carbonated beverages resembling champagne are made by fermenting the fruit with sugar (Morton, 1987; Pratheung et al., 2007). The leaves, roots and fruits have been used in folk medicine in Taiwan (Li et al., 1991) and main China (Yang et al., 1988) for the treatment of certain dermatological diseases, for instance, acute and chronic viral

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**Abbreviations:** BSA, Bulked segregant analysis; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA marker; CTAB, cetyl trimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid.

**Table 1.** Burliness in the cross combinations.

Cross	Non-woven fabric bag burliness rate (%)	Paper bag burliness rate (%)
Dajixin × yunan	34	15
Sweet jixin × yunan	15	10
Dajixin × sweet jixin	21	12
Yunan × dajixin	8	5
Yunan × sweet jixin	19	12
Sweet jixin × dajixin	28	19

hepatitis; the leaf decoction was used as a hair wash to remove dandruff and preserve hair colour (Perry, 1980). Recently, the extraction of seeds was found to possess antifungal and human immunodeficiency virus (HIV) reverse transcriptase inhibitory activities (Ng et al., 2003). The fruit is used in the Philippines for influenza, colds and abdominal colic pains (Stuart, 1977). In Thailand, the dried wampee is produced as a sweet preserved fruit (Pratheung et al., 2007). Seedless wampee is preferred by consumers. Conventional breeding methods for production of new seedless wampee cultivars in recent years are used. Breeding efficiency of these traditional methods is greatly limited due to a long juvenile phase of seedless progenies in F1 generation. Phenotype of seedless and seed plants was not obviously and difficultly distinguished.

Availability of tightly linked molecular marker for seedless gene would help to identify hybrid progeny carrying the gene. Polymerase chain reaction (PCR)-based marker techniques especially, random amplified polymorphic DNA marker (RAPD) in conjunction with bulked segregant analysis (BSA) had been used to tag agronomically important genes (Giovannoni et al., 1991; Michelmore et al., 1991). In an effort to identify molecular marker linked to seedless gene, we conducted BSA using several commercial cultivars. Hybrid progeny was identified by RAPD marker.

## MATERIALS AND METHODS

### Plant materials

Yunan seedless wampee and jixi seedless wampee are seedless cultivars, dajixin wampee and sweet jixin wampee are seed cultivars. Emasculation was conducted 2 days before anthesis, followed by immediate bagging with non-woven fabrics or paper bag of the inflorescence. Artificial pollination was conducted using a hairbrush with pollens collected earlier from the male parents (Table 1). Hybrid was grown in the College of Horticulture, South China Agricultural University, located in Guangdong Province of China.

### DNA extraction

Genomic DNA was extracted from the parents and the selected F1 plants using cetyl trimethylammonium bromide (CTAB) method (Dellaporta et al., 1983). Healthy leaves (2 - 3 g) from field-grown

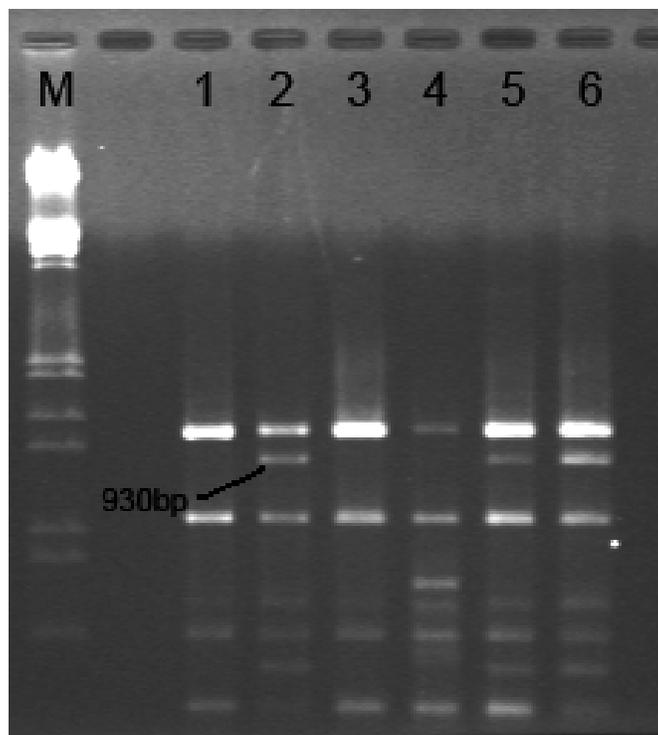
plants were excised, washed thoroughly and blot dried. The midrib was removed and the leaves were homogenized to a fine powder using liquid nitrogen before adding 10 ml of lysis buffer (freshly prepared 100 mM Tris-HCl, (pH 8.0); 20 mM ethylenediaminetetraacetic acid (EDTA), (pH 8.0); 1.4 M NaCl; 2% CTAB; 2% (w/v) polyvinylpyrrolidone-40,000; 2%  $\beta$ -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 and 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 mM Tris-HCl, pH 8.0; 1 mM 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 (pH 5.2) 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).

### Construct bulked segregant analysis

Two bulks, seedless and seed were prepared by pooling equal amount of DNA from each of the two seedless and two seed plants mentioned above. PCR amplification was carried out in 25  $\mu$ l of reaction mixture consisting of 5 ng of total genomic DNA, 1 $\times$ PCR buffer, 2 mM MgCl<sub>2</sub>, 0.4 mM of each dNTPs, 20 pm of random decamer primer and 1U of *Taq* polymerase. PCR was performed in a DNAEngine peltier thermal cycler (BIO-RAD). The PCR conditions were 94°C for 4 min, followed by 40 cycles of DNA amplification (45s at 94°C, 1 min at 37°C and 1 min 30s at 72°C ) and 8 min incubation at 72°C. PCR products were separated by electrophoresis at a constant voltage (2 V/cm) in a 1.2% (w/v) agarose gel. The EcoRI/HindIII  $\lambda$  DNA ladder plus molecular weight marker was used to compare the molecular weight of amplified products. The bands were visualized by staining with ethidium bromide (0.5  $\mu$ g l<sup>-1</sup>) and observed under a UV source.

## RESULTS AND DISCUSSION

Six cross combinations were successfully applied. Seed



**Figure 1.** Bulked segregant analysis with primer S06. M: Molecular weight marker (EcoRI/HindIII  $\lambda$  DNA). Lane 1, Seed bulk; lane 2, seedless bulk; lane 3, dajixin; lane 4, sweet jixin; lane 5, yunan seedless; lane 6, jiexi seedless.

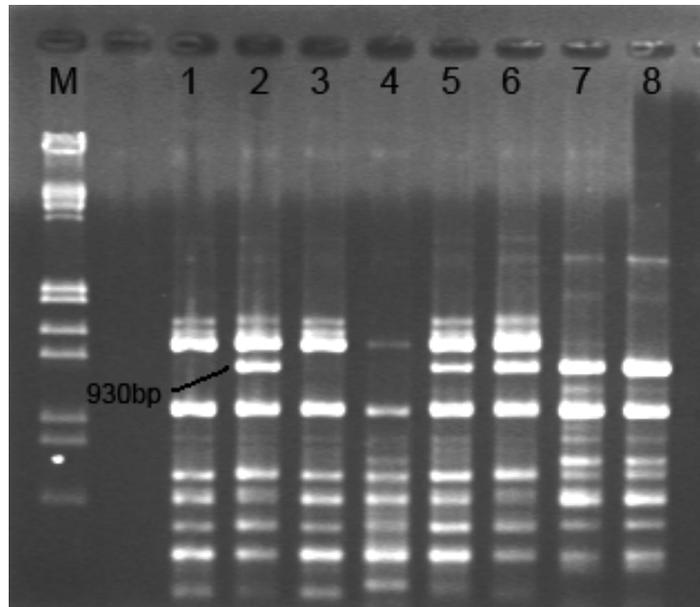
wampee as female parents and seedless wampee as male parents could get more fruits; every fruit contained 1 - 2 seeds. With the seedless as female parents and the seed as male parents, seeds were successfully gotten in the hybrid (data not show). Burliness rate in the hybrid was more increased in using non-woven fabrics bag than using paper bag. The cross combination of dajixin  $\times$  yunan produced the best results, while yuna'  $\times$  dajixin gave the lowest burliness rate (Table 1). These results showed that dajixin was more fit for female parent in hybridization. Both sweet jixin  $\times$  yunan and yunan  $\times$  sweet jixin got similar results.

The two bulks, seedless and seed were prepared by pooling DNA of two seedless and two seed plants and used in RAPD analysis. A total of 100 random decamer primers were tested for identifying primers yielding polymorphic amplification patterns between the bulks. With S06, all two seedless plants were given a 930 bp fragment, whereas out of the two, seed plants showed this amplicon (Figure 1). This fragment may be linked molecular marker for seedless gene. With this primer, hybrid was tested in two cross combinations. Seedless plants may be shown in hybrid of dajixin  $\times$  yunan and sweet jixin  $\times$  yunan (Figure 2).

Many bagging materials had been used for cross breeding of fruit trees. Kraft paper bags and newspaper bags are widely used in fruit trees hybridization. Non-

woven fabrics bag used in the hybridization have some merits. These include (i) Better gas permeability and reduce high temperature which affected pollen germination and growth in the bag; (ii) better lucency and inflorescence that could accept more sunlight; (iii) on raining days in flower season, could reduce water and humidity in the bag; (iv) good quality, could be used several years. In south China, non-woven fabrics bag could be used to improve fruit pigmentation of 'eizixiao litchi (Zhen et al., 2001).

Seedlessness was an important aim in some fruit breeding. Seedlessness in grapes was caused either by parthenocarpy or stenospermocarpy (Stout, 1936). In the stenospermocarpic grapes, fertilization occurs, but seed development stops at some stage, resulting in almost none to varying degrees of perceptible seed traces (Li et al., 2008). Yunan seedless wampee crossed as female parent, could get seeds, burliness rate was higher in sweet jixin as male parent. Dajixin as a female parent and yunan seedless as a male parent could get higher burliness rate. Chromosome numbers of yunan somatic cells are diploid, triploid and aneuploid. The aneuploid cell occupied the highest proportion of all the observed cells achieving 64.8% while the diploid cell had 28.8% (Feng and Liu, 2007). Yunan seedless wampee had more seedless rate and seedless characteristic was affected by weather, temperature and humidity. Conven-



**Figure 2.** Hybrid analysis by RAPD with primer S06. M: Molecular weight marker (EcoRI/HindIII  $\lambda$  DNA). Lane 1, Seed bulk; lane 2, seedless bulk; lanes 3 – 6, hybrid of dajixin  $\times$  yunan; lanes 7 - 8 hybrid of sweet jixin  $\times$  yunan.

tional breeding methods for the production of new seedless wampee combined with molecular markers could increase efficiency and shorten breeding period. Bulked segregant analysis coupled with RAPD had been successfully employed earlier to markers linked to resistance gene in rice (John et al., 2007), apomixes in buffelgrass (Ozias et al., 1993; Gustine et al., 1997), leaf rust resistance in barley (Poulsen et al., 1995), fruit skin color in Japanese pear (Eiichi et al., 2006).

In this study, out of 100 RAPD primers screened, only one primer, S06 gave special band when tested with individual plants. Our results showed that S06-930 bp may be molecular markers linked to seedless gene. This marker could be useful for rapid screening of seedless wampee in a hybridization population.

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