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Sexual genetic and simple sequence repeat (SSR) analysis for molecular marker development on the all hermaphrodite papaya

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ABSTRACT. The papaya (*Carica papaya* L.) is one of the most important economic tropical fruits in the world, and the hermaphrodite is the preferred type in field cultures. We analyzed the sexual ratio of offspring from the cultivar 'Taiwan Seed Station No. 7' (T7) by a self-cross and its cross with Taichung Sunrise (TS). Female progeny from the T7 self-crossing were not observed. This finding may be caused by a lethal gene that is linked to females. In this study, we selected 192 simple sequence repeats (SSRs) to analyze the polymorphism between T7 and TS. A total of 37 SSRs were identified for T7 and TS. In addition, 14 SSRs served as the molecular makers for identification

of T7, TS and their hybrid offsprings. Thus, the results show that the genetic similarity between T7 and TS is rather high. This suggests that T7 may be a mutant of TS. Phylogenetic analysis from the SSR polymorphisms of the above parent strains and 15 F_1 offspring revealed the genetic distance of the F_1 offspring located between T7 and TS. The results of this study may provide an opportunity for elucidating the genetic characteristics of all hermaphrodites via identification of molecular makers.

Key words: All hermaphrodites; Simple sequence repeat; Polymorphism; Phylogenetic analysis

INTRODUCTION

The papaya (*Carica papaya* L) belongs to the Caricaceae family. It is widely cultivated in tropical and subtropical areas. Its fruit is rich in vitamins, including vitamin A, vitamin C, and β-carotene (Eustice et al., 2008). In addition, the papain from its fruit is widely applied in medicine, food, and other industries (Nitsawang et al., 2006).

One of the most important biological characteristics of the papaya is that it is polygamous, with 3 different reproductive organ types, including the male and female flowers of the dioecious and hermaphroditic flowers of monoecious plants (Ming et al., 2007). The seeds obtained by self-fertilization of the hermaphroditic flowers usually present a 2:1 ratio of hermaphroditic flowers:female flowers (Storey, 1953). The family Caricaceae includes 5 genera and 34 species, and there are 21 species in the genus *Carica*. Most *Carica* are dioecious, with monoecious flowers only found in *C. papaya*, *C. pubescens*, and *C. monoica. C. papaya* has the longest planting history and highest value in the market, with many known cultivars (Aradhya, 1999). Among the hermaphroditic papayas, the pear-shaped fruit gains higher market preference. The hermaphroditic trait can only be confirmed after flowering by checking its inflorescence. Thus, farmers must spend longer time of planting until it grows to the reproduction phase to remove the female plants and save the hermaphrodites for fruit production, which may lead to higher costs (Ma et al., 2004).

Many crop cultivars have been successfully identified by different methods and the use of molecular makers in recent years, such as amplified fragment length polymorphism (AFLP). AFLP was previously used for a genetic diversity analysis of papaya species (Kim et al., 2002). Ranade et al. (2008) used single-primer amplified reaction (SPAR) for species identification of physic nuts (*Jatropha curcas*). Corazza-Nunes et al. (2002) used random amplified polymorphism DNA (RAPD) and simple sequence repeat (SSR) for species identification of the grapefruit (*Citrus paradisi*). Sondur et al. (1996) used the RAPD markers for developing a basic genetic linkage map for papaya. Kumchai et al. (2013) used inter-simple sequence repeat (ISSR) for identification of interspecific hybrids of the eggplant (*Solanum melongena* L.). The SSR is also a microsatellite, a repeated segment formed by 2-6 nucleotides that is frequently found in eukaryotes. SSR primers have been designed from the flanking regions of repeated sequences to detect variation among different cultivars. It has been widely applied, especially for species identification, and genetic diversity and phylogenetic relationship studies. It has also been used in the identification of Chinese jujube (*Ziziphus jujuba* Mill) germplasm (Ma et al., 2011), phylogenetic analysis of wine grapes (*Vitis vinifera*) (Bowers

et al., 1999), species diversity (Huang et al., 2010), phylogenetic analysis of Chinese orchids (*Cymbidium* spp.) (Moe et al., 2010), and construction of a genetic map for the papaya (Chen et al., 2007). Furthermore, it has been used for agronomic crops, such as soybeans [*Glycine max* (L.) Merr.], species identification (Narvel et al., 2000), and phylogenetic analysis of bread wheat (*Triticum aestivum* L.) (Gupta et al., 2003).

In this study, we used the all-hermaphroditic cultivar 'Taiwan Seed Station No.7 (T7)', the usual hermaphrodite plant of Taichun Sunrise (TS) (Chiu et al., 2003) and the offspring of both cultivars as materials for numerous analyses, including the investigation of sexual characteristics, SSR polymorphisms, and phylogenetic analyses. We aimed to identify the SSR molecular makers that could precisely distinguish T7 and TS and construct a phylogenetic relationship of the offspring and their parents to firmly establish the genetic characteristics of the T7 cultivars.

MATERIAL AND METHODS

Papaya cultivars

We used the all-hermaphrodite T7 and the typical hermaphrodite TS, which were grown at the Pingtung Branch Station of the Taiwan Seed Improvement and Propagation Station (Figure 1). The offspring between the 2 cultivars were obtained from the same place.

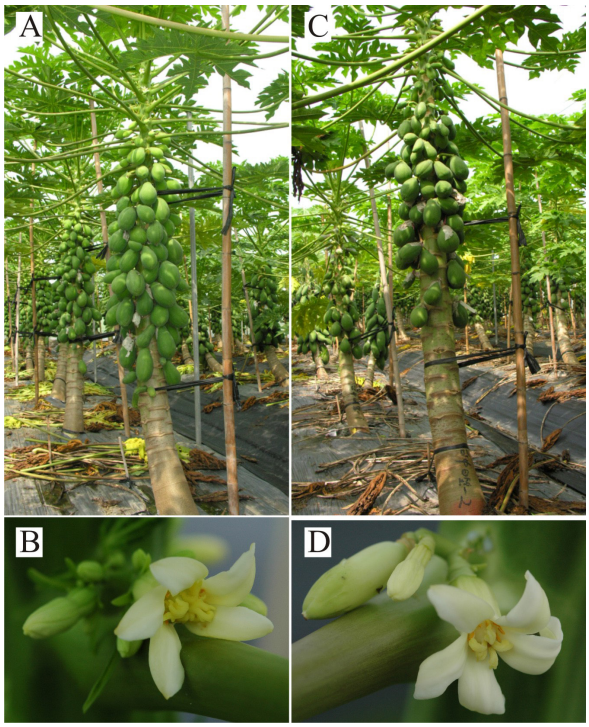

Figure 1. Morphology of T7 and TS papaya cultivars. (**A**) Mature fruiting plants, and (**B**) flower for the all hermaphrodite T7, (**C**) mature fruiting plants, and (**D**) flower for the hermaphrodite TS.

Papaya sex character analysis

Three cross pollinations were designed between the T7 and TS cultivars, including self-fertilization of the hermaphrodites of TS (TS x TS), self-fertilization of all T7 hermaphrodites (T7 x T7), and TS and T7 (TS x T7). Each combination was pollinated in tripilicate. From each pollination event, 70 seeds were selected and germinated from the mature fruits and then grown in the field until flowering; then, the sexual phenotype was recorded. We examined the expected value by the supposed 2:1 ratio of hermaphrodites:females by chi-square test.

Extraction of papaya genomic DNA

A total of 24 experimental materials, including 22 F_1 hybrid offspring and their parents, T7 and TS, were utilized. Fully developed leaves were sampled and immersed in liquid nitrogen and then stored at -80°C until use for genome purification by the DNeasy Plant Mini Kit (QIAGEN, USA) (after artificial trituration). The quality of the genome DNA solution was examined by a spectrophotometer (TECAN Infinite M200 PRO). Genome DNA solutions were adjusted to 20 ng/μL, and aliquots were stored at -80°C for further use.

SSR polymorphism analysis

SSR analysis followed the previously reported method by Chen et al. (2007). We selected the SSR with an average distance of 6 cM to design the primers. A total of 192 SSR primers were thus obtained (**Table S1**). We used a 20-μL polymerase chain reaction (PCR) mixture, including 20 ng papaya genomic DNA, 2 μL DMSO, 2 μL PCR buffer, 200 μM dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 U/μL Taq, and 9.2 μL water. The reaction condition was 95°C for 4 min for DNA denaturation. Then, we ran 35 cycles for DNA amplification at 94°C for 30 s, 58°C for 1 min, 72°C for 30 s, and a final extension at 72°C for 5 min. The amplified products were cooled down and stored at 4°C. A PCR product of 10 μL was separated by 2.5% agarose (SFR™, AMRESCO, USA) at 120 V for 70 min. After electrophoresis, the gel was stained by ethidium bromide and a photo image was captured by digital camera on the UV light box. The SSR markers, which showed polymorphisms, were chosen for phylogenetic analysis of the F_1 hybrid offspring of the 2 cultivars mentioned above.

Phylogenic analysis

The polymorphic SSRs were classified with bands belonging to the T7 and TS parents as 1 and 2, respectively. The binary data matrix was input into the NTSYS-pc software, and the unweighted pair group method with arithmetic mean (UPGMA) was used to run the phylogenetic analysis, which output a dendrogram.

RESULTS

Separation rate of sexual genetic analysis for different mating composition

Three mating combinations, TS x TS, T7 x T7, and TS x T7, yielded the following independent ratios for the number of hermaphroditic:dioecious female offspring: 122:68, 102:0,

and 68:36, respectively (Table 1). The results of the chi-square test supported a 2:1 ratio of hermaphrodites *vs* dioecious females in TS x TS and TS x T7. However, the offspring of T7 selfing did not match the expected sex rate. Meanwhile, dioecious females were not produced from this mating; therefore, we could not calculate the value of chi-square (Table 1). According to the data above, we can conclude that the self-fertilization of regular hermaphrodites (TS x TS) and the hybridization between normal hermaphrodites and all hermaphrodites (TS x T7) presented stable 2:1 isolation rates. However, the null dioecious female resulting from self-pollination of all hermaphrodites is an interesting finding that should be further elucidated.

Table 1. Sexual genetic analysis of different pollination combinations.

Combinations	Hermaphrodites	Females	r(H)	r(F)	χ^2	P value
TS x TS	122	68	2	1	0.561	0.499
T7 x T7	102	0	2	1	-	-
TS x T7	68	36	2	1	1.391	0.756

SR polymorphism analysis of TS, T7, and the F_1 hybrids

We selected a total of 192 SSRs with an average marker distance of 6 cM (Chen et al., 2007) (**Table S1**). The major bands amplified were located between 150-200 bp, and only rarely >30 bp, from the polymorphism analysis of the 2 cultivars (Figure 2; **Figure S1**). For instance, for primer P6K883CC, the major band was located at 110 bp for T7 but 300 bp for TS. The maximum variation in band distance was ~200 bp between the 2 parents (Figure 2). In summary, 37 SSRs with polymorphisms were identified for T7 and TS (Table 2). Based on this result, we conducted a polymorphism analysis of the F_1 offspring of T7 and TS by adopting the above 37 SSRs. The results showed that the major bands were located at 100-300 bp and a visible difference of 20-200 bp on the gel (Figure 3; **Figure S2**). A total of 14 SSR primers could be used for identifying the offspring (**Figure S2**) .

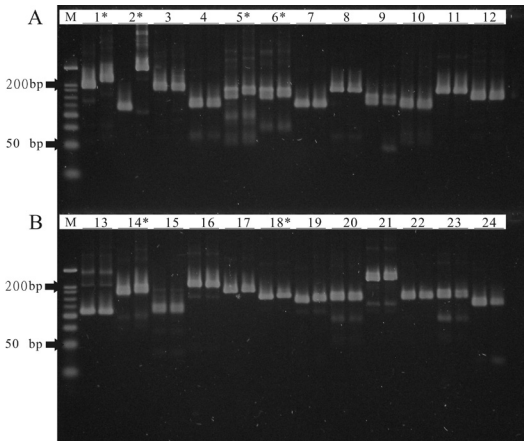

Figure 2. Simple sequence repeat (SSR) polymorphism analysis of papaya T7 and TS cultivars. *Lane M* = 100-bp ladder marker. *Lanes 1-24* present polymerase chain reaction (PCR) products of 24 different primers. The T7 cultivar was over the left side, and the TS cultivar was over the right side under the same primer number. The numbers labeled with an asterisk (*) indicate polymorphisms between 2 cultivars. The SSR numbers are referred to in Table 2. 1: P3K7483A5; 2: P6K883CC; 5: P6K900CC; 6: CPM1580CC; 14: P8K395CC; 18: P3K2937CC.

Table 2. Names and primer sequences of the 37 SSRs based on polymorphism analysis between T7 and TS cultivars (Chen et al., 2007).

Primer names	Forward sequences	Reverse sequences
P3K168CC	GCACAAGCGCTCTCCTTTCT	TTCCCCCTCTGATCTGGTCTC
CPM727CC	ACTTTTGTGGTGCAAAAGGC	CCAATTGTTAACTGTGGAAGG
P3K6378CC	TCGTTAAAGCGATTACCCCG	TGCTCTGAAAATAAACCGGCA
CPM1842A5	ACGTTGTTTCCTTCCCCCAT	TCCTCGCAATCCAATCATCA
P3K7483A5	GCAGCCCGTACAGAAGAGGA	CTCTGAGCAGCAAGCCAACA
P6K883CC	TGGCACACGTATGATTCCAA	TGGGTCTGAATTAAAAGGGAAAA
P6K900CC	TTTGGATCTTGTGGGTTCGG	GGAAGAGGAGATGTGAAAACGG
CPM1580CC	AGGCTAAATGAGAGAAGCAACACC	TCGAGCATTCAGGACATTGAGA
P8K395CC	CGACGTCGTTTCAATTTGAGC	AAACAAATGTATTCACACGCTTCG
P3K1024CC	GCATGATGAGATGGTGGTGA	TGAGGAGGAAAAAGTTGGTAACAT
ctg669CC	GGTTTTCAAATTCTGGAAAGTCCT	TCAGCAAATTAACCCTGCGA
P3K2937CC	TGCAATCACGAATAAGGGTCG	ATGCAGCATGGGAAAGGAAA
P8K534CC	CGGCTCTCAAATACAATTGTCAAA	TCAAATCCCCTCAGCATTGG
P3K149C0	TGGTGGATGTTGATGCATGTT	TCTGGTGGTCATGATGGTGG
P3K2214CC	TCTTTCTTCAAACCATGGAGCTG	CTGGCTTTGCTGGAGAGGAA
P3K1573CC	TGGTCCTTATAACTTTTCTCCCCT	AATCCCGTTCTATTCATCCCC
CPM1737CC	CCGAAATTGATCGTTACCCAA	CATTGAAATTCATGCCCGAC
P6K1268CC	GCAGTCTCTTCTCTCCAAGGTCA	GTCCCCTTAGCTGCATCCAC
P3K1433CC	TTCTGGACACTTTTACACCGGA	CCGCCAGTGATTCAAAACAA
ctg169CC	TCATCCAAAACCTCCCCTCA	GGCCGATGTCTTGAACTGCT
CPM746LCC	TTATGTTTGGGAAGAACGCC	GCCAATTCCCCACAAATCTA
P3K6057CC	GACGTTGGATCCTGCCGTTA	GCAAACAGACAACCCCCAAA
P3K2947CC	TGTCAACACCTCATTTTTGCTTG	GCATGCTCCTTTCGTCAACA
P3K5022CC	CGCTTTAACCCACATGGACA	TGTTGGAGAAAACGATGCGA
P8K62CC	TATATCAGCACCCACCCCCA	TTGTGTTCATTTGCTTTATCGGG
P8K662CC	TATCCATTTGGCAGCCATGC	GGTTCATGCTGGGGGAGATT
P3K3146CC	AACGGGGAATTTGGAGATGG	AGCGAGTTCGCTTGTGAGGA
CPM1525C0	AGGTATGTGAATTGGTCCTCAAAT	TCCTTCTCAACGACCTCGAA
P3K6604CC	AAGTTGATGGGAGCTGCGAC	GGGAGGAACCTGCACATCAG
P3K3974A3	AACAGTCACCATCTCTTCATGCC	TTCACATGCGTACTCTGTTTTTGA
P6K1129CC	CAAGATCTTCATGGCTGCCC	GGCATGGAAGACAATGAGGC
P3K156CC	TGTTGTGTTGATCGACTGATTGAT	TGCCCTAGCTTTCTAATCATCACA
P6K128CC	GCCGGCTCAGGAGGTTAAGA	CAATGACCAAACGCCACACA
CPM1573CC	GAAGAACCACTGGTGCCTGAA	GGCAGCAGCCTGGCTTATAG
P3K6947	ATTCTTCCTCCCTCCCTCCC	AAAAAGGGTGGGGGTTTGAG
P3K736	CTCTCTTCAAGGCCATCTCCA	GCCATGAAGTTGGCGAAGTATT
P3K2152	CTCCAAGAAAACTTTGACATGGG	CAATTATGAGCCGGTCACTAACAA

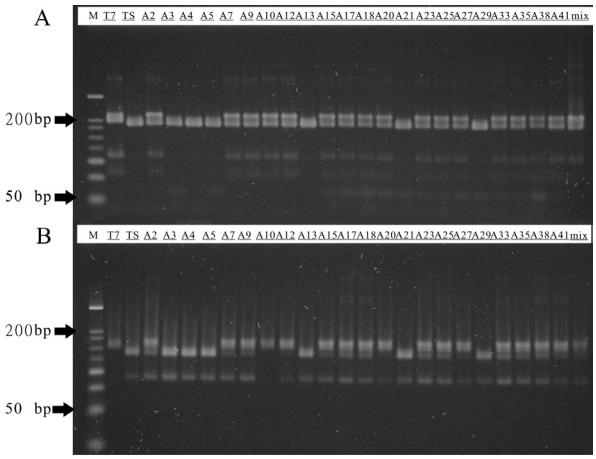

Figure 3. SSR polymorphism analysis using P3K2947CC (upper panel) and P3K149CC (lower panel) primer pairs. *Lane M* = 100-bp ladder marker. *Lanes T7* and *TS*, parents; *lanes A2*, *A3*, *A4*, *A5*, *A7*, *A9*, *A10*, *A12*, *A13*, *A15*, *A17*, *A18*, *A20*, *A21*, *A23*, *A25*, *A27*, *A29*, *A33*, *A35*, *A38*, and *A41* are 22 F_1 offspring; mix, genome mixture of the T7 and TS cultivars.

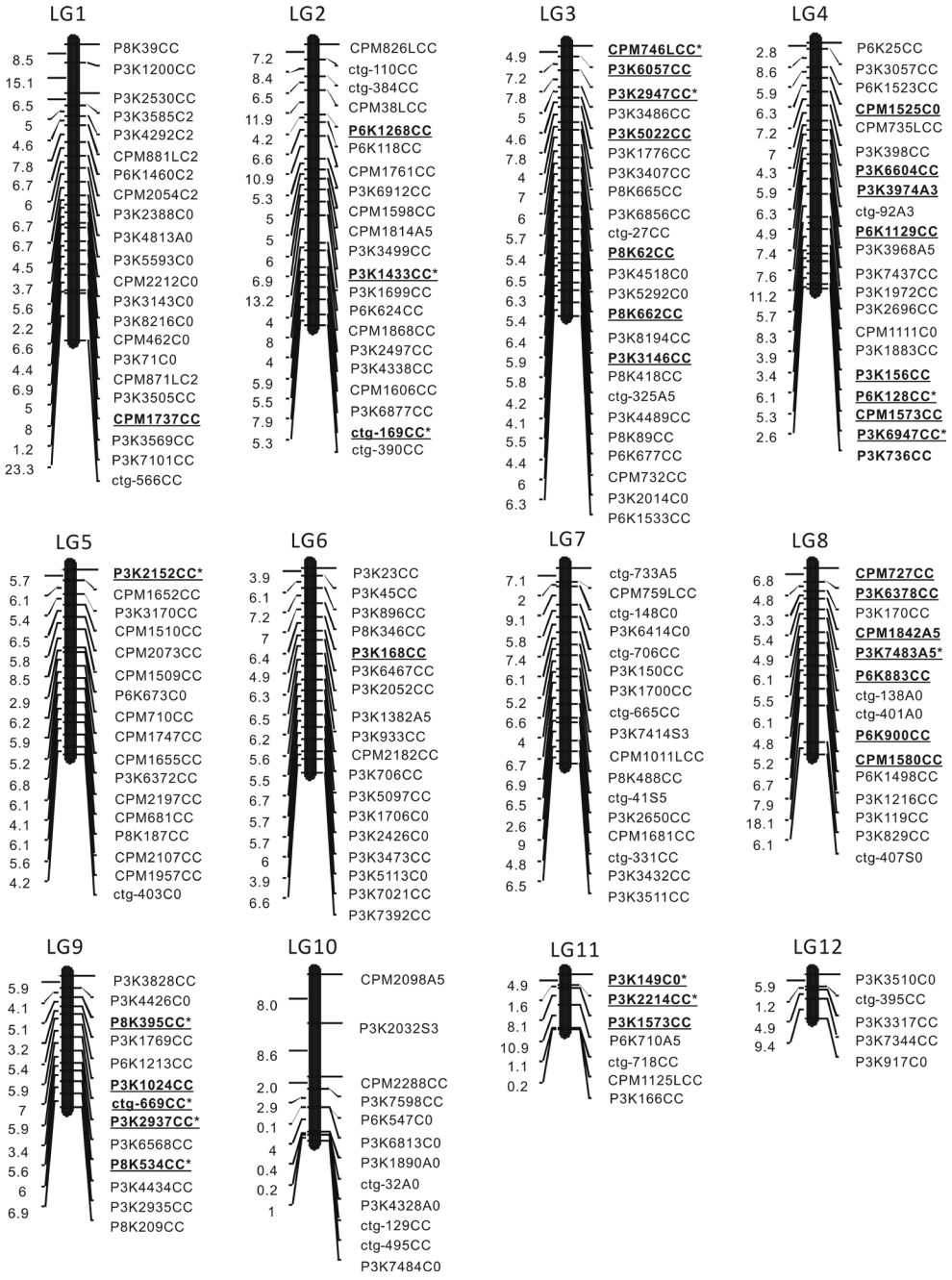

Figure 4. Locations of 192 SSRs selected based on equal genetic distance for linkage groups of papaya. The bold-typed and underlined labels: 23 SSR polymorphisms for parent identification. An asterisk (*) indicates: 14 SSR polymorphisms for identification of both parents and 14 F_1 offspring. LG: linkage group. Units: cM (after Chen et al., 2007).

We inferred, from the results published by Chen et al. (2007), assignment of the 192 SSRs on a linkage map. As shown in Figure 4, there were 24 SSRs that were virtually mapped to linkage group 3 (LG3). Among these, 7 SSRs (7/24 = 29%) could identify the 2 cultivars (labeled in green), and 2 SSRs (2/24 = 8%) could be used in the offspring analysis (labeled in red). The 13 SSRs assigned to LG9 showed the highest identification rate (5/13 = 38% for identification of the 2 cultivars; 4/13 = 30% for identification of the offspring and parents). Thus, in the current study, we chose 14 SSRs (14/192 = 7.3%) that could identify T7, TS, and their F_1 offspring from the selected 192 SSRs among the LGs.

Phylogenetic analysis of TS, T7, and their F_1 offspring

A total of 15 of the 22 F_1 offspring could be identified with reproducibility via polymorphism analysis of 14 SSRs. The offspring identifiable *via* phylogenetic analysis were labeled as follows: A2, A7, A9, A10, A12, A15, A17, A18, A20, A23, A25, A27, A33, A35, and A38. We obtained a dendrogram via UPGMA analysis. The F_1 offspring and both parents were distinctly divided into 3 groups. T7 was located at one end, while TS was located at the other end, and all F_1 offspring were located in the middle. There were numerous subgroups of the F_1 offspring. Eight offspring (i.e., A2, A7, A9, A15, A23, A25, A33, and A35) belonged to a single subgroup; they all had 100% similarity. The primers tested could not distinguish among these offspring. The other offspring subgroup was composed of A27 and A38. The remaining offspring (i.e., A10, A12, A17, A18, and A20) were divided into 5 subgroups. The distance between the F_1 offspring and both parents (T7 and TS) was minimal (coefficients of 0.45 and 0.48, respectively) (Figure 5).

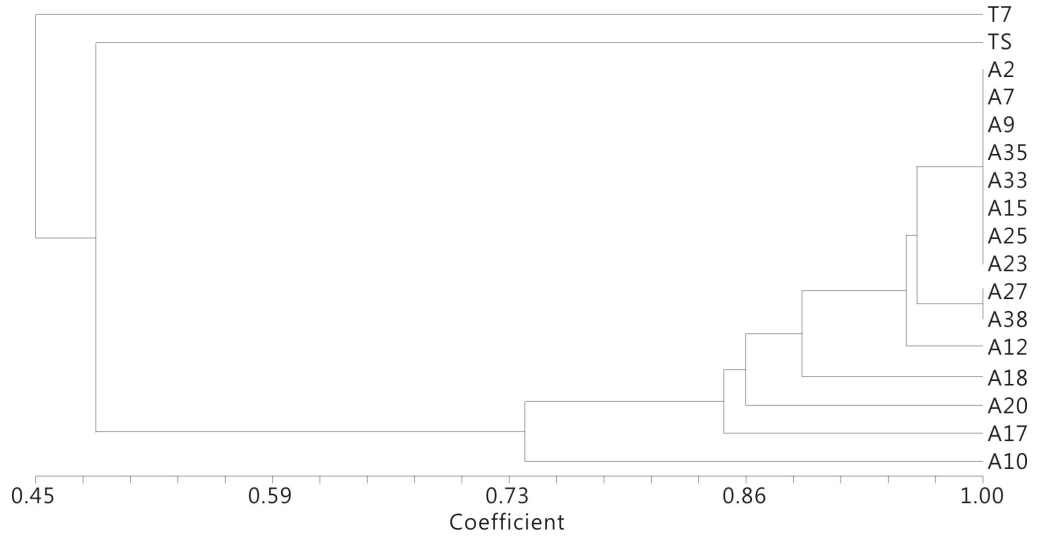

Figure 5. Phylogenetic tree of T7, TS, and their F_1 progenies. T7 and TS: parent cultivars; A2, A7, A9, A10, A12, A15, A17, A18, A20, A23, A25, A27, A33, A35, and A38: F_1.

DISCUSSION

Sex of the papaya is thought to be determined by a single locus carrying 3 alleles: *M*, M^h, and *m* (Storey, 1953). Because homozygosity of the 3 compositions of the same alleles (i.e., *MM*, M^hM, and M^hM^h) is lethal, the dioecious male possesses the heterozygous allele *Mm*. The hermaphrodite is M^hm. Meanwhile, the dioecious female possesses the homozygous recessive allele *mm* (Storey, 1953). According to Mendel's law, self-fertilization of hermaphrodites will produce 3 allele compositions as follows: M^hM^h (25%), M^hm (50%), and *mm* (25%). Since M^hM^h is lethal, only 2 compositions, M^hm and *mm*, will survive. The ratio of hermaphrodites: dioecious female is 2:1 (Storey, 1953). In this study, we found that the ratio of hermaphrodites: dioecious females of T7 selfing was 102:0, which appears to deviate from Mendel's law because dioecious female offspring were not obtained. Since the genotype of hermaphrodites is M^hm, and the dioecious female is *mm*, we reasoned that a lethal recessive gene may be linked to females in this cultivar. Thus, fatality in female offspring may be caused by this lethal allele. On the contrary, female fatalities were not observed for combinations obtained from the TS x TS or TS x T7 crossings. Because the offspring of T7 selfing were all hermaphrodites, we termed the T7 cultivar as 'all hermaphrodite'.

An SSR strategy applied to papaya cultivars was developed in this study. Fourteen of the 192 SSR markers were used for identification and polymorphism analysis of the T7 and TS cultivars and their F_1 offspring. Liu et al. (2014) found that 7 SSRs could identify 12 *Dendrobium* spp. following a polymorphism analysis of 53 SSRs. The rate was ~13.2%. Kumchai et al. (2013) also revealed 14 ISSRs that could identify true hybrid progenies between the eggplant (*Solanum melongena*) and its wild relative, *S. torvum*, from a polymorphism analysis of 33 ISSRs. The rate was ~42.4%. The genome of *Dendrobium* spp. is >2000 Mb (data not shown), and the genome of the eggplant is >1100 Mb (Doganlar et al., 2002). Both displayed higher SSR polymorphism ratios than those of the papaya cultivars T7 and TS. The genome size of the papaya is only 372 Mb (Eustice et al., 2008), which is smaller than that of the Japanese rice (*Oryza sativa* L. ssp. *Japonica*), with a genome size of only 420 Mb. In another study, we performed a polymorphism analysis of the Shiokari strain of rice and its near isogenic line (NIL) d-6 by using 192 SSRs. There were only 4 SSRs that exhibited polymorphisms, and the rate was ~2.1% (data not shown).

Based on the study above, we suggest that T7 and TS are rather similar with regard to their genetic background. The phylogenetic tree showed almost the same genetic distance between the F_1 offspring and the 2 parents. To sum up, we addressed the evidence for the phylogenetic relationship of TS, T7, and their F_1 progenies. In addition, we also genetically identified the sex of T7 as a complete hermaphrodite.

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Supplementary material

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