Development of Specific-Molecular Marker for Sex Determination among Papaya Cultivars Grown in Phitsanulok Province, Thailand

Kawee Sujipuli[1], Sittichai Urtgam[2], Narisa Kunpratum[3], and Touchkanin Jongjitvimol[2*]

[1] Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok 65000, Thailand
[2] Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok 65000, Thailand.
[3] Faculty of Sciences, Naresuan University, Phitsanulok 65000, Thailand
*Corresponding author, E-mail: touchkanin@psru.ac.th

ABSTRACT

Papaya (*Carica papaya* L.) is the one of important economical fruit plants in Thailand. Its sex-type determination in an early seedling stage is extremely significant for commercial production because it sufficiently accelerates the productive hermaphrodite plants in orchard. The aim of this research was to develop specific-molecular marker for sex determination among five different papaya cultivars (Holland, Khaek-Dam, Koko, Khak-Nuan, and Si-Sa-Ket), grown in Thailand. The result revealed that the SCAR-W11-F and SCAR-W11-R markers were very useful for detecting male and hermaphrodite of five studied papaya cultivars, by giving the accurate PCR product approximately 830bp length in male and hermaphrodite but not in female. Furthermore, the new designed markers (PH2-F and PH1-R), were successfully useful to determine only in hermaphrodite of all validated papaya cultivars, by generating the PCR product (approximately 101bp length). These indicated that both molecular markers were greatly beneficial and valuable to identify the sex types of papaya plants at an early seedling stage.

Key words: papaya, sex determination, PCR, molecular marker

INTRODUCTION

Papaya (*Carica papaya* L.) is the one of important economical fruit plants in Thailand, of which widely grown in all regions. Its unripe fruit is an important ingredient of very popular Thai-salad (called Som Tam). Milky latex in green fruit contains the proteolytic-enzyme papain used in many manufacturing industries such as hydrolyzing beer peptides (chillproofing), tenderizing meats, and medicinal applications (Ming *et al*., 2007). The ripe papaya fruit is mostly popular to consume as a fresh-cut fruit, containing the essential nutrients such as vitamin E, vitamin B, carotenoid, lycopene, calcium, potassium and fiber (Chandrika *et al*., 2003).

In papaya plants, the sex is one of mainly essential traits for papaya breeding program and fruit production, in that is trioecious with three basic sex forms: female, male and hermaphrodite on separate plants. Both female and hermaphrodite flowers can produce fruits, but not in male flower. However, the fruit

developed from hermaphrodite flower contains more flesh, less seed, and longer fruit shape (pyriform) than female flower. Therefore, only hermaphrodite plant results in more commercial value for the fruit production (Chan *et al*., 2003). For this reason, farmers have to plant at least three seedlings per hill to assure that there is one hermaphrodite plant in a hill, and both male and female plants were chopped down. However, the sexes can be identified by flower traits during their reproductive stage taking 3-4 months after transplanting (Storey, 1953; Hsu *et al*., 2012). This process is inefficient uses of time, labor, water, and nutrients (Ming *et al*., 2007).

Recent, advancements in DNA markers have provided techniques and resources for evaluating the sex determination in papaya during seedling stage, prior to their transplantation to the field. Several male-specific markers and hermaphrodite-specific markers were independently developed by Random Amplified Polymorphic DNA (RAPD) and Sequence-Characterized Amplified Region (SCAR) markers such as T12 and W11 markers (Deputy *et al*., 2002) and SCAR W11 and SCAR T12 (Deputy *et al*., 2002, Chaturvedi *et al*., 2014). However, none of these markers have been validated in the large population of papaya cultivars in Thailand. In this paper, SCAR-W11 (Deputy *et al*., 2002, Chaturvedi *et al*., 2014) and new-developed markers have been tested among papaya cultivars, grown in Phitsanulok province, Thailand.

MATERIALS AND METHODS

Sample collection and DNA extraction

In total, the males, females and hermaphrodites among five papaya cultivars including Holland (HO), Khaek-Dam (KD), Koko (KO), Khak-Nuan (KN), and Si-Sa-Ket (SSK) were collected from six private farms in Phitsanulok province, Thailand. Their young leaves were harvested and stored at -80^{0}C for further experiments.

The flash frozen young leaf tissue (50mg) was ground to powder in liquid nitrogen and total genomic DNA (gDNA) was extracted using the innuPREP Plant DNA Kit according to the manufacturer's instruction (Analytik Jena AG, Germany).

Their gDNA concentration was measured by a UV spectrophotometer (Nano-Drop ND-1000, Wilmington, DE, USA) and adjusted to a 50ng/µl final concentration. To assess the gDNA purity, it was run on 1.2% agarose gel electrophoresis and visualized by 0.001% ethidium bromide under the UV transilluminator (Gel Doc 2000, BIORAD).

Primer design

For sex identification in papaya, four new oligonucleotide primers (PH1-F, PH1-R, PH2-F, and PH2-R) were designed according to the published *Carica papaya* chromosome Y sequence of hermaphrodite (GenBank: AY428938.1) and male (GenBank: AY428939.1) (NCBI, 2014). Moreover, previously literature reports, SCAR-W11-F and SCAR-W11-R (Deputy *et al*., 2002, Chaturvedi *et al*., 2014) were used in this study (Table 1).

Table 1 The primers used in this study

Primer Name	Sequence (5'→3')
PH1-F	TTAGAGCTAGTTTTCAT**G**
PH1-R	GCGTTGCATTAATGCCACT**G**
PH2-F	CTGCCATGCACTAACCTCA**A**
PH2-R	TTGGGATGCGTGACAAGGC**T**
SCAR-W11-F	CTGATGCGTGATCATCTACT
SCAR-W11-R	CTGATGCGTGTGTGGCTCTA

Polymerase chain reaction (PCR) condition

PCR amplification profile was carried out in 20 mL volume containing 1µl each primer (10 mM), 1µl gDNA (50ng), 8µl Dnase free water and 10µl OnePCRTM Plus (GeneDireX, SGS, Taiwan).

The PCR condition was performed in a PCR machine (PCR T100^{TM} Thermal cycler, BIORAD) using the following temperature profile: an initial step of 3 min at 95^{0}C, amplified by 35 cycles of denaturing 30 sec at 95^{0}C, annealing 20 sec at 45^{0}C, and extension 1 min at 70^{0}C, a final step of 7 min at 72^{0}C. Amplified PCR products were detected by electrophoresis using 1.5% agarose gel.

RESULTS

Primer design for detecting papaya sex by PCR

To examine the sites of base substitution on chromosome Y between male and hermaphrodite *Carica papaya*, W11-Herm chromosome Y sequence (GenBank: AY428938.1 (H) and W11-Male chromosome Y sequence (GenBank: AY428939.1 (M)) (NCBI, 2014) were aligned using CLUSTAL2 program. The analysis revealed that three sites at 267th, 412th, and 474th in hermaphrodite had bases G, A and C respectively, whereas these same sites in male, the bases were substituted by A, G, and A respectively (Figure 1).

In this study, the primer design was based on the 3' terminal nucleotide of a primer corresponding to a specific site with a perfectly complemented base to only hermaphrodite DNA template. For these, new two forward (PH1-F and PH2-F) and two reverse (PH1-R and PH2-R) primers were designed and used to distinguish the hermaphrodite and male papaya by PCR. Moreover, the SCAR-W11 marker (Deputy et al., 2002) was used to distinguish between hermaphrodite and male group, and female group. The results found that two primer pairs designed for detecting hermaphrodite were based on AY428938.1 (H) sequence; (1) PH1-F and PH1-R, and (2) PH2-F and PH2-R with predicted length of PCR product as 101bp, and 182bp, respectively.

Morphology study of papaya flower in each sex type

Nine biological samples of papaya flower (Holland cultivar) were collected and classified as sex types according to flower structure (Figure 2). The result showed that a male flower was a small spoon-like shape, inflorescence flower on a long peduncle. Each floret consisted of five sympetalous and ten stamens (alternated

of five short and five long stamens) without pistil. A female flower was a bell-like shape, large and short pistil. Its flower consisted of five polypetalous, short peduncles, long styles without anthers. A hermaphrodite flower is a cone-like shape with five sympetalous. Its flower contained a long pistil and ten stamens. The result suggested that the flower morphology was very useful for sex type determination in papaya.

5'-CTGATGCGTGATCATCTACTAAATTCAAAGAGTTTGCCCTAATCACATTCACAAAGCAA
AGTCACCTAACAATCTACAAATGATCGTCTGTGAAATCTTATTATAATAATCATTACTAGA
GATCATTGAACCACCTTTTACAATTTACAAATCCATTAATCGATATAACTCTAAAACTAAG
GTTTTGAATGCTTACCATGGATGGGAATTGGGTCTCATGAAGAAACCCTTTCAGCTCATCG
TGTGGGGTTAGAGCTAGTTTTCAT**G(A)**CCCTGCATGTAGTTAAGAATAGTTTAAAAATGGT
CAGCCCATTTAAACCATGACTGGTGAAATGGACAAACTCACCTTTATGGCAGCAGAAAGA
AACATAAATCGTGGCAATCAACTTTCACTTACTGCCATGCACTAACCTCA**A(G)**GCCTTGTC
ACGCATCCCAACATGAGTAGTTGCTCTTGAAACCAACAAAGATTCCCCCCATG**C(A)**AGTG
GCATTAATGCAACGCATGTTAAAAACCTGCGGGTCCTACGAACCTAGAACATTTGATGCCT
ACAACACCACTTACAAAACACCCACTCTTCCTCTGCTAATTCTTGTAATTGTCAGCGTGCTT
GCCGAACATAGAGGCTTTCGGCCTCACTAACCTTTCTCCCCTCACACCCAATCCCATAAAT
CTCGTGGATCGTGCTCCTAGTGCTCATGGTGACACCCGCACATCACTAACCTCACATATAC
ACACTATGTCGCACAATCACTGACCTCCCTCATGCACGCGACACGGCACACACACCCTTAC
CCTTTCATTGCCCCTCACACATATAGATAGAGCCACACACGCATCAG-3'

Note: The bold letters represent the sites of base substitution; the letters in a bracket represent the bases found in a male sex papaya (GeneBank: AY428939.1) (NCBI, 2014)**.**

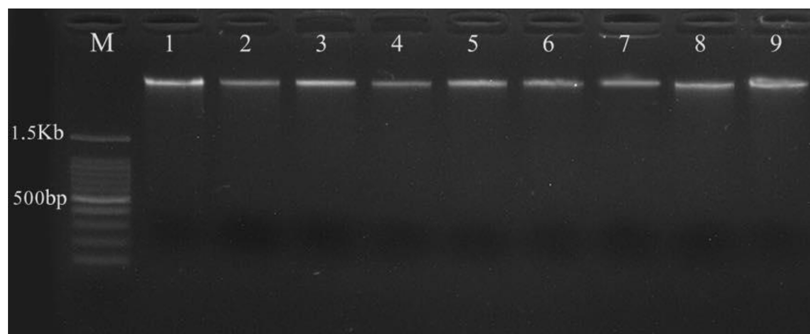
Figure 1 The sequence from hermaphrodite sex papaya (GeneBank: AY428938.1) used for primer design.

Figure 2 Determination of sex forms, male (A), female (B) and hermaphrodite (C), using morphological trait of papaya flower.

Genomic DNA qualification

The males, females and hermaphrodites among five papaya cultivars including HO, KD, KO, KN, and SSK were collected from six private farms in Phitsanulok province, Thailand. Their young leaves were harvested and the gDNA was isolated using innuPREP Plant DNA Kit.

Before starting any experiments, the quality of gDNAs was checked by an agarose gel electrophoresis. The gDNAs (approximately 0.5 µg) were run on a 1.2% agarose gel containing 0.001% (v/v) ethidium bromide for visualization of DNAs. As shown in Figure 3, bands corresponding to gDNAs were highly enriched, sharp, and without a smear. This indicated that gDNAs in the samples were high quality and purity without apparent RNA degradation.



Note: Lane M represents 100 bp marker, lanes 1-9 represent genomic DNA isolated from each papaya leaf sample.

Figure 3 The quality of a gDNA preparation was assessed by a 1.2% agarose gel electrophoresis (Naksing *et al*., 2015).

Sex type identification by PCR technique using W-11 primers

The SCAR-W11 primers were forward (5'-CTGATGCGTGATCATCTAC T-3'), and reverse (5'-CTGATGCGTGTGTGGCTCTA-3') which were used to identify sexes in the Holland papaya cultivar, represented three biological replicates of each male, female, and hermaphrodite. The results found that PCR products (expected to 830bp length), which were amplified by SCAR-W11-F and SCAR-W11-R, were presented in male and hermaphrodite samples, except in female samples (Figure 4). This result indicated that SCAR-W11 primers can distinguish male and hermaphrodite plants from female plant. However, these primers cannot distinguish between male plant and hermaphrodite plant. Therefore, the new primers should be further developed for determination between male and hermaphrodite papaya.

Papaya sex determination by new designed primers

In the study, new two-forward primers (PH1-F: TTAGAGCTAGTTTTCAT**G**, PH2-F: CTGCCATGCACTAACCTCA**A**), and two-reverse primers (PH1-R: GCGT TGCATTAATGCCACT**G**, PH2-R: TTGGGATGCGTGACAAGGC**T**) were designed

to determine the male- and hermaphrodite Holland papaya cultivar using PCR technique.

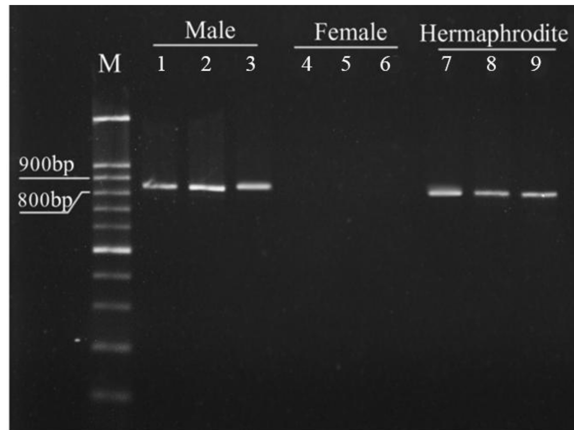


Figure 4 Amplification pattern of SCAR-W11 marker among male (lanes 1-3), female (lanes 4-6) and hermaphrodite (lanes 7-9) specimens of Holland papaya cultivar (Naksing *et al*., 2015).

Of these, the PH1-F and PH1-R primers gave only a band of 247 bp was observed in hermaphrodite samples whereas several bands of 247, 400, and 550bp were observed in male and female samples (Figure 5).

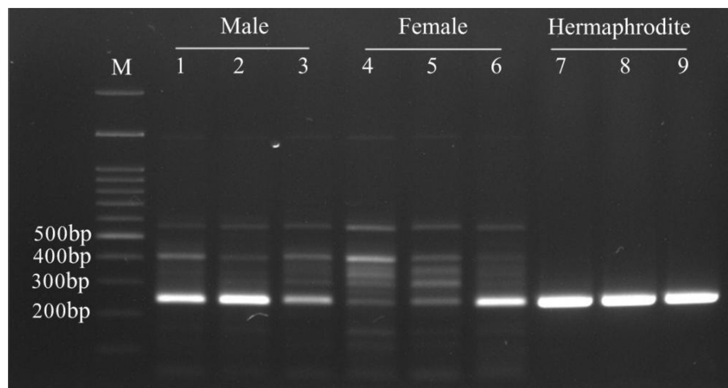


Figure 5 An amplification pattern of PH1-F and PH1-R primers among male (lanes 1-3), female (lanes 4-6) and hermaphrodite (lanes 7-9) specimens of Holland papaya cultivar.

Moreover, other tested primers (PH1-F and PH1-R) gave the PCR product size (expected to 182bp), which was presented in male and hermaphrodite samples, except in female samples (Figure 6).

For the last test, the PH2-F and PH1-R primer gave the PCR product size (expected to 101bp), which was presented only in hermaphrodite samples, except in male and female samples (Figure 7). Among the three primer pairs tested for their ability to generate the PCR product profiles which can clearly determine the hermaphrodite from male and female of Holland papaya cultivar.

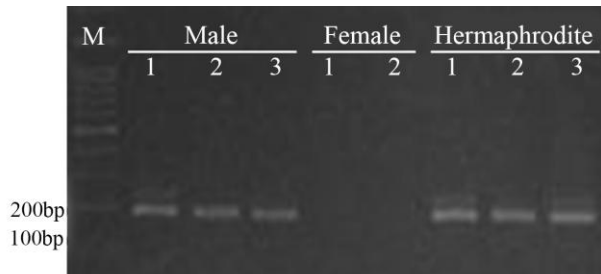


Figure 6 Amplification pattern of PH1-F and PH2-R primers among male (lanes 1-3), female (lanes 4-6) and hermaphrodite (lanes 7-9) specimens of Holland papaya cultivar.

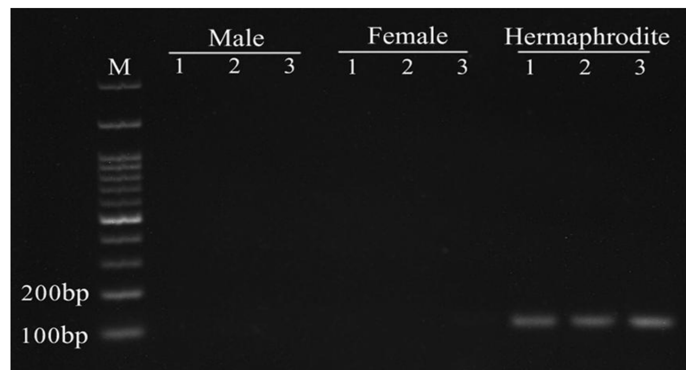


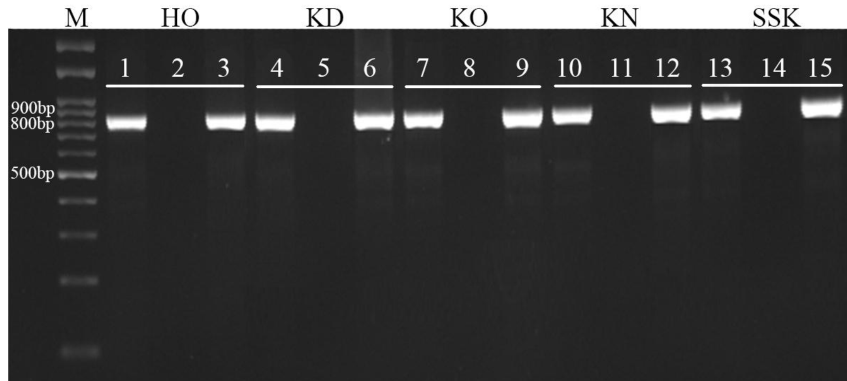
Figure 7 An amplification pattern of PH2-F and PH1-R primers among male (lanes 1-3), female (lanes 4-6) and hermaphrodite (lanes 7-9) specimens of Holland papaya cultivar.

Utilization of the two specific primers in different papaya cultivars

The previous results revealed that both two primer pairs, the SCAR-W11-F with SCAR-W11-R and the PH2-F with PH1-R, can be accurately identified sexes in Holland papaya cultivar. To further investigate it was possible whether these primer pairs might be used as molecular markers for male female and hermaphrodite identification in different papaya cultivars or not. Two individual primers were used the amplification polymorphism against DNA samples from male, female and hermaphrodite of five papaya cultivars: Ho, KD, KO, KN, and SSK.

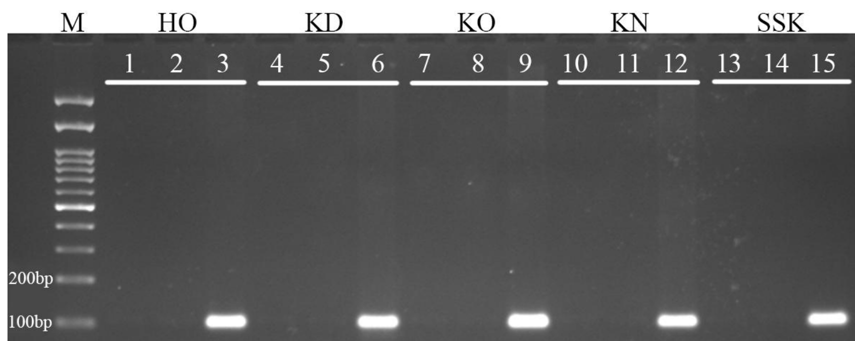
The results found that the SCAR-W11-F with SCAR-W11-R primers yielded clear amplified products, approximately 830bp length in male and hermaphrodite but

not in female of all five papaya cultivars (Figure 8). Moreover, the PH2-F with PH1-R primers gave characteristic amplified product, approximately 101bp length in hermaphrodite but not in male and female of all cultivars (Figure 9).



Note: Five papaya cultivars are represented by Holland (HO), Khaek-Dam (KD), Koko (KO), Khak-Nuan (KN), and Si-Sa-Ket (SSK). M represents 100bp ladder marker. Lanes 1, 4 ,7, 10, and 13 refer to male of different papaya cultivars. Lanes 2, 5 ,8, 11, and 14 refer to female papaya cultivars. Lanes 3, 6, 9, 12, and 15 refer to hermaphrodite papaya cultivars.

Figure 8 An amplification profile of the five papaya cultivars amplified with SCAR-W11 primer.



Note: Five papaya cultivars are represented by Holland (Ho), Khaek-Dam (KD), Koko (KO), Khak-Nuan (KN), and Si-Sa-Ket (SSK). M represents 100bp ladder marker. Lanes 1, 4 ,7, 10, and 13 refer to male of different papaya cultivars. Lanes 2, 5, 8, 11, and 14 refer to female papaya cultivars. Lanes 3, 6,9, 12, and 15 refer to hermaphrodite papaya cultivars.

Figure 9 An amplification profile of the five papaya cultivars amplified with PH2-F and PH1-R primer.

DISCUSSION

For commercial papaya production, efforts to identify only hermaphrodite of different papaya cultivars in a seedling stage is extremely important prior to its transplantation to the orchard. Generally, the papaya sexes cannot be examined either by seed shape or morphology at the juvenile developmental stage, but especially identified only after 4–6 months when the plant attains to reproductive maturity (Ma *et al*., 2004). Therefore the aim of this research was to develop specific- molecular markers for sex determination in different papaya cultivars, grown in Thailand.

In this study, the SCAR-W11 primers were very useful for detecting male and hermaphrodite of five studied papaya cultivars (Holland, Khaek-Dam, Koko, Khak-Nuan, and Si-Sa-Ket), by giving the precise PCR product approximately 830bp length in male and hermaphrodite but not in female. This result agreed with previous findings that the SCAR-W11 has been used to determine sex among different cultivars of papaya (*Carica papaya* L.) such as Sunrise and Kapoho (Deputy *et al*., 2002) including Arka Prabhat , Dwarf Lilly, Nigeria Shilong, and Surya (Chaturvedi *et al*., 2014). The reason may be that the range of DNA sequences within this primer pair, located on nearly centromere of the chromosome Y (Ming *et al*., 2007), shared some identical sequences between male and hermaphrodite, but not in female. This was supported by the result from alignment of AY428938.1-hermaphrodite sequence (831bp) and AY428939.1-mae sequence in male (834bp) using CLUSTAL2 program that had base substitution in a few sites along these sequences. This might cause a point mutation during the evolution of sex determination in papaya (Yu *et al*., 2007).

Moreover, the new primer pair (PH2-F and PH1-R) was successfully useful to determine only in hermaphrodite of all validated cultivars of papayas (Holland, Khaek-Dam, Koko, Khak-Nuan, and Si-Sa-Ket) grown in Thailand, generating the PCR product (approximately 101bp length). This developed molecular marker might be tightly linked to *Sex1* gene, determining papaya sex (Souder *et al*., 1996). Because the primers are located within region between SCAR-W11-F and SCAR-W11-R marker, it has genetic distance within 0.3cM from *Sex1* and no crossovers between SCAR-W11 and Sex1 as previously reported by Chaturvedi *et al* (2014).

CONCLUSION

In the present study, the results clearly illustrated that two primer pairs (SCAR-W11, and PH2-F–PH1-R) can be potentially and accurately used to identify papaya-sex specificity at seedling stage. The primer pair of SCAR-W11-F and SCAR-W11-R was able to identify male and hermaphrodite seedlings, by generation male- and hermaphrodite-specific PCR product approximately 830bp length, but not in females. The primer pair of PH2-F and PH1-R was able to identify hermaphrodite seedlings, by generation hermaphrodite-specific PCR product approximately 101bp length, but not in a male and females.

ACKNOWLEDGEMENTS

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