International Journal of Development and Sustainability
ISSN: 2186-8662 – www.isdsnet.com/ijds
Volume 6 Number 11 (2017): Pages 1621-1637
ISDS Article ID: IJDS17101603

Flora morphology and sex specific marker development of a Sarawak's specialty fruit species *Canarium odontophyllum*

Jen-Ching Ting [1*], Wei-Seng Ho [1], Cheksum Tawan [2]

[1] *Forest Genomics and Informatics Laboratory (fGiL), Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia*

[2] *Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia*

Abstract

Dabai or scientifically known as *Canarium odontophyllum* being a Sarawak's specialty fruit is well-known with its unique sexual characteristic. The hermaphrodite dabai trees bear fruits while male dabai trees do not and are normally removed from the field. Failure to determine gender at early stage can results in undesirable waste of resources and hence, there is a need to identify sex specific markers for sex determination using molecular DNA markers in dabai trees. The floral morphology of dabai was also documented in this study to further elucidate the molecular findings. A set of 64 RAPD primers were screened by using the bulk segregant analysis (BSA) approach and a unique RAPD fragment (DH_{272}) was detected in all the hermaphrodite samples. The DH_{272} fragment was then converted into SCAR marker and tested for its polymorphism. However, the SCAR marker did not differentiate between the two sex-type upon PCR amplification. To recover its polymorphism, sequences of two male-specific and one hermaphrodite-specific SCAR amplicons were subjected to SNP and CAPS identifications. Five SNPs were detected and one CAPS site, TaqI which is located with T-G base substitution on the recognition sequence was identified. It is of high possibility that the CAPS marker would discriminate hermaphrodite from male dabai trees via PCR amplification and further restricted with the TaqI restriction enzyme. More sequences are needed for additional SNP discovery and validation. However, this study demonstrated the usefulness of RAPD, SCAR, SNP and CAPS for sex determination in dabai and in future, a rapid dabai sex typing system could be established at the seedling stage.

Keywords: Dabai, *Canarium odontophyllum*, Hermaphrodite, RAPD, SCAR, SNP, CAPS, BSA, PCR, Floral Biology

Cite this article as: Ting, J.C., Ho, W.S. and Tawan, C. (2017), "Flora morphology and sex specific marker development of a Sarawak's specialty fruit species *Canarium odontophyllum*", *International Journal of Development and Sustainability*, Vol. 6 No. 11, pp. 1621-1637.

[*] Corresponding author. *E-mail address:* wsho@unimas.my

1. Introduction

Dabai, also called Sibu olive is known to be one of the most popular indigenous fruits in Sarawak. Scientifically named as *Canarium odontophyllum*, the dabai fruits can be found along the riverbanks in Sibu, Kapit and Sarikei (Jackson, 2008). The trees may grow to an altitude up to 700 metres above sea level. They are heavy fruit bearer and may yield up to 800 kilograms per tree. The dabai fruits are white in colour during their immature stage and subsequently turn purple when ripen. The olive-like fruits are oblong in shape, 35 to 40 mm long, 20 to 25 mm wide and have a thin edible skin. The whitish or yellow edible mesocarp inside is 6 to 8 mm thick and covers single, large three angled seed (Whitmore, 1972).

Dabai is a very nutritious fruit with high value of proximate composition and minerals (Voon and Kueh, 1999). To be certain, a 100g of edible portion constitutes up to 339kcal of energy, 3.8% of protein and 810mg of potassium. Study also reported the potential of dabai fruits to be exploited as functional food due to its high antioxidant properties (Azrina et al., 2010). Considering its beneficial properties, the Sarawak Agriculture Department is confident to venture into commercialization of dabai as Sarawak specialty fruit.

The sexual characteristic of dabai is still a concerned issue. Dabai trees have male and hermaphrodite flowers borne on different trees. Male trees will not bear fruit but to produce pollen for the hermaphrodite flowers. Therefore, only hermaphrodite trees are cultivated in the plantation and the male trees are normally removed from the field to increase profitability. To date, there is no way for the dabai growers to differentiate a dabai's sex based on the physical appearance of the seedlings. It only can be known after the dabai trees attain reproductive maturity which is approximately after 4 years of planting. Propagation by means of cutting and grafting is still difficult and therefore, seeds are still preferred choice in propagating the dabai trees. There is a 50:50 chance of obtaining hermaphrodite trees. The current practice is to grow dabai seedlings in one area, allowing them to grow for 4 years or more until the sex types are identified, and then to eliminate the undesired male trees to establish an orchard with only hermaphrodite trees.

Random amplified polymorphic DNA (RAPD) involving utilization of random primers to amplify a set of randomly distributed loci and further developing genetic markers for a variety of purposes (Williams et al., 1990). Using this approach, researchers have identified molecular markers for sex determination in various species such as *Trichosanthes dioica* (Singh et al., 2002), *Cannabis sativa* (Sakamoto et al., 2005), *Borassus flabellifer* (George et al., 2007) and *Commiphora wightii* (Samantaray et al., 2010). Although the RAPD method is relatively fast, cheap and easy to perform in comparison with other method, the issue of reproducibility is still a matter of debate. To overcome this drawback, a reliable assay called sequence characterized amplified region (SCAR) was established by cloning the RAPD fragment of interests and then sequencing of the cloned products (Agarwal et al., 2008; Ho et al., 2014). The sequence is thereafter used to synthesize two longer primers homologous to each end with approximately 15-30 base pairs (Shen et al., 2003). An advantage of SCAR markers is their potential for quick and robust assessment (Guérin et al., 2003). The strategy has been successfully used to generate genetic markers for plants such as *Olea europaea* (Hernandez et al., 2001), *Carica papaya* (Urasaki et al., 2002), *Eucommia ulmoides* (Xu et al., 2004) and *Salix viminalis* (Gunter et al., 2003). In sex determination of plant species, SCAR marker is developed to have genetically linked to sex determination loci. Single nucleotide polymorphism (SNP) requires sequencing of

the targeted fragment or gene of interest to identify the sequence variation (Ho et al., 2011; Tchin et al., 2012; Tiong et al., 2014; Tan et al., 2014). Meanwhile cleaved amplified polymorphic sequence (CAPS) polymorphisms are variations in restriction fragment lengths caused by SNPs that create or abolish restriction endonuclease recognition sites in PCR amplicons (Li et al., 2010).

Since there is no way to discriminate the gender of dabai at early stage or prior to the flowering stage, it is crucial to study its sex expression at the molecular level. Hence, the present study is aimed to identify the sex specific bands related to sex determination of dabai using RAPD, SCAR, SNP and CAPS markers. Little is known about the unique sexual characteristic of dabai also led us to examine and document the morphological characteristics of dabai flower and its flowering stages, whereby an outcome of which would have great contribution towards the evolutionary studies of this unique fruit species.

2. Materials and methods

2.1. Floral morphology of dabai

The fresh flowers of dabai were randomly collected from six different male and hermaphrodite trees in a population. In the laboratory, these flowers were observed under a dissecting microscope for characteristics such as symmetry of corolla; numbers of all four whorls; androecium characteristics; and gynoecium characteristics. Another dehiscence and stigma receptivity were also visually observed. Many flowers with receptacles still attached to plant stems were preserved in 90 % ethanol for histological purpose.

2.2. Plant materials and genomic DNA extraction

A total of 15 male and 23 hermaphrodite dabai fresh leaf samples were collected from a nursery farm in Serian, Sarawak. Total genomic DNA was extracted from fresh young leaves of dabai by using a modified CTAB procedures described by Doyle and Doyle (1990). The DNA samples were then purified using a Wizard Genomic DNA Purification Kit (Promega, USA). Spectrophotometric quantification was conducted to estimate the purity level of the DNA samples.

2.3. Bulk segregant analysis (BSA) and RAPD analysis

Pooled DNA samples were prepared for BSA (Michelmore et al., 1991) by mixing equal amount of purified DNA of the 15 male individuals (male bulk) and 23 hermaphrodite individuals (hermaphrodite bulk) separately. Prior pooling, male and hermaphrodite DNA of varying concentration were standardized and preset to 50ng/µl. Both male and hermaphrodite pooled DNA were subjected to PCR amplification using the Veriti™ thermal cycler (Applied Biosystems, USA) programmed to 1 cycle of 2 minutes at 94°C for initial strand separation, followed by 40 cycles of 1 minute at 94°C denaturation, 2 minutes at 35°C annealing, and 2 minutes at 72°C extension steps ending with 1 cycle of final extension at 72°C for 10 minutes. Total reaction volume for DNA amplification was 25 µl. Each reaction contained 5 µl of 1 × PCR buffer (200 mM Tris-HCl,

500 mM KCl and 2.5 mM $MgCl_2$, 0.2 mM each dNTPs (Promega, USA), 2.5 mM of $MgCl_2$, 10 pmol of RAPD primer (Operon Technologies, USA), 1.0 unit of *Taq* DNA polymerase (Invitrogen, USA) and 5 ng of DNA template.

Primer screening was done by screening 64 commercial decamer RAPD primers (Operon Technologies, USA). Amplified PCR products were resolved using 1.5% agarose gel electrophoresis followed by ethidium bromide staining and documented using the Geliance 200 Imaging System (Perkin Elmer, USA). The banding patterns of each primer were analyzed on the basis of presence and absence of band. RAPD bands that present in male bulk sample while absent in hermaphrodite bulk sample or, vice versa were selected and functioned as diagnostic bands for sex identification. To confirm the sex specificity of each diagnostic band, all putative RAPD primers were re-amplified using 15 male and 23 hermaphrodite DNA samples individually. The selected RAPD diagnostic band (DH_{272}) was converted into SCAR marker as described by Paran and Michelmore (1993).

2.4. SCAR marker analysis

Two oligonucleotide primer pairs (DH272(F): GTGAGGCGTCCACTTGACGC and DH272(R): TGTGAGGCGTCGGCGAAGAC) were designed for locus-specific amplification as identified by the RAPD marker. SCAR amplification was performed in 25µl reaction volumes containing similar components as described in RAPD analysis. PCR amplification using the Veriti™ thermal cycler (Applied Biosystems, USA) was programmed to 1 cycle of 2 minutes at 94°C for initial strand separation, followed by 40 cycles of 1 minute at 94°C denaturation, 2 minutes at 64°C annealing, and 2 minutes at 72°C extension steps ending with 1 cycle of final extension at 72°C for 10 minutes. Amplified PCR products were resolved through 1.5% agarose gel electrophoresis followed by ethidium bromide staining and documented using the Geliance 200 Imaging System (Perkin Elmer, USA).

2.5. SNP detection and CAPS analysis

Two male samples (M1 and M11) and one hermaphrodite sample (D10) were selected for SNP analysis. The sequences of DH_{272} were aligned by using ClustalW v2.1 software to identify single nucleotide polymorphisms and/or insertion-deletion (Indel) between male and hermaphrodite. Sequence comparison information was then used in CAPS analysis. Using NEBcutter V2.0 (Vincze et al., 2003), male and hermaphrodite sequences were used to detect restriction enzymes in NEB database as well as site of restriction. Polymorphic restriction site mutations were identified by comparing the enzymes found in all digested sequences. The corresponding restriction enzymes can be selected for CAPS marker analysis in future.

3. 3. Results and discussion

3.1. Flora morphology of dabai

The male flower of dabai was incomplete and unisexual with the presence of three whorls, i.e. calyx, corolla and androecium. The calyx on the lowest position on the receptacle was collectively green in colour and comprised three sepals. The rotated-shape corolla was parted and located in the interior of the sepals, with three distinctive white colored petals and red spot on the tip of each petal. Androecium characteristic was observed in term of region of attachment, in which the male reproductive organ was fused with petals at the base on a yellow disc-like tissue. Male reproductive organ of dabai comprised five stamens with typical anthers and filaments. Anther dehiscence was visually observed as the anther thecae had dehisced, exposing pollen grains for floral visits. Figure 1 illustrates a compilation of the microscopy images taken on the typical characteristics of the male flower.

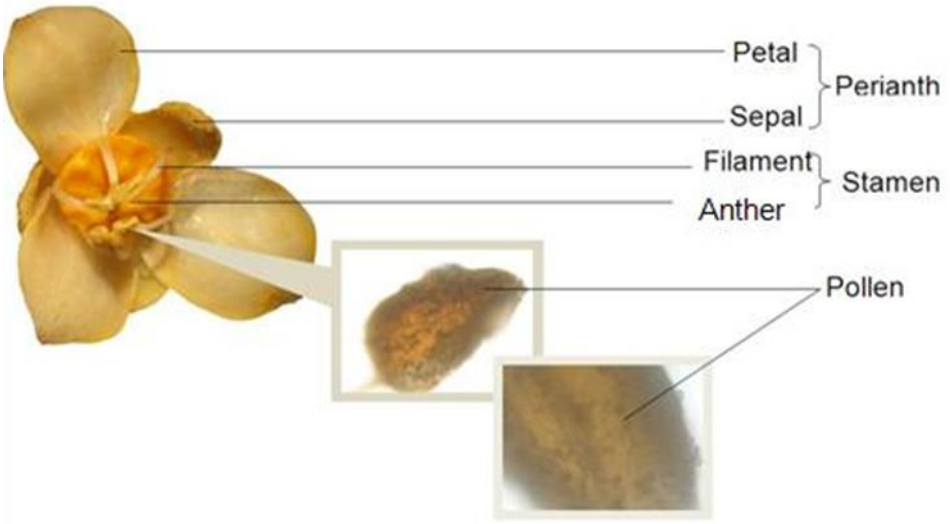

Figure 1. General morphology of male flower of dabai

The hermaphrodite flower of dabai was perfect, completed with all four whorls of a typical hermaphroditic flower (Figure 2). It shared homology with male flower within two whorls, i.e. the calyx and the corolla. Gynoecium characteristic was observed in term of region of attachment, in which the female reproductive organ was fused with petals at the base on a yellow disc-like tissue. Female reproductive organ of dabai comprised three carpels with three parted stigmas, fused style and a swollen part of ovary at lower part containing ovule. Stigmatic receptivity was noted visually as the stigmata are wet and shiny when they are receptive. As referred to be hermaphroditic, androecioum characteristic was also observed with six staminodes complete with anthers and filaments, surrounding the female ovary. However, anther dehiscence was not observed and therefore defined as sterile, reduced stamens.

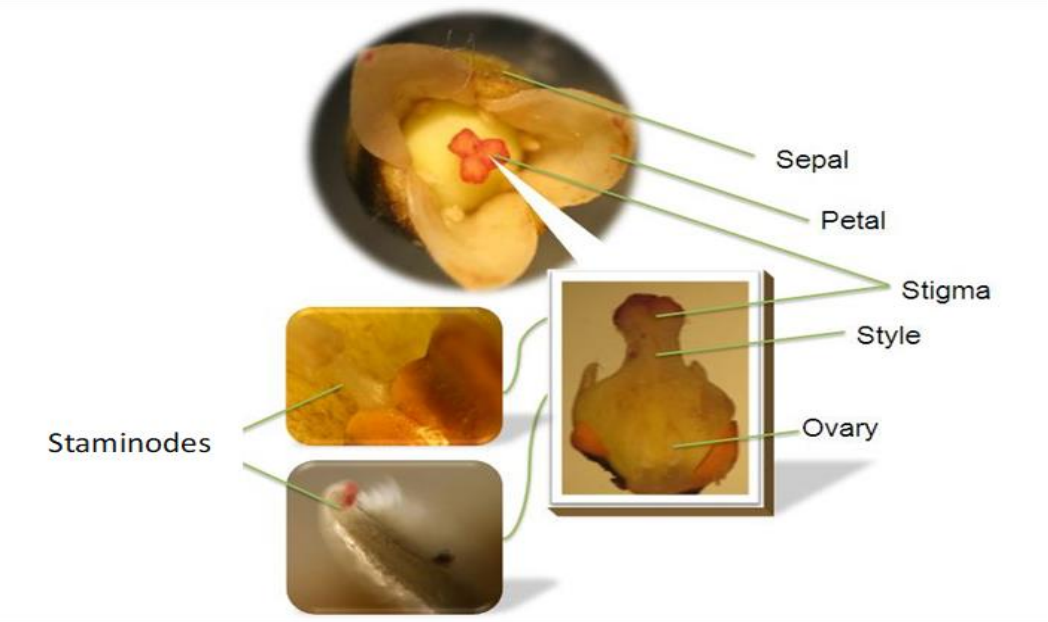

Figure 2. The hermaphrodite flower of dabai with typical female reproductive organ. The six sterile or abortive male reproductive organs are referred as staminodes with no pollen observed

Taxonomical classification had once defined dabai as dioecious species with staminate (♂) and pistillate (♀) flowers borne on different plants. Our study on floral morphological study revealed one extraordinary yet unique characteristic on the pistillate flowers. Ordinary pistillate flower comprises style, stigma and ovary, collectively known as a typical female organ. Microscopy observation discovered that the pistillate flower of dabai appear to have both male and female organs on the same flower, considerably hermaphrodite flower. Despite this firm finding that may revise the dioecy characteristic of dabai, floral modification via male sterility was another interesting discovery. Male organ exists in the hermaphrodite flower comprises of six staminodes, indicating self-incompatibility or male sterility with no observation on anther, filaments or pollen availability. This floral modification lies beneath a principle where all flowering plants originate as hermaphroditic that undergo gradual floral modification into a diversified floral form, with an intention to promote outcrossing or cross-pollination (allogamy) (Nelson et al., 1989). Thus, it can conclude that dabai populations are androdioecious with both hermaphrodite and androecious individuals.

The fruiting season of dabai is usually towards the year-end, together with the durian season. Flowering of this seasonal fruit requires both male and hermaphrodite flowers borne on separate trees. Sole function of the male flowers is to produce pollens for cross-pollination (allogamy) with the hermaphrodite flowers. Once pollinated, the hermaphrodite flowers will undergo several stages of floral development until the fruits set. Figure 3 outlines the flowering stages of hermaphrodite flower of dabai. In the initial budding stage, the lateral floral buds with small swelling are enclosed in protective bud scale and surrounded by immature perianth segment. In the calyx release stage, the buds are now enclosed by fused calyx in green where petal

growth is largely complete. Growth of inner ovary tissues pushes and splits the calyx between lobes, resulting in rapid release of the fully expanded petals. After release from the calyx, the 3 petals that are originally white in colour expand and acquire a yellowish lining with red spot on the tip. When unfold, it is clearly seen that hermaphrodite flower has three sepals, three petals, a pistil carrying a style with a three-lobed stigma and six staminodes. Once pollinated and fertilized, several changes in the flower take place. These include a) the sepals and petals dry out and wrinkle, but remain attached on the flower, and b) the petals fall and dabai fruits in white begin to set within the sepals. The style and stigma also wither and eventually fall off. Lastly the fruiting stage where sepals fall completely, leaving only white and continuously enlarging dabai fruits.

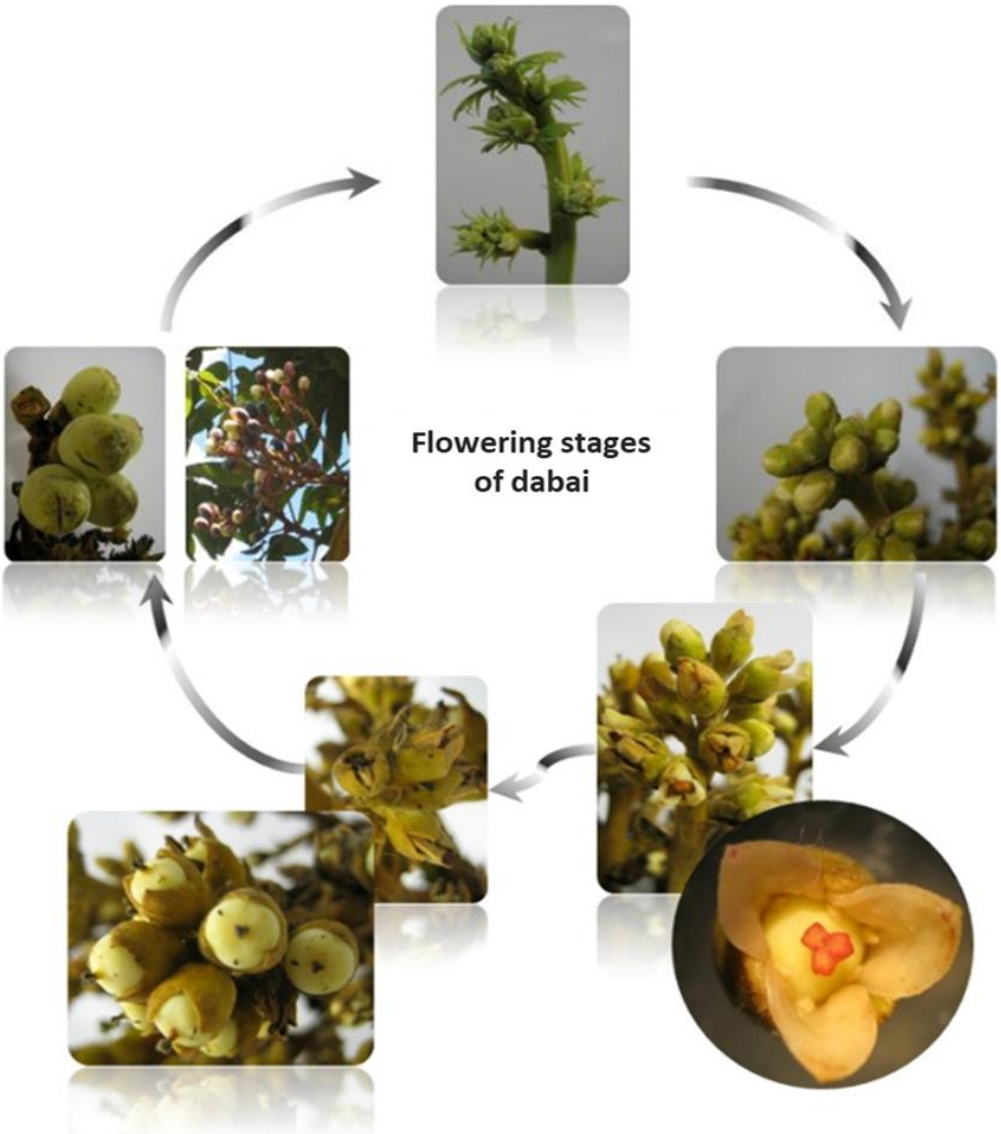

Figure 3. The flowering and fruiting stages of dabai

3.2. RAPD analysis

To the best of knowledge, the present study of sex determination in dabai using RAPD markers was the first to be reported. RAPD analysis for sex determination in dabai was performed following a novel protocol described by William et al. (1990). Instead of using segregating progeny as in *C. papaya* (Urasaki et al., 2002), male and hermaphrodite DNA pools were prepared by means of bulk segregant analysis. Without any modification on PCR profile and condition, both DNA pools were amplified using 64 random primers for potential detection of sex-related diagnostic bands. As mentioned by Wilkerson et al. (1993), primer screening on pooled DNA of opposite sex types provided a convenient approach to narrow down primers that express polymorphism, thus circumventing the need to run PCR on all individuals in each primer. Such method had been found successfully in species such as *T. dioica* Roxb. (Kumar et al., 2008); *G. biloba* (Jiang et al., 2003) and *B. flabellifer* L. (George et al*.,* 2007).

Of 64 random primers screening, 24 primers were found to amplify 18 male-specific diagnostic bands and 20 hermaphrodite-specific diagnostic bands. Most PCR reactions had resulted in monomorphic banding patterns, demonstrating a high genomic similarity between male and hermaphrodite of dabai. A similar result was also obtained by Sondur et al. (1996) who studied *C. papaya* with contrasting morphological traits in male, female and hermaphrodite cultivars. They tested 596 RAPD primers and only a relatively small number of polymorphic bands were obtained for constructing a genetic linkage map, with further determination of sex locus in papaya plants. The large genomic similarity may be explained by the fact that dabai is a narrowly distributed species. Hamrick and Godt (1989) stated that narrowly distributed species had higher genomic similarity than more widely spread or distributed species.

To reconfirm the sex specificity of all potential diagnostic bands, confirmation test was conducted on individual male and hermaphrodite samples. After series of PCR amplifications, a 272 bp RAPD fragment (DH_{272}) was found to have consistently amplified in all hermaphrodite individuals but completely absent in all male individuals tested (Figure 4). The exclusiveness of this hermaphrodite-specific polymorphic band thereby functioned as diagnostic band for selecting hermaphrodite seedlings from male seedlings.

Since random sequences were used as RAPD primers, identification of sex-related diagnostic band through RAPD analysis depend largely on the extent of chance (Bardakci, 2001). In the present study, 64 RAPD primers had been screened in dabai and found only one hermaphrodite-associated RAPD marker. Similar result was reported by Hormaza et al. (1994) whereby 700 primers were screened in *P. vera* for sex determination and only one female-associated marker was identified. This low frequency of sex-related bands may have indicated that genomes of male and hermaphrodite are rather homologous, and that the DNA locus involved in sex determination was rather small and probably controlled by single gene or few genes (George et al., 2007). According to Jiang and Sink (1997), sex determination system in *A. officinalis* was controlled by a single dominant gene. A total of 760 RAPD primers were tested on 63 *Asparagus* offsprings and one marker was found to be useful for sex determination in the plants. However, sex determination in *C. sativa* L. (Mandolino et al., 1999) and *C. papaya* L. (Chaves-Bedoya and Nuñez, 2007) utilized only 20 and 32 RAPD primers, respectively to identify a sex discriminating marker.

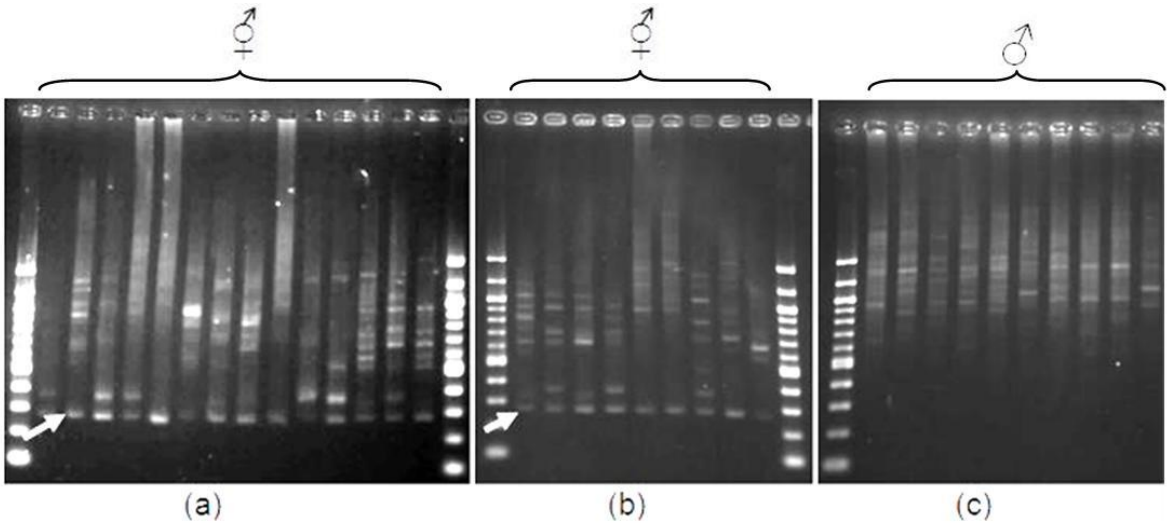

Figure 4. RAPD banding patterns of individual dabai amplified by primer OPC-02. The arrows indicate the unique band of 272 bp amplified. Panels (a) and (b): hermaphrodite DNA samples. Panel (c): male DNA samples.

3.3. Conversion of DH272 fragment to a SCAR marker

To establish a rapid and reliable PCR-based technique for dabai sex determination, the RAPD fragment of DH_{272} was converted to a SCAR marker. The SCAR primers were amplified on all 15 male and 23 hermaphrodite DNA samples. A single fragment of same size was found to be present in both male and hermaphrodite individuals (Figure 5). Modification of annealing temperatures and $MgCl_2$ concentrations were carried out to recover the polymorphic SCAR product (data not shown). However, the SCAR primers were unable to discriminate hermaphrodite from male samples.

Homology search in sequence database showed no significant similarity to the sequence of the hermaphrodite-specific fragment amplified (DH_{272}) by primer OPC02. This finding was in parallel with other plant species such as *G. biloba* (Jiang et al., 2003), *C. papaya* L. (Lemos et al., 2002; Urasaki et al., 2002) and *M. annua* L. (Khadka et al., 2002), where no homology was noted when comparing their sequence of interest with other sequences in the database. The lack of homologous characteristic could be explained in a way that DH_{272} sequence was probably located in an intergenic or non-coding region (Lemos et al., 2002). As in *E. ulmoides*, homology search of a 569 bp MSDE sequence linked to sex determination was rather contrasting with 53% homology with the *A. thaliana* chromosome 5. Further investigation later indicated that a single region on a homologous pair of chromosomes was responsible for sex determination in *E. ulmoides* (Xu et al., 2004).

The hermaphrodite-specific SCAR marker was then subjected to annealing temperature optimization. Khadka et al. (2002) stated that the range of optimal annealing temperature depend greatly on the level of polymorphic site between sex types, in which narrow range of optimal annealing temperature suggested low

polymorphism site and vice versa. Reviewing the gradient PCR profiles of the sex-specific SCAR markers, two possible outcomes could be presumed; that the SCAR marker would be hermaphrodite-associated thus justified the heterogametic mode of sex determination in dabai, or that the SCAR marker would be amplified in all the hermaphrodite individuals and some, or all the male individuals (Manoj et al., 2005). When verification test of SCAR marker proceed, rather strong indication could be made such that the hermaphrodite-specific sequence may have inherited from a male X chromosome or that it was closely linked with a female sex-determining gene (Ainsworth, 2000).

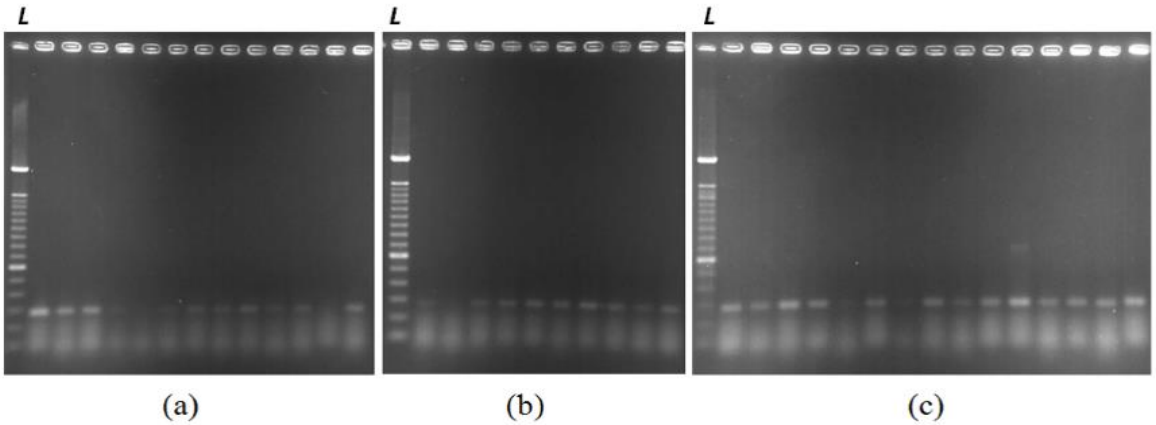

Figure 5. Agarose gel photos showing PCR products amplified by SCAR primer, DH_{272}. Panel (a) and (b): amplification on hermaphrodite individuals, panel (c): amplification on male individuals. L: 100 bp DNA ladder

Of all sex determination studies reviewed, male-specific association of SCAR markers was previously reported in *H. lupulus* (Polley et al., 1997), *P. longum* (Manoj et al., 2005) and *E. ulmoides* (Xu et al., 2004). *C. papaya* on the other hand, was widely investigated by researchers via SCAR analysis due to its hermaphrodite characteristic. Being a polygamous species with three sex types: male, hermaphrodite and female; many SCAR markers had been coincidently developed to discriminate male and hermaphrodite plants from female plants. These markers developed by different research groups were named as $OP\text{-}Y7_{900}$ (Chaves-Bedoya and Nuñez, 2007); OPC09-1.7 (Niroshini et al., 2008); SCARps (Urasaki et al., 2002); SCAR T12 and SCAR W11 (Deputy et al., 2002). Regardless of histological proofs if any, it was of rational suggestion that all above mentioned species were originated recently from some hermaphrodite ancestors (Manoj et al., 2005).

In the case of dabai, non-specificity of SCAR marker upon the process of verification was also encountered by species such as *C. sativus* (Mandolino et al., 1999), *C. papaya* (Sobir and Pandia, 2008) and *P. longum* (Manoj et al., 2005). During the optimization process of DH_{272}, non-polymorphic false-positive signals were observed as bands appeared in all hermaphrodite samples and even in all male samples. DH_{272} was unable to detect sex-specific polymorphism in an exact way the RAPD primer OPC-02 initially detected it. Inaccuracy of

SCAR marker in early PCR optimization stage was previously reported in *Agropyron elongatum* when developing SCAR marker for identification of leaf rust resistance gene *Lr24*. The non-polymorphic results were unable to attribute cultivars with outstanding leaf rust resistance to the gene *Lr24* (Uhrin et al., 2008).

Although most SCAR analysis had recorded success in other plant species, there were still studies documented total or partial loss of polymorphism, consequently provided new window for explanation and further analysis. For example, in *C. papaya*, Niroshini et al. (2008) obtained two RAPD fragments amplified by primer OPC-09 and OPE-03 that were specific to male and hermaphrodite plants. SCAR marker derived from the former showed sex specificity, however the SCAR designed from later failed to detect sex-specific polymorphism. Researchers thus referred this monomorphic condition to single base mutation at the primer annealing site during RAPD-PCR amplification. Since SCAR primer was designed with an extension of 10 bases at the 3' end of original RAPD primer, it was unable to amplify such single base polymorphism (Niroshini et al., 2008). Monomorphism of SCAR was also observed in *P. longum* when Manoj et al. (2005) conducted study to develop two sex-associated SCAR markers in the species. With one being strongly effective in amplifying male plants, the other MADP2 sequence-based SCAR marker failed to make the same achievement. A comparison of both male and female sequences showed three single base deletions in male plant, 744 bp and female plant was 747 bp in length. Theoretically, the marker could restore its polymorphism by incorporating nucleotide mismatches in the 3' end of the SCAR primer, or to be converted into a female-specific SCAR marker by means of base deletion. Whether these variations could represent a single PCR diagnostic assay was still unpredictable.

3.4. Conversion of SCAR to SNPs and CAPS

A conversion of SCAR into SNP marker was essential to recover the polymorphism detected in RAPD analysis, and could be applied in SCAR analysis on sex determination because certain sex-specific polymorphic SCAR markers might not possess identical sequences on or between two sex types. Such phenomenon was suggested in *M. annua* as researchers cloned and sequenced three SCAR fragments from strong, intermediate and weak male plants. Homology comparison showed 99.7% - 99.9% identity and located two base substitutions in strong male, four base substitutions and one insertion in intermediate male and a single base insertion in the sequence of the weak male (Khadka et al., 2002). Inaccuracy that could happen even in specific SCAR marker reflected the necessity to clone and sequence the monomorphic fragments in male and hermaphrodite samples of dabai. Paran and Michelmore (1993) also stated that despite the promise of SCAR being reliable and widely applied, monomorphic SCAR products and loss of initial polymorphism often happen.

When the SCAR DH_{272} sequences from male and hermaphrodite samples were compared, five base substitutions or single nucleotide polymorphisms (SNPs) were observed and there were no insertion/deletion (indel). Three A-G point mutations were found at 103 bp, 136 bp and 164 bp, respectively, one T-G point mutation at 79 bp and one A-T point mutation at 247 bp (Figure 6). Similarly, sequencing of monomorphic, same-sized SCAR fragments (750 bp) in *Lycopersicon esculentum* also revealed ten base substitutions and several indel mutations between two genotypes (Zhang and Stommel, 2001). In *Boehmeria*

spp., base substitutions were observed within 600 bp of SCAR fragments between two cultivars and two-point mutations were selected for CAPS assay (Li et al., 2010).

SNP could be converted into CAPS in all possibilities to generate a more reliable, simple, low-cost and robust assay. The principle of CAPS marker system is to use the SNP information available as causal and utilized an appropriate restriction enzyme to detect a SNP site, with a condition that its recognition sequence has been altered or introduced by that particular SNP (Thiel et al., 2004). Otherwise, development of CAPS markers could only be possible when mutation disrupted or created a restriction enzyme recognition site (Yeam et al., 2005). Among all studied species, development of CAPS markers in dabai was the first to be reported. Using NEBcutter V2.0, SNP information was examined manually to identify polymorphism that affected restriction enzyme site. The examination process was based on a principle that only enzymes that display a restriction site polymorphism in all aligned sequences are of interest (Thiel et al., 2004). In this study, one restriction enzyme (TaqI) had its four-base pair recognition sequence T/CGA targeted to digest at the same position of the T-G SNP (Figure 6). Therefore, restriction enzyme polymorphism with one altered base was reported and that the hermaphrodite sequence would not be able to find matches on the recognition sequence of TaqI as in the male sequence. Screening of restriction enzymes among aligned sequences might not always lead to a success. In attempts to recover SCAR polymorphism, Fang et al. (1997) screened 20 restriction enzymes and Naqvi et al. (1996) examined 11 restriction enzymes with both failed to detect polymorphism within cutting sites.

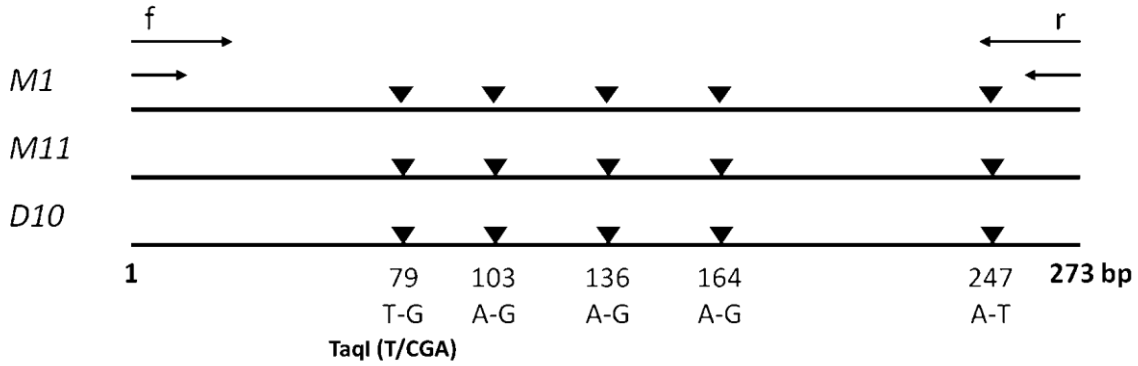

Figure 6. Schematic diagram of 273 bp fragments amplified by SCAR DH_{272} on M1, M11 and D10. Short arrows at termini represent the original 10-mer RAPD primers while longer arrows represent the location of 20-mer SCAR primers. f and r indicate forward and reverse SCAR primers, respectively. Solid triangles indicate position of SNPs along the sequences. The TaqI restriction enzyme that generated polymorphism was discovered by the NEBcutter V2.0

Although no previous CAPS assay was recorded in relationship with sex determination, the CAPS markers had been found to have enormous potential in identifying *Boehmeria nivea* variants (Li et al., 2010). This

Chinese herbal medicine of two variants (nivea and tenacissima) have its medicinal properties vary in between and required rapid and simple assay for identification. They found two restriction enzymes with their recognition sequences altered by single SNP. Upon PCR amplification, these CAP-based markers had successfully exhibited polymorphism with one variant being digested and one remained uncut (Li et al., 2010). Similar researches had been reported in sweet potato cultivars as researchers utilized 27 CAPS markers for genotyping of 60 cultivars (Tanaka et al., 2010). Cultivars such as strawberry and citrus (Kunihisa et al., 2005) were of success as well. Despite cultivar identification, 200 CAPS markers had been developed by Rostoks et al. (2005) to study the genetic diversity in barley and three CAPS markers were generated on recessive viral resistance alleles in *Capsicum* species (Yeam et al., 2005).

4. Conclusions

To the best of our knowledge, this is the first study to demonstrate the great potential of using molecular DNA markers, i.e. RAPD, SCAR, SNP and CAPS for sex prediction in dabai. The sex-specific markers can allow us to identify early enough which dabai seedlings are hermaphrodites and remove the male, saving on the investment that would be done in a plant with unknown sex, and consequently, reducing the production costs and end price of the fruits for the consumer. However, the PCR technique for sex prediction at this stage is still costly and therefore, there is a need to further develop a rapid, highly specific and reliable, and cost-effective dabai sex typing system for mass screening of dabai seedlings in future.

Acknowledgement

This work was supported by the funding from the Sarawak Timber Association and Universiti Malaysia Sarawak (Grant No. GL(F07)/06/2013/STA-UNIMAS(06) and the Ministry of Higher Education, Malaysia (Grant No. RACE/a(2)/884/2012(02).

References

Agarwal, M., Shrivastava, N. and Padh, H. (2008), "Advances in molecular marker techniques and their applications in plant sciences", *Plant Cell Rep.*, Vol. 27, pp. 617-631.

Ainsworth, C. (2000), "Boys and girls come out to play: the molecular biology of dioecious plants", *Annals of Botany*, Vol. 86, pp. 211-221.

Azrina, A., Nurul, N.M.N. and Amin, I. (2010), "Antioxidant properties of methanolic extract of *Canarium odontophyllum* fruit", *International Food Research Journal*, Vol. 17, pp. 319-326.

Bardakci, F. (2001), "Random amplified polymorphic DNA (RAPD) markers", *Turk J Biol.*, Vol. 25, pp. 185-196.

Chaves-Bedoya, G. and Nuñez, V. (2007), "A SCAR marker for the sex types determination in Colombian genotypes of *Carica papaya*", *Euphytica*, Vol. 153, pp. 215-220.

Deputy, J.C., Ming, R., Ma, H., Liu, Z., Fitch, M.M.M., Wang, M., Manshardt, R. and Stiles, J.L. (2002), "Molecular markers for sex determination in papaya (*Carica papaya* L.)", *Theoretical and Applied Genetics*, Vol. 106, pp. 107-111.

Doyle, J.J. and Doyle, J.L. (1990), "Isolation of plant DNA from fresh tissue", *Focus*, Vol. 12, pp. 1241-1251.

Fang, D.Q., Federici, C.T. and Roose, M.L. (1997), "Development of molecular marker linked to a gene controlling fruit acidity in citrus", *Genome*, Vol. 40, pp. 841-849.

George, J., Karun, A., Manimekalai, R., Rajesh, M.K. and Remya, P. (2007), "Identification of RAPD markers linked to sex determination in palmyrah (*Borassus flabellifer* L.)", *Current Science*, Vol. 93, pp. 8.

Guérin, G., Lerceteau-Kohler, E., Laigret, F., Roudeillac, P. and Denoyes-Rothan, B. (2003), "Development of SCAR marker linked to dominant gene conferring resistance *Colletotrichum acutatum* in Strawberry", *Acta Hort*., Vol. 626, pp. 85-91.

Gunter, L.E., Roberts, G.T., Lee, K., Larimer, F.W. and Tuskan, G.A. (2003), "The development of two flanking SCAR markers linked to a sex determination locus in *Salix viminalis* L.", *Journal of Heredity*, Vol. 94 No. 2, pp. 185-189.

Hamrick, J.L. and Godt, M.J.W. (1989), "Allozyme diversity in plant species" in Brown, A.D.H., Clegg, M.T., Kahler, A.L. and Weir, B.S. (Ed.), *Plant Population Genetics, Breeding, and Genetic Resources*, Sunderland, Sinauer, pp. 43-63.

Hernandez, P., Rosa, R., Rallo, L., Dorado, G. and Martin, A. (2001), "Development of SCAR markers in oliver (*Olea europaea*) by direct sequencing of RAPD products: applications in olive germplasm evaluation and mapping", *Theoretical and Applied Genetics*, Vol. 103, pp. 86-92.

Ho, W.S., Pang, S.L., Lau, P. and Ismail, J. (2011), "Sequence variation in the *cellulose synthase* (*SpCesA1*) gene from *Shorea parvifolia* ssp. *parvifolia* mother trees", *Journal of Tropical Agricultural Science*, Vol. 34 No. 2, pp. 323-329.

Ho, W.S., Yii, A.S., Lee, S.M., Cheksum, T., Isa, I., Pang, S.L. and Julaihi, A. (2014), "DNA Genotyping of Borneo Ironwood using M13 universal primer and SCAR marker development in *Eusideroxylon zwageri*", *Acta Biologica Malaysiana*, Vol. 3 No. 1, pp. 10-15.

Hormaza, J.I., Dollo, L. and Polito, V.S. (1994), "Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis", *Theoretical and Applied Genetics*, Vol. 89, pp. 9-13.

Jackson, C. (2008), "Commercializing dabai as Sarawak's speciality fruit", *The Star*, November 2008.

Jiang, C. and Sink, K.C. (1997), "RAPD and SCAR markers linked to the sex expression locus M in asparagus", *Euphytica*, Vol. 94, pp. 329-333.

Jiang, L., You, R.L., Li, M.X. and Shi, C. (2003), "Identification of a sex-associated RAPD marker in *Ginkgo biloba*", *Acta Botanica Sinica*, Vol. 45 No. 6, pp. 742-747.

Khadka, D.K., Nejidat, A., Tal, M. and Golan-Goldhirsh, A. (2002), "DNA markers for sex: Molecular evidence for gender dimorphism in dioecious *Mercurialis annua* L.", *Molecular Breeding*, Vol. 9, pp. 251-257.

Kumar, S., Singh, B.D., Sinha, D.P. and Rai, M. (2008), "Sex expression-associated RAPD markers in pointed gourd (*Trichosanthes dioica*)", in *Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae*, 2008. Avignon, France, pp. 543-550.

Kunihisa, M., Fukino, N. and Matsumoto, S. (2005), "CAPS markers improved by cluster-specific amplification for identification of octoploid strawberry (Fragaria × ananassa Duch.) cultivars, and their disomic inheritance", *Theoretical and Applied Genetics*, Vol. 110, pp. 1410-1418.

Lemos, E.G.M., Silva, C.L.S.P. and Actis, H. (2002), "Identification of sex in *Carica papaya* L. using RAPD markers", *Euphytica*, Vol. 127, pp. 179-184.

Li, C.I., Chiou, S.J., Tong, T.S., Lee, C.Y., Lee, L.T. and Cheng, C.M. (2010), "Development and validation of molecular markers for characterization of *Boehmeria nivea* var. niivea and *Boehmeria nivea* var. tenacissima", *Chinese Medicine*, Vol. 5, pp. 40-48.

Mandolino, G., Carboni, A., Forapani, S., Faeti, V. and Ranalli, P. (1999), "Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.)", *Theoretical and Applied Genetics*, Vol. 98, pp. 86-92.

Manoj, P., Banerjee, N.S. and Ravichandran, P. (2005), "Development of sex-associated SCAR markers in *Piper longum* L.", *Plant Genetic Resources Newsletter*, Vol. 141, pp. 44-50.

Michelmore, R.W., Paran, I. and Kesseli, R.V. (1991), "Identification of markers linked to disease resistant genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 88, pp. 9828-9832.

Naqvi, N.I. and Chattoo, B.B. (1996), "Development of a sequence characterized amplified region (SCAR) based on indirect selection method for a dominant blast-resistance gene in rice", *Genome*, Vol. 39, pp. 26-30.

Nelson, T. and Irish, E.E. (1989), "Sex determination in monoecious and dioecious plants", *The Plant Cell*, Vol. 1, pp. 737-744.

Niroshini, E., Everard, J.M.D.T., Karunanayake, E.H. and Tirimanne, T.L.S. (2008), "Detection of sequence characterized amplified region (SCAR) markers linked to sex expression in *Carica papaya* L.", *Journal of the National Science Foundation of Sri Lanka*, Vol. 36 No. 2, pp. 145-150.

Paran, I. and Michelmore, R.W. (1993), "Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce", *Theoretical and Applied Genetics*, Vol. 85, pp. 985-993.

Polley, A., Seigner, E. and Ganal, W.M. (1997), "Identification of sex in hop (*Humulus lupulus*) using molecular markers", *Genome*, Vol. 40, pp. 357-361.

Rostoks, N., Mudie, S., Cardle, L., Russell, J., Ramsay, L., Booth, A., Svensson, J.T., Wanamaker, S.I., Walia, H., Rodnguez, E.M., Hedley, P.E., Liu, H., Morns, J., Close, T.J., Marshall, D.F. and Waugh, R. (2005), "Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress", *Molecular Genetics and Genomics*, Vol. 274, pp. 515-527.

Sakamoto, K., Abe, T., Matsuyama, T., Yoshida, S., Ohmido, N., Fukui, K. and Satoh, S. (2005), "RAPD markers encoding retrotransposable elements are linked to the male sex in *Cannabis sativa* L.", *Genome*, Vol. 48, pp. 931-936.

Samantaray, S., Geetha, K.A., Hidayath, K.P. and Maiti, S. (2010), "Identification of RAPD markers linked to sex determination in guggal [*Commiphora wightii* (Arnott.)] Bhandari", *Plant Biotechnol Rep*., Vol. 4, pp. 95-99.

Shen, X, Guo, W., Zhang, T., Yu, J.Z. and Kohel, R.J. (2003), "Development of SCAR marker linked to a major QTL for high fiber strength and its usage in molecular-marker assisted selection in upland cotton", *Crop Science*, Vol. 43, pp. 2252-2256.

Singh, M., Kumar, S., Singh, A.K., Ram, D. and Kalloo, G. (2002), "Female sex-associated RAPD marker in pointed gourd (*Trichosanthes dioica* Roxb.)", *Current Science*, Vol. 82 No. 2, pp. 131-132.

Sobir, S.S. and Pandia, E.C. (2008), "Development of SCAR marker for detection of sex expression in papaya (*Carica papaya* L.) from several genetic backgrounds", *Bul. Agron*., Vol. 36 No. 3, pp. 236-240.

Sondur, S.N., Manshardt, R.M. and Stiles, J.I. (1996), "A genetic linkage map of papaya based on randomly amplified polymorphic DNA markers", *Theoretical and Applied Genetics*, Vol. 93, pp. 547-553.

Tan, C.J., Ho, W.S. and Pang, S.L. (2014), "Resequencing and nucleotide variation of sucrose synthase (SuSy) gene in a tropical timber tree *Neolamarckia macrophylla*", *International Journal of Scientific & Technology Research*, Vol. 3 No. 7, pp. 135-140.

Tanaka, M., Takahata, Y., Nakayama, H., Yoshinaga, M., Kumagai, T. and Nakatani, M. (2010), "Development of cleaved amplified polymorphic sequence (CAPS)-based markers for identification of sweetpotato cultivars", *Scientia Horticulturae*, Vol. 123, pp. 436-442.

Tchin, B.L., Ho, W.S., Pang, S.L. and Ismail, J. (2012), "Association genetics of the *cinnamyl alcohol dehydrogenase* (*CAD*) and *cinnamate 4-hydroxylase* (*C4H*) genes with basic wood density in *Neolamarckia cadamba*", *Biotechnology*, Vol. 11 No. 6, pp. 307-317.

Thiel, T., Kota, R., Grosse, I., Stein, N. and Graner, A. (2004), "SNP2CAPS: a SNP and INDEL analysis tool for CAPS marker development", *Nucleic Acids Research*, Vol. 32 No. 1, e5.

Tiong, S.Y., Ho, W.S., Pang, S.L. and Ismail, J. (2014), "Nucleotide diversity and association genetics of *xyloglucan endotransglycosylase/hydrolase* (*XTH*) and *cellulose synthase* (*CesA*) genes in *Neolamarckia cadamba*", *Journal of Biological Sciences*, Vol. 14 No. 4, pp. 267-275.

Uhrin, A., Láng, L. and Bedö, Z. (2008), "Comparison of PCR-based DNA markers for using different Lr19 and Lr24 leaf rust resistance wheat sources", *Cereal Research Communications*, Vol. 36 No. 4, pp. 533-541.

Urasaki, N., Tokumoto, M., Tarora, Y., Ban, Y., Kayano, T., Tanaka, H., Oku, H., Chinen, I. and Terauchi, R. (2002), "A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.)", *Theoretical and Applied Genetics*, Vol. 104, pp. 281-285.

Vincze, T., Posfai, J. and Roberts, R.J. (2003), "NEBcutter: a programme to cleave DNA with restriction enzymes", *Nucleic Acids Research*, Vol. 31, pp. 3688-3691.

Voon, B.H. and Kueh, H.S. (1999), "The nutritional value of indigenous fruits and vegetables in Sarawak", *Asia Pacific Journal of Clinical Nutrition*, Vol. 8 No. 1, pp. 24-31.

Whitmore, T.C. (1972), "Tree flora of Malaya", Longman Malaysia Sdn. Bhd, West Malaysia, pp. 121-136.

Wilkerson, R.C., Parsons, T.J., Albright, G., Klein, T.A. and Braun, M.J. (1993), "Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: Anopheles)", *Insect Molecular Biology*, Vol. 1 No. 4, pp.205-211.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, V. (1990), "DNA polymorphism amplified by arbitrary primers are useful as genetic markers", *Nucleic Acid Research*, Vol. 18, pp. 6531-6535.

Xu, W.J., Wang, B.W. and Cui, K.M. (2004), "RAPD and SCAR markers linked to sex determination in *Eucommia ulmoides* Oliv.", *Euphytica*, Vol. 136, pp. 233-238.

Yeam, I., Kang, B.C., Lindeman, W., Frantz, J.D., Faber, N. and Jahn, M.M. (2005), "Allele-specific CAPS markers based on point mutations in resistance alleles at the pvr1 locus encoding e1F4E in Capsicum", *Theoretical and Applied Genetics*, Vol. 112, pp. 178-186.

Zhang, Y.P. and Stommel, J.R. (2001), "Development of SCAR and CAPS markers linked to the beta gene in tomato", *Crop Science*, Vol. 41, pp. 1602-1608.