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Sex-linked Single Nucleotide Polymorphism (SNP) identification and molecular marker development of salacca (*Salacca zalacca* (Gaertn.) Voss)

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Abstract. *Prihatini R, Dinarti D, Sutanto A, Sudarsono. 2023. Sex-linked Single Nucleotide Polymorphism (SNP) identification and molecular marker development of salacca (*Salacca zalacca *(Gaertn.) Voss). Biodiversitas 24: 704-712. Salacca zalacca* (Gaertn.) Voss is a native tropical fruit species of Indonesia with three different sex types, i.e., female, male, and hermaphrodite. Early sex identification plays a vital role in the dioecious plant development, cultivation, and breeding program. The morphological and histological markers cannot differentiate salacca sex types during vegetative stages, during which molecular approaches are offered. Isolation of salacca's partial Transducin Beta Like-3 (TBL3) gene was done using *Phoenix dactylifera* L. TBL3-specific primers. After DNA sequencing and multiple sequence alignment, the putative TBL3 aligned data was used to construct a phylogenetic tree, find SNAP primers, and use the generated markers as predictors for sex-type phenotypes of salacca. Phylogenetic analysis showed that the dendrogram of aligned TBL3 partial sequences of salacca and other taxa showed that the salacca TBL3 is closely related to *P. dactylifera* and *Elaeis guineensis* Jacq. since the three palms belong to the same Arecaceae family. The partial sequence of the TBL3 gene of hermaphrodite salacca revealed ten single nucleotide polymorphisms (SNPs) compared to those of female and male plants. Protein alignment of the sequence also showed several differences, which may lead to different flower morphogenesis. The salacca SNAP sex-types markers were developed from one of the SNP sites and validated using samples from farmer plantations. The SNAP primer pairs of STBL1F-Ref - STBL1-Rev amplified a 300 bp fragment from hermaphrodite samples, whereas no band was amplified from the female and male samples. On the contrary, primers STBL1F-Alt - STBL1-Rev amplified a 300 bp fragment from female and male plants but not from the hermaphrodite plants. The salacca's TBL3 sequences and the SNAP markers are valuable tools for further salacca genomic exploration.

Keywords: Sex identification, sex polymorphisms, SNAP markers, snake fruits, TBL3-like gene

INTRODUCTION

The dioecious plants' sex types are often challenging to determine, particularly before flowering and vegetative development. On the contrary, recognizing male and female flowers is urgent for breeders and farmers, particularly before propagation, because this will optimize crop improvement and profits (Sarkar et al. 2017). Due to the fruit production capability in several plant species, female flower representation is more notable than male flower counterparts. These plants include *Carica papaya* L. (Abreu et al. 2015), *Pistacia vera* L. (Kafkas et al. 2015), and *Actinidia chinensis* Planch. (Zhang et al. 2015), *Cucumis sativus* L. (Pawełkowicz et al. 2019), *Phoenix dactylifera* L. (Al-Ameri et al. 2016; Ali et al. 2018).

Salacca, also known as snake fruit for its scally peel, is a tropical fruit belonging to the Arecaceae family. The *Salacca* genus includes 23 species, including *S. zalacca* (Zumaidar and Miftahuddin 2018). *Salacca zalacca* is a dioecious species, strictly outcrossing (Mazumdar et al. 2019). Salacca is categorized as an underutilized crop originating from South East Asia. The locals cultivated salacca as a backyard crop due to its unique taste and beneficial phytochemicals (Khoo et al. 2016).

Salacca palms can be grown from seed or vegetative offshoots (Ismail et al. 2018). Because half of the progeny are economically useless males, seed propagation is unsuitable for commercial salacca cultivation. However, sex discrimination is traditionally only achievable when flowering occurs after 3-4 years (Supapvanich et al. 2011). As a result, there is a great demand for effective early sex identification procedures based on genetic methodologies. Unfortunately, previous attempts to use molecular genetic markers to discriminate sex in salacca have met with limited success. There is still a general ambiguity and a lack of clarity in using genetic markers for sex determination in salacca, such as Random Amplified Polymorphic DNA (RAPD, Ediwirman et al. 2015) and Sequence Characterized Amplified Region (SCAR) (Li et al. 2017).

The genomic studies of salacca are not as extended as on other crops, and its genomic information does not exist in the public database. The most closely related dioecious plants to salacca is *P. dactylifera* - both belong to Arecaceae. However, many research groups have extensively studied sex determination in date palms (Cherif et al. 2016; Maryam et al. 2016; Intha and Chaiprasart 2018; Torres et al. 2018). Many methods and genes have been exploited to

develop markers for the sex-determination genes in date palms. One of the genes includes the transducin-beta-like-3 (TBL3).

The TBL3 gene is an ortholog to the tormoz-embryo defective (TOZ) gene, which is male-specific in European aspen *(Populus tremula* L.) and quaking aspen (*P. tremuloides* Michx.) and may be involved in the flower initiation phase (Pakull et al. 2015). In *Arabidopsis thaliana* (L.) Heynh mutation of the TOZ gene produced embryos with abnormal cell division patterns and detained embryos that develop not to have established regular planes. In addition, the mutant embryos have altered expression patterns of selected embryo patterning genes, implying that the positional indication needed for their appropriate expression is disrupted by the misoriented divisions (Griffith et al. 2007).

In this present research, we developed a novel comparative genomics approach to solve the sex determination problem in salacca, using a candidate sex-linked TBL3, which shows several single nucleotide polymorphisms between male and female date palms (Ali et al. 2018). This study aimed to find sex-linked single nucleotide polymorphism (SNP) identification and molecular marker development of salacca (*Salacca zalacca*) by isolating and sequencing partial TBL3 gene from nine salacca accessions, finding single nucleotide polymorphisms (SNPs), developing and confirming SNAP primers and evaluating their effectiveness for differentiating sex-type phenotypes of salacca palms.

MATERIALS AND METHODS

Salacca samples

Unopened young leaves samples were collected from salacca palms comprising three female, three male, and three hermaphrodite palms - planted as field collections in January 2021 at the Indonesian Tropical Fruit Research Institute (ITFRI), West Sumatera, Indonesia. The other thirty salacca palm samples consisting of ten females, ten males, and ten hermaphrodite palm samples, were obtained from the salacca farmer's garden. The 30 samples were used to confirm the effectiveness of the developed SNAP markers for differentiating salacca sex-type phenotypes.

DNA extraction and PCR amplification

The DNA extraction was conducted following the Plant Genomic DNA Mini Kit [Geneaid, Taiwan]. The DNA quality was checked using the Nanodrop spectrophotometer and diluted to a concentration of 30 ng/µL. A total volume of 12.5 µL reaction mix per sample was used for PCR amplification of the target DNA (i.e., a fragment of the TBL3 gene), consisting of 1 µL DNA sample, 4.5 µL ddH_2O, 6 µL Taq polymerase mix [Bioline, Germany], and 1 µL each of forward, and reverse primers at 30 µM.

The PCR amplification program was optimized for each primer using gradient PCR. Unless otherwise stated, the amplification uses the following steps, one cycle of initial denaturation at 95ºC for 1 min, followed by 35 cycles of denaturation at 95ºC for 15 s, primer annealing at 50-60ºC (5-10ºC below primer's Tm) for 15 s, primer extension at 72ºC for 10 s, and a final extension at 72ºC for 10 min. The TBL3-specific primer sequences used to isolate a fragment of the TBL3 gene from the salacca genome are presented in Table 1. The relative positions of the four TBL-3 specific primers from Ali et al. (2018) in this study to the TBL3 gene structure are presented in Figure 1A. The PCR products were fractionated in a 1.2% agarose gel electrophoresis using SB buffer at 50 V for 60 min.

TBL3 gene isolation and sequencing

The in-silico study was conducted to find the sex-linked candidate genes by exploring the NCBI DNA database (https://www.ncbi.nlm.nih.gov/). The fully annotated tubulin beta-like-3 (TBL3) gene of *Phoenix dactylifera* was used as the reference for the TBL3-specific primer design. The primer design was conducted using Genious Prime 2021.1.1 software (https://www.geneious.com/). The designed primers were used to amplify a fragment of the partial TBL3 gene using salacca genome templates.

The amplicon of putative partial TBL3 fragments was sequenced using automatic Sanger Sequencing systems and done by Base Asia, Singapore (http://base-asia.com), a DNA sequencing provider company. A total of 30 µL PCR products for each sample were sent to Base Asia, Singapore, and used for DNA sequencing. Subsequently, the sequences were further analyzed using Geneious Prime software to find the presence of single nucleotide polymorphisms (SNPs).

Phylogenetic analysis

The phylogenetic tree construction was done using orthologous partial TBL3 sequence data for diverse plant taxa. The DNA sequences were downloaded from the NCBI Genbank DNA database from the output of BLAST analysis using the determined putative salacca's TBL3 nucleotides as the query sequences. All sequences were edited using Genious Prime software. Subsequently, the MEGA11 software was used to calculate pair-wise genetic distances among accessions, and the phylogenetic tree was constructed (Tamura et al. 2021).

SNP analysis, SNAP primer design, and assays

The presence of SNPs was evaluated based on multiple sequence analysis (MSA) of partial TBL3 gene fragments from nine salacca accessions. The SNP showing sex-specific polymorphism was selected and used for designing SNAP primers to amplify approximately 300 bp SNAP markers. The allele-specific SNAP primer design was conducted using the online WebSNAPER software (http://pga.mgh.harvard.edu/cgibin/snap3/websnaper3.cgi, Drenkard et al. 2000). The melting temperature, primer length, and amplified product length were optimized using procedures suggested on the Websnapper website. Moreover, the developed primer sequences were assessed against the available *S. zalacca* genome to minimize mispriming.

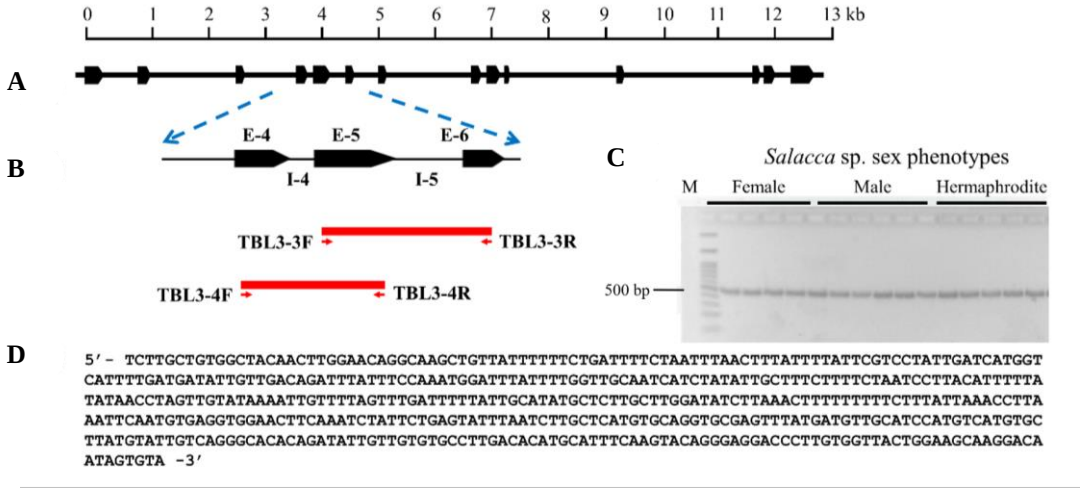

Figure 1. Two pairs of Transducin Beta Like-3 (TBL3)-specific primers were used in the present study to amplify putative TBL3 gene fragments from *S. zalacca* genomic DNA. The primer pairs (TBL3-3F - TBL3-3R and TBL3-4F - TBL3-4R) originated from *Phoenix dactylifera* (Ali et al. 2018). A. The general structure of the TBL3 gene with 14 exons (E) and 13 introns (I). B. Primer and PCR amplification positions using the TBL3-specific primers and the target amplicons. C. Photographs of agarose gel electrophoresis results were used to visualize PCR amplified product using TBL3-specific primers and salacca genomic DNA as the template. D. Representative DNA sequences of the putative partial TBL3 gene (~500 bp) from *S. zalacca* (Salacca accession No. 21, the hermaphrodite palm)

Table 1. List of primers pairs used to amplify partial TBL3 gene from salacca genome

Primer codes	Primer sequences	Amplified PCR product (bp) using the template of:	
		Date palm DNA	*S. zalacca* DNA
TBL3-3F*	TCTTGATATGAGGTTCTTGGGTG	473	~500
TBL3-3R*	ACACTATTGTCCTTGCTTCCAGT		
TBL3-4F*	TGTATTGTCAGGGCACACAGA	579	NA
TBL3-4R*	CAATGTCCCACACTCTCACCT		

Note: *Ali et al. (2018). NA: No amplicon was obtained from PCR amplification

The SNAP markers were generated using PCR, and their polymorphism was evaluated by agarose (1.2%) gel electrophoresis. The PCR was performed in a Mastercycler Nexus, PCR Cycler [Effendorf, Germany]. The PCR mix (12.5 uL total volume) included 30 ng DNA, 0.2 mM dNTP, 0.5 U Taq DNA Polymerase, and 5 pM of each forward and reverse primer. The PCR amplification steps include one cycle of initial denaturation for 1 min at 95ºC, followed by 35 cycles of denaturation for 15 s at 95ºC, primer annealing for 15 s at the proper Tm depending on the primers, primer extension for 10 s at 72ºC, and a final extension for 10 min at 72ºC. In addition, the agarose gel electrophoresis was run using SB buffer at 50 V for 60 min.

RESULTS AND DISCUSSION

Salacca zalacca is consumed for its unique fruit taste with sweet, sour, and astringent flavors. Figure 2 represents the *S. zalacca* morphology, such as the three-different sex-type phenotypes (Figure 2A and 2B for male, Figure 2C and 2D for female, and Figure 2E and 2F for hermaphrodite sex-types), and the representative fruit bunch (Figure 2G) and individual salacca fruits (Figure 2H). The salacca fruit is covered by brownish-black scaly peel. Thus, the salacca fruit is also known as the snake fruit. *S. zalacca* fruit bunch consisted of up to 10-30 individual fruits (Figure 2G). One individual fruit contains three fruit segments; each fruit segment has one brownish-black seed inside (Figure 2H), and the salacca fruit aril is creamy-white colored. The adult female-, male-, and hermaphrodite-type salacca palms can be differentiated by their flower inflorescences (Figure 2B, 2D, and 2F). Other morphologies among the three salacca sex-type palms are the same, other than the flower inflorescences (Figure 2A, 2C, and 2E).

The hermaphrodite salacca is originated from Bali and Maluku Island in Indonesia and known as the subspecies *S. zalacca* var. *amboinensis* (Atmowidi et al. 2021). Hermaphrodite is the sex salacca breeder wanted the most in commercial salacca production since cultivation practices for this type of salacca are less tedious than the dioecious plants. Hermaphrodite salacca is pollinated by wind or insects, and no man-hand pollination is needed in its cultivation (Mazumdar et al. 2019). Female palms are the second best salacca breeders needed since the plants produce fruit which is the most utilized part of the plant. Breeders do not want too many male palms because they are only needed as pollen donors on salacca pollination and will not produce fruits (Setiawan 2019).

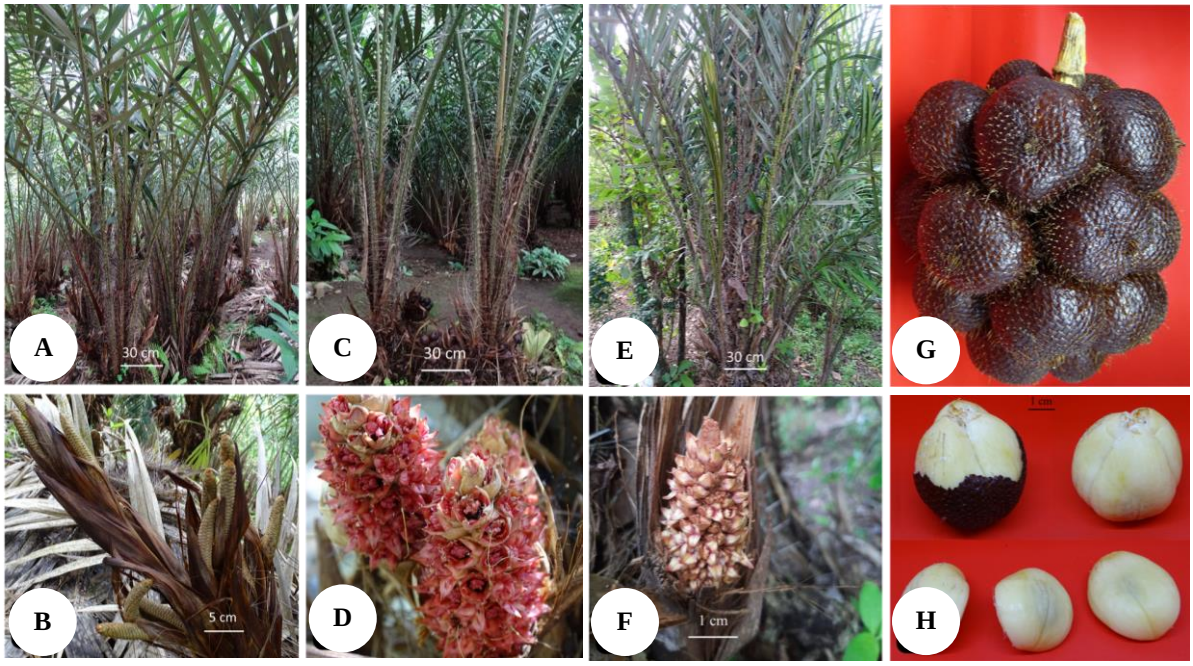

Figure 2. *Salacca zalacca*: A. Male palms, B. Male inflorescences, C. Female palms, D. Female inflorescences, E. Hermaphrodite palms, F. A hermaphrodite inflorescence, G. Fruit bunch, H. Individual fruit

Several popular varieties of *S. zalacca* var. *amboinensis* include *S. zalacca* var. *amboinensis* cv. Gula Pasir, Bali, Nangka, and Gondok have female and male flowers in a single inflorescence. Since salacca is outcrossing in nature, it has a wide range of genetic diversity (Budiyanti et al. 2015). For instance, a study in a district on Maluku Island showed that *S. zalacca* varied in morphology and molecular characteristics (Elly et al. 2018).

The pollination of *S. zalacca* var. *amboinensis* is characterized as cleistogamy, in which fertilization happens when the flowers are not fully open. Cleistogamy flower is never fully mature and considered to have evolved to encourage self-fertilization. Current research disclosed that cleistogamy occurred more frequently in zygomorphy or bilateral symmetry flowers (Sauquet 2021). It was also argued that cleistogamy was an adaptive response to pollen pollination and acted as reproductive assurance (Albert et al. 2011). In addition, cleistogamy was advantageous for pollination and fertilization by decreasing the high-temperature stress at flowering stages (Koike et al. 2015).

On the other hand, *S. zalacca* is a dioecious plant known to be xenogamy. In the commercial plantation of *S. zalacca* cv. Salak Pondoh, hand pollination is applied to maximize fruit production (Mazumdar et al. 2019). The advantages of hand pollination include the ability to control the number of male pollens, the frequency and timing of pollination, and independence from ecosystem variations (Toledo-Hernández et al. 2020). Farmers can increase both fruit quality and quantity, prevent fruit abortion, create more jobs, and ensure the sustenance of food security by applying hand pollination in salacca plantations. Hand pollination is limited by high labor efforts, high costs, and required expertise (Wurz et al. 2021). Nevertheless, hand pollination can be a valuable tool in crop systems where pollinators are either absent or unreliable for sustaining high-quality crop production (Colombo and Galmarini 2017).

Salacca partial TBL3 gene isolation and sequencing

In this experiment, the TBL3-specific primers used to amplify the partial TBL3 gene from salacca were derived from the TBL3 gene of *P. dactylifera* (Table 1). The TBL3-3F - TBL3-3R primer pairs successfully generated putative TBL3 amplicons (ca. 500 bp) using the salacca genomic DNA template (Figure 3). On the other hand, the same TBL3-3F - TBL3-3R primers only generated a 473 bp partial TBL3 amplicon from date palm DNA (Ali et al. 2018). However, the TBL3-4F - TBL3-4R primer pair did not generate any amplicon when amplifying salacca genomic DNA (Table 1). Meanwhile, when they were used to amplify date palm genomic DNA, the TBL3-4F - TBL3-4R primer pair generated a 579 bp partial TBL3 amplicon (Ali et al. 2018).

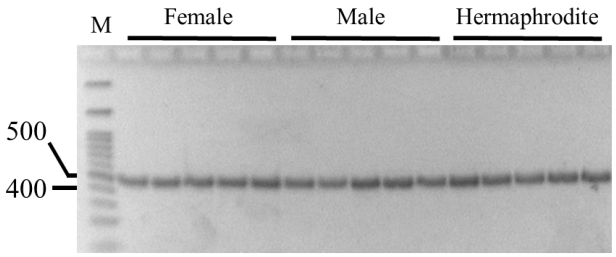

Figure 3. Representative photographs of agarose gel electrophoresis results were used to visualize PCR amplified product using TBL3-specific primers and salacca genomic DNA as the template

As many as nine putative TBL3 gene fragments (i.e., the fragment length is ~500 bp) were successfully isolated from nine salacca accessions (3 males-, 3 females- and 3 hermaphrodites palms) using TBL3-5F – TBL3-5R primer pair. The DNA sequences of the ~500 bp putative salacca TBL3 fragments were determined and then further analyzed to find the presence of single nucleotide polymorphisms (SNPs) among the amplicons originating from the three salacca sex types. Finally, the representative of the partial TBL3 gene sequences, amplified using TBL-specific primers from female snake fruit (*S. zalacca* accession No. 21), was displayed in Figure 1C.

The availability of public genomic databases has opened up new trends for developing molecular markers and prompted a shift in PCR-based techniques commonly used in plant science. Targeted fingerprinting marker technologies are based on well-established methods of arbitrarily amplified DNA techniques; nonetheless, they incorporate novel methodological innovations, such as incorporating gene or promoter elements in the primers. Because of the concurrent occurrence of dominant and co-dominant bands, these markers provide good reproducibility and increased resolution (Poczai et al. 2013). Taking advantage of date palm TBL3 sequences deposited on GeneBank, several pairs of primers were designed to amplify the salacca genome. The TBL3-3F and TBL3-3R amplified the most extended band (~500 bp) on every sample tested (3 of each female, male, and hermaphrodite palms).

TBL3 partial sequence phylogenetic analysis

The phylogenetic tree based on a multiple sequence alignment (MSA) of the putative partial TBL3 gene from nine salacca accessions and those representing various plant species was presented in Figure 4. Results of the phylogenetic analysis showed that nine of the putative TBL3 DNA sequences from salacca were clustered together in one cluster (Figure 4).

Due to the limitation of salacca sequence information on the public genome database (NCBI GeneBank), the TBL3-like gene in this study was referred to as the identified sex-associated SNPs in date palms (Ali et al. 2018). Several primers amplify the salacca genome (Figure 1, Table 1). One of the primer pairs to amplify *P. dactilyfera* proved to amplify the salacca DNA fragment. The primer pairs of TBL3-3F and TBL3-3R amplified similar fragment sizes (~500 bp) on *P. dactylifera* and *S. zalacca*. However, the TBL3-4F and TBL3-4R primer pairs did not produce any band on *S. zalacca*. The generated primer based on *P. dactylifera* TBL3 exon sequence (TBL3-5F and TBL3-5R primers) resulted in approximately 1000 bp DNA fragments of *S. zalacca*, whereas TBL3-6F and TBL3-6R, as well as pair of TBL3-7F and TBL3-7R which were explicitly designed in this present study, did not amplify any band. No further analysis was conducted yet for the 1000 bp amplicon obtained using the TBL3-5F and TBL3-5R primer pairs. However, preliminary sequence analysis verified that the sequence of TBL3-3F and TBL3-3R PCR products was an overlap region of TBL3-5F and TBL3-5R.

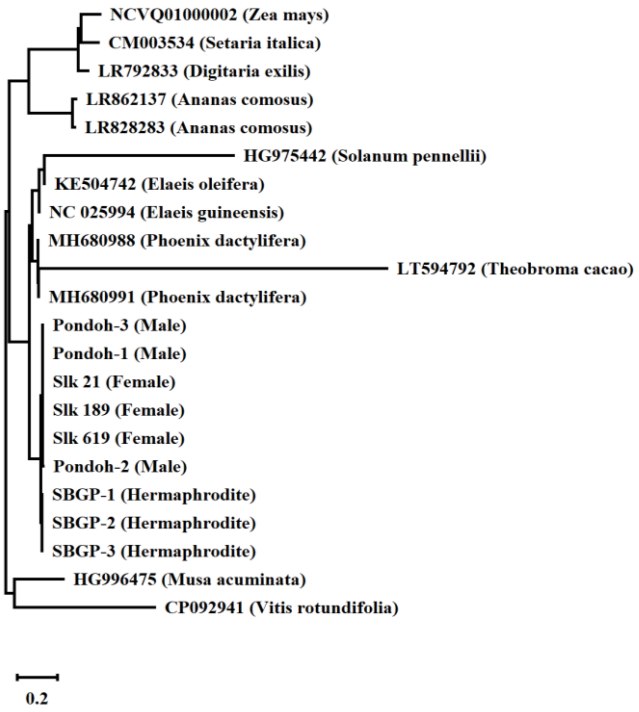

Figure 4. The phylogenetic tree was generated by the MEGA11 software (Tamura et al. 2021) using the Neighbor-Joining method (Saitou and Nei 1987) with 1000 bootstrap replicates (Felsenstein 1985) and the nucleotide sequence alignment of the partial Transducin Beta-like Protein 3 (TBL3) gene sequences of the different plant species. Except for salacca, the sequence data were obtained from NCBI DNA Database. The NCBI accession number and the species names are displayed directly in the figure. The representative salacca accessions are displayed in blue for hermaphrodites, red for females, and green for male plants

The phylogenetic tree of the alignment of salacca partial TBL3 gene sequences with other species showed that all salacca sequences were classified in the same cluster. In contrast, other Arecaceas, *P. dactylifera* and *E. guineensis*, were in different clades than the salacca. The earlier research revealed that *P. dactylifera* and *E. guineensis* clustered in the same clade (Ali et al. 2018) as in this salacca study. Twelve more taxa were included in the phylogenetic analysis in the present research. Thus, the phylogenetic relationship results among the taxa might differ from the previous report (Ali et al. 2018).

Putative TBL3 multiple sequence analysis and SNP identification

The Association of the TBL3 gene to sex types in date palms (*P. dactilyfera*) provides the opportunity for the TBL3 evaluation as the candidate gene associated with sex-type phenotypes for *S. zalacca* palms. Primer pairs were designed to amplify the highly variable regions of the TBL3 fragment. Multiple sequence alignment analysis among partial TBL3 genes from *S. zalacca* varieties showed 11 base substitutions (SNPs) in the intron sections. The list of the 11 SNP positions in the partial TBL3 gene of *S. zalacca* is presented in Table 2. Among the eleventh

SNPs, two SNPs (SNP position 77 and 268) were polymorphic for hermaphrodite (G for SNP position 68 and T for position 268) and male or female palms (A for SNP position 67 and G for position 268).

SNPs are single base-pair changes found in an individual's genome sequence. SNPs can be classified as transitions (G/A and C/T) or transversions (T/G, C/A, A/T, and C/G) based on nucleotide substitution. Single base changes, including SNPs that are insertion/deletions (InDel) in a single base, are common in mRNA. A single-nucleotide base is the smallest unit of inheritance, and SNP can provide the most specific and reliable markers (Nadeem et al. 2018). Even though SNPs are less polymorphic than SSR markers due to their biallelic nature, this disadvantage is easily offset by their abundance, ubiquitousness, and amenability to high- and ultra-high-throughput automation (Mammadov et al. 2012).

All identified SNPs were found in the intron, also known as intronic SNPs. It was suggested that intronic SNPs were accumulated at a faster rate than exonic SNPs. Although intronic SNPs are not directly involved in functional genes, it has been proposed that intron mutation in primary metabolic genes modifies protein versatility through alternative splicing and functional domain changes (Mukherjee et al. 2018). Thus, intronic SNPs may also cause changes in plant morphology in some cases (Sun et al. 2020).

The partial TBL3 sequence analysis results in salacca showed that female and male *S. zalacca* have similar sequences. However, the hermaphrodite salacca partial TBL3 sequences showed 11 SNPs compared to the female and male sequences, including seven transitions, three transversions, and one Insertion-Deletion (Table 2). The TBL3-based SNPs found among hermaphrodite male and female salacca palms may be developed as markers for salacca sex types.

Table 2. The identified SNPs in the partial STBL3 DNA sequences of nine salacca plants

SNP positions	Consensus nucleotide	Nucleotide among different sex-type phenotypes		
		Female	Herma-phrodhite	Male
77*	A	A	G	A
84	C	C/T	C/T	C/T
92	C	C/T	T	C/T
113	C	C/T	C/T	C/T
114	G	G/A	A	G
208	C	C/T	C/T	C/T
268	G	G	T	G
272	A	A/G	G	A
290	C	C/T	C/T	C/T
302	-	-	T	-
439	G	G/A	G/A	G/A

Note: *The SNP position was used to develop the STBL1-Ref and STBL1-Alt primers to generate SNAP markers for sex-type phenotypes in salacca

SNAP primer design and validation

The detected SNPs in the partial TBL3 gene of hermaphrodite salacca palms were then used to develop sex-type specific SNAP markers. In this study, the SNP-specific primers were designed using SNP position No. 77 (Table 2). The SNAP primer design was done using WebSnapper software https://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi), resulting in several primer pairs. Two forward, i.e., Reference (Ref) and Alternate (Alt) primers and reverse (Rev) primers were selected to generate hermaphrodite-specific SNAP markers (Table 3). Since only three primers were selected, the criteria for choosing specific primers include no self-complementary warning, no mismatch near the SNP site, slight Tm differences between either Ref and Rev or Alt and Rev primer combinations, fewer base numbers, and having the same Rev primer for both Refs - and Alt-forward primers.

For PCR amplification of the marker, the same Rev primer was paired with either the Ref or Alt primer. In addition, the *S. zalacca* variety, the source of candidate TBL3 gene amplification, was also used to validate the developed SNAP markers. The sex-types-specific SNAP primer validation results are presented in Figure 5. Subsequently, the developed SNAP marker was tested across ten accessions of female, male, and hermaphrodite salacca palms.

The results showed that the amplified SNAP marker generated using the STBL1_1Ref - STBL1-1Rev primer pair were specific to hermaphrodite salacca plants. On the other hand, the amplified marker using the STBL1_1Alt - STBL1-1Rev primer pair was specific to both female and male palms (Figure 5, Table 4).

A modest and low-cost marker is practical and valuable, especially for an early breeding activity with many populations (Lestari and Koh 2013). As a result, less expensive and simpler SNAP markers than genomic sequencing were developed in this study. The PCR program for generating SNAP markers differs from the standard PCR conditions. During PCR amplification, the annealing temperature and the cycle numbers were crucial in SNAP marker development (Lestari and Koh 2013). All primers corresponding to the total locus showed amplification by 28 cycles, with the annealing temperatures ranging from 35ºC to 40ºC considered optimum conditions for the SNAP markers amplification (Lestari and Koh 2013). Example of banding pattern generated by T-specific marker allele amplified genomic DNA of accessions with the T allele in the target locus.

Table 3. Developed salacca sex types specific SNP markers

SNAP primers	Number of bases	Tm (°C)	Expected amplicon size (bp)
STBL1-1Ref (forward)	24	48	300*
STBL1-1Rev (reverse)	19	47	
STBL1-1Alt (forward)	20	48	300**

Note: The amplicon was obtained using the STBL1-1Ref and STBL1-1Rev (*) and the STBL1-1Alt and STBL1-1Rev primer pairs (**)

The development of SNP markers could be conducted using several methods, including transcriptome analysis, Complexity Reduction of Polymorphic Sequences (CRoPS), and Restriction Site Associated DNA (RAD) (Mammadov et al. 2012). However, the SNAP marker in this present research was developed using more straightforward and less expansive methods by comparing the partial TBL3 gene sequences from salacca palms having different sex-type phenotypes. Such a straightforward method has already been used to generate SNAP markers associated with super nodulation in soybean (Kim et al. 2005), disease resistance in bananas (Sutanto et al. 2013), and genetic variability in coconut (Pesik et al. 2017).

SNAP marker diversity among *S. zalacca*

Table 4 represents the confirmation results of the TBL3-based SNAP markers using more female, male, and hermaphrodite samples. The results confirmed that the STBL1-1Ref and STBL1-1Rev primers only amplified a 300 bp amplicon from 10 hermaphrodite samples (Table 4). The female and male samples did not generate an amplicon (Table 4). Thus, the STBL1-1Ref - STBL1-1Rev could generate hermaphrodite-specific SNAP markers. On the other hand, the STBL1-1Alt and STBL1-1Rev amplified a 300 bp amplicon from ten female and ten male palms (Table 4). The STBL1-1Alt and STBL1-1Rev did not amplify amplicon from hermaphrodite palms (Table 4). Thus, the STBL1-1Alt - STBL1-1Rev primer pair could generate the male- and female-specific SNAP markers.

The sex-linked molecular marker as a detection tool was an alternative to other methods, such as morphology and cytogenetic markers. Before flowering, determining the sex of such economically important dioecious crops based solely on the external morphology of their embryogenic and juvenile forms is challenging. Massive cytogenetic research on such dioecious species disclosed the uncommon presence of sex chromosomes in such crops; if they are present, the procedure for the histo-cytological method is not exceptionally user-friendly. The same result was revealed for the salacca histological sex type study. The karyotype of both *S. zalacca* and *S. sumatrana* Becc. revealed the absence of sex chromosomes (Hadi et al. 2002; Friska 2019).

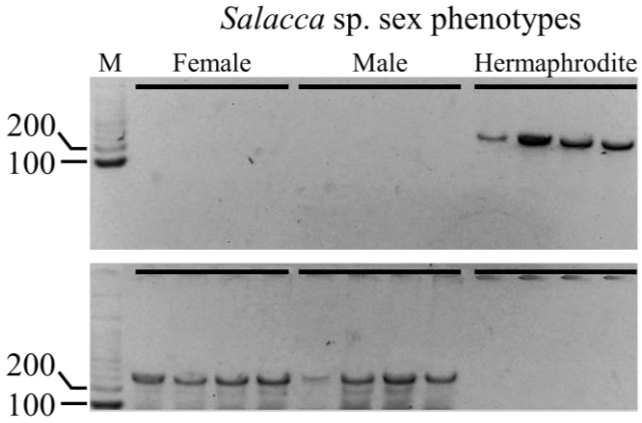

Figure 5. Gel image of PCR amplification product of female, male, and hermaphrodite *Salacca zalacca* using either the STBL1-1Ref - STBL1-1Rev primers (top) or the STBL1-1Alt - STBL1-1Rev (bottom). M: 100 bp DNA markers. The STBL1-1Ref - STBL1-1Rev primers only amplified 300 bp DNA fragments from hermaphrodite samples. No bands appeared from either female or male samples, whereas STBL1-1Alt - STBL1-1Rev primers amplified 300 bp DNA fragments from female and male samples and no band from hermaphrodite

Table 4. Validation results of Transducin beta like-3 (TBL3) based SNAP markers on 30 samples of *Salacca zalacca*

Salacca accessions (Sex type)	Primer pairs		Salacca accessions (Sex type)	Primer pairs	
	STBL1_1Ref - STBL1_1Rev	STBL1_1Alt - STBL_1Rev		STBL1_1Ref - STBL1_1Rev	STBL1_1Alt - STBL_1Rev
PD-1 (F)	-	+	PD-16 (M)	-	+
PD-2 (F)	-	+	PD-17 (M)	-	+
PD-3 (F)	-	+	PD-18 (M)	-	+
PD-4 (F)	-	+	PD-19 (M)	-	+
PD-5 (F)	-	+	PD-20 (M)	-	+
PD-6 (F)	-	+	SBGP-sha1 (H)	+	-
PD-7 (F)	-	+	SBGP-sha2 (H)	+	-
PD-8 (F)	-	+	SBGP-arp1 (H)	+	-
PD-9 (F)	-	+	SBGP-arp2 (H)	+	-
PD-10 (F)	-	+	SBGP-23.3 (H)	+	-
PD-11 (M)	-	+	SBGP-23.4 (H)	+	-
PD-12 (M)	-	+	SBGP-24.5 (H)	+	-
PD-13 (M)	-	+	Gading- sha1 (H)	+	-
PD-14 (M)	-	+	Gading-sha2 (H)	+	-
PD-15 (M)	-	+	Gading-sha3 (H)	+	-

Note: (-): amplicon is absence and (+): amplicon is presence

Furthermore, the telomere length of female and male salacca are equal. Thus, determining the salacca sex type based on the telomere length is impossible (Indhirawati and Purwantoro 2019). Early methods for determining the sex types in dioecious crops would alleviate breeders' difficulties, mainly when all superior parental selections are unknowingly composed of the commercially undesirable sex types. In addition, it would aid breeders and cultivators in saving field space, time, and other valuable resources that would otherwise be wasted in maintaining undesirable plants until flowering (Heikrujam et al. 2015).

The hermaphrodite SNAP marker developed in the present research was advantageous since two pairs of primers were designed. This approach was ideal for detecting male-female and hermaphrodite haplotypes (Milewicz and Sawicki 2012). It also may minimize false and unreliable results due to the poor quality of DNA that leads to the lack of PCR amplification (Ali et al. 2018). In addition, a male-specific SCAR marker was developed for *S. zalacca*; however, this SCAR marker cannot differentiate between male and hermaphrodite plants. Thus, the SNAP marker developed in this present research improved the detection of the sex polymorphism of *S. zalacca.*

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