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EVALUATION OF SCAR18 MARKER LINKED TO B-CAROTENE FOR EARLY SCREENING OF MANGO (*Mangifera indica* L.) PROGENIES

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ABSTRACT

A breeding program of Mango (*Mangifera indica*) was organized by a team of Brawijaya University since 2006 by cross pollination between Arumanis 143 (green skin) with yellow skin cvs. of Carabao, Haden, Podang and Swarnarika in vice versa. As early identification a molecular evaluation was conducted . Measurement were on carotene content, DNA analysis using PCR and sequencing. The result showed that mango cultivars having orange or yellowish skin contain β carotene higher than those mango cultivar having green skin. SCAR18 marker as a sign on the presence of beta (B) locus in tomato was not related with the differences of beta carotene accumulation in those manggo cultivars. SCAR18 marker that was amplified in mango produced specific amplimer of 320 bp in length. The similarity of $SCAR18_{320}$ sequence obtaining by sequences alignment among five parental cultivars and 12 hybrids was up to 100%. $SCAR18_{320}$ sequences that were generated by SCAR18 markers as predicted beta (B) loci had no significant similarity with sequences database relating to beta (B) gene in NCBI. $SCAR18_{320}$ match to 19 Ty3-gypsy retrotransposons and others sequences related transcriptional regulator in more dissimilar sequences (discontiguous megablast) category.

Key words: SCAR18, β carotene, skin color, *Ma - ngifera indica*

INTRODUCTION

Mango (*Mangifera indica*) is the most popular fruit in Indonesia and mostly are polyembrionic types (Vasanthaiah et al., 2007) that means each seed having more than one embryo. Therefore the breeding of the crop in Indonesia was lack behind. The mango consist of 200 cultivars spreaded in Indonesia (Purnomo *et al*., 1996) and mostly having green skin colour. In international market, it was less competitive than red or yellow skin mango cultivars. The exports of Indonesia mango were 2000 ton in 2009, out of total production of 1,621,997 tones (Direktorat Budidaya Tanaman Buah, 2009). However, Indonesia was the sixth world mango producer or 7.18% of world mango production, but were not the main world mango exporter (FAO-STAT, 2008). One of the reason of Indonesian mango especially the superior cultivar like Arumanis 143 **(**Ihsan and Sukarmin, 2008) is permanently green whereas the world consumer need yellow or red (Rebin *et al.,* 2002; Thimm, 2004).

To improve the skin colour, a hybridization since 2006 was conducted between Arumanis 143 (green, sweet taste, less fiber) with yellow or orange mango skin colour such as Haden, Carabao, Podang and Swarnarika in vice versa to obtain a new A 143 manggo having sweet taste, less fiber and orange/yellow skin colour was initiated. One hundred hybrids were resulted. However, the evaluation of hybrids consumed lot of time. Manggo, like most tree species, has a relatively long juvenile period of about 7 years, and the time to evaluate seedling trees can be up to 12 years (Gómez Lim and Litz, 2007). Thus, an efficient method that was not dependend on plant development stage was required.

One of DNA marker used as specific characteristics identification was SCAR (*sequenced characterized amplified regions*) marker (Zhang and Stomel, 2001; Li *et al*., 2008;

Shalahuddin (2009) used SCAR18 to characterized the differences of Arumanis 143 and Podang cultivars and their hybrids. SCAR18 primer used to mango, was developed by Zhang and Stomel (2001) to identify *Beta* gene accumulating beta carotene in tomato. In tomato, SCAR amplification produced 1067 bp polymorphism amplimer while monoporhism amplimer in mango consist of 350 bp, 600 bp, 750 bp and 950 bp. The inconsistency of amplimer for one gene target is interested problems. However, Both of them identified the gene for *β*-carotenoid pigment and yellow or orange in skin color. This differences predicted the relationship of annealing temperature. Tomato was amplified at 60^{o}C while mango at 36^{o}C. The lowering of annealing temperature increase the opportunity of mishybridization and produce non-specific amplimer (Van Pelt-Verkuil *et al.,* 2008).

Eventually, the result from Shalahuddin (2009), SCAR18 primers were evaluated to identify some other mango cultivars and several hybrids, expected to differentiate between Arumanis 143 has green skin and Carabao, Haden and Suvarnarekha which are yellow, reddish yellow and orange as well as previous research. The research objectives were (1) to study the relationship between differences of fruit skin color and β- carotene acumulation (2) to study and to obtain specific amplimer of SCAR18 primers that used as a marker of presence the *Beta* gene causing acumulation of β-carotene in manggo. (3) to study the sequences of parental cultivars and their hibrids that were generated by SCAR18 markers (4) to search the simililarity between mango sequences generating by primer SCAR18 in sequences database of NCBI (the US National Center for Biotechnology Information) related with *Beta* gene

MATERIALS AND METHODS

The research was conducted at laboratory of molecular biology, Department of Agronomy, Faculty of Agriculture Brawijaya University and laboratory of Eijkman, Jakarta from January until July 2009.

Plant Material

The plant material used in this study were 5 cvs.of mango Arumanis 143 (A), Carabao (C), Haden(H), Podang (P), Swarnarika (S) as parents, and 10 hybrids of AH, AP, AC, AS ,SA,PA,HA as the result of hybridization in 2006. Those plants were grown in Pohjentrek Experiment Station , Pasuruan ,East Java.

Analysis of Beta Carotene Content

A 5 g sample of pulp and skin fruit from each cultivar was crushed and the puree then poured into 125 ml erlenmeyer. 35 ml solution of acetone+ ether (50:50) was added to the puree, and homogenized using shaker at 164 rpm for 10 minutes or until smooth.. Residue was precipitated while extraction solution was filtered through No. 4 Whatman filter paper into 50 ml beaker glass. Filter and filtrate washed by adding petroleum ether+ acetone (50:50). Extraction repeated once and pellet was took out, filtrate redissolved up to 100 ml by adding 50:50 acetone: ether. 25 ml ubiquitous extraction solution entered into separation funnel. Washed with 25 distillation water, mixing, and waiting for phase separation. An aliquot of the beta carotene extract (upper phase) was evaporated under liquid nitrogen and redissolved in 50:50 acetone:ethanol to maintain consistency of solvent for subsequent analysis. Aliquot adding by 5-10g Na_2SO_4 per 100 ml eter, entered into column chromatography in dark room. 50:50 petroleum ether: acetone entered into column to elute beta carotene. Beta carotene concentration was determined by scanning UNICO Spectrophotometer UV/vis at 450 nm absorbance value.

DNA Extraction and PCR Program

Genomic DNA was extracted from young leaves following the DNeasy Plant Mini Kit method (Qiagen, 2006). PCR reaction was performed by GoTaq® Green Master Mix, 2X: GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400μM dATP, 400μM dGTP, 400μM dCTP, 400μM dTTP and 3mM MgCl2 following GoTaq® Green Master Mix methods (Promega Coorporation, 2005).

PCR amplification consist of 36^{o}C, 38^{o}C, 40^{o}C, 45^{o}C, 50^{o}C and 60^{o}C annealing temperatures for 45 second to compare the Shalahuddin (2009) and Zhang and Stommel (2001) methods. Initial denaturation was preserved at 95°C in 30 seconds. The extension reaction was typically performed at the optimal

temperature for Taq DNA polymerase, which is 72°C in 1 minute. A final extension is 5 minutes at 72°C.

Sequencing

Sequencing was run by SCAR18 primers. PCR products were then purified using Millipore purification microplates (Millipore, Molsheim, France) on a vacuum manifold (Millipore). Both 5 and 3 ends of cloned frag-ments were sequenced by means of M13 forward and reverse primers with Perkin-Elmer/Applied Biosystems AmpliTaq-FS DNA polymerase and Big Dye terminators in an ABI Prism 377 DNA sequencer (Perkin Elmer, Foster City, CA). The Compare and the Multiple Sequence Alignment functions were utilized for maximum matching of paired sequences and alignment of multiple sequences from different cultivars and hybrids, respectively. The gene finding was search by BLAST (Basic Local Alignment Search Tool) at NCBI.

RESULTS AND DISCUSSION

Beta Carotene Content

The reddish orange, orange or yellowish orange mango cvs. such as Haden, Podang and Swarnarika contain higher β-carotene content than green mango cultivar Arumanis 143 (Table 1). Mango's characteristic of both peel and pulp color development involves a progressive loss of chlorophyll in addition to an increase in carotenoid composition and content (Lizada. 1991).

This indicated that transition of mango peel and edible pulp from green to pale yellow , yellow , deep yellow or orange-yellow does not involve a simple unmasking of carotenoids, but synthesis as well (Fennema 1996). A major change in carotenoids during ripening involves development of β-carotene, the most prevalent carotenoid present at 50% of total carotenoids, the concentration of which is highest in fully ripe fruit (John *et al.,* 1970).

DNA Analyses

SCAR18 primer as a marker of the presence of beta (B) loci was not related with beta-carotene accumulation in mango (Figure 1). Haden that having highest beta carotene content produced shorter amplimer in length than Carabao and Podang, while Arumanis 143 that having lower beta carotene contents than Podang and Suvaranrekha also produced shorter amplimer. Not only in five parental cultivar but also in their hybrids showed inconsistency amplimer when reamplified in the same PCR reaction and thermocycling at 36^{o}C annealing temperature (data was not shown). Such was the case, AP 54.2 (Arumanis x Podang) produced two amplimer both of 300-400bp and 750bp at 36^{o}C in one event. Contrary in another event AP 54.2 produced three amplicons that were 750 bp, 600 bp and 100 bp These unstable polimorphic amplimers indicated that primer was not intended to binding site (non-specific primer hybridization) as a complementary region of the target DNA.

Table 1. Colour of fruits and β carotene contents of 5 manggo cultivars

Cultivars	Skin Colour based on RHS colour chart	Beta carotene content (ug/gram ± standard dev)
Haden	Light orange, brilian orange, strong radish orange, vivid reddish orang	30.22 ± 1.96 (a)
Podang Urang	Vivid yellow,light orangesh yellow, brilliant orange yellow	25.46 ± 4.96 (a)
Swarnarika	Strong greenish yellow, brilliant greenish, yellow, moderate yellow	24.03 ± 6.97 (a)
Carabao	Strong yellowish green, brilliant yellowish green	8.11 ± 4.96 (b)
Arumanis 143	Moderate bluish green, deep yellowish green, strong yellowish green,	9.81 ± 0.32 (b)

Thus, it may be necessary to optimizing new PCR protocol at higher annealing temperatures. When annealing temperature was increased from 36^{o}C to 38^{o}C the number of polymorphic amplimer were decreased. According to Figure 1 and 2, elevating annealing temperature allowed them to produce less polymorphic amplimer. The number of amplimer

of Arumanis 143 were decreased from three amplimers at 36°C (100, 300-400 and 500 bp) to monomorphic amplimer at 38°C (300-400 bp). Generally, the length of amplimer also became shorter. There were not any amplimer 1000 bp or more in length at 38°C annealing temperature. Podang amplimers that presented in 1500 bp at 36°C were absent in 38°C and generated only 800 and 100 bp.

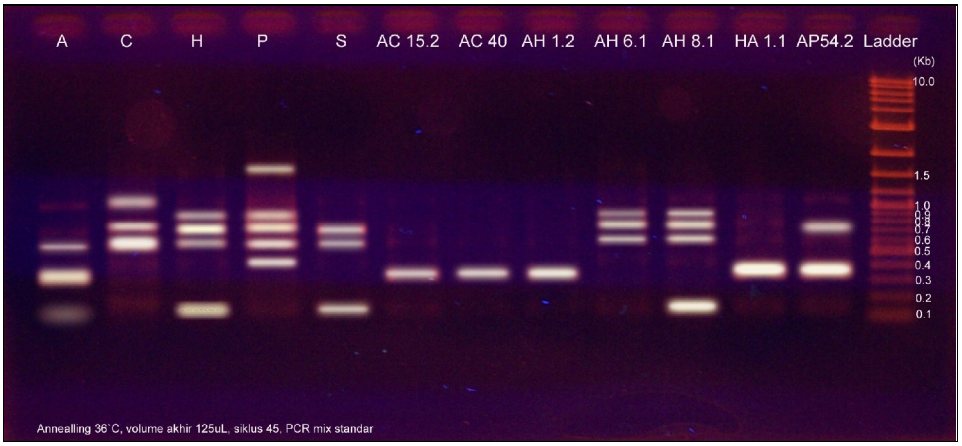

Figure 1. The amplimer of Arumanis 143 (A), Carabao (C), Haden (H), Podang (P) and Swarnarika (S). AC is the hybrids of Arumanis 143 (♀) x Carabao (♂), AH (Arumanis-143xHaden), HA (HadenxArumanis-143) and AP (Arumanis -143 x Podang) at annealing temperature of 36 °C in 125 µl PCR reaction consist of 62.5 µL *GoTaq® Green Master Mix* (2x), 10 µL primer SCAR18 r-f and 7 µL DNA template for 45 x cycles

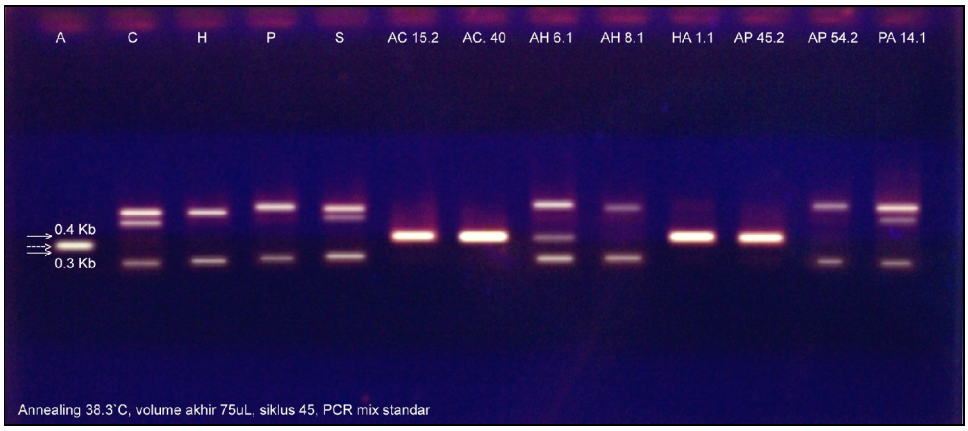

Figure 2. The amplimer of Arumanis 143 (A), Carabao (C), Haden (H), Podang (P) and Suvarnarekha (S). AC is the hybrids of Arumanis 143 (♀) x Carabao (♂), AH (Arumanis-143xHaden), HA (HadenxArumanis-143) and AP (Arumanis -143xPodang) at annealing temperature of 38.3 °C in 125 µl PCR reaction consist of 62.5 µL *GoTaq® Green Master Mix* (2x), 10 µL primer SCAR18 r-f and 7 µL DNA template for 45x cycl

Based on the yield of amplimer at 36°C and 38°C, annealing temperature was increased to 40°C. Surprisingly, all of cultivar and hybrids produced monomorphic amplimer at 300-400 bp in size (Figure 3). However, when annealling temperature was raised to 45°C and 50°C, the monomorpihic amplimer becomes more unreable. Moreover, amplimer was not visible at 60°C

annealing temperature (data not shown). According to amplimer appearance, 40°C was the best annealing temperature for PCR amplification of SCAR18 marker in mango.

Mangos characteristic of both peel and pulp color development involves a progressive change of chlorophyll in addition to an increase in carotenoid content (Lizada, 1991). The low content of carotenoid in Carabao was due to the physiological age of the fruit sample. During the analyses there were no ripe fruit, but halve riped therefore the colour was not fully yellow.

This indicated that transition of mango peel and edible pulp from green to pale yellow ,to yellow ,to deep yellow or to orange-yellow does not involve a simple unmasking of carotenoids, but synthesis as well (Fennema, 1996). A major change in carotenoids during ripening involves development of β-carotene, the most prevalent carotenoid present at 50% of total carotenoids, the concentration of which is highest in fully ripe fruit (John *et al.,* 1970)

Eventhough, the pattern of mango amplimer as monomorphic as tomato amplimer, both of them was different each other. In tomato the size of amplimer is 1067 bp, however, in mango was about 300-400 bp. This fact showed that any differences of target sequences amplifying by SCAR18. Furthermore, optimizing PCR parameters base on modification of *Taq* DNA polymerase and Mg concentrations, mango may not be produced 1067 bp amplimer in size (data not shown). Explicitly, this result shows that 300-400 bp is specific amplimer of SCAR18 in mango.

Sequencing

The analysis of PCR amplimer by nucleotide sequencing was the most accurate method for assessing whether the correct amplimer has been amplified or if the amplified region contains any nucleotide changes (Van Pelt-Verkuil *et al.,*2008). Certainly, each of amplimer resulting at each annealing temperature examined in sequencing. Material of sequencing consist of (1) sequencing of amplimer 1000,800 and 600 bp in lenght that were produced at 36°C (2) sequencing of amplimer 300-400 bp in length that was produced at 36; 38, and 40°C annealing temperatures.

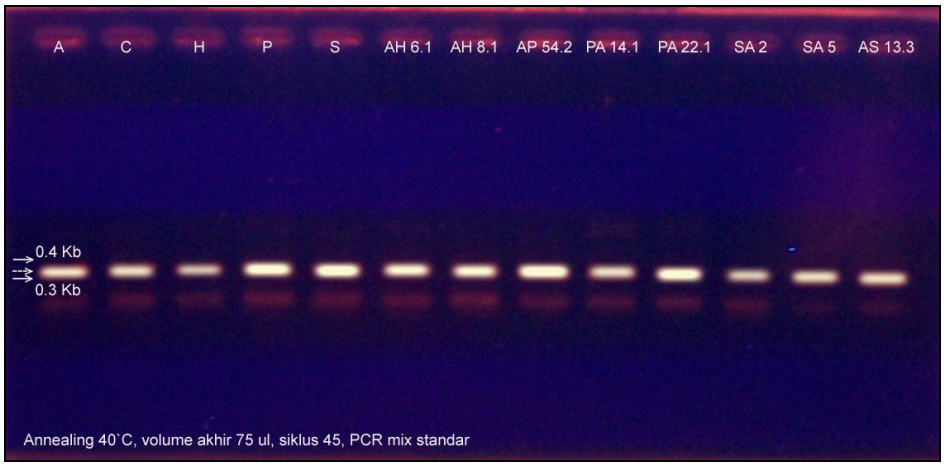

Figure 3. The amplimer of Arumanis 143 (A), Carabao (C), Haden (H), Podang (P) and Suvarnarekha (S). AC is the hybrids of Arumanis 143 (♀) x Carabao (♂), AH (Arumanis-143xHaden), HA (HadenxArumanis-143) and AP (Arumanis -143xPodang) at annealing temperature 40°C in 125 µl PCR reaction consist of 62.5 µL *GoTaq® Green Master Mix* (2x), 10 µL primer SCAR18 r-f and 7 µL DNA template for 45x cycles

The amplimers producing by Carabao 1000 bp show bad sequence with many non-descript nucleotides (N). Even in Carabao 800 and 600 bp also was found several N. These cases evidence that amplification at 36°C annealing temperature produced non specific hybridization and poor quality amplimer. In contrary, sequencing of band within 300-400 bp from varieties annealing

temperature produced good sequences when was run by SCAR18 forward or reverse. The full-length product sequences have 320 bases without gap like Carabao amplimer 1000, 800, 600 bp (Figure 4). Moreover, all of parental cultivar and their hybrids produced good sequences when run by SCAR18 f/r.

The similarity of SCAR18-mango sequences by alignment using BLAST-NCBI up to 100%. Generally, all of parental cultivar of mango sequences such as Arumanis 143, Carabao, Haden, Podang and Suvarnarekha and their hybrids have similarity in size and composition of bases (Figure 5).

Attentive, SCAR18 primer developed by Zhang and Stommel (2001) through *bulk segregant analysis* and linkage test to obtain RAPD amplimers relating beta acotene accuumulation. $OPAR18_{1100}$ was selected primer that produced amplimer in higher beta carotene accumulation tomato and was related to beta loci. Then it was developed as SCAR18 primer forward and reverse. SCAR18 primer f/r contain 10 original bases and 14 internal bases. However, mango may not be produced the same amplimer in size. Specific amplimer of mango is 320 bp in length.

Thus SCAR18 primer as DNA marker may not be used to characterized the differences of skin color of mango cultivars that contained various beta-carotene. Moreover, SCAR18 primer may not be used to identify gene causing beta carotene accumulation in mango hybrids.

Alignment of SCAR18 sequences to find the homology with other sequences in NCBI databases was conducted by BLASTN. According to *highly similar sequences* (megablast), there were no any similarity between SCAR18 sequences and NCBI sequences database. When the level of similarity was changed to *more dissimilar sequences* (*discontiguous megablast*), SCAR18 sequence similiar with *Ty3-gypsy retrotransposons* in *Citrus sinensis* (1) Musa ABB (2) *Malus x dometica* (17).

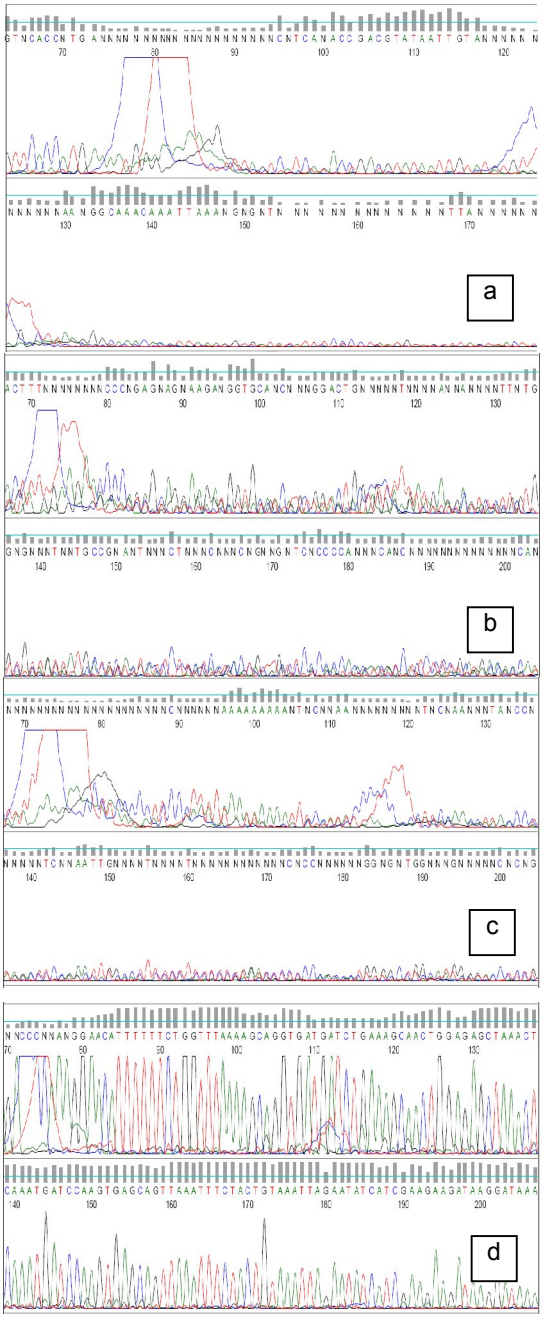

Figure 4. 1000 bp (a), 800 bp (b), 600 bp (c) of Carabao sequences was produced at 36°C annealing temperature and 300-400 bp (d) that was produced at 40°C.

```
Identities = 299/299 (100%), Gaps = 0/299 (0%)
Strand=Plus/Plus

Query  1    TGTCCACGCCCCTGCCACATTTCAAGCCCTAATAAATGAAATCTTTAGGACCCATTTACT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGTCCACGCCCCTGCCACATTTCAAGCCCTAATAAATGAAATCTTTAGGACCCATTTACT  60

Query  61   GCAGTTTATCCTTATCTTCTTCGATGATATTCTAATTTACAGTAGAAATTTAACTGCTCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GCAGTTTATCCTTATCTTCTTCGATGATATTCTAATTTACAGTAGAAATTTAACTGCTCA  120

Query  121  CTTGGATCATTTGAGTTTAGCTCTCCAGTTGCTTTCAGATCATCACCTGCTTTTAAACCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CTTGGATCATTTGAGTTTAGCTCTCCAGTTGCTTTCAGATCATCACCTGCTTTTAAACCA  180

Query  181  GAAAAAATGTTCCTTTGGGGTCTCTCGCTTGGAATATTTGGGTCACATTATTTCTACTGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GAAAAAATGTTCCTTTGGGGTCTCTCGCTTGGAATATTTGGGTCACATTATTTCTACTGA  240

Query  241  CAAAATGGCTGTTGATCCCCAGAAGATCCAGTGTATGATTGATTTGCCGGTGCCGGTAG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CAAAATGGCTGTTGATCCCCAGAAGATCCAGTGTATGATTGATTTGCCGGTGCCGGTAG  299
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Figure 5. The result of sequences *alignment* was used by BLASTN between Arumanis 143 (*query*) and Podang (*subjct*)

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Citrus sinensis retrotransposon Ty3-gypsy like, partial sequence
Length=1272
Score = 80.6 bits (88),  Expect = 4e-12
 Identities = 94/127 (74%), Gaps = 0/127 (0%)
 Strand=Plus/Plus

Query  131  CTGTAAATTAGAATATCATCGAAGAAGATAAGGATAAACTGCAGTAAATGGGTCCTAAAG  190
            |||||||  ||||| || || || || |  || |||||||   | | ||  |   | ||
Sbjct  329  CTGTAAACAAGAATGTCGTCAAAAAAAACTAGAATAAACTTACGAAGATAAGGTTTGAAA  388

Query  191  ATTTCATTTATTAGGGCTTGAAATGTGGCAGGGGCGTTGGACAGCCCAAAGGGCATTACC  250
            ||    || |||  ||||||||||||||  ||||| ||||||||||||||||||||||||
Sbjct  389  ATAGAGTTCATTGTGGCTTGAAATGTGGAGGGGGCATTGGACAGCCCAAAGGGCATTACG  448

Query  251  AGAAACT  257
            |||||||
Sbjct  449  AGAAACT  455
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Musa ABB Group Ty3-gypsy-like retrotransposon (partial) and Radka3-like repeat
(partial), clone B5-2
Length=808
 Score = 66.2 bits (72),  Expect = 8e-08
 Identities = 95/134 (70%), Gaps = 0/134 (0%)
 Strand=Plus/Minus

Query  130  ACTGTAAATTAGAATATCATCGAAGAAGATAAGGATAAACTGCAGTAAATGGGTCCTAAA  189
            |||||| |||| ||||||||| ||||| |  |  | |||||   | | ||| | || |||
Sbjct  330  ACTGTATATTAAAATATCATCAAAGAAAACCAAAACAAACTTACGGAGATGAGCCCGAAA  271

Query  190  GATTTCATTTATTAGGGCTTGAAATGTGGCAGGGGCGTTGGACAGCCCAAAGGGCATTAC  249
             || ||||| ||||     || || || |  || || ||||  | |||||| ||||||||
Sbjct  270  TATATCATTCATTAAACTCTGGAAGGTAGAGGGCGCATTGGTTAACCCAAAAGGCATTAC  211

Query  250  CAGAAACTGATGAT  263
             ||||||| || ||
Sbjct  210  TAGAAACTCATAAT  197
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```
Malus x domestica isolate GyRTFJ-44 retrotransposon Ty3-gypsy
nonfunctional reverse transcriptase gene, partial sequence
Length=432
 Score = 66.2 bits (72),  Expect = 8e-08
 Identities = 95/134 (70%), Gaps = 0/134 (0%)
 Strand=Plus/Minus

Query  130  ACTGTAAATTAGAATATCATCGAAGAAGATAAGGATAAACTGCAGTAAATGGGTCCTAAA  189
            ||| |||| || ||||||||| ||||| |  || | ||||||  | |||  ||   | ||
Sbjct  333  ACTATAAACTAAAATATCATCAAAGAATACCAGAACAAACTGACGCAAAAAGGGATTGAA  274

Query  190  GATTTCATTTATTAGGGCTTGAAATGTGGCAGGGGCGTTGGACAGCCCAAAGGGCATTAC  249
             || ||||| || |||   ||||| || |  || |||||||  || | ||| ||||||||
Sbjct  273  AATCTCATTCATGAGGCTATGAAAGGTTGTTGGAGCGTTGGTGAGGCTAAACGGCATTAC  214

Query  250  CAGAAACTGATGAT  263
             || |||| || ||
Sbjct  213  AAGGAACTCATAAT  200
```

Figure 6. The similarity between SCAR18 sequence and sequences on NCBI database in *more dissimilar sequences* (*discontiguous megablast*).

Based on Figure 6 may be observed that within 1272 bp of *Citrus sinensis retrotransposon Ty3-gypsy* like was found 127 bases identified similarly. Also compared by *Ty3-gypsy retrotransposons* of Musa ABB, SCAR18 sequences had 70% similarity and *Malus x domestica isolate GyRTFJ-44 retrotransposon Ty3-gypsy nonfunctional reverse transcriptase gene*. Gen *Ty3-gypsy* is one of class of *retrotransposon* (RTN) *long terminal repeats* (LTRs) (Zuccolo *et al.*, 2008). *Long terminal repeat* (LTR*) retrotransposons*, (LTR-RTNs) is the main groups of RNA element from Repetitive sequences play significant roles in the evolution of genome architecture, gene expression, and speciation. LTR-RTs can also act as potential controlling elements. Through insertion, they can inactivate or modify genes (Leprinc *et al*., 2001; Varagona *et al.*, 1992) and can also have genome wide effects contributing to transcriptional interference by producing sense or antisense transcripts of adjacent genes (Kashkush, 2003).

CONCLUSIONS AND SUGGESTION

SCAR18 primer as DNA marker may not be used to characterized the differences of skin color of mango cultivars that contained various beta-carotene. Moreover, SCAR18 primer may not be used to identify gene causing beta carotene accumulation in mango hybrids. It is suggested to develop specific primer to characterize beta-carotene of mango by analyzing DNA using RAPD and SSR.

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