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Stearoyl-ACP Delta 9 DesaturaseMight not be the only Factor Affecting Unsaturated Fatty Acids Concentration in Oil Palm (*Elaeis* Sp.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author HAS designed the study, performed experiments, performed the statistical, bioinformatics analyses and wrote the first draft of the manuscript. Authors HAS and AY developed the concept and designed experiments. Authors AY and MTS supervised its analysis and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Oil palm (*Elaeis guineensis*) is currently the major oil-producing plant in the world. *E. oleifera* is another species of oil palm that is not widely cultivated due to lower productivity. Oil extracted from *E. guineensis* showed a balance ratio of saturated to unsaturated fatty acids level, while oil from *E. oleifera* showed higher unsaturated fatty acids. Long chain-saturated fatty acids are more harmful to health than unsaturated fatty acids. In this research gene encoding steraoyl-Δ9-acyl carrier protein (ACP) desaturase enzyme (*SAD*, EC 1.14.19.2) was studied, as this is one of the responsible enzyme for biosynthesis of unsaturated fatty acids. Analysis were conducted on *SAD* gene and fatty acids content of *E. guineensis, E. oleifera* represented by Suriname and Brazil origin and the Hybrids (offspring of crossing between *E. guineensis* and *E. oleifera*). The result shows

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that no amino acid variations among *SAD* gene were found within *E. guineensis* and Hybrids, whereas two variations were found between *E. oleifera*. However, the mutations were found not within the core domains that were the polypeptides, substrates, or ions binding site of the enzyme. Concentration of oleic fatty acid (OLA, C18:1) of *E. guineensis, E. oleifera* and Hybrids showed variation. This variation might be indirectly influenced by other enzymes that acting prior to *SAD* such as β-ketoacyl ACP synthase (*KAS* II) enzyme that result in variation of stearic fatty acid (STA, C18:0) accumulation and thus further OLA content.

Keywords: Fatty acid; oil palm; palm oil; enzyme; oleic acid.

1. INTRODUCTION

Plant is the world's largest producer of oil such as *Ricinus communis*, *Brassica napus*, *Euonymus alatus* and *Tropaeolum majus*, and these oils are also a major food for humans and are increasingly used for non-food applications [1]. Generally, high-fat food would potentially increase the risk of heart attack. Furthermore, it is known that long chain-saturated fatty acids are more harmful for human health than unsaturated fatty acids [2].

Oil palm has two widely known species that have different morphological characteristics and composition of fatty acids namely, *Elaeis guineensis* and *E. oleifera*. Crude palm oil extracted from *E. guineensis* have relatively balanced ratio of saturated fatty acids to unsaturated fatty acids level, while *E. oleifera* have higher levels of oleic acid (OLA, C18:1) and linoleic (LNA, C18:2) fatty acids but lower for any saturated fatty acids [2].

Fatty acid biosynthesis in plants requires a series of reactions catalyzed by a set of enzymes consist of β-ketoacyl ACP synthase (*KAS*), Steroyl-acyl carrier protein (ACP) desaturase (*SAD*), acyl-ACP thioesterase, fatty acid elongase, and fatty acid desaturase [3]. *SAD* is one of the most important enzymes responsible for the production of OLA and might act as primary determinant for composition of unsaturated fatty acids in plant. It is a soluble enzyme and found in the plastid. This enzyme introduces a cis double bond into acyl-ACP at the Δ9 position through the release of H atoms to form monounsaturated oleoyl-ACP. This process requires stearoyl-ACP as substrate, reduced acceptor and O_2 to produced oleoyl-ACP, acceptor and water [4].

In plants, *denovo* fatty acid biosynthesis takes place in the plastids and starts with carboxylation of acetyl-CoA or malonyl-CoA. Then a series of condensation reactions by *KAS* enzyme type I, II, and III allow elongation of the carbon chain of butiryl acid (C4:0) until PAM and stearic (STA, C18:0) [5]. Afterwards, desaturase enzyme produces carbon chain unsaturation such as OLA and LNA [6].

The objective of this study was to conduct a comparative genomic analysis of *SAD* genes from *E. guineensis,* Brazilian and Suriname *E. oleifera* and Hybrids and to find its correlation with the unsaturated fatty acids concentration phenotype.

2. MATERIALS AND METHODS

2.1 Plant Material and DNA Extraction

DNA sources were from oil palm collection of Indonesian Oil Palm Research Institute, Medan - North Sumatera, Indonesia. Six genetic samples was used as follow, *E. guineensis* (Eg) represented by Lame and Langkat varieties; *E. oleifera* (Eo) represented by Brazilian and Suriname origin; Hybrid represented by Brazilian Eo x Eg and Suriname Eo x Eg. Total DNA was isolated from leaf tissue and purified using optimized DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) as described by the manufacturer.

2.2 Primer Design and PCR Amplification

Exons of *SAD* gene were amplified using a specific primer pairs designed by PerlPrimer v1.1.21. Primer pairs were designed based on BLASTN algorithm using accession U68756.1 (*E. guineensis*) and FJ940768.1 (*E. oleifera*) as the query against public databases at NCBI WGS oil palm(www.ncbi.nlm.nih.gov/assembly/GCA_000442705.1 and GCA_000441515.1). DNA aliquots were used in the standard PCR amplification protocol using GoTaq Green Master Mix (Promega). For internal control we used oil palm elongation factor (*OPEF*) gene with forward primer (5'-GGTGTGAAGCAGATGATTTGC-3') and reverse primer (5'-CCTGGATCATGTCAAGAGCC-3'). Amplification

program was as follows: initial denature step for 3 min at 95℃, followed by 35 cycles for 30 seconds at 95℃, 30 seconds at 58℃, 60 seconds at 72℃ and a final extension step for 9 minutes at 72℃ [7]. The PCR products from each gene/genotype were pooled and aliquots were visualized by 1% agarose gel electrophoresis to verify amplicons specificity followed by sequencing.

2.3 DNA Sequencing

Aliquots of the purified PCR amplicons were sequenced with Big-Dye V3.1 Terminator chemistry (Applied BioSystems) using amplicon-specific primers designed to span the entire amplicons with overlap in both orientations. The chromatogram from Applied Biosystems contained single exon fragments that were then assembled.

2.4 Genetic Diversity Analysis

DNA trace files were assembled, identification of open reading frames, translation and multiple sequence alignment with MUSCLE were conducted using GENEIOUS 7.1.5. Chloroplast transit peptide was annotated using TARGETP 1.1 SERVER and the enzyme active site by the NCBI database accession O24428 and ACQ41834.

2.5 Phylogenetic Analysis

The phylogenetic tree was constructed based on sequence alignment of full-length mRNA of the samples and some other oil crop species. GENEIOUS TREE BUILDER application was used with Kimura substitution models and methods of neighbor joining. The level of significance was tested on 1000 bootstrap.

2.6 3D Structure Prediction

Modeller and Chimera 1.9 were used to predict and visualized protein 3D structure. Theoretical model of oil palm *SAD* protein (target) was built based an identical protein that has been well studied, accession no. 1afr (template) from the PDB database for *R. communis SAD* protein.

2.7 Phenotyping of Fatty Acid Compositions and Statistical Analysis

Fatty acid composition was determined using GC on a DB 23 J&W Scientific column according to AOCS Official Method Ce 1b-89. A total of 3 ripe bunches were taken from every tree samples. Then 30 fruits from the middle of bunch were randomly selected for oil extraction. Oil sample was weighed as much as 0.025 g. Then inserted into the flask vial. As much as 0.5 mL of Na-methilate 5 N was added and the tubes were sealed then vortexed for 2 minutes. 1 mL Iso-Octane was added then further vortexed for 1 minute. Centrifugation was conducted for 10 minutes then as much as 1 mL of the top layer was used and inserted into the GC instrument. The fatty acids level observed was lauric (C12:0), myristic (C14:0), palmitic (PAM, C16:0), palmitoleic (C16:1), STA, OLA, LNA, α-linolenic (18:3), arachidic (C20:0), and eicosenoic (C20:1) acids.

GC conditions were as follows: type of column: DB 23 J & W Scientific, gas carrier: hydrogen, temperature of detector: 260℃, temperature of injector: 260℃, temperature of first column: 70℃, ramp rate: 20℃/min, temperature of second column: 180℃, iso time: 1℃/min, temperature of third column: 182℃, iso time: 10℃/min, final column temperature: 220℃ and hold time: 2 minutes.

One-way analysis of variance analysis (ANOVA) was used to examine the differences between fatty acids contents in the samples followed by the Duncan test probability at 0.05 levels. Analyses were performed using SPSS 16.0 software.

3. RESULTS

In this research, *SAD* gene sequences of *E. guineensis*, *E. oleifera* and Hybrids were successfully obtained and have been submited to GenBank database with accession number KM979555, KM979556, KM979557, KM979558 and KM979559. BLASTN and BLASTX algorithms were used against the NCBI non-redundant database to identify coding regions, open reading frame, exons and introns as well as the deduced amino acid sequence of each DNA sample. Multiple sequence alignment of the DNA and its translation using MUSCLE were analyzed and a phylogenetic tree was constructed using the neighbor-joining method. Mutations and indels of any alignment combinations were correlated against NCBI annotation and fatty acids phenotype. Finally, comparative (homology) modeling was conducted to predict the protein three-dimensional structure.

3.1 SAD Gene DNA

The *SAD* gene of six samples has similar DNA sequence structure that composed of three

exons and two introns (Figs. 1 and 2). *SAD* gene DNA of *E. guineensis* and Hybrids were 8555 bp. Total size of *SAD* exons for *E. guineensis* and Hybrids were 1,182 bp while those for the Brazilian and Suriname *E. oleifera* were 1,299 bp and 1,296 bp respectively. *E. guineensis* and Hybrids were 99% identical to accession U68756 at mRNA levels as well as the *E. oleifera* to accessions FJ940768. The alignments among samples were found identical at 99-100% (Table 1). DNA translation size for *E. guineensis* and Hybrids were 393 amino acids, while those of the Brazilian and Suriname *E. oleifera* were 432 and 431 amino acids respectively. *E. guineensis* and Hybrids were 99% identical to accession O24428 at their amino acid level, 93% and 95% for Brazilian and Suriname *E. oleifera* respectively to accession ACQ41834. Meanwhile identical values among samples at the amino acid level ranged from 99-100% (Table 2).

There was no mutation observed from the alignment between *E. guineensis* whereas two silent mutations were observed between Hybrids at mRNA sequence. Meanwhile, two missenses and three silent mutations and four indels were observed from the alignment between *E. oleifera* at mRNA sequence (ESM 1). Alignment of *E. guineensis* against the Hybrids produced one missense and three silent mutations (ESM 2). On the other hand, when compared against *E. oleifera* there were four missenses, and four silent mutations at mRNA sequence (ESM 3). Four missense and eight silent mutations were observed from the alignment between *E. oleifera* against Hybrids at mRNA sequence (ESM 4). All missense mutations were found from the alignment among samples revealed four *SAD* gene isoforms: 1. *E. guineensis*; 2. Brazilian *E. oleifera*; 3. Suriname *E. oleifera*; and 4. Hybrids (Fig. 3).

Amino acid motifs EENRHG and DEKRHE were found in all samples (Fig. 3). The motifs are highly conserved in the *SAD* gene [8-11] to form a site of the diiron center where oxygen activation and substrate oxidation take place [9]. There appear to be deletions and mutations in the motif DEKRHE compare to enzyme accession ACQ41834 (*E. oleifera*) (Fig. 3).

3.2 Phylogeny of *SAD* Genes

All samples were in a same clade with other monocots and separated from the dicots plant. Similar result has been reported that the tree can be divided into two major groups: one group for monocotyledonary plants and the other group for dicotyledonary plants [7]. *E. guineensis, E. oleifera* and Hybrids were clearly separated in a single different clade. The separation of *E. oleifera* sample between Suriname and Brazilian origin came as a surprised. It could imply that the different clade between samples is due to the different origin. *E. guinensis* was derived from the West African region between Angola and Gambia, while *E. oleifera* was derived from the South American rainforest in Brazil and Suriname. We observed also that *E. oleifera* accessions FJ940768 was likely derived from Brazil since they were in the same clade with Brazilian *E. oleifera* sample (Fig. 4).

3.3 Prediction of Protein 3d Structure

PDB accession no. 1afr (*R. communis*) showed the highest similarity to the four *SAD* isoforms samples and in addition to having the most excellent level of resolution by x-ray diffraction among other identical proteins. Modeller9.13 generated some auto-model protein 3D structures based on alignment against the accession 1afr. Best auto-model structure determination was performed based on minimum DOPE scores (Discrete Optimized Protein Energy).

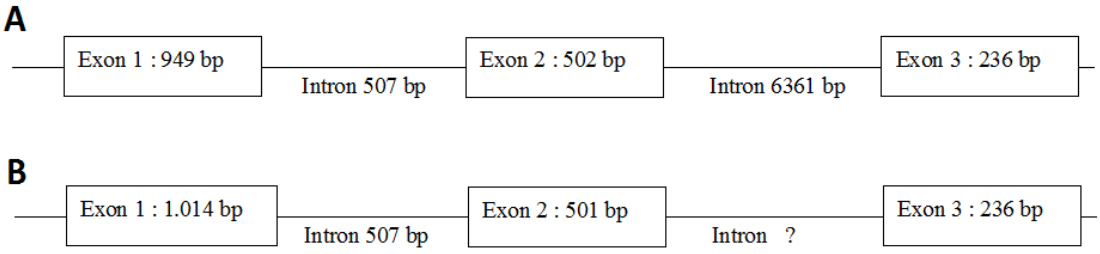

Fig. 1. Structures of *SAD* gene scheme (a) *E. guineensis* and Hybrid (b) *E. oleifera*

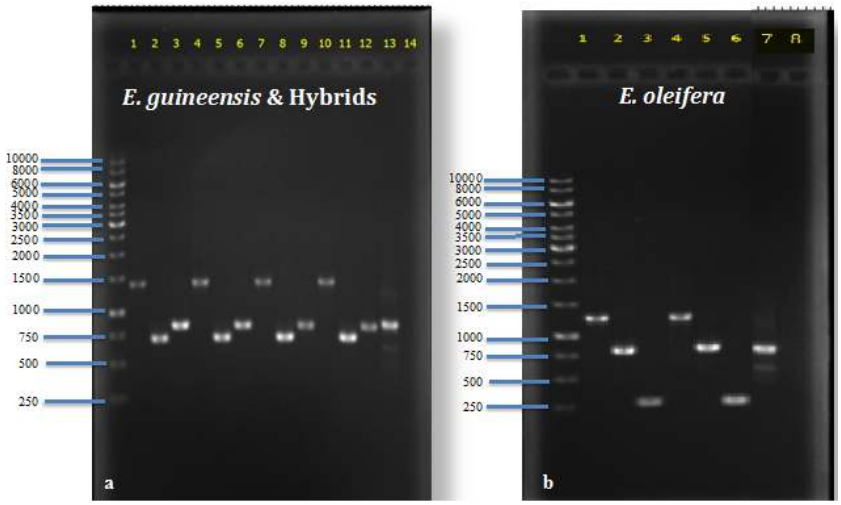

Fig. 2. PCR amplification of *SAD* gene (a) *E. guineensis* and Hybrid and (b) *E. oleifera*. Agarose gel (a): Exon 1 was on band number 1, 4, 7, and 10; exon 2 was on band number 2, 5, 8 and 11; exon 3 was on band number 3, 6, 9, and 12; band numbers 13 and 14 were internal control OPEF gene and negative control respectively. Agarose gel (b) : exon 1 was on band number 1 and 4; exon 2 was on band number 2 and 5; exon 3 was on band number 3 and 6; band numbers 7 and 8 were internal control OPEF gene and negative control respectively. Several hundred base pairs of intron was added to each exon for primer design

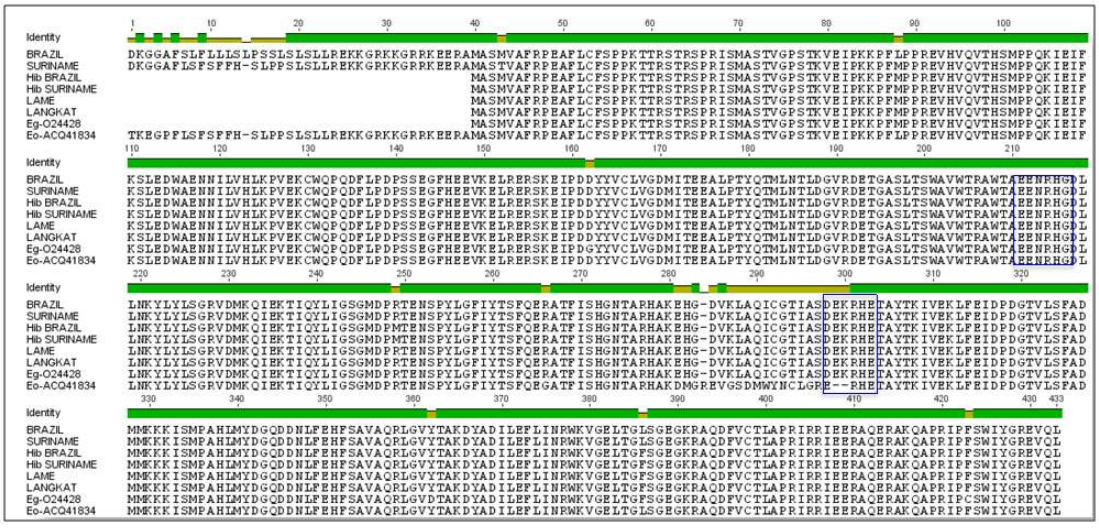

Fig. 3. Multiple sequence alignment using MUSCLE among samples, *E. guineensis* (O24428) and *E. oleifera* (ACQ41834). EENRHG and DEKRHE motifs were in transparent blue box. Green and brown box showed 100% match and mismatch respectively. Image alignment was generated using Geneious ® 7.1.5

Residues at the position 102 - 111 have a relative high energy compared to the template (Fig. 5). This relates to some mismatch at alignment between template and model. Structure prediction at the mismatches residues had low quality or does not have a native-like model (Figs. 6 and 7). Generally the four selected auto-model isoforms showed similar structure profile to each other.

Through the predicted three-dimensional structure, the position of amino acid mutations that occur can be revealed. All mutations of amino acids such as M-T; M-L; M-R or F-L were

located entirely on the protein coil of the predicted three-dimensional structures. Indels were located deep at the N-terminus and appears to be outside of the domain mature chain of *SAD* gene.

3.4 Association among Fatty Acid Composition and *SAD* Isoforms

Most of all fatty acids phenotype were significantly ($p<0.05$) different among the samples except for lauric acid. Palmitate was the highest fatty acid among all the saturated fatty acids phenotype. As for the highest and the lowest Langkat *E. guineensis* concentration was 43.80% and Suriname *E. oleifera* was 27.09%, respectively. OLA was the highest fatty acid among all the monounsaturated fatty acids phenotype. Suriname *E. oleifera* and Langkat *E. guineensis* were being the highest (66.37%) and the lowest (37.66%), respectively. For polyunsaturated fatty acids, LNA was the highest fatty acid. Brazilian Hybrid and Suriname *E. oleifera* were being the highest (13.54%) and the lowest (2.61%), respectively (Fig. 8).

4. DISCUSSION

4.1 SAD Enzyme

The important role of desaturase enzyme in biosynthesis of healthy fatty acids is known in many plant species. The enzyme is responsible to introduce the cis-double bond between carbons in a certain position on the acyl site of fatty acid chain. In this study, analysis of the DNA sequences encoding *SAD* enzyme have been conducted on six genetic material of oil palms *E. guineensis, E. oleifera* and Hybrids. Correlations were made among these isoforms against the saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids phenotypes of the genetic material planted at different locations.

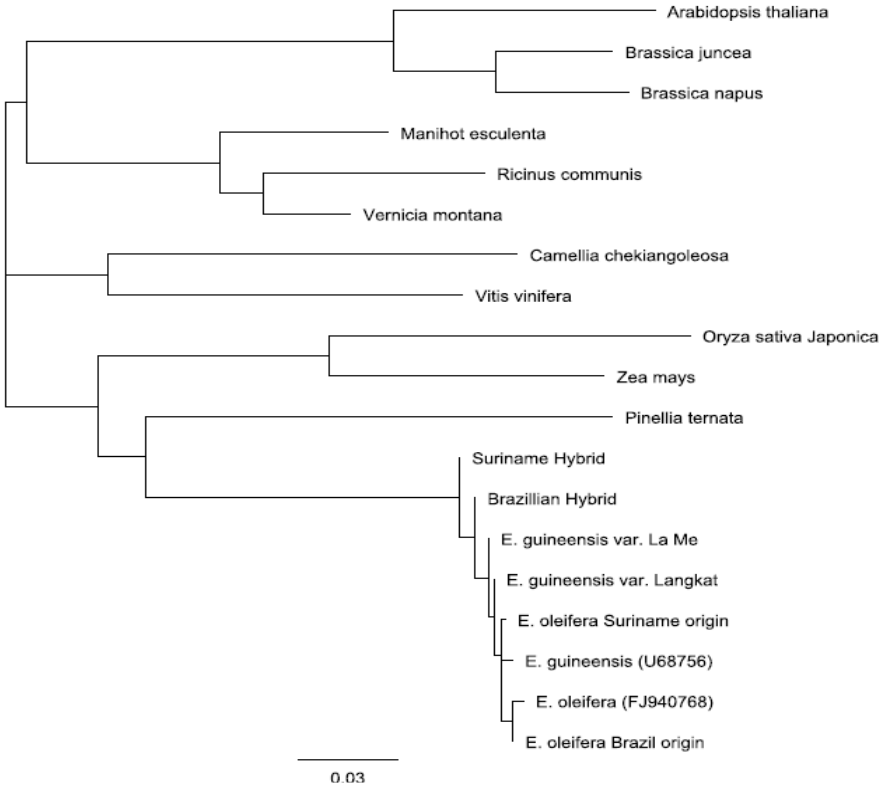

Fig. 4. Phylogenetic tree of *SAD* gene full-length mRNA sequences of all samples (red box) and some oil crops from monocots and dicots*. Arabidopsis thaliana (NM_129933.3), Brassica juncea (AF153420), B.napus (X97325), Camellia chekiangoleosa (KC342958), Elaeis guineensis-NCBI (U68756), Elaeis oleifera-NCBI (FJ940768), Manihot esculenta (JX513389), Oryza sativa Japonica (NM_001051750.1), Pinellia ternata (JQ390410), R. communis (XM_002531843), Vernicia montana (EU072353), Vitis vinifera (XM_003635330),* and *Zea mays (EU960917).* Only bootstrap values more than 50 were displayed. Image was generated using Geneious ® 7.1.5

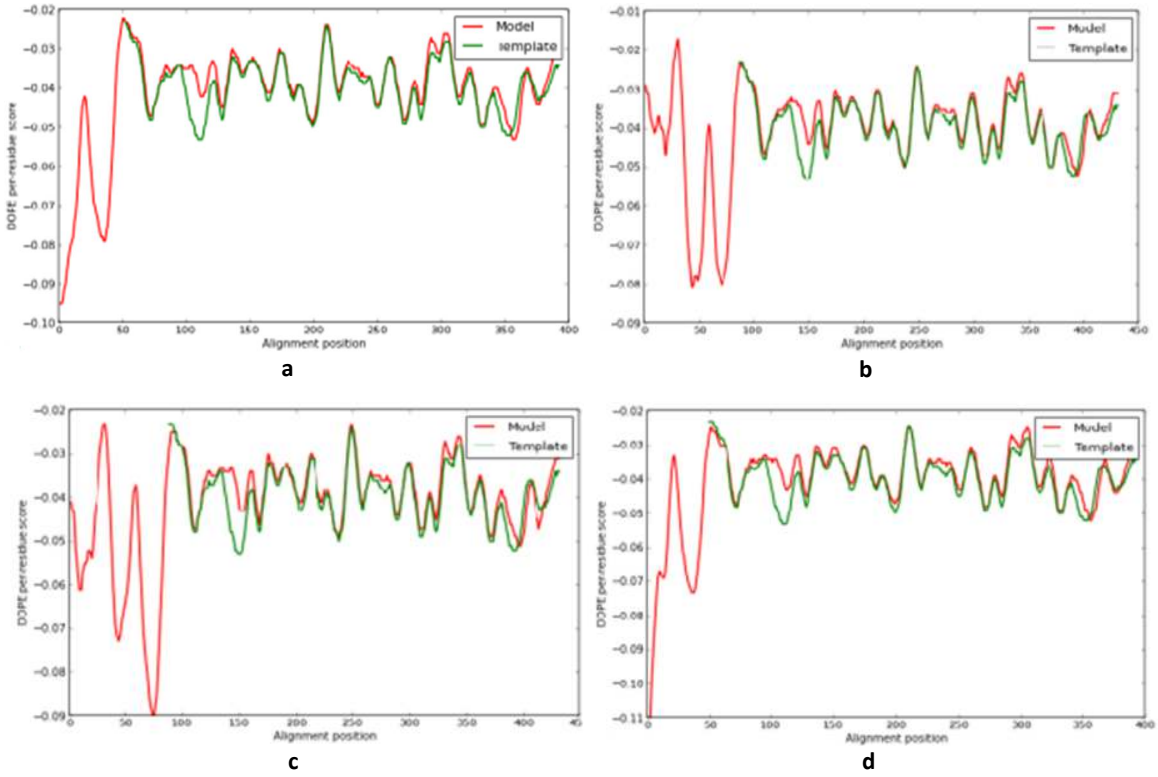

Fig. 5. Plotting DOPE scores of (a) *E. guineensis*; (b) Hybrids; (c) Suriname *E. oleifera* and (d) Brazilian *E. oleifera* against the template 1afr (*R. communis*)

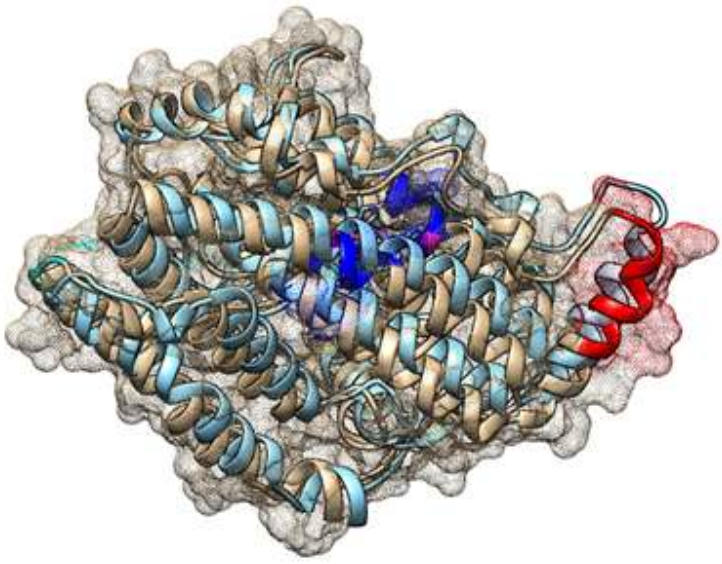

Fig. 6. Superimposed predicted protein 3D structure of *SAD* gene Suriname *E. oleifera* (brown) against 1afr (blue). Red: mismatch with the template 1afr, dark blue: *SAD* motifs, pink: diiron-center and cycan: mutation. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) http://www.ncbi.nlm.gov/pubmed/15264254

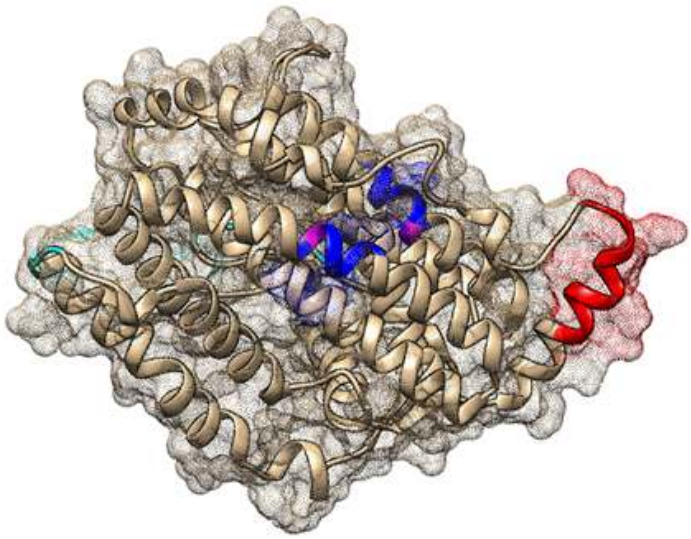

Fig. 7. One of the four predicted 3D protein isoform structures of *SAD* gene. Red: mismatch with the template 1afr, dark blue: *SAD* motifs, pink: diiron-center and cycan: mutations. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) http://www.ncbi.nlm.gov/pubmed/15264254

Table 1. Identical percentage among *SAD*gene at mRNA level

	***Eo.* Brazil**	***Eo. Suriname***	***Eg.* Langkat**	***Eg.* Lame**	**Suriname hybrid**	**Suriname hybrid**
Eo. Brazil						
Eo. Suriname	99.31					
EG. Langkat	99.58	99.75				
Eg. Lame	99.58	99.75	100			
Suriname Hybrid	99.41	99.58	99.83	99.83		
Brazillian Hybrid	99.53	99.45	99.70	99.70	99.87	

Note: Eg: Elaeis guineensis; Eo: Elaeis oleifera

Table 2. Identical percentage among *SAD*gene at amino acid level

	***Eo.* Brazil**	***Eo. Suriname***	***Eg.* Langkat**	***Eg.* Lame**	**Suriname hybrid**	**Brazillian hybrid**
Eo. Brazil		M-T (44) M-L (89)	M-L (88) F-L (385)	M-L (88) F-L (385)	M-L (88) M-R (249) F-L (385)	M-L (88) M-R (249) F-L (385)
Eo. Suriname	96.99		M-T (42) F-L (384)	M-T (42) F-L (384)	M-T (42) M-R (248) F-L (384)	M-T (42) M-R (248) F-L (384)
Eg. Langkat	99.49	99.49		-	M-R (210)	M-R (210)
Eg. Lame	99.49	99.49	100		M-R (210)	M-R (210)
Suriname hybrid	99.24	99.24	99.75	99.75		-
Brazillian hybrid	99.24	99.24	99.75	99.75	100	

Note: Eg: Elaeis guineensis; Eo: Elaeis oleifera. Number in bracket indicated relative amino acid position. Letter indicated variation in amino acid residue

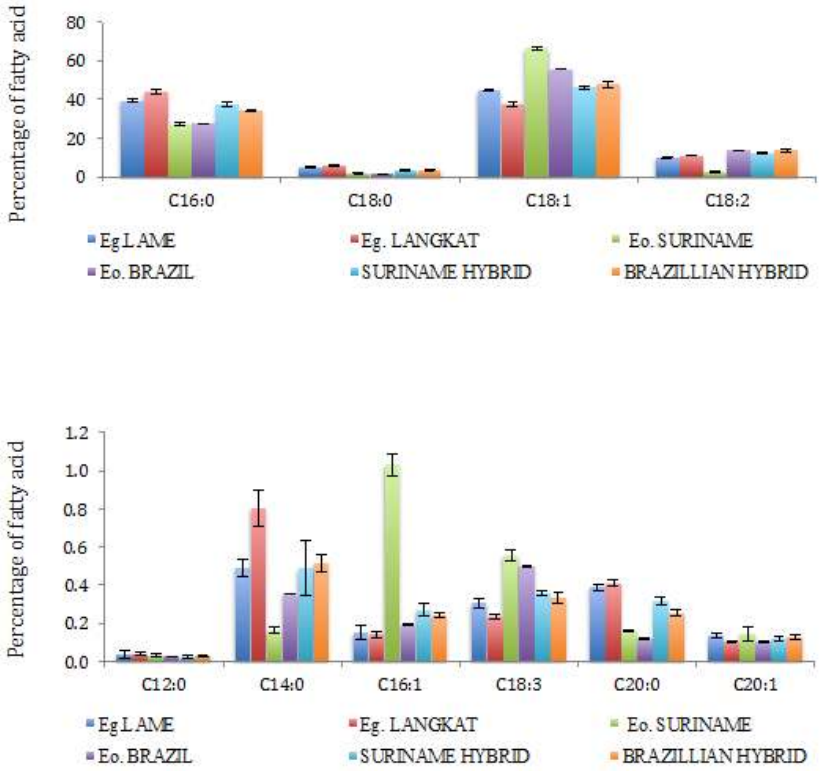

Fig. 8. Fatty acids content of *E. guineensis, E. oleifera* and Hybrid samples. Fatty acids were analyzed by GC method. Fatty acids with (a) higher and (b) lower concentration
Eg: Elaeis guineensis, Eo: Elaeis. oleifera

Researchers have been interested to alter the unsaturated OLA in oil crops, through modification of stearoyl-ACP desaturation by *SAD* enzyme [12]. However, modifications of OLA synthesis can disrupt membrane fluidity since it plays an important role in membrane integrity and function as cellular signaling, and adaptation thermal energy storage in plants [13]. The complexity of *SAD* multi-gene families is another indication that shows its importance in plants [12].

Spectroscopic analysis and X-ray diffraction study showed that *SAD* enzyme belongs to the class of diiron-oxo proteins, characterized by Fe atoms being ligated by two histidine residues and carboxylic acid (aspartate or glutamate) and bridged by oxygen [14]. Analysis of amino acid sequence of *SAD* enzyme in this study showed the presence of motifs EENRHG and DEKRHE on all samples. The motifs are known to exist in the *SAD* to form part of the diiron center as a site for of oxygen activation and substrate oxidation [8-11]. The same motifs on peanut [8], palm oil [11], rice [10] and castor oil [9] have also been reported. In our study, both of the motifs are located in the α-helix of the *SAD* protein 3D structure predicted using MODELLER9.13 (Fig. 6 and 7). The motifs are also located at the same position in the *SAD* proteins 3D structure of olive crop [15].

4.2 Mutation of *SAD* gene

Genetic variation of desaturase enzyme affects accumulation of the fatty acid final product [16] and genetic variation in SCD-1, FADS1, and FADS2 enzyme could modify desaturase activities [17]. DNA sequence analysis of *SAD* gene in this study showed some variation between samples such as missense mutations, silent mutations and indels in locations other than the core domains. Amino acid alignment between enzymes from *E. guineensis* and between Hybrids did not show any mutation and indels, but the alignment between both of them produced a missense mutation, R and M. Analysis of amino acid sequences between *E. oleifera* showed two mutations, L-M and M-T, and the M-T variation was occured in transit

peptide domain. Mutation at the transit peptide domain was shown in Suriname *E. oleifera* only. Some mutations were also found in the following alignment such as *E. oleifera* against *E. guineensis* and *E. oleifera* against Hybrids. Some of the mutations involve nonpolar residues (hydrophobic) such as the M, L and F, while T is uncharged polar residues and R are positively charged (Table 2). Amino acids sequence annotation of *SAD* gene is based on accession O24428 for Hybrids and *E. guineensis,* accessions ACQ41834 for *E. oleifera*. Based on these annotations, all of these mutations did not occur in the polypeptide, substrate, or ion binding sites of the enzyme (ESM 5).

4.3 Correlation with Phenotype

Factors that affect activity of an enzyme include substrate(s) concentration, pH, temperature, ionic strength and natural of salt present [18]. In this study, measuring the remaining substrate concentration and/or the quantity of the products, namely fatty acids in palm oil, indirectly assessed the activity of SAD enzyme. The composition of each fatty acid in this study however showed variation between samples, which in part due to differences in the activity of several related enzymes. This study thus, suggested that there were variations in the enzyme activities involved in formation and conversion measured.

Sequence analysis between Lame and Langkat *E. guineensis* did not show any mutations or indels in the *SAD* gene but both of the samples have significantly ($p<0.05$) different concentrations of OLA although STA as *SAD* substrates is not significantly different ($p<0.05$). This strongly suggested that the *E. guineensis SAD* enzyme in both samples have the similar activity. *Ssi2* alleles of Arabidopsis that encode *SAD* gene mutant reduced the activity of the gene product by increasing concentrations of STA and decreasing OLA [19]. Another fact is that OLA then converted by the oleate desaturase enzyme (oleoyl-phosphatidylcholine Δ12-desaturase) into LNA that also affected the concentration of OLA [20]. Different concentrations of miristic and PAM in the early biosynthesis was negatively correlated to the OLA and other unsaturated fatty acids except for LNA (Fig. 8). *KAS* I enzyme catalyze elongation cycle of 4:0-ACP to 16:0-ACP, then the *KAS* II enzyme catalyze next elongation process of 16:0-ACP to 18:0-ACP in plastids [1]. *KAS* I and II as well as oleate desaturase enzymes unfortunately were not (yet) characterized genetically in this study. We assumed there could be a variation in the activity of these enzymes that lead to variation accumulation of STA and OLA observed in the *E. guineensis.*

Similar to *E. guineensis*, sequence alignment of amino acid between Hybrids did not show any mutations or indels. However, STA and OLA concentration was not significantly different ($p<0.05$). PAM concentration was a little different but the concentration of STA, OLA and some other unsaturated fatty acids were not significantly different ($p<0.05$) except for LNA (Fig. 8). Similar to the *E. guineensis*, our results imply that *KAS* II enzyme activities might be varied within the Hybrid samples. Hybrid that is the offspring between *E. guineensis* and *E. oleifera* showed fatty acid accumulation point between both parents. The proportion of fatty acids in interspesific crossed of *E. oleifera* x *E. guineensis* showed intermediate values between the parents, which proved the theory of co-dominant gene in biosynthetic palm oil [21].

Suriname and Brazilian *E. oleifera* samples showed the most significant different in the fatty acid profiles compared to other samples except for palmitoleic acid. *KAS* type I, II and III and *SAD* enzymes of this samples may have been highly active to catalyze the substrate(s) conversion compared with other samples, and this lead to the highest concentration of some unsaturated fatty acid as observed in our analysis. Concentration of STA substrate in these samples was the lowest, suggesting a high *SAD* enzyme activity (Fig. 8). Generally OLA is high of concentration but not STA when *SAD* enzyme is very active, although *KAS* II enzyme activity is decreased [22]. Although there were mutations observed but we believed that Suriname and Brazilian *E. oleifera's SAD* enzyme have similar activity. This is infered to the fact that STA concentration was not different ($p<0.05$). In addition, the mutations occur not on the important/critical part of the *SAD* enzyme.

In this study, six genetic material samples revealed only five *SAD* gene alleles, which subsequently lead into 4 isoforms consisted of *E. guineensis,* Suriname and Brazilian *E. oleifera* and Hybrids. Finally, we found that there was significant difference substrate concentration of STA as substrat for *SAD* enzyme. Several studies have concluded that the oil content in the seeds is a complex quantitative trait that is regulated by a number of genes and possibly several environmental factors [23]. Two isoforms

of *SAD* gene isolated from *E. guineensis* did not give different expression in mesocarp tissue [11].

4.4 Protein 3D Structure and Phylogenetic Tree

Protein 3D structures are usually highly conserved among homologs compared to the amino acid sequence and DNA/RNA [24]. Protein 3D structure of *SAD* enzyme was predicted using the MODELLER9.13 to understand its molecular mechanism. The well-studied known of *SAD* proteins 3D structure from *R. communis* 1AFR accession was used as the template to predict the *SAD* protein 3D structure of the sample in our experiment. *R. communis SAD* protein contains 363 amino acids and is composed of 11 α-helix domain. Subunit enzyme has a diiron center with iron ions that bind symmetrically [14].

Protein 3D structure was used as a model in the analysis of amino acid residues that are needed in the process of *SAD* enzyme catalysis and electron transport. *SAD* enzyme reaction mechanisms are similar to the enzyme ribonucleotide reductases and bacterial monooxygenases due to similarity in conformation and orientation of iron-binding residues [25].

Mutations found in this study was only located on the coil that may not alter the direction of the *SAD* protein 3D structure folding. In addition, there were no mutations in the core domains, which are the polypeptide, substrates, or ion binding sites (Figs. 6 and 7). Such mutations allegedly not affecting the protein 3D structure folding such that the effects of specificity and speed of reaction including activity of each *SAD* enzyme isoform were not significantly different ($p<0.05$) in this study.

Variation of *SAD* mRNA among samples were tested and illustrated by constructing a phylogenetic tree (Fig. 4). Neighbor-Joining phylogenetic method divided the sample into three clades. This is in accordance with the pedigree of samples and place of origin that were divided into three groups: *E. guineensis* originated from Africa, *E. oleifera* from South America and the Hybrids. The three groups differ markedly in morphology and composition of fatty acids.

5. CONCLUSION

The specific primers designed in this study were effective to isolate the *SAD* gene and revealed four *SAD* isoforms, namely Brazilian and Suriname *E. oleifera* isoform, *E. guineensis* isoform and Hybridisoform identified from the six genetic material. The unsaturated fatty acids concentration phenotype especially the OLA is significantly ($p<0.05$) higher in *E. oleifera* isoform. Although there were genetically variations, but *SAD* enzyme was not the only factor that influence unsaturated fatty acids concentration in palm oil. These results provide useful information for the next development of palm oil with unique and useful oil profiles using marker-assisted selection breeding program.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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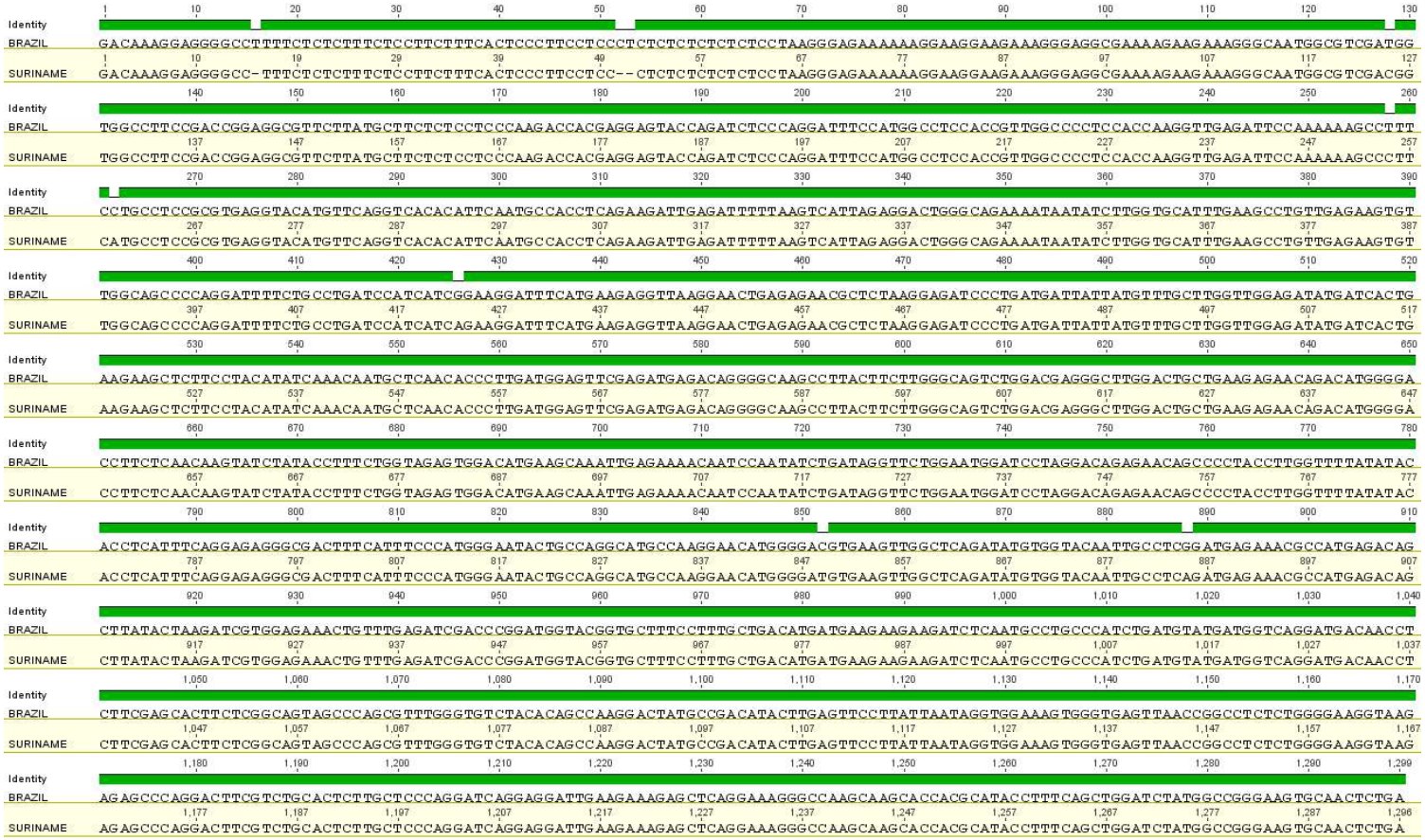
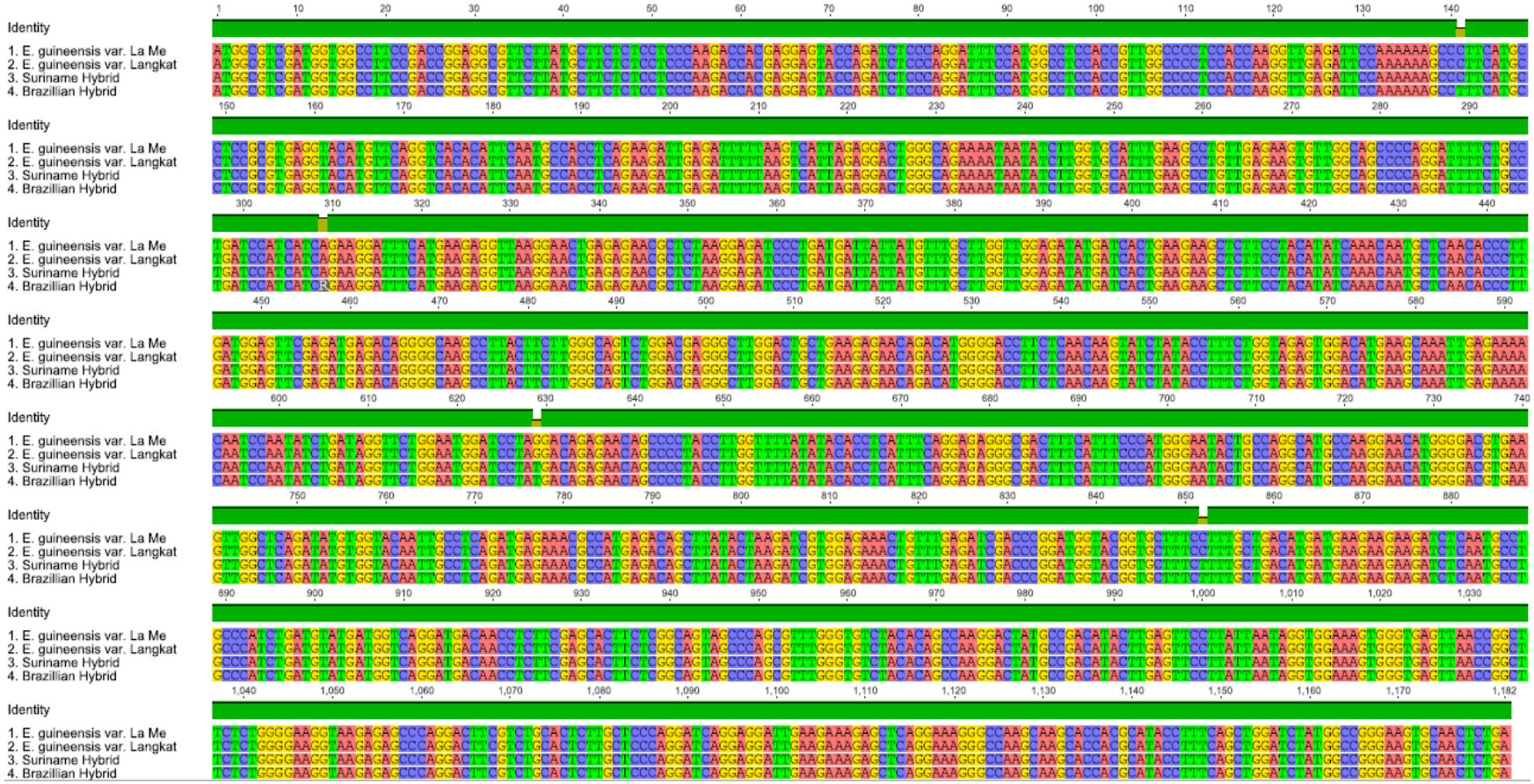
APPENDIX

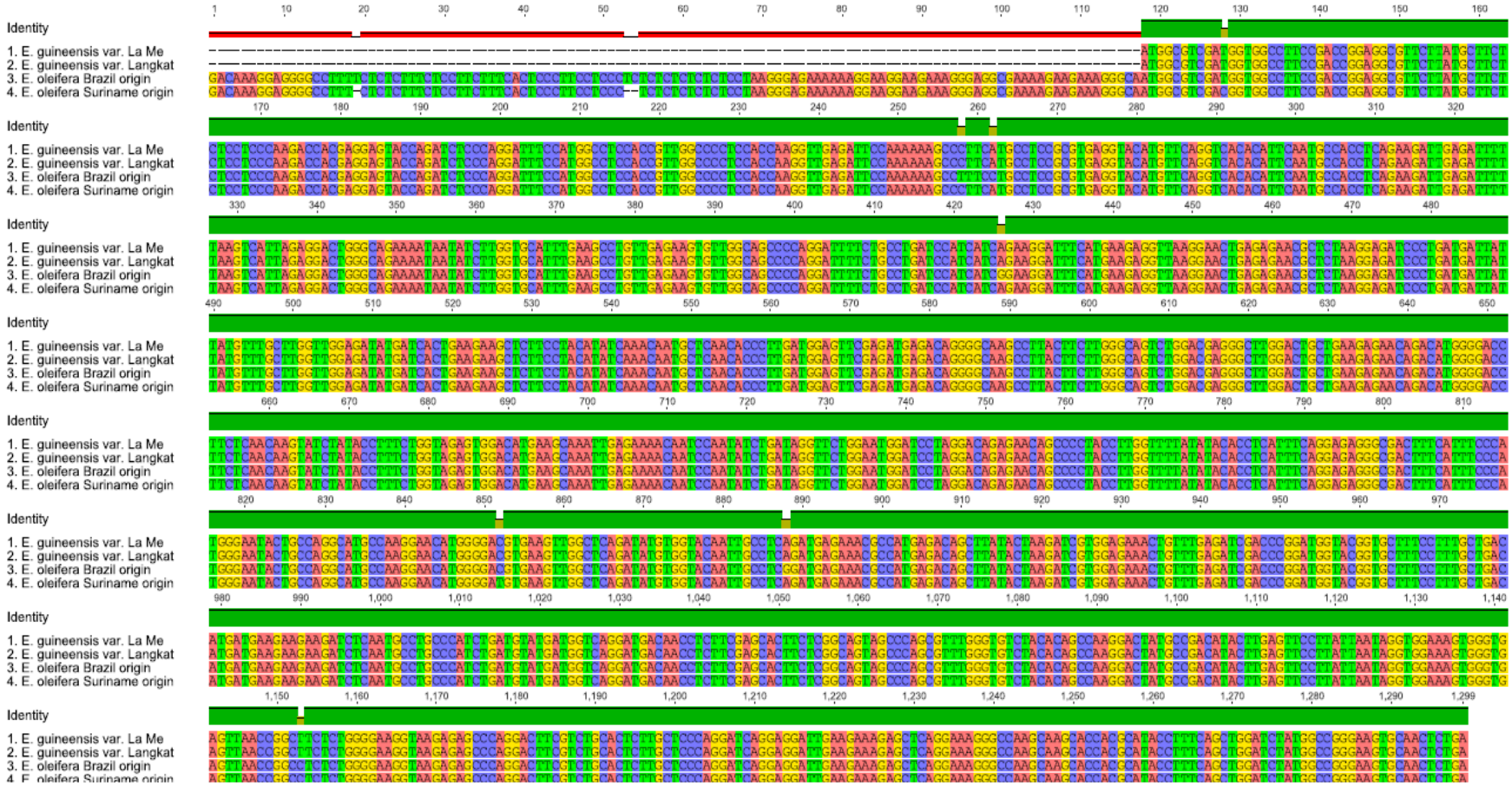
Alignment between *E. oleifera* at mRNA sequence (ESM 1).



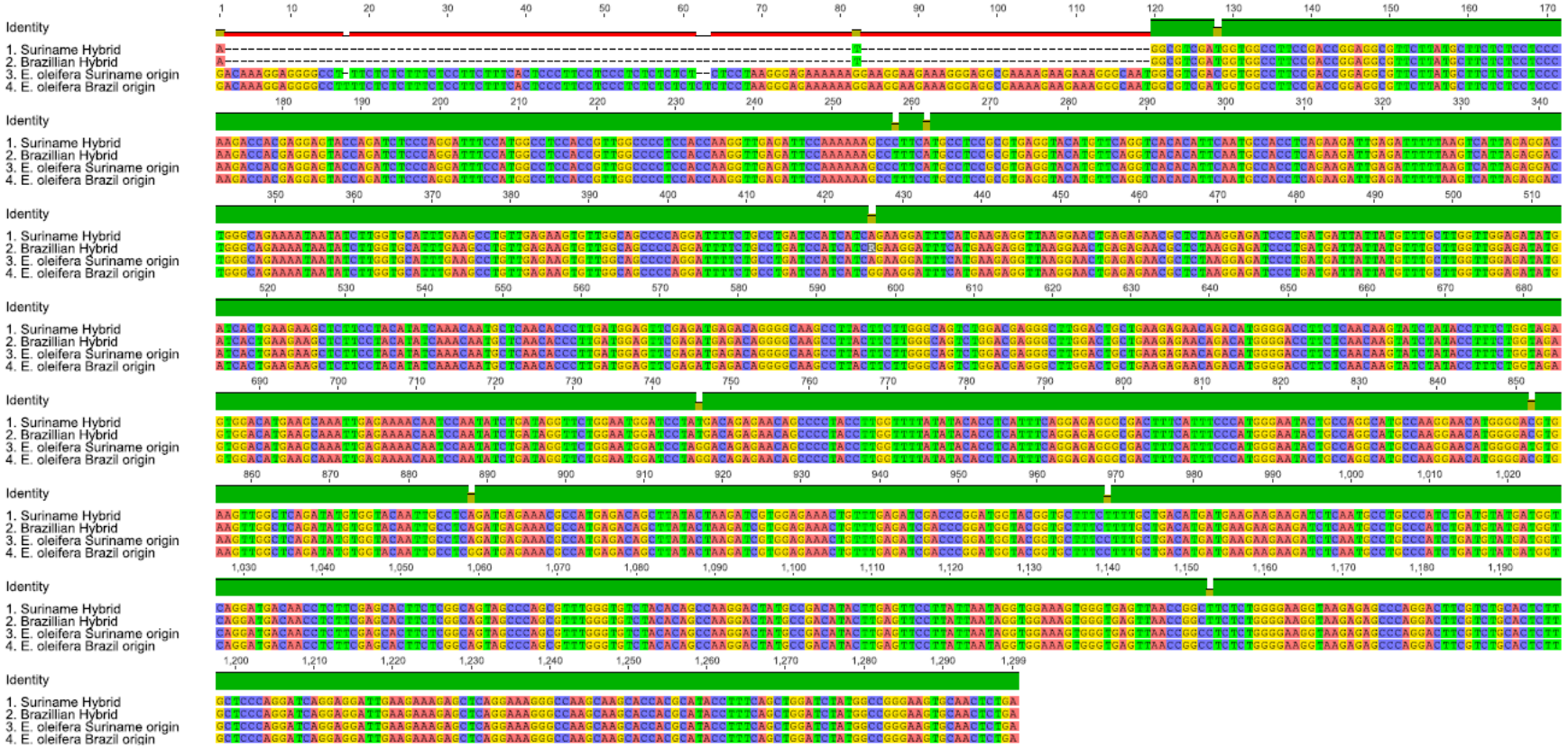
Alignment of *E. guineensis* against the Hybrids at mRNA sequence (ESM 2).



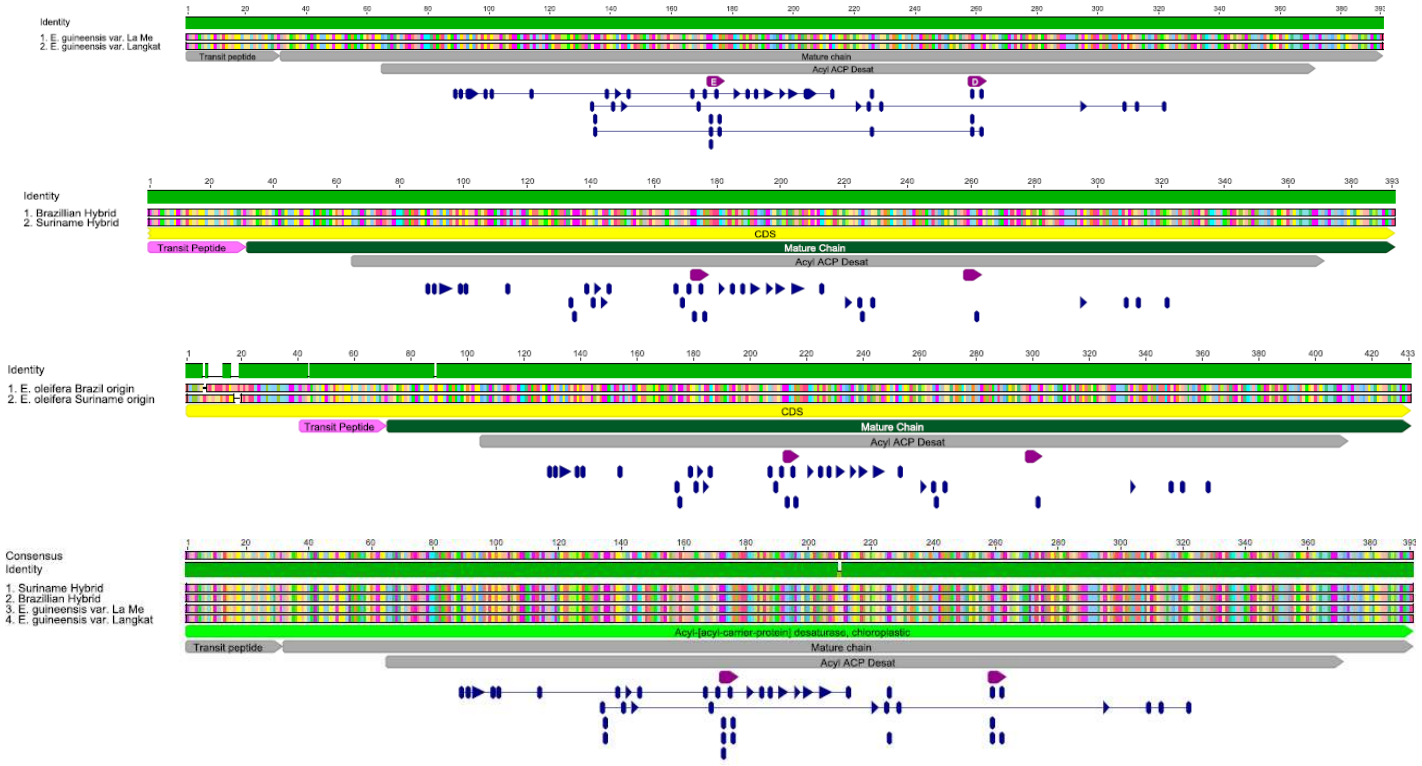
Alignment of *E. guineensis* against *E. oleifera* at mRNA sequence (ESM 3).



Alignment between *E. oleifera* against Hybrids at mRNA sequence (ESM 4).



These mutations did not occur in polypeptide, substrate, metal or ion bindings (ESM 5).



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