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Molecular characteristic on intra-species of *Metroxylon sagu* from Papua, Indonesia by *nad*2 and *mat*K genes

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Abstract. *Abbas B, Mawikere NL, Tjolli I, Arsyad M, Munarti. 2021. Molecular characteristic on intra-species of* Metroxylon sagu *from Papua, Indonesia by nad2 and matK genes. Biodiversitas 22: 5302-5310.* Sago palm (*Metroxylon sagu* Rottb.) is a palm that is capable accumulated a lot of starch for food in the trunk. The molecular characterization of sago palm is very important as identities of biological existence in the certain areas. This study aimed to reveal molecular characteristic on intra-species of *M. sagu* from Papua based on NADH dehydrogenase subunit 2 (*nad*2) gene and maturase K (*mat*K) gene. 15 accessions were used in this study. Sequences of *nad2* gene on some accessions of *M. sagu* were shown that no differences. The *nad*2 gene on some accessions of *M. sagu* was inferred only one haplotype and the *mat*K gene were inferred two haplotypes. The highest frequencies of the nucleotide in both *nad*2 and *mat*K gene were calculated thymine (T). The amino acid leucine was the most common, accounting for 11.44% of haplotype-1 and 11.50% of haplotype-2. The amino acids alanine, cysteine, and methionine have the lowest rates, with 1.99% for haplotype-1 and 2.00% for haplotype-2. The evolutionary relationships were shown no mutation rates occur in the *nad*2 gene and lower mutation rates occur in the *matK* gene on *M. sagu*. Based on the genotype-2 (Sagu01, Sagu02, Sagu08, Sagu12, and Sagu15) is proposed to be new variety.

Keywords: Chloroplast, *mat*K gene, *Metroxylon sagu*, mitochondrial, *nad*2 gene

INTRODUCTION

Sago palm (*Metroxylon sagu* Rottb.) is a palm that is capable accumulated a lot of starch in the trunk. *Metroxylon sagu* is one of the well-known palm species producing sago which is the main food staple for certain tribes in the eastern part of Indonesia. Abbas (2015) and Abbas (2018) documented that the strach resulting from sago palm can reaches 49 tons dry starch ha^{-1}, while other varieties of *M. sagu* in Papua, Indonesia based on local names such as Para, Panne, Yebha, Wanny have an average dry starch production of 674 kg, 576 kg, 512 kg, 491 kg $tree^{-1}$ respectively (Yamamoto 2011). Despite this, sago palm forest production was only around 230 kg $tree^{-1}$ dried starch (Yater et al. 2019). Based on morphological and genetic analysis, the sago palm exhibits a lot of variation (Abbas et al. 2017; Abbas 2018; Eksomtramage and Duangpan 2018). Genetic assessment using RAPD markers from *M. sagu* seeds showed significant variety (Riyanto et al. 2018).

Mitochondrial genomes (mt genomes) are circular DNA organelles that are maternally inherited (Castro et al. 1998) and range in size from 222 to 773 kb for angiosperm (Kitazaki and Kubo 2010). The *nad2* is one of many genes in mitochondrial genome that is possibility used for genetic characterization and description of organism. The mt genome structure was not influenced by the presence of crosses pollination. Dissociation mitochondrial DNAs (mtDNA) in the plant genome were stated that caused by the evolution through insertion or deletion in a long time, approximately 10,000 to100,000 years (Mower et al. 2007). Pervaiz et al. (2015) stated that the mt genome of *Prunus* species has a high conservative level. In the maternal inherited marker such as mtDNA and cpDNA markers, genetic differentiation occurs in extremely modest levels (Petit et al. 2005). The mitochondrial genome of plants ranges in size from 200 to 2,000 kbp (Morley and Nielsen 2017). Mitochondrial genomes have a low mutation rate (Christensen 2013), making them an effective molecular marker for determining sago palm phylogeny. DNA barcoding is an important approach for recognizing the genetic characteristics of plants and other organisms. Singh and Banerjee (2018) showed that DNA barcoding was a reliable approach for intra-species rice identification, distinguishing 54 percent of 286 species using matK and rbcL as markers (Kuzmina et al. 2012). DNA barcoding is a method of identifying species using one or more short gene sequences extracted from standard genomic sections. The chloroplast genome contains several genes that can be utilized for DNA coding in plants, including the accD, *matK*, *ndhJ*, *rpoB*2, *rpoC*1, and *ycf*5 genes (Chase et al. 2007) and *nad2* gene is located in the electron transport complex I that possessive conserve protein (Alberola et al. 2019).

The Plant Working Group of the Consortium for the Barcoding of Life (CBoL) (2009) recommended three genes, namely rbcL, *mat*K, and ITS. In the field of plant taxonomy and phylogenetics, DNA barcoding may be used

to classify plants more correctly than morphological identification. *Mat*K, a chloroplast gene situated on the trnK intron, is the DNA coding used in this study. It is roughly 1500 base pairs long (bp). In most Angiosperms, *mat*K has a base length of up to 1500 bp and is found between exons 5 and 3 of the tRNA-lysine gene (Kar et al. 2015). According to Hollingsworth et al. (2011), the matK gene is now an important tool for studying intra-species and inter-species genetic variability.

The aims of this study were to explore nucleotide sequence of *nad2* gene associated in the genome mitochondrial and sequence plastid associated with *mat*K genes in the sago palm to reveal molecular characteristic and evolutionary relationship of sago palm accessions for subjected to new variety of *M. sagu*.

MATERIALS AND METHODS

Plant materials

Sago palm used in the studies were obtained from several regions in Papua that has been collected by the Sago Palm Research Center (SRC), University of Papua (UNIPA), Indonesia in 2017. DNA analysis was conducted in 2019. Leaf samples were taken from accessions of sago palm in a growth russet stage. The names of accessions are Sagu1, Sagu2, Sagu3, Sagu4, Sagu5, Sagu6, Sagu7, Sagu8, Sagu9, Sagu10, and Sagu11, Sagu 12, Sagu 13, Sagu14 and Sagu15. The surface of the young leaf samples was cleaned with an alcoholic tissue and brought to the Laboratory of Biotechnology, University of Papua, Indonesia for DNA isolation.

DNA isolation

Leaf tissue dissociation was done by grind the sample to a fine powder. As much as 20 mg fine powder of the sample were transfer to a 1.5 ml micro centrifuge tube. DNA isolation was done by following the procedure of Plant Genomic DNA Mini Kit (Geneaid 2012). The procedures of DNA isolation using Geneaid protocols are tissue dissociation, lysis, DNA binding, wash, and DNA elution. The genomic DNAs is stored at -20°C freezer before PCR preparation.

PCR and sequencing

The *nad*2 and *mat*K primer sets were used in this study it is adopted from Duminil et al. (2002) and Kuzmina et al. (2012), respectively. The primer sets were synthesized by Integrated DNA technology (IDT), Singapore 117610. Primer sets for *nad*2 gene as follows forward 5' TTC ATA TAG AAT CCA TGT CC 3'and reverse 5' CTA TTT GTT CTT CGC CGC TT 3' and for *mat*K gene as follows MatK-1RKIM-f 5'-ACCCAGTCCATCGAAATCTTGGTTC-3 and MatK-3FKIM-r 5'-CGTACAGTACTTTTT GTGTTACGAG-3'. PCR mixtures and cycles condition of both *nad*2 and *mat*K genes were followed by 25 µl total volume that contains: 1 x PCR buffer contained 1.5 mM MgCl 2 (KAPA 2G Robust HotStart), 10 mM dNTP mix, 10 µM of forward and reverse primer, 1 x KAPA Enhancer, 0.5 U KAPA 2G Robust Hotstar polymerases and 10 ng genomic DNA. PCR cycles condition as follows: initial denaturation for 15 seconds at 94°C, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 50°C, for 45 seconds extension at 72°C. PCR amplification fragments were separated on 1% agarose gels by electrophoresis, staining was done using Ethidium Bromide and visualization by using UV illumination apparatus. Sequencing and purification of DNA PCR product were performed by 1st Base Asia, Singapore 117610.

Data analysis

Both mitochondrial *nad*2 and chloroplast *mat*K DNA sequences in the form of electropherogram were edited and checked to obtain correct DNA sequence. Editing and proofreading sequences were performed by comparing the peak color of both forward and reverse the electropherogram to the sequence of nucleotides produced using Molecular Evolutionary Genetic Analysis (MEGA) version 11 software (Tamura.et al. 2021). Each sequence in this study was obtained from the forward and reverse sequences of each sample. The editing result of a nucleotide sequence is stored in the fasta file format. Cluster alignment were performed based on Clustal W with MEGA 11 version software. Comparison of sample sequences with GenBank database (NCBI) is done using Basic Local Alignment Search Tools (BLAST) available on the National Center for Biotechnology Information (NCBI) web (Zhang et al. 2000; Morgulis et al. 2008). Evolutionary analysis of sago palm accession based on *nad*2 gene and *mat*K gene were calculated by using Mega 11 software (Tamura et al. 2021).

RESULTS AND DISCUSSION

Nucleotide sequence editing

Sequencing results of nucleotide were edited and alignment by using MEGA 11 version for obtaining the true nucleotide sequence and ensure that the DNA from the PCR results is not contaminated. Editing and alignment of *nad*2 nucleotide sequence on *M. sagu* show that fifteen accessions were observed, confirmed and declared eight perfect sequences for *nad2*-gene, others seven accessions were predicted contamination (Table 1). Editing and alignment of *mat*K nucleotide sequences on intra-species of *M. sagu* show overall fifteen accessions confirmed perfect sequences. The numbers of *nad*2 and *mat*K nucleotide sequence on *M. sagu* were identified 1304 and 603-767 bases length respectively.

The nucleotide sequences as molecular characteristic identities on intra-species of *M. sagu* that were inferred from mitochondrial *nad2* gene and chloroplast *mat*K genes were registered in the GenBank, NCBI (Table 1). Mitochondrial sequences associated with *nad2* genes in the sago palm accessions showed that no differences. The results are in agreement with Abbas et al. (2019) and Wulandari et al. (2021) reported.

Table 1. Identities of *Metroxylon sagu* accessions based on mitochondrial *nad*2 gene and chloroplast *mat*K gene

Accession	Nucleotide of *nad2* and *mat*K gene					
	nad2 gene			*mat*K gene		
	Nucleotide ID	Protein ID	bp Number	Nucleotide ID	Protein ID	bp number
Sagu1	NA	NA	NA	MT557585.1	QWN59107.1	603
Sagu2	NA	NA	NA	MT557586.1	QWN59108.1	603
Sagu3	KY849955.1	Intron	1304	MK860160.1	QGJ03632.1	767
Sagu4	KY849956.1	Intron	1304	MK860161.1	QGJ03633.1	767
Sagu5	KY849957.1	Intron	1304	MK860162.1	QGJ03634.1	767
Sagu6	KY849958.1	Intron	1304	MK860163.1	QGJ03635.1	767
Sagu7	KY849959.1	Intron	1304	MK860164.1	QGJ03636.1	767
Sagu8	KY849960.1	Intron	1304	MT557587.1	QWN59109.1	603
Sagu9	KY849961.1	Intron	1304	MK860165.1	QGJ03637.1	767
Sagu10	KY849962.1	Intron	1304	MK860166.1	QGJ03638.1	767
Sagu11	NA	NA	NA	MK860167.1	QGJ03639.1	767
Sagu12	NA	NA	NA	MT557588.1	QWN59110.1	603
Sagu13	NA	NA	NA	MK860168.1	QGJ03640.1	767
Sagu14	NA	NA	NA	MK860169.1	QGJ03641.1	767
Sagu15	NA	NA	NA	MT557589.1	QWN59111.1	603

Note: not available (NA), protein presented in Table 2 (*), identity (ID), base pair (bp), NADH dehydrogenase subunit 2 (*nad*2), and maturase K (*mat*K)

Fifteen accessions for sequencing of sago palm were performed, but other seven accessions were figured out that their sequence contaminated. The peaks of electropherograms of those seven accessions were not clear separation. The *nad2* gene sequences of eight intra-species of *M. sagu* showed no differences event the morphological different (Abbas et al. 2017). Based on investigation of mitochondrial genome in the previous studied such as beet showed that plant mitochondrial genome possesses a low mutation rate, a little compactness, large size, and high rearrange structure (Darracq et al. 2011). Furthermore, it was reported that mt genome of plants have a mechanism of base excision repair pathway (Boesch et al. 2009) so that the nucleotide structure is very conservative, even though morphologically different. Morphological differences were probably controlled by multigenic function which is associated in the nucleus and mitochondrial, or chloroplast genome, such as young petiole color, spine types, and spear color. Genes associated with mitochondrial genome such as *nad*2 gene were generally known their function as energy regulation in biological metabolism. Chen et al. *(*2017) reported that the mitochondria is responsible as primary source of cellular energy for growth, development, and reproduction of organism.

The mitochondrial sequences of *nad2* gene in sago palm were observed in this study it does not different. This mean sagu3 to Sagu10 is the same based on on *nad2* genes, others Sagu01, Sagu02, Sagu11, Sagu12, Sagu13, Sagu14, and Sagu15 data does not avalaible. The chloroplast sequences associated with *mat*K genes on *M. sagu* were shown two haplotypes based on the *mat*K gene sequence (Table 2). Haplotype-1 was identified on Sagu03, Sagu04, Sagu05, Sagu06, Sagu07, Sagu09, Sagu10, Sagu11, Sagu13, and Sagu14, and Haplotypes-2 was identified on Sagu01, Sagu02, Sagu08, Sagu12 and Sagu15 (Table 2). Nucleotide characteristic of Haplotype-1 was identified by Thymine (T) base in the position of nucleotide 5th base and 16th base. On the other hand, nucleotide substitutions of Haplotype-2 were identified by adenine (A) base in the position site of nucleotide base 5th and nucleotide deletion in the position site of nucleotide base 16th (Table-3). These characteristics revealed that the *matK* gene in the sago palm genome is highly conserved, with only two nucleotide tide changes of 604 to 767 base nucleotide sequences. Provan et al. (1999) observed very low mutation rates in the range of 3.2 x 10-5 to 7.9 x 10-5 in the chloroplast genome. According to Selvaraj et al. (2008) both chloroplast genome and mitochondrial genome of sago palm belong to highly conserved DNA sequences. The nuclear genome of the sago palm from the Papua Islands, on the other hand, was found to be highly variable using the RAPD marker (Abbas et al. 2009; Abbas 2018; Riyanto et al. 2018). Sagu01, Sagu02, Sagu08, Sagu12, and Sagu15 have mutated in the location of two nucleotides base in the DNA sequence organization, according to the *mat*K sequence of sago palm genotypes-2. Thymine to adenine base mutation is the first nucleotide base mutation, and thymine base deletion is the second nucleotide base mutation (Table 3). One of the explanations for the low mutation rate of the chloroplast genome is that it is inherited uniparentally (Savolainen et al. 1995; Viard et al. 2001).

Nucleotide characteristic of *nad*2 and *mat*K gene

The Nucleotide sequences in the *nad*2 gene were alleged as intron and the nucleotide sequences in the *mat*K gene were observed as exon. The nucleotide *mat*K sequence on intra-species of *M. sagu* was observed and declared two haplotypes (Table 2). The arrangement and nucleotide combination of the *nad*2 gene in intra-species *M. sagu* showed that only one haplotype was found, meaning there was no difference at the intra-species level of *M. sagu*. The nucleotide arrangement and combination of the *mat*K gene is divided into two genetic categories, namely haplotype-1 and haplotype-2.

The observations of nucleotide frequency from *nad*2 gene showed that thymine (T) and cytosine (C) were 27.1% and 25.9%, respectively, in Sagu03, Sagu04, and Sagu05,

while for Sagu06, Sagu07, Sagu08, Sagu09, and Sagu10 had the frequency thymine (T) and cytosine (C) nucleotides were 27.0% and 26.0%, respectively. The frequency of guanine (G) was 22.3%, and adenine was 24.7% for all individual samples (Table 4). The observation of nucleotide frequency from the *mat*K gene showed that thymine (T) in haplotype-1 differed from the nucleotide frequency (T) in haplotype-2. The frequency (T) of Haplotype-1 is 36.1% and Haplotype-2 is 36.4%. The nucleotides frequency of cytosine (C) and guanine (G) was the same for haplotype-1 and Haplotype-2, namely 17% and 16.3%, respectively. Adenine (A) nucleotide frequency was 30.6% for Haployipe-1 and 30.4% for haplotype-2 (Table 4). The highest frequencies of the nucleotide in both *nad*2 and *mat*K gene were calculated thymine. Louie et al. (2003) reported that nucleotide composition may use as mark for identifying eukaryotic organism. There is a clear GC and TA bias in introns, with an excess of G over C and T over A. This bias can be found in all noncoding, transcribed genes. The action of the transcription-coupled DNA repair system is most likely to blame (Green et al. 2003). The bias can be detected up to 1000 bp after the transcription ends. This is because the fact that transcription usually continues past the poly(A) signal, and the pre-mRNA is cleaved at the signal sequence before the poly(A) tail is added (Dye and Proudfoot 2001). Polypyrimidine tracts (PPT) and branch sites are known to be found at the 3′ intron ends (Louie et al. 2003). Within the last 40 nucleotides, there is an increase in C and T content. Aside from that, both intron ends appear to be roughly symmetrical. The endpoints are C and G-rich, owing to an overabundance of GGG (involved) at least in part. PPT and branch sites are known to be found at the 3′ intron ends. McCullough and Schuler (1997) reported that AU-rich intronic sequences, AG-rich exonic sequences and the 5′ splice site itself collectively define 5′ intron boundaries in dicot nuclei.

Table 2. Sequence alignment of *nad*2 gene and *mat*K gene on accessions of *Metroxylon sagu*

Gene	Accession	Sequence alignment
nad2 gene	Haplotype-1	TCCGAACCGCAGGAGATAGTTGCCCATCATACGGCTCACCAACTTCACTTGCCTCTAAGGGGGGCTCGCT
	Sagu03	CCGGGCAGGTTCGGATCACTTACAATACACGGCTCTACGAAGGGGTTAGGAGCGTTTTCAAGATGATTCT
	Sagu04	TTCTTTGTCGAGACGAAAAAGGAACCCATTTTTTCGACTGGAAAATGGGAGTCTGTTTTGTCTACTTTAT
	Sagu05	CCATCCCCCTCTATCAAAATGATCAAAAAGGAAGGTGAGCTTGCTTCTTATTCCCGTGTTTGATCTTTTC
	Sagu06	CATCTCTGCCCCGCTTCCATGTGGGCAGAGACCCCTGTAGAGAATGAAGAGGGGCCAAGGATCTTCCTCT
	Sagu07	CAAGAGTGCTTCTCGAGGCTCCACTCTCTCCCCTGAATAAGTAAGGCTCCGTTAGCCTGGGCTGAGATGG
	Sagu08	GGATAAGGAGTCAGGATTGAAGCCCCCAACGTTCTGCCAGACACTGGACAGGGGTTAGCTCTGTAAATGT
	Sagu09	GTAGAGCCAAGTGTAGTGTGGTGTAGTAGTAGGCACTTCTAGGCCCCTTCCCGGCTACTGGATCACTCCA
	Sagu10	GTGCTTCGGGTACTACGGACCCTCTGCCATCCATTGCAGCAGAGCCGTTTCATGAGCGGGGGGGCTAAGC
		GCAGTTCTTTGAATCAAACGTTGAATGAAATCGAAATCGATTCTTTTTTAGATATCCGGATAGATGGATG
		GATCTATCTTTCTATTCATATATATTTTGCAAGAAGCCCCAAATCCTTGATTTGGCCAGGAAACAAAGCA
		CTGCTTTGGGCCCAGGAAGCGAAGGGAATGAGCTCGGCTGCTTCTCCTCCACACTTCTTTATTTCTCCGT
		GCCCGTTCCGCATGCGCTTCGCGCGCCATTGGCGCTTTGCTCTCCTCTTATTTCTTCATTGGACGGTTCG
		GATGGACTTCGCCGTTCTTTCCCAACGAAAATGGAAAGGGCTGTATCACATCGAGATGTCGATTCGTTTT
		CCGCCCCAAATGAGATGGGGAATTAGTCACCTCTGTCCCTTCATCTTTCTGAATTGAATCGAGGCCCGGC
		CCGGCTCGCGTCGTTCCAACAACCGACGGGGAGCACCTCAGTATACGATCGCGCGCAGTAACTGGGAGTC
		CTATTACACCGGCGGCCAACTTCCATTCACCAAACCCAGGTTCATCTCGTGTAGTGATTGTGGACTCGTA
		CAAGGATATGGAGTCGACGGTTGATGTATCAGACTCGACCCTGTCTTTCGTAGCATGCATTCCCATCCGT
		GTCGCAACTGATTCGGTAAGCTACGTGTCCGGTGCACGGAAAAC
*mat*K gene	Haplotype-1	TTATTGCGATTCTTTCTTCACGAATATCATAATTGGAATAGTCTTATTACTCCGAATAATTCTATTTTTT
	Sagu3	TTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTTCCTATATAATTCTTATGTATCTGAATGCGA
	Sagu4	ATTTGTATTAGTTTTTCTTCGTAAACAATCTTCTTATTTACGATTAACATCTTCTGGAGCTTTTCTTGAG
	Sagu5	CGAACACATTTCTATGGAAAAATAGAACATCTTATAGTAGTGCGCCATAATTATTTTCAGAGGACCCTAT
	Sagu6	GGTCCTTCAAGGATCCTTTCATGCATTATGTTCGATATCAAGGAAAAGCAATTCTGGTTTCAAAGGGGGC
	Sagu7	TCATCTTCTGATGAAGAAATGGAAATGTCACCTTGTCAATTTCTGGCAATATCATTTTCACTTTTGGTCT
	Sagu9	CAACCGTACAGGATCCATATAGACCAATTATCAAACTGTTCTTTCTATTTTCTAGGTTATCTTTCAAGTG
	Sagu10	TATTAATAAATCTTTCGACGGTAAGGAATCAAATGCTAGAGAATTCATTTCTAATGGATACTGTTACTAA
	Sagu11	AAAATTCGATACCAGAGTCCCAGTTATTCCTCTTATTGAATCATTGTCTAAAGCAAAATTTTGTACCGTA
	Sagu13	TCGGGGCACCCTATTAGTAAGCCGATCTGGACCGATTTATCAGATTGCGATATTATTGATCGATTTGGTC
	Sagu14	GGATATGTAGAAATCTTTCTCATTATCACAGTGGATCCTCAAAAAAACAGAGTTTGTATCGAATAAA
	Haplotype-2	TTATTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTTCCTATATAATTCTTATGTATCTGAATGC
	Sagu01	GAATTTGTATTAGTTTTTCTTCGTAAACAATCTTCTTATTTACGATTAACATCTTCTGGAGCTTTTCTTG
	Sagu02	AGCGAACACATTTCTATGGAAAAATAGAACATCTTATAGTAGTGCGCCATAATTATTTTCAGAGGACCCT
	Sagu08	ATGGTCCTTCAAGGATCCTTTCATGCATTATGTTCGATATCAAGGAAAAGCAATTCTGGTTTCAAAGGGG
	Sagu12	GCTCATCTTCTGATGAAGAAATGGAAATGTCACCTTGTCAATTTCTGGCAATATCATTTTCACTTTTGGT
	Sagu15	CTCAACCGTACAGGATCCATATAGACCAATTATCAAACTGTTCTTTCTATTTTCTAGGTTATCTTTCAAG
		TGTATTAATAAATCTTTCGACGGTAAGGAATCAAATGCTAGAGAATTCATTTCTAATGGATACTGTTACT
		AAAAAATTCGATACCAGAGTCCCAGTTATTCCTCTTATTGAATCATTGTCTAAAGCAAAATTTTGTACCG
		TATCGGGGCACCCTATTAGTAAGCCGATCTGGACCGATTTATC

Note: The length of haplotype one for *nad*2 gene is 1304 bp and the length of haplotype-1 and haplotype-2 for *mat*K gene are 767 bases and 603 bases, respectively

Table 3. An example of nucleotide sequence in the mutation sites and deletion sites

Genotypes	Accessions	Alignment nucleotide sequence
Genotype-1	Sagu03	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu04	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu05	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu06	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu07	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu09	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu10	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu11	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu13	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu14	TTTTTTTTTTTTTTTTGAAAAAGAAAATAAAAGACTATTTCGGTT
Genotype-2	Sagu01	TTTTATTTTTTTTTT-GAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu02	TTTTATTTTTTTTTT-GAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu08	TTTTATTTTTTTTTT-GAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu12	TTTTATTTTTTTTTT-GAAAAAGAAAATAAAAGACTATTTCGGTT
	Sagu15	TTTTATTTTTTTTTT-GAAAAAGAAAATAAAAGACTATTTCGGTT

Table 4. Nucleotide frequencies of *nad*2 gene and *mat*K gene on accessions of *Metroxylon sagu*

Accession	Nucleotide frequency (%)							
	***nad*2 gene**				***mat*K gene**			
	T	**C**	**A**	**G**	**T**	**C**	**A**	**G**
Sagu 01	NA	NA	NA	NA	36.1	17.0	30.6	16.3
Sagu 02	NA	NA	NA	NA	36.1	17.0	30.6	16.3
Sagu 03	27.1	25.9	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 04	27.1	25.9	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 05	27.1	25.9	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 06	27.0	26.0	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 07	27.0	26.0	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 08	27.0	26.0	22.3	24.7	36.1	17.0	30.6	16.3
Sagu 09	27.0	26.0	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 10	27.0	26.0	22.3	24.7	36.4	17.0	30.4	16.3
Sagu 11	NA	NA	NA	NA	36.4	17.0	30.4	16.3
Sagu 12	NA	NA	NA	NA	36.1	17.0	30.6	16.3
Sagu 13	NA	NA	NA	NA	36.4	17.0	30.4	16.3
Sagu 14	NA	NA	NA	NA	36.4	17.0	30.4	16.3
Sagu 15	NA	NA	NA	NA	36.1	17.0	30.6	16.3

Notes: not available (NA)

Amino acid characteristic of *nad*2 and *mat*K gene

This work does not describe *nad*2 protein caused by the nucleotide allegedly as intron, so in the process transcription just until precursor messenger RNA (pre-mRNA) and removing when the transcription and translation process to be mature messenger RNA (mRNA) as well as protein translation respectively. Intron sequences do not participate in protein-coding sequences (Jo and Choi 2015). According to Amini et al. (2014), intron remove by catalytic RNAs (ribozymes). Amino acid frequencies of *mat*K gene were presented in Table 4. There were as many as 20 different types of amino acids found in this investigation, namely Ala, Cys, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, and Tyr. The composition of haplotype-1 was found to be different from that of haplotype-2. Leucine was the amino acid with the highest frequency, at 11.44% for Haplotype-1 and 11.50% for Haplotype-2. The amino acids Alanine, Cysteine, and Methionine have the lowest rates, with 1.99% for Haplotype-1 and 2.00% for Haplotype-2 (Table 4). Gorissen et al. (2018) stated that large differences in essential amino acid (EAA) contents and amino acid (AA) composition between various plant-based protein isolates. The EAAS were observed in this study that are Leucine, Isoleusine, Valine, Lysine, Histidine, Phenylalanine, Methionine, and Threonine. The highest proportion of the EAAs in the chloroplast *mat*K gene of *M. sagu* was found EAA Isoleucine (Table 4). Determine characteristics of plant may be inferred from the amino acid. Chauhan et al (2020) found that the diversity of plant by using AA sequence and Brooks et al. (2002) explained the evolution of amino acid frequencies over long time. Mutation within exon may be resulting amino acid change. In the previous study was shown that change Cysteine to Phenylalanine at codon 706 in cell demonstrated was sufficient to inhibit phosphorylation (Kaye et al. 1990). Proteins from the Last Universal Ancestor (LUA) were discovered to be usually richer in amino acids that are thought to have been most abundant in the prebiotic environment, and poorer in amino acids that are thought to have been unavailable or sparse (Brooks et al. 2002).

Table 5. Amino acid frequencies (%) in *mat*K gene on accessions of *Metroxylon sagu*

Genotype	*Accession*	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Haplotype-1	Sagu03	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagou4	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu05	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu06	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu07	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu09	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu10	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu11	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu13	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
	Sagu14	1.99	1.99	2.49	3.98	10.95	2.99	4.98	4.98	7.46	11.44	1.99	3.98	2.99	3.48	5.47	9.45	4.48	6.97	2.49	5.47	201
Haplotype-2	Sagu01	2.00	2.00	2.50	4.00	10.00	3.00	5.00	5.50	7.50	11.50	2.00	4.00	3.00	3.50	5.50	9.50	4.50	7.00	2.50	5.50	200
	Sagu02	2.00	2.00	2.50	4.00	10.00	3.00	5.00	5.50	7.50	11.50	2.00	4.00	3.00	3.50	5.50	9.50	4.50	7.00	2.50	5.50	200
	Sagu08	2.00	2.00	2.50	4.00	10.00	3.00	5.00	5.50	7.50	11.50	2.00	4.00	3.00	3.50	5.50	9.50	4.50	7.00	2.50	5.50	200
	Sagu12	2.00	2.00	2.50	4.00	10.00	3.00	5.00	5.50	7.50	11.50	2.00	4.00	3.00	3.50	5.50	9.50	4.50	7.00	2.50	5.50	200
	Sago15	2.00	2.00	2.50	4.00	10.00	3.00	5.00	5.50	7.50	11.50	2.00	4.00	3.00	3.50	5.50	9.50	4.50	7.00	2.50	5.50	200

Notes: Alanine (Ala) Cysteine (Cys), Aspartic (Asp), Glutamic (Glu), Phenylalanine (Phe), Glycine (Gly), Histidine (His), Isoleucine (Ile), Lysine (Lys), Leucine (Leu), Methionine (Met), Asparagine (Asn), Proline (Pro), Glutamine (Gln), Arginine (Arg), Serine (Ser), Threonine (Thr), Valine (Val), Tryptophan (Trp), Tyrosine (Tyr)

Table 6. Genetic distance based on Amino acid composition in *mat*K gene among accessions of *Metroxylon sagu*

Accessions	Sagu01	Sagu02	Sagu03	Sagu04	Sagu05	Sagu06	Sagu07	Sagu08	Sagu09	Sagu10	Sagu11	Sagu12	Sagu13	Sagu14	Sagu15
Sagu01	0.0000														
Sagu02	0.0000	0.0000													
Sagu03	0.0039	0.0039	0.0000												
Sagu04	0.0039	0.0039	0.0000	0.0000											
Sagu05	0.0039	0.0039	0.0000	0.0000	0.0000										
Sagu06	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000									
Sagu07	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000								
Sagu08	0.0000	0.0000	0.0039	0.0039	0.0039	0.0039	0.0039	0.0000							
Sagu09	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0039	0.0000						
Sagu10	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0039	0.0000	0.0000					
Sagu11	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0039	0.0000	0.0000	0.0000				
Sagu12	0.0000	0.0000	0.0039	0.0039	0.0039	0.0039	0.0039	0.0000	0.0039	0.0039	0.0039	0.0000			
Sagu13	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0039	0.0000	0.0000	0.0000	0.0039	0.0000		
Sagu14	0.0039	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0039	0.0000	0.0000	0.0000	0.0039	0.0000	0.0000	
Sagu15	0.0000	0.0000	0.0039	0.0039	0.0039	0.0039	0.0039	0.0000	0.0039	0.0039	0.0039	0.0000	0.0039	0.0039	0.0000

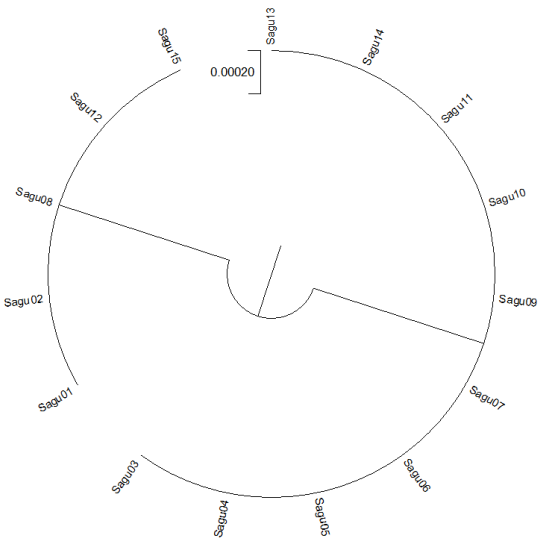

Figure 1. Evolutionary relationships infer by using *mat*K gene on accessions of *Metroxylon sagu*

Genetic distance based on amino acid composition is show among haplotype differentiation with genetic distances equal 0.0039 (Table 6). The previous study was reported that nucleotide differences in intra-species of sago palm by using chloroplast *mat*K gene with genetic distances equal 0.002 (Abbas et al. 20020a). The differentiation of *M. sagu* in the level intra-species show lower and just single nucleotide different as well as indicated mutation. Segregations in the DNA plastid of the *mat*K gene in the sago palm Haplotype-2 were consistent with Dipterocarpaceae phylogenetics based on the *matK* gene (Harnelly et al. 2018), and corresponded to the *Pandanus* (palms) DNA barcode (Zebua et al. 2019). DNA barcoding studies on intra-specific mangroves revealed 0.2% variabilities using the *mat*K marker (Saddhe et al. 2016), and vascular plants revealed 0.04% variabilities using the *mat*K marker (Saddhe et al. 2016) and sand rice (*Agriophyllum squarroum*) revealed 1.8% variabilities using the *mat*K marker (Genievskaya et al. 2017).

Evolutionary relationships based on *nad*2 and *mat*K genes

The *nad*2 gene was observed not differentiated, so clearly that no occurrence site of the DNAs evolution in the *nad*2 gene of *M. sagu*. Otherwise, *mat*K gene was calculated differentiation. The branch lengths are in the same units as the genetic distances used to estimate the evolutionary relationships, and the tree is drawn to scale. The evolutionary distances were calculated using the Maximum Composite Likelihood technique (Tamura et al. 2004), and are in base substitutions per site unit. There were 15 accessions of *M. sagu* sequences in this study. 1st+2nd+3rd+Noncoding codon locations were included. For each sequence pair, all unclear locations were deleted (pairwise deletion option). In the end, there were 605 positions in the dataset. MEGA11 was used to perform evolutionary analysis (Tamura et al. 2021). The evolutionary relationships in the nucleotide plastid *mat*K gene among intra-species of *M. sagu* are presented in Figure 1. The results are shown that only two haplotypes were recorded, which mean that lower mutation rates are inferred on intra-species of *M. sagu* (Figure 1). Haplotype-1 and haplotype-2 were observed ten and five samples respectively. The chloroplast mutation rate is lower than mitochondrion mutation rates (Smith 2015). Using the plastid *mat*K gene, the DNA Barcode can be utilized to establish inter- and intra-genera as well as inter-species relationships in the palm family (Abbas et al. 2020b).

In conclusion, molecular characteristics on intra-species of *M. sagu* were shown that the *nad2* gene on of *M. sagu* was inferred only one haplotype and the *matK* gene were inferred two haplotypes. The highest frequencies of the nucleotide in both *nad2* and *matK* genes were calculated thymine (T). The amino acid leucine was the most common, accounting for 11.44% of haplotype-1 and 11.50% of haplotype-2. The amino acids Alanine, Cysteine, and Methionine have the lowest rates, with 1.99% for haplotype-1 and 2.00% for haplotype-2. The evolutionary relationships were shown that no mutation rates occur in the *nad2* gene and lower mutation rates occur in the *matK* gene on intra-species of *M. sagu*. The genotype-2 (Sagu01, Sagu02, Sagu08, Sagu12, and Sagu15) is suggested and proposed to be new variety based on molecular characters.

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