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The Phylogenetic Relationship Among Varieties of *Lansium domesticum* Correa Based on ITS rDNA Sequences

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Abstract

Lansium domesticum Corr. with vernacular name in Indonesian *duku* has been reported containing therapeutic bioactive compounds, and some of these compounds shown to be potent antitumor, anticancer, antimalaria, antimelanogenesis, antibacteria, and antimutagenic activities. This plant is commonly known as *duku*, *kokosan* and *langsat* by the local community in Indonesia. The morphological appearance of all varieties is nearly the same, and identification of the varieties is very difficult for growers. Variation of DNA sequences of the ITS (Internal *transcribed spacer*) region can be used as a molecular character to determine the phylogenetic relationship of different varieties of *L. domesticum*. The aims of this study were to determine taxonomy status of *duku*, *kokosan*, and *langsat*, also phylogenetic relationship among varieties of *L. domesticum* based on ITS rDNA sequencing. DNA was isolated from leaves of plant and then amplified using F1 and R1 primers. Nucleotide sequences were identified using Sequence Scanner Software Programm version 1.0, nucleotide sequences from 18S, ITS1, 5.8S, ITS2 and 26S region, that has been mergered using EditSeq and SegMan in software Suite for Sequence Analysis DNASTAR Lasergene DM version 3.0.25. The results of study showed that DNA fragments ranging in size from 782-810 bp. Different pattern of DNA fragments indicated polymorphism among *duku*, kokosan, and *langsat*. Based on the results of the ITS rDNA sequencing and phylogenetic tree analysis. It was determined that *Lansium* and *Aglaia* are a separated genus with the similarity index value of 0.98. *Duku*, *kokosan* and *langsat* were divided into two cluster, namely cluster kokosan-langsat and cluster duku with the similarity index value of 0.996.

Keywords : Phylogenetic relationship, ITS region, *L. domesticum, duku, kokosan, langsat*

Introduction

Lansium domesticum is an important fruit tree and a highly variable species, with different forms that have been classified by some taxonomists as distinct species. In Southeast Asia, the plant has numerous common names, that is known as *duku*, *kokosan* and *langsat* (Indonesia); *duku*, *langsak* (Burmese); *buahan*, *lansone*, *lansones*, *lanzon*, *lanzone* (Philippine); *langseh*, *langsep*, *lansa* (Malay); *duku*, *langsat*, *longkong* (Thai) and *Bòn-bon* (Vietnamese). It still occurs wild or naturalized in these area and is one of the major cultivated fruits. The greatest producers of *L. domesticum* are Malaya, Thailand, the Philippines and Indonesia. On a small scale, this plant is also cultivated in Vietnam, Burma, India, Sri Lanka, Hawaii, Australia, Surinam and Puerto Rico (Yacob and Bamroongrugsa, 1991; Lim, 2012). There are numerous varieties of *L. domesticum*, both the plants and the fruit. Overall, there are three main varieties of these fruits, in Indonesia i.e *duku*, *kokosan* and *langsat* (Yulita, 2011; Hanum *et al.*, 2012).

Several parts of the plant have medicinal

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uses. The fruit peel is dried and burned to repel mosquitoes, it is also used to treat intestinal parasites and diarrhea. Powdered seeds are used to reduce fever, and the bark is used to treat malaria and scorpion stings (Naito, 1995; Loekitowati and Hermansjah, 2000; Saewan *et al.*, 2006). Skin and leaf extracts of fruit of *L. domesticum* interrupt the lifecycle of *Plasmodium falciparum*, and are active towards a chloroquine-resistant strain of the parasite (T9) *in vitro*. Study indicates extracts of *L. domesticum* are potential source for compounds with activity against chloroquine-resistant strains of *P. falcifarum* (Yapp and Yap, 2003)

Cosmeceutical value from its antioxidant, moisturizing, whitening and lightening effects. Dry extract of fruit is used for skin depigmentation and as a moisturizer (Tilaar *et al.*, 2008; Manosroi *et al.*, 2012). Extracts of this plant showed that strong inhibition of melanin production of B16 melanoma cells without significant cytotoxicity, presenting as a potential ingredient for skin-whitening cosmetics (Arung *et al.*, 2009).

The air-dried fruit peel of *L. domesticum* yielded five onoceroid triterpenes; the air-dried seeds yielded one onoceroid triterpene (lansionic acid) and germacrene D. Studies of the compounds showed various degrees of activity against *P. aeruginosa, B subtilis, C albicans, A niger* among others (Ragasa *et al.*, 2006).

Phylogenetic relationship among duku, kokosan, and langsat in infraspecies level are still not clear. At the level of infraspecies, there are two different taxonomic status of *duku*, *kokosan* and *langsat*. Ridley (Lim, 2012) suggested that *duku*, *kokosan* and *langsat* (pisitan) belonging to two varieties which were *L. dommesticum* var. *typica* (*duku*) and *L. dosmesticum* var. *pubescens* (*pisitan* and *kokosan*), and Morton (1987) suggested that *L. domesticum* Corr. divided into two varieties, namely *L. domesticum* var. *domesticum* (duku) and *L. domesticum* var. *pubescent* (pisitan/langsat). Another study by Yee *et al.* (1993), based on the observation of the anatomy of leaves, flowers, and fruits of duku separated them as varieties within the same species, namely *L. domesticum*. Morton (1987) and Yee *et al.* (1993) did not mentioned kokosan taxonomic status at the level of varieties. Lim (2012) stated that duku, kokosan and pisitan were grouped into two groups namely *L. domesticum*'duku group' and *L. domesticum*'langsat-longkong group'. According to Brandenburg (1986) and Hettercheid *et al.* (1996), the term group on crop can be equated varieties with the formal classification.

Molecular approach is an effective technique in genetic analysis, that can be used to determine genetic relatedness among duku, kokosan, and langsat in Indonesia. DNA sequences of the internal transcribed spacer (ITS) of the ribosomal RNA transcription unit have proven useful in resolving phylogenetic relationship of closely related taxa and in distinguishing species in plant (Hershkovitz and Zimmer, 1996; Muellner *et al.*, 2008; Pandey and Ali, 2012).

Ribosomal DNA (rDNA) genes are organized in clusters of tandemly repeated units, each of which consists of coding regions (18S, 5.8S, and 28S) and 2 internal transcribed spacers (ITS) and 1 non-transcribed spacer (NTS) region. ITS sequences have been used successfully in studying phylogenetic and genomic relationships of plants at lower taxonomic levels and the ITS regions are therefore valuable in more discrete phylogenetic separation of closely related species, recognition of new species, determination of conspecificity between isolates, discrimination within a species, and differentiation between species and subspecies (Samigullina *et al.*, 1998; Aktas *et al.*, 2007; Song *et al.*, 2012).

ITS an interesting subject for evolutionary/phylogenetic investigations, that which are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. The

nucleotide sequence variation found in both of ITS-1 and ITS-2 is used extensively for the systematic analysis of closely related taxa, at least in part due to the speedy rate of evolutionary change characterizing these DNA regions. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (Baldwin *et al.*, 1995; Hershkovitz and Zimmer, 1996). The aims of this study to determine taxonomy status of *duku*, *kokosan*, and *langsat*, also phylogenetic relationship within different varieties of *L. domesticum* based on squencing of ITS rDNA.

Materials and Methods

Plant materials

Samples used in this study were 10 samples of *duku*, *kokosan*, and *langsat* leaves from eight province in Indonesia with all of their vernacular names, summarized in Table 1.

DNA isolation

Total DNA was isolated from leaves using the Nucleon Phytopure Plant and Fungal DNA kit exraction RPN-8511/GE (Healthcare, U.K.) following the procedure described by Daryono and Natsuaki (2002).

Amplification condition and agarose gel electrophoresis

DNA amplification was conducted based on Kasmdari *et al.* (2002) with slight modifications. DNA were amplified by polymerase chain reaction (PCR) using forward primer F1 5'GATCGCGGCGGCGACTTGGGCGGTT C3' and reverse primer R1 5'GGTAG TCCCGCCTGACCTGGG3' (Muellner *et al.*, 2008). Each sample was prepared by PCR reaction mixture, PCR kit (Megamix-Blue PCR Master mix: the enzyme Taq polymerase, 2.75 mM MgCl2, 220 µM dNTPs, and blue agarose loading dye) 22 µl, 1 µl primer F1 (100 pmol), 1 µl primer R1 (100 pmol), DNA samples of 1 µl (10 ng/ml). PCR reaction was performed by 30 cycles consisting of 3 phases, namely (i) pre-denaturation for 5 minute denaturation at 95°C, (ii) denaturation for 1 minutes at 95°C, (iii) annealing for 1 minutes at 60°C, (iv) elongation for 2 minutes at 72°C, and (v) post-elongation for 10 minutes at 72°C. All PCR products were separated by electrophoresis on 1.5 % w/v agarose gel in 1xTBE, stained with 2.5 µl GoodView (Fermentas), viewed under ultraviolet light and photographed using digital camera (Cannon).

Purification and Sequence Analysis of ITS rDNA region.

Purification and *sequencing* of ITS rDNA LOKI, LSle, LHat, LPung, LTan, LMat, DDre, DKom, DSle, and KKal was conducted by First BASE Laboratories (Singapore).

Data Analysis

Analysis of nucleotide sequences was

Table 1. List of samples used in the study

No	Vernacular Name	Sample Code	Source of Materials	Province
1.	Duku Komering	DKom	Ogan Komering Ilir; Ogan Komering Ulu	South Sumatera
2.	Duku Sleman	DSle	Sleman	Yogyakarta
3.	Duku Dendan	DDre	Bengkalis	Riau
4.	Langsat OKI	LOKI	OKI	South Sumatera
5.	Langsat Sleman	LSle	Sleman	Yogyakarta
6.	Langsat Matesih	LMat	Karanganyar	Central Java
7.	Langsat Tanjung	LTan	Tabalong	South Kalimantan
8.	Langsat Punggur	LPung	Kubu Raya	West Kalimantan
9.	Langsat Hatu	LHat	Ambon	Ambon
10.	Kokosan Kaliurang	KKal	Kaliurang	Central Java

conducted by using *Sequence scanner* v1.0 (Applied Biosystem) *program* and Lasergene (DNASTAR Inc.). Program of *basic local alignment search tool* (BLAST) in website of *DNA Data Bank of Japan* (DDBJ) was used to find homolog sequences with data in GenBank. *Multiple sequence alignment* using ClustalW program in website DDBJ with using *L. domesticum* voucher Muellner130 (AY695587.1), *L. domesticum voucher* MWC2113 (AY695586.1); *Aglaia rugulosa* (AY695578.1); 2. *Aglaia coriacea* (EF491263.1); 3. *Aglaia spectabilis* (AY695580.1) 4. *Aglaia korthalsii* (EF491264.1); 5. *Aglaia teysmanniana* (AY695582.1) (outgroup) registered in *GenBank* as comparator.

Similiarities sequence analysis between samples were carried out using BioEdit programs (Hall, 1999) followed by construction of phylogenetic tree. Phylogenetic analysis was performed by *Neighbor-Joining* (NJ) methods using ClustalIX2 and MEGA5 programs, whereas genetic distance analysis relied on parameter Kimura-2 model. Grouping stability was calculated using 1000 *Bootstrap* value (analysis formation of branch of phylogenetic tree) (Tamura *et al*., 2011)

Result and Discussion

Amplification of Duku, Kokosan, and Langsat ITS region

PCR amplification using primers (F1 and R1) specifically recognized ITS region. The PCR products were visualized by agarose gel electrophoresis under UV light to chek the presence of amplified bands. Figure 1 showed clearly amplified bands (*single band*) of ~800 bp that was generated from

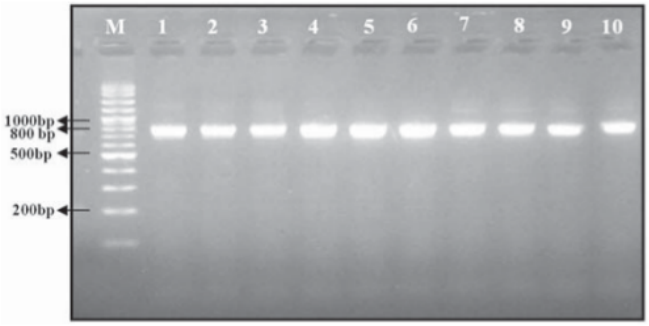

Figure 1. DNA fragments resulted from amplification using *forward* primer (F1) and *reverse* primer (R1). M: DNA Marker (Ladder Vivantis 100 bp); 1. DKom; 2. DSle; 3. DDre; 4. LOKI; 5. LSle; 6. LHat; 7. KKal; 8. LMat; 9. LTan; 10. LPung.

Table 2. Fragment Sizes of *Duku*, *Kokosan*, and *Langsat* ITS region

No	Sample	18S (bp)	ITS 1 (bp)	5.8S (bp)	ITS2 (bp)	28S (bp)	ITS region (ITS1; 5.8S; ITS2) (bp)	Total (bp)
1.	DDre	27	367	164	223	28	754	809
2.	DKom	28	367	164	230	24	761	813
3.	DSle	27	367	164	228	24	759	810
4.	KKal	28	365	164	224	24	753	805
5.	LMat	28	352	164	226	23	742	793
6.	LPung	26	365	164	226	24	755	805
7.	LOKI	26	366	164	224	28	754	808
8.	LHat	27	346	164	223	22	733	782
9.	LTan	26	364	164	226	22	754	802
10.	LSle	28	366	164	228	24	758	810

all samples (DKom; DSle; DDre; LOKI; LSle; LHat; LMat; LTan; LPung; and KKal) using PCR with specific primers.

DNA sequencing of ITS region using *forward* primer (F1) and *reverse* primer (R1) produced nucleotide sequences with size ranged from 782-810 bp which consisted of 18S; ITS 1; 5.8S; ITS 2; and 28S region (Table 2).

The results in Table 2 also showed that ten samples of plant has produced size of varied fragment ITS region, ITS 1 ranged from 346-367 bp and ITS 2 ranged from 223 - 228 bp while 5.8S region was more stable at the size of 164 bp. The results obtained in this studies, the same the report Muellner *et al.* (2005) on *Aglaia* is for ITS 1 ranging from 263-274 bp and ITS 2 ranged from 221-227 bp, while 5.8S was stable at the size of 164 bp.

Variation of fragment size resulted from amplification of ITS region indicated that there was variation on the length of ITS region. Aktas *et al.* (2007) reported that the ITS1 length varied more than the ITS2, ITS1 varied in length from 482 to 1634 bp and was longer than the ITS2 region (268-525 bp) in all *Theileria* isolates. Some rRNA genes were organized in clusters of tandemly repeated units, each of which consisted of coding regions (18S, 5.8S, and 28S) and 2 internal transcribed spacers (ITS) and 1 non-transcribed spacer (NTS) region. While the coding regions were evolutionarily conserved and had been utilized for phylogenetic inferences for major phyla (Hills and Dixon, 1991).

In this experiment, region of 5.8S rRNA had similar fragment size of 164 bp in all samples. Accoding to Hidayat and Pancoro (2001), region of 5.8S rRNA was more constant because of these gene encode rRNA which part constitute of ribosom small subunit to be a benefit to synthesis of protein. Ritland *et al.* (1993) reported that 5.8S region was relatively unvaried and ITS region do not encode an rRNA subunit and showed the expected greater sequence variation than that in the 5.8S.

Fragment sizes of ITS region (ITS 1, 5.8S, and ITS2) in this study with size ranged from 733-761 bp were different with fragment sizes that had been reported by Muellner *et al.* (2005), in species of *Aglaia* (Meliaceae) including *L. domesticum* with size ranging between 627-664 bp in size. The fragment sizes of ITS regions resulted from this research were similar to fragment sizes that commonly found in Angiospermae. Baldwin *et al.* (1995) reported that generally fragment sizes of ITS region of Angiospermae approximately 700 bp with the sizes of ± 300 bp, ± 165 bp, and ± 300 bp, respectively.

Nucleotide sequence analysis for comparing genetic information of *L. domesticum* and some species from *Aglaia* genus on ITS region was done compaty with foreknown sequences of DNA in *GenBank* databases. The homologous nucleotide sequences of each samples were analysed using BLAST (*Basic local alignment search tool*)

Table 3. Homology Search (Searching for highest similiaritas) on Duku, Kokosan, and Langsat relied on DDBJ.

No	Sample	High Similarity	No Acession	*Total Score*	*Query Coverage*	*Identity value*
1.	DDre	*L. domesticum* voucher MWC2113	AY695586.1	1277	92%	99%
2.	DKom	*L.domesticum* voucher Muellner130	AY695587.1	1321	91%	99%
3.	DSle	*L. domesticum* voucher Muellner130	AY695587.1	1290	92%	99%
4.	KKal	*L. domesticum* voucher MWC2113	AY695586.1	1266	92%	99%
5.	LMat	*L. domesticum* voucher MWC2113	AY695586.1	1279	92%	99%
6.	LPung	*L. domesticum* voucher MWC2113	AY695586.1	1029	92%	99%
7.	LOKI	*L. domesticum* voucher Muellner130	AY695587.1	1297	92%	99%
8.	LHat	*L.domesticum* voucher Muellner130	AY695587.1	1343	92%	99%
9.	LTan	*L. domesticum* voucher MWC2113	AY695586.1	1310	92%	99%
10.	LSle	*L. domesticum* voucher Muellner130	AY695587.1	1354	92%	99%

Table 4. Values of Index of Similarity sequences of Duku, Kokosan, dan Langsat ITS rDNA region

Sample	Similarity																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1																	
2	0,997																
3	0,995	0,998															
4	0,998	0,992	0,998														
5	0,996	0,997	0,997	0,990													
6	0,987	0,987	0,987	0,987	0,987												
7	0,987	0,987	0,987	0,987	0,987	0,996											
8	0,987	0,987	0,987	0,987	0,987	0,998	0,996										
9	0,987	0,987	0,987	0,987	0,987	0,996	0,996	0,996									
10	0,987	0,987	0,987	0,987	0,988	0,996	0,996	0,997	0,996								
11	0,987	0,987	0,987	0,987	0,988	0,997	0,996	0,996	0,996	0,998							
12	0,987	0,987	0,987	0,987	0,987	0,996	0,996	0,996	0,998	0,996	0,992						
13	0,987	0,987	0,987	0,987	0,987	0,996	0,997	0,996	0,998	0,996	0,992	0,998					
14	0,987	0,987	0,987	0,987	0,988	0,997	0,996	0,997	0,996	0,998	0,998	0,996	0,998				
15	0,987	0,987	0,987	0,987	0,987	0,998	0,998	0,996	0,997	0,996	0,995	0,997	0,997	0,997			
16	0,987	0,987	0,987	0,987	0,988	0,996	0,996	0,997	0,997	**0,999**	0,998	0,996	0,998	0,998	0,996		
17	0,987	0,987	0,987	0,987	0,988	0,996	0,998	0,998	0,996	0,997	0,995	0,996	0,996	0,997	0,998	0,997	

1. *Aglaia rugulosa*; 2. *Aglaia coriacea*; 3. *Aglaia spectabilis*; 4. *Aglaia korthalsii*; 5. *Aglaia teysmanniana*; 6. *L. domesticum* voucher MWC2113; 7. L. Tanjung; 8. *L.domesticum* voucher Muellner130; 9. DDre; 10. DKom; 11. LOKI; 12. KKal;13. LMat; 14. LHat; 15. LPung; 16. DSle; 17. LSle.

software versi 2.2.24 on server of DDBJ (*DNA Data Bank of* Japan) as mention in Table 3.

The result showed that all samples produced 99% similarity with *L. domesticum* voucher MWC2113 (AY695586.1) and *L. domesticum* voucher Muellner 130 (AY695587.1) but had no similiarity to another species (Table 3).

Similarity index values of DKom, DSle, DDre, LOKI, LSle, LHat, LMat, LTan, LPung, KKal, *L. domesticum* voucher MWC2113, *L. domesticum* vouchers Muellner130, and *A. rugulosa, A. coriacea, A. spectabilis, A. korthalsii, and A. teysmanniana* as outgroup were given on Table 4. The highest and lowest sequence similarity index values were 0.999 and 0.987, respectively. Highest sequence similarity index value was found on DKom and LOKI meanwhile the lowest sequence similarity index values was found in all types of Aglaia was used as an out group with of duku, kokosan, langsat, *L. domesticum* voucher MWC2113 and *L.domesticum* voucher Muellner130.

Grouping pattern and relatedness among *duku*, *kokosan*, and *langsat* with *A. rugulosa, A. coriacea, A. spectabilis, A. korthalsii,* and *A. teysmanniana* as outgroup based on sequence similarity index value were analyzed with MEGA5 resulted as phylogenetic tree (Figure 2).

Figure 2 showed that the phylogenetic tree had two main clusters namely cluster I, which consisted of *duku*, *kokosan*, and *langsat* and cluster II consisted of several species of *Aglaia*. Based on the clustering pattern suggested that *Lansium* and *Aglaia* is a monophyletic group that had a high similarity among the members. According to Hidayat and Pancoro (2008) in a phylogenetic approach, a group of organisms whose members have a lot of similarities of character is considered to have a very close relationship and estimated descended from a common ancestor.

Of these groupings could be defined separation between genus *Lansium* and

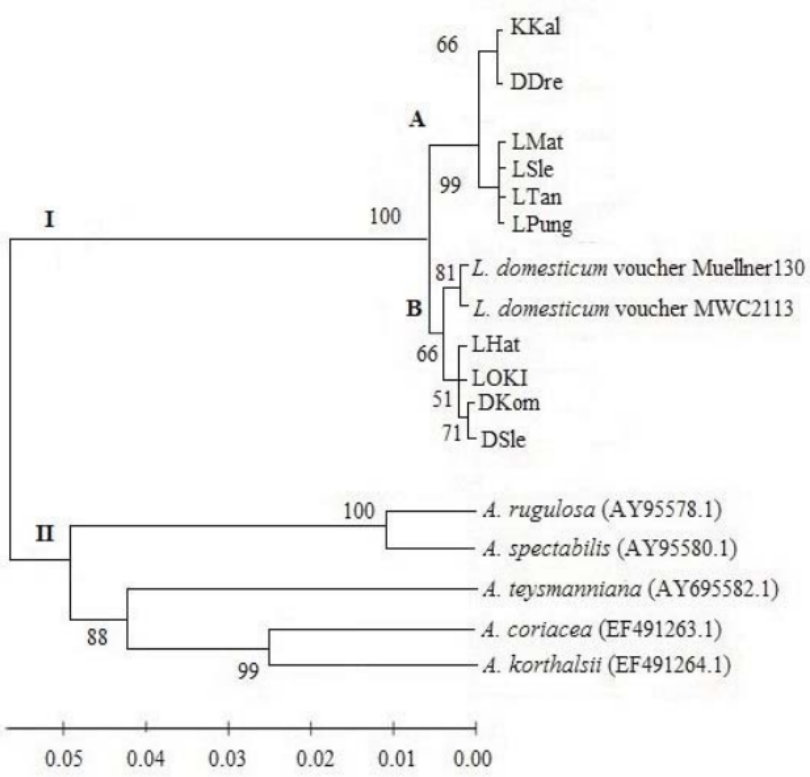

Figure 2. Phylogenetic tree of duku, kokosan, dan langsat based on ITS rDNA. The values on each of branch refers to values of *bootstrap*. Scala underneath of tree refers to genetic distance among samples.

Aglaia. Our results were different from previously described by Kostermans (1966), which stated that the placement of *Lansium* into *Aglaia* with three species, namely *A. dookkoo* Griff. (*duku*), *A. aquea* (Jack) Kosterm. (*kokosan*), and *A. domestica* (Corr. Emend. Jack) Pellegrin (*langsat*), and placement of *Lansium* as well as sectio of the genus *Aglaia* Lour. Our studies supported studies that had been reported by Pennington & Style (1975) which stated *Lansium* and *Aglaia* were different genus. Muellner *et al*. (2005) also suggested that *Lansium* as a separate genus from *Aglaia* based on 16rps intron regions and secondary metabolites. This statement was reinforced by the results obtained by Muellner *et al*. (2008) based on the ITS region of Meliaceae plants, also got the same grouping pattern, that was *Lansium* and *Aglaia* separated into different groups and put *Lansium* and *Aglaia* on tribus Aglaieae.

Cluster I was divided into two sub-clusters, namely subcluster A and subcluster B. Sub cluster A consisted of two clusters, the first cluster consisted of KKal and DDre with the similarity index value of 0.998. The inclusion of DDre in group kokosan is possibly occured by mistake in vernacular name by local communities. DDre was collected from Bengkalis Island, Pekan Baru. Recently, it is known that kokosan has only been recognized and is found in Java Island. Although based on fruit morphology DDre has similarities with kokosan but DDre has sweet fruit flavors like duku, unlike the kokosan that taste is very sour. Hanum *et al* (2012) reported based on RAPD approach stated DDre into the group kokosan. Based

on our reseach of nucleotide sequences of the ITS region further strengthens position of DDre in the group of kokosan. The second cluster consisted of langsat namely LMat, LSle, LTan, and LPung with the similarity index value of 0.998.

Subcluster B consisted of two sub-clusters, the first cluster consisted of *L. domesticum* voucher Muellner130 and *L. domesticum* voucher MWC2113 to the value of similarity index of 0.998. The second cluster consists of DSle, LHat, DKom, and LOKI. Similarity index values between DKom with LOKI are 0.999, while the value of similarity index between DSle with LHat are 0998. The inclusion of LOKI and LHat in group of *duku* is possibly occurred by mistake in vernacular name and genetic variation occurred on LOKI and LHat. According to Suryanto (2003), genetic variation can occur because of alteration in nucleotides constituent of DNA. Genetic variation of *duku*, *kokosan*, and *langsat* likely occurred because there has been a cross-pollination and vegetative propagation.

Dispersal by humans can indirectly cause genetic variation. Genetic variations may occur due to natural mutations due to the influence of environmental stress the place of origin of the plant. Plants survive by adapting to their environment, breed and pass on their genes to the next generation. This process has been going on for decades causing genetic changes in LOKI dan LHat. Pandin (2010) stated that alteration occur due to the changes in reimbursement mechanisms and alteration in DNA nucleotide bases does not necessarily change the morphological characters, so that the use of markers that directly integrates with the genetics system will be better able to describe the actual state of the genome. Hanum *et al.* (2012) reported based on the RAPD approach of *duku*, *kokosan*, and *langsat* was known that LOKI and LHat were included in the group of *duku*.

Based on the results of the ITS rDNA sequencing and phylogenetic tree analysis, it can be determined that *Lansium* and *Aglaia* are separate genera with the similarity index value of 0.98, and *duku*, *kokosan* and *langsat* were divided into two clusters, namely cluster *kokosan-langsat* and cluster *duku* with the similarity index value of 0.996.

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