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DIVERSITY AND RESPONSE TO GAMBOGE DISORDER OF MANGOSTEEN (*Garcinia mangostana* L.) IN SWAMPY AREAS OF TEMBILAHAN, INDONESIA

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SUMMARY

Gamboge disorder is a major problem in mangosteen (*Garcinia mangostana* L.). Based on indigenous knowledge of growers in swampy areas of Tembilahan, Riau Province-Indonesia, there are different types of individual mangosteen which differently respond to gamboge disorder. The objectives of this study were to identify genetic variation of mangosteen cultivated in Tembilahan, to analyze their genetic relationships and association with gamboge disorder. Thirty one mangosteen accessions from this region were evaluated using 11 morphological characters, ISSR (inter simple sequence repeat) and RAPD (randomly amplified polymorphism DNA) markers, and their response to gamboge disorder. Twenty one of them were amplified successfully on each primers used and then grouped through cluster analysis. The cluster analysis separated the accessions into two different groups at similarity of 0.84. Similarity coefficients of the accessions ranged from 0.84 to 0.99. Both groups were different in morphology, molecular profiles, and response to gamboge disorder. The first group included popular cultivar for growers, due to the number of fruit segments (4 to 11), firm flesh, and absence of gamboge disorder in the flesh, called as Tembilahan or non-gamboge disorder (non-GD) type. The second group included common mangosteen and sometimes contained gamboge disorder, which was called non-Tembilahan or gamboge disorder (GD) type. The unique markers, consisted of OPH12-1600 bp, OPH 13-2400 bp, and OPH18-950 bp, and PKBT2-350 bp, respectively were amplified in non-Tembilahan types and absent in Tembilahan types. These results were important to develop specific molecular markers for selection a new cultivars and genotypes for conservation.

Key words: Diversity, *Garcinia mangostana* L., gamboge disorder, ISSR, RAPD, swampy area

Key findings: The research identified two types of mangosteen in swampy areas of Tembilahan, consisting of Tembilahan as non-gamboge disorder (non-GD) type and non-Tembilahan as gamboge disorder (GD) type, respectively. The specific morphological and molecular characters, and response to gamboge disorder are distinctive for the two types of mangosteen.

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INTRODUCTION

Mangosteen *(Garcinia mangostana* L.*)* is a native fruit to Indonesia and South East Asia (Almeyda and Martin, 1976), East Indies (Indonesia) and South-East Asia (Campbell, 1966), and Peninsular of Malaysia (Ochse *et al*., 1961). The mangosteen is an obligate apomictic fruit species, from which the seed develops without fertilization and believed that all of its progenies may have the same genetic background as their mother plant (Richards, 1990a; Koltunow, 1993). Several studies reported the occurrence of genetic variation in

mangosteen accessions, from Java and Sumatra (Mansyah *et al.,* 2003), Tasikmalaya, West Java (Sinaga *et al.,* 2007), and as well as Indonesia and Malaysia (Ramage *et al.,* 2004).

The current research showed phenotypic and genotypic variability among mangosteen populations. Variation in fruit morphology is important for plant fitness because it influences dispersal capabilities (Bolmgren and Eriksson, 2005). Phenotypic variation found in mangosteen include: canopy shape, mature leaf color (green and variegated), flowers and fruits number per cluster, pedicel length, fruit shape, fruit-base shape, stigma lobe shape, size, and thickness, the fruit segments number, and rind thickness (Mansyah *et al.,* 2010). The variegated leaves character is found in certain plants while other characters are commonly found in mangosteen plants in the population. Similar to these results, a mangosteen clone survey was conducted in the Eastern region of Thailand showed differential morphology and could be distinguished in 6 characters i.e. small leaves and small fruits, oblong fruit shaped, thin stigma lobe, aborted seed of fruit, variegated leaves in whole tree, and variegated leaves in partial tree (Chaiwat *et al.,* 2015).

Genetic variation also occurred among the progenies themselves and between the progenies and the mother plant both from polyembryonic and monoembryonic seedlings (Mansyah *et al.,* 2013). A mangosteen genetic study revealed by randomly amplified DNA fingerprinting (RAF) found diversity among 37 of *G. mangostana* accessions, with 9 different genotypes were clustered into three distinct groups (Ramage *et al.,* 2004). Among the different genotypes of mangosteen, there was no information on the genotypic response to gamboge disorder. Gamboge disorder is a physiological disorder, indicated by symptoms of yellow gum in fruit aril and endocarp, which causes the fruits to be unfit for fresh consumption and processing (Yaacob and Tindall, 1995). One of the factors causing gamboge disorder is the breakdown of epithelial cells in the cell wall surrounding the yellow latex secretory duct (Dorly *et al.,* 2008).

Mangosteen is generally grown on dry land up to 900 meters above sea level. Good soil texture, structure, drainage, moisture, high organic matter content, and optimum pH (5.5 to 7.0), are favorable for mangosteen growth. Mangosteen requires annual rainfall distribution above 1270 mm/year. In Indonesia mangosteen also grows well in swampy areas such as Tembilahan Riau Province, South Kalimantan, and South Sumatera. The objectives of this study were to identify the diversity of mangosteen cultivated in swampy area of Tembilahan, analyze their genetic relationships, and association with fruit gamboge disorder.

MATERIALS AND METHODS

This section should describe the origin and nature of the materials used. Procedures used, experimental design included level of replication, and methods of data analysis should be presented, giving references to papers describing new or unusual techniques. Software used for data analysis should be indicated. This section should be concise but contain sufficient detail for other researchers to repeat the experimental work.

Plant material

Plant materials used were leaves and fruits of 31 mangosteen trees which were selected randomly from Pulau Palas, the swampy area of Tembilahan Hulu, Indragiri Hilir Regency, Riau, Indonesia. The coordinates of the location are 0°25'12.5"S to 103°6'17.44"E, and elevation about 16 meters above sea level. This area is located on the banks of Indragiri Hilir river and always flooded with fluctuating water levels (Figure 1). The mangosteen plants in this area are planted in the land with drainage channels in the form of small trenches. In this way, the mangosteen plant roots are protected from floods. The study was conducted for about four months during the fruit season of 2010.

Morphological observations

The morphological characters observed were leaf and twig size, sepal colour, petal size and thickness, pedicel length, fruit shape, stigma

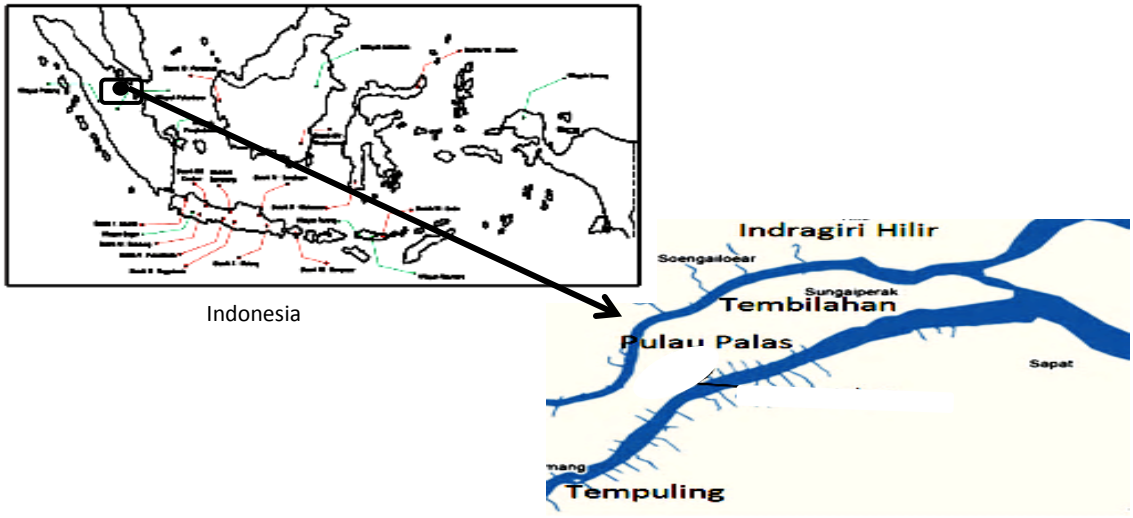

Figure 1. Location of mangosteen samples in the Pulau Palas village of Tembilahan district, Riau Province.

lobe shape, number of fruit segments, water content (juicy or not juicy) and flesh texture (firm or soft). This morphological observations were based on descriptors of mangosteen (IPGRI, 2003; Mansyah *et al*., 2010).

Gamboge disorder observation

About 50 fruit samples per tree were observed for presence or absence of gamboge disorder. The flesh of fruits with gamboge disorder are covered by a yellow gummy, and sometimes undergo translucent flesh disorder (TFD). The gamboge disorder can be categorized as non GD or very low GD (0 to 1%), low GD (more than 1% to 20%), moderate GD (20 to 30%) and high GD (more than 30%). Responses of mangosteen accessions to gamboge disorder could be divided into two categories, gamboge disorder (GD) genotype and non gamboge disorder (non-GD) genotype. GD genotypes were described as mangosteen plants with gamboge disorder incidence degree from low to high level. The non-GD genotypes were indicated by their fruit flesh almost free from yellow gummy contamination (0 to 1 %).

Molecular marker analysis

DNA extraction was conducted using a modified CTAB extraction method (Doyle and Doyle, 1990) that included add 2% PVP-40 in the CTAB extraction buffer. PCR amplifications of the DNA regions were conducted using five ISSR and eight RAPD primers. ISSR primers were obtained from Pusat Kajian Buah Tropika (PKBT) laboratory, Bogor Agricultural University (Table 1). About 0.1 g fresh mangosteen leaves were grinded for DNA extraction. DNA quantity and quality were determined by electrophoresis in 1% of agarose gel and 1X TAE buffer solution, stained in ethidium bromide solution and visualized using a ultraviolet (UV) trans-illuminator. PCR reactions were carried out in a total volume of 25 µl consisting of 2 µl (20 ng) of DNA template, 12.5 µl Go Taq Green Master Mix (Promega, Cat No. M7122), 1 µl primer (20 µM), and 9.5 µl nuclease-free water. Amplification for RAPD and ISSR markers were performed under the following conditions: one cycle of pre denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94 °C for 0.5 min, 0.5 min at annealing temperature (depend on primers used, Table 1), and extension at 72 °C for 1 min, and one of final extension at 72 °C for 5 min. PCR products were separated on 1.2% agarose gel and 1X TAE buffer solution, stained with ethidium bromide and visualized with UV light. Marker alleles were then scored as present (1) or absent (0).

Table 1. Details of primers used in this study

No	Primer Name	Primer Sequences (5'---3')	Marker Type	Annealing temperature (°C)
1	PKBT2	(AC)8-TT	ISSR	51
2	PKBT3	(AG)8-T	ISSR	51
3	PKBT 7	(GA)9-A	ISSR	51
4	PKBT10	(GT)9-A	ISSR	54
5	PKBT11	(GT)9-C	ISSR	54
6	SB13	AGTCAGCCAC	RAPD	36
7	SB19	CAGCACCCAC	RAPD	36
8	OPH12	ACGCGCATGT	RAPD	36
9	OPH13	CACGCCACAC	RAPD	36
10	OPH18	GAATCGGCCA	RAPD	36
11	P1	GGTGCGGGAA	RAPD	36
12	P3	GTAGACCCGT	RAPD	36
13	P5	AACGCGCAAC	RAPD	36

Molecular analysis

Genetic diversity and the relationship among accessions were calculated using Jaccard's coefficient and a sequential, agglomerative, hierarchical, and nested (SAHN) cluster analysis. This analysis was performed using the un-weighted pair group method with arithmetic means (UPGMA) algorithm computed by NTSYS-pc (Numerical Taxonomy and Multivariate Analysis) software version 2.1 (Rohlf, 2000).

RESULTS

Morphological and gamboge disorder observations

Eleven morphological characters and one physiological trait (response to gamboge disorder) were investigated in this study. Based on those characters the mangosteen in Tembilahan area could be divided into two types. The first type was characterized by ellipsoid fruits, short pedicel, small petals, ellipsoid stigma lobe, 4 to 11 fruit segments, yellow sepal with slightly red margin, not juicy and firmly flesh, smaller leaves and twigs than the other one, and free or very low of gamboge disorder incidence (0 to 1%). The second type was characterized by round fruits, medium pedicel, large petals, round stigma lobe, 4 to 8 fruit segments, red colour in the margin and yellow color in the center of sepal, juicy and soft flesh, bigger leaves and twigs than the other one, and contained gamboge disorder (low to high gamboge disorder incidence).

The first type was present at fourteen or 70% samples used (individual number 1, 2, 3, 5, 7, 8, 9, 10, 13, 19, 21, 34, 35, and 36). This type resolved as ancestral for the Tembilahan type and called as non-gamboge disorder (non-GD). The second type consisted of seven samples (individual number 14, 22, 24, 25, 26, 28, 29) or approximately 30% of mangosteen samples in this area. This type belongs to non-Tembilahan type or common mangosteen as gamboge disorder (GD) genotypes. The morphology of the two mangosteen types was described in Table 2, and Figures 2 and 3.

Polymorphism of individual mangosteen revealed by the molecular markers

The ISSR and RAPD analysis generated 113 scorable DNA fragments of which 63 (55%) polymorphic and 50 (45%) monomorphic bands. The amplified products ranged from 250 to 2400 bp in length. ISSR primers show higher polymorphism and number of DNA fragments than RAPD primers. The number of polymorphic bands and number of DNA fragments per primer were 64% and 11.8 for ISSR, respectively and 46% and 6.8 for RAPD, respectively (Table 3.).

Table 2. Morphological and gamboge disorder characteristics of the two types of mangosteen in Tembilahan region.

No.	Morphological Characters	Mangosteen types	
		Type 1 (non-GD)	Type 2 (GD)
1	Fruit shape	Elliptic	Round
2	Pedicel length	Short (less than 1 cm)	Medium to long (1 to 3 cm)
3	Stigma lobe shape	Elliptic	Round
4	Number of fruit segments	Four to eleven	Four to eight
5	Sepal color	Yellow with slightly red margin	Red colour in the margin and yellow colour in the center
6	Petal size	Small (about 2 cm x 3 cm)	Large (about 3 cm x 6 cm)
7	Petal thickness	Thin (less than 1 mm)	Thick (about 1 to 1,2 mm)
8	Water content	Not juicy (78 to 80%)	Juicy (more than 80%)
9	Flesh texture	Firm	Soft
10	Leaf size	Small (20 to 25 cm in ength, 12 to 14 cm in width)	Large (28 to 30 cm in length, 14 to 16 cm in width)
11	Twig size	Small (0.5 cm in diameter)	Large (0.7 cm in diameter)
12	Gamboge disorder	Absent (very low, 0 to 1 %)	Present (low, 2-20% to high , more than 30 %)

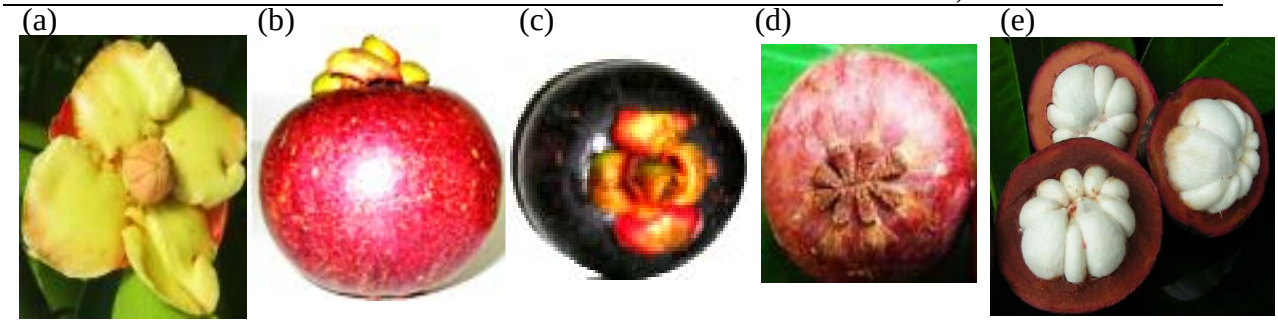

Figure 2. Fruit morphology characteristics of Tembilahan or non-gamboge (non-GD) type: (a) yellow sepal with slightly red margin; (b) elliptical fruit with short pedicel; (c) small petal, (d) elliptical stigma lobe; (e) 8 to 11 fruit segments and free from gamboge disorder in flesh.

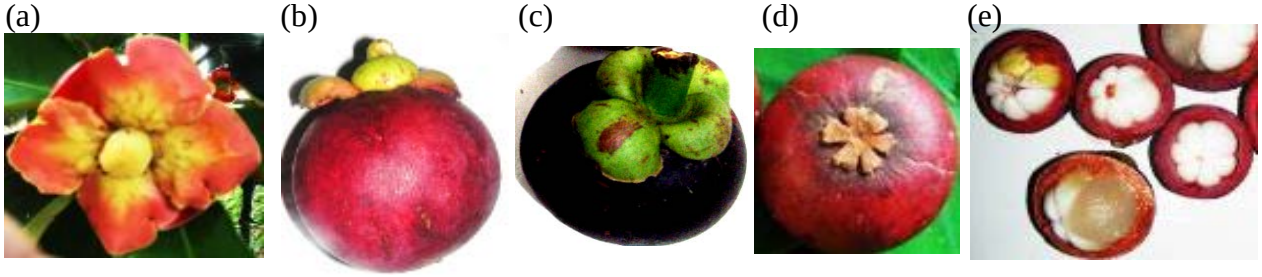

Figure 3. Fruit morphology characteristics of non-Tembilahan or gamboge (GD) type: (a) the sepal with red margin and yellow center; (b). round fruit with medium pedicel; (c) large petal; (d) round stigma lobe; (e) 4 to 8 fruit segments and some fruit flesh contaminated with gamboge disorder and translucent fruit disorder.

Table 3. Polymorphism data from RAPD and ISSR markers.

Marker type	Band total	Monomorphic bands	Polymorphic bands	Average of DNA fragments per marker
RAPD	54	29 (54%)	25 (46%)	6.8
ISSR	59	21 (36%)	38 (64%)	11.8
Total	113	50 (45%)	63 (55%)	9.3

ISSR and RAPD analysis of Tembilahan population produced some common and specific patterns. Parts of PCR analysis results by ISSR primer PKBT2 and RAPD primer OPH12, OPH13, and OPH18 were showed in Figure 4. The presence of specific marker bands of PKBT2-350 bp, OPH12-1600 bp, OPH13-2400 bp and 950 bp, and OPH18-1800 bp, 950 bp and 850 bp in some individuals Tembilahan population is a fascinating phenomenon. The DNA fragments of PKBT2-350 bp, OPH12-1600 bp and OPH18-950 bp respectively were consistently existed in the individual numbers of 14, 22, 24, 25, 28, and 29. The DNA fragments OPH13-2400 bp were shown in the individual numbers of 19, 21, 22, 25, and 28. These specific DNA fragments needed further analysis by sequencing to determine the DNA sequence and predict the traits associated to the markers. The other primers showed random variation of DNA banding patterns among the individuals in the population of Tembilahan mangosteen.

Cluster analysis

Cluster analysis was performed for 21 of 31 mangosteen samples used, which were successfully fingerprinted using all primers. Based on dendrogram in Figure 5 the mangosteen from Tembilahan area was divided into two groups at similarity coefficient of 0.84. The similarity coefficients of the accessions ranging from 0.84 to 0.99. Group I consisted of 14 accessions of Tembilahan or non-GD type and group II consisted of 7 non-Tembilahan accessions or GD types. The two groups were different in morphology, genetically diverse and their response to gamboge disorder. The extent of relationship between gamboge disorder and the two mangosteen types has been explored primarily and providing evidence of different response of mangosteen trees to this physiological disorder. Diversity of DNA banding patterns in Figure 4 and cluster analysis in Figure 5 revealed that the bands of PKBT2-350 bp, OPH12-1600 bp, OPH13-2400 bp, and OPH18-950 associated with group II.

DISCUSSION

Morphological and gamboge disorder variation

Mangosteen is commonly known as an allotetraploid apomictic plant (Richards, 1990b; Matra *et al*., 2016). Morphological variation in apomictic plants is correlated with epigenetic rather than to genetic variation (Rois *et al.,* 2013). Epigenetic markers in plants are partially heritable (Verhoen *et al.*, 2010) and can be directly influenced by abiotic environments (Bossdof *et al.,* 2008; Zhang *et al*., 2013). Other factors that can cause morphological diversity are spontaneous mutations and epigenetic influences triggered by transposable elements in plants with high ploidy levels (Wegscheider, 2009). Retrotransposons produce various modifications depending on the place of insertion. If they insert into the coding regions, this may cause changes in gene expression and phenotypic variability (Bennetzen, 2000).

Mutations in single genes or many genes resulted in large or small phenotypic effects (Pritchard and Rienzo, 2010). The rapid genetic and epigenetic changes can affect all traits of plants such as flowering time, morphology of leaves and flowers. (Wendel and Doyle 2005). Mangosteen in Tembilahan has specific morphological characters and response to gamboge disorder. The main morphological differences were found in this study have pedicel length, petal size, stigma lobe size and shape, number of fruit segments, sepal colour, and the texture of flesh. The accessions with different phenotypic are potential for further selection. The results of this study strengthen the information of morphological diversity in mangosteen (Table 2, Figures 2 and 3).

Gamboge disorder incidence occurred at various levels from very low (0 to 1% or almost free from gamboge disorder) to high (more than 30%). The variation of gamboge disorder incidence may be caused by variation in the strength of cell walls and the environment and genetic interaction.

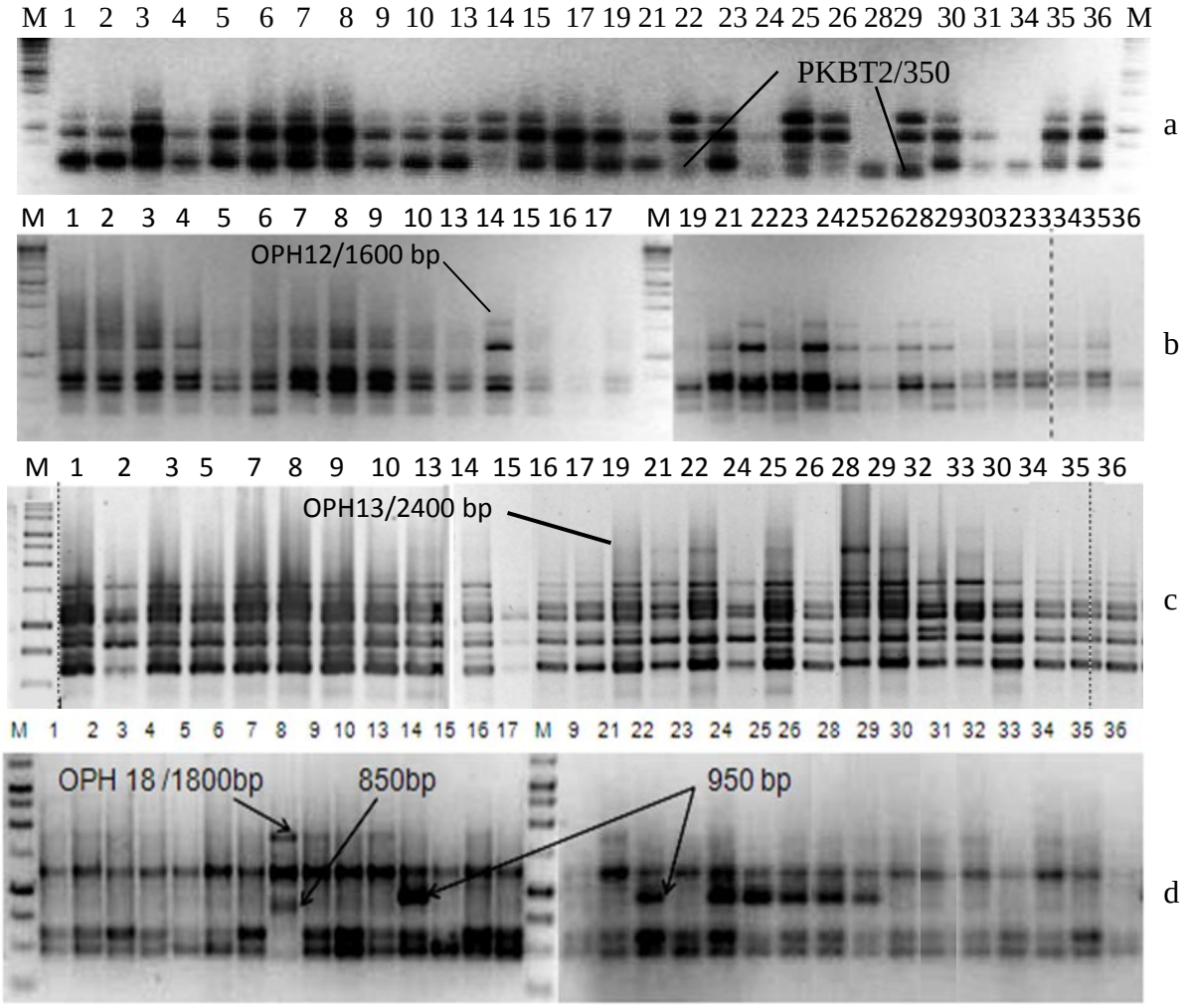

Figure 4. Agarose gel electrophoresis of mangosteen from Tembilahan population amplified by the primers, PKBT2 (a), OPH12 (b), OPH13 (c) andOPH18 (d). The arrows are show specific bands for non-Tembilahan (GD) type.

The mechanisms that regulate the mechanical strength and cell wall biosynthesis is very complex and requires coordination of a number of metabolic pathways involving genes (Li *et al*., 2003).

Molecular variation

The results indicated that ISSR and RAPD markers can be used to identify genetic variation in mangosteen. The percentage of polymorphic bands and average number of amplification products of ISSR markers were higher (64% and 11.8 bands) than RAPD markers (46% and 6.75 bands) (Table 3). This result is similar to the study in the apomixis plant, *Psammochloa villosa* (Poaceae). The ISSR markers resulted in about 70.52% of polymorphic bands (Li and Ge, 2001). The higher polymorphysm of ISSR markers was also observed on genotype identification and genetic diversity among barley cultivars by Fernandez *et al.* (2002) and among wild barley (*Hordeum vulgare* subsp. spontaneum) populations from West Turkey (Tanyolac, 2003).

RAPD and ISSR marker applications were also useful for the genetic diversity studies in *Vigna umbellata*, and identification of variation within landraces (Saraladevi *et al*., 2008). These two marker systems were found to be useful for genetic diversity studies in pistachio and identification of variation within *Pistacia vera* cultivars (Tagizad *et al*., 2010). In this study, 21 mangosteen samples used were

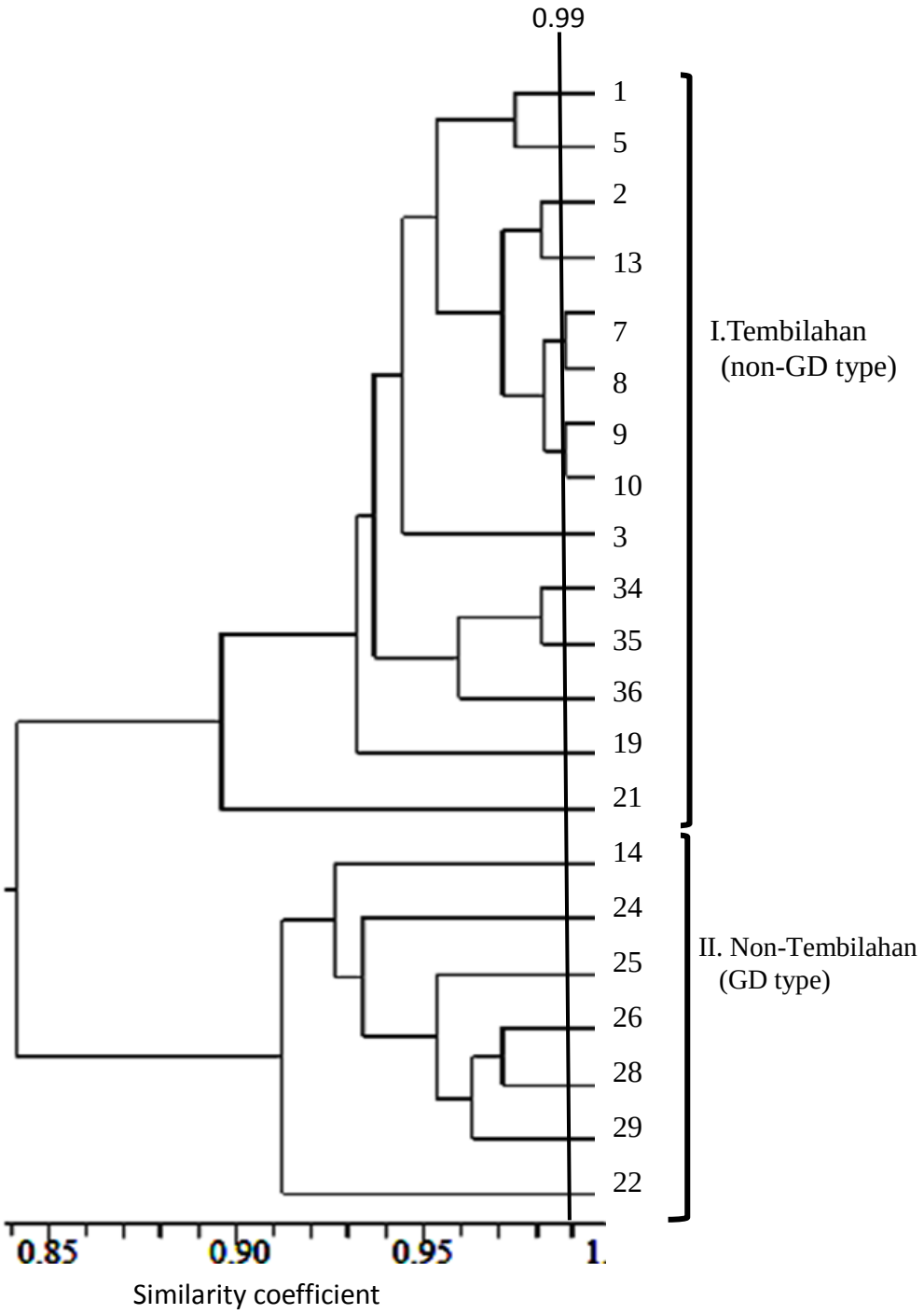

Figure 5. Dendrogram of 21 mangosteen trees from Tembilahan based on 5 ISSR and 8 RAPD primers.

from an entirely different genetic background. Although the total number of polymorphic bands detected by eight RAPD primers was lower than that of the five ISSR primers, these results suggested that the RAPD markers were more reliable than ISSR markers in discriminating genetic variability of the mangosteen. In this case RAPD primers such as OPH12, OPH13 and OPH18 gave specific bands for certain genotypes which were clearly associated with non-Tembilahan genotypes and separating the accessions into two groups. While most of the ISSR primers produced non-specific or random polymorphic bands. The use of RAPD analysis for variability studies on apomixis plants has been done in cassava. The results indicated facultative apomixis at low frequency in cassava (Nassar *et al*., 1998). It has also been used in the apomictic plant *Hypericum perforatum*, and allows for identification of individual differences (Pilepic *et al.*, 2008).

Genetic relationships of the 21 samples used in this study indicated that the mangosteen population in Tembilahan have high genetic similarity: from 0.84 to 0.99 (Figure 5). The high similarity reflected that the mangosteen plant have narrow genetic variation as an apomictic plant. The low genotypic variation could help asexual plants to adapt to various environmental conditions, new microhabitats, and abiotic and biotic conditions. These conditions seem to be related to epigenetic variation (De-Walt and Hamrick, 2004; Poulin *et al*., 2005; Hardesty *et al.*, 2012; Roiloa *et al.*, 2014).

These studies showed variation in DNA banding patterns between Tembilahan and non-Tembilahan types, as well as the presence and absence of gamboge disorder in the two types. Generally, the first type is dominant in the Tembilahan area and rarely found in other areas. The findings suggested that differences in the morphology and molecular are associated with differences between non-GD and GD genotypes. Banding patterns data of Tembilahan (non-GD) and non Tembilahan (GD) types generate candidate markers and need to be further analysis via sequence analysis. The markers identified based on these results are PKBT2-350 bp, OPH12-1600 bp, OPH13-2400 bp, and OPH18-950 bp. This markers can enrich the preliminary results about the association between molecular markers specific to cell wall strength in mangosteen, MCWS 1000 and 1200 bp as new approach for detecting GD and non GD genotypes of mangosteen (Mansyah *et al.*, 2014) .

Genetically 21 mangosteen samples from Tembilahan population were entirely different from each other. Genetic variation observed in apomictic tetraploid mangosteen can be caused by the importance of gene and genome duplications (Wendell, 2000). The study of allelic diversity using microsatellite markers found that mangosteen had more than two alleles per locus as evidence of tetraploidy in mangosteen (Matra *et al.*, 2016). Many polyploids are formed from unstable genome and undergo rapid repatterning (Adams and Wendell, 2005; Otto, 2007). The level of molecular changes associated with phenotypic evolution (Papp and Erzberger, 2011). Understanding the molecular mechanisms of phenotypic change has been a long-standing goal in evolutionary biology (Jacob, 1977). Thus not only genotypic but also epigenetic variations are factors enhancing genotype plasticity and adaptation (Verhoeven and Preite, 2013; Douhovnikoff and Dodd, 2015).

In many cases, polyploid plants have broad variation due to formation from different genome constitution. Cytology studies showed that the mangosteen may be an allotetraploid derivative of the two close related species *G. hombroniana* and *G. malaccensis* (Richards, 1990b). The two different sources of nuclear genomes may lead to genome reorganization and influence genetic instability in polyploids (Wendel, 2000; Liu and Wendel, 2002). These results can be used as a model for the study of morphological and physiological features in mangosteen. Additionally, because mangosteen is an apomictic plant it is possible to investigate the evolution process through studying variation of fruit types. Further studies will provide a level of phylogenetic investigation of mangosteen that includes both DNA sequence data and morphological characters.

The existence of different mangosteen genotypes in the swampy area of Tembilahan indicated that the mixture of native plants and introduced plants from other regions has

occurred. Although mangosteen is unlikely to disperse for long-distances naturally, the spread most probably occurred by migration. The first group of mangosteen accessions with unique of morphological and molecular patterns was probably introduced by the South Kalimantan community, which is indicated by the diversity of ethnicity and the languages used by people living in this area. Most of the people living in Tembilahan are a community of Banjar, the swampy area of South Kalimantan. The Banjar community had migrated to Tembilahan area at the end of 18th century (Hasanzainuddin, 2007). In the South Kalimantan, we also found this mangosteen type.

CONCLUSION

There are two types of mangosteen in the swampy region of Tembilahan based on morphology, molecular and response to gamboge disorder. The first type originated from Tembilahan which is characterized by the number of fruit segments (4 to 11), its firm flesh, and the most importantly no gamboge disorder in its flesh (non GD genotypes). The second type is the common mangosteen which is generally found in other areas and sometimes contained gamboge disorder (GD genotype).

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