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Comparison Analysis of Genetic Diversity of Indonesian Mangosteens (*Garcinia mangostana* L.) and Related Species by Means Isozymes and AFLP Markers

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ABSTRACT

Mangosteen *(Garcinia mangostana*) belongs to a large genus of *Garcinia* that native in South East Asia, as well as Indonesia, and in order evaluate genetics diversity of mangosteen and their close relatives, we employed isoenzyme and AFLP marker on 13 accessions of mangosteen and their close relatives. Isoenzyme marker using four enzyme systems produced 25 bands and 88% out of them were polymorphic and elucidate genetic variability at similarity level ranged between 0.38-0.89. AFLP markers with three primer system produced 220 polymorphic bands and revealed genetic variability at similarity level ranged between 0.38-0.89 successfully produced high polymorphism bands and elucidates genetic variability at similarity coefficient ranged between 0.21-0.77. Both markers exhibited similar clustering pattern, and group successfully *G. mangostana* accessions in one clustering group. Furthermore *G. malaccensis* and *G. porrecta* consistently showed closer genetic relationship to *G. mangostana* clustering group in both markers, in comparison to *G. hombroniana,* which implies the assumption they may be the progenitor of *G. mangostana*, and should be reviewed with more accurate data.

Key words: genetic diversity, mangosteen, isozymes, AFLP.

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) belongs to family *Guttiferae,* genus *Garcinia* (Verheij, 1991). *Garcinia* is a large genus that consists of about 400 species, and originated from East India, Malay Peninsula and South East Asia, as well as Indonesia (Campbell 1966). Based on morphological and cytological studies, Yaacob and Tindall (1995) suggested that mangosteen originated from South East Asia; subsequently Almeyda and Martin (1976) proposed that mangosteen is an inhabitant Indonesian fruit.

Some species of *Garcinia,* including *G. mangostana* produce fruit without pollination, the phenomenon is referred to as agamospermy, which is the production of seed without fusion of gametes (Koltunow et al., 1995; Thomas 1997). The process of embryo formation in *G. mangostana* was first studied by Treub (1911) who reported that the early development of woodiness in the endocarp soon after anthesis made the observation of embryo development difficult (Tixier, 1955). However, Lan (1989) provided a detailed account of mangosteen embryology and reported that the embryo of *G. mangostana* is derived from tissue of integument instead of from the egg.

An understanding of genetic diversity and its phylogeny among cultivated plant accession significantly influence on the quality increase and the results, and it also improves the management of germplasm conservation (Roldan-Ruiz et al., 2001). Plant genetic improvement highly depends on the available genetic resources. Wide genetic diversity will give higher opportunity in the selection process of the best characters. Some research on the genetic diversity using some markers could explain the phylogeny within and among population (Fajardo et al., 2002; Hurtado et al., 2002; McGregor et al., 2002).

Genetic variability analysis can be done by using many manner of markers, such as morphology (Talhinhas et al., 2006), isoenzymes (Ayana et al*.,* 2001), and molecular markers (Assefa et al*.,* 2003; Cavagnaro et al*.,* 2006), such as AFLP marker (Vos

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et al., 1995). Recently, due to burgeoning in biotechnological technique, the molecular markers have been widely used to elucidate genetic information in the molecular level (Roy et al*.,* 2006).

Each marker system has the advantages and disadvantages, so that the assessment of the markers system is an important step to decide the most suitable marker regarding to research purpose. The comparison of several markers has been done with comparative study of some molecular markers with PCR base such as Palombi and Damiano (2002) which compared RAPD and SSR markers to detect genetic variability of kiwi plant, Ferdinandez and Coulman (2002), compared the efficiency of RAPD, SSR, and AFLP to identify plant genotypes. Saker et al. (2005), has used different markers to characterize the barley.

The study is aimed to distinguish the advantages of isoenzyme and AFLP markers in elucidating genetic variability and phylogenetic relationships among the mangosteen (*Garcinia mangostana* L.) and the close relatives, and to study the suitable molecular to develop specific molecular markers in characterization of mangosteen and its close relatives.

MATERIALS AND METHODS

Plant material

This research was conducted in the laboratory of Biotechnology and Tree Breeding BIOTROP Bogor, Molecular Laboratory and Plant Biology the Research Center for Biological Resources and Biotechnology IPB Bogor, and Laboratory of Tropical Fruit Research Center IPB Bogor. Thirteen (13) leaf samples of mangosteen and its close relatives were collected from several locations in Indonesia, namely: Pandeglang (Banten), Sukabumi, Purwakarta (West Java), Ponorogo (East Java), Lampung Regency, Palangkaraya (Central Kalimantan), Kendari (South East Sulawesi), Ambon (Maluku), *G. rigida, G. hombroniana,* and *G. celebica* (Bogor Botanical Gardens), and *G. malaccensis, G. porrecta,* and *G. benthami* (Mekarsari Tourism Park Bogor).

Isoenzymes analysis

Thirteen fresh samples were taken for isozyme analysis following Soltis and Soltis (1989). The enzymes analyzed are peroxidase (PER), phosphatase acid (ACP), malic dehydrogenase (MDH), and esterase (EST). The separation of isoenzyme bands was done with electrophoresis by using agarose gel with concentration of 10% for 4 hours, and 100 volt.

AFLP analysis

Extraction and DNA purification

The DNA of Leaf samples were extracted for AFLP analysis the same as for isoenzyme analysis. DNA extraction followed CTAB (Doyle and Doyle, 1987) with some modifications. DNA concentration was tested with electrophoresis and immigrated with standard DNA (DNA lambda) 10 and 100 ng/mL on agarose gel 1.2%.

Restriction-ligation

Approximately 0.5 µg genomic DNA was cut 1 unit *Mse*I and 5 unit *Eco*RI. At the same time it is ligated with 5 pmol *Eco*RI and 50 pmol *Mse*I adaptor with 1 U T4 DNA ligase. The adaptor sequence *Eco*RI is 5´-CTCGTAGACTGCGTACC-3´, 3´-CTGACGCATGGTTAA-5´ and the adaptor sequence *Mse*I is 5´-GACGATGAGTCCTGAG-3´, 3´-TACTCAGGACTCAT-5´.

Preselective amplification

Primers for preselective amplification are *Eco*RI+A and *Mse*I+C as homologous adaptor *Eco*RI and *Mse*I, each with one additional nucleotide at 3' end. PCR reactions were carried out in reaction mix containing of 4 µl restriction-ligation DNA, 2.5 pmol primer *Eco*RI +A and 2.5 pmol *Mse*I primer +C, 0.4 U *Taq* polymerase DNA, 0.2 mM each dNTP and 1x buffer PCR 20 µL. The PCR amplification was programmed for 20 cycles at 94° (1 second), 56°C (30 seconds), and 72°C (2 minutes). The PCR products 10 uL was tested w on 1.5% agarose gel. The amplified fragments range from 100-1500 bp.

Selective amplification

The selective amplifications were conducted by using primer *Eco*R1+ ANN and *Mse*1+CNN. The PCR reaction was performed using DNA pre-amplification 3 µL, 1 pmol primer *Eco*RI + ANN, 5 pmol primer *Mse*I + CNN without labeling, 0.4 U Taq polymerase DNA, 0.2 mM each dNTP and 1 x buffer PCR with a total volume of 20 µl. PCR reaction was programmed with 1 cycle for 30 seconds at 94°C, 30 seconds 65°C, 2 minutes at 72°C, followed by eight cycles of variable annealing temperature with a decrease of 1°C each cycle, and terminated with 23 cycles of 1 second at 94°C, 30 seconds at 56°C, 2 minutes at 72°C.

PAGE electrophoresis

The selective amplification products were displayed using PAGE electrophoresis, and presented as a diagram. Approximately 2 µL PCR product mixed with 0.15 µL 6-carboxy-Xrhodamin (ROX)-labeled internal standard length GeneScan-500 ROX and *dye* 0.85 µL formamide, denaturized for 3 minutes at 90°C and cooled in ice. Electrophoresis using 5% gel denaturing polyacrylamide (Long RangerTM, FMC Bioproducts) in buffer electrophoresis 1x TBE by using ABI PrismTM 377 DNA sequencer (Applied Biosystems) at 2500 V for 4 hours. The raw data was obtained using ABI PRISMTM V.1.1 software. Next, the AFLP fragments were analyzed with GENESCANTM version 2.1 (Applied Biosystems).

Data analysis

The bands of the isozyme technique and AFLP were translated into the binary data. These data were used to arrange the genetic similarity matrix based on the formula of Nei and Li (1979) with UPGMA (*Unweighted Pair-Group Method Arithmetic*) method using NTSYS (*Numerical Taxonomy and Multivariate System*) version 2.02 (Rolf, 1998). Genetic similarity between all pairs of accessions was calculated according to Nei and Li (1979).

RESULTS AND DISCUSSION

Variability analysis with isozyme marker

Isozymes analysis on 13 accessions of mangosteen and their close relatives showed that the four isoenzyme systems of esterase (EST), peroxidase (PER), acid phosphatase (ACP), and malic dehydrogenase (MDH) produced 25 bands and 22 bands (88%) out of them were polymorphic band (Table 1).

Table 1. The number of bands and polymorphism level of 5 isoenzyme on 13 accessions of mangosteen and their close relatives.

Isoenzymes	Band number	Polymorphic bands	Monomorphic Band
EST-1	4	4 (100%)	0
EST-2	3	3 (100%)	0
EST-3	3	3 (100%)	0
PER-1	2	2 (100%)	0
PER-2	3	3 (100%)	0
PER-3	1	0 (0%)	1
ACP-1	1	1 (100%)	0
ACP-2	3	2 (66,7%)	1
MDH-1	1	0 (0%)	1
MDH-2	4	4 (100%)	0
	25	22 (88%)	3

Cluster analysis based on isoenzyme assay revealed, that genetics distance among 13 accessions of mangosteen and their close relatives ranged between 0.38-0.89 of similarity coefficient (Figure 1). The similarity matrix correlation value MxComp r = 0.902 indicated that the dendrogram produced with *goodness of fit* highly compatible which depict the cluster (Rolf, 1998). Presentation accumulation of the three main first components on the 13 accessions of mangosteen and its relatives represent 63,5% genetic diversity that explained by 25 isozyme characters, and 70% genetic diversity was obtained from accumulation of four main components.

Subsequently, isozyme analysis showed that mangosteen accessions and *G malaccensis* are clustered at 0.68 of similarity coefficient (32%) separated to other close relatives (Figure 1). The genetic diversity resulted from similarity analysis was relatively high for the obligate apomictic compared to *Taraxacum* (19%) (Ford and Richards, 1985). Variation in apomictic plants occurred faster in mutation (Hughes and Richards, 1985). This results indicated that isozyme analysis successfully grouped mangosteen out of their close relatives, and *G malaccensis* closer to mangosteen than other close relatives. However, further analysis showed that *G. porrecta* has closer genetic relationship to *G. mangostana* clustering group at 0.61 of similarity coefficient, compare to *G. hombroniana* which is assumed as another progenitor of mangosteen (Richards, 1990), indicated that isozyme assay not yet confirmed *G. hombroniana* as G *mangostana* progenitor.

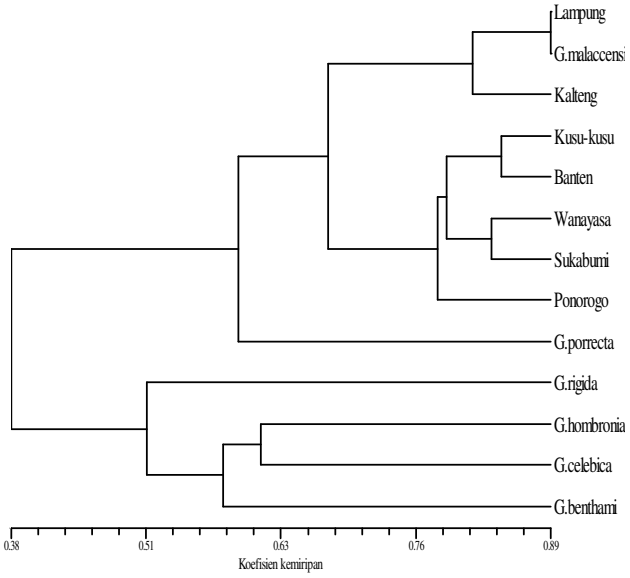

Figure 1. Dendogram of 13 accessions based on isozyme marker.

Variability analysis with AFLP

AFLP analysis on 13 accessions of mangosteen and their close relatives using three primer combinations of ACC_CAG, ACT_CAA and ACT_CAC produced 220 polymorphic bands at band size ranged between 50-500 bp. The number of bands resulted from each primer combination varied between 19-94 bands or at average 73.3 bands for each primer combination. The primer combination of ACT_CAA produced the highest number of polymorphic (94 bands) followed by primer combination of ACT_CAA 70 bands and primer ACC_CAG 56 bands (Table 2).

Cluster analysis results based on AFLP markers, showed that genetics distance among 13 accessions of mangosteen and their close relatives ranged at between 0.21-0.77 (Figure 2). Based on the AFLP dendrogram, this hypothesis can be accepted. With value r = 0.977, meaning that the dendrogram resulted with *goodness of fit* very suitable to depict the grouping. Principle component analysis indicated that the three main first components represented 47.2% genetic diversity, and 70% genetic diversity of 612 characters was obtained from accumulation of six main components.

Table 2. The number of bands and polymorphism of 3 pairs of primer AFLP on 13 accessions of mangosteen and close relatives.

Primer AFLP	Band number	Polymorphic bands
ACC_CAG	94	100%
ACT_CAA	70	100%
ACT_CAC	56	100%
Total	220	100%

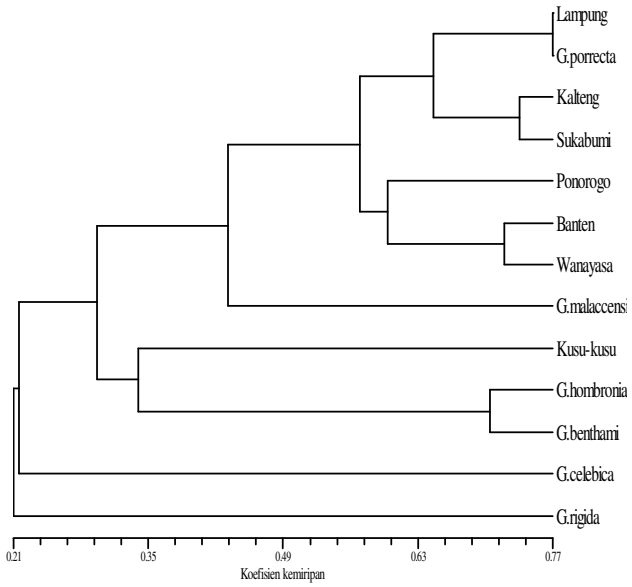

Figure 2. Dendogram of 13 accessions based on AFLP marker.

Further analysis on dendrogram constructed from AFLP marker indicated that mangosteen accessions clustered in one group with *G. porrecta*, separated with other close relatives at similarity coefficient of 0.58. Subsequently, AFLP marker results confirmed that among evaluated close relatives of mangosteen *G. malaccensis* and *G. porrecta* consistently closer to mangosteen accessions clustering group compare to other close relatives.

Discussions

Since AFLP markers produced higher polymorphic characters (220 bands) compare to those of resulted by isozyme marker (22 polymorphic bands), AFLP marker revealed higher genetic diversity 79% compare to 62% that explained by isozyme marker. Cophenetic correlation value of both markers as high as 90% showed that the dendrogram generated from both markers have equal clustering pattern descended from the symqual matrix. The highest cophenetic correlation resulted by AFLP marker was 0.978. This value showed correlation between grouping and similarity matrix was fit, and gave best value to construct the grouping and arrangement similarity matrices (Table 3). However, grouping pattern in isozyme marker was slightly different to those of AFLP marker, in terms of the number of groups, since isoenzymes generated four clustering groups compared to AFLP marker that generated six clustering groups (Table 3).

The occurrence of genetic variability between and within individuals, within population and between cultivars in cultivated species occurred by mutation, introgression, recombination, adaptation to new environment, and selection which occurs continually (Geleta et al*.,* 2007). Genetic diversity within cultivated and wild plants is important to prevent some problems associated with cultivation failure. Cultivated plants can be improved by introduction of wild relatives especially in the center of distribution, such as the mangosteen which is distributed in Indonesia and Malay Peninsula (Harlan and de Wet, 1971; Hawkes, 1977).

High genetic diversity as represented by polymorphic band percentage is not common for mangosteen as an apomictic obligate, this might due to several factors as accumulation of natural mutation, repeated hybridization among mangosteen progenitors Carman (2001), and ploidy developmental processes. High variation among mangosteen genotype is a genetic potential to obtain high potential genotypes for specific purpose, which could be done through selection approach among superior trees in the field (Sobir and Poerwanto, 2007).

Since *G. malaccensis* consistently showed closer genetic relationship with *G. mangostana* clustering group in isozyme and AFLP markers, we conducted bands similarity proportion analysis that contributed by *G. malaccensis, G. porrecta* and *G. hombroniana* which were estimated as mangosteen progenitor against the mangosteen based AFLP markers. *G. malaccensis* shared 53% similar band with *G.*

Table 3. Similarity coefficient value, cophenetic correlation, mangosteen group and close their relatives with isoenzyme and AFLP markers in similarity 58%.

Isoenzim				AFLP			
Similarity coefficient	**Value**	**Group**	**Accession**	**Similarity coefficient**	**Value**	**Group**	**Accession**
Polymorphism (%)	88%	I	M, GM, GP	Polymorphism (%)	100%	I	M, GP
Highest value (%)	0.889	II	GR	Highest value (%)	0.773	II	GM
(Accessions)	GM vs. L	III	GH & GC	(Accessions)	GP vs. L	III	MK
Lowest value (%)	0.2	IV	GB	Lowest value (%)	0.169	IV	GH & GB
(Accessions)	GB vs. W			(Accessions)	GR vs. S	V	GC
Cophenetic correlation (r)	0.902			Cophenetic correlation (r)	0.978	VI	GR

Notes: M = mangosteen (*G. mangostana*), GM = *G. malaccensis,* L = Lampung mangosteen*,* GB = *G. benthami,* W= Wanayasa mangosteen, GP = *G. porrecta*, GR = *G. rigida,* GH = *G. hombroniana,* and S = Sukabumi mangosteen.

G. porrecta shared 61.5 % similar band with *G. mangostana,* while *G. hombroniana* shared 50% similar band with *G. mangostana.* Moreover, if *G. malaccensis* and *G. hombroniana* simulated as progenitor of *G. mangostana*, 33% of G mangostana bands could not explained by G. *malaccensis* and *G. hombroniana,* while if *G. malaccensis* and *G. porrecta* simulated as progenitor of *G. mangostana*, 29 % of *G. mangostana* bands could not explained by G. *malaccensis* and *G. porrecta.*

These result of above indicated that the proposal of G. *malaccensis* and *G. hombroniana* were progenitor *of* G. *mangostana* should be reviewed carefully with more accurate evidences, since fruit morphology of *G. mangostana* to fruit morphology of *G. porrecta*, compare to those of *G. hombroniana* fruit characters (Sobir et al., 2009, unpublished data).

CONCLUSION

Isoenzyme assay employed four enzyme systems and three primer combinations of AFLP marker on 13 accessions of mangosteen and their close relatives successfully produced high polymorphism band and elucidate genetic variability at similarity coefficient of 0.38 and 0.21 respectively. Both markers exhibited similar clustering pattern, and grouping G mangostana accessions in a clustering group. *G. malaccensis* and *G. porrecta* consistently in both markers showed closer genetic relationship to *G. mangostana* clustering group compare to *G. hombroniana* that implies the assumption of progenitor of *G. mangostana*, should be reviewed with more accurate data.

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