PHYLOGENETIC RELATIONSHIPS AMONG SEVERAL SALACCA SPECIES (*SALACCA* SPP.) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Sri Hadiati*, Sukartini, Riry Prihartini, & Ellina Mansyah

Indonesia Tropical Fruits Research Institute, Jl. Raya Solok-Aripan Km.8 Po.box 5 Solok West Sumatra 27301

*Corresponding author: shadiati@yahoo.com

ABSTRACT

Of 23 salacca species found in the world, 15 species are found in Indonesia. The high salacca diversity is caused by open pollination systems, frequent usage of seeds as planting material, and breeding practices. This research which was conducted at the Molecular Laboratory of the Indonesian Tropical Fruit Research Institute, aimed to determine the level of primer polymorphism and phylogenetic relationships among salacca species. Five species of salacca (10 accessions) and 9 progenies from their crossing were used as samples. Eleven Rapid Amplified Polymorphism DNA markers were utilized for molecular analysis. All primers were given ranges of 57.1-100% polymorphic level. The range of genetic similarity coefficients were 0.512 - 0.937. Observed accessions could be separated into four groups. The highest genetic similarity coefficient was determined between PH-K and PH-MJ accessions (0.937), meanwhile the lowest value was indicated on MW-AFN hybrid and *S. wallichiana* (0.512). For ensuring the efficiency in germplasm management, only accessions which showed high genetic similarities are chosen as representation of the group while eliminating others. On the contrary, accessions which showed low genetic similarity are used as crosses between parents to obtain wide genetic variability and high heterosis effects.

Keywords: *Salacca* spp., Phylogenetic relationship, RAPD

1. INTRODUCTION

Salacca plants in the world are known to be very diverse. 15 out of the 23 species of salacca in the world are found in Indonesia (Zumaidar & Miftahuddin, 2018). Kalimantan has the largest diversity (11 species) of salacca (Ramella *et al.*, 2005). Natural open pollination environment, use of seeds as material for planting, and breeding programs contribute to the high diversity in salacca (Suskendriyati *et al.*, 2000). Inter and intra-species diversity are genetic sources for germplasm collections and producing new superior varieties. Phenotypic and genotypic variabilities and phylogenetic relationship information are needed to determine the parental crossing. Progenies with high heterosis can be obtained from parents with a wide genetic distance to a certain extent (Bruel, 2006; Nandariyah, 2010).

Genetic diversity for breeding programs can be obtained through the use of molecular markers. Random amplified polymorphic DNA (RAPD) is one way to know genetic diversity based on Polymerase chain reaction (PCR) (Rauf *et al.*, 2010). RAPD techniques have been used to study genetic diversity, genetic distance and species identification of salacca (Nandariyah *et al.*, 2004; Nandariyah, 2010; Budiyanti *et al.*, 2015; Elly *et al.*, 2018). However, the number of primers used in past study is still limited (3-6 primers). The number of primers determine the pattern of DNA bands.

Varied and many DNA bands will increase the accuracy of phylogenetic analysis (Das *et al.*, 2009). Polymorphisms resulting from RAPD amplification are random (Kumar & Gurusubramanian, 2011) and more suitable for the purpose of characterization and determination of genetic diversity (Das *et al.*, 2009). Genetic diversity is useful as a reference in breeding programs (Kelly & Miklas, 1998). The reference can be used before crossing to assemble new varieties, or to evaluate the results of crossing. One such reference is genetic information obtained from phylogenetic analysis (Theanphong *et al.*, 2016). The purpose of this study was to determine the level of primary polymorphism used and grouping between species and varieties of salacca (phylogenetic) based on RAPD analysis.

2. MATERIAL AND METHODS

2.1. Material

The sample for this research consisted of 5 species (10 accessions) and 9 progenies (Table 1). The morphological characters of the accessions can be seen in Appendix 1.

Table 1. List of salacca accessions used as research materials

No.	Accession	Individual plant codes	Species
1.	Sidempuan Merah	SDM (44-3)	Salacca sumatrana
2.	Sidempuan Putih	SDP (31-26)	Salacca sumatrana
3.	Pondoh	PH	Salacca zalacca var. zalacca
4.	Sanjung	SJG (18-11)	Salacca zalacca var. zalacca
5.	Mawar	MWR (27-18)	Salacca zalacca
6.	Gading Bali	GB	Salacca zalacca var. amboinensis
7.	Gula Pasir	GP (23-3)	Salacca zalacca var. amboinensis
8.	Affinis	AFN	Salacca affinis
9.	Wallichiana	WLC	Salacca wallichiana
10.	Glabrescens	GBC	Salacca glabrescens
11.	Sidempuan Merah x Sanjung	SDM x SJG (36-15)	S. sumatrana x S. zalacca
12.	Pondoh x Sanjung	PH x SJG (41-7)	S. zalacca x S. zalacca
13.	Pondoh x “M”	PH x “M” (49-19)	S. zalacca x S. zalacca
14.	Pondoh x “K”	PH x “K” (48-21)	S. zalacca x S. zalacca
15.	Pondoh x “MJ”	PH x “MJ” (17-4)	S. zalacca x S. zalacca
16.	Pondoh x Mawar	PH x MWR (6-28)	S. zalacca x S. zalacca
17.	Mawar x S. affinis	MWR x AFN (12-4)	S. zalacca x S. affinis
18.	Mawar x Sidempuan Putih	MWR x SDP (35-13)	S. zalacca x S. sumatrana
19.	Bali x Pondoh (Sari Intan 541)	B x PH	S. zalacca var. aboinensis x S. zalacca var. zalacca

2.2. Methods

Eleven primers namely AST2 L, AST2 R, AST9 L, AST9 R, AST12 R, OPA17, OPA 18, OPX 17, RAPD3, RAPD4, and RAPD6 were used for amplification in the RAPD technique. Genomic DNA was extracted using the CTAB method. Total volume (12,5 µl) for amplification consisted of 4,5 µl dH2O; 1,25 µl 10 pM primer; 6,25 µl Green go taq; and 0,5 µl 20 ng genomic DNA. PCR machines were set according to 45x cycles; preheating = 95 °C (2 min); denaturation = 95 °C (1 min); annealing = 36 °C (1 min); elongation = 72 °C (2 min); and final elongation = 72 °C (10 min). An amplification product (3 µl) was electrophoresed on 1.2% agarose gel with 1x SB buffer (50 volt; 60 min). Furthermore, the gel was immersed in a solution of ethidium bromide (40 µl 1%

ethidium bromide/litre dH2O) for 10 minutes and dH2O for 15 minutes. A UV transilluminator was used for viewing DNA band profile images. Binary data was obtained from scoring based on the presence or absence of the band. Scored binary data was used to calculate:

a. Polymorphism/primer: (∑ polymorphic bands/∑ total bands)x100%
b. Phylogenetic analyzed using *NTSYSpc* versi 2.1 and *UPGMA*.

3. RESULT

The amplification results showed the diversity of the number of polymorphic and monomorphic DNA bands (Table 2).

Table 2. Polymorphism levels of 11 primers based on the salacca DNA banding patterns

No.	Primer	Sequences 5' - 3'	Total bands	Polymorphic bands	Monomor-phic bands	Polymor-physm level (%)
1.	AST2 L	ATATGGTTGCAGAGCGGATG	5	4	1	80
2.	AST2 R	GCAAAACAGTGCTTGCTTCC	5	4	1	80
3.	AST9 L	ACATCGCAGGGGTCTTGA	13	10	3	76,9
4.	AST9 R	CAACCATTGTGGGGATGTG	8	6	2	75
5.	AST12 R	TGAATCCCATTCCTGTCAGC	7	4	3	57,1
6.	OPA17	GACCGCTTGT	8	8	0	100
7.	OPA 18	AGGTGACCGT	9	9	0	100
8.	OPX 17	GACACGGACC	7	5	2	71,4
9.	RAPD3	GTAGACCCT	4	3	1	75
10	RAPD4	AAGAGCCCGT	10	9	1	90
11	RAPD6	CCCGTCAGCA	7	6	1	85,7
	Total		83	68	15	

UPGMA cluster analysis showed that the 19 salacca accessions were divided into four major groups at 0.71 genetic similarity coefficient (Figure 1).

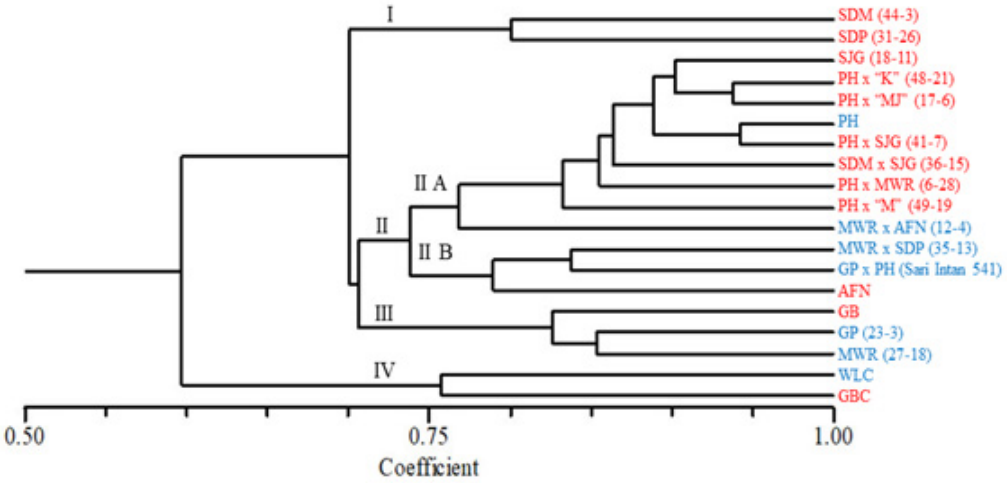

Fig. 1: Dendrogram of 19 salacca accessions based on UPGMA cluster analysis using 11 primers)

Notes : SDM='Sidempuan Merah', SDP='Sidempuan Putih', PH='Pondoh', SJG='Sanjung', MWR='Mawar', GB='Gading Bali', GP='Gula Pasir', AFN=*S. affinis*, WLC= *S. walliciana*, GBC= *S. glabrescents*. The name of the red accession=astringent taste, the name of the blue accession=non-astringent taste.

The genetic similarity of 19 salacca based on 11 RAPD primers ranged from 0.512 to 0.937. The greatest genetic similarity coefficient (0.937) was between the PHxK accession and the PHxMJ accession, while the smallest was between MWRxAFN accession and WLC (*S. wallichiana* (0.512)) (Table 3).

Table 3. Matrix of genetic similarities between species or accessions of salacca

Acc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1,000																		
2	0,800	1,000																	
3	0,725	0,682	1,000																
4	0,747	0,808	0,848	1,000															
5	0,681	0,687	0,848	0,855	1,000														
6	0,706	0,710	0,903	0,865	0,942	1,000													
7	0,723	0,703	0,835	0,804	0,824	0,813	1,000												
8	0,707	0,711	0,889	0,871	0,851	0,905	0,860	1,000											
9	0,674	0,681	0,915	0,876	0,895	0,929	0,845	0,937	1,000										
10	0,737	0,714	0,738	0,716	0,716	0,742	0,667	0,729	0,711	1,000									
11	0,630	0,667	0,691	0,652	0,674	0,698	0,619	0,699	0,690	0,805	1,000								
12	0,730	0,732	0,756	0,688	0,71	0,736	0,706	0,714	0,727	0,846	0,853	1,000							
13	0,683	0,667	0,828	0,816	0,857	0,891	0,844	0,876	0,86	0,699	0,675	0,741	1,000						
14	0,595	0,609	0,739	0,738	0,816	0,784	0,737	0,766	0,755	0,636	0,729	0,698	0,813	1,000					
15	0,732	0,684	0,785	0,711	0,689	0,738	0,756	0,790	0,776	0,693	0,722	0,767	0,718	0,747	1,000				
16	0,747	0,723	0,771	0,787	0,745	0,773	0,698	0,776	0,764	0,734	0,710	0,779	0,756	0,759	0,838	1,000			
17	0,720	0,699	0,699	0,723	0,660	0,705	0,698	0,776	0,719	0,683	0,715	0,675	0,732	0,667	0,784	0,795	1,000		
18	0,629	0,590	0,590	0,562	0,584	0,602	0,519	0,600	0,571	0,676	0,620	0,667	0,571	0,512	0,609	0,658	0,685	1,000	
19	0,647	0,605	0,605	0,574	0,552	0,593	0,532	0,615	0,561	0,583	0,551	0,571	0,587	0,550	0,597	0,648	0,676	0,758	1,000

Notes: 1).SDM; 2) SDP; 3). SJG; 4). SDMxSJG; 5). PH; 6). PHxSJG; 7). PHxM; 8). PHxK; 9). PHxMJ; 10). GB; 11). GP; 12). MWR; 13). PHxMWR; 14). MWxAFN; 15). MWRxSDP; 16). Sari Intan 541 PNG; 17). AFN; 18). WLC; 19). GBC

4. DISCUSSION

The main target expected is polymorphic bands. Polymorphism can be caused by several factors including: a primary mismatch, the modification or change of the primary, and differences in the amplified genomic DNA region (Kumar & Gurusubramanian, 2011). The presence of monomorphic bands shows the high conserved region of the genome (Singh *et al.*, 2006).

Based on Figure 1, Group I consisted of accessions of the species *S. sumatrana*, namely red-fleshed (SDM) and white-fleshed (SDP). *S. sumatrana* has superior characters (thick fruit flesh and large fruit), but astringent fruit taste (Hadiati *et al.*, 2012). In addition, it has a larger plant size compared to the Javanese salak and Balinese salak (Hadiati *et al.*, 2008). Group II was divided into two sub groups. Sub-group IIA consisted mainly of *S. zalacca* var. *zalacca* (Javanese Salak) and the results of the crossing. In this group, most of them produced astringent fruits, except for 'Pondoh; (PH) and MWRxAFN. Group IIB consisted of MWRxSDP; GPxPH and AFN (*S. affinis*). In this group, most of them had non-astringent fruit taste, except for AFN. Groups IIA and IIB could not be categorized explicitly based on astringency. Group III consisted of accessions belonging to the species *Salacca zalacca* var. *amboinensis* (GP and GB). The inclusion of MWR in group III was acceptable because one of the MWR's parental is GP. This group had specific leaflet characteristics. The curled leaflet edge and the smaller leaflet size are clear differentiators compared to *S. sumatrana* and *S. zalacca* var. *zalacca* (Appendix 1). Group IV consisted of two species namely *S. wallichiana* and *S. glabrescens*. Based on morphological characters, the two

species are different. The two species should be separated into different groups. *S. wallichiana* had a spineless petiole and the number of fruits per bunch can reach > 300 pieces per bunch.

The inaccuracy of salacca grouping in this study can be improved by increasing the diversity of the band (polymorphism of the amplification results). Addition of primers is one way to increase polymorphism (Sall *et al.*, 2000). Another way that can be taken is the use of specific primers for species/variety differentiation (Ramella *et al.*, 2005). The genetic similarity of 19 salacca based on 11 RAPD primers ranged from 0.512 to 0.937 (Table 2). The similarity coefficient above 50% shows the relationship between accessions is quite close (Zumaidar *et al.*, 2015). Except for MWR x SDP, progenies have a large genetic similarity coefficient to one of their parents.

5. CONCLUSION

All primers were given a range between 57.1-100% polymorphic level. The range of genetic similarity coefficients was between 0.512 - 0.937. Observed accessions could be separated into four groups. The highest genetic similarity coefficient was determined between PH-K and PH-MJ accessions (0.937), meanwhile the lowest value was indicated on the MW-AFN hybrid and *S. wallichiana* (0.512).

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Appendix 1. Morphological characters of salacca

No.	Accession	Curled leaflet edge	Petiole	Fruit skin	Flesh color	Astringent taste
1.	Sidempuan Merah/ SDM (44-3) (*S. sumatrana*)	absent	spiny	spiny	Reddish white (red 45 D, yellow 4 D)	present
2.	Sidempuan Putih/ SDP (31-26) (*S. sumatrana*)	absent	spiny	spiny	Cream (Yellow white 158 A)	present
3.	Pondoh/PH *S. zalacca*	absent	spiny	spiny	White (White NN 155 B)	absent
4.	Sanjung/SJG (18-11) (*S. zalacca*)	absent	spiny	spiny	Light cream (Yellow white 158 B)	present
5.	Mawar/ MWR (27-18) (S. zalacca)	present	spiny	spiny	White (White NN 155 A)	absent
6.	Gading Bali/GB (*S. zalacca* var *amboinensis*)	present	spiny	spiny	Cream (Yellow white 158 A)	present
7.	Gula Pasir/GP (23-3) (*S. zalacca* var *amboinensis*)	present	spiny	spiny	White (White NN 155 A)	absent
8.	*S. affinis*/AFN	absent	spiny	spineless /smooth	Cream (Yellow white 158 A)	present
9.	*S. wallichiana*/WLC	absent	spineless	spiny	Yellow orange 22 C	absent

10.	*S. glabrescents*/GBC	absent	spiny	spiny	Yellow orange 20 D	present
11.	SDM x SJG (36-15) (Sidempuan Merah x Sanjung)	absent	spiny	spiny	Cream (Orange white 159 B)	present
12.	PH x SJG (41-7) (Pondoh x Sanjung	absent	spiny	spiny	Cream (Yellow white 158 A)	present
13.	PH x "M" (49-19) (Pondoh x "M")	absent	spiny	spiny	Cream Yellow white 158 A	present
14.	PH x "K" (48-21) (Pondoh x "K")	absent	spiny	spiny	Cream (Yellow white 158 A)	present
15.	PH x "MJ" (17-6) (Pondoh x "MJ")	absent	spiny	spiny	Cream (Yellow white 158 A)	present
16.	PH x MWR (6-28) (Pondoh x Mawar)	absent	spiny	spiny	Cream (Yellow white 158 B)	present
17.	MW x AFN (12-4) (Mawar x *S. affinis*)	absent	spiny	spiny	White (White NN 155 C)	absent
18.	MWR x SDP (35-13) (Mawar x *S. sumatrana*)	absent	spiny	spiny	White (White NN 155 A)	absent
19.	Sari Intan 541 (GP x Pondoh)	absent	spiny	spiny	White (White NN 155 A)	absent