RAPD Salak Fragment

by Jamsari Jamsari

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SPECIFIC RAPD FRAGMENTS RELATED WITH SEX TYPE IN SALACCA (*Salacca edulis* L.)

Ediwirman[1,2]*, Irfan Suliansyah[1], Gustian[1] and Jamsari[1]

[1]Plant Breeding and Biotechnology Department, Faculty of Agriculture Andalas University, Padang, 25163. West Sumatera Indonesia
[2]Faculty of Agriculture. Tamansiswa Univ3ersity, Padang-West Sumatera Indonesia.

*Corresponding author : ediwirman_tamsis@yahoo.co.id

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ABSTRACT

Salacca (*Salacca edulis* L.) is a tropical plant with a high economic value. The sexes of salacca can be grouped as dioecious, monoecious, dan hermaphrodite. Farmer's success in cultivation is especially determined by the male to female ratio. The present ratio of male and female plants is 1:4. Determining sex based on morphological characteristics is not effective and difficult for young plants (seedlings), also to lysozim marker and chromosome. Marker Assisted Selection is a strategy to determine sex using a marker. Random Amplifed Polymorfism DNA (RAPD) is an amplification technique that uses markers. Of 305 primers tested, 4 primers, OPO-17 produced 297 bp product, OPAP-20 produced 554 bp as the specific fragment for the female and UBC-454 produced 934 bp as the specific fragment for the female and UBC-454 produced 946 bp as male, primer UBC-78 with the size of fragment 562 bp as the specific fragment for the female.

Keywords: Sex determination, dioecious, RAPD molecular marker, Marker Assisted Selection (MAS)

INTRODUCTION

Salacca (*Salacca edulis* L.) is a native Indonesian fruit commonly found in Sumatra, Kalimantan and Java. To date, 22 species of salacca have been identified. Four cultivars, namely Pondoh, Bali, Condet and Sidempuan, are very popular varieties cultivated in Indonesia. Among them, Pondoh is the most cultivated because of its superior characteristics compared to the three other varieties. However, some constraints are faced by farmers in cultivating this variety particularly due to its high "male bias" in the population. Moreover, the ratio of male : female will determine the success of salacca cultivation, with the ratio of male and female 4:1 (Schuiling and Mogea, 1992). Therefore, identifying the sex of seedlings prior to field cultivation is a key factor in the production of salacca fruit per hectare.

Sex of dioecious salacca can normally be determined after 5 years. A Strategy to determine sex has been developed using Marker Assisted Selection (MAS). The using maker is a startegy with approach morphologycal and biochemical to determination of sex plant. Some researchers have identified sex in salacca by using a marker. Hadi *et al*, (2002) argued that a morphological identification at the seed stage is not accurate. It is possible to distinguish chromosomes but this is difficult and depends on the phase of plant growth, and thus makes it unpractical. Fajriani *et al*. (2009) also showed that it was possible to use a isozim (lysozym) marker to distinguish the sex of pondoh salacca. The morphological dan isozim marker is very influenced by the enviroment.

The advent of DNA based molecular techniques has opened opportunities for marker system development. This approach has been applied in many organisms including humans (Jeffreys *et al*., 1985), animals (Parker *et al*., 1991) and plants (Helentjaris et al., 1986). In DNA is genetic material that can reveal the sex. DNA markers are genetically not influenced by the environment. A molecular marker using PCR (Polymerase Chain Reaction) based technology is used to identify and characterize dioecious plants (Kafkas, *et al*., 2001). The molecular marker linked to sex has been done with RAPD technique in dioecious plants such as jojoba (Hosseini *et al*., 2011) and asparagus (Li *et al*., 2012). The SCAR marker has been used to determine the sex of asparagus (Jamsari *et al*., 2004). Parjanto *et al*., (2006) reported that it is possible to determine the sex of male salacca plants using a RAPD marker.

It is important to develop additional sex linked molecular markers for the salacca dioecious plant, because sequence markers vary among cultivar. A PCR based approach using a RAPD analysis is one of the simplest and easiest methods to retrieve markers. This study investigated the newly identified sex linked to RAPD markers in combination with Bulked Segregant Analysis (BSA).

MATERIAL AND METHODS

The plant materials used were taken from the plantation of a farmer who had flowers in two locations. This location is zone development salacca in West Sumatera-Indonesia. It is fail by do not male plant. The first location was Batu Hampar (latitude 00°31'15.4"; longitude 100°17'59.8"), and the second location is Batu Kalang (latitude 01°15'38.1"; longitude 100°25'56.8"). The samples size collected was based on the male:female ratio in each location. The first location consisted of 6 plants comprising of 3 males and 3 females from the plantation of a farmer in Sicincin, West Sumatra while the second group comprised of 4 plants consisting of 2 males and 2 females from the plantation of a farmer in Tarusan, West Sumatra. The sample is collected was based on the population number from each location. Leaf samples were taken and kept in the depository room at 4C until the DNA would be extracted.

DNA Isolation and Extraction

Total genomic DNA was isolated from the young leaves of plants. The DNA isolation was carried out using a CTAB method with minor modifications as described by Doyle and Doyle (1987). A total of 100 – 150 mg of leaf were ground to a fine powder using liquid nitrogen and put into heated eppendorf (1.5 ml) containing 400 µl buffer CTAB (1 M Tris HCl pH 8.5; 5 M NaCl, 0.5 M EDTA; 10% CTAB; -mercaptoethanol). The extraction was performed using a vortex and later incubated at 64C for 30 min. Later, 700 µl Chloroform, Phenol and Isoamilalcohol were added to the solution (25:24:1) and mixed for 10 minutes, and finally centrifuged at 14.000 rpm for 10 minutes. The supernatant was transferred to a new eppendorf and 500 µl of a Chloroform Isoamilalkohol (24 : 1) solution was added and centrifuged at 14000 rpm for 10 minutes. The Supernatant was then transferred again to the eppendorf and 500 µl of cold ethanol 96% was added and later dried before a further 70% solution of ethanol was added. The DNA pellet was re-suspended in 100 µl 1 x TE and stored at a temperature of 15^{0}C. The quantity of DNA was checked and determined using 1% electrophoresis agarose gel. An aliquot of 25 ng/µl was prepared for further analysis.

Bulked Segregant Analysis was conducted according to procedures described by Michelmore et al. (1991). DNA Bulk were prepared by pooling an equal quantity, each of the DNA bulk on the nucleotide concentration balance (1 g/1 µL) per genotype. The DNA from 5 individual males (male bulk) and 5 individual females (female bulk) was used. Bulked Segregant Analysis was used to screen RAPD marker associated with the reproductive trait, male and female. A total of 305 of 10 mer RAPD primers (Operon Technology Inc. Alamaeda) were used in Table 1.

Table 1. RAPD primer used for screening the sex of salacca dioecious plants.

No.	Primer	Sekuen	TM (°C)	No.	Primer	Sekuen	TM (°C)
1	Moh1	TGGCGACCTG	43,5	7	OPA-06	GGTCCCTGAC	34,0
2	Moh7	GTTCCGCTCC	43,5	8	OPA-07	GAAACGGGTG	34,0
3	F1	AGGACTCGGA	37,0	9	OPA-08	GTGACGTAGG	32,0
4	OPA-01	CAGGCCCTTC	34,0	10	OPA-09	GGGTAACGCC	34,0
5	OPA-02	TGCCGAGCTG	34,0	11	OPA-10	GTGATCGCAG	32,0
6	OPA-05	AGGGGTCTTG	32,0	12	OPA-11	CAATCGCCGT	32,0

Table 1 (Continuous)

No.	Primer	Sekuen	TM (°C)	No.	Primer	Sekuen	TM (°C)
13	OPA-12	TCGGCGATAG	32,0	49	OPF-03	CCTGATCACC	37,0
14	OPA-13	CAGCACCCAC	34,0	50	OPF-04	GGTGATCAGG	37,0
15	OPA-15	TTCCGAACCC	34,0	51	OPF-08	GGGATATCGG	37,0
16	OPA-19	CAAACGTCGG	34,0	52	OPF-09	CCAAGCTTCC	37,0
17	OPA-20	GTTGCGATCC	34,0	53	OPF-13	GGCTGCAGAA	38,0
18	OPB-01	GTTTCGCTCC	32,0	54	OPF-14	TGCTGCAGGT	38,0
19	OPB-06	TGCTCTGCCC	34,0	55	OPG-04	AGCGTGTCTG	37,0
20	OPB-11	GTAGACCCGT	32,0	56	OPG-06	GTGCCTAACC	37,0
21	OPB-13	TTCCCCCGCT	34,0	57	OPG-14	GGATGAGACC	37,0
22	OPB-14	TCCGCTCTGG	34,0	58	OPG-17	ACGACCGACA	38,0
23	OPB-15	GGAGGGTGTT	32,0	59	OPH-04	GGAAGTCGCC	38,0
24	OPB-17	AGGGAACGAG	34,5	60	OPH-12	ACGCGCATGT	38,0
25	OPB-20	GGACCCTTAC	34,0	61	OPH-15	AATGGCGCAG	38,0
26	OPC-02	GTGAGGCGTC	34,0	62	OPH-16	TCTCAGCTGG	38,0
27	OPC-04	CCGCATCTAC	34,0	63	OPI-01	ACCTGGACAC	38,0
28	OPC-05	GATGACCGCC	34,0	64	OPI-02	GGAGGAGAGG	38,0
29	OPC-08	TGGACCGGTG	34,0	65	OPI-11	ACATGCCGTG	34,0
30	OPC-12	TGTCATCCCC	34,0	66	OPI-12	AGAGGGCACA	34,0
31	OPC-14	TGCGTGCTTG	34,0	67	OPI-19	AATGCGGGAG	34,0
32	OPC-15	GACGGATCAG	38,0	68	OPI-20	AAAGTGCGGG	34,0
33	OPC-16	CACACTCCAG	34,0	69	OPJ-01	CCCGGCATAA	37,0
34	OPD-02	GGACCCAACC	34,0	70	OPJ-06	TCGTTCCGCA	37,0
35	OPD-04	TCTGGTGAGG	35,0	71	OPJ-09	TGAGCCTCAC	34,0
36	OPD-06	ACCTGAACGG	34,0	72	OPK-02	GTCTCCGCAA	39,5
37	OPD-07	TTGGCACGGG	34,0	73	OPK-06	CACCTTTCCC	34,0
38	OPD-10	GGTCTACACC	37,0	74	OPK-09	CCCTACCGAC	39,5
39	OPD-14	CTTCCCCAAG	37,0	75	OPK-12	TGGCCCTCAC	34,0
40	OPE-02	GGTGCGGGAA	37,0	76	OPK-13	GGTTGTACCC	34,0
41	OPE-04	GTGACATGCC	37,0	77	OPK-15	CTCCTGCCAA	34,0
42	OPE-05	TCAGGGAGGT	37,0	78	OPK-18	CCTAGTCGAG	39,5
43	OPE-08	TCACCACGGT	37,0	79	OPL-09	TGCGAGAGTC	37,0
44	OPE-14	TGCGGCTGAG	37,0	80	OPL-10	TGGGAGATGG	37,0
45	OPE-16	GGTGACTGTG	37,0	81	OPL-11	ACGATGAGCC	37,0
46	OPE-17	CTACTGCCGT	37,0	82	OPL-13	ACCGCCTGCT	37,0
47	OPE-19	ACGGCGTATG	39,5	83	OPL-17	AGCCTGAGCC	43,5
48	OPF-02	GAGGATCCCT	37,0	84	OPL-19	GAGTGGTGAC	39,5

Table 1 (Continuous)

No.	Primer	Sekuen	TM (°C)	No.	Primer	Sekuen	TM (°C)
85	OPM-02	ACAACGCCTC	39,5	121	OPT-12	GGGTGTGTAG	39,5
86	OPM-04	GGCGGTTGTC	37,0	122	OPT-16	GGTGAACGCT	39,5
87	OPM-05	GGGAACGTGT	37,0	123	OPT-17	CCAACGTCGT	39,5
88	OPM-07	CCGTGACTCA	39,5	124	OPU-03	CTATGCCGAC	39,5
89	OPM-09	GTCTTGCGGA	39,5	125	OPU-09	CCACATCGGT	39,5
90	OPM-19	CCTTCAGGCA	39,5	126	OPU-11	AGACCCAGAG	39,5
91	OPN-01	CTCACGTTGG	38,0	127	OPV-02	AGTCACTCCC	39,5
92	OPN-11	TCGCCGCAAA	38,0	128	OPV-20	CAGCATGGTC	39,5
93	OPN-12	CACAGACACC	38,0	129	OPW-02	CATCGCCGCA	39,5
94	OPN-13	AGCGTCACTC	38,0	130	OPW-03	GTCCGGAGTG	39,5
95	OPN-14	TCGTGCGGGT	38,0	131	OPW-14	CTGCTGAGCA	39,5
96	OPN-16	AAGCGACCTG	38,0	132	OPW-20	TGTGGCAGCA	39,5
97	OPN-19	GTCCGTACTG	38,0	133	OPX-07	GAGCGAGGCT	39,5
98	OPO-09	TCCCACGCAA	39,0	134	OPX-15	CAGACAAGCC	39,5
99	OPO-10	TCAGAGCGCC	43,6	135	OPY-02	CATCGCCGCA	39,5
100	OPO-12	CAGTGCTGTG	48,7	136	OPY-03	ACAGCCTGCT	37,0
101	OPO-13	GTCAGAGTCC	39,5	137	OPY-08	AGGCAGAGCA	37,0
102	OPO-17	GGCTTATGCC	39,5	138	OPY-10	CAAACGTGGG	37,0
103	OPO-18	CTCGCTATCC	39,5	139	OPY-20	AGCCGTGGAA	37,0
104	OPP-04	GTGTCTCAGG	34,0	140	OPZ-05	TCCCATGCTG	37,0
105	OPP-06	GTGGGCTGAC	34,0	141	OPZ-11	CTCAGTCGCA	37,0
106	OPP-07	GTCCATGCCA	39,5	142	OPZ-12	TCAACGGGAC	37,0
107	OPP-12	AAGGGCGAGT	34,0	143	OPZ-18	AGGGTCTGTG	37,0
108	OPQ-11	TCTCCGCAAC	39,5	144	OPAB-12	CCTGTACCGA	37,0
109	OPQ-12	AGTAGGGCAC	39,5	145	OPAB-19	ACACCGATGG	37,0
110	OPQ-13	GGAGTGGACA	49,1	146	OPAC-06	CCAGAACGGA	38,0
111	OPQ-17	GAAGCCCTTG	39,5	147	OPAC-12	GGCGAGTGTG	37,0
112	OPR-11	GTAGCCGTCT	39,5	148	OPAD-03	TCTCGCCTAC	39,5
113	OPR-12	ACAGGTGCGT	39,5	149	OPAD-08	GGCAGGCAAG	38,0
114	OPR-14	CAGGATTCCC	39,5	150	OPAD-12	AAGAGGGCGT	39,5
115	OPR-17	CCGTACGTAG	39,5	151	OPAD-14	GAACGAGGGT	39,5
116	OPS-01	CTACTGCGCT	39,5	152	OPAD-19	CTTGGCACGA	39,5
117	OPS-03	CAGAGGTCCC	38,0	153	OPAE-08	CTGGCTCAGA	39,5
118	OPS-18	CTGGCGAACT	38,0	154	OPAE-12	CCGAGCAATC	39,5
119	OPT-03	TCCACTCCTG	39,5	155	OPAE-14	GAGAGGCTCC	39,5
120	OPT-08	AACGGCGACA	32,0	156	OPAE-18	CTGGTGCTGA	39,5

Table 1 (Continuous)

No.	Primer	Sekuen	TM (°C)	No.	Primer	Sekuen	TM (°C)
157	OPAF-02	CAGCCGAGAA	39,5	194	OPAP-20	CCCGGATACA	39,5
158	OPAF-05	CCCGATCAGA	39,5	195	OPAQ-03	GAGGTGTCTG	39,5
159	OPAF-08	CTCTGCCTGA	39,5	196	OPAQ-05	ACGGAGCTGA	39,5
160	OPAF-12	GACGCAGCTT	39,5	197	OPAQ-12	CAGCTCCTGT	39,5
161	OPAF-14	GGTGCGCACT	43,6	198	OPAR-01	CCATTCCGAG	39,5
162	OPAG-04	GGAGCGTACT	39,5	199	OPAR-05	CATACCTGCC	39,5
163	OPAG-11	TTACGGTGGG	39,5	200	OPAR-16	CCTTGCGCCT	39,5
164	OPAH-18	GGGCTAGTCA	39,5	201	OPAS-20	TCTGCCTGGA	39,5
165	OPAH-19	GGCAGTTCTC	39,5	202	OPAT-02	CAGGTCTAGG	39,5
166	OPAI-19	GGCAAAGCTG	39,5	203	OPAT-05	ACACCTGCCA	39,5
167	OPAJ-02	TCGCACAGTC	39,5	204	OPAT-16	CTCTCCGTAG	39,5
168	OPAJ-03	AGCACCTCGT	39,5	205	OPAT-18	CCAGCTGTGA	39,5
169	OPAJ-10	GTTACCGCGA	39,5	206	OPAU-08	CACCGATCCA	39,5
170	OPAJ-17	ACCCCCTATG	39,5	207	OPAU-18	CACCACTAGG	39,5
171	OPAK-11	CAGTGTGCTC	39,5	208	OPAV-07	CTACCAGGGA	39,5
172	OPAK-12	AGTGTAGCCC	39,5	209	OPAV-14	CTCCGGATCA	39,5
173	OPAK-15	ACCTGCCGTT	39,5	210	OPAV-16	GACAAGGACC	39,5
174	OPAL-06	AAGCGTCCTC	39,5	211	OPAV-20	TCATGCGCAC	39,5
175	OPAL-12	CCCAGGCTAC	39,5	212	OPAW-04	AGGAGCGACA	39,5
176	OPAL-13	GAATGGCACC	39,5	213	OPAW-05	CTGCTTCGAG	39,5
177	OPAL-16	CTTTCGAGGG	39,5	214	OPAW-13	CTACGATGCC	39,5
178	OPAM-08	ACCACGAGTG	39,5	215	OPAW-16	TTACCCCGCT	39,5
179	OPAM-13	CACGGCACAA	38,0	216	OPAW-20	TGTCCTAGCC	39,5
180	OPAM-14	TGGTTGCGGA	39,5	217	OPAX-11	TGATTGCGGG	39,5
181	OPAM-15	GATGCGATGG	39,5	218	OPAY-03	TTTCCGGGAG	39,5
182	OPAN-01	ACTCCACGTC	39,5	219	OPAY-08	AGGCTTCCCT	39,5
183	OPAN-14	AGCCGGGTAA	39,5	220	OPAY-09	CCGATCCAAC	39,5
184	OPAN-15	TGATGCCGCT	39,5	221	OPBA-09	GGAACTCCAC	39,5
185	OPAO-01	AAGACGACGG	39,5	222	OPBB-06	CTGAAGCTGG	39,5
186	OPAO-04	AACAGGGCAG	39,5	223	OPBB-15	AAGTGCCCTG	39,5
187	OPAO-05	TGGAAGCACC	39,5	224	OPBB-20	CCAGGTGTAG	39,5
188	OPAO-08	ACTGGCTCTC	39,5	225	OPBC-10	AACGTCGAGG	39,5
189	OPAP-01	AACTGGCCCC	39,5	226	OPBC-17	CCGTTAGTCC	39,5
190	OPAP-02	TGGTCATCCC	39,5	227	OPBD-13	CCTGGAACGG	43,5
191	OPAP-03	GTAAGGCGCA	39,5	228	OPBD-18	ACGCACACTC	39,5
193	OPAP-17	ACGGCACTCC	43,5	229	OPBE-01	CACTCCTGGT	39,5

Table 1 (Continuous)

No.	Primer	Sekuen	TM (°C)	No.	Primer	Sekuen	TM (°C)
230	OPBF-15	ACGCGAACCT	39,5	269	UBC-346	TAGGCGAACG	39,5
231	OPBF-17	CAAGCTCGTG	39,5	270	UBC-354	CTAGAGGCCG	35,4
232	OPBG-06	GTGGATCGTC	39,5	271	UBC-368	ACTTGTGCGG	39,5
233	OPBH-17	CTCTTACGGG	39,5	272	UBC-369	GCGCATAGCA	39,5
234	OPBH-19	GTCGTGCGGA	43,5	273	UBC-388	CGGTCGCGTC	35,4
235	UBC-14	CCTGGGTTTC	39,5	274	UBC-411	GAGGCCCGTT	35,4
236	UBC-21	ACCGGGTTTC	39,5	275	UBC-429	AAACCTGGAC	35,4
237	UBC-78	GAGCACTAGC	39,5	276	UBC-433	TCACGTGCCT	39,5
238	UBC-79	GAGCTCGTGT	39,5	277	UBC-440	CTGTCGAACC	39,5
239	UBC-97	ATCTGCGAGC	39,5	278	UBC-444	GCAGCCCCAT	43,6
240	UBC-98	ATCCTGCCAG	50,2	279	UBC-452	CTAATCACGG	35,4
241	UBC-114	TGACCGAGAC	39,5	280	UBC-454	GCTTACGGCA	39,5
242	UBC-131	GAAACAGCGT	39,5	281	UBC-464	CACAAGCCTG	39,5
243	UBC-139	CCCAATCTTC	35,4	282	UBC-476	TTGAGGCCCT	39,5
244	UBC-141	ATCCTGTTCG	35,4	283	UBC-497	GCATAGTGCG	39,5
245	UBC-150	GAAGGCTCTG	39,5	284	UBC-499	GGCCGATGAT	39,5
246	UBC-181	ATGACGACGG	35,4	285	UBC-502	GCATGGTAGC	39,5
247	UBC-188	GCTGGACATC	39,5	286	UBC-514	CGGTTAGACG	39,5
248	UBC-190	AGAATCCGCC	35,4	287	UBC-526	AACGGGCACC	43,6
249	UBC-195	GATCTCAGCG	39,5	288	UBC-535	CCACCAACAG	39,5
250	UBC-209	TGCACTGGAG	39,5	289	UBC-553	TCCGAGATCG	39,5
251	UBC-220	GTCGATGTCG	39,5	290	UBC-558	CGATATCCGG	39,5
252	UBC-225	CGACTCACAG	39,5	291	UBC-559	GAGAACTGGC	39,5
253	UBC-226	GGGCCTCTAT	39,5	292	UBC-560	CACTGCTGTC	39,5
254	UBC-248	GAGTAAGCGG	39,5	293	UBC-566	CCACATGCGA	39,5
255	UBC-255	TTCCTCCGGA	39,5	294	UBC-578	GGTGTCCACT	39,5
256	UBC-259	GGTACGTACT	35,4	295	UBC-580	GCGATAGTCC	39,5
257	UBC-268	AGGCCGCTTA	39,5	296	UBC-590	CCGGCATGTT	39,5
258	UBC-271	GCCATCAAGA	35,4	297	UBC-618	CGGACTATGT	35,4
259	UBC-278	GGTTCCAGCT	39,5	298	UBC-642	GTGGTCTCGA	39,5
260	UBC-288	CCTCCTTGAC	39,5	299	UBC-662	GGCTACGTCT	39,5
261	UBC-307	CGCATTTGCA	35,4	300	UBC-663	CGTATAGCCG	39,5
262	UBC-310	GAGCCAGAAG	35,4	301	UBC-670	CCCTTGAGAC	39,5
263	UBC-323	GACATCTCGC	39,5	302	UBC-674	ATCGATCCGG	39,5
264	UBC-327	ATACGGCGTC	39,5	303	UBC-676	GCTAACGTCC	39,5
265	UBC-330	GGTGGTTTCC	39,5	304	UBC-686	CGTGACAGGA	39,5
266	UBC-331	GCCTAGTCAC	39,5	305	UBC-794	GAGGGGAAAG	39,5
267	UBC-339	CTCACTTGGG	39,5	306	UBC-45	TTAACCCCGG	39,5
268	UBC-342	GAGATCCCTC	39,5	307			

PCR Amplification

PCR reaction was carried out using Ready to Go PCR (RTG-PCR) (GE Healthcare-UK). PCR reaction was performed in 15 µl of a final volume : 3 µl DNA bulk, 3 µl (20 mol), and 9 µl ddH2O PCR PCR reaction was performed on Biometra thermalcycler machine (Biometra-Germany). The amplification program was; 1 cycle of initial denaturation at 94^{0}C for 1 minute, followed by 45 cycles of denaturation at 95^{0}C for 30 seconds, annealing at 37C for 30 seconds, and extension at 72C for 90 seconds, and 1 cycle of final extension at 72^{0}C for 8 minutes. Analysis of the PCR product was conducted by a gel electrophoresis technique using 1,5% agarose gel stained with ethidium bromide. One kb ladder (Fermentas-USA) was used as size standard reference. Visualization was done using Gel Doc System supplied by Biometra (Biometra-Germany).

RESULTS AND DISCUSSION

Characterization of Sampling Area

A sample of *salacca dioecious* was collected from 2 farms. The first location was Lubuk Bonta (latitude 00°31'15.4; longitude 100°17'59.8) at an altitude of 241 m (upland). The second location, Batu Kalang (latitude 01°15'38.1; longitude 100°25'56.8) has an altitude of 5 m (lowland). This location has been zoned for the development of *salacca dioecious* in West Sumatra, with the age of plants being 10 years old and have produced fruit. At this second location, far fewer male plants are found compared to females, and as a result plant productivity is low.

DNA Isolation

DNA isolation produced good DNA. This is indicated by the fragment quantity, that can be deduced by the concentration on extracted DNA after comparing with DNA-λ as a reference (Figure 1).

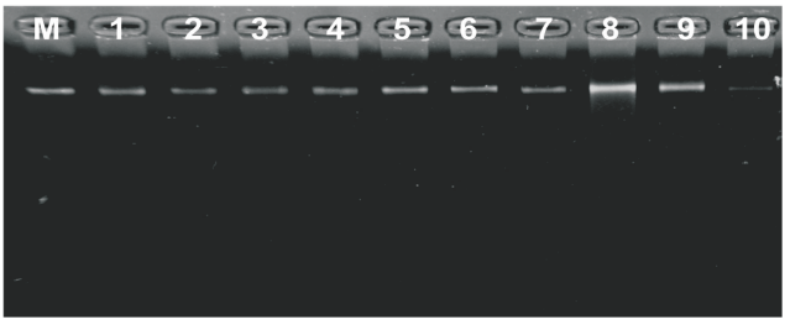

Figure 1. Result of DNA isolation process using CTAB buffer in salacca plant (M) -DNA (50 ng/µL), female plants (line 1 – 5) and male plant (line 6 - 10).

Figure 1 shows that the isolation DNA genomic has produced relative high DNA concentration in the range between 45 to 120 ng/µL. DNA isolated using the methodology described by Doyle and Doyle (1987) with minor modifications, can produce high quantity DNA. DNA Isolation from young leaves produced good DNA quality. Dorea *et al*. (2014) reported that young leaves produce good quality DNA compared to adult leaves. Young leaves have secondary active metabolic compounds such as polyfenol and polysaccharides. Weising (2009) using a compound phenolic, obtained highly viscous DNA, indicating the presence of pectin like polysaccharides that were water soluble and tend to co-isolate with DNA. Polysaccharides also interfere with activity enzymes, such as, polymerase, restriction endonucleases, ligases, therefore resulting in unsuccessful amplification. Mathew *et al*. (2014) also report that young leaves of large cardamon plants produced 120 µg/g.

Differential Screening of Primers

Bulked Segregant Analysis (Michelmore *et al*., 1991) is a method used for rapidly identifying markers linked to specific genes or a genomic region. Bulked Segregant Analysis (BSA) was conducted using 5 genomes from female and male plants, and mixed together to balance the concentration. Screening was done to use 305 primer with DNA bulked. The determination of the marker related to the sex controlling region in the RAPD analysis depended on the primer sequence used. Figure 2 and 3 shows an example of RAPD fragment patterns of the female and male bulked and samples were identical for each primer with patterns fragment is variety.

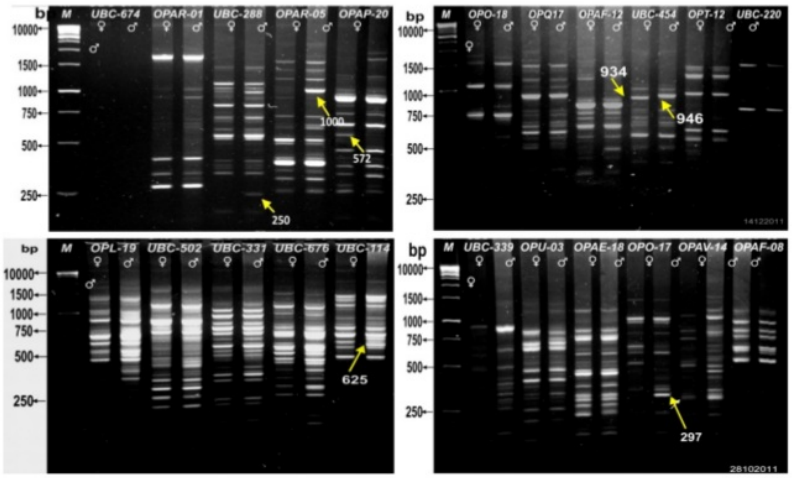

Figure 2. RAPD fragment patterns from bulked DNA samples from male and female plants of salacca dioecious. ♀ and ♂ presented DNA bulked from female and male, respectively. The specific fragments linked to females and males are indicated by arrowheads.

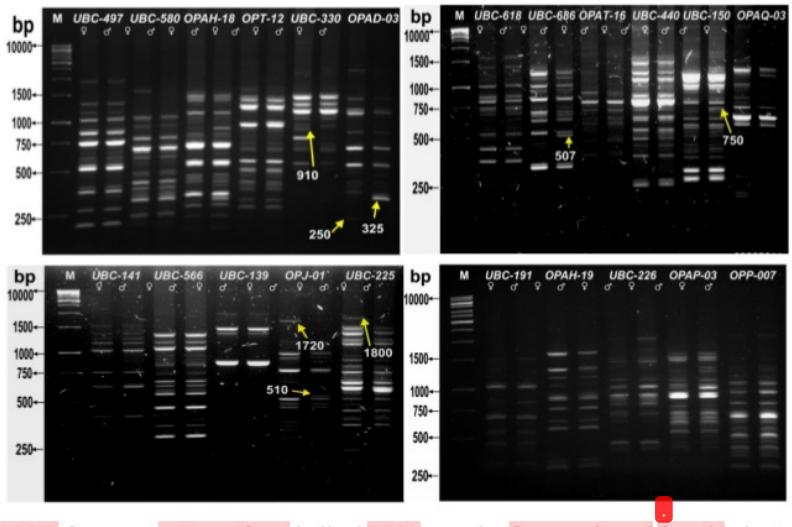

Figure 3. RAPD fragment patterns from bulked DNA samples from male and female plants of salacca dioecious. ♀ and ♂ presented DNA bulked from female and male, respectively. The specific fragments linked to female and males are indicated by arrowheads.

The use of 305 RAPD primers with Bulked Segregant Analysis is sex linked in the dioecious salacca and 266 of the primers generated fragments while the remaining did not. There were 3223 fragments in total with 1 – 7 fragments for each primer. The fragment size was 125 – 1800 bp. Out of 35 primer it is among to result fragment sex linked to used as candidate primer with 454,5 fragment. There were 14 fragments linked to female plants from 13 primers, 16 primers linked to male plants with 20 specific fragments, and 6 primers with 6 specific fragments linked with female plants and 8 specific fragments with male plants. The fragment patterns of 35 primers are shown in Table 2.

Table 2. Number of fragments from 35 RAPD primers sex linked with Bulked Segregant Analysis.

No.	Primer	Sequences 5'.... 3'	Number of fragment				Average Fragment
			Polymorfism		Spesific Fragment		
			Female	Male	Female	Male	
1.	UBC-114	TGACCGAGAC	17	18	1	0	17.5
2.	UBC-225	CGACTCACAG	25	24	1	0	24.5
3.	UBC-330	GGTGGTTTCC	16	15	1	0	15.5
4.	UBC-346	TAGGCGAACG	13	12	1	0	12.5
5.	UBC-553	TCCGAGATCG	18	17	1	0	17.5
6.	UBC-78	GAGCACTAGC	17	16	1	0	16.5
7.	OPS-01	CTACTGCGCT	13	11	2	0	12.0
8.	OPAF-12	GACGCAGCTT	13	12	1	0	12.5
9.	OPAG-04	GGAGCGTACT	21	20	1	0	20.5
10.	OPAT-18	CCAGCTGTGA	16	15	1	0	15.5
11.	OPAX-11	TGATTGCGGG	15	14	1	0	14.5
12.	OPQ-17	GAAGCCCTTG	16	16	1	0	16.0
13.	OPAP-20	CCCGGATACA	17	19	1	2	18.0
14.	UBC-150	GAAGGCTCTG	23	24	0	1	23.5
15.	UBC-188	GCTGGACATC	23	22	0	1	22.5
16.	UBC-288	CCTCCTTGAC	18	19	0	1	18.5
17.	UBC-323	GACATCTCGC	18	19	0	1	18.5
18.	UBC-327	ATACGGCGTC	18	19	0	1	18.5
19.	UBC-497	GCATAGTGCG	16	17	0	1	16.5
20.	UBC-558	CGATATCCGG	27	28	0	1	27.5
21.	UBC-686	CGTGACAGGA	23	24	0	1	23.5
22.	OPAJ-03	AGCACCTCGT	10	11	0	1	10.5
23.	OPAQ-03	GAGGTGTCTG	18	19	0	1	18.5
24.	OPAR-05	CATACCTGCC	14	15	0	1	14.5
25.	OPAV-14	GACAAGGACC	21	17	0	3	19
26.	OPM-09	GTCTTGCGGA	10	11	0	1	10.5
27.	OPQ-11	TCTCCGCAAC	9	10	0	1	9.5
28.	OPR-12	ACAGGTGCGT	16	18	0	2	17
29.	OPR-17	CCGTACGTAG	12	15	0	2	13.5
30.	UBC-21	ACCGGGTTTC	13	14	1	2	13.5
31.	UBC-663	CGTATAGCCG	14	15	1	1	14.5
32.	OPAD-03	TCTCGCCTAC	14	14	1	1	14
33.	OPJ-01	CCCGGCATAA	24	24	1	1	24
34.	OPO-17	GGCTTATGCC	13	14	1	2	13.5
35.	UBC-454	GCTTACGGCA	11	11	1	1	11
	Total				20,0	30,0	454,50
	Average		16,63	16,83			16,38

Results of amplification of DNA genomes with RAPD primer as marker demonstrate a linkage with female and male salacca dioecious. The fragment patterns as differential female and male plants are varied, as an indicator of polymorfism of fragments. A marker being linked togen/genomic regions through RAPD analysis depends to large extent on chance. In BSA, most primers failed to amplify reproducible RAPD markers occurring on sex type alone. The chances of any RAPD markers being linked to a gene or a genomic region of interest is mainly dependent on genome size, type of gene or genomic region and on the type of population used for marker analysis. Agrawal et al. (2007) worked with 72 markers in jojoba and found only sex-specific marker RAPD. Hosseini et al. (2011) tested 20 primers in jojoba and found one sex-specific marker for males and females. Ehsanpour and Arab (2009) also tested 30 RAPD primers and 2 primers were differentiated male and female in *Pistacia vera* L. Adawy et al. (2014) tested 122 random primers and found 4 differentiated primers in date palms. Banerjee *et al*. (1999) together with Xu *et al*. (2004) also report that the DNA marker linked to sex can be used if information about the genetic mechanism of sex linking is unknown.

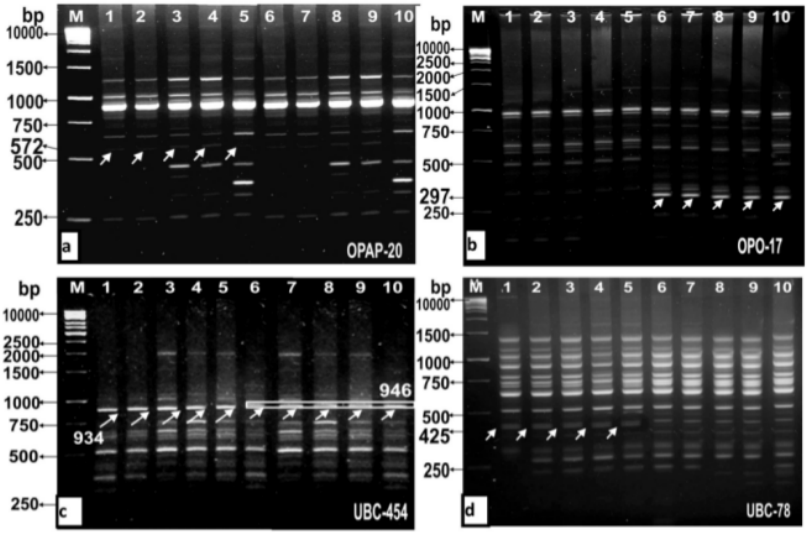

Figure 4. Verification of the female and male-specific RAPD markers in salacca dioecious. RAPD primers OPAP-20 (a); OPO-17 (b), UBC-454 (c), and UBC-78 (d). Female (line 1 – 5) and male (line 6-10), 1 kb ladder (M).

Figure 4 shows that primers OPAP-20 and UBC-78 successfully generated a differential fragment that can be used to distinguish between females and males - fragment specific results are all in females and not males. While primer OPO-17 and UBC-454 only revealed one differential fragment present in all males and absent in all females. To verify these results, these primers revealed sex-specific markers.

The results of this study demonstrate that RAPD markers can differentiate female and male types. Using more primers will increase the probability of finding a specific fragment. A specific fragment still needs to be verified as individual. Pourkazemi and Razikazemi (2011) showed an absence of molecular markers linked to sex determination may be due to the shortcomings of RAPD techniques, the most important of which are hypersensitivities to pollution, difficult scoring of the produced bands on the gel, dominance of markers, ambiguity of identifying the allele system, unspecified position of RAPD markers on genetic maps and unclear consanguinity and similarities of bands which have similar migrations on the gel. Welsh and McCelland (1990) explained that the disadvantages of this technique include weak reproducibility of the bands, scoring of the produced bands, extreme sensitivity to pollution and its dominant inheritance.

Determining a suitable primer for a character related to the sex is an important step. The RAPD technique is a method with low difficulty level but in order to obtain specific products, a lot of primers (markers) are required. The use of UBC-78 primers identified specific fragment for female sized 465 bp which is not found in males. In Figure 3a, using the timed-running electrophoresis, separation between the specific and non-specific fragments in males were unclear. However, by extending the running time to 3 hours, the separation became clear. This matter was carried out using a RAPD marker with an unknown genome background but expected to provide information related to the sex, based on previous research. As explained by Ming and More (2007), sex in dioecious plants was determined by the very specific sexual chromosome. This matter will affect the obtained accuracy. Moreover, Banerjee *et al*. (1999) and Xu et al. (2004) demonstrated the use of DNA markers for differentiating male and female sexes in plants where the sex was still unknown. The use of molecular markers in determining the sex has increased, such as using Amplified Fragment Length Polymorfism (AFLP) in *Asparagus officinalis* (Spada *et al*., 1998; Reamon-Büttner *et al*., 1998), and *Ficus fulva* (Parrish *et al*., 2004), and RAPD in papaya, *Cannabis sativa and Populus tomentosa* (Lemos *et al*., 2000; Deputy *et al*., 2002; Hou *et al*., 2009). Vaidya and Naik (2014) also demonstrate from 85 primers, which 5 primers can differential male, female, and hermaphrodite in *Simarouba glauca*. One primer can also produce specific male and female fragments with different fragment sizes.

In dioecious plants, it has been postulated that many genes are involved in the differentiation of male and female flowers but that sex differentiation could be controlled by a single locus acting as a trigger. In such a scenario, genes having the genetic information for carpel or stamen development would be present in both male and female plants, with one major gene being the only difference between the two sexes. The use of a RAPD marker related to the determination of a gene or genome depends on the type and size of the gene or region. Early detection of male and female sexes in salacca is important before the plant is relocated to the plantation. Several molecular markers in differentiating the sex of dioecious plants such as papaya (Urasaki, *et al*., 2002); Pisticia (Hormaza *et al*., 1994), attempting to differ the sex of several Phoriisticia varieties but cannot differ the male and female. Based on the results, it can be concluded that a new marker is required to distinguish the sex type. Molecular marker based technology has been proved a reliable strategy for detection of sex-associated markers in dioecious and bisexual plants. The RAPD marker technique is the cheapest, most user friendly and reliable tool to use for efficient fingerprinting of many plants.

CONCLUSION

The RAPD marker can be used to differentiate male and female salacca dioecious. Out of 305 primers tested, 4 primers, OPO-17 produced 297 bp product, OPAP-20 produced 554 bp as the specific fragments for the female and UBC-454 produced 934 bp as the specific fragments for the female and UBC-454 produced 946 bp as male, primer UBC-78 with the size of fragment 562 bp as the specific fragment for the female. Four primers still require further testing in order to determine the sequence that determines sex determination genes in male and female salacca.

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