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Genetic diversity of sago palm in Indonesia based on chloroplast DNA (cpDNA) markers

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ABSTRACT

Abbas B, Renwarin Y, Bintoro MH, Sudarsono, Surahman M, Ehara H (2010) Genetic diversity of sago palm in Indonesia based on chloroplast DNA (cpDNA) markers. Biodiversitas 11: 112-117. Sago palm (*Metroxylon sagu* Rottb.) was believed capable to accumulate high carbohydrate content in its trunk. The capability of sago palm producing high carbohydrate should be an appropriate criterion for defining alternative crops in anticipating food crisis. The objective of this research was to study genetic diversity of sago palm in Indonesia based on cpDNA markers. Total genome extraction was done following the Qiagen DNA isolation protocols 2003. Single Nucleotide Fragments (SNF) analyses were performed by using ABI Prism GeneScan^R 3.7. SNF analyses detected polymorphism revealing eleven alleles and ten haplotypes from total 97 individual samples of sago palm. Specific haplotypes were found in the population from Papua, Sulawesi, and Kalimantan. Therefore, the three islands will be considered as origin of sago palm diversities in Indonesia. The highest haplotype numbers and the highest specific haplotypes were found in the population from Papua suggesting this islands as the centre and the origin of sago palm diversities in Indonesia. The research had however no sufficient data yet to conclude the Papua origin of sago palm. Genetic hierarchies and differentiations of sago palm samples were observed significantly different within populations (P=0.04574), among populations (P=0.04772), and among populations within the island (P=0.03366), but among islands no significant differentiations were observed (P= 0.63069).

Key words: genetic diversity, sago palm, chloroplast DNA, haplotype, Indonesia.

INTRODUCTION

Sago palm (*Metroxylon sagu* Rottb.) is capable to accumulate high carbohydrate content in its trunk. The potential of sago palm accumulating high carbohydrate content will be good choice crops for anticipating carbohydrate crisis in the world. Increasing utilities of sago palm need genetic diversity information within the species. The genetic diversity of this plant is important to comprehend for germplasm conservation and breeding program in the future. Up to present information on genetic diversity of sago palm is very limited. Previous studies examined the levels diversity of sago palm by using RAPD markers (Ehara et al. 2003; Barahima et al. 2005) and AFLP (Celiz et al. 2004). One of the most important markers for assessing genetic diversity of plant is cpDNA.

The chloroplast genome of eucaryotes evolved from endosymbiotic an ancestral cyanobacterium (Douglas 1998). Most chloroplast genes of higher plants are organized in clusters and co-transcribed as polycistronic pre-RNAs, which are generally processes into shorter overlapping RNA species (Sugita and Sugiura 1996). The circular double-stranded DNA contains a pair of inverted repeats of 25,156 bp which are separated by a small and a large single copy region of 18,271 bp and 81,936 bp, respectively (Kato et al. 2002). A total of 84 predicted protein-coding genes including 7 genes duplicated in the inverted repeat regions, 4 ribosomal RNA genes and 37 tRNA genes (30 gene species) representing 20 amino acids species were assigned on the genome based on similarity to genes previously identified in other chloroplasts (Kato et al. 2002).

Chloroplast DNA (cpDNA) diversity was exhibited in several species. Intraspecific variation of cpDNA sequences was detected in *Fagopyrum cymosum* (Yamane et al. 2003). Estimated interspecific sequence divergence of *Astragalus* was reached 3.92% (Liston 1992). Amplification specific chloroplast genes of Conifers by polymerase chain reaction (PCR) were detected at 23, 26, 38, 48, 67, and 25 site changes in *frx*C, *rbc*L, *psb*A, *psb*D, *trn*K, and 16S, respectively among species of Conifer (Tsumura et al. 1995). A molecular phylogeny of dipterocarpaceae was constructed which was revealed by 141 site changes in different specific chloroplast genes: *rbc*L, *psb*A, *psb*D, *rpo*B, *rpo*C, *pet*B, *atp*H, 16S, *psa*A, *pet*A, and *trn*K (Tsumura et al. 1996).

Chloroplast DNA are suitable marker for assessing genetic relationship among individuals of plant or plant species such as *Prunus* species (Badenes and Parfitt 1995),

Pinus species (Wang et al. 1999), *Fagopyrum* species (Yamane et al. 2003), among cultivars of Swichgrass, *Panicum virgatum* L. (Hultquist et al. 1996), and among accessions of *Syringa* (Kim and Jansen 1998). The chloroplast genome have been demonstrated that is maternal inheritance in apples (Savolainen et al. 1995; Ishikawa et al. 1992), and largely conserved sequences such as chloroplast gene *rbc*L encoding the large subunit of the RuBisCo of Cucumber, Pumpkin, and Rose (MacKenzie et al. 2002). The chloroplastic *atpB* and *rbc*L coding sequences were found only five divergence sequence in 904 base pairs chloroplast DNA of 40 apples cultivars (Savolainen et al. 1995).

The molecular markers which are very conservative markers and preferable for revealing genetic diversities should be chloroplast DNA (cpDNA) markers. In the previous study, the cpDNAs were already applied for revealing genetic diversities of barley (Russel et al. 2003), potato (Bryan et al. 1999), alfalfa (Mengoni et al. 2000), and several species of plant (Raamsdonk et al. 2003; Kormuak et al. 2003; Viard et al. 2001; Besnard et al. 2002; Parducci et al. 2001). The objectives of research were to reveal the diversities and the differentiations of sago palm in Indonesia based on cpDNA markers.

MATERIALS AND METHODS

Total genome extraction

Leaf tissue samples were preserved by using silica gel granules (Chase and Hill 1991). DNA extraction was following Qiagen DNA isolation protocols 2003. A total 97 samples of sago palm were collected from six islands and nine populations of sago palm centre in several islands in Indonesia. The location and the vernacular name of samples that were used in this experiment were presented in Table 1 and Figure 1.

Table 1. The populations and the vernacular name of sample used

Island	Population	Vernacular name of sample
Papua	Jayapura	Bharahabow, Bharahabow-1, Bharawalisa, Bharawalisa-1, Bharawalisa-2, Folo, Folo-1, Folo-2, Hobolo, Osukhulu, Osukhulu-1, Osukhulu-2, Phane, Phane-1, Phane-2, Rondo, Rondo-1, Ruruna, Ruruna-1, Ruruna-2, Wani, Wani-1, Yakhalobhe, Yebha, Yerirang, Yerirang-1, and Yerirang-2
	Serui	Aming, Aming-1, Animpeun, Awa, Awa-1, Awa-2, Huworu, Huworu-1, Huworu-2, Kurai, Kurai-1, Kurai-2, Sunare, Sunare-1, Sunare-2, Weun, Owawu mambai, Owawu Ureifasei, Owawu-1, Umar, Umar-1, Umbeni, Woru), and Woru-1
	Manokwari	Antah, Anandong, MKW, MKW-1, MKW-D1, and MKW-D2
	Sorong	Bosairo, Bosairo-1, Bosairo-2, Igo, Kororo, Kororo-1, Raimamare, Raimamare-1, and Raimamare-2
Maluku	Maluku	Hihul, Tuni, and Makanalu
Sulawesi	Palopo	Tawaro-1, Tawaro-2, Tawaro-3 and Tawaroduri
Kalimantan	Pontianak	Sagu-1, sagu-2, sagu-3, sagu-4, sagu-5, sagu-6, sagu-7, sagu-8, sagu-9, sagu-10, sagu-11, sagu-12, sagu-13, sagu-14, sagu-15, sagu-16, sagu-17, and sagu18.
Java	Bogor	Kirai-1 and Kirai-2
Sumatra	Selat Panjang	Molat, Riau-1, Riau-2, Riau-D1, Riau-D2, Rotan, and Tuni-R

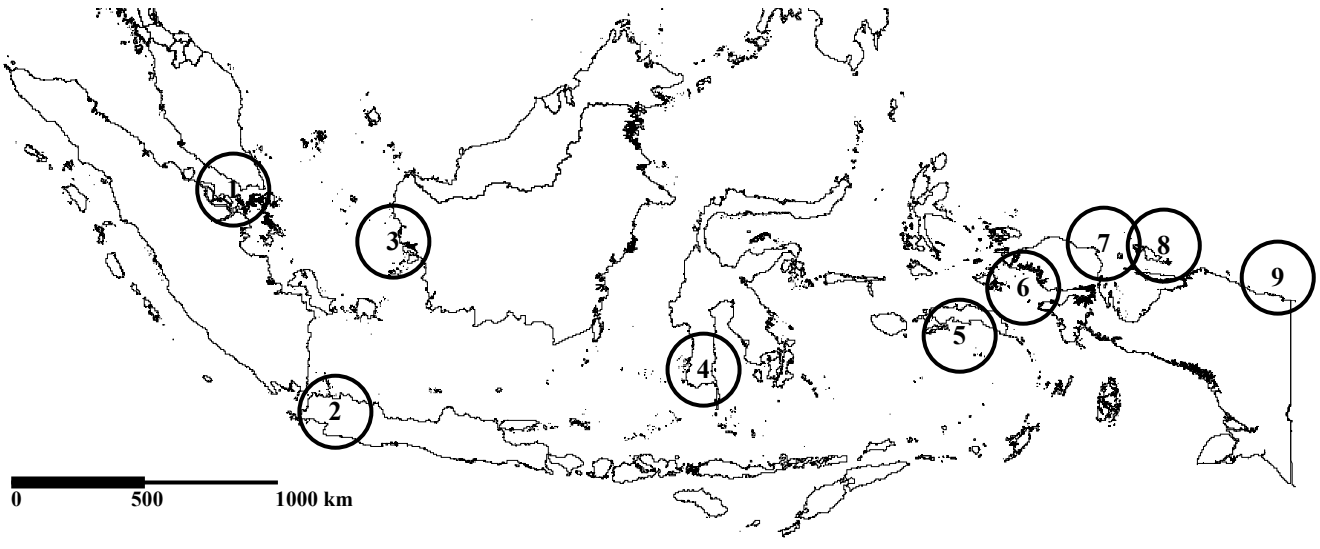

Figure 1. The map of sampling sites of sago palm used. The cycles represent the population sampling: 1. Selat Panjang, 2. Bogor, 3. Pontianak, 4. Palopo, 5. Ambon, 6. Sorong, 7. Manokwari, 8. Serui, 9. Jayapura

Chloroplast DNA Amplification

The cpDNA of sago palm was amplified by using three pairs of primer (rpl1671, NTCP21 and NTCP22) and performed by using polymerase chain reaction (PCR). The PCR reaction mix consisted of 2.5µl of 10x buffer containing 15 mM MgCl2, 0.5µl of 2.mM dNTP (GeneAmp^R mix, Warrington, UK), 10µg of BSA, 0.25µl each of NTCP21 primer pairs (forward primer 44.8 nmol and reverse primer 54.8 nmol) and NTCP22 primer pairs (forward primer 40.8 nmol and reverse primer 49.4 nmol), 0.42 U Ampli Taq Gold^{TM} (Applied Biosystems) and 10ng genomic DNA. The PCR cycle conditions were as follows: initial denaturation step of 4 min at 94^{o}C, followed by 35 cycles of 30 second denaturation at 94^{o}C, 1 min annealing (9 level touchdown) at 59^{o}C for the first cycle, decreasing by 1^{o}C per cycle until the annealing temperature reached 51^{o}C, then continuing 26 cycles at 51^{o}C, 1 min extension at 72^{o}C, and an additional 5 min extension at 72^{o}C at the end of 35 cycles.

Single nucleotide fragment (SNF) analysis

The analysis was performed by using ABI Prism GeneScan^R 3.7. The three primer sets used (rpl1671, NTCP21, and NTCP22) were labeled with FAM, HEX, and NED respectively which synthesized by Qiagen and the PCR reaction was perform as describe above. The total volume cocktail needs for Gene Scan reaction were 11µl as follows: mixed Hi-Di^{TM} Formamide and GeneScane^{TM} – 500 Rox^{TM} size standard (20: 1) and 1µl DNA PCR which have been amplified by fluorescent primer then denatured at 95 µl for 2 min before the plate well insert into Gene Scan tools.

Data analysis

The genetic diversities were calculated by using Infinite Allele Model (IAM, Kimura and Crow 1964). The effective number of haplotypes (ne = $1/\sum P_i^2$) and the haplotypic diversity (H_E = [n/(n-1)][1- $\sum p_i^2$], n is indicated the number of individual analyzed in a population and *p*i is the frequency of the i-*th* haplotype in the the populations, Nei 1987). The Genetic Hierarchies and differentiations were estimated by the Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) by using *Arlequin* software (ver. 2.000, Schneider et al. 2000, University of Geneva, Switzerland). This test can estimate sources of variance (SV) such as among individuals, within populations, among populations within groups, and among groups. Significant values were calculated by a permutation test from 16000 permutated matrices. The AMOVA was based on distances between cpDNA haplotypes calculated from the sum of the squared number of repeat differences between two haplotypes with formula: $d_{xy} = \sum(a_{xy} - a_{yi})^2$, a_{xy} and a_{yi} are the number of repeats for the *i*th locus in haplotype x and y.

RESULTS AND DISCUSSIONS

Polymorphism of cpDNA

Primers rpl1671, NTCP21 and NTCP22 generated PCR products of 100 samples, but no-polymorphism were detected on 3% agarose gels. SNF analysis by using fluorescence primers observed polymorphic on 97 of 100 samples whereas other three samples failed to detect amplification fragments. The characteristic of the fluorescence primer sets were showed in Table 2. The performance of SNF analyses can be seen in Figure 2. The nucleotide detections clearly appeared in each individual sample. Primer pairs of rpl1671-FAM produced 4 alleles in the ranges of the sizes 147 to 406 base pairs (bp), primer pairs of NTCP21-HEX produced 5 alleles in the ranges of the sizes 76 to 406 bp, and primer pairs of NTCP22-NED produced 2 alleles in the ranges of 75 to 160 bp (Table 2). In the previous study, Primer pairs of NTCP21 and NTCP22 also detected alleles polymorphism in potato (Bryan et al. 1999).

Table 2. Characteristic of three cpDNA loci were observed on sago palm

Primers name	Primer Sequences (5'-3')	Location in Tobacco	Numbers of alleles	Base pairs (bp) sizes
rpl1671-FAM	F: gct atg ctt agt gtg tga ctc; R: tca tat agt gac tgt ttc tt	No information	4	147- 406
NTCP21-HEX	F: aaa aag atc cca caa aga aaa; R: ctt atc gat tcc tgt caa aaa g	ORF74A exon	5	76 - 406
NTCP22-NED	F: tat cag aaa aag aaa aag aag g; R: gtc aaa gca aag aac gat t	ORF74A/*trn*S intergenic region	2	75 - 160

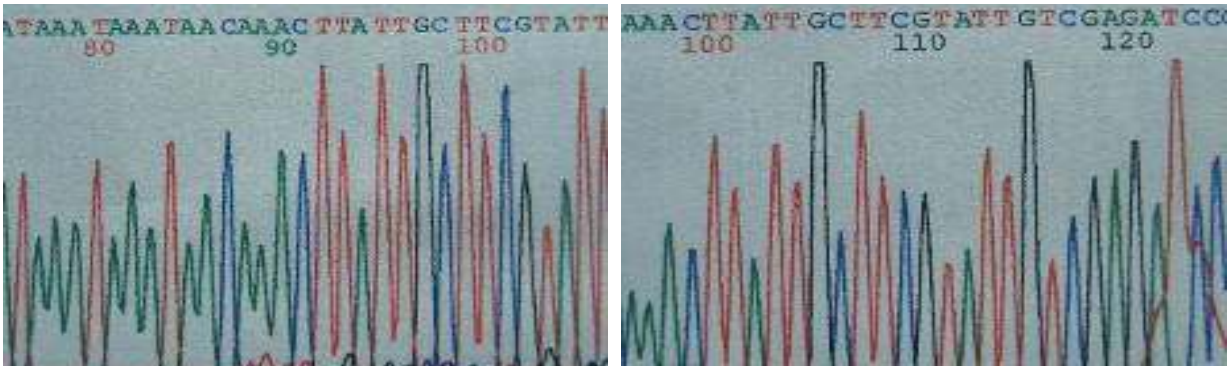

Figure 2. Sample of single nucleotide fragment analyses by using ABI PRISM GeneScan^{R}3.7

Haplotypes identification and composition

In the three cpDNA loci (rpl1671, NTCP21 and NTCP22) of sago palm were observed ten haplotypes and eleven alleles in the populations of sago palm (Table 3). Haplotypes H01 and H02 were located in two cpDNA loci and haplotypes H03 to H10 were located in three cpDNA loci. Composition of haplotypes frequencies in the populations was showed in Table 4. Haplotypes H01, H02, H07, and H09 were found spread into two or more than two populations. Only small individual of sago palm were moved from certain population to others because only four of ten haplotypes were shared into two or more than two populations, which indicated that four haplotypes only of sago palm were estimated to migrate by many ways. In the related studies by using cpDNA markers were also reported that there were refugee population in the species of *P. sylvestris* L. and *A. alba* Mill. (Provan et al. 1998; Vendramin et al. 1999).

Haplotype H05 was found only in the population of Palopo with 0.25 frequencies and haplotype H10 was found only in the population of Pontianak with 0.06 frequencies. Haplotypes H04, H06, and H08 were only found in the population of Jayapura with 0.04, 0.04, 0.12 frequencies respectively. Haplotype H03 was found only in the population of Serui with 0.04 frequencies, so that we found four specific haplotypes in the Papua islands. In these observations we found six specific haplotypes which were distributed in Papua, Sulawesi, and Kalimantan islands. Specific haplotypes phenomenon was also found in the species of *Pinus sylvestris* L. (Provan et al. 1998), *Alyssum* spp. (Mengoni et al. 2003), and *Cunninghamia* spp. (Hwang et al. 2003). Specific haplotypes in the populations indicated that population should be origin of diversities, but the research can not mention the populations of sago palm were diverse because the populations also have a haplotype which shared overall population (H07 in Table 4). Populations from Papua island (Jayapura and Serui), Sulawesi island (Palopo), and Kalimantan island (Pontianak) which belongings specific haplotypes should be origin of sago palm diversities. The degree of haplotypes numbers can be used as one of many indicators to show the centre of diversities. Vendramin et al. (1999) and Mengoni et al. (2003) reported that high number of haplotypes indicated high level of variation. The presence of the widespread common haplotype was indicated a major ancient population (Provan et al. 2001). Papua islands should be origin and centre of sago palm diversities because it has the highest specific haplotypes and the highest numbers of haplotypes, as well as existence of wild types. Haplotypes were described in this study should be existed in a long time in the past because cpDNA markers highly conservative sequences (Provan et al. 2001), very low mutation rates which range from 3.2×10^{-5} to 7.9×10^{-5} (Provan et al. 1999), no recombinant DNA (Ishikawa et al. 1992; Provan et al. 2001) and uniparentally inherited (Savolainen et al. 1995; Viard et al. 2001). The highest numbers of haplotypes indicated the highest variation in the population such as occurred in the population of *Abies alb*a Mill. (Vendramin et al. 1999). Mengoni et al. (2003) documented that the differentiation of chloroplast haplotypes in the population reflected genetic entity. The other report was similar with our observation as follows: Flach (1983) and Flach (1997) were documented that Papua islands are the highest of sago palm diversities based on morphological characters and the widest wild stands. Based on the diversities and vast wild stands the Papua islands should be considered as the centre of diversity (Flach 1997). In Papua also were recognized the highest sago palm varieties based on morphological characters and environmental adaptability (Yamamoto et al. 2005).

Table 3. Haplotypes identification of 97 samples of sago palm by using cpDNA markers

No. of Haplo-types	rpl1671-FAM allels (bp)				NTCP21-HEX allels (bp)					NTCP22-NED allel (bp)	
	147	158	159	406	76	99	160	161	406	75	160
H01	0	0	1	0	0	0	1	0	0	0	0
H02	0	0	1	0	0	0	0	0	0	0	1
H03	0	1	0	0	0	0	1	0	0	0	1
H04	1	0	0	0	0	0	1	0	0	0	1
H05	0	0	1	0	0	0	1	0	0	0	1
H06	0	1	0	0	0	0	0	1	0	0	1
H07	0	0	1	0	0	0	0	1	0	0	1
H08	0	0	0	1	0	0	0	0	1	0	1
H09	0	0	1	0	1	0	0	0	0	1	0
H10	0	0	1	0	0	1	0	0	0	0	1

Table 4. Composition of haplotype frequencies based on 11 polymorphic alleles from 3 cpDNA loci

Haplo-type	1	2	3	4	5	6	7	8	9
H01	-	-	-	-	-	-	-	0.04	0.08
H02	0.14	0.50	0.31	-	0.33	-	-	0.12	-
H03	-	-	-	-	-	-	-	0.04	-
H04	-	-	-	-	-	-	-	-	0.04
H05	-	-	-	0.25	-	-	-	-	-
H06	-	-	-	-	-	-	-	-	0.04
H07	0.86	0.50	0.63	0.75	0.67	1.00	1.00	0.77	0.58
H08	-	-	-	-	-	-	-	-	0.12
H09	-	-	-	-	-	-	-	0.04	0.15
H10	-	-	0.06	-	-	-	-	-	-

Note: Population from Selat Panjang (1), Bogor (2), Pontianak (3), Palopo (4), Ambon (5), Sorong (5), Manokwari (7), Serui (8), dan Jayapura (9), and Number of haplotypes (H01 to H10).

Genetic diversity

The genetic diversities within the island showed that Papua island has the highest values of haplotype numbers, polymorphic sites numbers, and percentages of polymorphic haplotype than any other islands. Java island has the highest mean pairwise differences (Table 5). Haplotype diversity values (H_E) among individual were relatively high than overall populations. H_E value of one indicated that no haplotype sharing in the sample (single haplotype) or samples different from the others such as occurred in the island of Java. These features occurred probably by the sizes of samples were very limited and criteria of sampling in the population it based on phenotypic differences. In the related cases were also

recorded in individual tree (*Pinus sylvestris* L.) within the Woodland (Provan et al. 1998). In the previous study of sago palm using RAPD markers high variance in individual level of sago palm was observed (Ehara at al. 2003) and was also observed using AFLP (Celiz et al. 2004).

Table 5. Genetic diversity based on 10 haplotypes and 11 polymorphic alleles

Island	Genetic variabilities						
	No. of populations	R_{IP}	$\sum_H$	S	π	H_E	P
Papua	4	16	8	9	0.9216	0.4544	81.8182
Maluku	1	3	2	1	0.6667	0.6667	9.0909
Sulawesi	1	4	2	1	0.5000	0.5000	18.1818
Kalimantan	1	17	3	2	0.6029	0.5221	27.2727
Java	1	2	2	1	1.0000	1.0000	9.0909
Sumatra	1	7	2	1	0.2857	0.2857	9.0909

Note: average individual number per population (R_{IP}), haplotype number ($\sum_H$), polymorphic site number (S), haplotype diversity (H_E) mean pairwise differences (π), percentage of haplotype polymorphism (P)

Genetic hierarchy and differentiation

AMOVA values of sources of variance (SV) indicated that among islands (-3.88% and $F_{CT=}$ -0.03884) were no significantly different, but SVs values of within populations (95.39% and $F_{ST=}$0.04610), among populations irrespective of islands (5.91% and $F_{CT=}$0.05054), and among populations within the island (8.49% and $F_{SC=}$0.0817) were significantly different. The highest level of variation percentages was observed within the population (95.39% and $F_{ST=}$0.04610) (Table 6). The largest part of genetic variation in the sample populations was attributed to variation within the population then following by among populations within the island and among populations irrespective of the islands (among populations). Among populations in the Papua island contributed the largest part variation to the total variant. The probabilities of over all SV were significantly different, except among islands were no significantly different. In the previous study on the *Pinus sylvestris* L. low percentages of variant among populations (3.24%) was observed, but probability value was significantly different (Provan et al. 1998). The negative values were observed for among the islands (Table 6). It means that the islands did not contribute to the total variance. These features similar to the tetraploid alfalfa populations were observed by Mengoni et al. (2000). The biological meanings of negative values of coefficient correlation (F_{CT}) were samples among the islands more related than samples within the islands (Schneider et al. 2000). No significant differences among the island can be estimated that the geographical differences were not contributed to population variation. The small percentage of variant among populations were observed (5.91%) in sago palm. This was similar to Parducci et al. (2001) observed in *Abies* sp. Parducci et al. (2001) reported that variation among populations was low (6.10%), but variation within population or among individuals was high (74.66 %). Another low genetic variation was found in *Pinaceae* sp. (Viard et al. 2001) and tree species (Austerlitz et al. 2000). These observations showed that the site of within and among populations of sago palm should be the main focus for conservation and sustainable used rather than the site of islands.

CONCLUSIONS

cpDNA markers were used for accessing genetic diversities of sago palm in Indonesia it showed polymorphism. Ten haplotypes and eleven alleles were found into on 97 samples of sago palm. Specific haplotypes were detected in the population from Papua, Sulawesi, and Kalimantan islands. Sulawesi and Kalimantan islands will be source of sago palm diversities and Papua islands will be origin and centre of sago palm diversities in Indonesia based on cpDNA data. The haplotype numbers were ranged from 2 to 8, the polymorphic sites were ranged from 1 to 9, the mean numbers of pairwise differences were ranged from 0.2857 to 1.0000, the haplotype diversities were ranged from 0.2857 to 1.0000, and the percentages of polymorphic haplotype were ranged from 9.0909 % to 81.8182 %. Genetic hierarchies and differentiations of sago palm samples were estimated by AMOVA which showed significantly differences among individuals and among population. Population from Jayapura was significantly different with population from Palopo and Pontianak. Genetic differentiations of sago palm samples were observed significantly different within the population, among populations, and among populations within the island, but no significantly different was observed among islands.

Table 6. Analysis of molecular variance (AMOVA) based on eleven alleles and ten haplotypes

Source of variation (SV)	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Index	*P*
Among island	5	2.382	-0.01573	-3.88	$F_{CT=}$-0.03884	0.63069^{ns}
Among populations within an island	3	2.634	0.03440	8.49	$F_{SC=}$0.08177	0.03366*
Within populations	88	33.995	0.38630	95.39	$F_{ST=}$0.04610	0.04574*
Total	96	39.010	0.40497			
Among populations irrespective of island	8	5.016	0.02428	5.91	$F_{ST=}$0.05914	0.04772*

Note: AMOVA calculation performed by 10,000 permutations. Degree of freedom (d.f), Probability (*P*) Fixation Index of samples among island levels (F_{CT}), Fixation Index of samples among population within an island levels (F_{SC}), Fixation Index of samples among individual levels or within population levels (F_{ST}), not significantly different (ns), and significantly different (*)

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