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Research Article
Analysis of Genetic Diversity of Sago Palm (*Metroxylon sagu* Rottb.) in West Kalimantan Province, Indonesia Using SSR Markers

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Abstract

Background and Objective: Sago palm (*Metroxylon sagu* Rottb.) is one of the largest carbohydrate-producing plants in the world. SSR has an essential role in the genome and is widely used to determine plant diversity compared to other molecular markers. This research focused on identifying the diversity of sago palms in the West Kalimantan region using SSR markers. The benefit of this research is to obtain EST-SSR information on sago palms that can be used to design SSR primers so that it can be used to study the diversity of sago populations in the West Kalimantan Region. **Materials and Methods:** DNA isolation was obtained by extracting young leaves of sago palms originating from West Kalimantan. The DNA concentration and quality were measured using Nanodrop. SSR amplification using PCR was carried out on sago palm accessions with polymorphic primers from sago accession selection. **Results:** DNA concentration measurements were 33.8-1380.3 ng μL^{-1} and DNA purity was 1.8. The results of the SSR analysis using seven primers on 27 sago accessions produced an average number of alleles and the percentage of polymorphic primers 3.57 and 0.538, respectively. The highest number of alleles and PICs produced by sV2006 primers are 5 and 0.721. Analysis of the diversity of 27 accessions of sago with seven primers in the form of phylogenetic trees forming three different groups. **Conclusion:** There was a high diversity of 37 accessions of sago Kalimantan in our study with 7 primers and the phylogenetic trees formed three groups. The sago accession from West Kalimantan has different variations between Pontianak and the accession from Mempawah, while the accession from Sambas has several similarities with Mempawah and Singkawang.

Key words: Genetic diversity, sago palm, SSR markers, Sambas, accessions

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesia is known as the centre of sago palm diversity. Various accessions with wide genetic variation have been found in the provinces of Papua, West Papua and Maluku[1,2]. The palm was then spread to Sulawesi and Western Indonesia such as Kalimantan, Java and Sumatra. Sago is one of the palm trees that produce starch. Sago starch, which was initially a staple food for people on the island of Papua, has now started to be consumed by many people in other areas, especially because of its benefit for non-gluten and low glycemic index. Sago food has also become the main choice for people with diabetes[3,4] and autism[5,6]. Nowadays various food industries made from sago have started to rise and develop, with the demand for products at home and abroad that continues to increase.

Sago forest is found in the lowlands to an altitude of 1.000 m above sea level, in swamps, on the banks of rivers, banks of lakes and in moist areas whereas wood waste from sago can be used as raw material for pulp and paper[7]. Sago is commonly found in West Kalimantan and grows naturally in wetlands and peatlands[8]. The local sago varieties found in West Kalimantan are *bemban* and *buntal*[8,9]. Sago palms found in the West Kalimantan Region grow naturally and irregularly. The extent of reaching around 2,430 ha spread across Sambas, Pontianak, Ketapang and Sanggau Regencies. Sago in this area is spread out in small clumps along rivers, marshes and some grow side by side with rubber trees. Sago palms in some districts in West Kalimantan only have their leaves taken for use as roofing material and have not been used as a source of starch. However, in some areas of sago centres in West Kalimantan, there are also traditional industries that process sago stems to obtain starch[7]. According to Maherawati *et al.*[8], sago starch from West Kalimantan has high amylose content.

One of the ways to protect Indonesian germplasm, especially sago palms, should be an inventory and characterization of both phenotypes and genotypes. Genetic markers are known to have a vital role in uncovering and studying plant diversity and population genetics with techniques to detect genetic variability between individuals, populations and species. Knowledge of genetic variability is a prerequisite for studying the evolutionary history of a species and also for breeding and conservation programs for plant genetic sources. Genetic diversity data is needed to protect sago palms and their genetic components, which are thought to be native to Indonesia so that other countries do not exploit them. The genetic diversity of sago in Indonesia can be identified by an approach based on molecular biology methods based on sequences of deoxyribonucleic acid (DNA) and polymerase chain reaction (PCR) techniques.

So far, the characterization of sago palms was done phenotypically[8,10] based on differences in morphology, odour, taste, colour, texture and size. Identification of plants that is done phenotypically has limitations, including the existence of variations between subjective analysis of raw materials and the confusion of the character of each plant. The development of molecular characterization techniques based on PCR is a solution to detect and determine the authenticity of raw materials for sago palms. DNA marking techniques have been widely used to determine the authenticity of valuable species of a plant. DNA markers are very accurate because they can provide polymorphism information, as a unique genetic composition in each species, which does not depend on age and physiological conditions such as environmental factors. One DNA marker that is often used is SSR (Simple Sequence Repeat). Xiao *et al.*[11] suggest several reasons for the use of SSR namely: The abundant site SSR, distributed with uniform in the plant genome, highly polymorphic, codominant, expected in producing quickly through PCR and is easily accessible by other laboratories through publications primer sequences.

The purpose of this study was to identify SSR markers in sago palm EST sequences, design and synthesize SSR primers and study the diversity of sago palms in the population of West Kalimantan. The benefit of this research is to obtain EST-SSR information on sago palms (*Metroxylon sagu* Rottb.) that can be used to design SSR primers so that they can be used to study the diversity of sago populations in the West Kalimantan region.

MATERIALS AND METHODS

Study area: A total of 37 sago palm individual trees originally collected from West Kalimantan were used in this study. All these sago palm samples were selected based on geographical different places, where spread in seven districts (Pontianak, Landak, Kubu Raya, Bengkayang, Mempawah, Singkawang and Sambas). The processing of research results was carried out from March, to November, 2019 at the Gene Technology Laboratory, Laboratory for Biotechnology, the National Research and Innovation Agency (BRIN), in the Puspiptek Area, Serpong, Banten.

The sample used in this study was young leaves of sago palms, each location was recorded using GPS. Sago leaf DNA isolation method is based on the method developed by Purwoko *et al.*[12] with some modifications. The quality and quantity of DNA were determined by electrophoresis on a 1% agarose (w/v) gel in TAE buffer at a voltage of 100 V for 30 min using eXU MUPID and using a NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

Table 1: Quality and quantity of total DNA derived from 37 accessions of sago palm

Sample origin	Code sample	Concentration (ng uL^{-1})	Purity (260/280)
Kuala Mandor 1, Pontianak	P1	237.8	1.86
Kuala Mandor 2, Pontianak	P2	357.5	1.83
Desa Sungai Enau, Pontianak	P3	172.5	1.79
Rantau Panjang, Sebangki, Kabupaten Landak	P4	396.3	2.03
Sul Ambawang Kuala, Sungai Ambawang, Kabupaten Kubu Raya	P5	136.3	1.92
Sul Ambawang Kuala, Sungai Ambawang, Kabupaten Kubu Raya	P6	207.9	1.86
Gg. Limbung, ArangLimbung, Sungai Raya, Kabupaten Kubu Raya	P7	201.7	1.69
Sungai Ambangah, Sungai Raya, Kabupaten Kubu Raya	P8	117.1	1.43
Sungai Ambangah, Sungai Raya, Kabupaten Kubu Raya	P9	336.1	1.71
Sungai Ambangah, Sungai Raya, Kabupaten Kubu Raya	P10	113.3	1.46
Sungai Ambangah, Sungai Raya, Kabupaten Kubu Raya	P11	377.6	1.91
Bengkayang	BKY1	771.6	1.95
Singkawang	SKW1	563.8	1.53
Singkawang	SKW2	1380.3	1.91
Mempawah	MPW1	861.6	1.68
Mempawah	MPW2	45.1	1.81
Mempawah	MPW3	356.7	1.49
Mempawah	MPW4	308.4	1.79
Sambas (Utara)	SBS1	33.8	1.92
Sambas (Utara)	SBS2	49.6	1.66
Sambas (Utara)	SBS3	432.9	1.57
Sambas (Utara)	SBS4	361	1.66
Sambas (Utara)	SBS5	591.2	1.59
Sambas (Utara)	SBS6	1013	1.52
Sambas (Utara)	SBS7	193	1.77
Sambas (Utara)	SBS8	465.6	1.53
Sambas (Utara)	SBS9	499.7	1.69
Sambas	SG01	499.8	1.98
Sambas	SG02	514.16	1.76
Sambas	SG03	1406.6	1.91
Sambas	SG04	1541.2	1.89
Sambas	SG05	698	1.93
Sambas	SG06	724.73	2.04
Sambas	SG07	766.46	1.96
Sambas	SG08	832.7	1.97
Sambas	SG09	305.03	1.79
Sambas	SG10	358.66	1.79

Furthermore, the DNA of all samples was visualized with UV transilluminator Vilber Lourmat and documented using a digital camera.

Research protocol: SSR amplification was carried out using seven SSR markers from Purwoko *et al.*[12] in Table 1. DNA amplifications were performed in a PCR machine Takara PCR Thermal Cycler Dice® (http://catalog.takara-bio.co.jp/product/basic_info.php?unitid=U100004192). Amplifications were carried out in 25 µL reaction solution containing Taq buffer (5 µL), KAPA Taq Extra HotStart(0.25 µL), 25 mM $MgCl_2$ (1.5 µL), 10 mM dNTP (0.5 µL) primer forward (0.5 µL), reverse primers (0.5 µL) and ddH_2O to a volume of 25 µL. The program used was as follows: One cycle of 95°C for 3 min, 35 cycles of 95°C for 30 sec for denaturation, 35 cycles of Tm-5°C for 30 sec for annealing and 35 cycles of 72°C for 30 sec and final extension 72°C for 60 sec and ended with a temperature of 4°C. The PCR products for each sample were separated on 1.5% metaphor agarose gel in TAE buffer at 50 V for 45 min and then stained with Sybr safe gel stain. These were visualized and photographed under a UV Light Transilluminator (Biorad, USA). The sized DNA products were calculated by comparison with the 100 bp DNA ladder (Vivantis, Malaysia).

The dissimilarity matrix was calculated based on allele data for level two ploidy and a simple dissimilarity index. Dissimilarity matrix calculation using bootstrap analysis at 10,000 iterations. Factorial analysis based on dissimilarity was determined using options from 37 axes to be edited and factorial analysis was selected after the default axis was determined. We perform phylogenetic tree construction using dissimilarities matrix, which is calculated by the method of approach neighbour-joining. Dissimilarity matrix, bootstrap, factorial analysis and tree construction are phylogenetic for accession sago done using dissimilarity analysis and representation for Windows (Darwin) 6:05 version[13].

Several population parameters were calculated (allele numbers, expected and observed heterozygosity and polymorphism information content) for each SSR marker locus using Cervus software version 3.0[14] and GENALEX software version 6.501[15]. STRUCTURE software version 2.3.4[16] (https://pritch.Bsd.uchicago.edu/structure.html) was used to analyze population structure and differentiation by allele frequencies. For the calculation of the estimated ideal population (K), each individual in the mixed model was run with K = 1 to 10 and each K was replicated 20 times. Each replication is applied with a 100,000 step burn-in period followed by 250,000 Monte Carlo Markov Chain (MCMC) replications. Ad-hoc statistics are evaluated to estimate changes in log data probabilities according to K values as suggested by Evanno *et al.*[17]. Whereas the ideal population is determined based on the highest K value estimated using the harvester structure at (http://taylor0.biology.ucla.edu /struct_harvest/)[18].

RESULTS AND DISCUSSION

Total genome DNA isolation: DNA isolation of sago palms was performed by extracting the young leaves from 37 accessions of sago palms from West Kalimantan. The measurement of the quality and quantity of the isolated DNA was revealed in Table 1.

DNA concentration and quality were measured using Nanodrop. The measurement results showed that the concentration varied from 33.8-1541.2 ng μL^{-1}. The ratio of optical density (OD) at the wavelengths of 260 and 280 nm is generally used to detect the purity level of DNA. In general, the acceptable DNA purity is at a ratio of 1.8. When the ratio is low, it's indicated the presence of protein, phenol or other contaminants that absorb strongly at a wavelength of 280 nm[19]. The results quality of DNA isolation in our study also varied from 1.43 to 2.04. Some of the DNA purity was low, however, all of them were used for the next stage, i.e., PCR amplification.

SSR primer selection: The SSR primers used for the identification of sago palm diversity from West Kalimantan can be seen in Table 2. The sago palm accessions used in this study consisted of 5 populations. Seven primers succeeded in forming amplicons on 1% agarose gel. Unfortunately, the separation of amplification results on 6% agarose gel for 3 hrs was not able to show polymorphic alleles. The separation of the SSR amplification results in the sago genome was then continued by using 3% metaphor agarose gel for 3 hrs. Furthermore, 7 polymorphic loci were used for the analysis of sago palm diversity.

Amplification was carried out for 7 loci using 7 pairs of primers in 37 accessions of sago palms. The primers used had allele sizes from 247-596 bp, five of which had allele sizes of around 300 bp. Five out of seven primers synthesized flanking the di-nucleotide SSR motif. The other two primers flanked the tri-(AAG) and tetra-nucleotide (AAAT) motifs. The polymorphic alleles in this study were mostly found in the di-nucleotide motif, especially the AG motif. A similar result was also reported by Ting *et al.*[20] that di-nucleotide produces the most polymorphic alleles. The results of PCR amplification were then visualized on 11% agarose gel for scoring in Fig. 1. The SSR profile of sV67385 showed a polymorphic pattern in the samples from Mempawah (No. 15 and 16, marked in circle line) which can be seen from the size of the resulting bands. A similar result was also found in the SSR sV2006 profile which showed a polymorphic pattern in samples from Sambas (No. 20 and 21).

Data analysis of SSR marker: A total of 35 alleles were detected with a range of 4-7 alleles per locus based on the results of the analysis using 7 SSR markers. The statistical summary of the analysis of polymorphisms produced by SSR markers is presented in Table 3. The number of total alleles obtained in our study is more than that conducted by Abbas *et al.*[21] which only obtained 3 polymorphic alleles using SSR primers from coconut plants. Our result is even higher than that of Purwoko *et al.*[12] which detected 24 alleles based on 7 SSR markers in 41 sago accessions. The difference in the number of alleles obtained is thought to be related to differences in genetic material, including the number and genetic background, the number and types of SSR markers, whether functional markers or universal markers[22,23], as well as the resolution of DNA separation.

The results of SSR analysis using 7 primers in 37 accessions of sago yielded the average number of alleles and the percentage of polymorphic primers (PIC) of 5 and 0.654, respectively. The highest number of alleles and PIC produced by primers sV2006 were 7 and 0.798 (Table 3). The average PIC value of 0.654 indicated that the loci used were very informative. According to Mateescu *et al.*[24], the PIC values are grouped into three, namely the uninformative group with a PIC value <0.3, quite informative with a value of 0.3-0.59 and very informative with a value of >0.6. The theoretical PIC value can range from 0 to 1. A value of 0 means that the locus has only one allele (monomorphic) while a PIC value of 1 means that the locus has unlimited alleles[25]. A low PIC value indicates a low ability to differentiate genotypes[26].

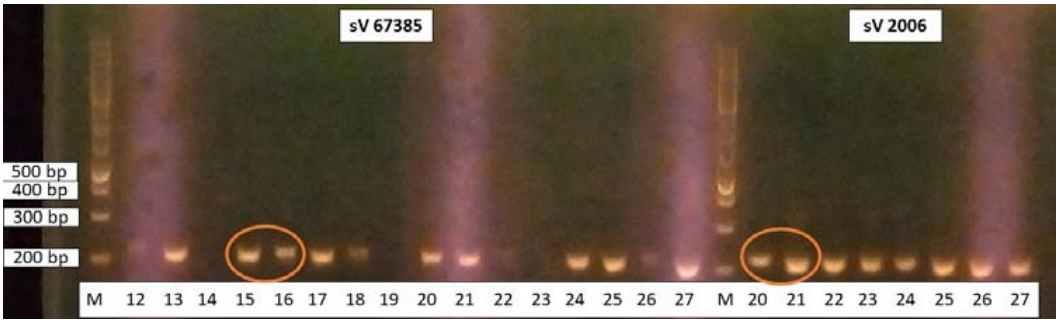

Fig. 1: Visualization of PCR primer products sV67385 and sV2006 on 1.5% agarose gel visible polymorphic bands are circled

Table 2: Characteristics of 7 pairs of primers used[12]

Primer	Source	Primary sequences (5'-3')	Motifs	Product size (bp)	*Oryza sativa* chromosome	E-value
sV2006	NGS-Illumina	F: GTATAGATGGAAAGCGTTGG R: CCGCTCCTTATCCTAGTCTT	AT	247	Chr 2	3.00E-28
sV400785	NGS-Illumina	F: ACTCCGCTCACTTGCACA R: GCACGCCTAAGGATGGAA	AG	300	Chr 5	8.00E-08
sV513907	NGS-Illumina	F: GGCGGAGCTTCAAGAACA R: TCAATGCCAGACAAAGATGC	AG	312	Chr 6	0.016
sV67385	NGS-Illumina	F: AGCACCGAAGGAAACAACC R: AGCCGAAAAGCCGAGTCT	AG	310	Chr 7	1.00E-05
sV109470	NGS-Illumina	F: CCCATGCCTTATGCTGGA R: CTTGCTGGCTAGTGCCAAT	AAG	360	Chr 9	4.00E-24
sV100242	NGS-Illumina	F: TTGAGCCAGGTATCATCCAA R: ATCGTGGCAGAAGGTGGT	AAAC	308	Chr 10	0.028
sV2283	NGS-Illumina	F: ACGGACCAGTCGGCATTA R: TCGGGGAGAGAGCGATTA	AG	596	Chr 2	2.00E-63

Table 3: Allele size, chromosomal synthesis, number of alleles per primer (N), percentage of polymorphic primers (PIC), observed heterozygosity (Ho) and expected heterozygosity (He) in 27 sago accessions based on 7 SSR primers

Primary	Allele size (bp)	Chromosome *Oryza sativa*	E-value	N	PIC	Ho	He
sV2006	247	Chr 2	3.00E-28	7	0.798	0.378	0.834
sV400785	300	Chr 5	8.00E-08	4	0.51	0.784	0.588
sV513907	312	Chr 6	0.016	4	0.612	0.459	0.688
sV67385	310	Chr 7	1.00E-05	4	0.67	0.568	0.733
sV109470	360	Chr 9	4.00E-24	4	0.653	0.297	0.719
sV100242	308	Chr 10	0.028	6	0.663	0.649	0.725
sV2283	596	Chr 2	2.00E-63	6	0.675	0.351	0.727
Average				5	0.654	0.498	0.716

The observed heterozygosity value (0.498) has a lower mean than the expected heterozygosity value (0.716). According to Boonsrangsom *et al.*[27], high He indicates that the locus has a high level of diversity and is the most informative in plant identification. At locus sV400785, the observed heterozygosity was higher than the expected heterozygosity. This may be due to the small sample size and the presence of null alleles. The heterozygosity value indicates the probability of a marker used to distinguish two randomly selected alleles in a population if the heterozygosity value is higher, most of the samples tested are heterozygous, thus showing diversity in a population[28]. The higher the level of heterozygosity, the higher the ability of a marker to differentiate between genotypes genetically. The heterozygosity value resulting from genotype testing using SSR markers can detect high differences in genotypes in a population with almost the same morphological characteristics[29].

Phylogenetic analysis: Phylogenetic analysis was carried out to identify the genetic distance between varieties[30]. Analysis of the diversity of 37 accessions of sago Kalimantan using 7 SSR markers was carried out based on observations of the overall frequency of alleles that appeared and the results of dendrograms using the method of Unweighted Neighbor-Joining (UNJ). The phylogenetic tree formed three different groups in Fig. 2. The first group collected various accessions from various regions in Kalimantan, namely Pontianak (P1, P4, P5, P6, P9, P11), Sambas (SBS1, SBS2, SBS3, SBS4), Mempawah

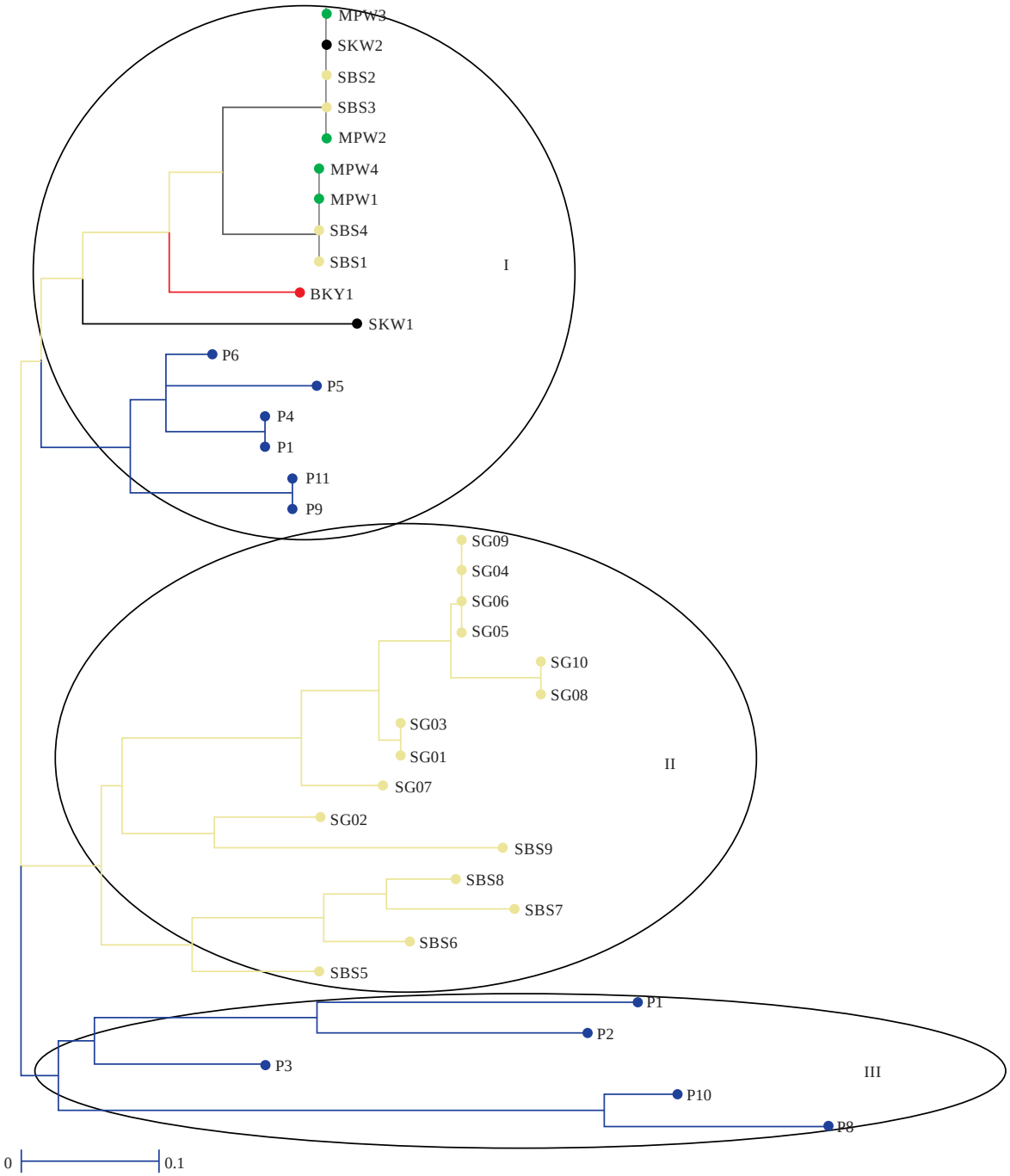

Fig. 2: Phylogenetic tree of 37 sago accessions with 7 SSR markers using unweighted neighbour-joining

(MPW1, MPW2, MPW3, MPW4), Singkawang (SKW1, SKW2) and Bengkayang (BKY1). The second group collected accessions only from Sambas (SBS5, SBS6, SBS7, SBS8, SBS9, SG01, SG02, SG03, SG04, SG05, SG06, SG07, SG08, SG09, SG10) while the third group collected accessions only from Pontianak (P2, P3, P7, P8, P10). The sago accession from Kalimantan has different variations between the Pontianak accession and from Mempawah, while the accession from Sambas has some similarities with Mempawah and Singkawang.

PCoA is a method that explores the dissimilarity of data through a dissimilarity matrix and assigns each accession a location in a low-dimensional space[31]. The results of the PCoA analysis showed agreement with the results of the phylogenetic analysis which revealed three separate groups

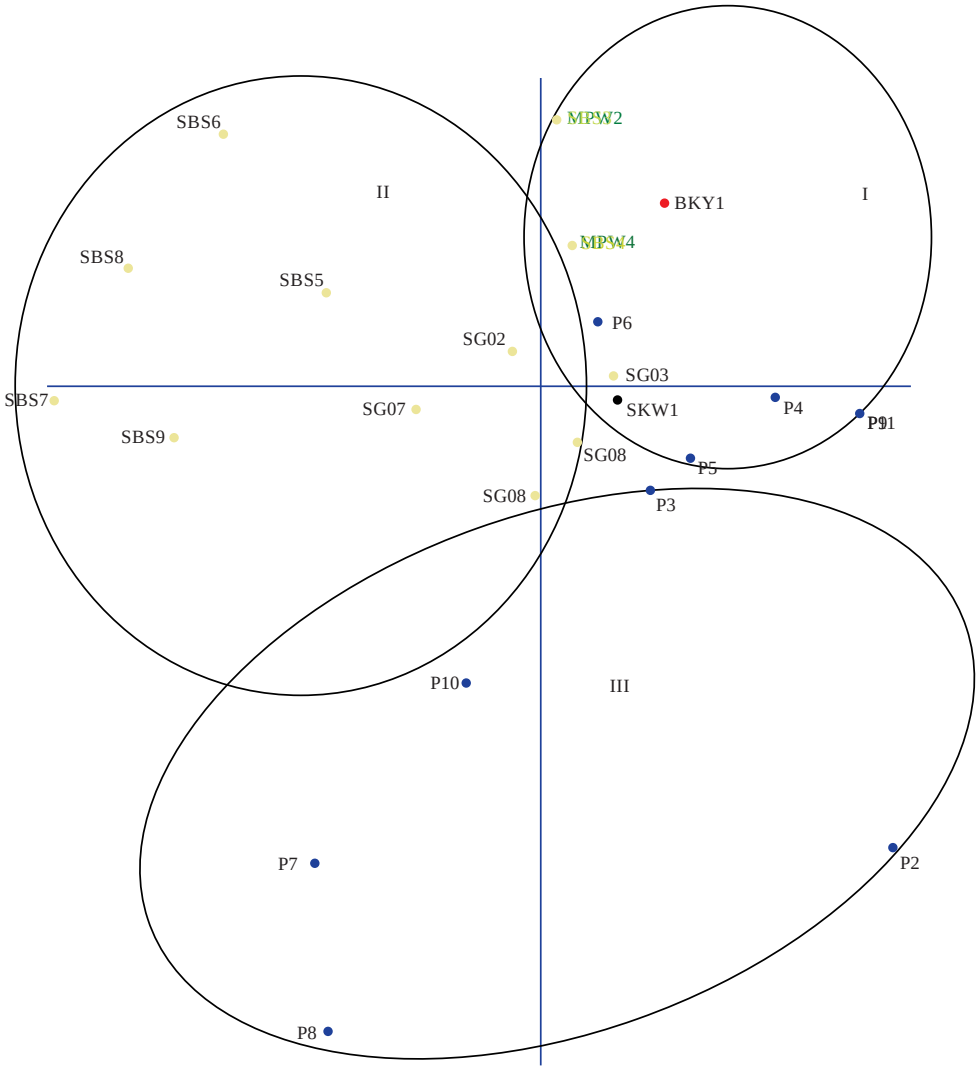

Fig. 3: Principle coordinate analysis (PCoA) 37 sago accessions with 7 SSR markers

among the Kalimantan sago accessions in Fig. 3. The first and largest cluster contains 17 accessions (P1, P4, P5, P6, P9, P11, SBS1, SBS2, SBS3, SBS4, MPW1, MPW2, MPW3, MPW4, SKW1, SKW2, BKY1). The second cluster consists of 15 accessions from Sambas (SBS5, SBS6, SBS7, SBS8, SBS9, SG01, SG02, SG03, SG04, SG05, SG06, SG07, SG08, SG09, SG10) and the third contains accessions from Pontianak (P2, P3, P7, P8, P10). The first cluster has a high diversity consisting of accessions from Sambas, Pontianak, Singkawang, Mempawah and Bengkayang. The results of PCoA analysis showed a similar trend with phylogenetic analysis on all accessions.

CONCLUSION

The level of genetic diversity of 37 accessions of sago Kalimantan in the current study was quite high based on the results of cluster analysis and main coordinates using 7 SSR markers that were able to distinguish accessions based on location. Analysis of the diversity of 37 accessions of sago with 7 primers in the form of phylogenetic trees formed three groups, namely: In the first group gathered accessions from Pontianak and Sambas, the second group gathered accessions from Mempawah and Sambas, the third group gathered accessions from Mempawah and Sambas. The sago accession from Kalimantan has different variations between Pontianak and the accession from Mempawah, while the accession from Sambas has several similarities with Mempawah and Singkawang. This study found that there is the genetic diversity of the Kalimantan sago plant can be beneficial for conservation and plant breeding activities. This research will help researchers to uncover critical areas of information on the genetic diversity of sago palms based on SSR markers that are not widely known.

SIGNIFICANCE STATEMENT

This study discovered the genetic diversity of sago palm in Kalimantan Island that can be beneficial for conservation and preservation of sago palm, as well as, starch composition studies linked to markers, which in the end can be used for the development of sago palm by using marker-assisted breeding.

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