GENETIC VARIABILITY OF ARROWROOT (*Maranta arundinacea* L.) IN YOGYAKARTA PROVINCE, INDONESIA BASED ON ISSR ANALYSIS

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ABSTRACT

The cultivation of arrowroot (*Maranta arundinacea* L.) in Indonesia, particularly in Yogyakarta Province has a great potential to be developed. This study aimed to determine genetic variability and analyze the intraspecific relations of arrowroot using Inter Simple Sequence Repeats (ISSR) markers to be used as basic information in considering characters selection of arrowroot cultivation in Yogyakarta. Exploratory survey method was conducted in Gunungkidul, Kulon Progo, Sleman, and Bantul Districts to collect cultivar accessions. Accessions were replanted in Sawitsari Research Station. DNA isolation from the leaves of 7-month-old accessions was carried out using CTAB buffer solution. The DNA fingerprinting analysis was carried out using the result of DNA amplification with 4 ISSR markers. The polymorphism data were then used for phenetic analysis using UPGMA algorithm and Baroni-Urbani Busser similarity coefficient to form a dendrogram. A total of 5 local cultivars were found, identified as 'Sili', 'Sembowo', 'Sugo', 'Kebo', and 'Teropong'. Each cultivar showed distinct rhizome morphological characteristics. The ISSR-PCR analysis resulted in high polymorphism with a 68.17% polymorphism mean. The mean of polymorphic band was 6.75. The dendrogram was developed based on the analyses and consisted of 4 clusters with 80% similarity index. Cluster A, B.I. and B.II.b consisted of 'Sili', 'Teropong', and 'Kebo' cultivars, respectively, while cluster B.II.a gathered 'Sugo' and 'Sembowo' cultivars.

Keywords: cluster analysis, intraspecific classification, ISSR markers, polymorphism

INTRODUCTION

Arrowroot cultivation in Indonesia has a great potential to be developed. Flour made from arrowroot rhizome has high economic and nutritional values (Madineni *et al.* 2012; Guilherme *et al.* 2017). Arrowroot flour contains high protein, even greater than that of cassava flour (Aprianita *et al.* 2014), low glycemic index value and high fiber content. Therefore, food products made from arrowroot flour are easier to digest compared with those made from other types of flour (Lestari *et al.* 2017). Food products made by arrowroot fluor is also recommended for people with digestive disorder and the elderdy (Silva *et al.* 2000; Heredia-Zárate & Vieira 2005; Mason 2009; Silveira *et al.* 2013) Furthermore, arrowroot flour has long been used traditionally by the household and by the food industries as a basic ingredient for baby food. Further research revealed that arrowroot flour is potential to be used as a thickening agent in various industries, such as cosmetics, pharmaceutical and food industries (Kitahara *et al.* 2007). In addition to these benefits, the widespread arrowroot population in Indonesia, especially in Yogyakarta Province, is the main reason for developing arrowroot cultivation (Djaafar *et al.* 2010; Masitoh 2014).

Information obtained from molecular analysis of a plant population can identify genotype differences among individuals as a selection step in plant breeding programs. An efficient character selection process by relying on the information about the genetic variation of a wild plant population with a suitable

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breeding method has succeeded in developing modern cultivars, which has increased the yield of various food crops since the mid-20th century. Molecular markers applied to reveal genetic variabilities can thus, provide promising information in agriculture as well as in protecting crop variabilities (Govindaraj *et al.* 2015; Perez-de-Castro *et al.* 2012; Faraldo *et al.* 2003).

Inter Simple Sequence Repeat (ISSR) is one of molecular markers commonly used in the study of genetic diversity, phylogeny, gene detection, genome mapping and evolutionary biology in various types of plants (Reddy *et al.* 2002). The combination of using ISSR in PCR has been done in genetic fingerprinting (Blair *et al.* 1999), cultivar identification (Wang *et al.* 2009), phylogenetic analysis (Gupta *et al.* 2008) and assessment of hybridization (Wolfe *et al.* 1998). The ISSR marker uses a 16-25 bp single primer that is specific to target identical regions among microsatellites. Primers composed of eight repeating dinucleotide units, six repeating trinucleotide units or several repeating tetra- or pentanucleotide units with or without nucleotide anchorage system that targets the microsatellite region (Zietjiewicz *et al.* 1994).

Various types of molecular markers were used to reveal the genotype diversity of arrowroot, especially between the local varieties and cultivars. Two of the molecular markers are RAPD and ISSR markers (Pinto 2015; Asha *et al.* 2015). This study aimed to determine genetic variability and analyze the intraspecific relationship of arrowroot population in Yogyakarta Province, Indonesia by using the ISSR markers. The expected results were the diversity of arrowroot germplasm in Indonesia which can be used to strengthen the phenotypic characters variations that have been used in arrowroot cultivation and breeding.

MATERIALS AND METHODS

Plant Materials

Sample collection was carried out during the dry season, from August to September 2019 in 18 subdistricts in Sleman, Gunungkidul, Kulon Progo, and Bantul Districts in Yogyakarta Province using the exploratory survey method. The whole arrowroot rhizome was taken from each accession and then replanted in the Sawitsari Research Center Plantation, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. Species identification was carried out using the key of determination from van Steenis (1975), Woodson and Schery (1980), Wu and Kennedy (2000), Hammel *et al.* (2014), Lim (2016). Meanwhile, the cultivar identification was carried out through interviews with arrowroot farmers. Fresh leaf from each 7-month-old accession was used for DNA extraction.

DNA Isolation

Genomic DNA from the finely ground leaves (0.5 g) was extracted using the modified CTAB method (Deswina *et al.* 2019). Extraction buffer consisted of 50 mM Tris-HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol, 10 mM EDTA, 0.1% cetyltriethylammonium bromide (CTAB). The DNA quality was checked in an agarose gel through the electrophoresis and then quantified by measuring the OD at 260 nm and 280 nm through the spectrophotometry. Purity of DNA sample was calculated from OD at a 260/280 ratio.

ISSR Analysis

In this recent study, a total of 4 ISSR primers were used for analysis. The primers are UBC 811, UBC 827, UBC 818 and UBC 825 (Table 1).

Table 1 ISSR markers details

Markers	Sequence (5' - 3')	Annealing temperature (°C)	Size range (bp)
UBC 811	$(GA)_8C$	53	300-2,000 (Asha *et al.* 2015; Kambale *et al.* 2018)
UBC 827	$(AC)_8G$	52	300-1,600 (Asha *et al.* 2015; Kambale *et al.* 2018)
UBC 818	$(CA)_8G$	45	350-1,600 (Asha *et al.* 2015)
UBC 825	$(AC)_8T$	52	400-2,000 (Asha *et al.* 2015; Kumar *et al.* 2010)

The extracted DNA was amplified in a Thermal Cycler (Bio-Rad). The reaction mixture consisted of 8.5 μL ddH_2O, 2.0 μL of each primer, 12.5 μL MyTaq polymerase and 2 μL DNA sample. The total reaction volume was made up to 25 μL using sterile distilled water. PCR was carried out in a master cycler gradient with a certain temperature setting (Table 2).

Table 2 PCR amplification setting (Asha *et al.* 2015)

Steps	Temperature (°C)	Time (minutes)	Cycles
Pre-denaturation	94	4	1
Denaturation	94	1	
Annealing	45-55	0.5	40
Extention	72	1	
Post Extention	72	4	1
Hold	4	∞	1

The annealing temperature was standardized for each primer. The results of amplification were separated by gel electrophoresis in 2% agarose gel with 1X TBE buffer and were imaged using gel documentation with observation through UV vis transilluminator.

Data Analysis

The analysis was carried out only with the unambiguously and reproducibly amplified ISSR bands. Those DNA bands were scored as present (1) or absent (0). Smeared and weak bands were excluded. The resulting binary data matrix was analyzed using MVSP (MultiVariate Statistical Program) version 2.0. The analysis aimed to examine genetic relationship among accessions by estimating the similarity index using the Baroni-Urbani Busser similarity coefficient. Cluster analyses were carried out on the same similarity coefficient with the Unweighted Pair-Group Method of Average (UPGMA) (Rohlf 2000).

RESULTS AND DISCUSSION

Plant Collection and Identification

Identification of local cultivars succeeded in revealing the existence of 5 cultivars, namely 'Sembowo', 'Sili', 'Sugo', 'Kebo', and 'Teropong'. The 'Sili' and 'Sembowo' cultivars were found to be widely distributed in 4 districts (Table 3).

DNA Extraction

DNA extraction using the CTAB method was carried out on the accessions 'Sembowo' (represented by SENG1), 'Sili' (represented by SIPE3), 'Sugo' (represented by SUBR1), 'Teropong' (represented by TENG1) and 'Kebo' (represented by KEKE1). The quantity of isolated DNA was tested by using spectrophotometry and showed a fairly high level of purity in four of the five accessions. KEKE1 accessions were known to be contaminated, but based on the results of the electrophoresis tests, this DNA sample could still be used. Results of DNA concentrations varied. Each accession had a good DNA concentration and met the standards. The results of the spectrophotometric test are shown in Table 4.

Table 3 Sampling collection

No	Sampling location			Accessions type	Accessions code
	District	Subdistrict	Village, Subvillage		
1	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (besar)	SIBB1
2	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (kecil)	SIKB1
3	Bantul	Sedayu	Argodadi, Brongkol	'Sili' (bengkok)	SIEB1
4	Bantul	Sedayu	Argodadi, Brongkol	'Sugo'	SUBR1
5	Sleman	Pajangan	Triwidadi, Ngincep	'Sembowo'	SENG1
6	Sleman	Pajangan	Triwidadi, Ngincep	'Sili'	SING1
7	Bantul	Pajangan	Triwidadi, Ngincep	'Teropong'	TENG1
8	Bantul	Pleret	Wonolelo, Kedungrejo	'Kebo'	KEKE1
9	Gunungkidul	Wonosari	Gari, Gondangrejo	'Sembowo'	SEGO2
10	Kulon Progo	Pengasih	Pengasih, Pengasih	'Sembowo'	SEPE3
11	Kulon Progo	Pengasih	Pengasih, Pengasih	'Sili'	SIPE3
12	Kulon Progo	Pengasih	Sendangsari, Gegunung	'Sembowo'	SEGE3
13	Kulon Progo	Pengasih	Sendangsari, Gegunung	'Sili'	SIGE3
14	Sleman	Moyudan	Sumberagung, Kaliduren III	'Sembowo'	SEKD4
15	Sleman	Prambanan	Sumberharjo, Sengir	'Sembowo'	SESE4

Table 4 Spectrophotometry results

Accession Code	OD 260	OD 280	OD at 260/280 ratio	Concentration (ng/μL)
SENG1	7.03	3.67	1.929	365.52
SIPE3	2.54	1.35	1.898	127.32
SUBR1	4.62	2.43	1.917	231.11
KEKE1	5.57	9.95	1.371	678.87
TENG1	3.82	1.95	1.970	191.00

Each sample of isolated DNA concentrations showed various values ranging from 127.32 ng/μL to 678.87 ng/μL. The results indicated that isolated DNA from 'Sili' leaves produced the lowest concentration. However, the results also showed a fairly high absorbance ratio value of 1.898 at OD ratio 260/280. The OD value shows the purity of the DNA sample. There was only DNA sample of the local cultivar 'Kebo' which had a very low absorbance ratio value of 1.371 at OD ratio 260/280. The quantity of DNA concentration and quite low purity could be caused by polyphenol contamination. The quality of the DNA sample from 'Kebo' cultivar was still good and clearly showed the DNA bands.

The absorbance purity ratio of 260/280 is a very important measurement for estimating polyphenol contamination in the extracted DNA samples. Ratio of A260/A280 below 1.8 shows a poor DNA extraction result and is not recommend for molecular analysis (Sambrook & Russell 2001). A high purity ratio of A260/A280 also indicates the purity of DNA from RNA contamination (Koetsier & Cantor 2019). The use of high enough mercaptoethanol can replace the role of RNase in degrading and removing RNA from DNA samples, even though this reducing agent is toxic and pungent (Mommaerts *et al.* 2015).

Isolation of plant DNA using the CTAB protocol has many advantages and is proven to produce more sample volumes with high DNA concentrations. CTAB buffer solutions generally play a role in damaging cell structures, from cell walls to cell membranes and also the nuclear membrane. This is solely done to release the genetic material from the nucleus (Amani *et al.* 2011). CTAB buffer solution contains 2-β-mercaptoethanol which has been shown to completely remove polyphenol components in cells (Horne *et al.* 2004; Li *et al.* 2007). In this study, a buffer solution with a concentration of 0.1% mercaptoethanol was used and proved to be optimal in removing polyphenol contamination. Meanwhile, another study revealed that the use of 0.3% mercaptoethanol could improve the quality of DNA pellets from precipitation (Suman *et al.* 1999).

Analysis of ISSR-PCR

Molecular analysis used four ISSR markers, namely UBC 811, UBC 818, UBC 825 and UBC 827. PCR temperature optimization for these four markers resulted in different optimum annealing temperatures between UBC 827 & UBC 825 and UBC 818 & UBC 811. UBC 827 and UBC 825 markers require an annealing temperature of 50 °C, while the other two markers require an annealing temperature of 55 °C.

Electrophoresis of PCR-ISSR results showed variations in the number of DNA bands, polymorphic bands and different monomorphic bands among ISSR markers (Table 5).

Table 5 Number of DNA bands and polymorphism (%) in each molecular marker

ISSR markers	Sequences (5'- 3')	Total DNA Bands	Total Polymorphic DNA Bands	Polymorphisms (%)
UBC 811	$(GA)_8C$	11	8	72.7
UBC 818	$(AC)_8G$	8	4	50
UBC 825	$(CA)_8G$	10	8	80
UBC 827	$(AC)_8T$	10	7	70
	Average	9.75	6.75	68.17

Visualization of the electrophoresis results using the Gel-Doc Transilluminator showed variations among these molecular markers (Fig. 1). The average polymorphism appeared was 68.17% with the mean number of polymorphic bands of 6.75 and the average number of DNA bands of 9.75. UBC 811 primer produced the highest number of DNA bands, while UBC 811 and UBC 825 primers produced the highest number of polymorphic DNA bands with eight bands. Amplification using UBC 825 primer resulted in the highest polymorphism at 80%.

Electrophoresis using a DNA ladder with a size of 100 bp and a buffer solution of 2% TBE 1X showed that the size of the DNA bands in each primer was different, but there were several monomorphic bands with the same size between one marker and another. The DNA bands were 750 bp, 600 bp, and 450 bp. Overall, the size of DNA bands appeared to be ranging from 180 bp to 1,000 bp. Meanwhile, the polymorphic bands showed by each marker were diverse. In UBC 811, polymorphic DNA bands were found to have sizes of 200-300 bp, 300-400 bp and 800 bp. UBC 818 primer amplification resulted in the lowest polymorphism value, with only 50% polymorphism showed by the DNA bands 250 bp, 650 bp and 500-600 bp.

ISSR marker is an ideal genetic marker for various studies, most notably on genetic variation (Shafiei-Astani *et al.* 2015) and DNA fingerprinting (Shen *et al.* 2006). As a good genetic marker, ISSR produces high genetic variability and shows multilocus data due to the use of the highly variable microsatellite sequences that are ubiquitously distributed across the genome (Anne 2006; Tautz & Renz 1984; Wolff *et al.* 1995). The addition of one or more nucleotide anchors in ISSR marker to target the end of the microsatellite region can prevent primer dimerization (Bani *et al.* 2017). Based on comparison with other molecular markers, the ISSR marker has higher reproducibility compared to the Random Amplified Polymorphic DNA (RAPD). The ISSR marker is more time and money efficient compared to the Amplified Fragments Length Polymorphism (AFLP) (Phong *et al.* 2011; Ng & Tan 2015).

Electrophoresis of ISSR-PCR products generally uses 1.5-2.0% agarose gel by weight/volume (w/v) in order to achieve good DNA band separation. When the agarose gel concentration is higher, reaching 3% w/v or more, the gel will break more easily as it hardens (Ng & Tan 2015). According to Bornet & Branchard (2001), 2.0% w.v agarose gel showed the best performance in resolving the ISSR band compared to other concentrations of agarose gel.

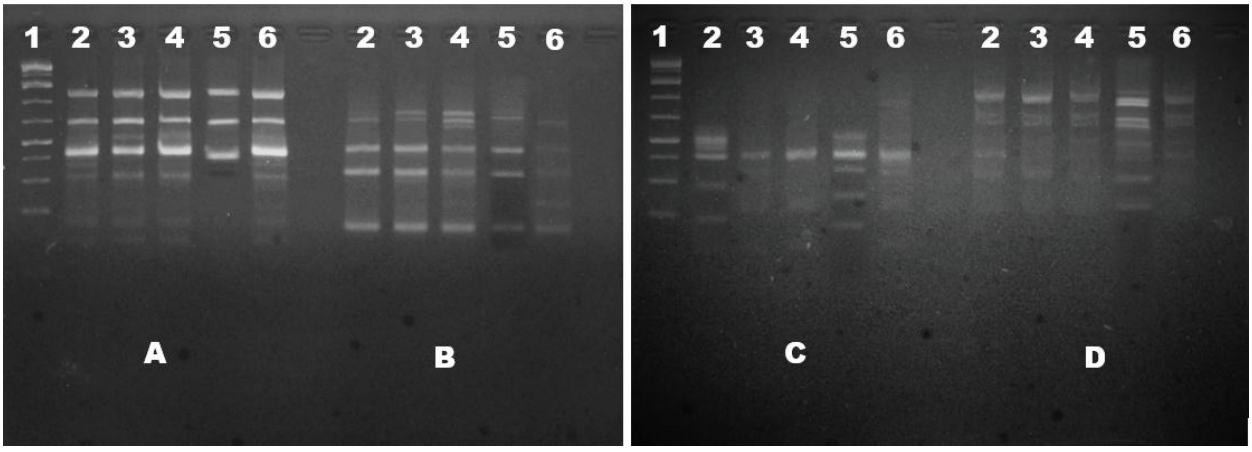

Figure 1 Amplification results
Notes: Amplification with 5 accessions (SENG1 (lane 2); SIPE3 (lane 3); TENG1 (lane 4); KEKE1 (lane 5); and SUBR1 (lane 6)) using ISSR markers (UBC 811 (group A); UBC 818 (group B); UBC 825 (group C); and UBC 827 (group D)) with 100 bp DNA ladder (lane 1).

Plant systematic studies of several plant taxa based on the molecular characteristics using ISSR-PCR revealed a greater number of polymorphic loci than the use of RAPD-PCR, because the abundant formation of ISSR primer target sequences throughout the eukaryotic genome, which evolves rapidly (Ansari *et al.* 2012; Phong *et al.* 2011; Moulin *et al.* 2012). Previous study on genetic variability of arrowroot in Yogyakarta using RAPD markers showed a low polymorphism and required further study (Setyowati 2013). In this recent study, the use of ISSR was proven to show the genetic variability of arrowroot in Yogyakarta. Therefore, ISSR marker proved to be better and more suitable in the analysis of arrowroot genetic variability than RAPD.

ISSR is a recent genetic marker that overcomes many technical limitations in other methods such as RFLP and RAPD analyses. ISSR markers have higher reproducibility than that of RAPD and have been successfully used to estimate the extent of genetic diversity at intra and inter-specific levels in a wide range of crop species (Asha *et al.* 2015). Hence, the ISSR markers were used in our study to assess the genetic diversity of arrowroot.

The amplification results of the 4 primers with 7 arrowroot accessions in India showed high polymorphism values (Asha *et al.* 2015), which were in agreements with the results of this study. However, the number of total DNA bands and the average percentage of polymorphisms obtained were lower than the previous research conducted by Asha *et al.* (2015). The differences were due to genetic diversity among plant population influenced by geographical condition and also appeared as molecular characteristics of each sample. Based on both studies, the polymorphic DNA bands obtained from ISSR analysis ranged from 10 to 60 fragments from various loci.

The use of ISSR UBC 825 primer in this study resulted in the highest polymorphism values and the greatest number of polymorphic DNA bands. This showed the effectiveness and the high reproducibility of these primers in the analysis of genetic diversity of arrowroot plants. The primary use of UBC 825 in the previous study conducted by Asha *et al.* (2015) was also known to produce the greatest number of polymorphic DNA bands and the highest polymorphism. Based on the electrophoresis, UBC 825 indicated the presence of microsatellite DNA sequences with sizes ranging from 800 bp to 180 bp.

Phenetic Analysis Based on Molecular Characteristics

Phenetic analysis based on the molecular characteristics can separate five local cultivars of arrowroot in Yogyakarta into four clusters. Dendrogram branching resulted in four clusters which were determined with the help of phenon lines at 80% similarity index. Three of the four clusters were outliers, each consisted of accessions of SIPE3, TENG1 and KEKE1. One cluster consisted of accessions SENG1 and SUBR1 (Fig. 2).

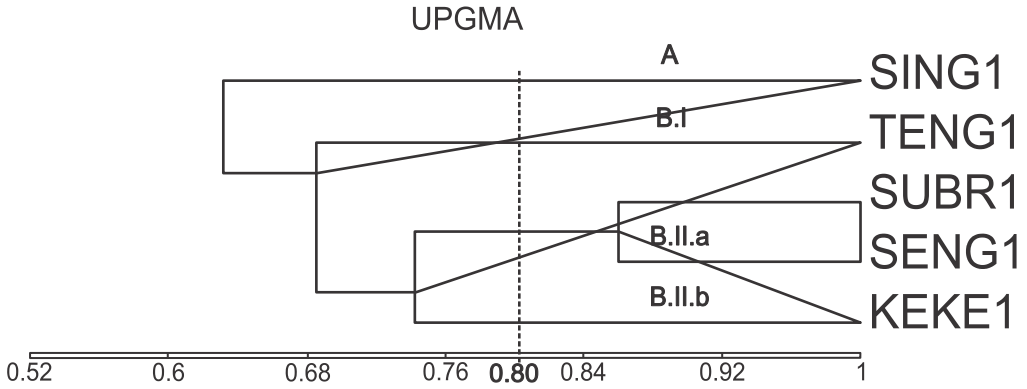

Figure 2 Dendrogram of arrowroot phenetic relationship based on molecular characteristics
Note: Molecular characteristics were the result of DNA fingerprinting analysis with ISSR markers, using the Baroni-Urbani Buser similarity coefficient.

Table 6 Similarity matrix for cluster analysis of arrowroot based on molecular characteristics

	KEKE1	SENG1	SUBR1	TENG1	SING1
KEKE1	1				
SENG1	0.741	1			
SUBR1	0.745	0.86	1		
TENG1	0.698	0.672	0.629	1	
SING1	0.654	0.676	0.726	0.534	1

Dendrogram branching was determined by the similarity matrix between accessions. The similarity values between one accession and the other accessions were different (Table 6). Analysis using the Baroni-Urbani Buser similarity coefficient showed that the accessions of SUBR1 and SENG1 had the highest similarity coefficient, which was 0.86. The lowest similarity coefficient was shown by SING1 and TENG1, which is 0.534.

The dendrogram showed 4 branches (Fig. 2). The first branch separated the SING1 accession, representative of the local cultivar 'Sili', from the other four local cultivar accessions at 0.64 point. It indicated the high dissimilarity between 'Sili' local cultivar with the other five local cultivars based on its molecular genetics. The genotypic characters obviously made distinct phenotypic characters of 'Sili' local cultivars. Based on interviews with arrowroot farmers, it was found that the 'Sili' cultivar was not very attractive for cultivation because it produced rhizomes containing higher fiber and smaller size than the other four local cultivars rhizomes. The second branch of the dendrogram separated the cultivar of 'Teropong' (TENG1) from 'Sugo', 'Sembowo', and 'Kebo' cultivars at 0.68 point. Then, the KEKE1 accession separated from the cluster consisting of SUBR1 and SENG1 accessions at 0.86 point. The highest similarity that showed by 'Sugo' cultivar and 'Sembowo' cultivar was supported by the similarity of rhizome morphologies from both cultivars, which information was provided by the farmers during the interviews. However, around 10% of dissimilarity index obtained based on molecular characteristics still showed great differences on the morphological and or anatomical characters.

Further studies of the use of ISSR markers in DNA fingerprinting techniques, cultivation or revealing genetic variation can provide several advantages. Genotype-specific ISSR markers can be sequenced to be used as a basis for the synthesis of Sequence Characterized Amplified Region (SCAR) primers, which can then be used to determine genotype taxonomy. In addition, markers associated with high agronomic value characters can be sequenced to be used as Sequence Tagged Sites (STS) markers that are useful in cultivation and breeding programs. Meanwhile, although the microsatellites that play a role in ISSR-PCR are probably nonfunctional, they are known to be associated with region coding. Therefore, ISSR can be used to construct gene-rich regions (Vijayan 2005).

CONCLUSION

The study used 5 local cultivars of arrowroot (*M. arundinacea* L.) from four districts in the Yogyakarta Province consisting of five local cultivars namely 'Sembowo', 'Sili', 'Kebo', 'Teropong' and 'Sugo'. Molecular analysis using ISSR markers was successful in showing high polymorphism and explaining genetic variabilities in the five local arrowroot cultivars. The analysis of the phenetic relationship using the MVSP program, the UPGMA algorithm method and the Baroni-Urbani Buser similarity coefficient showed the formation of four clusters on the dendrogram based on molecular characteristics. Clustering based on molecular characteristics was able to separate local cultivars from one another, but showed a close relationship between the local cultivars 'Sembowo' and 'Sugo'.

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