RESEARCH ARTICLE

SABRAO Journal
of Breeding 129-144, 2018

GENETIC VARIABILITY AND CLASSIFICATION OF GANDARIA (*Bouea*) IN INDONESIA BASED ON INTER SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

T. HARSONO[1]*, N. PASARIBU[2], SOBIR[3], FITMAWATI[4] and E. PRASETYA[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Indonesia
[3]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
[4]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Riau, Indonesia
*Corresponding author's email: triharsonounimed@gmail.com
Email addresses of coauthors: nursahara@usu.ac.id, fitmawati2008@yahoo.com, sobir@ipb.ac.id, ekoprasetya.biologi@gmail.com

SUMMARY

The genus *Bouea* is a member of the Anacardiaceae family whichis widespread in the Malesian Region. This genus consists of two species, namely *Bouea oppositifolia* (Roxb.) Adelb. and *Bouea macrophylla* Griffit. This study aims to analyze the genetic diversity *Bouea* in Indonesia. A total of 75 accessions of *B. macrophylla* and 30 accessions of *B. oppositifolia* were analyzed using inter simple sequence repeat (ISSR) markers. The results on *B. macrophylla* showed that the species were divided into three clusters with a similarity coefficient of 0.35. Group I consists of 53 accessions from Ambon, South Kalimantan, West Kalimantan, Banten, Bogor (Loji, Pandeglag, Leuwisadeng and Jasinga), Cibinong, and Bogor Botanical Garden, whereas Group II consists of 17 accessions from Riau (Batu Sangkar), West Sumatra, Cibinong, Aceh, Medan, Jambi, Palembang, Lampung, and Bangka Belitung. Group III consists of 5 accessions from west Sumatra Province (Batu Sangkar), Bogor Botanical Garden, Jambi, and South Kalimantan. *B. oppositifolia* is divided into four groups with a similarity coefficient of 0.84. Group I consists of one accession from Bogor Botanical Garden, Group II consists of 25 accessions from North Sumatera, Bogor Botanical Garden and Bangka Belitung, Group III consists of two accessions from North Sumatra, whereas group IV consists of two accessions from the Bogor Botanical Garden. The ISSR marker could separate *B. macrophylla* and *B. oppositifolia* with a similarity coefficient of 0.34. It was determined that *B. macrophylla* and *B. oppositifolia* were ancestors based on ISSR markers.

Key words: *Bouea,* Indonesia, intraspecies, ISSR

Key findings: Genetic variability and classification of gandaria (*Bouea*) based on inter simple sequence repeat (ISSR) markers provide useful information to analyze genetic diversity and can be used for the conservation of gandaria in Indonesia.

Manuscript received: January 9, 2018; Decision on manuscript: March 17, 2018; Accepted: April 7, 2018.

Communicating Editor: Prof. Dr. Naqib Ullah Khan

INTRODUCTION

Bouea is a genus of *Anacardiaceae* widely distributed in the Malesian region (Ghazali and Mohammad, 2014). Malesian region is an area with distinctive flora and fauna and have the highest levels of vegetation diversity in the world (Bass *et al*., 2012). *Bouea* distribution covers the territory of West Malesian, including the islands of Sumatra, Java, Kalimantan, Brunei Darussalam, Singapore, and Malaysian Peninsula (Rifai, 1992). *Bouea* consists of two species: *Bouea oppositifolia* (Roxb.) Adelb. and *Bouea macrophylla* Griffith (Hou, 1974). Another species of *Bouea* is reported to originate from Trang Bom, Vietnam under scientific name of *Bouea poilanei* Evr. ("Xoai Mu" and "Xoai Muc") with the distinct characteristics of having red-colored fruits (Le and Hancock, 1999).

The classification of *Bouea* into two species, namely *Bouea macrophylla* and *Bouea oppositifolia*, Hou (1974) is the only grouping, which becomes the main reference in discussing this genus. Classification of *Bouea* was performed using morphological data. Harsono *et al*. (2016) reported that *Bouea* showed high morphological variations. Morphological variation of *B. oppositifolia* is higher compared to those of *B. macrophylla.* Variation of the genus *Bouea* in Peninsular Malaysia using the molecular marker of ISSR indicates a considerable variation between accessions that are morphologically indistinguishable (Ghazali *et al*., 2015).

Linking characteristics between the varieties and the magnitude of the plasticity of morphological features has made it difficult to determine the limits of existing cultivars. Thus, it needs to be supported by data sources which are obtained through other approaches (Fitmawati and Hartana, 2010). Morphological markers often cause different perceptions among researchers because of their high level of plasticity and sensitivity to environmental factors (Tanksley and Bematzy, 1989). Identification of familial relationship of a plant can be carried out by combining morphological with molecular markers (Waugh, 1997). The researchers used molecular markers to support identification with morphological markers because they are more stable (Yunus, 2007) and less sensitive to changes in the environment and aging process, rendering the data obtained relatively more accurate (Julisaniah *et al*., 2008). One of the methods that can used to minimize environmental influence on species or cultivars is the use of molecular markers (Finkeldey *et al*., 2010).

The inter simple sequence repeat (ISSR) marker is based on PCR amplification products with a size of about 100-3000 bp near microsatellite area that forms the basis of some

simple sequence repeat (SSR) motifs. The ISSR technique is very useful in determining genetic instability in the early stages of *in vitro* culture, genetic diversity evaluation, cultivar identification, and monitoring of somaclonal variation (Trojanowska and Bolibok, 2004). ISSR is more informative than RAPD in wheat, fruit crops (strawberries and apples) and pea (Trojanowska and Bolibok, 2004; Korbin *et al*., 2002). This marker system is quite reproducible and has been used for rapid characterization on many cultivars such as poplars (Gao *et al*., 2006), common beans (Gonzales *et al*., 2005), *Cycad* (Xiao *et al*. 2005), study of kinship between *ginger* relatives (Wahyuni *et al*., 2004), and *Fusarium culmorum* isolate (Mishra *et al*., 2003). This study aims to analyze the genetic variation of *Bouea* using ISSR markers.

MATERIALS AND METHODS

Plant Material

Two different species of Gandaria (*Bouea*) i.e., *B. oppositifolia and B. macrophylla* were used to study the genetic variability and classification among different accessions based on inter simple sequence repeat (ISSR) markers. Fresh samples of *B. oppositifolia* were obtained from North Sumatra, Riau, Bangka Belitung, and Bogor Botanical Garden, which still have *B. oppositifolia* collection from Lampung, Bangka Belitung, and West Sumatra. Fresh samples of *B. macrophylla* were obtained from Ambon, Banten, West Sumatra, Bogor, Jambi, West Kalimantan, South Kalimantan, Bogor Botanical Gardens, Palembang, Lampung, Bangka Belitung, Medan, and Aceh (Figure 1). Fresh samples used in observations using the ISSR molecular markers include 75 accessions of *B. macrophylla* and 30 accessions of *B. oppositifolia* (Table 1).

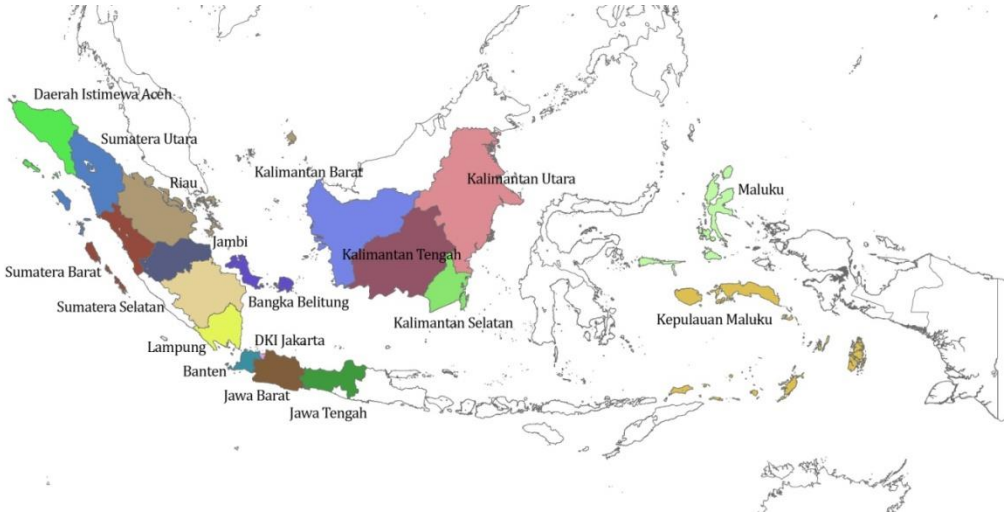

Figure 1. *Bouea* sampling area based on field studies.

Table 1. Fresh samples of *Bouea* obtained from the territory of Indonesia.

No.	Type	Province	Total
1	*B. oppositifolia* (Roxb.) Adelb.	North Sumatra	5
2	*B. oppositifolia* (Roxb.) Adelb.	Riau	1
3	*B. oppositifolia* (Roxb.) Adelb.	Isles of Bangka Belitung	19
4	*B. oppositifolia* (Roxb.) Adelb.	Bogor Botanical Gardens	5
5	*B. macrophylla* Griffit.	Ambon	14
6	*B. macrophylla* Griffit.	Banten	8
7	*B. macrophylla* Griffit.	West Sumatra	5
8	*B. macrophylla* Griffit.	Bogor	13
9	*B. macrophylla* Griffit.	Jambi	2
10	*B. macrophylla* Griffit.	West Kalimantan	6
11	*B. macrophylla* Griffit.	South Kalimantan	18
12	*B. macrophylla* Griffit.	Palembang	2
13	*B. macrophylla* Griffit.	Lampung	1
14	*B. macrophylla* Griffit.	Bangka Belitung	1
15	*B. macrophylla* Griffit.	Medan	1
16	*B. macrophylla* Griffit.	Aceh	1
17	*B. macrophylla* Griffit.	Bogor Botanical Gardens	3
Total			105

DNA Extraction

Total DNA was isolated from fresh leaves using CTAB method of (Doyle and Doyle, 1987) with modification. DNAs were suspended in TE buffer.

ISSR Amplification

DNA amplification was performed using seven ISSRs primers (Table 2) which had been selected from eleven ISSRs primers with high polymorphic band rates. PCR reaction volume was 25 µl, which consists of 2 µl of DNA genome, 1 µl of each *reverse* and *forward* primers (10 pmol), 12,5 µl *Taq* polymerase (KAPA2GTM Fast ReadyMix (2x) with Loading Dye) and 9.5 µl of ddH2O (aquabidest).

PCR Program for ISSR were as follows: (1) initial denaturation at 97 ^{o}C for 4 minutes (1 cycle); (2) PCR which consists of denaturation at 97 ^{o}C for one minute, annealing at 55 ^{o}C for one minute and at 72 ^{o}C for 2 minutes (35 cycles); and (3) final extension at 72 ^{o}C for 4 minutes (1 cycle), followed by (4) cooling at 4 ^{o}C. The amplified PCR product was visualized through electrophoresis using 1% agarose gel in TBE buffer and stained with 4 µl of SYBR® Safe DNA Gel Stain (Invitrogen). 7 µl of PCR product was added with 1 µl of loading dye when running along with 100 bp DNA Ladder marker using electrophoresis machine at 100 Volts for 45 minutes. Visualization of marker bands was carried out using gel documentation equipped with UV illumination.

Data recording and analysis

Scoring of DNA band polymorphism was done using Gel Pro Analyzer program and the creation of a dendogram and genetic distance analyses were done using NTSys PC (version 2.02). Individual grouping patterns based on genetic similarity matrices were reflected in the shape of dendrogram with a genetic similarity range of 0.00 (0%) to 1.00 (100%). The average number of allele counts, the average number of effective alleles, genetic diversity, the

Table 2. Primer to be used in ISSR analysis.

No	Name of Primer	Sequence	Sequence	T_m (°C)
1	PKBT3	(AG)8T	AGAGAGAGAGAGAGAGT	55
2	PKBT4	(AG)8AA	AGAGAGAGAGAGAGAGAA	55
3	PKBT5	(AG)8TA	AGAGAGAGAGAGAGAGTA	55
4	PKBT7	(GA)9A	GAGAGAGAGAGAGAGAGAA	55
5	PKBT9	(GA)9T	GAGAGAGAGAGAGAGAGAT	55
6	PKBT10	(GA)9A	GTGTGTGTGTGTGTGTGTA	55
7	PKBT12	(GT)9T	GTGTGTGTGTGTGTGTGTT	55

(Source: Tomar, et.al 2011)

Table 3. Analysis of *B. macrophylla*'s genetic diversity using ISSR markers.

Primers	Number of Effective Alleles	Heterozygosity	Polymorphic Information Content (PIC)	Shannon's Information Index
PKBT3	1.4342	0.2625	0.915	0.4075
PKBT4	1.3970	0.2392	0.885	0.3723
PKBT5	1.2878	0.1952	0.835	0.3223
PKBT7	1.4761	0.2916	0.925	0.4485
PKBT9	1.3663	0.2365	0.883	0.3804
PKBT10	1.3894	0.2431	0.880	0.3803
PKBT12	1.6092	0.3602	0.933	0.5391
Average	1.4228	0.2611	0.893	0.4072

Shannon information index, the number of polymorphic loci, and the percentage of polymorphic loci were analyzed using the program POPGENE (version 1.32). Analysis of molecular variance (AMOVA) was used to measure genetic diversity in populations and outside populations and was analyzed using GenAlex 6.5.

RESULTS AND DISCUSSION

Allelic variation among *B. macrophylla* Griffit accessions

Polymorphic ISSR markers from seven primers ISSR are listed in Table 3, where primer of PKBT10 produced the highest polymorphic information content (PIC). PIC values obtained ranged from 0.880 to 0.933 with an average value of 0.893. This indicates that the primary ISSR marker used on observations is capable of producing high polymorphic alleles. Polymorphic information content (PIC) is used to determine the level of polymorphism of a molecular marker. According to Botstein *et al*. (1980), PIC value is the index used to measure the value of polymorphism value. According to Gou and Elston (1999), the PIC is defined as the probability of genotype markers of a given offspring that allows detection in the absence of crossing-over from the two-marker alleles of the affected parents it received. PIC for dominant markers have a maximum value of 1.0 (De Riek *et al*., 2001; Bolaric *et al*., 2005). Polymorphism is considered high if the PIC value is ≥ 0.5, medium if PIC = 0.25 < PIC < 0.5, and low if the PIC value is ≤ 0.25 (Botstein *et al*., 1980).

The number of alleles for each ISSR primer ranges from 14 (PKBT5) to 21 (PKBT3) with an average of 18

alleles per primer. The highest number of effective alleles (1.6092) went to PKBT12 primer and the lowest effective alleles detected went to PKBT5 primer (1.2878). PKBT12 primer (0.3602) shows the highest heterozygosity value and the highest Shannon information index is shown by PKBT12 primer (0.5391). Shannon's information index is a measure of gene diversity (Lewontin, 1974). The heterozygosity value is one of the parameters to measure the level of genetic diversity in a population. Heterozygosity is the result of calculation of the frequency of genes in each locus (Nei, 1978). The higher the heterozygous frequency in a population, the higher the level of diversity (Vilas *et al*., 2015)

Heterozygosity value of the total population (H_T) is 0.2219, which indicates that there is considerable genetic variation among individuals within the *B. macrophylla* population. The coefficient of genetic differentiation (G_{ST}) in *B. macrophylla* has a value of 0.5750, which indicates that this figure has a higher value than the amount of standard genetic differentiation proposed by Nybom and Bartish (2000) with a value of 0.23 (for cross-cultivated crops) and 0.19 for endemic plants. Geographical isolation is the main factor of the high value of genetic differentiation coefficient of *B. macrophylla* because the geographical isolation has inhibited the gene flow. This condition is evidenced by the low value of gene flow (0.3681). Gene flow is a collective term encompassing all mechanisms, which cause movement of genes from one population to another (Slatkin, 1995). Fischer and Matthies (1998) stated that the greater geographical isolation, the lower the flow of genes.

Cluster analysis of *B. macrophylla* Griffit.

Cluster analysis based on ISSR marker data in *B. macrophylla* and the outgroup (*Mangifera indica* and *Anacardium occidentale*) has similarity coefficients ranging from 0.18 to 0.83 and is classified into four main groups at a coefficient of 0.35 (Figure 2). Group I is the largest group consisting of 53 accessions with a similarity coefficient of 0.36 consisting of accessions from Ambon, South Kalimantan, Banten, West Kalimantan, Bogor (Loji, Pandeglag, Leuwisadeng, and Jasinga), Cibinong, and Botanical Gardens. Group II consists of 10 accessions with a similarity coefficient of 0.37 consisting of accessions from Batu Sangkar, West Sumatra, Cibinong, Aceh, Medan, Jambi, Palembang, Lampung, and Bangka Belitung. Group III with a similarity coefficient of 0.35 consists of five accessions from accessions derived from Batu Sangkar (West Sumatra), Kebun Raya (Bogor) (KR7 origin Jambi and KR8 origin Peninsula Malaysia), and South Kalimantan). Group IV consists of the following outgroup *Anacardium occidentale* and *Mangifera indica*.

Group I is divided into seven subgroups again, group I has 11 accessions from Ambon with a similarity coefficient of 0.51. Group II has eight accessions originating from South Kalimantan and Ambon with a similarity coefficient of 0.56. Group III has 11 accessions originating from South Kalimantan and Ambon with a similarity coefficient of 0.54. Group IV has nine accessions from Banten and West Kalimantan with a similarity

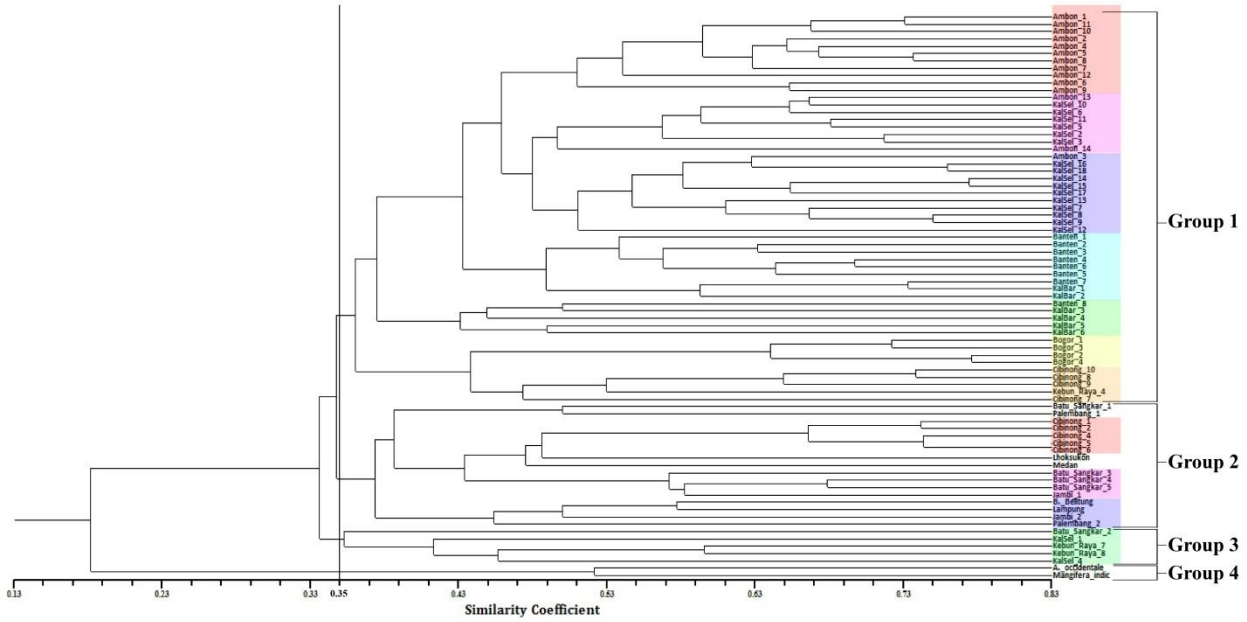

Figure 2. Dendogram of *B. macrophylla* using ISSR data with Unweighted Pair Group Method with Arithmetic Average (UPGMA).

index of 0.49, group V has five accessions from West Kalimantan and Banten with a similarity index of 0.43. Group VI has four accessions from Bogor (Loji, Pandeglang, Leuwisadeng, and Jasinga) with a similarity coefficient of 0.64, and group VII has five accessions from Cibinong and four from Botanic Garden with a similarity coefficient of 0.47.

The similarity index within 75 accessions of *B. macrophylla* ranged between 0.6429 - 0.994 with the highest observable similarity between Ambon and South Kalimantan populations, whereas the lowest observable similarity is found among the population of Medan and Lampung. Similarity index matrix between populations using ISSR markers in 14 populations of *B. macrophylla* is presented in Table 4. The observable genetic distance ranged from 0.0509 - 0.4418, where the furthest distance is found between the population of Lampung and Medan and the nearest genetic distance exists between the Ambon and South Kalimantan populations.

Analysis of molecular variance (AMOVA) *B. macrophylla*

The results of AMOVA analysis shows that genetic variation in the population (86%) is greater than the genetic variation between populations (14%). This suggests that the variations present in *B. macrophylla* are largely due to the influence of variation in the population compared to the variations caused by differences in geographical conditions. This is probably due to the high level of distribution of these plants in a population, which caused high level of variation. Despite its vast geographical distribution, it is likely that *B. macrophylla* can only live in the same environmental conditions between one population and another.

Table 4. Matrix of similarity index and genetic distance among *B. macrophylla* populations using ISSR marker.

Pop	AM	BA	BL	BS	EP	JB	KB	KR	KS	PLB	LP	MDN	SN	BO
AM	*****	0,9134	0,8204	0,9035	0,9090	0,8695	0,9171	0,8880	0,9504	0,8474	0,7928	0,7665	0,8016	0,8858
BA	0,0905	*****	0,7925	0,8823	0,8829	0,8347	0,9319	0,8543	0,9268	0,8139	0,7486	0,7507	0,8166	0,8442
BL	0,1979	0,2326	*****	0,8340	0,8234	0,8502	0,7762	0,7679	0,8195	0,8456	0,8254	0,6905	0,7540	0,7927
BS	0,1014	0,1253	0,1815	*****	0,8821	0,9123	0,8759	0,8705	0,9028	0,8700	0,8166	0,7626	0,8079	0,8666
EP	0,0954	0,1245	0,1943	0,1255	*****	0,8626	0,8712	0,8859	0,8976	0,8189	0,8072	0,7717	0,8041	0,8835
JB	0,1398	0,1807	0,1623	0,0918	0,1478	*****	0,8233	0,8629	0,8519	0,8475	0,8165	0,7576	0,7913	0,8594
KB	0,0865	0,0706	0,2533	0,1325	0,1378	0,1944	*****	0,8640	0,9140	0,8317	0,7381	0,7498	0,8026	0,8696
KR	0,1188	0,1575	0,2641	0,1387	0,1211	0,1475	0,1462	*****	0,8729	0,8394	0,7679	0,7136	0,7707	0,8980
KS	0,0509	0,0760	0,1991	0,1022	0,1080	0,1603	0,0899	0,1360	*****	0,8315	0,7957	0,7413	0,8096	0,8697
PLB	0,1656	0,2059	0,1677	0,1392	0,1997	0,1654	0,1843	0,1751	0,1846	*****	0,8202	0,7187	0,7864	0,8820
LP	0,2321	0,2896	0,1919	0,2026	0,2142	0,2027	0,3037	0,2641	0,2285	0,1982	*****	0,6429	0,6587	0,8322
MDN	0,2659	0,2867	0,3704	0,2710	0,2592	0,2776	0,2879	0,3374	0,2993	0,3303	0,4418	*****	0,7302	0,7532
SN	0,2211	0,2026	0,2824	0,2133	0,2180	0,2341	0,2198	0,2604	0,2112	0,2403	0,4174	0,3145	*****	0,7663
BO	0,1213	0,1693	0,2323	0,1431	0,1239	0,1515	0,1397	0,1076	0,1396	0,1256	0,1837	0,2835	0,2661	*****

AM = Ambon, BA = Banten, BL = Bangka Belitung, BS = Batu Sangkar, West Sumatra, EP = Cibinong, KB = West Kalimantan, KR = Botanical Gardens, KS = South Kalimantan, PLB = Palembang, LP = Lampung, MDN = Medan, SN = Lhoksukon, Aceh, BO = Bogor

Above diagonal	Similarity Index
Below diagonal	Genetic Distance

Table 5. Results of AMOVA on 75 accessions of *B. macrophylla* in 14 populations using ISSR markers.

Source of Variation	*df*	SS	$\bar{X}$ SS	Est. Var.	%	F_{st}	*P-value*
Inter Population	10	325.519	29.59	2.423	14%	0.140	0.001
In Population	64	965.884	14.86	14.86	86%		
Total	74	1291.403		17.283	100%		

Table 6. Analysis of *B. oppositifolia*'s genetic diversity using ISSR markers.

Primers	Number of Effective Alleles	Heterozygosity	Polymorphic Information Content (PIC)	Shannon's Information Index
PKBT3	1.0711	0.0622	0.6799	0.1225
PKBT4	1.1165	0.0207	0.7596	0.0408
PKBT5	1.0345	0.0322	0.4987	0.0731
PKBT7	1.2168	0.2056	0.6254	0.3001
PKBT8	1.3369	0.2286	0.7559	0.3599
PKBT9	1.1330	0.1590	0.7789	0.2815
PKBT10	1.1126	0.0861	0.6684	0.1521
PKBT12	1.2484	0.2147	0.8405	0.3524
Average	1.1587	0.1261	0.7009	0.2103

High level of variation in populations indicates genetic diversity in the population. The genetic diversity in high populations indicates considerable population differentiation. Results of AMOVA also show that variation in population and between populations are significant (*P-value* < 0.01). AMOVA can be used to separate the variation when there is adequate genetic distance to describe the difference of an allele with another (Holsinger *et al*. 1996). Results of AMOVA within 75 accessions of *B. macrophylla* in 14 populations using ISSR markers are presented in Table 5.

Allelic variations among the accessions of *B. oppositifolia*

The results of diversity analysis on 30 accessions of *B. oppositifolia,* using seven ISSR primers are presented in Table 6. PIC values ranged from 0.4987 – 0.8405 with an average value of 0.7009, which indicates that the primary ISSR used in this study is capable of producing high polymorphic data, except for the PKBT five primers with PIC value below 0.5, specifically 0.4987. The highest number of effective allelic observed is found in PKBT12 (1.2484) primer and the highest heterozygosity value is obtained from PKBT8 (0.2286) primer. The Shannon information index varies between 0.0408-0.3524, with the highest value indicated by the PKBT8 primer (0.3599). Shannon information index measures the level of diversity, the appropriateness of markers, and the emergence of genetic polymorphisms (Kesari *et al*., 2010). The observed heterozygosity is expected to be able to define the probability that certain randomly selected individuals from the population will be heterozygous at a particular loci and observed heterozygosity will be lower than expected (Mishra, 2013). This information and heterozygosity index also support the diversity between populations, in comparison with the population (Sirkar, 2017)

The number of effective alleles in Table 6 shows the frequently similar size of alleles taken to achieve a certain level of gene diversity. This means that it is possible to compare when the number and distribution of alleles differ significantly (Hartl and Clark, 1989). The concept of polymorphism used to define genetic variation in the population. PIC has become the most commonly used formula for genetic studies in measuring the information content of a molecular marker (Botstein *et al*.,

1980). A high PIC value indicates the high informative value of a primer. The results shows that the value of PIC from the observations ranged from 0.4987 to 0.8405 with an average of 0.7009, which means that the informative value of data generated by the molecular marker used in this study is quite high.

The heterozygosity value of the total population (H_T) is 0.1528, which indicates that there are considerable genetic variations among individuals within the *B. oppositifolia* population. The genetic differentiation coefficient (G_{ST}) in *B. oppositifolia* has a value of 0.4316, which indicates that this figure has a high value based on the standards determined by (Nybom and Bartsih, 2000). Geographical isolation is the main factor of low value of genetic differentiation coefficient of *B. oppositifolia* because geographical isolation has determined the gene flow. This condition has evidenced by the high value of gene flow (0.6584). Fischer and Matthies (1998) stated that greater geographical isolation, the flow of genes diminishes, whereas adjacent geography will cause high gene flow.

Cluster analysis of *B. oppositifolia*

Cluster analysis based on ISSR marker data in *B. macrophylla* and its outgroup (*Mangifera indica* and *Anacardium occidentale*) has similarity coefficients ranging from 0.56 to 0.99 and is classified into 5 main groups at a coefficient of 0.84 (Figure 3). Group 1 is an *outgroup* which consists of *M. indica* and *A. occidentale.* Group 2 is a group with 1 accession, namely the Botanical Gardens (KR1). Group 3 is the largest group with 23 accessions, namely accession from North Sumatra (GT, HA, SO), Riau, Botanical Gardens (KR2 and KR3), and Bangka Belitung. Group 4 is a group with 2 accessions from North Sumatra (SP and LG). Group 5 is a group with 2 accessions from the Botanical Gardens (KR5 and KR6).

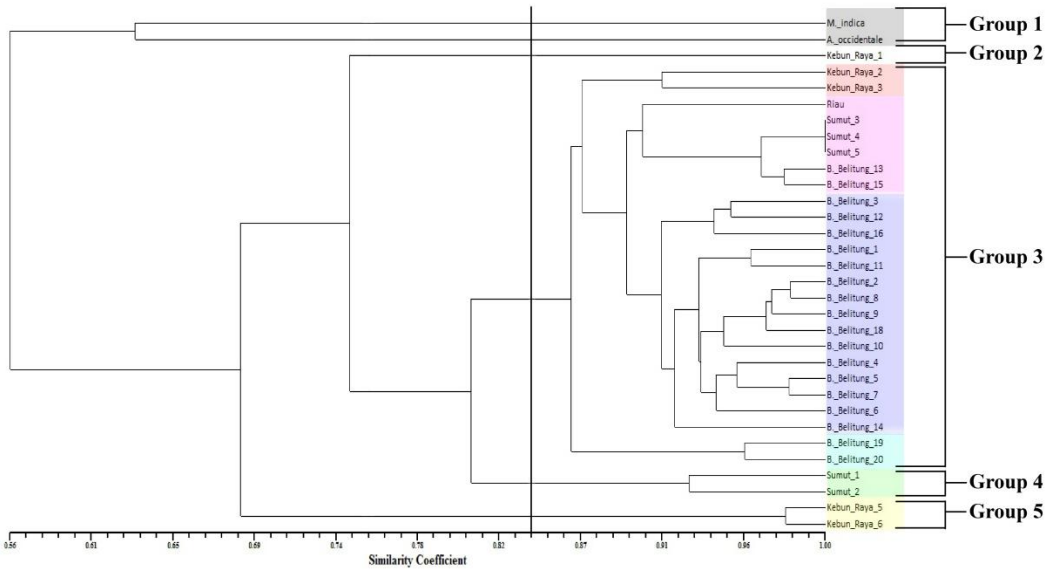

Figure 3. Dendogram of *B. oppositifolia* using ISSR data with Unweighted Pair Group Method with Arithmetic Average (UPGMA).

Table 7. Matrix of similarity index and genetic distance among populations using ISSR marker.

Populations	Botanical Gardens	North Sumatra	Riau	Bangka Belitung
Botanical Gardens	******	0.9164	0.8946	0.9428
North Sumatra	0.0873	******	0.9288	0.9317
Riau	0.1113	0.0739	******	0.9186
Bangka Belitung	0.0589	0.0707	0.0849	******

Above diagonal Similarity Index
Below diagonal Genetic Distance

Table 8. AMOVA on 30 accessions of *B. oppositifolia* in 4 populations using ISSR markers.

Source of Variation	*df*	SS	$\overline{X}_{SS}$	Est. Var.	%	F_{st}	P-*value*
Inter Population	2	47.629	15.876	2.054	40%	0.400	0.001
In Population	27	86.246	3.080	3.080	60%		
Total	29	133.875		5.134	100%		

The similarity index on 30 accessions of *B. oppositifolia* ranged between 0.8946 and 0.9428 with the highest observable similarity between Botanical Gardens and Bangka Belitung populations, whereas the lowest observable similarity is found among the populations of Botanical Gardens and Riau. Similarity index matrix between populations using ISSR markers in four populations of *B. oppositifolia* is presented in Table 7. The observable genetic distance ranges from 0.0589-0.1113, where the furthest distance is found between the populations of Botanical Gardens and Riau and the nearest genetic distance exists between Botanical Gardens and Bangka Belitung populations. Genetic distance matrix between populations using ISSR markers in four populations of *B. oppositifolia* is presented in Table 7.

Analysis of molecular variance (AMOVA) of *B. oppoositifolia*

The results of molecular variance analysis indicate that the genetic variation in the population is 60%, whereas genetic variation among populations is 40%. This suggests that the variations present in *B. oppositifolia* are largely due to the influence of variation in the population compared to the variations caused by differences in geographical conditions. This is probably due to the low level of distribution of these plants in a population, which caused low level of variation. The variation index between populations and in nearby populations appears to be quite comparable between geographic influences and genetic influences from within the population. Results of AMOVA also show that variation in population and between populations are very significant ($P < 0.01$). Results of AMOVA on 30 accessions of *B. oppositifolia* in four populations using ISSR markers are presented in Table 8.

Cluster analysis among accessions of *B. macrophylla* and *B. Oppositifolia*

The results of cluster analysis of 30 accessions of *B. oppositifolia*, 75 accessions of *B. macrophylla*, and two *outgroups* (*M. indica* and *A. occidentale*) are presented in Figure 4. Thirty accessions of *B. oppositifolia* and 75 accessions of *B. macrophylla* added with 2 outgroups (*Mangifera indica* and *Anacardium occidentale*) have similarity coefficients ranging from 0.17 to 1.00 and are classified into 3 main groups at a coefficient of 0.34 (Figure 4). The results of the analysis show that the results data using ISSR markers are able to distinguish both *Bouea* genus and its outgroups.

Studies using molecular markers related to the genus of *Bouea* are still very rare, and therefore the search for comparative data for molecular analysis in this study proves to be very difficult. The results in this study are quite different from those of Ghazali *et al*. (2015) who examined the relationship between species in the genus *Bouea* in the Malaysian peninsula based on ISSR markers, which indicated that the similarity coefficient of *B. macrophylla* ranged from 0.659-0.955 and similarity coefficient of *B. oppositifolia* ranged from 0.591-0.977. This is due to the differences in sample split distance. Among the *Bouea* genus originating from Indonesia, most samples were far in between the provinces and islands, whereas *Bouea* originating from the Malaysian peninsula has low level of distribution. This geographic position may affect the genetic variations, which emerged from each member of the *Bouea* genus.

According to Harsono *et al*. (2016), based on the diversity analysis of *Bouea* based on morphological characters, *B. oppositifolia* has a similarity coefficient between 0.49-1.00, whereas *B. macrophylla* has a similarity coefficient between 0.77-1.00. This indicates that the morphological variation of *B. oppositifolia* is higher compared to that of *B. macrophylla.* Morphological variation is influenced by the level of individual plasticity. Phenotypic plasticity is influenced by the interaction between the individual and his environment (Mboumba and Ward, 2008). Phenotypic plasticity and local adaptation are considered important mechanisms in the adaptation of plants to new environments (Sexton *et al*., 2002). This is inversely proportional to the results obtained using ISSR markers. This difference may be due to the differences in geographical distribution. The geographical distribution of *B. macrophylla* is more vast than that of *B. oppositifolia.* The vast distribution of *B. macrophylla* causes low gene flow due to the long distances between populations. According to Harsono *et al*. (2017), based on the genetic analysis using cpDNA sequence of *trnL-F intergenic space*, *B. oppositifolia* is considered as the ancestor of *B. macrophylla.* This research can be used by breeders to identify the diverse genotypes of different groups and use them in future breeding programs. Based on all information obtained in this study, the existence of *Bouea* as an endemic plant in western Indonesia should always be preserved. This study provides a baseline data for *Bouea* conservation programs in Indonesia.

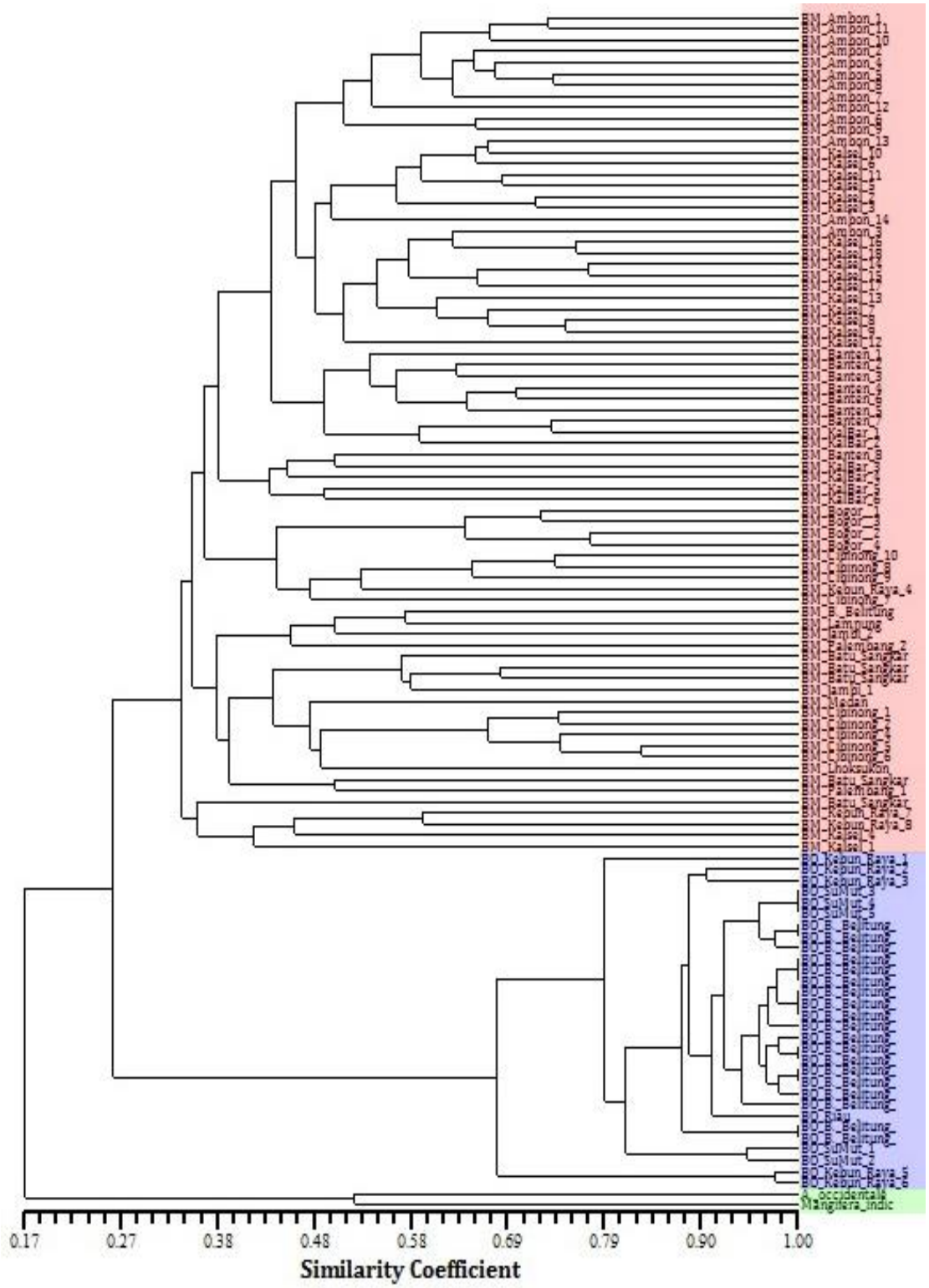

Figure 4. Dendogram of Joint ISSR data of *B. macrophylla* and *B. oppositifilia* using ISSR data with Unweighted Pair Group Method with Arithmetic Average (UPGMA). Red (*B. macrophylla*), Blue (*B. oppositifolia*), and Green (*M. indica* and *A. occidentale*) backgrounds show cluster differences.

CONCLUSION

ISSR markers can be used to distinguish species from the *Bouea* genus originating from Indonesia. *B. macrophylla* (0.18-0.83) has a greater genetic variation compared to *B. oppositifolia* (0.56-0.99). The value of gene flow (N_M) in *B. oppositifolia* (0.6584) is higher compared to that of *B. macrophylla* (0.3691). The primers used in this study resulted in greater polymorphic information content value in *B. macrophylla* (0.893) compared to *B. oppositifolia* (0.7009). *B. macrophylla* is grouped into 3 clusters with a similarity coefficient of 0.35 whereas *B. oppositifolia* is grouped into 4 clusters with similarity coefficient of 0.84. The ISSR marker is capable of separating *B. macrophylla* and *B. oppositifolia* with a similarity coefficient of 0.34. This indicates that *B. macrophylla* and *B. oppositifolia* came from the same ancestor. The ISSR marker can used to distinguish members of the *Bouea* genus.

ACKNOWLEDGEMENT

This research was supported by KEMENRISTEK DIKTI through HIBAH FUNDAMENTAL 2017. Thanks to all parties involved in this study.

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