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CITRUS DIVERSITY OF WEST SUMATRA BASED ON MORPHOLOGY, INTER-SIMPLE SEQUENCE REPEATS AND ITS COMBINED ANALYSIS

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ABSTRACT
Citrus species in West Sumatra is very diverse, however, the analysis of diversity based on either morphology or genetic or both combined is limited. Analysis was conducted on 27 cultivars derived from exploration activity in four districts of West Sumatra in October - November 2014 along with four germplasm collections cultivars as the controls. The morphological characterization was done based on IPGRI Descriptor List, while the genetic analysis was done using Inter-Simple Sequence Repeats (ISSR) markers. The data of morphological characters was analyzed first using Principal Component Analysis (PCA), for grouping plants Cluster Analysis was used, and the dendogram was calculated according to UPGMA SAHN method on NTSys program. The results showed that the plants diversity based on morphological and genetic are grouped into two and four groups with the degree of similarity are 21-100% and 58% - 96%, respectively; while based on its combined analysis, there are two major groups with a degree of similarity ranged between 56-94%. Of all citrus samples from West Sumatra, there are 3 types which could be genetically identified properly, the Siam-1 is similar to Siam Bangkinang, GF-1 is related to Kontrol-4 (G88.1/Grape Fruit) and Keprok-1 is related to the Kontrol-1 (KKO/Kacang Mandarin) with their levels of genetic similarity are 96%, 93.4% and 77.8%, respectively. Meanwhile, based on combined data, there is a 94% degree of similarity between Siam-1 with S. Bangkinang and JC-1 with Limau Kuning. The diversity of West Sumatra Citrus is very high; this is evidenced by some species that composed of several genetically different individuals.

KEY WORDS
Citrus, West Sumatra, diversity, morphology, molecular, genetic.

Citrus species cultivated in West Sumatra are mandarin and tangerine in general. Of tangerine varieties, ‘Madu’ and ‘Gunung Omeh’ are commercially cultivated the most in the region of Limapuluh Kota, Agam and Pasaman. Of the mandarin types, ‘Kacang’ derived from Solok regency, is widely grown in the region of Solok, also in small number in Agam and Pasaman (Diperta, 2014). In addition to these types, based on field observation in several regions, there are many other non-commercial citrus. Morphologically, these citrus can be grouped into two major groups with characters that have a high degree of similarity i.e. leaf color, stem shape, shape and number of petals; while the main distinguishing characters are leaf shape, petioles and fruit shape (Hardiyanto et al., 2015). At tangerine species, based on the morphology of the fruit, the 20 cultivars from all over Indonesia can be categorized into two type of fruit shape, obloid and spheroid (Martasari et al., 2012).

However, those morphological markers are only used to determine the variation in agronomic traits covering both within and between species (Koehler-Santos et al., 2003). In general, the main morphological characters used as markers are leaves, flowers and fruits (Dorji and Yapwattanaphun, 2011; Oliveira et al., 2002), while the location or origin of the plant does not affect the results of the classification (Susandarini et al., 2013).

Molecular analysis can also be used for grouping variations and for creating phylogenetic relationships among species of citrus (Golein et al., 2012). According to Biwas

et al. (2011), dendograms obtained from analyses using 4 markers, namely Amplified Fragment Length Polymorphism (AFLP) (Ernesto et al., 2005), Sequence-Specific Amplified Polymorphism (S-SAP), Selectively Amplified Microsatelite Polymorphic (SAMPL) and Simple Sequence Repeat (SSR) showed a high degree of resemblance. In the study by El-Mouei et al. (2011), SSR primer can be used to determine intraspecific variation of citrus, where the highest diversity found in tangerines (0.513) and the lowest in the Grapefruit (0.074).

In addition to those markers, the Inter-Simple Sequence Repeat (ISSR) is also widely used. This method is a PCR-based DNA marker using microsatellite sequences. ISSR marker has several advantages, such as simple, easy, and fast application, only needs a low quantity DNA template (10-30 bp), repeatable and consistent, it does not require a lot of information for the primers and is able to distinguish individuals which have very close relationship (Zietkiewicz et al., 1994); could distinguish wild citrus (C. indica Tanaka) with other commercial citrus species (Marak and Laskar, 2010); distinguish mandarin 'SoE' with its mutant derived from Gamma Rays treatment (Yuliati et al., 2010). This marker could also be used for identification of relationship among citrus cultivars (Fang et al., 1997) as well as allotetraploid characterization of somatic hybrids in plants (Scarano et al., 2002), however among citrus spesies, according to Filho et al. (1998) the similarity within the mandarin group is high. The purpose of this study was to classify citrus in West Sumatra based on analysis of morphological characters of leaves, fruit and genetics.

MATERIALS AND METHODS OF RESEARCH

Plant materials were obtained from local exploration activity conducted in South Solok, Agam, Solok and Sawahlunto, West Sumatra in 2014, representing citrus which are grown commercially and grown without maintenance. For control, four citrus accessions with definite identity originating from the collection of germplasm of Indonesian Citrus and Subtropical Fruit Research Institute (Balitjestro) were used. The names of the local as well as species are presented in Table 1.

Table 1 – Local name, species and its origin of citrus in West Sumatera

Sample No.	Local name	Species	Origin
1	Siam Super	*Citrus nobilis*	Kab. Solok Selatan
2	Siam-1	*Citrus nobilis*	Kab. Solok Selatan
3	Jeruk Bali-1	*Citrus maxima*	Kab. Solok Selatan
4	JC-1	*Citrus reticulata*	Kab. Solok Selatan
5	Jeruk Bali-2	*Citrus maxima*	Kab. Solok Selatan
6	Sunkis-1	*C. sinensis*	Kab. Solok Selatan
7	Limau Nipis	*C. aurantiifolia*	Kab. Solok Selatan
8	Limau Purut	*C. hystrix*	Kab. Solok Selatan
9	Limau Sundai	*C. aurantium*	Kab. Solok Selatan
10	Siam Bangkinang	*Citrus nobilis*	Kab. Solok Selatan
11	Limau Kuning	*Citrus reticulata*	Kab. Solok Selatan
12	L. Lunda/Bodong	*C. medica*	Kab. Solok Selatan
13	Lemon	*C. limon*	Kab. Agam
14	Limau Karatan	*Citrus reticulata*	Kab. Agam
15	Siam Bangkinang	*Citrus nobilis*	Kab. Agam
16	Asam Cuka	*C. aurantium*	Kab. Agam
17	Limau Kabau	*Citrus reticulata*	Kab. Agam
18	Purut	*C. hystrix*	Kab. Agam
19	GF-1	*C. paradisi*	Kab. Agam
20	GF-2	*C. paradisi*	Kab. Agam
21	Keprok-1	*Citrus reticulata*	Kab. Solok
22	Keprok-2	*Citrus reticulata*	Kab. Solok
23	Siam-2	*Citrus nobilis*	Kab. Solok
24	Keprok-3	*Citrus reticulata*	Kab. Solok
25	GF-3	*C. paradisi*	Kab. Solok
26	Nipis Sawah Lunto	*C. aurantiifolia*	Kab. Sawahlunto
27	Sambal	*C. amblicarpa*	Kab. Solok
28	Kontrol 1: KKO (Keprok Kacang)	*Citrus reticulata*	Balitjestro
29	Kontrol 2: MWO (Manis Waturejo)	*Citrus sinensis*	Idem
30	Kontrol 3: KUI (Satsuma)	*Citrus unshiu*	Idem
31	Kontrol 4: G 88 1 (Grape Fruit)	*C. paradisi*	idem

Morphological characters were observed with reference to standards issued by the International Plant Genetic Resources Institute (IPGRI) Descriptors of Citrus (IPGRI, 1999). Total of 14 morphological characters were observed on leaves and fruit. Data were analyzed using Principal Component Analysis (PCA) and Cluster Analysis using Multivariate Analysis software in Minitab 16.

Genetic analysis performed by isolating and amplification of DNA with ISSR marker. The analysis can only be conducted on 26 samples as well as four controls. Extraction, isolation and quantification of DNA was carried out based on the method of Doyle and Doyle (1990) which has been modified, while the amplification of the DNA sample with marker ISSR was carried out by method of Scarano et al. (2002).

The amplification of DNA samples was done in the following order:

1. The PCR program was as follows: a predenaturation temperature of 94°C for 3 minutes, followed by 28 cycles which each consisted of a denaturation step at 94°C for 45 seconds, annealing at 53°C for 1 minute, an extension step at 72°C for 2 minutes. The amplification using ISSR marker was terminated with a final extension cycle at 72°C for 10 minutes.

2. PCR profile for amplification of DNA with LTR-retrotransposon described as follows a denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 46°C for 1 minute, and an extension at 72°C for 2 minutes. Cycle PCR for LTR marker was ended with a final extension at 72°C for 10 minutes.

3. Each DNA sample was mixed with 20 mL of a mixture containing 10 ng genomic DNA as template, 0.25 mM dNTP (dATP, dCTP, dGTP, and dTTP), 0.5 pmol primer (ISSR 4 and ISSR LTR) (Table 2), 1 unit of Taq DNA polymerase in 1x buffer solution and 3 mM MgCl 2. Separation of DNA amplification products was conducted by electrophoresis on 2% agarose gel containing ethidium bromide (10 mg / l) in a solution of 0.5 x TBE for 40 minutes at 100 volts. Detection of DNA bands performed with gel documentation system.

D. Scoring and Dendrogram Analysis: Scoring was based on the presence of DNA in each plant. Each DNA band was considered as one character representing one DNA locus. DNA bands with the same migration rate were assumed to be homologous loci. DNA profiles were then translated into binary data based on the presence of DNA (1) and absence (0) to construct the similarity matrix. Grouping in UPGMA dendrogram calculated according to the SAHN method in NTSYS-PC version 2.10 (Rohlf, 1992).

RESULTS AND DISCUSSION

Principal Component Analysis. PCA analysis results obtained from five major components is able to explain the cumulative diversity of 76.1% of the 14 variables observed. The main component was determined based on the value of Total Initial Eigenvalues (Table 2). Value less than one was not used in calculating the amount of the main components formed, thus there were five major components obtained from the analysis that affect on plant diversity. These components are leaf lamina attachment, wing petiole shape, leaf lamina margin, fruit shape, shape of fruit apex, and fruit diameter, which are influential in grouping the citrus.

Table 2 – Egeinvalues for 31 citrus accessions

Component	Eigenvalues		
	Total	Explained Variance (%)	Accumulative explained variance (%)
1	3,019	21,6	21,6
2	2,548	18,2	39,8
3	1,877	13,4	53,2
4	1,776	12,7	65,9
5	1,435	10,2	76,1

Clustering based on morphological characters of leaves and fruits. The results of cluster analysis of 31 samples showed five major components that contribute to the 76.1%

cumulative diversity of 14 variables observed. Based on the result, accessions were clustered into 2 groups with level of similarity from 21.7 to 100% (Figure 1).

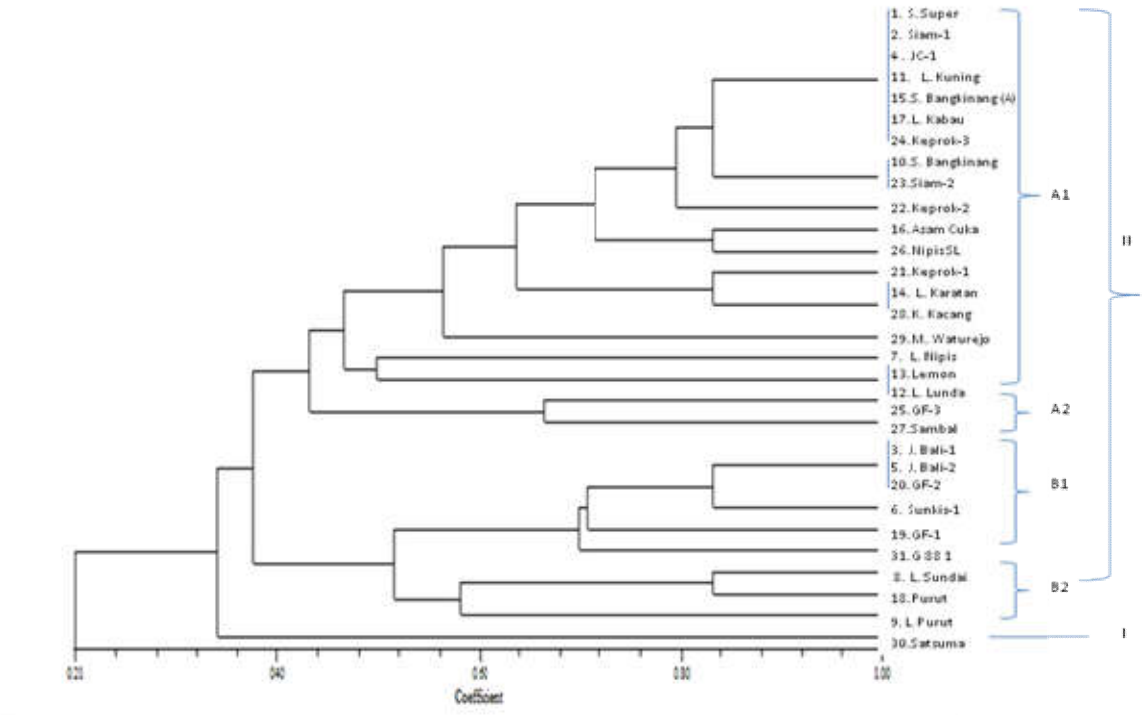
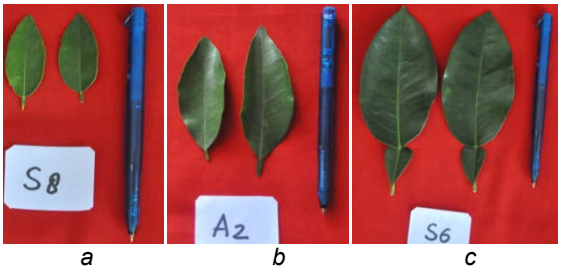


Figure 1 – Dendogram of 31 citrus accessions based on six main morphological characters of leave and fruit

Group I consists of 1 accession, namely Satsuma Mandarin that has brevipetiolate leaf lamina attachment, obcordate petiole wing shape, obloid fruit shape, depressed fruit apex and large size fruit, while Group II consists of two sub-groups. Accessions in sub group IIA in general have sessile leave, without petiole wing; fruits are spheroid, truncate apex and small to moderate size. Meanwhile, sub Group IIB generally has brevipetiolate leaf lamina attachment, obcordate petiole wing shape; fruit shape and apex are similar to subgroup IIA with large size fruit.

Separately, based merely on the morphology of the leaves, the plant will be divided into three major groups, namely 1. accessions with sessile leave (without petiole), small sized fruit with various leaf lamina shape; 2. accessions with sessile leave, medium sized fruit with ovate leaf lamina shape in general; and 3. accessions with brevipetiolate leave (petiole smaller than the leaf lamina), large fruit and the lamina is ovate (Figure 2a, b and c).

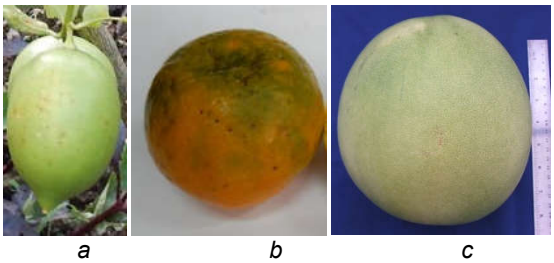


a *b* *c*

Figure 2 – Morphological characters citrus leaf shape (a) Sessile, (b) ovate shaped lamina, (c) Brevipetiolate

Based on the character of the fruit, clustering analysis produced three large groups, in which group 1 has ellipsoid fruit shape with convex fruit base and mammiform fruit apex,

group 2 has obloid shape, truncate base and apex and group 3 has spheroid shape with mostly convex base and truncate apex (Figure 3a, b and c).



a *b* *c*

Figure 3 – Shapes of citrus fruit (a) Ellipsoid, (b) Obloid, (c) spheroid

Clustering of citrus accessions based on genetic characterization. Based on the results of DNA amplification, ISSR and LTR-retrotransposon markers were able to recognize microsatellite sequences that exist in the citrus genomes in different frequencies and quantities. From 30 DNA samples (without sample no 24), 27 bands were generated and all of them were polymorphic (Table 3); banding pattern is presented in Figure 4 and 5.

Table 3 – Marker and number of loci amplified by ISSR and LTR-retrotransposon

Marker	Total Loci	Polimorfic loci	Monomorfic loci
ISSR 4	13	13	0
LTR-Retrotransposon	14	14	0
TOTAL	27	27 (100 %)	0

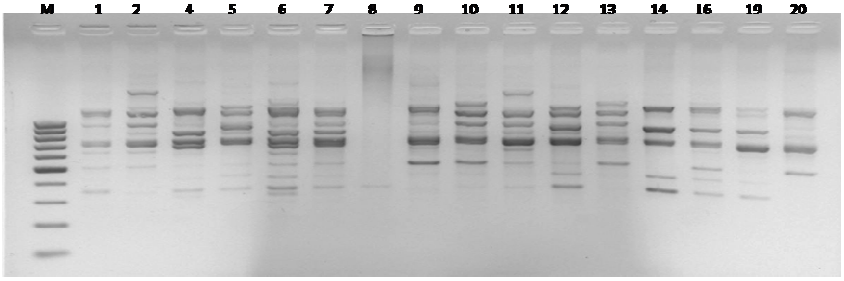


Figure 4 – DNA banding pattern amplified by ISSR4 (sample no. 1 – 20)

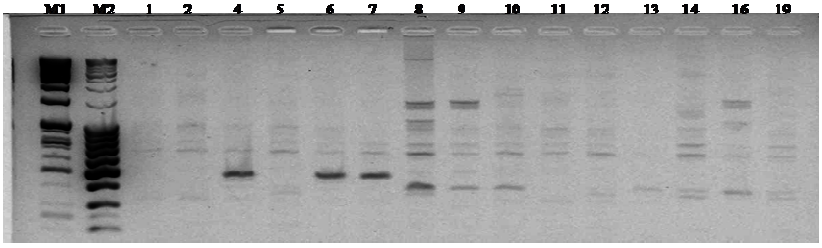


Figure 5 – DNA banding pattern amplified by LTR-Retrotransposon (sample no 1-19)

Based on the analysis, citrus accessions were divided into four groups (Figure 6). Group IA and IB have genetic similarity of 85-89%, Group IIA and IIB have genetic similarity about 72.5% - 77.5% and 72.5% - 89%, respectively, while Group III and IV respectively have genetic similarity of 77% - 92% and 59.5% - 85%.

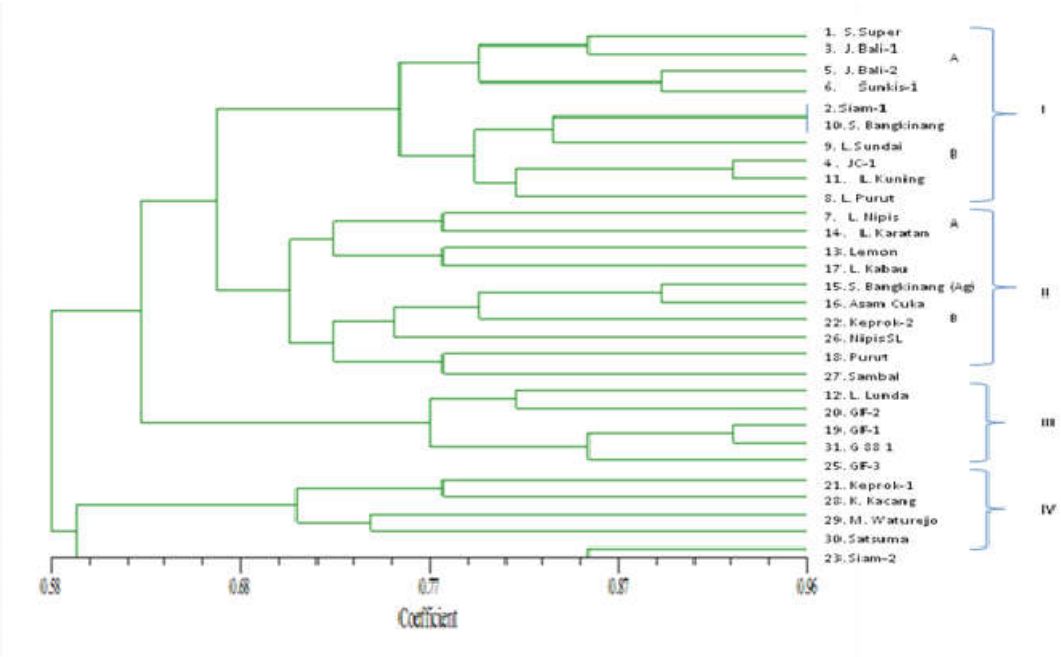


Figure 6 – Dendrogram of 30 citrus accessions based on combined data from ISSR and LTR makers

Clustering based on combined data of morphology and genetic analysis. From the clustering analysis, in general there are two major groups, in which each Group I and II consists of two sub-groups. In Group I, there are *C. reticulate, C. unshiu* and *C. sinensis* consists of Keprok-1, Kacang, and Satsuma (sub group A) along with Manis Waturejo (sub group B) with level of similarity ranged from 66.6 to 78.8%. In Group IIA there are 7 species, *C. nobilis, C. reticulata, C. hystrix, C. aurantium, C. maxima, C. sinensis, C. limon* and C. *aurantifolia* which consist of Siam-1 looks similar to S . Bangkinang, JC-1 is similar to L. Yellow, L. Purut, L. Sundai, pummelo, Sunkis, Lemon, L. Karatan and L. Kabau (IIA-1) as well as Purut, Lime, Keprok-2, S. Bangkinang (Agam) and Asam Cuka in IIA-2 with level of similarity between 63.8 to 94%. In Group IIB, there are *C. medica, C. amblicarpa,* and *C. paradise* which are L. Lunda / Bodong, Sambal, GF-1, G 88 1, GF-2 and GF-3 with level of similarity between 63.1 - 88.0% (Figure 7).

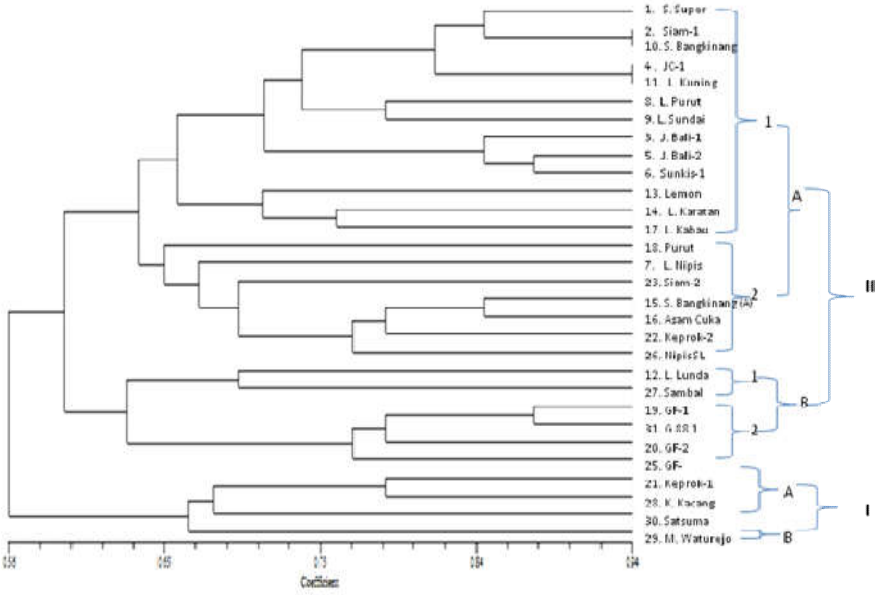


Figure 7 – Dendrogram of 30 citrus accessions based on combined data of morphology and genetic analysis

DISCUSSION OF RESULTS

Leaves are a major part with absolute existence in plants beside roots and stems. Thus to identify a plant, leaf morphology is one of the characters that can be used to characterize it. According to Padoan et al. (2013), in addition to the shape, leaf size can be used as an identifier of diploid or triploid citrus. In triploid plants, leaf size tends to be smaller than that of diploid, but with higher leaf index. From the morphology analysis results, clusters that indicated specific species could not be obtained, either by leaf, fruit or both characters combined. So according to Koehler-Santos et al. (2003), the analysis of morphological markers can only be used to determine the variation in agronomic traits covering one species or between species. To determine the relationship in one taxa, the more in-depth analysis on the anatomy of leaves and petioles should be done (Ogundare and Saheed, 2012).

Based on cluster analysis, only Siam-1 with S. Bangkinang (origin of Kab. Solok Selatan) showed 100 % genetic similarity while others have enormous variation (59.5% - 93.4%). This is proven by the type of Siam that could be grouped into either group I, II and VI, as well as Purut that could be put in group I and II. When compared to the four controls, of the 27 citrus obtained from exploration in West Sumatra, there are only two accessions, origin of Agam and Solok, that have a high degree of similarity, namely GF-1 similar to Grapefruit / G88.1 and Keprok-1 with Kacang/KKO (31); each with a degree of genetic similarity of 93.4% and 77.5%.

The high genetic variation is assumed to be the effect of intra- and inter-species hybridization and mutation in the field. This is easily detected using ISSR marker because it can detect more DNA bands better than RAPD so the accuracy of the identification and exploration of polymorphism is higher. The same results were obtained in a study of genetic relationship on 19 tangerine types derived from several regions in Indonesia (Agisimanto et al., 2007). Kumar et al. (2010) were able to obtain 4 major groups of 53 accessions from 4 species (*C. indica* Tanaca, *C. medica* L, *C. latipes* dan *C. spesies*) based on genetic, not by the origin of the samples.

Of the three kinds of the analysis, it was found that the results of classification of morphological, genetic and combination of morphological and genetic showed different clusters. This is in accordance with the opinion of Koehler-Santos et al. (2003), wherein when the molecular and morphological analysis used to determine the diversity of mandarin is done all together, the results will show different cluster. This is because the morphological characters that exist are complementary to genetic characters in these plants. From these results, it can be concluded that citrus in West Sumatra can be grouped based on the morphology of leaves and fruit as well as genetic into two and four groups respectively by the degree of similarity of 21-100% and 58% - 96%, while based on the combined analysis of morphological and genetic characters, there are two major groups with a degree of similarity ranged between 56-94%. The diversity of West Sumatra Citrus is very high; this is evidenced by some species that composed of several genetically different individuals.

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