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Genetic variability of lemon basil (*Ocimum* × *africanum* Lour.) from Indonesia based on morphological characters and ISSR markers

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Abstract. *Makmur K, Chikmawati T, Sobir. 2020. Genetic variability of lemon basil (*Ocimum × africanum *Lour.) from Indonesia based on morphological characters and ISSR markers. Biodiversitas 21: 5948-5953.* Lemon basil (*Ocimum* × *africanum* Lour.) or *kemangi* is a highly beneficial plant, yet still underutilized. Therefore, providing necessary information on genetic diversity of this species is essential for further utilization. The aim of this study was to elucidate genetic diversity of 33 accessions of *O.* × *africanum* collected from four Islands of Indonesia along with three accessions of *Ocimum basilicum* L. Morphological observation was conducted on 37 morphological characters following the International Union for the Protection of New Varieties of Plants descriptor guidelines; subsequently, 13 ISSR primers were employed in molecular analysis. Both morphological and molecular data were analyzed based on simple matching similarity index using UPGMA method. Morphologically, *O.* × *africanum* and *O. basilicum* were clearly separated at the similarity index of 0.52, and among two species were divided into two groups according to two either character. ISSR analysis using 13 ISSR primers produced 111 DNA bands, and 108 of them (97.29%) were polymorphic. Cluster analysis based on ISSR data could not explicitly separate *O.* × *africanum* and *O. basilicum* accessions. Besides, *Ocimum* accessions collected from the same area did not always cluster into one group.

Keywords: Lemon basil, genetic diversity, ISSR, morphology, *Ocimum* × *africanum*

INTRODUCTION

The genus *Ocimum* belongs to the family *Lamiaceae*, subfamily *Nepetoideae*, and tribe *Ocimae*. This genus consists of 30 species distributed in tropical regions of America, Africa, and Asia (Paton et al.1999). *Ocimum* × *africanum* and *O. basilicum* are the two widely consumed species. Both are herbaceous, annual plants with square stem, decussate leaves, lips-shaped flower, with strong lemon scent (Paton and Putievsky 2014). These two species can be easily distinguished from their plant color.

O. basilicum is known as purple basil, contained high anthocyanin contents which cause most of the plant parts colored purple. This species is very popular and has been massively cultivated for culinary purposes and industrial raw materials of cosmetics, perfumes, and instant food. On the other hand, *O.* × *africanum* (lemon basil) is more widely known in Indonesia, and used as vegetables, spices, and food supplement, but it has not been intensively managed. This species is a natural hybrid of *O. basilicum* and *O. americanum* (Conn 2014). It was reported that *O.* × *africanum* is rich in antioxidants and contained other metabolic compounds such as phenols, flavonoids, and terpenes; therefore, it can be developed to be a high economic value commodity (Tahira 2013).

For the development efforts in the future and management of genetic resources, genetic diversity information is essential. However, currently, the genetic diversity data of *O.*× *africanum* in Indonesia is still very limited. To obtain such valuable data, plant analysis using morphological and molecular markers can be used to generate the information wanted. Morphological data can be obtained by characterizing the morphological features of plants using descriptor guidelines. Morphological features are flexible and easily observed, but the data generated are often influenced by environment; thus it needs to be supported by other data.

Analysis of genetic diversity using molecular approaches offers several advantages, such as providing data that can be objectively analyzed, and it takes a relatively short time to obtain (Jonah et al. 2011). Several molecular markers have been used to study molecular genetic diversity at the species level, and one of them is *Inter-Simple Sequence Repeat* (ISSR) marker. The ISSR marker is a molecular marker-based on *Polymerase Chain Reaction* (PCR), which offers more efficient and accurate results. This marker has many advantages, including relatively low cost, no prior sequence, and high polymorphism. Data generated from ISSR marker can be used for genetic selection in the context of the maintenance

and improvement of genetic diversity that can support plant breeding programs (Lal et al. 2012). ISSR has been used to determine various plants such as *Daemonoropsdraco* (Asra et al. 2014) and *Duriokutejensis* (Handayani and Rahayu 2017). This study was aimed to describe the genetic diversity of *O.* × *africanum* based on morphological characters and ISSR markers as basic information for the management of genetic resources.

MATERIALS AND METHODS

Plant materials

The plant materials used in this study consisted of 33 accessions of *O.* × *africanum* and three accessions *O. basilicum* which then be used as the outgroup. The *kemangi* seeds were generously provided by the Center for Tropical Horticultural Studies (PKHT), IPB University, Bogor, Indonesia (Table 1). The seedling and morphological characterization was processed at the PKHT field station Tajur II, Bogor, Indonesia and molecular characterization was performed at the PKHT Laboratory at Baranangsiang Campus, IPB University, Bogor, Indonesia.

Morphological characterization

Morphological characterization was conducted at the Center for Tropical Horticultural Studies (PKHT) Field Station Tajur II. The procedure was started by sowing the seeds in the tray. The germinated seeds were then transferred into polybags. A month after planting, the seedlings were transferred from polybag to the experimental field (40 cm x 50 cm). Each accession was planted as many as 12 seedlings. The morphological observations were conducted after the plants produced flowers. As many as 37 characters were examined based on the International Union for the Protection of New Varieties of Plants descriptors guidelines (UPOV 2003).

Inter-Simple Sequence Repeat Marker characterization

The molecular characterization was undertaken at the Center of Tropical Horticulture laboratory, IPB University. The molecular procedure consisted of three main activities, namely DNA isolation, DNA amplification, and visualization of PCR products.

DNA was isolated using the CTAB method (cetyl trimethyl ammonium bromide) (Doyle and Doyle 1987), which had been modified by adding washing buffer on samples that have been pulverized.

PCR amplifications were carried out in GeneAmp PCR brand Applied Biosystems 2720. The PCR reactions were performed using 13 ISSR primers to amplify the *kemangi*'s DNA, in a final volume of 13 mL consisted of 6 mL Taq® Go Green, 1 mL of ISSR primer, 1 mL of sample DNA, and 5 mL of deionized water. PCR reaction was carried out for 35 cycles. Each cycle has consisted of an initial denaturation at 94˚C for 4 min, denaturation 94˚C for 30 sec, annealing at 41˚C - 54˚C for 35 sec, extension primer at 72ºC for 1 min, final extension at 72ºC for 5 min, and the cooling temperature of 4˚C for 10 min.

PCR products were separated in 1.5% agarose gels for 45 minutes at a voltage of 50 volts in buffer TAE 1X solution. The gel first wells were filled with 1.5 mL marker DNA 1 kb as a DNA size standard. The results of electrophoretic gels were stained with ethidium bromide 1.0 µg L^{-1}, then was visualized under UV light (Aghaei et al. 2012). The migrated bands on an agarose gel were photographed using Canon HD PowerShot A2500 digital camera.

Genetic variability analyses

Morphological characters were scored as multistate data (1, 2, 3, etc.), and bands were scored as binary data, (1) for the presence or (0) for the absence of the homologous band. Genetic similarity was analyzed using Similarity of Qualitative Data (SIMQUAL) with Simple Matching (SM) similarity coefficient. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) method. All statistical analysis was performed using NTSYSpc version 2.11a program (Rohlf 1998).

Table 1. Species name, accession code, and origin of basil used in this study

Species	Acc. code	Origin
O. × *africanum* Lour.	KP9	Kasiguncu, Poso, Central Sulawesi
O. × *africanum* Lour.	KP10	Kasiguncu, Poso, Central Sulawesi
O. × *africanum* Lour.	KT8	Dayeuh luhur, Tempuran, West Java
O. × *africanum* Lour.	KL3	Terbaya, Kota Agung, Lampung
O. × *africanum* Lour.	KL5	Terbaya, Kota Agung, Lampung
O. × *africanum* Lour.	KU	Nabire, Papua
O. × *africanum* Lour.	KY1	Gunungketur, Yogyakarta, Central Java
O. × *africanum* Lour.	KY3	Gunungketur, Yogyakarta, Central Java
O. × *africanum* Lour.	KW	Warung Loa, Bogor, West Java
O. × *africanum* Lour.	KA1	Jaya Baru, Banda Aceh
O. × *africanum* Lour.	KA2	Jaya Baru, Banda Aceh
O. × *africanum* Lour.	KS11	Sukamerta, Rawamerta, West Java
O. × *africanum* Lour.	KS10	Sukamerta, Rawamerta, West Java
O. basilicum L.	KCO6	Cisondari, Bandung, West Java
O. basilicum L.	KCO4	Cisondari, Bandung, West Java
O. × *africanum* Lour.	KC10	Ciaruteun ilir, Bogor, West Java
O. × *africanum* Lour.	KC12	Ciaruteun ilir, Bogor, West Java
O. × *africanum* Lour.	KTL	Telagasari, Karawang, West Java
O. × *africanum* Lour.	KPL2	Palasan, Ciater, Subang, West Java
O. × *africanum* Lour.	KPL3	Palasan, Ciater, Subang, West Java
O. × *africanum* Lour.	KR10	Kuningan, West Java
O. × *africanum* Lour.	KR6	Kuningan, West Java
O. × *africanum* Lour.	KT1	Dayeuh Luhur, Tempuran, West Java
O. × *africanum* Lour.	KT3	Dayeuh Luhur, Tempuran, West Java
O. × *africanum* Lour.	KT5	Dayeuh Luhur, Tempuran, West Java
O. × *africanum* Lour.	KT10	Dayeuh Luhur, Tempuran, West Java
O. basilicum L.	KCO1	Cisondari, Bandung, West Java
O. × *africanum* Lour.	KR4	Kuningan, Kramatmulya, West Java
O. × *africanum* Lour.	KM6	Bonto baddo, Makassar, South Sulawesi
O. × *africanum* Lour.	KM12	Bonto baddo, Makassar, South Sulawesi
O. × *africanum* Lour.	KTS	Tamansari, Karawang, West Java
O. × *africanum* Lour.	KCI6	Cipetir, Bogor, West Java
O. × *africanum* Lour.	KCI10	Cipetir, Bogor, West Java
O. × *africanum* Lour.	KR1	Kuningan, Kramatmulya, West Java
O. × *africanum* Lour.	KR11	Kuningan, Kramatmulya, West Java
O. × *africanum* Lour.	KT9	Dayeuh Luhur, Tempuran, West Java

RESULTS AND DISCUSSION

Ocimum × *africanum* Lour. morphological variation analysis

Based on the observations of 37 morphological characters, there are some similar morphological characters among 33 *O.* × *africanum* accessions from rounded and erect shaped (intermediate) canopy pattern; indumentum types on stem, branches, inflorescence, bract, and petiole; petal, pistil color, anther color, and the number of flowering branches. Despite their similarity, they also showed several different phenotypes, i.e., the leaf shape, leaf size, depth of serration on the leaf margin, the presence of undulation on the leaf margin, petiole length, inflorescence length, flowering time, presence of anthocyanin pigments in the branch, and inflorescence. The most prominent morphological characters among them were leaf size, the presence of anthocyanin pigments in young lateral branches, and inflorescence.

Ocimum × *africanum* can be easily distinguished from *O. basilicum* by the presence of puberulent indumentum on the stem and branches, compound verticillaster of inflorescence, shorter inflorescence length (8-16 cm), longer flowering period (57 days after planting), the purple color on the stem and leaves, and high intensity of purple color on the lateral branches thoroughly (Table 2, Figure 1).

Cluster analysis based on morphological data generated a dendrogram with similarity coefficients ranged from 0.39 to 0.97 (Figure 2). At a similarity coefficient of 0.39, all accessions were classified into one group that was clustered together by some common characters i.e., semi-circular of canopy shape or between round and erect *(intermediate)*, more than three flowering branches, and fimbriate indumentum on branch. At the similarity coefficient of 0.52, *O.* × *africanum* can be clearly divided from *O. basilicum* but at the similarity coefficient of 0.65, all accessions of *O.* × *africanum* observed were separated into two major groups (Figure 4). The groups were divided by the presence of anthocyanin pigment on young lateral branches and undulation on the leaf margin.

Table 2. The differences of morphological characteristics between *Ocimum* × *africanum* Lour. and *O. basilicum* L. species

Characteristic	*O.* × *africanum* Lour.	*O. basilicum* L.
Indumentum type on the stem and branches	Pubescent	Puberulent
Inflorescence type	Verticillaster	Compound verticillaster
Young lateral branch color	Green-purplish spot	Purple overall
The purple color on the costa and the entire surface of the leaf	Absent	present
The length of the inflorescence (cm)	14 - 38.5	8 - 16
Flowering time (Days after planting/ HST)	21-42	57

Figure 1. The morphological variation of *Ocimum basilicum* and *O.* × *africanum* (A-E): *O. basilicum*, A. Habit erect-rounded with a loose branch density, B. Inflorescence colored purple with average distance short internode, C. Pigment anthocyanin on the leaf surface and overall the stem and branches purple, D. Leaf margin is not undulation, E. Leaf margin serrate dept. (F-J): *O.* × *africanum*, F. Habit erect-rounded stem density middle, G. Inflorescence green colored with average distance of internodes medium-long, H. Leaf blade and stem have no anthocyanin pigment and branches young lateral colored green with purple spots, I. Undulation Leaf margin, J. Leaf margin serrate shallow

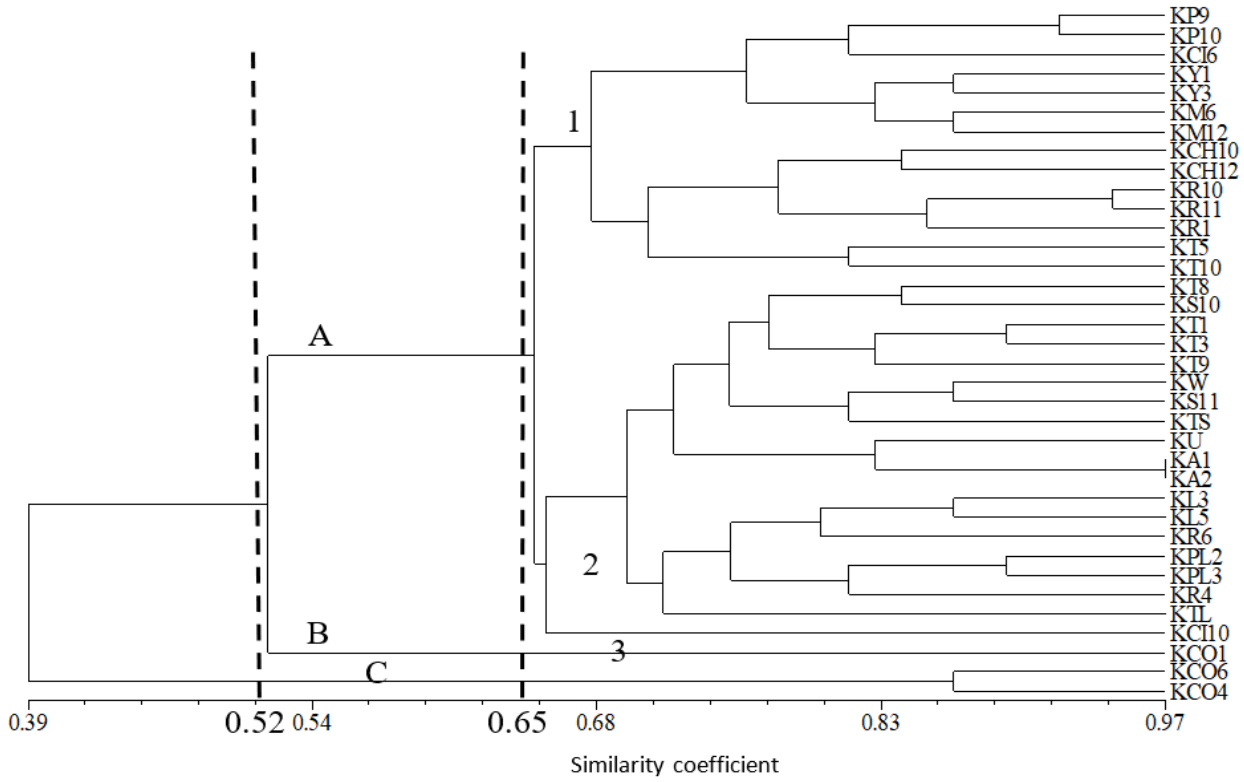

Figure 2. Dendogramof 33 accessions of *Ocimum* × *africanum* and three accessions *O. basilicum* constructed using UPGMA method based on morphological data. A: the main group of *O.* × *africanum*, B and C: the main groups of *O. basilicum*, 1 & 2: *O.* × *africanum* groups, 3 & 4: *O. basilicum* groups

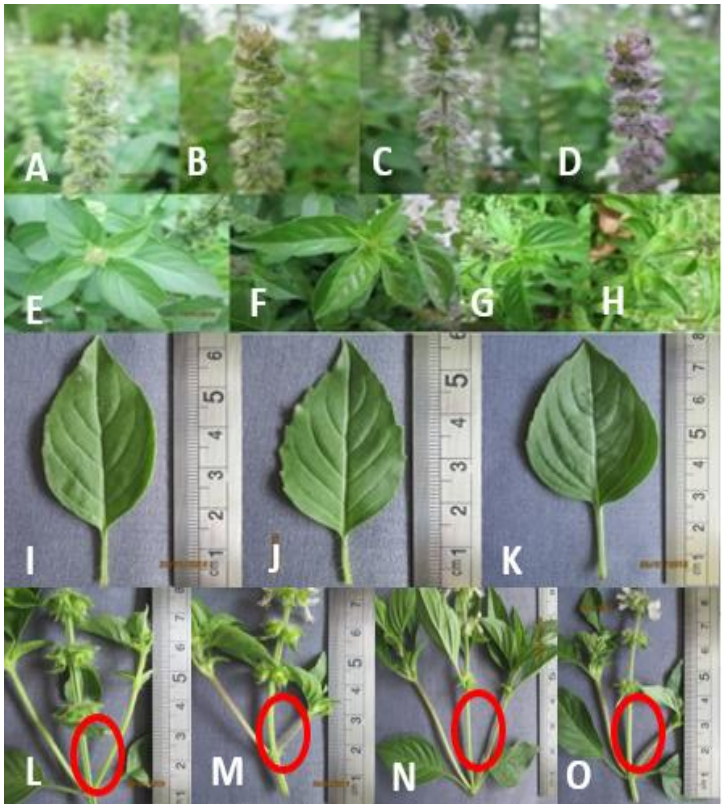

Figure 3. The morphological variation of *Ocimum* × *africanum* Lour. on A-D) anthocyanin pigments variation in the inflorescence, E-H). Undulation variation on the leaf margin, I-K). The shape, size, green color, and the serration's depth on the leaf margin variation, L-O). Anthocyanin pigments variation in the young lateral branches

The first group consisted of fourteen accessions originating from different areas, namely Poso, Yogyakarta, Makassar, Cirutenhilir, three Kramatmulya accessions (KR10, KR11, and KR1), two Tempuran accessions (KT5 and KT10), and one Cipetir accession (KCI6). The first group members shared two characters, i.e., undulation at young leaf margin and no anthocyanin pigment in young lateral branch. The KT10 accession had anthocyanin pigment in its young lateral branch. The second group shared some similar characters, i.e., undulation at the margin of young and mature leaves and anthocyanin pigment in their branches (Figure 3). The presence of anthocyanin pigments on young lateral branches is a good character that can be used to find good accessions in crop breeding programs because plants containing anthocyanin are beneficial plants that can be used as raw food material (Euloge et al. 2012; Bassole et al. 2005; Behera et al. 2012; Aluko et al. 2013; Kumari and Jain 2012).

Morphological analysis grouped some accessions from different populations with different origin and geographical distances into the same group; therefore, in this research, the grouping is affected by morphological features, which is different among accessions. Morphological differences can arise due to differences in gene expression resulting in different appearance or range of phenotype differences (Crowder 2010). The expressed differences of phenotypes may affect the variation in a population.

All accessions of *O.* × *africanum* observed using morphological features were clustered into two major groups, 1 and 2 (Figure 2). Each group has its characteristics, which are distinguished from another group; thus, the accession of each group may have the potential to be developed as a cultivar. However, further research needs to be carried out to ensure that the group's inherent characteristics are completely distinct, uniform, and stable following the International Code of Nomenclature for Cultivated Plants.

Ocimum × *africanum* Lour. variation based on ISSR marker analysis

Thirteen universal ISSR primers for plant were successfully amplified and produced 111 DNA bands. As much as 108 of them were polymorphic, with polymorphic level of 97.29%. Number of bands amplified by each primer ranged from 6 to11 bands, while the band length ranged from 250-2000 base pairs. The level of polymorphism ranged from 88.88 to 100% (Table 3).

The Primer PKBT 10 amplified the highest band number, which generated 11 bands and 100% polymorphic. A large number of DNA bands amplified by each primer indicates that the primer sequences are abundant in the genome of *O.* × *africanum.* Primers that amplified the less amount of DNA were PKBT 4 and PKBT 9. The number of amplified bands is not always correlated with the polymorphic level (Table 3). For example, this primer sequence of the primer ISSRED 4 is not only abundant in an accession but also abundant in the genomes of other accessions; thus, the produced band is not entirely polymorphic (Figure 4).

Cluster analysis based on 108 polymorphic bands produced a dendrogram with similarity coefficient values ranged from 0.65-0.97 (Figure 5). At a similarity coefficient of 0.72, the dendrogram could not strictly separate species *O. basilicum* from *O.* × *africanum*. However, at the same coefficient, all accessions observed, including accession *O. basilicum*, were separated into five groups. Presumably, it was alleged that the two observed species belong to the same complex of species, therefore, the gene flow may occur between two species by natural hybridization, resulting in both species showing high similarity (Erum et al. 2011). This result also supported the previous studies that showed *O. basilicum* and *O.* × *africanum* had high similarity value, which was reasonably close (Vieira et al. 2003).

Table 3. ISSR primers names and profiles of *Ocimum* × *africanum* and *O. basilicum*

Primer names	Primer Sequences	Band (bp)	JP	JPP	PPP (%)
ISSRED 4	$(GAG)_6G$	350-2000	10	9	90
ISSRED 6	$(GT)_8C$	250-1000	9	8	88.88
ISSRED 7	$(GTC)_6$	375-1500	9	9	100
ISSRED 11	$(GTGC)_4$	250-1500	7	7	100
ISSRED 14	$(GACA)_4$	375-1500	10	10	100
PKBT 2	$(AC)_8TT$	250-750	8	8	100
PKBT 4	$(AG)_8AA$	250-850	6	6	100
PKBT 7	$(GA)_9A$	250-1000	8	8	100
PKBT 8	$(GA)_9C$	250-1000	9	9	100
PKBT 9	$(CTC)_5GC$	250-1000	6	6	100
PKBT 10	$(GT)_9A$	325-1300	11	11	100
PKBT 11	$(GT)_9C$	375-1200	8	8	100
PKBT 12	$(GT)_9T$	250-1400	10	9	90
Total			111	108	97.29

Note: JP: Total band, JPP: Total polymorphic band, PPP: Percentage of polymorphic band

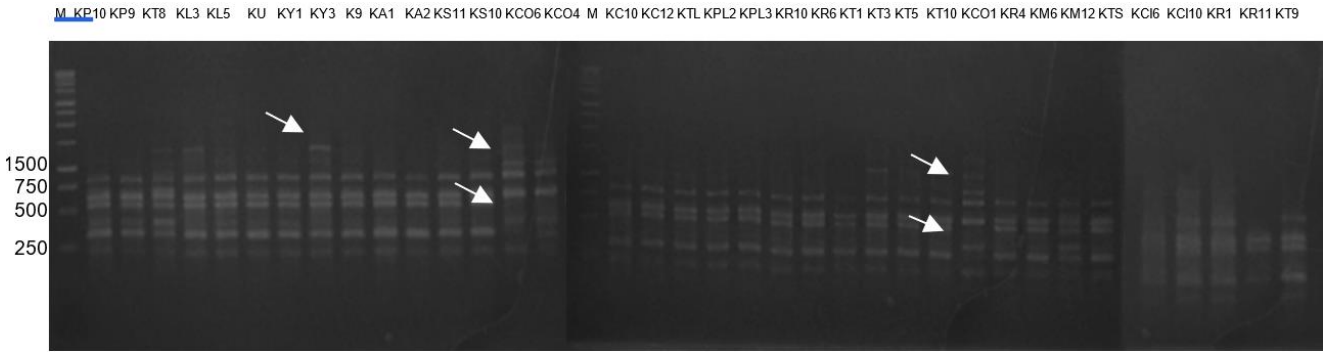

Figure 4. Variation of DNA band pattern produced by primer ISSRED 4, which checked on 1.5 % agarose gel. M: DNA Marker 1 kb, KP10 & KP9: Poso, KT8: Tempuran, KL3 & KL5 : Lampung, KU: Papua, KY1 & KY3: Yogya, KW: Warungloa, KA1 & KA2: Aceh, KS11 & KS10: Sukamerta, KCO6, KCO4, & KCO1: Cisondari, KC10 & KC12: Cirutenhilir, KTL: Telagasari, KPL2 & KPL3: Palasan, KR10, KR6, KR4, KR1, & KR11: Kramatmulya, KT1, KT3, KT5, & KT10: Tempuran, KM6 & KM12: Makassar, KTS: Tamansari, KCI6 & KCI10: Cipetir

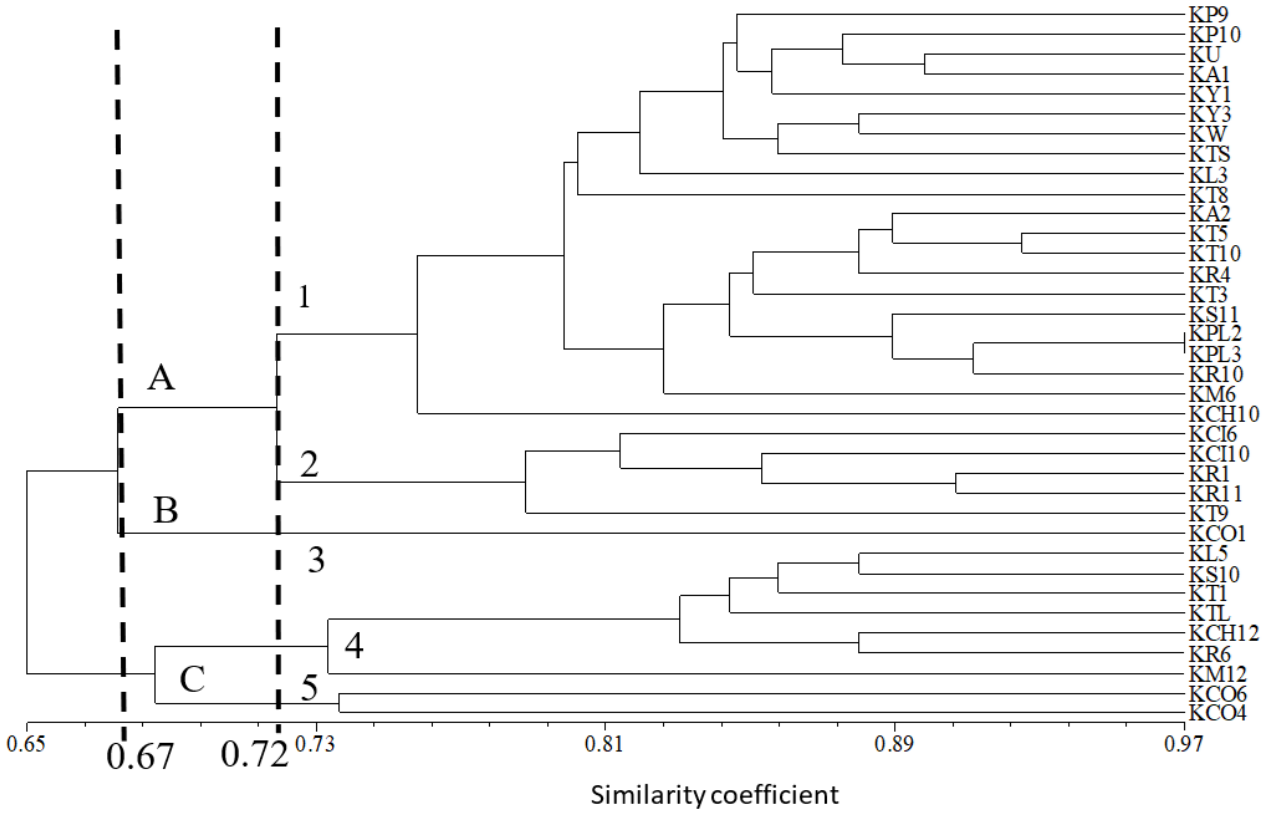

Figure 5. Dendrogram of 33 accessions of *Ocimum* × *africanum* and three accessions *O. basilicum* constructed using UPGMA method based on ISSR data. Group A: the main group of *O.* × *africanum*, Group B and C: the main mixed groups of *O.* × *africanum* and *O. basilicum*, 1 & 2: *O.* × *africanum* groups, 3 & 5: *O. basilicum* groups, 4: *O.* × *africanum* group

The opportunity to exchange genes between different species can produce offspring inherited genetically from each of the two parents, result in both species can not be divided clearly genetically. *Ocimum* × *africanum* is a hybrid of *O. basilicum* and *O. americanum*, thus, they have many similar genetic properties to their parental (Paton 2012). Besides, *Ocimum* species are known as cross-pollinated plants. Cross-pollination using pollinating agents can produce higher variation than a self-pollinated plant (Nation et al. 1992). Cross-pollinated plants also have higher chance of recombining genes and higher gene flow in the population (Atweel et al. 1999).

The similarity coefficient value from ISSR marker (0.67-0.97) was higher than that of morphological marker (0.52-0.97), indicating that variability by morphological marker is higher than that variability at DNA level by ISSR

marker. It can be due to the different allele frequencies generated using several ISSR primers observed. The observation results found that ISSRED 4 primer is one of the primers with less informative allele frequency (Figure 5). Such less informative allele frequency leads to lower diversity, although ISSR marker primers used to have high percentage of polymorphic band (Singh 2015). Previous studies on several types of *Ocimum* using multiple markers indicated that ISSR markers have the highest polymorphism level with lower allele informative levels in a primer (Chen et al. 2013). Besides, ISSR marker is a dominant marker, making only a few informative alleles formed. Dominant markers only generate 0-50% informative alleles (Shete 2000).

In conclusion, similarity coefficient of *O.* × *africanum* was ranged from 0.52-0.97 based on morphological marker, thus successfully divided *O.* × *africanum* from *O. basilicum.* At the similarity coefficient of 0.65, *O.* × *africanum* was grouped into two major groups by the presence and absence of anthocyanin pigments on the young lateral branches and undulation at the whole of leaf margin. ISSR primers produced 111 bands with polymorphic level of 97.29%. Clustering analysis based on ISSR data revealed the level of similarity coefficient of 0.67-0.97; therefore, it could not separate *O.* × *africanum* from *O. basilicum*. Both morphological and ISSR markers showed that the grouping does not follow the origin of the accessions. These indicated that the genetic variability among accessions was not correlated with environmental factors from the original habitat. Diversity of characteristics in each accession formed variety within a species (infraspecies). It can be utilized in breeding program to select the accession. Thus, the selected accessions need to be replanted to determine their distinctness, uniformity, and stability of characters.

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