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Short Communication: Genetic diversity of patchouli cultivated in Bali as detected using ISSR and RAPD markers

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Abstract. *Pharmawati M, Candra IP. 2015. Genetic diversity of patchouli cultivated in Bali as detected using ISSR and RAPD markers. Biodiversitas 16: 132-138.* Patchouli is a bushy herb that has strong scent. Patchouli's oil is extracted from patchouli leaves and used as perfumes, incense and traditional medicines. Centre of patchouli cultivation in Bali is in Badung District, however it is also grown in other area such as Buleleng District. The patchouli plant from Aceh ((*Pogostemon cablin* (Blanco) Benth) is considered as a better plant species due to its high quality oil. Java patchouli (*Pogostemon heyneanus*) has lower quality. Molecular marker was used to detect diversity of patchouli grown in Bali. Leave samples of patchouli from 12 areas in Badung and Buleleng District were collected. The areas included Lemukih, Wanagiri, Pupuan, Belok, Mekarsari, Nungnung, Plaga, Sidan, Mengwi, Lukluk, Abiansemal and Jegu. Patchouli samples of Lhokseumawe, Tapak Tuan, Sidikalang and Java were obtained from Research Institute of Spices and Medicinal Plants, Bogor, Indonesia. DNA was extracted using CTAB buffer. Seven ISSR primers and five RAPD primers produced scorable bands and used for diversity and cluster analyses. The dendrogram showed that patchoulis grown in Bali are group together, separated from Java patchouli. This support observation based on leaf morphology, that they belong to Aceh patchouli. The patchouli grown in Bali showed low genetic diversity with Nei and Li's similarity in the range of 0.857 to 0.989.

Keywords: Bali, genetic diversity, ISSR, patchouli, RAPD

INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.) belongs to mint family, Lamiaceae. Patchouli is believed to be a native of the Philippines; however it also grows wildly in Indonesia, Malaysia and Singapore (Ramya et al. 2013). Patchouli is also cultivated in India (Kumara and Anuradha 2011). The main product of patchouli plant is essential oil known as patchouli oil. This oil is used as perfume, scents and medicine (Kalra et al. 2006; Yang et al. 2013). Besides that, patchouli oil can be used as insect repellant (Maia and Moore 2011).

The patchouli cultivation in Indonesia was initially developed in Aceh, North Sumatra, West Sumatra and Bengkulu (Haryudin and Maslahah 2011). Cultivation of patchouli then developed to other areas of Indonesia including Java, Kalimantan and Bali (Nuryani 2006). The productivity of patchouli in Indonesia is 87.20 kg/ha, this is a relatively low production mean (Setiawan and Rosman 2013). The low productivity of patchouli is caused by traditional cultivation technology, pest and disease attack, and the use of unidentified patchouli varieties or the used of non superior varieties (Setiawan and Rosman 2013). Patchouli is best grown in humid climate condition, up to an altitude of 800-1000 m above sea level (m asl.) (Ramya et al. 2013).

In Indonesia, there are three patchouli species which can be differentiated by morphological characters, oil quality and resistance to biotic and abiotic stress. The three species are *P. cablin* Benth. or *P. patchouli* Pellet van Suavis Hook also known as aceh patchouli, *P. heyneatus* Benth. also known as java patchouli and *P. hortensis* Becker also known as soap patchouli. Three superior quality of patchouli varieties (Tapak Tuan, Lhokseumawe and Sidikalang) have been resealed by Indonesia Research Institute of Spices and Medicinal Plants, Bogor, Indonesia. The names of those varieties are based on their provenance. Tapak Tuan is superior for its production; Lhokseumawe has high oil content, while Sidikalang is tolerant to bacterial wilt and nematode (Nuryani 2006).

Patchouli has also been cultivated in Bali. It is considered that Bali will become a new production center for patchouli (Setiawan and Rosman 2013). The cultivation of patchouli in Bali is driven by rapid development of spa industries, where there is high demand of patchouli oil. The purity and identity of patchouli varieties is important for germplasm characterization (Kumara and Anuradha 2011). Common molecular markers used for detection of genetic diversity are RAPD (Random Amplified Polymorphism DNA) and ISSR (Inter Simple Sequence Repeat). The RAPD marker successfully identified variation of *Jatropha curcas* in India (Ikbal et al. 2010), assess diversity of medicinal plant *Catharanthus roseus* (Shaw et al. 2009) as well as determine genetic diversity of spice plant *Ocimum basilicum* (Ibrahim et al. 2013). The ISSR marker was shown to be able to detect genetic variation between *Allium*

species (Son et al. 2012), and *Capsicum* species (Thul et al. 2012).

This study aims to evaluate genetic diversity of patchouli grown in Bali using PCR-RAPD and PCR-ISSR. *Patchouli cablin* does not flower (Bhaskar and Vasanthakumar 2000), thus it is propagated using stem cutting or in vitro multiplication (Swamy et al. 2010). The diversity may have resulted from a long adaptation time to climatic condition and cultivation system (Chacko 2009). Information on genetic diversity of patchouli cultivated in Bali will be useful for breeding of patchouli. Patchouli plant with good adaptation to environment can be used as genetic source in patchouli breeding.

Materials and Methods

Sample collection

Leaves of patchouli were collected from cultivations areas in Bali. Leaf was collected from one plant in each cultivation area. The areas included Lemukih, Wanagiri, Pupuan and Belok which are highland areas with altitude of 1000 m asl, Mekarsari, Nungnung, Plaga and Sidan with altitude of 500-1000 m asl, Mengwi, Lukluk, Abiansemal and Jegu wich are lowland areas with altitude of 500 m asl. (Figure 1). As comparisons, Aceh patchouli varieties (Sidikalang, Lhokseumawe, Tapak Tuan) and Java patchouli were used and collected from Research Institute of Spices and Medicinal Plants, Bogor, Indonesia. Voucher specimens of all samples were deposited at Herbarium Biology Udayana (HBU) at Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Denpasar, Bali, indonesia.

DNA extraction

DNA was extracted according to Khanuja et al. (1999). Leaf samples (0.1 g) were ground using mortar and pestle, and 600 µL of extraction buffer (2% w/v CTAB, 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), 2% (v/v) 2-mercaptoethanol) was added and incubated at 60°C for 1 h. After that, 500 µL chloroform : isoamylalcohol (24:1) was added and vortexed, and centrifuged at 12,000 rpm for

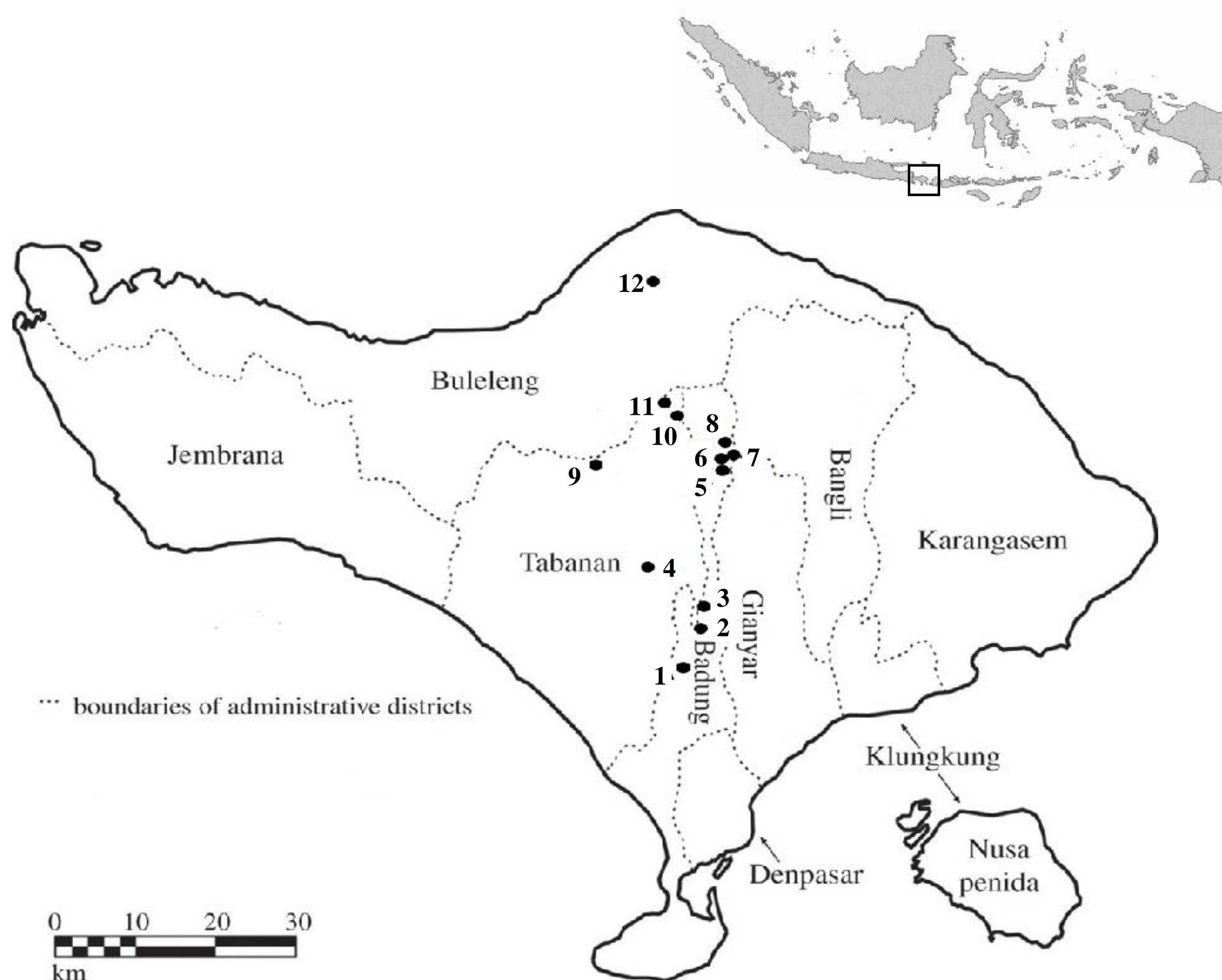

Figure 1. Study sites of sampling collection in Bali Island (●). Patchouli leaves were collected from 1. Lukluk, 2. Abiansemal, 3. Mengwi, 4. Jegu, 5. Nungnung, 6. Plaga, 7. Belok, 8. Sidan, 9. Pupuan, 10. Mekarsari, 11. Wanagiri, and 12. Lemukih. Insert is map of Indonesia. Map of Bali was modified from Lansing and Fox (2011).

10 min. The supernatant was transferred to a new microtube and 250 µL of 5 M NaCl was added and mixed. Then, 0.6 volume of cold isopropanol was added and incubated at -20ºC for 1 h. Following incubation, the sample was centrifuge at 12,000 rpm for 10 mins. Pellet was washed with 500 µL 70% ethanol and centrifuged at 12,000 rpm for 3 mins. Pellet was air dried for 15 mins and dissolved in 500 µL buffer TE. RNase A (5 µL) was added and incubated at 37 °C for 30 mins. Then same volume of chloroform: isoamylalcohol (24:1) was added and centrifuged. The supernatant was transferred to a new tube and 2 volume of cold ethanol was added. Sample was then centrifuged and pellet was washed with 70% etanol. Pellet was air dried and 100 µL ddH_2O was added.

PCR-ISSR and PCR-RAPD

The PCR-ISSR and PCR-RAPD were conducted in 25-µL PCR reaction. The mixtures contained 20 ng DNA, 1.5 mM $MgCl_2$, 1·x PCR buffer (MoBio), 0.5 µM primer, 200 µM of each dNTP (Promega) and 1 unit of Taq DNA polymerase (MoBio). To reduce background amplification, 5% (final concentration) glycerol was added (Grunenwald 2003). Amplifications were carried out using a thermocycler (MyGenie) with an initial denaturation/ activation step of 4 min at 95°C, followed by 40 cycles of 1 mins at 94°C, 1 min at annealing temperature (36°C for RAPD, 48°C and 50°C for ISSR) and 2 min extension at 72°C. The 40 cycles were followed by a final extension for 10 min at 72°C. Twelve ISSR primers (University of Bristish Columbia, Canada) and seven RAPD primers (Operon Technology, USA and University of British Columbia, Canada) were tested.

The amplification products were analysed using 1.8% agarose electrophoresis in 1 x TAE buffer for 50 min at 100 V, and then stained with ethidium bromide at final concentration of 0.5 µg/mL for 30 min. As a size marker, 1 kb DNA ladder (Fermentas) or 100bp ladder (GeneAid) was included in the gel. Visualization was done using GelDoc UV transilliminator.

Data analysis

The presence of the band was scored 1 and the absent of band was scored 0. The genetic differences were calculated using Nei and Li's coefficient of similarity by Unpaired Group Method of Average (UPGMA) using Multi-Variate Statistical Package (MVSP) software version 3.1. and a dendrogram was developed using the same software.

Results

Patchouli is now grown extensively in Bali. Based on leaf morphology, the patchouli grown in Bali is similar to Aceh patchouli. Patchouli samples collected from 12 cultivation areas have leaf shape that is categorized as ovate with pointed apex and double serrate margin. Leaf is green-purple color with smooth surface. These characters are main leaf characters of Aceh patchouli (*P. cablin*) (Fatriana 2011). The leaf length varied from 5.75-8.25 cm and leaf width varied from 4.70-6.75 cm. In shaded area, the length of leaf is in the range of 9.15-13.70 cm and leaf width is in the range of 4.70-6.75 cm.

Molecular testing of patchouli grown in Bali using ISSR and RAPD markers were conducted to evaluate their genetic diversity. The amplification products of PCR-ISSR using primer UBC 855 is shown in Figure 2, while products of PCR-RAPD using OPB 04 primer are presented in Fugure 3. Among the 12 ISSR primers tested, seven primers resulted in 32 clear and scorable bands, while other primers resulted in smear pattern. From seven scorable primers, 3 primers (UBC 808, UBC 820 dan UBC 888) resulted in monomorphic bands. Among the seven RAPD primers used, five primers produced 32 scorable bands, and all bands were polymorphic. The number of bands, the size of PCR products in each primer as well as the percentage of polymorphism is presented in Table 1 and 2.

Using PCR-ISSR only, low polymorphism was detected (Table 1). Therefore, to analyzed genetic diversity of patchouli cultivated in Bali, combined RAPD and ISSR markers were used. Based on PCR-ISSR and PCR-RAPD profiles, the Nei and Li's similarity coefficient was performed to group the patchouli samples. The Nei and Li's similarities of patchouli grown in Bali are in the range of 0.857 to 0.989. The matrix of Nei and Li's coefficient of patchouli cultivated in Bali as well as Aceh and Java patchouli is shown in Table 3.

Cluster analysis showed that Java patchouli is separated from other patchoulis and form group A, while samples of patchouli grown in Bali and samples of Aceh patchouli are in group B (Figure 3). Based on Nei and Li's coefficient of similarity of 0.92, group B can be further divided into subgroup B1, B2, B3 and B4. Subgroup B1 consists of patchouli sample from Lukluk. Subgroup B2 consists of samples from Belok and Lemukih, and subgroup B3 consists of samples from Jegu and Wanagiri. Sample from Sidan, Plaga, Mertasari, Tapak Tuan, Pupuan, Sidikalang, Abiansemal, Mengwi Nungnung, Abiansemal and Lhokseumawe are clustered into group B4. When similarity coefficient of 0.94% was used as a baseline, samples become more separated and form smaller groups.

Discussion

Leaf morphological characters of patchouli cultivated in Bali were similar to those of Aceh patchouli. However, the size of leaf differed when they grow in open area and in shaded area. This indicates that morphological characters are influenced by environment as stated by Sugimura et al. (2006) which resulted in difficulty of botanical classification. Therefore it is hard to determine whether patchoulis cultivated in Bali belong to the three high quality patchoulis (Lhokseumawe, Sidikalang and Tapak Tuan).

Both using PCR-ISSR and PCR-RAPD, Java patchouli showed different banding patterns as compared to other patchoulis tested. For examples, using primer UBC 855, band of 320 bp was present in Java patchouli, while using primer OPB 04, bands of 510 bp and 790 bp were present in Java patchouli but absent in other patchouli samples. Java patchouli is a different species than Aceh patchouli. These results showed that both PCR-ISSR and PCR-RAPD were able to differentiate the two species In the dendrogram, Java patchouli was separated from other.

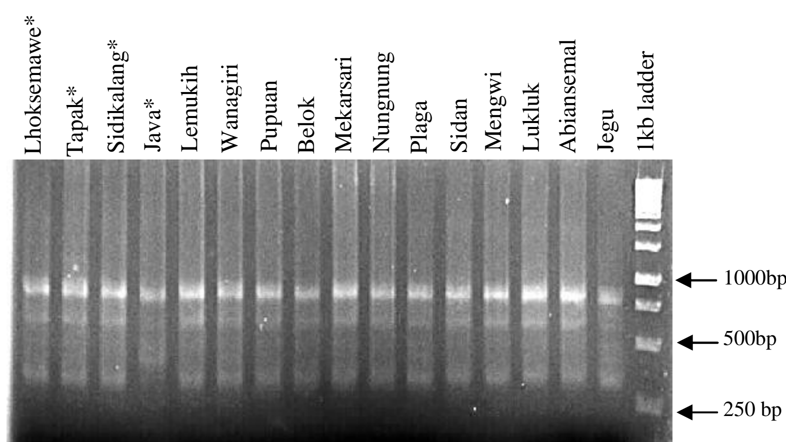

Figure 2. Amplification products of patchouli using UBC 855. Patchouli samples from Research Institute of Spices and Medicinal Plants, Bogor (*) and samples from cultivation areas in Bali were shown across the top of figure

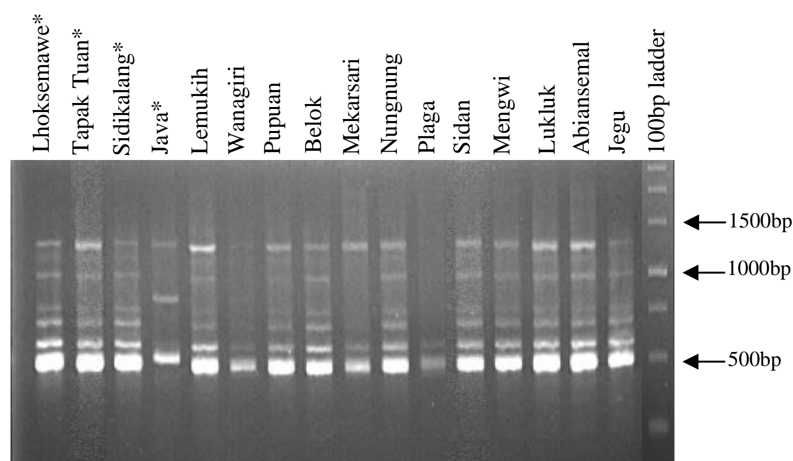

Figure 3. Amplification products of patchouli using OPB 04. Patchouli samples from Research Institute of Spices and Medicinal Plants, Bogor (*) and samples from cultivation areas in Bali were shown across the top of figure

Table 1. ISSR primers, numbers and sizes of bands amplified and the polymorphisms produced

Primer name	Primer sequence	Bands size (bp)	Number of band	Number of polymorphic band	Polymorphism (%)
UBC 810	(GA)8T	505-792	3	1	33
UBC 848	(CA)8 RG	500-1508	9	4	44.4
UBC 855	(AC)8YT	352-810	4	2	50
UBC 891	HVH(TG)7	436-1000	6	3	50
Average			5.5	2.5	44.35

Table 2. RAPD primers, numbers and sizes of bands amplified and the polymorphisms produced

Primer name	Primer sequence	Bands size (bp)	Number of band	Number of polymorphic band	Polymorphism (%)
OPA 04	AATCGGGCTG	150-750	6	5	83.3
OPB 04	GGACTGGAGT	500 -1305	9	6	66.7
OPD 11	GTAGACCCGT	178-1030	5	4	80
OPD 14	TCCGCTCTGG	245-2140	8	6	75
UBC 250	CGACAGTCCC	350-895	4	3	75
Average			6.4	4.8	76

Table 3. Matrix of Nei and Li's Coefficients of patchouli cultivated in Bali as well as Aceh and Java patchouli based on PCR-ISSR and PCR-RAPD

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.000															
2	0.943	1.000														
3	0.944	0.909	1.000													
4	0.649	0.658	0.667	1.000												
5	0.944	0.932	0.889	0.615	1.000											
6	0.925	0.891	0.936	0.659	0.894	1.000										
7	0.978	0.944	0.954	0.633	0.945	0.947	1.000									
8	0.941	0.881	0.884	0.622	0.930	0.867	0.920	1.000								
9	0.943	0.930	0.909	0.658	0.909	0.913	0.944	0.881	1.000							
10	0.967	0.956	0.935	0.625	0.957	0.938	0.989	0.909	0.956	1.000						
11	0.943	0.953	0.886	0.658	0.932	0.913	0.944	0.881	0.977	0.956	1.000					
12	0.941	0.929	0.907	0.676	0.884	0.911	0.943	0.878	0.952	0.932	0.929	1.000				
13	0.957	0.923	0.946	0.617	0.925	0.928	0.957	0.899	0.945	0.968	0.923	0.921	1.000			
14	0.920	0.884	0.909	0.605	0.886	0.870	0.899	0.905	0.860	0.889	0.860	0.857	0.923	1.000		
15	0.955	0.897	0.921	0.649	0.899	0.903	0.956	0.894	0.920	0.945	0.897	0.941	0.957	0.920	1.000	
16	0.925	0.870	0.936	0.683	0.872	0.939	0.926	0.876	0.891	0.917	0.870	0.889	0.928	0.891	0.925	1.000

Note: 1 = Lhokseumawe, 2 = Tapak Tuan, 3 = Sidikalang, 4 = Java, 5 = Lemukih, 6 = Wanagiri, 7 = Pupuan, 8 = Belok, 9 = Mekarsari, 10 = Nungnung, 11 = Plaga, 12 = Sidan, 13 = Mengwi, 14 = Lukluk, 15 = Abiansemal, 16 = Jegu

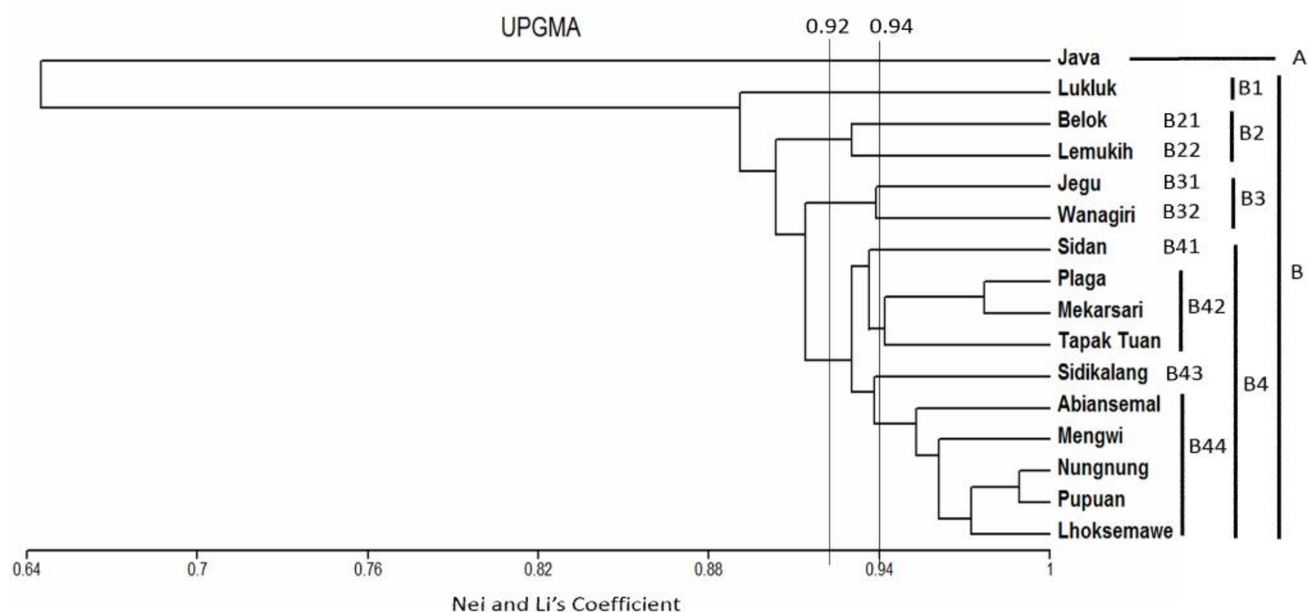

Figure 3. Dendrogram of patchouli cultivated in Bali and three superior patchouli varieties based on PCR-ISSR and PCR-RAPD analyses

patchouli samples. This result supported leaf morphological observations which found that patchouli grown in Bali has similar characteristics with Aceh patchouli.

Comparing PCR-ISSR and PCR-RAPD, this study found that PCR-RAPD resulted in more polymorphism. This is in discordance with several reports which stated the robustness and the highest ability of ISSR to detect variations compared to RAPD (Datta et al. 2010; Yadav et al. 2014). High efficiency of RAPD in detecting polymorphism in plant was reported (Sadeghi and Cheghamirza 2012). According to Sadeghi and Cheghamirza (2012), this may due to the use of primer with high GC content that resulted in higher stability. This is in agreement with our finding.

The Nei and Li's similarities of patchouli cultivated in Bali were in the range 0.857 to 0.989. This indicates that the patchouli grown in Bali had low genetic diversity. A study of patchouli genetic diversity of Johor variety (Malaysia), Singapore variety (Singapore) and Bangalore variety (India) using 10 RAPD primers found that the varieties have significant diversity (Kumara and Anuradha, 2011). According Kumara and Anuradha (2011), the varieties studied in their research may have originated from different regions.

Samples of patchouli from Bali did not group into the altitude of their growing locations, for example Mengwi, Lukluk, Abiansemal and Jegu are lowland areas with altitude of 500 m asl., however, the patchouli samples from those areas were scattered in the dendrogram. Furthermore, samples from nearby areas did not cluster together. In our study, sample from Belok was far separated with sample from Sidan and Plaga, while those areas are geographically close to one another. A study reported by Wu et al. (2011), using RAPD marker, showed that patchouli from adjacent areas was classified together. According to Wu et al. (2011), this might have been due to the possibility that the chosen populations lived in the regions for rather a long time and were seldom transplanted. They further explained that patchouli was introduced to China for long time. However, this is not the case for patchouli in Bali. The commercial cultivation of patchouli in Bali only started in 2006 (Bali Post, 8 December 2007).

The diversity of patchouli grown in Bali could be because of different source of seedling or different varieties. The patchouli samples from Plaga and Mekarsari are genetically closer to Tapak Tuan, while patchouli from Nungnung and Pupuan are closer to Lhokseumawe. Personal communication with patchouli farmers at each location obtained information that seedlings of patchouli grown at Pupuan, Nungnung, Mekar Sari, Mengwi, Sidan and Abiansemal came from Yogyakarta. Patchouli seedlings grown at Lemukih, Plaga, Wanagiri and Jegu came from Bogor, while seedling of patchouli at Belok and Lukluk were from East Java. The diversity could also be due to natural mutation caused by biotic or abiotic stress at plant nursery. Aceh patchouli does not flower, therefore the diversity does not come from hybridization (Swamy et al. 2010).

This study revealed that patchouli cultivated in Bali is Aceh patchouli which is genetically similar to those grown in plantation areas. This study indicates low genetic variation of patchouli in Bali. Genetic improvement of patchouli requires wide variation of germplasm which can be obtained through induced mutation (Rekha et al. 2009) or somaclonal variation (Swamy et al. 2010; Ravindra et al. 2012).

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