



Genetic and Leaf Characteristic Diversity on 10 Mutant Progenies of Patchouli (*Pogostemon cablin*) Provide Insights to Selection Strategies

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ABSTRACT

The importance of patchouli plants to produce essential oil has established this medicinal herb as one of the Indonesia's main economic contributor. To date, Indonesia can only provide 5 varieties i.e. Sidikalang, Tapak Tuan, Lhokseumawe, Patchoulina 1 and Patchoulina 2. In order to increase national patchouli production, several efforts needs to be done by providing new superior patchouli lines. This experiment was undertaken to reveal diversity of mutant progenies derived from gamma ray irradiation in patchouli. Six SSR primers were used to determine genetic diversity on MV3 generation of the mutants. Molecular analysis revealed a moderate to high polymorphism based on tested primers, which amplified 73 bands, and resulting of 0.705 PIC value. Dendrogram analysis based on Euclidean dissimilarity showed broad diversity that ranged from 0.23 to 0.82. Phenotypic tree gave clear separation that all mutant clones were clustered into three different group, with Euclidean dissimilarity coefficient ranging from 0.02 to 0.13. This report indicates that Patchouli vegetatively-propagated mutants were able to maintain their variability continuously from their parental lines (M0). Furthermore, our findings offer comprehensive information on breeding strategies of patchouli plants, giving an opportunity to assess important traits at early generation.

INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.) is the main raw materials in the pharmaceutical industry and essential oils of the world (Huang et al., 2016) and it has been grown for medicinal use for more than 1000 years in China and Southeast Asia. There are limited data to underpin the genetic and genomic resource management for Patchouli. Herein, we used specific-locus amplified fragment sequencing to generate a genetic delineation of *P. cablin* collected from Vietnam, South China, and Indonesia (Sumatra, to produce medicinal drugs, perfumes, and soaps (Wu et al., 2011). Until now, there is no volatile substance that can substitute patchouli oil (Ramya, Palanimuthu, & Rachna, 2013). To date, the domesticated crops and their wild relatives are cultivated on a large scale to meet the industrial requirements. World patchouli's

essential oil production is recorded at 800 t/year (National Horticulture Board, 2014). Indonesia is the main producer that supplies more than 2/3 total world demand. However, based on data released by the Indonesian Ministry of Agriculture (2017) there was a diminishing trend of patchouli oil productivity over the past few years, which is 154 kg/ha in 2014 to 149 kg/ha.

In contrast from those in Indonesia, the production of Patchouli in China and India has increased by 536 kg/ha since 2010 (FAO, 2017). The decrease of oil quality and yield is assumed to be caused by the poor genetic materials used by farmers, since many patchouli plantations were converted into other crops and no improvement concerning the oil quality (Indonesian Ministry of Agriculture, 2015). An imperative action was needed to support the increasing demand of patchouli

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essential oil (Kusuma & Mahfud, 2017). As reported by Singh, Singh, Srinivas, Rao, & Puttanna (2015) currently there is a need to increase the productivity by providing distinguishable varieties to meet the demand at both domestic as well as international market.

To enhance national or world essential oil requirements, producing the national superior patchouli varieties, with high productivity of fresh herbage and essential oil quality is an indispensable effort. The problem to develop improved varieties of patchouli in Indonesia is the narrowness of genetic variability. This matter will complicate the selection process, which is caused by the absence of hybridization process (Yan, Xiong, Zhang, & He, 2016). In addition Swamy & Sinniah (2016) reported that vegetative propagation on Patchouli can lead to bottleneck diversity. As a result of narrow genetic base, an imperative requirement such as mutation is indispensable.

Several reports related to the efforts in broadening the genetic variability of Patchouli had been done by Mo, Zeng, Huang, Chen, & Yan (2012), Nuryani (2004), and Yan, Xiong, Zhang, & He (2016), by utilizing in vitro fusion of protoplasts and colchicine induction. It is proven by using chemical and physical mutagens can induce phenotypical and genotypic variability. Compared to the effort of broaden genetic variability of Patchouli by physical mutation, results were remain insufficient. Previously, gamma ray irradiation mutations have been conducted in various patchouli clones and putatively has broaden the genetic base of the materials, with morphologically obvious on several leaf properties (Tahir & Rofiq, 2013), and putatively will broaden the genetic base.

The utilization of molecular markers on patchouli plants remains limited, while Sandes *et al.* (2013) developed an SSR (Simple Sequence Repeat) markers to classify two Patchouli species. Examination on patchouli diversity with molecular markers were reported by Wu *et al.* (2011) on genetic diversity through ISSR and SRAP markers, genome sequences on chloroplasts (He *et al.*, 2016) and molecular characterization (Sandes *et al.*, 2016). Utilization of molecular marker as a tool of selection in Patchouli is not widely known as reports concerning the selection of Patchouli in Indonesia remains insufficient. Recent successful report concerning utilization of SSR on Patchouli selection was reported by Ouyang *et al.* (2018) confirming that SSR encoded unigenes flanked by their biological functions.

Molecular markers together with their phenotypic appearance (agronomic characters) were

used in this study to identify patchouli clones that have linked to the productivity and quality characteristics of patchouli oil. Selection strategies on early generations of vegetatively propagated plants can accelerate the varieties implementation to farmers as a main user. By performing marker-assisted breeding (MAB) technique, selected progenies were obtained without being influenced by environmental factors, plant growth stages and tissue types, as well as have high accuracy and relatively easy to conduct. Current work on application of MAB were successfully performed on essential oil-produced crops (De Pasquale *et al.*, 2006; Eduardo *et al.*, 2013; Kusuma, Ahsan, Setiawan, Abdullah, & Tahir, 2018; Labra *et al.*, 2004) Cumulative response to mutagens of early generation is occasionally greater than parental lines (Yang, 2009). Effectiveness of selection on early generation were previously reported by Bernardo (2003) and Figueiredo *et al.* (2015). Objectives of this work were to estimate genetic diversity of mutated patchouli plants at MV₃ generations and to determine potential patchouli clones as parental source material.

MATERIALS AND METHODS

An experiment was conducted on Politeknik Negeri Lampung from March to November 2017. A total of 10 plant material coded as NPL 1 to NPL 10, the NPL 1 and NPL 2 were the control accessions, while the rest were mutants. Each accessions consisted with 10 samples. The mutants and control plants of Patchouli were grown at Green House of Politeknik Negeri Lampung with an optimum environmental condition, with 24°C daily degrees and well watered condition. Plant materials were generated from in vitro of MV₂ lines of Patchouli mutant clones, which previously obtained and selected from physical mutations with various gamma-ray doses, ranged from 50 Gy to 100 Gy (Tahir & Rofiq, 2013). These accessions were actually local Lampung patchouli germplasms, and collected from South Lampung, Indonesia.

To determine genetic diversity, nuclear DNA were extracted from Patchouli mutant young leaf with a modified CTAB method by Sandes *et al.* (2016) by addition of MATAB 4%. PCR reaction were consisted with 3.0 µL 5 ng/µL DNA, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, 1.6 µL 2.5 mM dNTPs, 1.0 µL 1 U/µL Taq DNA polymerase, 2.0 µL 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 0.8 µL 25 mM MgCl₂, and 10.6 µL H₂O. To ensure the DNA quantity, spectrophotometer was performed as well as DNA quality were checked by using 1 %

agarose gels with a comparison from EcoRI+HindIII as marker ladder. All DNA samples were purified by using RNase and proteinase K to avoid unwanted proteins and RNA contaminations according to He et al. (2016) by using RNase and proteinase K.

Six primers were chosen in this experiment and picked from GenBank® using sequence available from Huang et al. (2016) and it has been grown for medicinal use for more than 1000 years in China and Southeast Asia. There are limited data to underpin the genetic and genomic resource management for Patchouli. Herein, we used specific-locus amplified fragment sequencing to generate a genetic delineation of *P. cablin* collected from Vietnam, South China, and Indonesia (Sumatra and developed by Sandes et al. (2013). The primer characteristics are available in Table 1. Amplification was done with 94 °C denaturation stage for 5 minutes and 33 cycles and extension at 72 °C for 10 minutes. The PCR product is then electrophorized in 7 % denaturing polyacrylamide. Visualization results were analyzed based on the presence or absence of bands. These data were subjected to percentage polymorphism calculation, as well as polymorphic information content (PIC). PIC value were calculated with the following formula:

$$PIC = 1 - \sum_{i=1}^n P_i^2 - 2 \sum_{i=1}^{n-1} \sum_{j=i+1}^n P_i^2 P_j^2 \quad (1)$$

Where P_i = allele frequency of each markers and n = number of different alleles

Dendrogram tree were constructed by using UPGMA (Unweighted Pair Group Method Arithmetic) of the program NtSys ver. 2.1. To ensure relation from genotypic information, phenotypical analysis by using leaf anatomical characters were observed. Traits

observed were leaf turgidity, specific leaf area, Σ stomata, leaf angle, leaf thickness, xylem thickness, and stem diameter. All measurements were conducted at the end of vegetative stage. All traits were subjected to analysis of variance (ANOVA) to determine the significance of variation among the mutant clones and subjected to Tukey's test 5 %, with an assistance of CropStat Ver. 2.7 software. Thereafter, anatomical characteristic diversity was analyzed by cluster dendrogram analysis, and data were standardized previously.

RESULTS AND DISCUSSION

SSR-Diversity Analysis

The diversity index based on the polymorphism level of the SSR locus used is presented in Table 2. PIC or Polymorphism information content from each locus has been assessed to see how far SSR markers are used to provide polymorphic information. Based on the average score, the PIC value is 0.705 which is the high value, indicating all SSR markers were highly informative. Lowest PIC were found in *Pca11* and *Pca3* markers, where both loci have 0 value. This occurs because of the possibility of unfavorable amplification process, resulting DNA band was monomorphic. The locus which has the highest PIC value is *Pca2* with a value of 0.874. This high value indicates that *Pca2* locus is capable of being used as a selection marker on the next MV generation. In contrast with Huang et al. (2016) and it has been grown for medicinal use for more than 1000 years in China and Southeast Asia. There are limited data to underpin the genetic and genomic resource management for Patchouli.

Table 1. SSR-primer characteristics of *P. cablin*

Locus	Primer sequence (5' – 3')	Repeat motif	Size (bp)	Source
<i>Pca2</i>	F:GTCTGAAGGTTTCAGCCTCTT R:TCGGAACATCAGCAATGAG	CAATG(3)	125-130	Sandes et al. (2013)
<i>Pca5</i>	F:CCCTTTACAATAACCTCGAC R:ATCAACACAGCACACCGTAGA	TATT(3)	130-134	
<i>Pca11</i>	F:TTCCCCTTAGATTGTGCAAA R:AGACAAAAGTGAGGCATCTG	TTGA(3)	232-238	
<i>Pca12</i>	F:AAAAAGCTCACGCCCTTCT R:CTCGTTATCAATGGGATCA	AACC(3)	250-254	
<i>Pca1</i>	F:ACACACTCCCCCACCATAC R:CCACCTGTTTCTTTCACTTC	GA(16)	228-240	
<i>Pca3</i>	F:CCATTCGTCACCTCTC R:AAACAGGCAAGTGAAAGT	CA(8)	164-168	

Table 2. Allele frequencies, PIC and diversity index based on six sets of amplified SSR primers

Locus	A	Major Allele	Size (bp)	H _E	H _O	PIC
<i>Pca2</i>	2	0.63	120bp - 200 bp	0.777	0.721	0.87499
<i>Pca5</i>	2	0.41	130bp - 140bp	0.765	0.587	0.71300
<i>Pca11</i>	2	1	112 bp - 128 bp	0	0	0
<i>Pca12</i>	2	0.58	240bp - 264 bp	0.588	0.622	0.67959
<i>Pca1</i>	3	0.44	230bp - 240bp	0.868	0.429	0.57571
<i>Pca3</i>	2	1	122 bp - 130 bp	0	0	0
Mean	3.33					0.705

Remarks: A = allele; H_E = expected heterozygosity; H_O = observed heterozygosity; PIC = polymorphism information content.

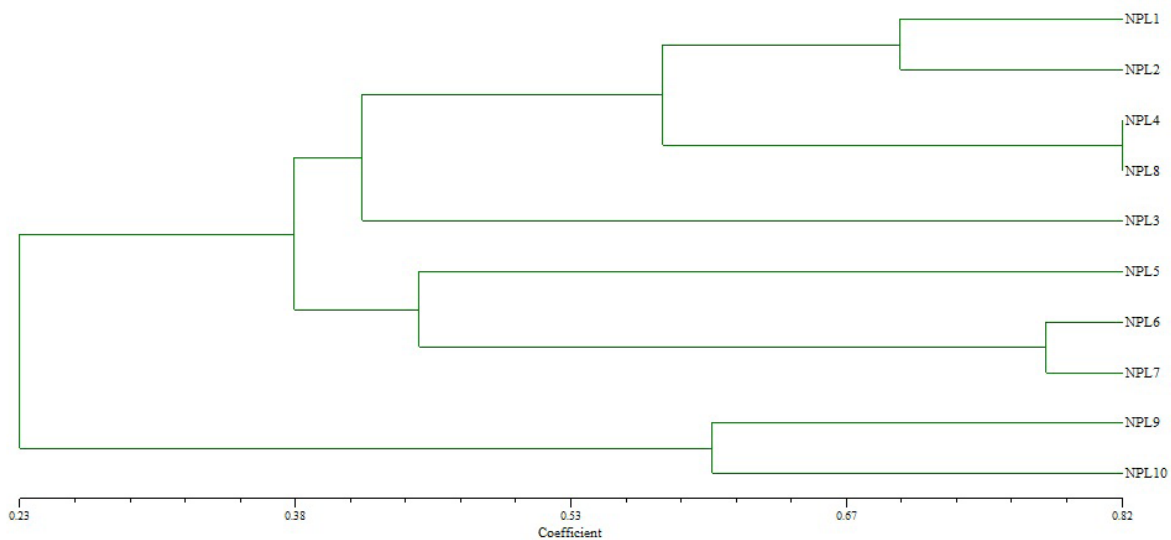


Fig. 1. Dendrogram tree of molecular analysis from 10 patchouli mutants

Herein, we used specific-locus amplified fragment sequencing to generate a genetic delineation of *P. cablin* collected from Vietnam, South China, and Indonesia (Sumatra, average genetic diversity on group of cultivated Patchouli accessions from South East Asia was reported has a low PIC score, ranged from 0.102 to 0.152, with an average value 0.252. This could assuming that Patchouli experienced a series of bottlenecks during the history its cultivation. Further report showed that several Indonesian local accessions were experienced low polymorphism, that obtained only 44 % of PIC from three ISSR markers (Pharmawati & Chandra, 2015).

The heterozygosity value at each locus ranges from 0 to 0.868. This means that each locus tested on these 10 mutants illustrates a wide genetic

diversity. The highest heterozygosity values can be found at *Pca1* locus with a score of 0.868. Based on the report from He et al. (2016). *Pca1* locus is associated with leaf thickness and turgidity on Patchouli plants. Heterozygosity values on two other markers, consisting *Pca11* and *Pca3* were not available due to presence of monomorphic bands.

Furthermore, a cluster analysis was conducted to determine the genetic diversity of each patchouli mutant genotype (Fig. 1), so that the selection direction could be estimated more specifically. Dendrogram graph indicates that 10 mutant clones were divided into 2 main clusters, with a Euclidean dissimilarity coefficient ranging from 0,23 to 0,82. Cluster 1 shows the diversity of NPL 9 and NPL 10 with a value of 0.60. Cluster 2 consists of NPL 1 to NPL 8 genotypes.

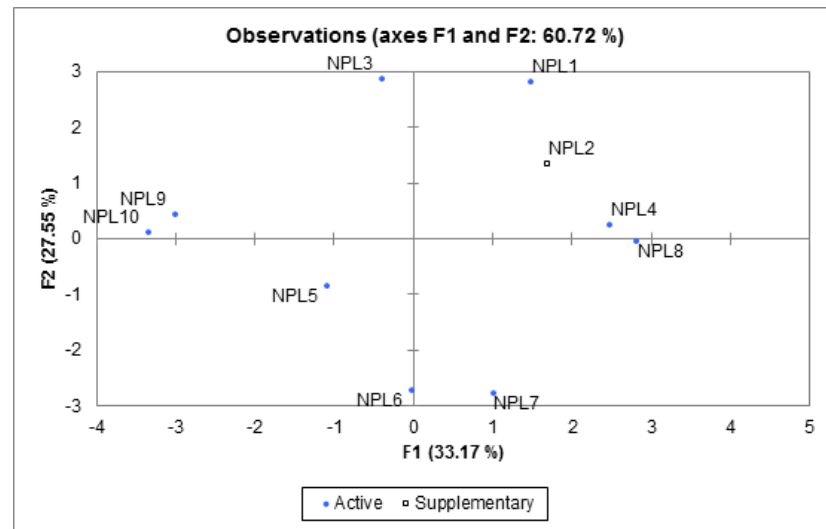


Fig. 2. Biplot interpretation of 10 patchouli mutants based on SSR analysis

This suggests an increasing number of gamma-ray dose is thought to be consistent with the increase in the genetic diversity coefficient, although previous study was successfully determined that LD_{50} for Patchouli was 120 Gy (Tahir, Ersan, Riniarti, & Kusuma, 2018). However, it appears in cluster 2 that increasing doses of gamma-ray radiation can lead to the dynamics of genetic diversity. This is shown in genotypes of NPL 4 and NPL 8 which have the closest genetic relationship among genotypes.

Estimation of genetic diversity in patchouli mutants is important to determining the direction of selection of these mutants. Mutation breeding, physical mutation in particular, occur randomly and molecular or phenotypical confirmation is necessary on each generation to allow the extension of genetic variation in order to gain stability (De Vetten et al., 2003; Fu, Li, & Shu, 2008). Assessment by using molecular markers on patchouli mutants has been shown to determine genetic diversity. The SSR marker used is a marker associated with the quality character of patchouli plants and several other phenotypic characters (Sandes et al., 2013).

A biplot analysis was performed to determine the population distribution of the tested genotype (Fig. 2). The graph shows consistency according to dendrogram analysis, where F1 shows 33.17 % diversity and F2 shows 27.55 % diversity. It can be seen on the graph that the patchouli mutations NPL 4 and NPL 8 have close relationship in cluster 2, similar to NPL 9 and NPL 10 which have close genetic kinship. This information can provide sufficient

information to determine distinguishable clones in early generation, thus potential clones can be implemented to farmers as soon as possible. Other findings compared to this population distribution in this study were exposed by Huang et al. (2016) and it has been grown for medicinal use for more than 1000 years in China and Southeast Asia. There are limited data to underpin the genetic and genomic resource management for Patchouli. Herein, we used specific-locus amplified fragment sequencing to generate a genetic delineation of *P. cablin* collected from Vietnam, South China, and Indonesia (Sumatra, by using SNP markers to infer genetic structure and migration history. It is proven that Indonesian Patchouli (Sidikalang, Tapaktuan, and other minor accessions) were mostly located on same group.

Phenotypic Analysis Based on Leaf Characteristics

Statistical summary of anatomical data are presented on Table 3. Range value, mean, and standard deviation were observed and ANOVA was also performed to determine the significance of the variation between mutant clones. Seven leaf characteristics – leaf turgidity, specific leaf area, Σ stomata, leaf angle, leaf thickness, xylem thickness, and stem diameter – represents significance of variation among mutant clones. Majority of range value of each trait were wide, leaf thickness in particular (401.75 to 590.55). Variation within mutant clones can provide effectiveness for breeder to perform selection.

Table 3. Statistical summary and P-values of Anatomical data on 10 patchouli mutants

Variable	Min	Max	Mean	SD	P
Leaf Turgidity (bar)	77.300	86.225	83.246	2.831	0.000
Specific Leaf Area (cm ²)	13.548	28.952	21.062	4.301	0.001
Σ Stomata	43.050	53.500	47.039	3.551	0.000
Leaf Angle (°)	38.330	67.500	52.037	8.948	0.025
Leaf Thickness (mm)	401.750	590.555	486.589	58.954	0.000
Xylem Thickness (mm)	0.235	0.410	0.324	0.067	0.000
Stem Diameter (cm)	2.025	3.065	2.596	0.347	0.000

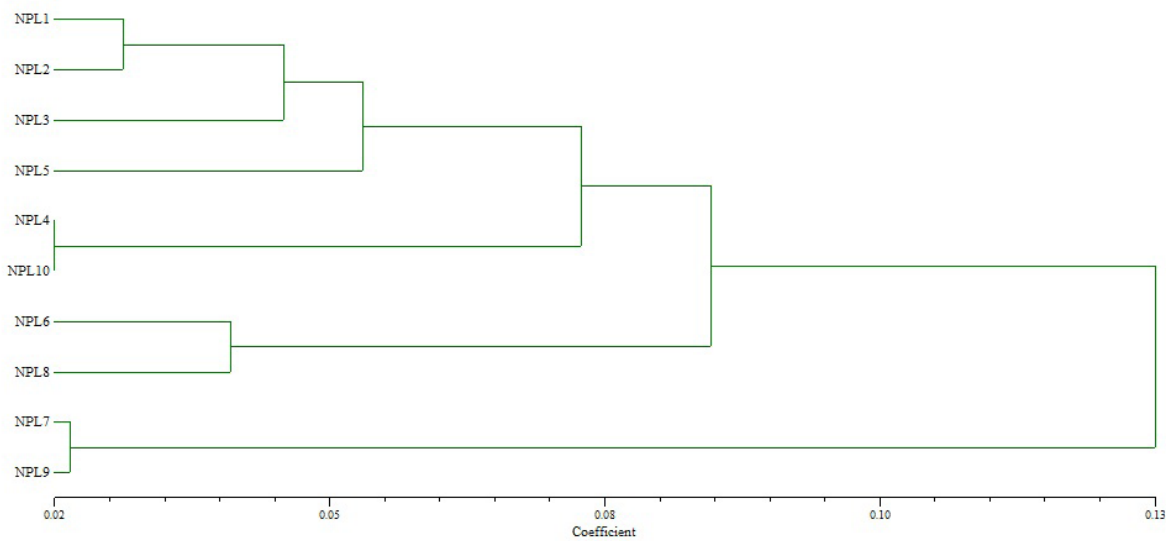


Fig. 3. Phenotypic tree of 10 patchouli mutant lines based on dendrogram analysis

This wideness value could lead into an addition of essential oil yield, as similar correlations on tree basil was reported by Vieira, Grayer, Paton, & Simon (2001), yet further experiment needs to be done. Swamy & Sinniah (2016) explained that weight of fresh herbage yield were derived from leaf composition and positively correlated with yield of essential oil. Several leaf traits on varieties of Patchouli, both wild and cultivated were found likely similar, including leaf thickness (Swamy & Sinniah, 2016). Based on our result, leaf thickness on mutant lines (NPL 5 and NPL 8, in particular), outperformed the parental lines which can reach 590,55 mm. Additionally, SSR markers that were used in this study provide identical trait existence between phenotypic and genotypic examinations. It is confirmed that physical mutation can transform leaf thickness into a thicker one. Yet also anatomical traits of Patchouli leaves revealed the existence of

diverse morphologies under different environmental conditions (Li, Wu, & Guo, 2011).

Variation based on dendrogram tree gave clear separation, and clustered Patchouli clones into two groups (Fig. 3), with a Euclidean similarity coefficient from 0.02 to 0.13. NPL 7 and NPL 9 (group 2) were placed as different group among the other clones. Group 1 which consisted NPL 1, NPL 2, NPL 3, NPL 4, NPL 5, NPL 6, NPL 8, and NPL 10 were also divided into two sub-group. NPL 6 and NPL 8 are considered as sub-group 1A, while the other clones are placed as sub-group 1B. Variation on leaf characteristics were not exactly consistent with dendrogram on molecular analysis. This occur probably the SSR markers used were not specifically flanked with leaf traits observed. Sandes et al. (2013) reported that 6 primers of nuclear SSR were used to study primary and secondary metabolites in Patchouli plants.



Fig. 4. Leaf Anatomical differences between NPL2 and NPL 8

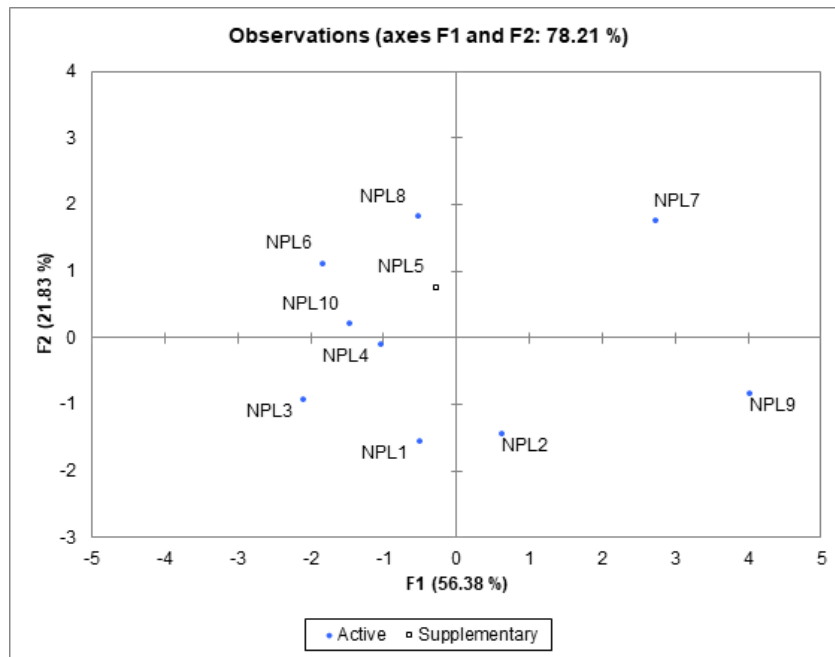


Fig. 5. Biplot interpretation of 10 patchouli mutants based on leaf characteristics

However, differences between mutant clones appear on leaf shape characteristics (Fig. 4). Mutational changes can occur on anatomical or morphological traits as explained by Swamy & Sinniah (2016). Anatomical traits of Patchouli, such as trichome, stem color, and leaf shape will differentiate along with climatic and nutrient changes (Li, Wu, & Guo, 2011). Yet it still needs a molecular confirmation to study leaf shape differentiation of Patchouli mutants by using specific markers and metabolomic studies.

According to comparison of biplot analysis based on molecular markers and vegetative anatomy

characteristic (Fig. 5), the patchouli mutants were divided into three different group. It is consisted with Group 1 (NPL 4, NPL 5, NPL 6, NPL 8 and NPL 10), while Group 2 consisted with NPL 1, NPL 2, and NPL 3. The rest were located on Group 3 (NPL 7 and NPL 9). The first axis explained 56.38 % of total variation while the other axis accumulated 21.83 % value. Variation on Group 1 were contributed from all variables observed which revealed that all mutant clones shared a various range from each traits. Group 2 were contributed from stem diameter, xylem thickness, and Σ stomata with narrow margin

of range value. Group 3 were characterized from leaf thickness and leaf angle, which presenting a widest range value of each clones.

Combined to recent study of mutational progenies on Patchouli (Lal et al., 2018), our findings were validated by molecular markers. All genetic and phenotypical diversity values were confirmed with biplot and cluster analysis to decide which clones will be used for further selection. By revealing mutant clones on phenotypical perspective, it will be very useful to confirm variation on certain clones. As a result of combined diversity analysis, there is a wide prospect for emerging superior varieties.

Further Prospects of Mutational Effects on Patchouli Plants

The impact of broadening diversity of Pogostemon species, would be a key to underpin essential oil industry, since Indonesian patchouli plants were reported has a narrow diversity (Tahir, Ersan, Riniarti, & Kusuma, 2018). The availability of various and different Patchouli genotypes can make a significant contribution to the enhancement effort of Patchouli breeding. According to the result of our study, molecular and Anatomical differences could determine a yield of essential oil from each selected mutants. Several associated leaf characters recorded on this study, such as leaf thickness, and xylem thickness, can be correlated to essential oil yield, as reported by Ravindra et al. (2012) that there is a positive correlation between fresh herb yield to essential oil yield. Molecular approach on this study can be utilized as a tool to confirm particular essential oil on Patchouli plants. This work were strengthen by previous work of Tahir, Ersan, Riniarti, & Kusuma (2018) which tested a cytogenetical studies on MV₂ generation of Patchouli plants. Proven that mutational changes were still appear in MV₂ and MV₃ generations.

Future challenges of Patchouli breeding shall be protecting and maintaining their genetic variation. Simultaneous propagation in industrial scale without maintaining the variability, can lead to declining genetic variation. As reported by Li, Wu, & Guo (2011) and Paul et al. (2010). Pogostemon cablin (Blanco that extensive vegetative propagation has contributed to declining genetic diversity in Patchouli. In contrast with Zingiber species, they were vegetatively propagated but has a wide range of diversity (Wicaksana, Gilani, Ahmad, Kikuchi, & Watanabe, 2011). It means that strategies to maintain genetic diversity of Patchouli has to be relatively different

with another vegetatively propagated plants (e.g., *Zingiber barbatum*), since species vegetative propagation does not correlated species wide variation. Those prospects can be a challenges to Patchouli breeders, as they need to achieve wide genetic diversity, as well as determine the conservation strategies to maintain variation.

CONCLUSION AND SUGGESTION

Direct selection can be performed by using the experiment's findings. All results indicates that mutational changes were still inherited continually as a residual effects in vegetatively-propagated plants. Successful attempt to broaden Patchouli genetic variability as shown by molecular dendrogram analysis provide insight to perform selection. Continuous effort to reveal diversity and essential oil yield needs to be done, as well as a new method of maintaining the diversity of Patchouli plants.

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