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MOLECULAR IDENTIFICATION AND MORPHOLOGICAL CHARACTERIZATION OF PATCHOULI *(Pogostemon* sp.) FROM BATANG REGENCY, CENTRAL JAVA PROVINCE

Identifikasi Molekuler dan Karakterisasi Morfologi Nilam (*Pogostemon* sp.) dari Kabupaten Batang, Provinsi Jawa Tengah

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ABSTRACT
*Patchouli (*Pogostemon cablin *Benth.) is an essential oil-producing plant from Batang Regency that has excellence in patchouli alcohol contents and highly survives in any condition. Molecular identification has been done in the ITS region because DNA sequences in the ITS rRNA region evolved faster than in other areas. This study aimed to get the molecular and morphological identity of patchouli (*Pogostemon *sp.) from BPP Kabupaten Batang. The study consisted of sample preparation, DNA isolation, amplification, electrophoresis, sequence data analysis, and phylogenetic analysis using MEGA X. All parts of plant were morphologically identified and compared with patchouli from Sidikalang, Aceh, Java, and China. Extraction DNA produced 301.7 ng μL^{-1} concentration and 1.93 purity. Amplification of ITS fragment patchouli produced a 670 bp-sized single band. Phylogenetic analysis showed patchouli BPP related to* Pogostemon cablin *(KR608752.1) with 98% coverage identity. BPP patchouli showed 62,5% morphological similarity with Sidikalang patchouli compared to Java patchouli. In conclusion, BPP patchouli is a Sidakalang patchouli P. cablin that has undergone environmental adaptation.*

Keywords: *identification, ITS, molecular, morphology, nilam*

ABSTRAK
Nilam (*Pogostemon cablin* Benth.) adalah tanaman atsiri dari Kabupaten Batang dengan keunggulan berupa kandungan patchouli alcohol dan ketahanan hidup yang tinggi. Identifikasi molekuler dilakukan pada daerah ITS karena sekuens DNA pada daerah ITS rRNA berevolusi lebih cepat dibandingkan daerah gen lain sehingga akan bervariasi pada setiap spesies. Penelitian ini bertujuan memperoleh identitas molekuler dan morfologi tanaman nilam (*Pogostemon* sp.) dari BPP Kabupaten Batang. Penelitian ini terdiri dari penyiapan sampel, isolasi DNA, amplifikasi fragmen ITS, elektroforesis, sekuensing, analisis data sekuens, analisis filogenetik menggunakan MEGA X. Morfologi seluruh bagian tanaman diidentifikasi dan dibandingkan dengan nilam dari Sidikalang Aceh, Jawa, dan Cina. Isolasi DNA nilam menghasilkan konsentrasi 301,7 ng μL^{-1} dan kemurnian 1,93. Amplifikasi fragmen ITS nilam menghasilkan pita tunggal 670 bp. Hasil identifikasi molekuler diikuti dengan analisis filogenetik menunjukkan nilam BPP Kabupaten Batang berkerabat dekat dengan *P. cablin* (KR608752.1) sebesar 98%. Hasil identifikasi morfologis nilam BPP memperlihatkan 62,5% persamaan morfologi dengan nilam Sidakalang dibandingkan dengan nilam Jawa. Kesimpulan, nilam BPP merupakan nilam Sidakalang *P. cablin* yang telah mengalami adaptasi lingkungan.

Kata Kunci: identifikasi, ITS, molekuler, morfologi, nilam

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INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.) or “nilam” (in Indonesian) is an essential oil-producing plant that is widely needed in the medical, industrial, and aromatic fields (Swamy and Sinniah 2016, Febriyenti et al. 2019). Patchouli oil is also used in the perfumes, soaps industry and medicines (Tahir et al. 2019). The essential oil of this plant is obtained through the distillation of the leaves and stems. Patchouli plants are often found in Indonesia, but only a few can produce essential oils that meet export standards. The patchouli alcohol content requirements issued by the Indonesian National Standard (SNI 06-2385-2006) are 30%. The GCMS analysis of patchouli from the Agricultural Extension Center (BPP) of Batang Regency showed that the patchouli oil content reached 30.99% (Kusumaningrum et al. 2016). In addition, patchouli plants from BPP Batang Regency can survive throughout the year. In contrast, patchouli plants from other areas tend to have a shorter lifespan (less than a year) even though the need of patchouli essential oil exports is demanded constantly. It is challenging to identify patchouli plants based only on their morphology. Therefore molecular identification should also be performed. This effort was made to find patchouli plant species that stably produce high-quality essential oils as a superior breed in its area. Patchouli plants grown in the BPP area of Batang Regency are generally known as the Sidakalang variety, originating from Aceh. Local patchouli from Batang itself is thought to be Javanese patchouli. These patchouli plants that adapted to the environment at BPP Batang Regency have shown advantages such as resistance to bacterial wilt and drought while maintaining high patchouli oil content (Kusumaningrum et al. 2016).

BPP Batang Regency is a place to cultivate patchouli plants where superior seeds from various regions that are generally used in several cultivation areas like the Aceh Sidakalang variety are developed (BPS Batang 2019). Superior patchouli can be distinguished in morphology, oil content, and resistance to biotic and abiotic stresses (Parmawati and Chandra 2015). The process of cultivation and acclimatization of patchouli at BPP Batang Regency is one of the efforts to make BPP Batang Regency a center for superior patchouli seeds to break the chain of dependency for patchouli seeds from outside Batang Regency. Currently, patchouli seeds from BPP Batang Regency have also been used to supply areas outside Batang Regency.

The process of cultivation and environmental adaptation at BPP Batang Regency has developed patchouli plants capable of producing good quality essential oils, such as patchouli from East Java (Disbun Jatim 2013). The adapted patchouli plants will be identified molecularly to obtain genetic information. Identification is the process of finding the identity of an unknown plant. Identification of patchouli species and varieties is crucial to obtain various information and descriptions of these varieties to develop their potential. The description of a variety can facilitate the plant breeding process based on the genetic information as the primary material for the plant breeding process (Supriyanti et al. 2015).

Molecular identification in plants using ITS fragments has a high degree of accuracy and has been carried out by many researchers (Michel et al. 2016, Kusumaningrum et al. 2018, Ouédraogo et al. 2019, Vijayakumar et al. 2019). Molecular identification can also be carried out using 18S rDNA and the chloroplast *rbcl* gene. The advantage of ITS rDNA is the higher speed of evolution compared to other gene regions so that each species will have variations in its sequence (White et al. 1990). Eukaryotic organisms generally have two ITS regions, namely ITS-1, which is located between the 18S gene and 5.8S gene, and ITS-2 is located between the 5.8S and 28S genes (Mulyatni et al. 2016). The molecular identification of patchouli using ITS primers was carried out to obtain genetically accurate results. This was done because the morphological identification of various patchouli plants in the BPP Batang Regency tends to obtain subjective results, so it needs to be followed by molecular identification.

Patchouli varieties from Sidikalang Aceh have shown lower survival rates, susceptibility to pests, and patchouli alcohol content less than the standard of SNI 06-2385-2006 when it was planted in Batang Regency. This is different from patchouli from BPP Batang Regency, which has a higher

content of patchouli alcohol, pest resistance, and survival rate. These advantages have caused uncertainty between their identities, considering that patchouli in Batang Regency generally comes from Sidikalang Aceh. Molecular identification is expected to obtain more accurate results as a source of superior seeds for the Batang Regency and other areas and develop their potential. Thus, the research objectives are molecular identification using the ITS area and patchouli morphology from the BPP Batang Regency.

MATERIALS AND METHODS

Location and time

This research was conducted at the Biotechnology Laboratory, and the Integrated Laboratory, Diponegoro University, Semarang. Research activities were carried out from September 2020 to February 2021.

Materials

Patchouli sampling locations were located between 7º 4' 0" and 7º 3' 0" South Latitude and between 109º 49' 30" and 109º 51' 0" East Longitude. This was done by making a location map using the ArcGIS application in Figure 1.

DNA isolation

The patchouli plants were taken using the cutting method on plants grown in the BPP area. Patchouli plants taken are patchouli plants with green and healthy leaves. The overall morphology was observed before the cutting process. Isolation of DNA from patchouli samples was performed using the cetyltrimethylammonium bromide (CTAB) method with slight modifications without using mercaptoethanol (Doyle and Doyle 1990). Fresh patchouli leaves of around 0.5 grams were ground with a mortar and pestle while added with CTAB during grinding. The solution was incubated at 60 ºC for 30 minutes. Every 10 minutes, the solution was homogenized, followed by using vortex for 1 minute. The solution was added with chloroform and isoamyl alcohol (CIA) [24:1] in a ratio of 1:1 with the sample, then centrifuged at 8000 rpm for 10 minutes. The supernatant was transferred to a new tube, added isopropanol in a ratio of 1:1, and incubated at −20 ºC overnight. The sample was centrifuged for 10 minutes at 8000 rpm. The supernatant was separated and added Tris and ethylenediamine tetraacetic acid (TE) pH 8 as much as 250 µL, sodium acetate 3M 20 µL, and absolute ethanol 500 µL then

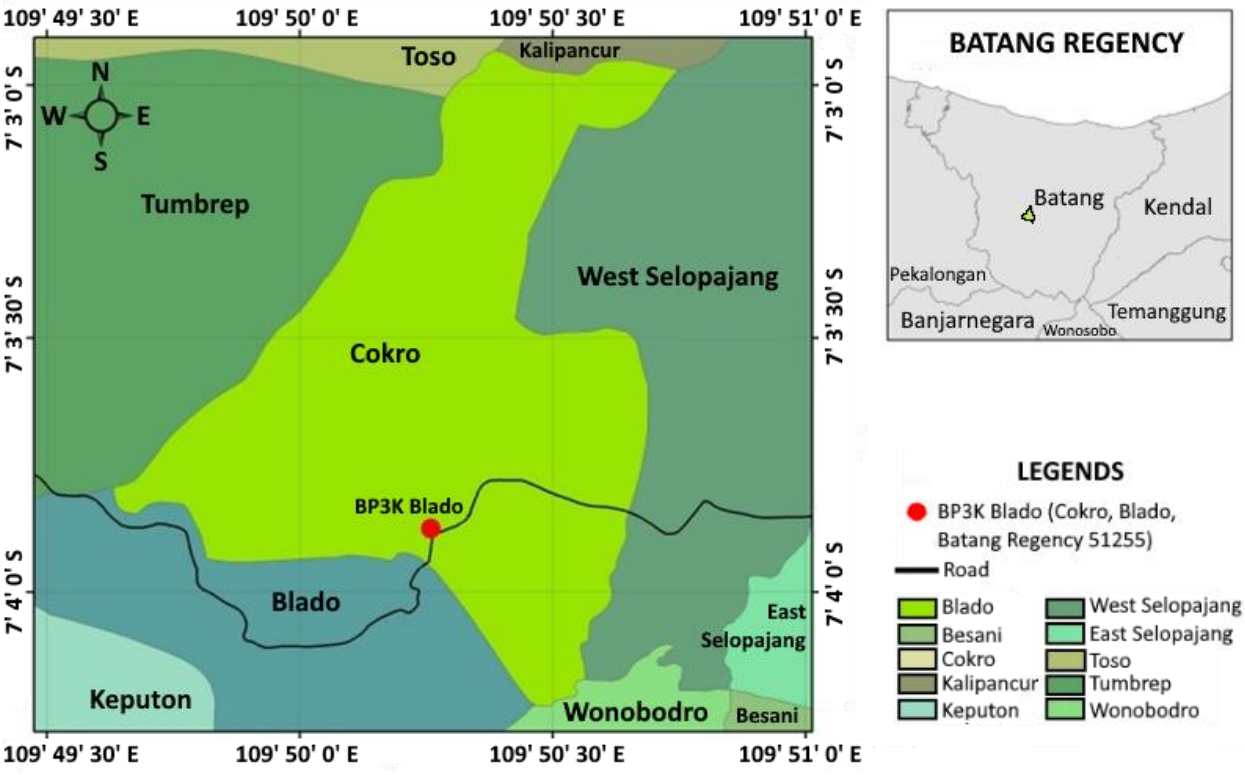

Figure 1. Map of patchouli sampling locations at BPP Batang Regency

Table 1. Results of patchouli DNA quantitative test using nanodrop

No.	Sample ID	Nucleic Acid Conc. (ng/µL)	A_{260}	A_{280}	260/280
1	Tube 1	301,7	6,034	3,131	1,93
2	Tube 2	321,4	6,428	3,287	1,96
3	Tube 3	176,1	3,522	1,799	1,96
4	Tube 4	221,4	4,428	2,298	1,93

incubated for 30 minutes at −20 ºC. The solution was centrifuged for 10 minutes at a speed of 8000 rpm. The supernatant was rinsed with 200 µL of 70% alcohol. Then the sample was centrifuged for 10 minutes at 8000 rpm. The pellet was added with 50 µL of TE and incubated at 20 ºC.

Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique that uses enzymatic activity to amplify specific DNA fragments. Each PCR test requires a DNA template, primers, nucleotides, and DNA polymerase, formed into a PCR mixture (Garibyan and Avashia 2013). The PCR mixture was 25 µL in one PCR tube for 4 PCR tubes. The PCR mixture for each tube consisted of 2 µL of DNA samples with a concentration of 301.7 ng μL^{-1}; 12.5 µL MyTaq Polymerase Master Mix consisting of Taq Polymerase, PCR Buffer, dNTPs, and Mg^{2+}; 1 µL of forward primer, 1 µL of reverse primer and 8.5 µL of ddH_20. Amplification was carried out using a gradient PCR thermal cycler for 30 cycles with pre-denaturation steps at 95 ºC for 1 minute, denaturation for 15 seconds at 95 ºC, annealing for 30 seconds at 54 ºC, 55 ºC, 56 ºC, 57 ºC (White et al. 1990) and the extension at 72 ºC for 10 seconds. The amplification process was terminated with the final extension phase at 72 ºC for 10 minutes and 4 ºC holding temperature. PCR result was checked by electrophoresis with 1% agarose gel in TAE buffer with 100 V for 30 minutes.

Phylogenetic analysis

DNA sequences obtained after the sequencing process by 1st Base Laboratory were aligned using BioEdit software to build the consensus sequence. The consensus was then analyzed with the Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database. The sequence data from BLAST NCBI were aligned in Clustal X. A phylogenetic tree was constructed using MEGA X using the Neighbour-Joining method with the Bootstrap number of 1000 replications (Saitou and Nei 1987).

Morphological characterization

The profile of patchouli plants can be identified as follows (Haryudin and Maslahah 2011, Setiawan and Sukamto 2016):

1. Number of leaves
   The number of leaves is calculated from the intact leaves, particularly those that have completely opened.
2. Leaf length and width
   Leaf length and width can be measured using a ruler on patchouli leaves that have fully opened from the leaf diameter as surface area.
3. Plant height
   Plant height is measured from the base of the stem to the tip of the patchouli plant.
4. Color of stems and leaves
   The stems and leaves on the patchouli plant are observed from mature leaves and stems.
5. Observation of the tips and leaves edges

RESULTS AND DISCUSSION

DNA quantitative and qualitative test results

Isolation of patchouli DNA using the modified Doyle and Doyle method obtained qualitative and quantitative results. Table 1 shows the quality of patchouli DNA by the purity level of 1.945. Similar results were acquired in previous studies on several herbal plants, which obtained DNA purity from 1.8 to 1.9 (Tiwari et al. 2012).

The purity of the sample, as seen from $\lambda_{260}/\lambda_{280}$ with a ratio of ~1.8, was accepted as 'pure' DNA that was not contaminated with phenol or other chemical residues. In contrast, DNA purity of ~2.0 can be considered contaminated by RNA (Aboul-Maaty and Oraby 2019). Abdullah et al.

Table 2. Comparison of the morphology of patchouli Java, Sidikalang, and *P. cablin* (KR608752.1) Yao et al. (2016)

Morphology identity	BPP patchouli	Jawa	Sidakalang	*P. cablin* (KR608752.1)
Plant height (cm)	70,6 - 73,3	70-	70,70 - 75,69	70 – 76
Stem color	Greenish-purple	Greenish- purple	Greenish- purple	Greenish- purple
Leaf color	green	green	green	green
Leaf shape	*deltoideus*	*ovatus*	*deltoideus*	*deltoideus*
Leaf length(cm)	6,20 – 6,50	6,0 – 6,2	6,23 – 6,75	6 – 7
Leaf width (cm)	4,90 - 6,30	5,0 – 6,0	5,16 – 6,36	5 – 6
Leaf tip	*acutus*	*acuminatus*	*acutus*	*acuminatus*
Leaf edge	*serratus*	*serratus*	*serratus*	*serratus*
Patchouli alcohol (%)	30,99 (Kusumaning-rum dkk, 2016)	30,28 (Nuryani 2004)	34,41 (Nuryani, 2004)	-

Description : acutus (pointed, having a short sharp apex angled less than 90°), serratus (saw-toothed; with asymmetrical teeth pointing forward), acuminatus (tapering to a long point in a concave manner), deltoideus (shaped like Greek letter Delta, triangular, stem attaches to side), ovatus (egg-shaped, with a tapering point and the widest portion near the petiole)

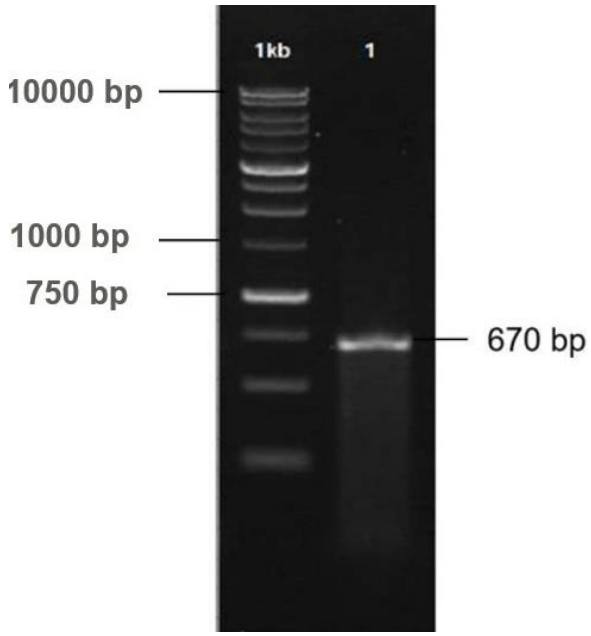

Figure 2 . Results of amplification of the patchouli ITS from BPP Batang (1 = DNA Ladder 100 bp; 2 = ITS patchouli)

(2016) and Lin (2021) stated that the most minor concentration of DNA that a UV-Vis machine can measure is 2.5 ng μL^{-1}. The quantity of patchouli DNA showed a result of around 255.15 ng μL^{-1}. This concentration has met the sufficient value to be carried on the amplification step.

DNA amplification results

The results of amplification using ITS molecular markers are shown in Figure 2. Amplification using gradient PCR obtained several different effects. BPP patchouli DNA produced DNA bands of about 670 bp using an annealing temperature of 54 ºC, while the other samples that used annealing temperatures of 55 ºC, 56 ºC, and 57 ºC did not produce any bands. White et al. (1990) stated that the annealing temperature in plants for ITS 1 area was 63 ºC and ITS 2 area was 58 ºC. However, this result proved that ITS DNA band from the patchouli plant could also be obtained at the annealing temperature of 54 ºC.

The results of ITS amplification in this study obtained a DNA band length of about 670 bp. The band obtained is longer than other researches using ITS 1 and ITS 2 primers for patchouli ITS amplification, 424 bp and 380 bp (Kool et al. 2012, Swamy and Sinniah 2016). In addition to using ITS, molecular identification of patchouli plants can use other markers. Michel et al. (2016) used the *mat*K chloroplast region as an alternative molecular marker for aromatic plants. Zhang et al. (2019) analyzed the gene sequences for patchouli plants from several regions with 18S rRNA, which produced a sequence length of 1803-1805 bp, and *mat*K chloroplasts with a sequence length of 1245 bp.

Phylogenetic analysis result

The patchouli phylogenetic trees were constructed using MEGA X with references to

several species obtained from the BLAST results in GenBank from the NCBI site. Furthermore, the genetic distance was calculated using the Jukes-Cantor model in the MEGA X software. The patchouli phylogenetic tree is shown in Figure 3.

The analysis results using BLAST showed that patchouli ITS sequences were 99% similar to *Pogostemon cablin* (KR608752.1) from China. In addition, BLAST analysis showed that BPP patchouli had a similarity of 98.38% with Javanese patchouli (*P. heyneanus*) and 97.42% with *P. septentrionalis* from China. The phylogenetic tree in Figure 3 also shows that the BPP patchouli plant is not the same group as the Javanese patchouli plant. Nevertheless, they remain in one monophyletic group. Genetic relationships in plants grouped into one species generally have a 99% similarity level, 96% identity, and 90% genome coverage (Ciufo et al. 2018).

The relationship between BPP patchouli and *P. cablin* from China based on evolutionary lines in the phylogenetic tree falls into one clade. According to Wright et al. (2011), a clade is an information that represents a phylogenetic relationship. Yao et al. (2016) stated that using chloroplast DNA molecular markers and ITS can show a phylogenetic relationship between the *Pogostemon* subgenus group.

Morphological characterization results

Morphological profile observed between patchouli samples cultivated in BPP and *P. cablin* as shown in Table 2. There were differences in leaf number, leaf width, stem color, leaf color, leaf margin, leaf tip, leaf shape, and plant height (Tjitrosoepomo 2018). BPP and Sidakalang patchouli had more leaves than *P. cablin* from China. The number of leaves could be affected by light intensity. High sunlight intensity will induce many leaves (Buntoro et al., 2014). BPP patchouli has a similar plant height to Sidakalang patchouli, mainly because plants grow in the tropics. However, BPP patchouli had a different mean plant height from *P. cablin* from China. Chen et al. (2015), and Manda and Mataa (2020) stated that light is one of the environmental factors that have an essential role in plant growth, which correlates with plant height. Based on the leaf area and width comparison, the measurements seen from the biggest to smallest were Sidakalang patchouli, BPP patchouli, and *P. cablin* Cina, respectively.

Kusumaningrum et al. (2016) stated that patchouli from China contains patchouli alcohol of 37.53% in leaves where the place of cultivation influences the patchouli alcohol content. Patchouli plants are less resistant to drought because they have shallow roots, like the Tapaktuan patchouli plant originating from Aceh (Nasruddin et al. 2018). Patchouli plants

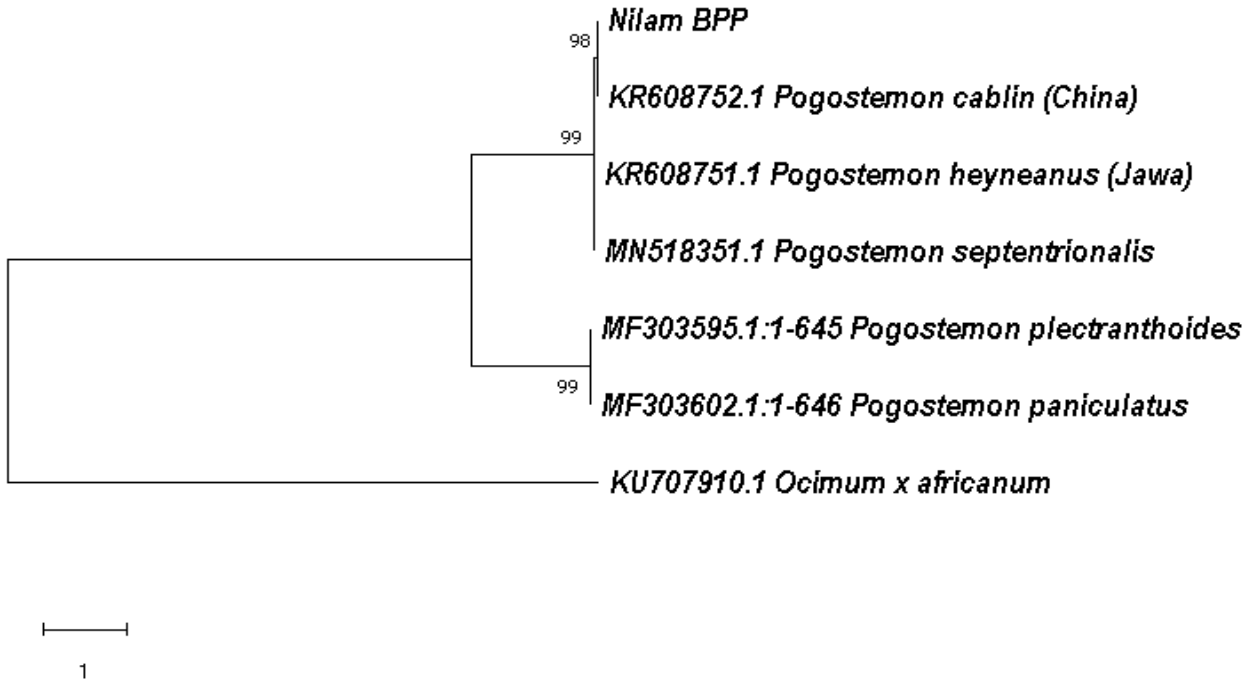

Figure 3. Phylogenetic tree of BPP Batang patchouli using Maximum-likelihood tree

are less resistant to drought because they have shallow roots, like the Tapaktuan patchouli plant originating from Aceh. BPP Batang patchouli can withstand drought, although it still requires shade at about two weeks of growth. After that, BPP Batang patchouli was able to survive without shade. According to Setiawan and Sukamto (2016), patchouli grown in full sunlight, such as Sidakalang plants, have higher levels of patchouli alcohol than the plants in the shade. A different result was stated by Raai et al. (2020), in which shaded plants have larger leaf sizes to optimize light absorption. This phenomenon is supported by several other researchers, who found that eucalyptus (Degani et al. 2016) and lemon balm plants (Oliveira et al. 2016) in the shade would produce more essential oils. Moreover, Sidakalang patchouli is known to have susceptibility towards nematode at the beginning of the planting period than BPP patchouli, which has more resistance (Kusumaningrum et al. 2016).

Based on the research results obtained, the patchouli plant from Batang was identified as *Pogostemon cablin,* which could adapt to the Batang region environment. The patchouli plant can be further developed based on its ability to survive in the Batang area throughout the year and the high production level of patchouli alcohol. Furthermore, with these advantages, patchouli plants from the Batang area thus have the potential to be used as superior patchouli seeds for the local and surrounding areas.

CONCLUSION

The molecular identification of patchouli from BPP Batang Regency using ITS markers was 98% similar to *Pogostemon cablin* (KR608752.1) from China. The morphological identification of BPP patchouli showed 62.5% morphological similarities with Sidakalang patchouli compared to Javanese patchouli. Thus, patchouli in the Batang Regency BPP is a *Pogostemon cablin* that could adapt to the Batang region environment.

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