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Sequence and Phylogenetic Analyses of *Bebara* Medicinal Plant (*Timonius flavescens* (Jacq.) Baker) Based on *matK*, *rbcL* and *tnrL* Genes in Central Kalimantan

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Bebara medicinal plant (*Timonius flavescens* (Jacq.) Baker.) is a plant used by local communities in Central Kalimantan as a medicine for health recovery and increasing the production of breast milk after giving birth. Information and publications regarding the introduction of species and classification of *bebara* plant still does not yet exist so far and this is the new studies topic and interesting points to be studied further. This research aims to analyze the sequence variation of *matK rbcL, tnrL* genes and to construct phylogenetic trees in *bebara* plant through the use of a PCR (Polymerase Chain Reaction)-based method. The analysis of sequence variation shows a difference between *bebara padang* and *bebara natai*. The analysis results of the *matK* gene reveals 5 nucleotide differences; the *rbcL* gene has 3 nucleotide differences and there is a cytosine (C) base insertion on *bebara padang*; and the *tnrL* gene has 2 nucleotide differences. *MatK* gene is used to identify this plant because it has more nucleotide variations than the *tnrL* and *rbcL* genes. Phylogenetic trees with *matK* gene show that *bebara padang* and *bebara natai* are grouped in the family of *rubiaceae*. By using pairwise distance values, it obtains the results that *bebara natai* has a genetic distance value of 0.001 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*. On *bebara padang* has a genetic distance value of 0.017 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*. The genetic distance value of *bebara padang* and *bebara natai* is 0.016. This study recommends the importance of further research on species identification, plant morphological characteristics, the content of bioactive compounds and cultivation efforts conducted.

Keywords: Genetic variations, *matK, rbcL, tnrL* genes, *bebara* (*Timonius flavescens* (Jacq.) Baker.), Central Kalimantan.

INTRODUCTION

Bebara medicinal plant *Timonius flavescens* (Jacq.) Baker.) is a plant used by local communities in Central Kalimantan as a medicine for health recovery and increasing the production of breast milk after giving birth. Information and publications regarding the introduction of species and classification of *bebara* plant still does not yet exist so far. Its scientific/latin name is given based on the results of herbarium sample identification of *bebara* plant sent to UPT Balai Konservasi Tumbuhan Kebun Raya Purwodadi (Technical Implementation Unit for Plant Conservation Center of Purwodadi Botanical Garden) and LIPI (Indonesian Institute of Sciences) “Herbarium Bogoriensi”, the Botanical Field of Biology

Research Center. Accurate species identification using traditional methods can take a long time due to lack of knowledge about plants and/or lack of flower and fruit characteristics needed for the identification (Drouet et al., 2018; Wäldchen et al., 2018; Baird et al., 2018; Seethapathy et al., 2018; Nagarajan et al., 2018; Sogbohossou et al., 2018; Cope et al., 2012; Costion et al., 2011; Schippmann et al., 2006; Techen et al., 2004; Mohammadi & Prasanna, 2003; Schippmann et al., 2011, and Colpaert et al., 2005). Molecular approach is one of the right and fast ways that can be done to determine genetic potential, one of them is through the use of PCR (Polymerase Chain Reaction)-based method.

Identification of DNA sequence-based species is a method that is considered fast, accountable, and consistent, thus, it is important in conservation biology and diversity research (Waugh, 2007). Identification with DNA sequence is carried out using molecular markers. One of the molecular markers currently used in expressing taxonomy is DNA barcoding which is a short DNA sequence that can show genetic variation in a species (Siccha et al., 2018; Baetscher et al., 2018; Freed et al., 2018; Manzanilla et al., 2018; Badotti et al., 2018; Porter & Hajibabaei, 2018; Hongsanan et al., 2018; Mills et al., 2017; Allio et al., 2017; Yang et al., 2017; Therkildsen et al., 2017, and Chippindale et al., 1999). In the DNA Barcoding process, certain genes can be used as markers in the genetic division of species and phylogenetic reconstruction (Yan et al., 2018; Krehenwinkel et al., 2018; Thézé et al., 2018; Manzanilla et al., 2018; Raj et al., 2018; Song et al., 2018; Kress & Erickson, 2007; Newmaster et al., 2006; Pons et al., 2006; Kress et al., 2005, and Hebert, 2004).

Phylogenetic trees are used to see and determine kinship/ genetic relationships based on genetic proximity between organisms based on analysis of certain genes. In this case, genes that are often used are *matK* (Maturase within trnK intron), *rbcL* (ribulose-1,5-bisphosphate carboxylase) and *tnrL* (tRNA-Leucosine (UAA) 5'exon intron).This research aims to analyze the sequence variation of *matK rbcL, tnrL* genes and to construct phylogenetic trees in *bebara* plant based on differences in growing places (*padang* and *natai*) in Sukamara Regency, Central Kalimantan.

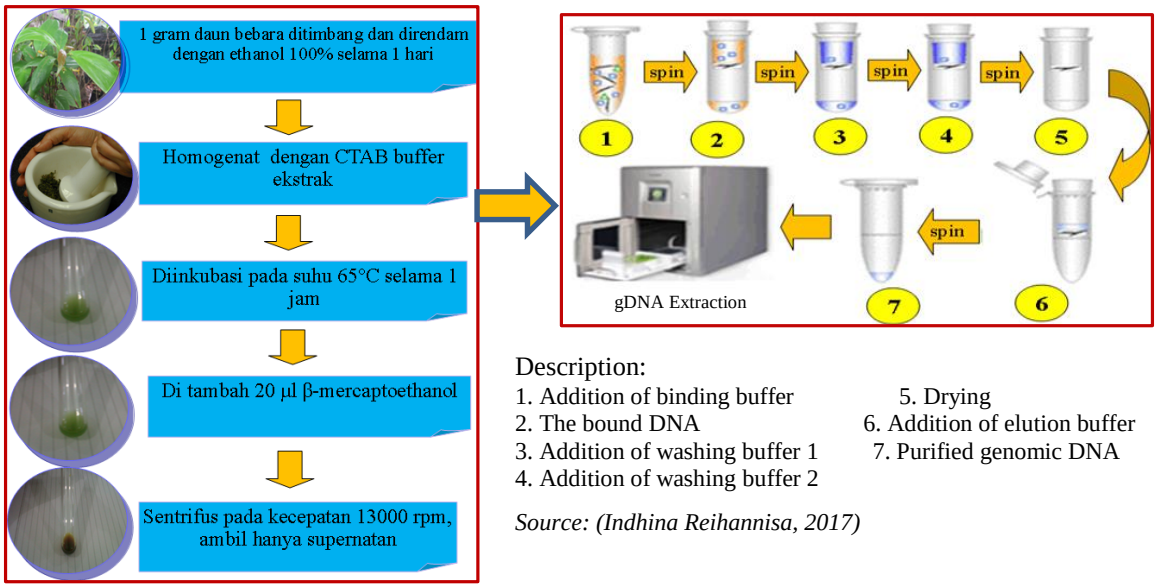
MATERIALS AND METHODS

Figure 1, DNA analysis began by extracting 1 gram of total small pieces of young *bebara* leaf which was soaked in 100% ethanol for 1 day, 0.25 gram of leaves was weighed and isolated using CTAB extraction buffer by using the doyle and doyle method (1987).

The *homogenate* was then added with 20µl β-mercaptoethanol and then incubated at 65°C for 1 hour. The homogenate was centrifuged at a speed of 13000 rpm for 10 minutes at room temperature to form pellets and supernatants. The supernatants were transferred to a 1.5 ml tube and added with a PCI solution (Phenol: chloroform: isoamylalcohol) with a ratio of 25:24:1 as many as 1:1 with supernatant and vortex volume until it became homogeneous. The homogenate was centrifuged at a speed of 13000 rpm for 10 minutes at room temperature, then the supernatants were transferred to a new 1.5 ml tube and added with ethanol absolute 2.5 times of supernatant volume. The solution was then incubated at -20°C for 1 hour or overnight. The solution was centrifuged at 13000 rpm for 10 minutes at 4°C to form pellets and supernatants. Supernatants were discarded and pellets were added with 500 µl of 70% ethanol. Then the pellets were centrifuged at a speed of 13000 rpm for 10 minutes at 4°C. Next, 70% ethanol solution was discarded and the pellets were dried at 55°C. The pellets were added with 50µl of TE buffer pH 7.5.

Visualization of DNA Isolation Results and PCR Results

A total of 0.15 grams of agarose was added with 15 ml of 1x TBE pH ~8.3 solution in the Erlenmeyer tube and it was heated until boiling. Then leave it at room temperature to a temperature of around 50°C and then it was added with 1 µl of Ethidium Bromide. Next, the solution was poured into a mold that has been installed with a comb. After the gel became solid, then the comb was pulled. Then, it was inserted into the electrophoresis chamber containing a 1x TBE pH ~8.3 solution. A total of 2 µl of DNA was inserted into the well. Then, it was run at a voltage of 50 volts for 30 minutes. The gel was then visualized on the UV transiluminator and photographed using a camera. The same was done to visualize the PCR results with the use of 0.3 grams of agarose.



Description:

1. Addition of binding buffer
2. The bound DNA
3. Addition of washing buffer 1
4. Addition of washing buffer 2
5. Drying
6. Addition of elution buffer
7. Purified genomic DNA

Source: (Indhina Reihannisa, 2017)

Figure 1. DNA Isolation Process (Dioxyribose Nucleic Acid)

Description:

1. 1 gram of *bebara* leaves was weighted and soaked in 100% ethanol for 1 day
2. Homogenate with CTAB extraction buffer
3. Incubated at 65°C for 1 hour
4. Added with 20µl β-mercaptoethanol
5. Centrifuges at a speed of 13000 rpm, take only the supernatants

PCR sample

PCR was conducted to the total DNA of isolation results by using the primers of *matK* (5`CGATCCTTTCATGCATT-3 and 5'-ATCTGGGTTGCTAACTCAATG-3, *rbcL* (5'-ATGTCACCACAAACAGAGACTAAAGC-3' and 5'-TCGCATGT ACCTGCAGTAGC-3') and *tnrL* (5'-CGAAATCGGTAGACGCTACG-3' and 5'-GGGGATAGAGGGACTTGAAC-3').

The composition of the PCR reaction consisted of 2 µl of dH2O, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 5 µl of Intron Mastermix and 1 µl of DNA. The composition was inserted into the PCR tube and run on the PCR machine with the following PCR program : *predenaturation* with a temperature of 94°C for 5 minutes then followed by 35 cycles, with denaturation with a temperature of 94°C for 30 seconds, annealing with a temperature of 55°C for 30 seconds and extension with a temperature of 72°C for 30 seconds, then followed by the final extension with a temperature of 72°C for 10 minutes. The amplification results were then stored at -20°C and sequenced *at FirstBase Laboratories Sdn Bhd Selangor*, Malaysia.

Statistical analysis

Sequencing results were analyzed using a sequencer scanner and the data was entered in fasta format and analyzed using Bioedit and phylogenetic trees formed by the Maximum likelihood method. The genetic distance was calculated using the Kimura's 2-parameter method integrated in the MEGA6 software developed by Tamura et al., (2013).

RESULTS

Local people call this medicinal plant as "bebara" which is believed to be hereditary to restore health and increase the production of breast milk (ASI). Scientifically, there is no information about the identification of these types of *bebara* plant and it is only known by local people. Its scientific/latin name was obtained by sending herbarium samples to UPT Balai Konservasi Tumbuhan Kebun Raya Purwodadi (Technical Implementation Unit for Plant Conservation Center of Purwodadi Botanical Garden), *bebara* medicinal plant is classified as follows:

Division : *Magnoliophyta*

Family : *Rubiaceae*
Class : *Magnoliopsida*
Genus : *Timonius* sp
Subclass : *Asteridae*
Species : *Timonius* sp.
Order : *Rubiales*

Furthermore, the herbarium sample was also sent to LIPI (Indonesian Institute of Sciences) "Herbarium Bogoriensi", the Botanical Field of Biology Research Center, in which it obtained the following results:

Family : *Rubiaceae*
Species : *Timonius flavescens* (Jacq.) Baker

Accurate species identification using traditional methods can take a long time due to a lack of data and information about plants. Molecular approach is one of the right and fast ways that can be done to determine genetic potential. One of them is through the use of PCR (Polymerase Chain Reaction)-based methods.

Molecular Characterization

Molecular characters are very useful in determining the taxonomy of plants. Some studies make plant phylogenetic trees based on molecular characters and combine them with morphological data. based on research of Magallonet al., (2018); Kusuma et al., (2018); Braukmann et al., (2017); Azani et al., (2017); Kawahara et al., (2017); Wu et al., (2006); Bremer et al., (1999); Bremer (1996), and Robbrecht & Manen (20006) that Family of *rubiacea* is a large family of angiosperms which even though it is easily recognizable, it is difficult to classify. In addition, there is the existence of intrafamilial phylogeny (Bremer, 1996). *Timonius* is included in the genus of *guetttardeae* and family of *rubiaceae* (Costion et al,. 2016). The results of isolation using the modified method of doyle and doyle (1987) are shown in Figure 2. From the DNA isolation results, then the DNA of *bebara padang* and *bebara natai* were amplified using *matK*, *rbcL* and *tnrL* genes shown in Figure 3. Based on DNA test results using the primers of *matK , rbcL,* and *tnrL* from the steps above, it obtained the following results.

Whole genomic and PCR results

The results of total DNA amplification using 1% agarose showed that the whole genomic DNA of *bebara natai* and *bebara padang* in figure 2 was greater than 1000 basepair. Figure 3 shows that the amplicon length of the primers of *matK* (*matK*-F 5´-TAATTTACGATCAATTCATTC-3´,*matK* R5´GTTCTAGCACAAGAAGTCG-3´) was 1000 bp; the amplicon length of the primer of *rbcL* (*rbcL*1b: 5`-ATGTCACCACAAACAGAAAC-3` and rbcL-724R: 5`-TCGCATGTACCTGAGTAGC-3`) was 700 bp the amplicon length of the primer of *tnrL* (5'-CGAAATCGGTAGACGCTACG-3' and 5'-GGGGATAGAGGGACTTGAAC-3') was 400 bp.

DNA barcoding in plants involves sequences of mitochondrial or chloroplastic genomes using reference sequences from the database. The database can be obtained from gene banks (http: www.ncbi.nlm.nih.gov) and BOLD (www.Boldsystem.org). DNA sequences have a high similarity in species when compared and there are more variations in nucleotide composition for different species. The findings of the research conducted by Yan et al., (2018); Dormontt et al., (2018); McManus et al., (2018); Fitzpatrick et al., (2018); Epp et al,. (2018); Feliner& Rosselló (2007); Qiu et al., (1999) and Palmer (1992) show that study of plants through variations in DNA sequence of chloroplasts, mitochondria and core DNA is very useful to determine the systematics of plants. Plant systemic gene sequences such as *rbcL, matK* and *tnrL* can be used as markers to identify plants (Vannozzi et al., 2008, and Wahyudi et al., 2013). *matK*gene is an ideal gene for determining plant taxonomy because it has a very high substitution rate, a very large level of base nucleotide variation and has a very low transition/transversion level. Currently, the *matK* gene has been used as an important tool for examining intraspecies and interspecies genetic diversity because it has a high substitution rate (Mader et al., 2018; Chrungoo et al., 2018; Schwarz et al., 2017; Khederzadeh et al., 2017; Dizkirici et al., 2018; Nowicki et al., 2018; Olmstead et al., 2018; and Yao et al., 2010, Hollingsworth et al., 2011; and Selvaraj et al., 2008). *matK* gene (Maturase K), is a chloroplast gene measuring around 1500 base pairs (bp) located in *trnK*introns (Selvaraj et al., 2008).

Marquina et al., (2018); Tabssum et al., (2018); Xu, eta al., (2018) and Weger et al., (2018) show that determination of barcoding among plant species has several problems. For example, in some cases, DNA barcoding is incapable to act as a means of identification and there is a difficulty in determining the history of a species that has an impact on the rate of molecular evolution. Therefore, in determining the barcoding of a species, the combination of several genes in determining the tested species is very important (Kress et al., 2009).

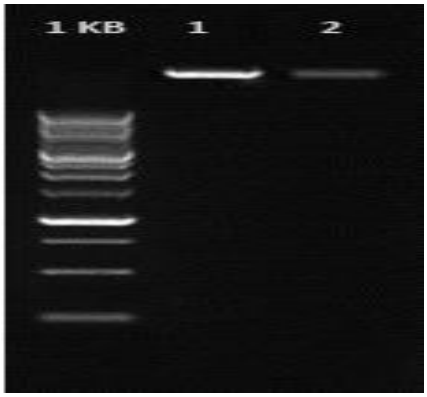


Figure 2. Whole genomic DNA sample of *bebara natai* (1) and *bebara padang* (2)

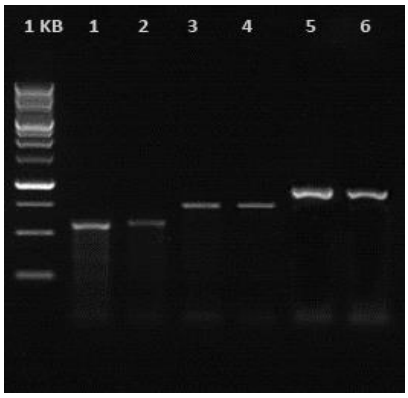


Figure 3. Amplification results of *bebara natai* and *bebara padang* by using *tnrL* gene(1&2), *rbcL* gene (3 & 4) and *matK* gene (5&6)

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                    100       110       120       130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
bebara natai        TCTTCCTTGCATTTATTACGATTCTTTTTCCACGAGTATTGGAATTGGAATACTCTTATTGCTACAAAGAAACCCAGTTTTGATTTTTCA
bebara padang       ....................................................................................T.......
                    280       290       300       310       320       330       340       350       360
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
bebara natai        TTTCATTTGCGATCAACATCTTTTGGAGTCTTTCTTGAACGAATCTATTTCTATGGAAAAATAGAACGTCTTGTAGAAGTTTTTGCTAAG
bebara padang       ..................................................................T.....................
                    460       470       480       490       500       510
                    ....|....|....|....|....|....|....|....|....|....|....|....|....
bebara natai        CTCCCTTTGATGAATAAATGGAAATATTACCTTGTCAATTTTTGGCAATGTTATTTTGATCTGTGGTTT
bebara padang       ........T.....................A..............TT-......A
```

Figure 4. Results of nucleotides alignment of *bebara natai* and *bebara padang* using *matK* gene

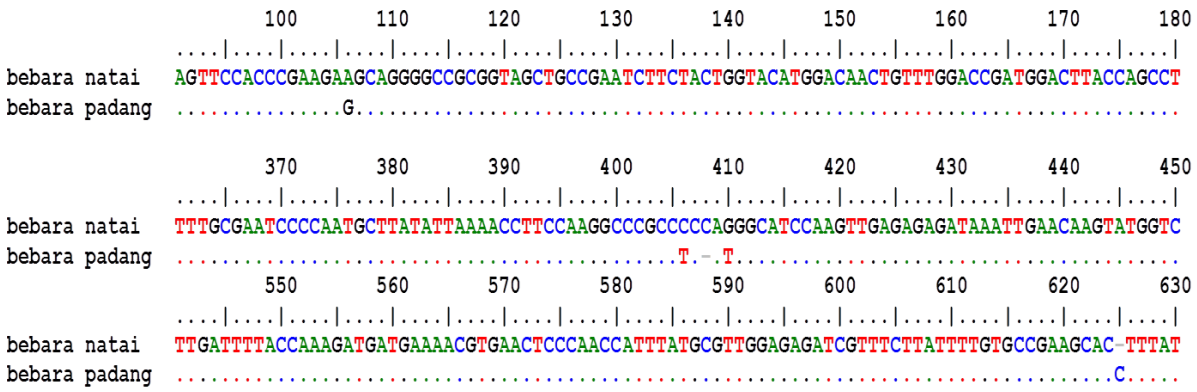

Figure 5. Results of nucleotides alignment of *bebara natai* and *bebara padang* using *rbcL* gene

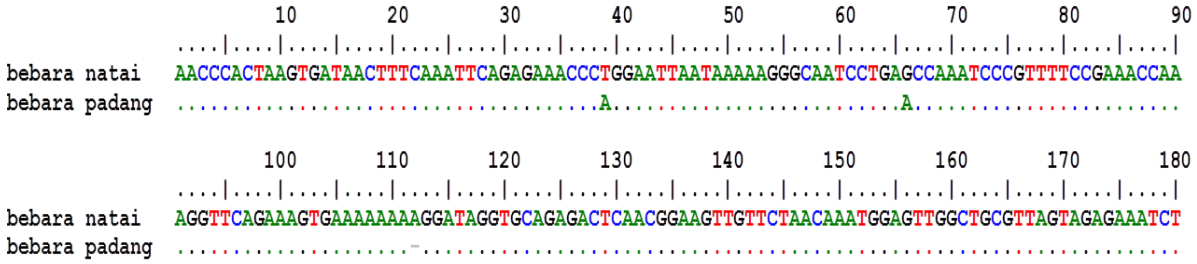

Figure 6. Results of nucleotides alignment of *bebara natai* and *bebara padang* using *tnrL* gene

The alignment between the sequences of *bebara natai* and *bebara padang* habitats using the *matK, rbcL* and *tnrL* genes can be seen in Figures 4, 5 and 6.

The picture above shows that in the alignment of *bebara natai* and *bebara padang* using the *matK* gene, there was a change in Guanine (G) to Thymine (T), Cytosine (C) to Adenine (A), Cytosine (C) to Thymine (T), Adenine (A) to Thymine (T), and Thymine (T) to Adenine (A). Alignment results with the *rbcL* gene contained base changes in Adenine (A) to Guanine (G), Cytosine (C) to Thymine (T), Guanine (G) to Thymine (T) and there was Cytosine (C) base insertion on *bebara padang*. Alignment with the *tnrL* gene contained base changes in Thymine (T) to Adenine (A) and the base of Guanine (G) to Adenine (A). *matK* gene can be used to identify these plants because they have a greater variety of nuleotides when compared with *tnrL* and *rbcL* genes. High nucleotide variations in nucleotide sequences can be used to determine variations in one genus or family of plant. In the nucleotide sequence, there are conserved regions and mutated regions where conserved areas will be relatively stable in plants and passed on to their offspring/ descendant.

Taxonomic studies on plants can use DNA sequences. The characterization of plastid genes such as the *rbcL* gene that encodes a large subunit of *ribulase biphosphate* carboxylase (Rubisco) which is located in the chloroplast genome and its functions as a photosynthetic gene is very important in knowing plant photosynthesis patterns (Sun et al., 2016; Savolainen and Chase, 2003).

Phylogenetic tree

The phylogenetic tree is a logical approach to show the evolutionary relationship between organisms (Williams, 2018; Strelin et al., 2018; Faith, 2018; Quintero et al., 2018; Schweiger et al., 2018; Schmidt, 2003, and Woese et al., 1990). Phylogenetics is interpreted as a model to represent ancestrall organism relations, molecular sequences or both (Washburne et al., 2018; Tan et al., 2018; Cibois et al., 2018; Li et al., 2018, and Brinkman and Leipe, 2001). One of the objectives of phylogenetic preparation is to properly construct relationships between organisms and estimate differences that occur from one ancestor to offspring/ descendant (LI et al., 1999).

The results of the phylogenetic tree using the *matK* gene showed that *bebara padang* and *bebara natai* were grouped in family of *rubiaceae (*Figure *7)*.

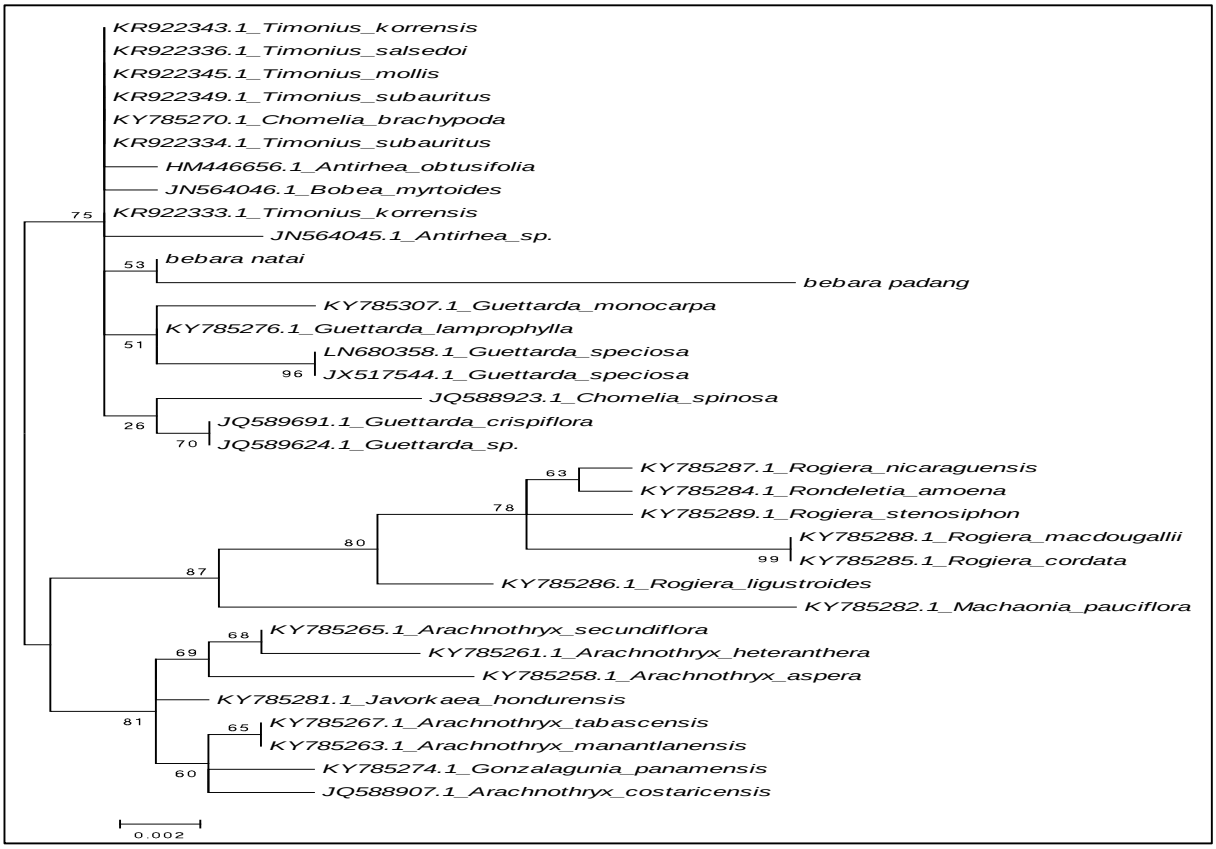

Figure 7. Phylogenetic tree of *bebara natai* and *bebara padang* with *matK* gene using maximum likelihood and Tamura-nei approach

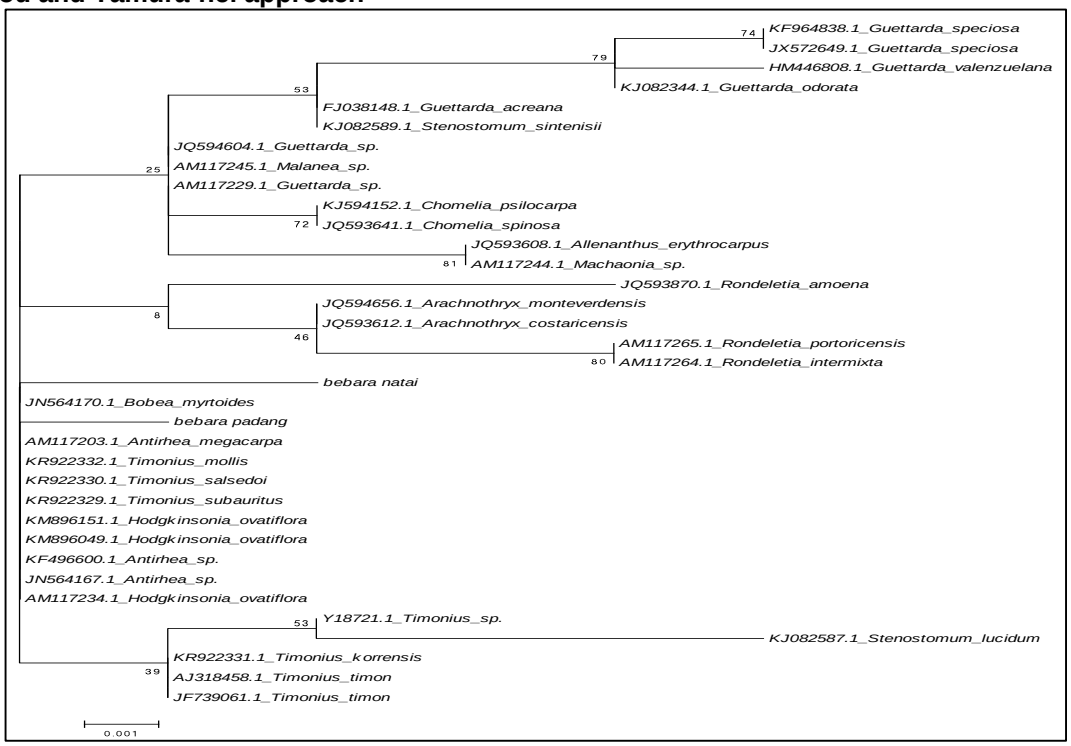

Figure 8. Phylogenetic tree of *bebara natai* and *bebara padang* with *rbcL* gene using maximum likelihood and kimura-2 parameter model

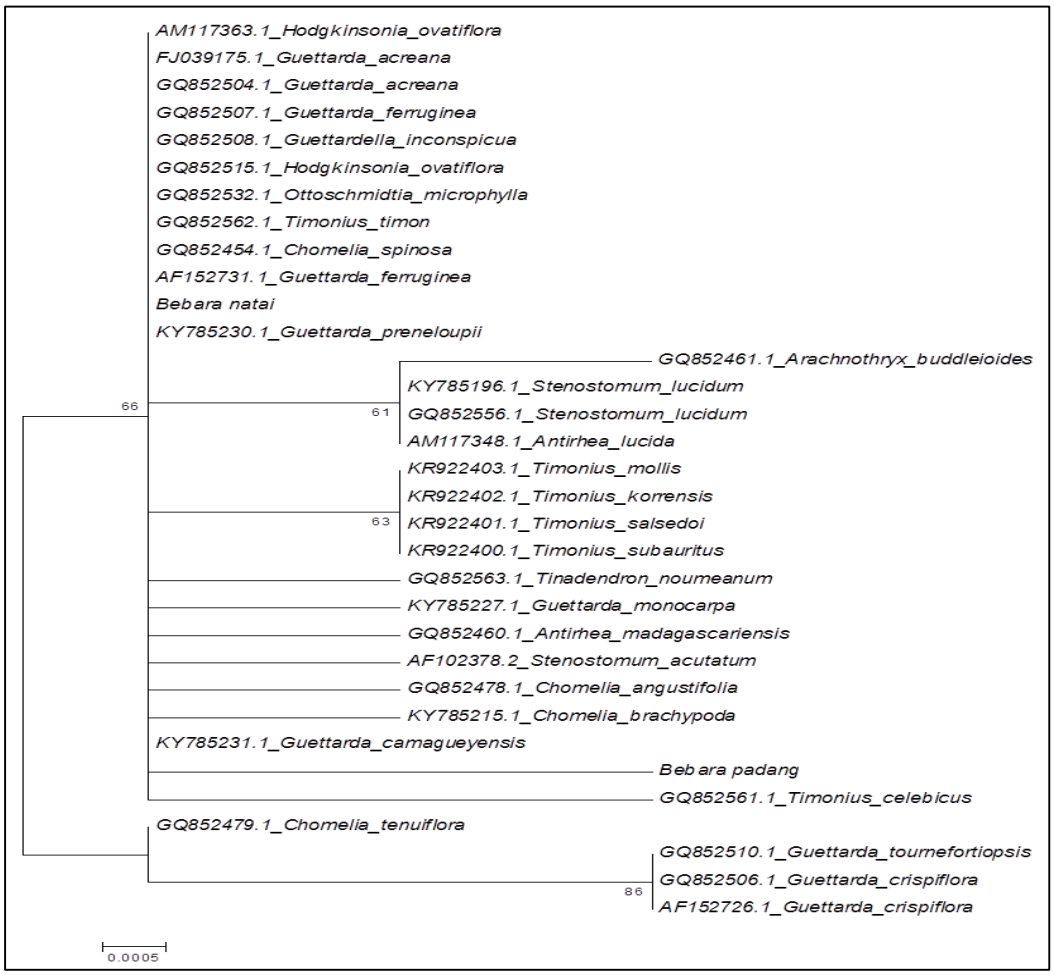

Figure 9. Phylogenetic tree of *bebara natai* and *bebara padang* with *tnrL* gene using maximum likelihood and kimura-2 parameter model

By using pairwise distance values, it obtained the results that *bebara natai* had a genetic distance value of 0.001 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*. On the other hand, *bebara padang* had a genetic distance value of 0.017 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*.

Figure 8, The genetic distance value of *bebara padang* and *bebara natai* was 0.016. The data of genetic distance from *bebara natai* and *bebara padang* by using the *rbcL* gene of *bebara natai* had a genetic distance value of 0.004 with *Timoniusmollis, Timoniusselsedoi, Timoniussubauritius, Hodgkinsonovatiflora, Antirhea sp., Antirheamegacarpa* and *Bobeamyrtoides*.In *bebara padang*, it had a genetic distance value of 0.002 with *Timoniusmollis, Timoniusselsedoi, Timoniussubauritius, Hodgkinsonovatiflora, Antirhea sp., Antirheamegacarpa* and *Bobeamyrtoides*. The genetic distance value of *bebara natai* and *bebara padang* was 0.006.

Figure 9, The genetic distance value of *bebara natai* and *bebara padang* by using *tnrL* gene, *bebara natai* had a genetic distance value of 0.000 with *Chomelia spinosa, Timonius timon, Ottoschmidtia microphylla, Hodgkinson ovatiflora, Guettardella inconspicua, Guettarda ferruginea, Guettarda acreana, Guettarda preneloupii* and *Guettarda camagueyensis*. Bebara padang had a genetic distance of 0.004 with *Chomelia spinosa, Timonius timon, Ottoschmidtia microphylla, Hodgkinson ovatiflora, Guettardella inconspicua, Guettarda ferruginea, Guettarda acreana, Guettarda preneloupii* and *Guettarda*

camagueyensis. The genetic distance value of *bebara padang* and *bebara natai* was 0.004. According to Johansson et al., (2018); Hendrickson et al., (2018), and Tallei et al., (2016); that the smaller the genetic distance value between the two organisms, the closer the relationship between them is.

Phylogenetic results based on the *matK, rbcL* and *tnrL* gene showed that the use of the *matK* gene in identification of *bebara padang* and *bebara natai* using the Maximum likelihood tree construction with the kimura-2 parameter model with 1000 bootstrap value formed a separate branch with confident level of 53%. In the phylogenetic tree using the *matK* gene, there were two large clusters with confident level of 75%. The *rbcL* gene can be used for phylogenetic analysis in the family and subclasses of angiosperms and seed plants (Kang et al., 2017).

CONCLUSION

The resulting analysis of sequence variation shows a difference between *bebara padang* and *bebara natai*. The analysis results of the *matK* gene reveals 5 nucleotide differences; the *rbcL* gene has 3 nucleotide differences and there is a cytosine (C) base insertion on *bebara padang*; and the *tnrL* gene has 2 nucleotide differences. *MatK* gene is used to identify this plant because it has more nucleotide variations than the *tnrL* and *rbcL* genes. Phylogenetic tree with *matK* gene using the Maximum likelihood tree construction with the kimura-2 parameter model with 1000 bootstrap value forms a separate branch with confident level of 53%. In the phylogenetic tree using the *matK* gene, there are two large clusters with confident level of 75%. It shows that *bebara padang* and *bebara natai* are grouped in the family of *rubiaceae*. By using pairwise distance values, it obtains the results that *bebara natai* has a genetic distance value of 0.001 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*. On the other hand,*bebara padang* has a genetic distance value of 0.017 with *Chomelia brachypoda, Timonius subauritius, Timonius mollis, Timonius korrensis* and *Timonius selsedoi*. The genetic distance value of *bebara padang* and *bebara natai* is 0.016.

RECOMMENDATION

This study recommends the importance of further research on species identification, plant morphological characteristics, the content of bioactive compounds and cultivation efforts conducted so that local knowledge of the society which empirically hereditary are believed it thruth and scientifically proven to contain bioactive compounds that are beneficial to health. Morphology characteristics identification in the future will be useful to the community of users in order to get to know this plant so that it is not wrong to use it. Cultivation is carried out as a conservation effort towards the preservation of this plant which is starting to be difficult to find around forest areas caused by land fires and the conversion of forest land into other uses such as oil palm plantations.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

The article is part of the Dissertation of Doctoral and all the authors have contributed: MM conducted experiments, data collection, data analysis and writing manuscript, Prof. S contributed to the experimental design, the determination of the research treatment, Dr. RS and Prof. EN contributes to experimental design, determination of research treatment and review of manuscripts.

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