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Morphological and Molecular Identification of Nutmeg (*Myristica* Sp) in West Halmahera Using The Barcode *MatK* Gene

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Abstract: ***The morphological and molecular identification of nutmeg (Myristicasp) in West Halmahera district using the matK gene has been carried out. The purpose of this study was to determine the morphological and molecular characters of nutmeg (Myristicasp) with matK markers from West Halmahera. The method used is in three stages, namely a field survey for observations, data collection, analysis of morphological diversity and molecular analysis using the matK gene DNA marker. Molecular data analysis was performed with MUSCLE on the Geneious V5.6 program identified in the BOLD system (www.boldsystems.org). The results showed that morphologically the nutmeg (Myristicasp) in West Halmahera has different morphological characters and diversity of around 5 kinds, namely small yellow mace nutmeg (RDKK), large yellow mace nutmeg (RDKB), red mace nutmeg. oblong (RDML), red mace nutmeg (RDFM), and large red mace nutmeg (RDMB). The results of molecular analysis through sequencing showed that the matK gene had a DNA length of 837 base pairs. Nutmeg (RDKK) and (RDKB) are 100% identical to Sangihe nutmeg (TT&BK) and Indian Nutmeg (KT445278.1-India), in 837 nucleotide base sequences, whereas nutmeg (RDKB) is 98.3% identical with different nucleotide sequences in 13 (A-C), 16 (G-A), 17 (G-T), 18 (A-G), 19 (T-G), 812 (T-A) and 834 (T-A), 98.8% identical nutmeg (RDFM), different in nucleotide sequences 11 (C-T), 12 (T-C), 14 (C-T) and 23 (A-C), nutmeg (RDMB) is identical 99.9% different on the order of 834 (T-A). Samples with morphological characters of nutmeg with yellow mace were M.fragrans. The morphological characteristics of nutmeg with red mace, both large and small, need further research.***

Keywords: Nutmeg; the *MatK* gene; Morphology; West Halmahera, Fuli; Molecular Analysis

1. Introduction

Indonesia has a large natural resource for the nutmeg group called the Myristicaceae tribe. Myristicaceae or nutmeg group originally came from the Banda Islands in the Maluku Islands (Purseglove et al., 1995). According to Rukmana (2018) there are 6 types of nutmeg, namely *M. fragrans* Houtt, *M. argentea* Warb. *M. succedanea* Reinw., And *M. facttu*Lam, *M. specioga* Ware, *M. malabarica* Lam. According to the results of research by Das et al., (2012) in North Maluku, four species of nutmeg were found through morphological and agronomic markers, namely: *M. fragrans* Houtt, *M. argentea* Warb. *M. succedanea* Reinw., And *M. facttu* Lam (Das et al. 2012). Nutmeg found in North Maluku is not only *M. fragans*, but there are species that appear morphologically different (Das et al., 2012).

Morphological variations need to be supported by molecular identification because they are very precise or have minimal errors. Methods of identifying living species have evolved from morphological identification to molecular identification based on short pieces of DNA called "DNA barcodes" (Hebert et al. 2003). DNA barcode has applicative functions, for example for ecological surveys (Dick and Kress 2009), identification of cryptic taxons (Lahaye et al. 2008), and confirmation of medicinal plant samples (Xue and Li, 2011), identification of morphological characters with markers. The molecule that is commonly done is the matK DNA marker (Kolondam et al., 2012, Talley and Kolondam, 2014, Pandiangan et al., 2019b).

The morphological markers of nutmeg in North Maluku have been reported by Das et al., 2012, which found 4 types of nutmeg, but it is different from what Rukmana (2018) reported on 6 types of nutmeg. This difference is still limited to observations (Das et al., 2012) in Central Halmahera and Tidore. West Halmahera Regency is an area located in the western part of North Maluku Province. The types of vegetation developed in West Halamahera Regency are agriculture, plantations and forestry and especially nutmeg. Therefore, there is a need for research on morphological and molecular identification by using the matK gene for nutmeg from West Halmahera.

2. Method

Location and Time of Research

This research will be conducted in March-November 2020. Morphological identification with nutmeg samples taken from West Halmahera, while molecular identification by extracting DNA, PCR, and electrophoresis was carried out at the Biotechnology Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Sam Ratulagi University Manado and Sequencing was conducted at First Base Malaysia

Morphological Identification

Morphological identification was carried out by surveying the fruit morphology field, collecting data on morphology and fruit diversity, analyzing the morphological data of the nutmeg, then molecular analysis of the morphologically different nutmegs with the DNA marker of the matK gene. Observations were made based on the Tropical Fruit

Description standard (IPGRI, 1980; Marzuki, 2008 in Robert et al, 2012). Observations of morphological characters include: 1) Fruit length, 2) Fruit diameter, 3) Thickness of fruit flesh, 4) Fresh weight of seeds, 5) Length of seeds, 6) Diameter of seeds, 7) Fresh weight of mace, 8) Fresh weight of fruit, 9) Shape of fruit 10) Color of fruit skin, 10) Color of flesh of fruit, 11) Color of mace.

DNA Extraction

The DNA extraction process uses a modified Multisource Genomic DNA Miniprep Kit (Axygen) based on Pandiangan et al., (2019a). The leaf tissue was cut and weighed as much as 10 mg, put into an Eppendorf tube, 350 µL of PBS and 0.9 µL of Rnase A were added then crushed with pestel (homogenized). About 20 µL of Proteinase K and 150 C-L buffer (lysis buffer) were added and then vortexed for 1 minute. Then incubated for 1 hour at 56˚C. Then to the sample was added to the P-D buffer (protein precipitation buffer) 350 µL, vortexed for 30 seconds, then centrifuged at a speed of 12,000 x g for 10 minutes. The miniprep column that has been provided is placed in a 2 mL Eppendorf tube, the sample supernatant is piped into the column, then centrifuged at a rate of 12,000 x g for 1 minute. The filtrate in the 2 mL tube was removed and the miniprep column was placed back in its original position. Buffer W1 (washing buffer) as much as 500 µL was pipette into the column and centrifuged at a speed of 12,000 x g for 1 minute. The filtrate was removed and the miniprep column was placed back in its original position, then 700 µL of Buffer W2 was piped into the column and centrifuged (this step was repeated 2 times). The filtrate in the 2 mL tube was removed and the miniprep column was placed back in its original position and then centrifuged. The miniprep column was transferred to a new tube, namely the 1.5 mL Eppendorf tube, the DNA was eluted by adding 100 µL of deionized water to the center of the membrane and centrifuged. The total DNA that can be retained by the silica membrane is 20 µg, the final concentration is approximately 0.2 µg / µL (Pandiangan et al, 2019a).

Amplification of the Matk Gene Using PCR Technique

The matK gene primers used were matK-3F (5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3 ') and matK-1R (5'ACC CAG TCC ATC TGG AAA TCT TGG TTC-3'). The matK gene primers that are still in powder form are rehydrated with nuclease-free water. The concentration for the stock primer solution will be diluted to 100 pmol / µL and then extracted to 10 pmol / µL. The optimal condition of the reaction will use an optimization from previous research by Kolondam (2012). The components mixed were 10 µL 5X Master Mix, 1 µL forward primer, 1 µL reverse primer, 3 µL DNA template, and 35 µL deionized water. The final condition for each 50 µL PCR reactions will be 1.25 Taq DNA polymerase units, 0.2 mM each dNTPs, 1.5 mM MgCl2, 0.2 mM each primer and approximately 0.6 µg DNA samples. The temperature setting for the PCR machine started with initial denaturation at 95˚C for 2 minutes, followed by 35 cycles of denaturation, primary attachment and DNA extension stages. The temperature and time were 95˚C for 30 seconds, X˚C for 30 seconds, and 72˚C for 1 minute, respectively (Pandiangan et al, 2019a).

DNA electrophoresis

DNA electophoresis was carried out according to (Pandiangan et al, 2019a), namely 0.75 grams of agarose in 50 mL of 1X TBE Buffer (Tris-Boric EDTA) boiled to get 1% agarose gel to be used. Then the agarose gel is printed along with a comb (comb) to make wells. After hardening, the gel was placed in an electrophoresis device and flooded with 1X TBE Buffer. A total of 5 µL of PCR products containing loading dye were piped directly into agarose gel wells. Approximate size of DNA bands in agarose gel used 1 kb DNA Ladder. 5 µL of Ladder DNA was piped into the gel well. The electric voltage was given through an electrophoresis device of 100 volts for 30 minutes and visualized using a UV-Transiluminator. Documentation is done using a digital camera and a UV light filter. The remaining PCR product is sent with its primary partner for sequencing. All processes including PCR clean up (extraction of DNA from the gel) are performed by a sequencing service provider.

3. Data Analysis

The sequenced DNA chromatogram was edited using Geneious v5.6 software (Drummond et al., 2012). The initial portion of the DNA was removed at about 30 bp and erroneously corrected nucleotide readings based on the degree of accuracy of the reads. A reverse and d complement process is performed for the sequencing results using reverse primers then combined with the forward primary sequencing results using MUSCLE (Multiple Sequence Comparison by Log-Expectation) which is integrated in Geneious. The accuracy of the target gene amplification was tested by predicting the amino acid sequence based on the matK sequence. The aim is to see the presence of a stop codon in the middle of the sequence of these active genes so that it is known for sure that what is amplified is not a pseudogene gene that is in another part of the cell. The sequences that have been tested are converted into a FASTA (fast alignment) format for comparison. DNA and amino acid sequences were aligned with each other to reveal the uniqueness of each, which included suspected insertions / deletions and point mutations resulting in changes in amino acid sequence. The matK gene chunks in the FASTA format of each sample were identified via BOLD (Barcode of Life Database) Systems (www.boldsystems.org). The results of the hit search resulted in a table describing the 20 species that had the highest coverage scores and percentage similarity sequences. An alignment of the sample sequences with the search results is shown to illustrate the location of the differences and gaps between the two DNA sequences (Pandiangan et al, 2019a).

4. Results and Discussion

Results of Identification of the Morphology of West Halmahera Nutmeg (*Myristica* sp)

The results of the identification of morphological characters showed that the fruits of nutmeg (*Myristica* sp) found in West Halmahera were large yellow mace (*Myristica* sp), small yellow mace (RDKB), small yellow mace (RDKK), large red mace (RDMB), oval red mace (RDML), and red mace nutmeg (RDFM) (Figure 1)

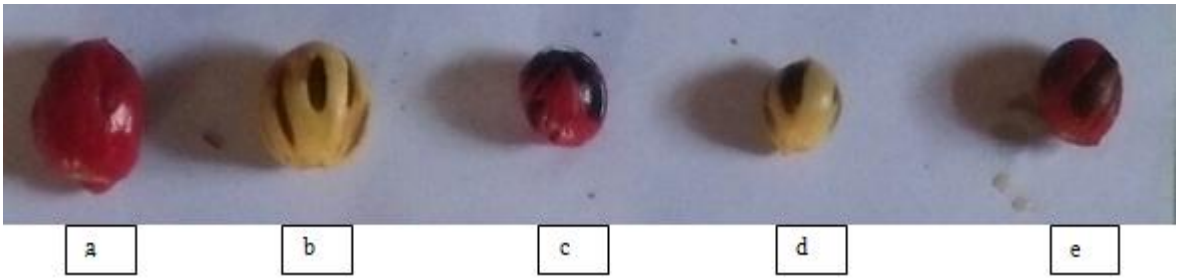

Figure 1: Morphology of large red mace nutmeg (RDMB), large yellow mace nutmeg (RDKB), red mace nutmeg (RDFM), small yellow mace nutmeg (RDKK), and oblong red mace nutmeg (RDML)

The results of characterization of the shape of the nutmeg in this area showed that the fruit was oval, round and oval in shape. The results of characterizing the size of the nutmeg in this area were medium in size because they had a multiplication of length x width between 20 cm2 - 29 cm2. The results of this characterization are in accordance with Robert et al. (2015). Nutmeg with medium fruit size is also found in the nutmeg population in Lampung (Rosyali, 2016).

The results of characterization of the nutmeg skin color in West Halmahera Regency were yellow and the color of the nutmeg flesh in Sangihe was white. This shows that the character of the skin color and the color of the nutmeg in this area is not different from the Banda nutmeg based on the description of Banda nutmeg, the color of the white flesh and the color of the yellow fruit skin (according to the Decree of the Minister of Agriculture Number 4059 / Kpts / SR.120 / 12/2009, dated December 28, 2009) The size of the nutmeg is the quality criterion for the nutmeg. The nutmeg sizes found in West Halmahera Regency are medium and large. Most of the seed sizes observed were medium and large seeds, which were scattered in the villages of Sahu and Tabaru Districts.

The result of mace color characterization showed that the mace color character was the same among all observed accessions, namely red and yellow. The color character of mace mace in West Halmahera is different from the released mace of nutmeg varieties, namely the Banda, Tidore 1, Ternate 1 and Tobelo 1 varieties (Minister of Agriculture Decree number: 4061 / Kpts / SR.120 / 12/2009). The color of the mace is a character that is not influenced by the environment as stated by (DAS, et al. 2017) that the character of the mace color is a character that is not influenced by environmental factors.

Based on the observed fresh weight and wet weight of mace, large red mace (RDMB) had a higher average weight than the other accessions, namely 3 grams. The nutmeg mace has thicker and denser mace so that it has a greater weight. Large yellow mace nutmeg (RDKB) has an average weight of 2.1 grams, small mace nutmeg (RDKK) has an average weight of 1 gram, red mace nutmeg (RDFM) has an average weight of 1.3 grams and oblong red mace nutmeg (RDML) the average weight is 1 gram and has a thinner mace. This has similarities with Patani nutmeg; the fulcrum only covers most of the seeds. This was also stated by Bermawie, et al. (2018) that in addition to diversity in size, mace nutmeg diversity was also found in the percentage of seed cover.

Results of Molecular Identification of nutmeg using the matK gene marker

The results of amplification of DNA extract through PCR can be seen in Figure 1. The results of dielectrophoresis amplification in agarose gel were then observed under a UV lamp showing a lot and thick results (Figure 2). On the left of the image is a DNA standard with a specific base pair. The DNA of the matK gene for nutmeg was produced around 835 bp after electrophoresis and compared with standard DNA.

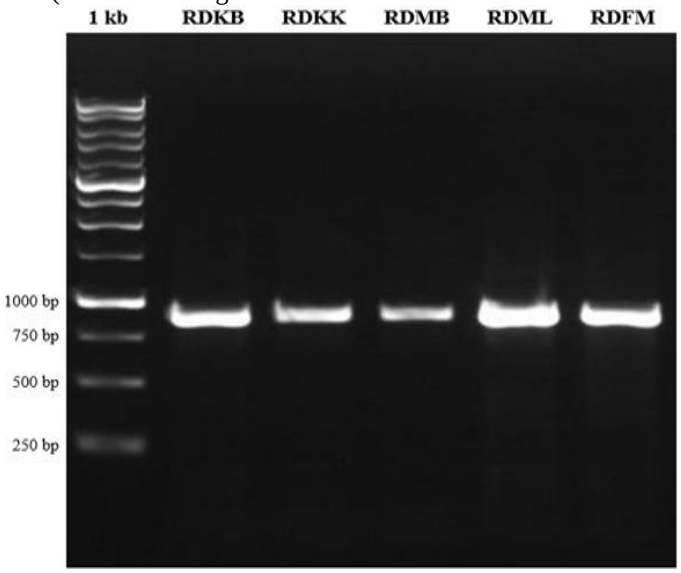

Figure 1: Visualization of the results of DNA amplification of the MatK gene samples of RDKB, RDKK, RDMB, RDML, and RDFM nutmeg using 0.8% agarose gel electrophoresis

The sample sequences of nutmeg (*Myristica* sp) taken from West Halmahera were large yellow mace (RDKB), small yellow mace nutmeg (RDKK), large red mace nutmeg (RDMB), oval red mace nutmeg (RDML), and red mace nutmeg. (RDFM) was successfully amplified using PCR techniques analyzed using the Geneious v5.6.4 program, then converted into FASTA format and used in the search for similar sequences in GenBank using BLAST. DNA samples from the isolation and amplication of the matK gene showed a clear and thick band pattern as indicated by the results of electrophoresis visualized with UV-Transluminator light (Figure 1).

The results of DNA amplification using PCR techniques using matK-1F-R and matK-724R-F primers showed that the RDKB, RDKK, RDMB, RDML, and RDFM samples had a nucleotide sequence with a length of about 800 bp as expected.

The sequence in the FASTA format of the nutmeg plant was compared with the five closest relatives in NCBI and the juxtaposition was carried out using the Multalin program. The sequences of large yellow mace nutmeg (RDKB), large yellow mace nutmeg (RDKK) and oblong red mace nutmeg (RDML) have a similarity to the sequence in NCBI, namely *Myristica fragrans* (100%), while large red mace nutmeg (RDMB), and red mace (RDFM) have a similar level (98%).

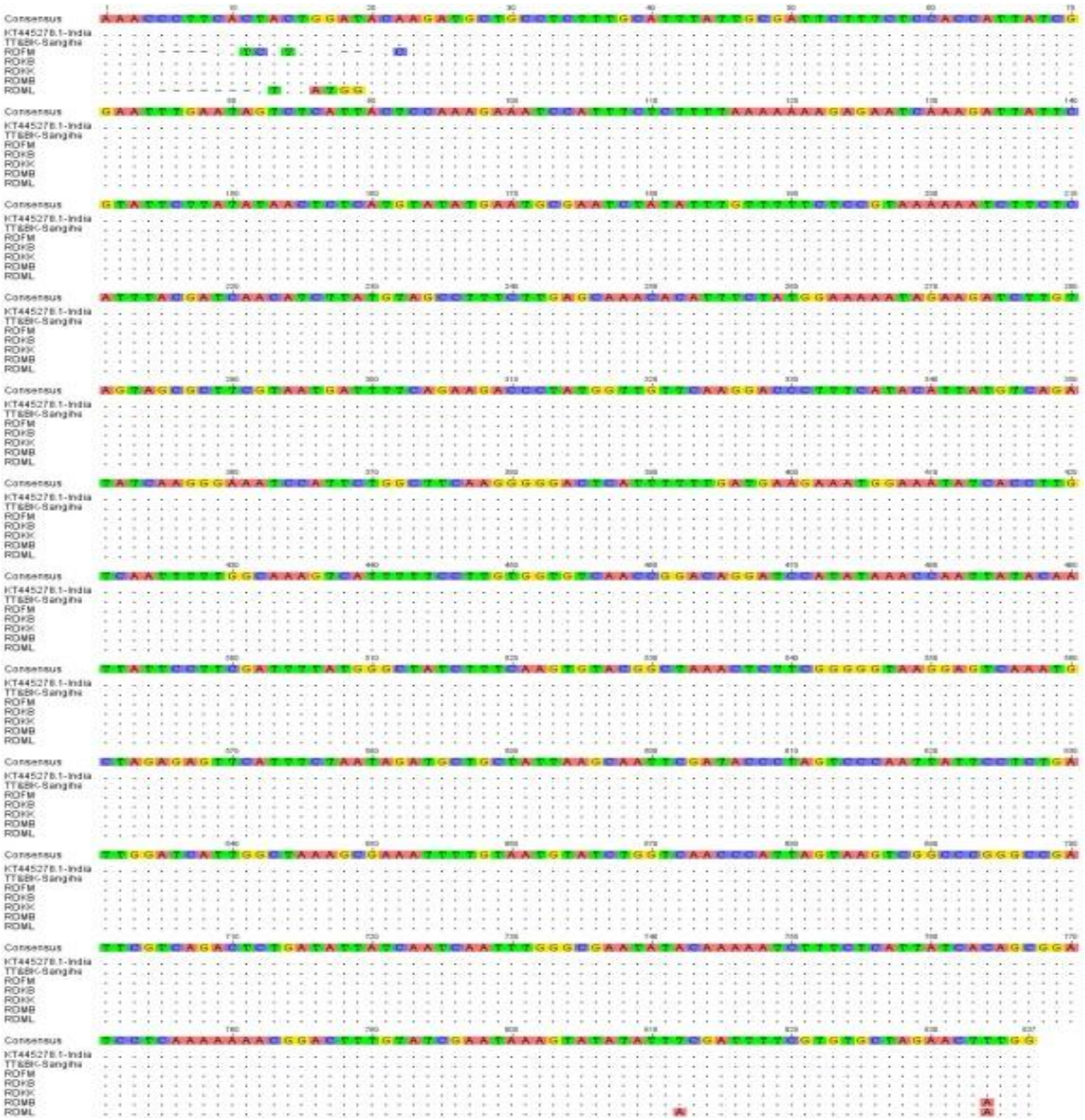

Figure2: The Nutmeg Sequence of West Halmahera RDKB, RDKB, RDKK, RDMB, RDML, and RDFM with Sangihe Nutmeg (TT & BK) and Indian Nutmeg (KT445278.1-India)

The results of molecular analysis through sequencing showed that the matK gene had a DNA length of 837 base pairs sequence. After comparing with Sangihe nutmeg (TT&BK) and Indian Nutmeg (KT445278.1-India), the small yellow mace (RDKK), large yellow mace nutmeg (RDKB) are identical or 100% in 837 nitrogen base sequences, red mace identical (RDML) only 98.3% or there is a difference of six different nucleotide bases on the

sequence 13 (AC), 16 (GA), 17 (GT), 18 (AG), 19 (TG), 812 (TA) and 834 (TA), identical red mace (RDFM) of 98.8%, there is a difference in the nucleotides of four different nucleotide bases on sequences 11 (CT), 12 (TC), 14 (CT) and 23 (AC), large red mace (RDMB) is 99.9% different at 1 nucleotide in sequence 834 (TA).

Table 2: Matrix comparison of West Halmahera RDKB, RDKB, RDKK, RDMB, RDML, and RDFM nutmeg Sangihe (TT&BK) and Indian Nutmeg (KT445278.1-India)

No.	Nama Sampel	1	2	3	4	5	6	7
1	KT445278.1-India	100						
2	TT&BK-Sangihe	100	100					
3	RDFM	98.8	98.8	100				
4	RDKB	100	100	98.8	100			
5	RDKK	100	100	98.8	100	100		
6	RDMB	99.9	99.9	98.7	99.9	99.9	100	
7	RDML	98.3	98.3	98.4	98.3	98.3	98.4	100

The difference between West Halmahera nutmeg calculated with Geneious software (Table 2) shows that the genetic distance of large yellow mace (RDKB) and small yellow mace (RDKK) is 100% similar to Indian nutmeg (KT445278.1) and Sangihe nutmeg (TT&BK), while red mace nutmeg (RDFM) (98.8%) red mace nutmeg (RDMB) was 99.9%, red mace nutmeg (RDFM) (98.8%) red mace nutmeg (RDML) 98.4%. The sequence pair that has the smallest amount of change between them is called neighbors, meaning that the pair has a very close relationship (Darus, 2016).

Figure 3: Phylogenetic Tree Comparison of West Halmahera nutmeg RDKB, RDKB, RDKK, RDMB, RDML, and RDFM with Nutmeg Sangihe (TT&BK) and Indian Nutmeg (KT445278.1-India)

Phylogenetic trees (Figure 3) show nutmeg (*Myristica* sp) from West Halmahera Regency, large yellow mace nutmeg (RDKB) and small yellow mace nutmeg (RDKK) are a monophyletic group with Sangihe and Indian nutmeg and are closely related to small red mace. (RDFM). Monophyletic groups are groups of organisms that consist of a common ancestor and all their offspring have a very close relationship (Hidayat and Puncoro, 2006). Small red mace nutmeg (RDFM) Large red mace nutmeg (RDMB) and oblong red mace nutmeg (RDML) are included in a separate group.

5. Conclusion

Based on the results of this study, it can be concluded that different morphologies are not necessarily different in molecular terms or DNA sequences, but can also differ molecularly or in DNA sequences. Nutmeg with morphological characters with yellow mace originating from West Halmahera is the same type of *Myristica fragrans* nutmeg as Sangihe (TT&BK) and Indian Nutmeg (KT445278.1-India). The sample with the morphological character of the nutmeg with yellow mace was included in the type of nutmeg *Myristica fragrans*. The morphological characteristics of nutmeg with red mace, both large and small, need further research.

6. Suggestion

There needs to be further research on this nutmeg for a wider area in Indonesia, especially North Maluku, to reach a wider range of species to find out the type or species of the morphological characters of the nutmeg with red mace, both large and small fruits by comparing them with gene bank (NCBI).

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