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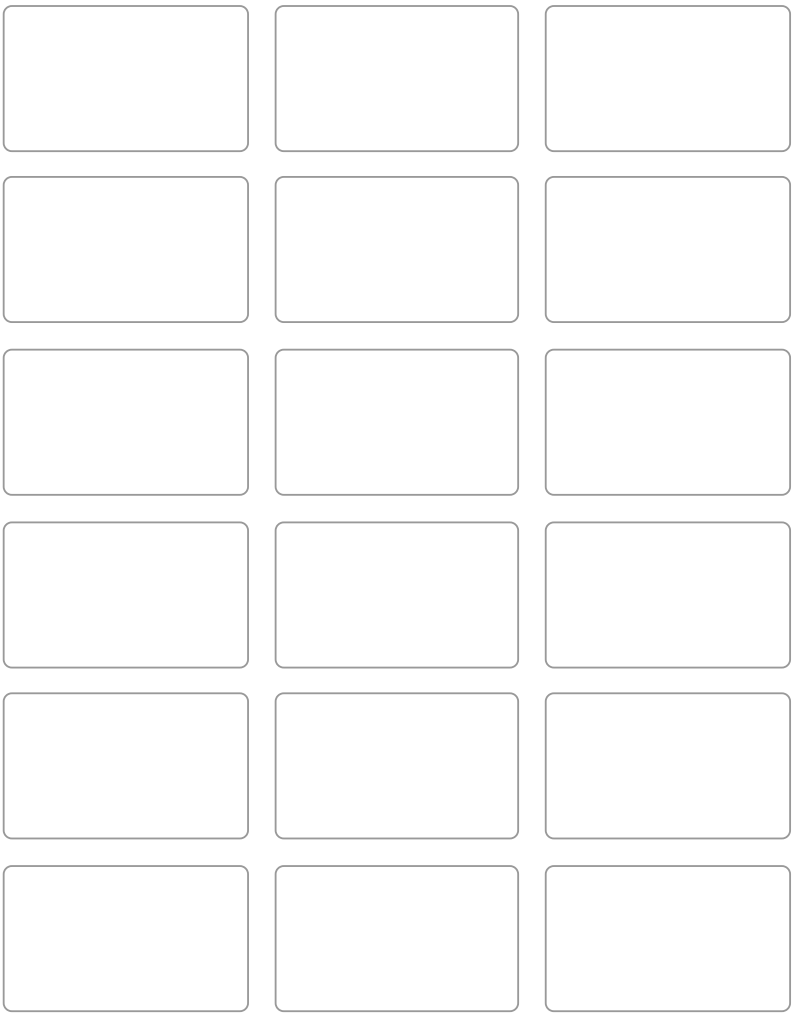

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Assistant Professor
Department of Biotechnology, Bharathidasan University, Constituent College, Perambalur, Tamil Nadu, India
Email: drkraajkumar@gmail.com

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The position of species and the reconstruction of phylogeny trees of Tahuna nutmeg based on the *rbcL* gene DNA chloroplast

[1] Fanny Nella Nanlohy, [2] Mokosuli Yermia Semuel, [3] Aser Yalindua, [4] Elne Vieke Rambi

[1, 2, 3] Departement of Biology, Faculty Mathematics and Science, State University of Manado, Kampus UNIMA di Tondano, Sulawesi Utara, Indonesia

[2] Laboratory of Molecular Biology, Faculty Mathematics and Science, State University of Manado, Kampus UNIMA di Tondano, Sulawesi Utara, Indonesia

[3] Health Polytechnic, Ministry of Health Republic of Indonesia, Manado, Sulawesi Utara, Indonesia

Abstract
Nutmeg (*Myristica* sp.) is a plant originating from the Banda archipelago, Maluku, Indonesia. Nutmeg species and diversity of varieties could be assessed by observing morphological and phenotypic variabilities in the field. Secondary metabolites of this plant have a high value because of their benefts for the health, food, and pharmacy industries. The objectives of this research were to obtained position of species and phylogeny trees of Tahuna nutmeg based on *rbcL* gene DNA chloroplast. Universal *rbcL* primer pairs were used to amplify 591 bp DNA fragment. BLAST serach from NCBI site showed that Tahuna nutmeg has 99% identity with *Mauloutchia chapelieri*, *Horsfieldia amygdalina*, *Staudtia kamerunensis*, *Virola michelii*, *Virola kwatae* and *Myristica fragrans.* It can be inferred from this study that partial of *rbcL* gene cannot be used to differentiate species in *Myristica*; it can only be used to differentiate the genus levelwithin family Myristicaceae.

Keywords: *rbcL* gene, tahuna nutmeg, DNA barcode, myristica

1. Introduction

Nutmeg (Myristica sp) is native to Indonesia. The genus Myristica by many experts is considered as native plants of Indonesia (Soeroso, 2012) [12]. Nutmeg plants originated from the Banda Islands, Maluku (De Guzman and Siemonsma, 1999; Sasikumar *et al.* 1999; Soeroso, 2012) [2, 11, 12]. Nutmeg has ecological and etnomedical sigificances (Steeves, 2011; Tallei and Kolondam, 2015) [13, 15], and is amongst older group within angiospermae division, which consists of recently evolved species (Newsmaster & agupathy, 2009; Tallei and Kolondam, 2015) [9, 10, 15]. Characterization based on morphological markers of species from nutmeg family is difficult considering the similarity of their leaves, so identification relies heavily on the characteristics of small flowers (1-4 mm in size), which can only be obtained when the plant is mature sufficiently (Newsmaster & Ragupathy 2009; Soeroso, 2012) [9, 10, 12].

Based on conventional identification, nutmeg plants in Indonesia consist of several sub species. Nutmeg ternate (*Myristica succadewa* BL), Forest Nutmeg or also called Bacan Nutmeg (*Myristica speciosa* Warb), Nutmeg Gosoriwonin or Nutmeg Onin (*Myristica schefferi* Warb), Nutmeg of Papua (*Myristica argantea* Warb), Nutmeg of Burung (*Myristica sylvetris* Houtt) and Nutmeg of Banda (*Myristica fragrans* Houtt) (Juwahir, 2015) [5]. However, information on plant diversity and types of nutmeg varieties in Indonesia is still very limited. Nutmeg plant research originating from North Sulawesi is underestimated. Meanwhile, Tahuna nutmeg has high quality and special economic value in the trade of International today (Soeroso, 2012) [12]. The genetic diversity of nutmeg plants is well known, for the conservation and protection of indigenous biodiversity of Indonesia. The conservation of nutmeg germplasm is also needed to prevent genetic erosion due to human actions at nutmeg growing sites.

The most widely used plant identification today is the DNA barcoding method. DNA barcoding is identified using a molecular approach based on short DNA fragments (Hebert *et al.*, 2003) [4]. An organism can be identified and differentiated by barcode DNA from species stage to subspecies (Lahaye *et al.* 2008) [7]. The advantage of this technique is to identify and characterize a variety of species that can not be distinguished morphologically (Tudge, 2000) [14]. In addition, this technique is also used to identify an organism even though the DNA of the organism is not in whole or pure form, even its DNA has been degraded (Hajibabaei *et al.* 2006) [3]. In animals, the DNA of the micondria is used as the basis for the preparation of the DNA barcode, whereas in plants due to the relatively small number and variation of the mitochondrial sequence, the DNA plastids (chloroplasts) are used as the material for making DNA barcodes (Hebert *et al.*, 2003; Mokosuli *et al.* 2016) [4].

The use of two plastid genes of *rbcL* and matK is recommended by The Consortium for the Barcode of Life (CBOL) as the standard barcode for plants and COI gene for animals (Hollingsworth *et al.* 2009). However, a group of researcher at the CBOL recommends two loci combination, *rbcL* and *mat*K, as standard DNA barcode for plants (Steeves,

2011) [13]. These two regions in chloroplast DNA were chosen because of having high degree of differentiation between species (Bafeel *et al.* 2011). Furthermore, these two genes play important role in phylogenetic reconstruction for land plants (Kuzmina *et al.* 2012). The *rbcL* and matk genes have been used as DNA markers for plant species (Hebert *et al.* 2003; Hajibabaei *et al.* 2006) [4, 3] Compared with the chromosomal genes (chromosomal genes), chloroplast DNA has a 1-10x faster evolution rate (Avisa *et al.* 1987) [1]. Research aimed at obtaining species status and reconstructing the phylogenetic trees of nutmeg plant originating from Tahuna North Sulawesi.

2. Materials and Method

Samples

The nutmeg samples came from two locations in Tahuna namely: Mahena (MHNB) and Kulur (KLRB) (Figure 1). Fresh leaf and fruit samples were determined in the Laboratory of Biology, Faculty Mathematics and Science, State University of Manado, before being used for DNA analysis.

Fig 1: Leaf and Fruit of Tahuna Nutmeg.

DNA Extraction and Purification

Frozen leaf of Tahuna nutmeg was extracted using Geneiad DNA Mini Kit Plant according to the protocol given by the company. Fifty mg of fresh leaf was cut into small pieces and freeze with liquid nitrogen and grind to fine powder. Sample placed in a 1.5 mL Eppendorf tube. Into the tube, 400 mL Lysis Solution GP1 Buffer and 20 mL Proteinase K were added. The tube was vortexed vigorously for 5 seconds and incubated at 50 oC for 30 min. Tube was centrifuged at 12,000 rpm for 1 min and supernatant was transferred into new 1.5 mL tube. Into the supernatant, 200 mL Binding Solution W1 was added to lyse the sample then vortexed. Sample was applied to Spin Filter located in a 2.0 mL Receiver Tube and centrifuged at 12,000 rpm for 1 min. Into the Spin Filter, 400 mL Washing Solution was added and centrifuged at 12,000 rpm for 30 sec. Receiver Tube with fltrate in it was discarded and Spin Filter was placed in a new Receiver Tube. Into the Spin Filter, 750 mL Washing Solution W1 was added and the tube was centrifuged at 12,000rpm for 30 sec. The Receiver Tube containing flter was discarded and Spin Filter was again placed in a new Receiver Tube. The tube was centrifuged for maximum speed for 2 min to remove all traces of ethanol. The Receiver Tube was discarded. The Spin Filter was added to Elution Tube and 100 mL of Elution Buffer was added. The tube was incubated for 1 min at room temperature and centrifuged at 8,000 rpm for 1 min. The eluted DNA was used for DNA amplification

DNA Amplification

Primers used for DNA amplifcation of *mat*K gene were *rbcL*aF (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3') and *rbcL*aR (5'-GTA AAA TCA AGT CCA CCR CG-3') (Kress and Erickson, 2007). DNA was amplifed using 5x Firepol PCR Master Mix Ready-to-Load (Solis BioDyne). Total volume for amplifcation was 40 mL consisted of 2 mL DNA sample and 1.5 mL of each primer (10 mM). DNA amplifcation was carried out in Rotorgene Cycler (Qiagen) as follows: predenaturation at 95oC for 4 min followed by 35 cycles of denaturation at 95oC for 30 sec, annealing at 50 oC for 40 sec, elongation at 72 oC for 50 sec, and one cycle of fnal extention at 72 oC for 2 min. The PCR products were separated by 1.0% agarose gel electrophoresis. Amplicon was bidirectionally sequenced at First BASE (Singapura).

3. Data Analysis

Sequence chromatogram was edited using Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand) and assembled into bidirectional contig. The *rbcL* gene sequence was employed in Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to query for highly similar sequence. Correct identification means identity percentage of BLAST is the highest searched sequences derived from expected species, or species from expected genus. In contrary, ambiguous identifcation means identity percentage of BLAST is the highest of the searched sequenced derived not from expected species or genus, or family. A final alignment for *mat*K sequences was generated using Multalin V.5.4.1 developed by Corpet (1988) (http:// multalin.toulouse.inra.fr/ multalin/). Sequences were trimmed at both ends of the alignment to avoid many missing data at the end of the sequences so the final characters were 707 sequences. Phylogenetic tree was constructed using software MEGA 6.0.

4. Results and Discussion

4.1 Results

Total DNA extract from leaves of Nutmeg plant successfully done. The DNA purity of Mahena samples and Kulur samples are in the optimal purity range of 1.7-2.0. Furthermore, the total DNA concentration is also at the optimum concentration for DNA templete amplification of rbc gene (Table 1).

Table 1: The concentration and purity of total leaf DNA of Nutmeg

No.	Origin of sample	Concentration (1µg/ml)	Purity (A_{260}/A_{280})
1	Mahena (MHNB)	24,50	1,86
2	Kulur (KLRB)	22,18	1,68

Fragments of rbc gene that were successfully amplified, were visualized with electrophoresis using 0.8% agarrose gel. The *rbcL* genes of the Kulur sample were visualized at 571 bp while the Mahena samples were 573 bp long (Fig. 2).

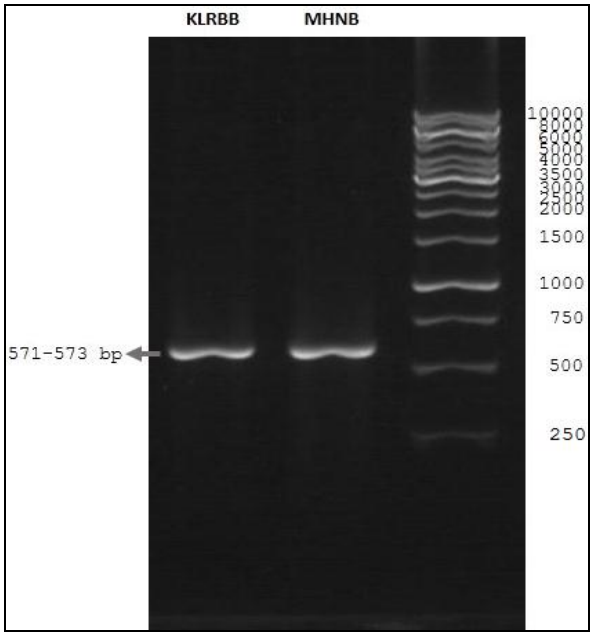

Fig 2: Agarose gel of amplifed partial *rbcL* gene (571-573 bp) of Tahuna nutmeg (KLRB and MHNB) and 1 kb marker.

The sequencing process of sample, yielding a high quality chromatogram (HQ), where the HQ value of KLRB is 89.2% and MHNB: 90.2%. Both KLRB and MHNB samples showed the highest composition of timin (28.3% and 28%, respectively). Samples of KLRB have a percentage of A-T (55%) and G-C (45%), while MHNB samples have an AT percentage (54.6%) and GC (45.4%) (Table 1).

Table 2: Nucleotide Composition of *rbcL* gene of Tahuna Nutmeg

Nucleotide	KLRB (Kulur)	MHNB (Mahena)
Adenin	26,7%	26,6%
Timin	28,3%	28,0%
Sitosin	21,6%	21,7%
Guanin	23,4%	23,6%

The alignment analysis results, on the NCBI website, obtained 100 data sequences of identical *rbcL* gene, which have been recorded on Gen Bank NCBI. Of the 100 sequences, 20 sequences of *rbcL* gene were obtained with the highest identical level (www.ncbi.nih.gov) with the *rbcL* gene of Tahuna nutmeg (Table 2).

Table 3: Twenty Similarity Sequens of BLAST Results of Tahuna Nutmeg

Species	Query cover	E value	% Identic	Accession Number
Mauloutchia chapelieri	97%	0.0	99%	AF197594.1
Horsfieldia amygdalina isolate G216	97%	0.0	99%	KR529437.1
Staudtia kamerunensis voucher PM4941	97%	0.0	99%	KC628429.1
Staudtia kamerunensis voucher PM5269	97%	0.0	99%	KC628405.1
Virola michelii voucher NL110149	97%	0.0	99%	FJ038132.1
Virola michelii voucher NL110074	97%	0.0	99%	FJ038130.1
Virola kwatae voucher NL110070	97%	0.0	99%	FJ038129.1
Myristica fragrans	97%	0.0	99%	AY298839.1
Knema latericia	97%	0.0	99%	L12653.2
Myristica fragrans	97%	0.0	99%	AF206798.1
Knema tenuinervia isolate J616	97%	0.0	99%	KR529477.1
Knema globularia isolate J385	97%	0.0	99%	KR529464.1
Myristica cf. sepicana GW2419	97%	0.0	99%	FJ976152.1
Compsoneura atopa isolate 1374	97%	0.0	99%	EU090508.1
Compsoneura capitellata isolate 835	97%	0.0	99%	EU090509.1
Virola sebifera isolate 779	97%	0.0	99%	EU090543.1
Coelocaryon preussii	97%	0.0	99%	AY743437.1
Horsfieldia amygdalina isolate G111	95%	0.0	99%	KR529436.1
Compsoneura ulei isolate 6192	97%	0.0	99%	EU090541.1
Compsoneura mutisii isolate 914	97%	0.0	99%	EU090532.1

4.2 Discussion

The use of nutmeg leaf samples for DNA extraction, showed good concentration and purity. This is evidenced by the successful amplification of *rbcL* genes, both in KLRB and MHNB samples. Amplification also proceeds well, evidenced by the results of sequencing with chromatograms, which have high HG. The result of aligning the rough sequences of the nutmeg genes from Tahuna by the BLAST method on the NCBI site, yields a sequence of 99% identical levels with *Mauloutchia chapelieri* [AF197594.1]. However, the sequences of the *rbcL* gene from the Tahuna also have identic with 19 other sequences, with the same percentage identic (99%) (Table 3). Thus, the *rbcL* gene sequence has not been able to separate the grouping of Nutmeg Tahuna, at the species level.

Reconstruction of phylogeny based on *rbcL* gene, placing samples of KLRB and MHNB having the closest kinship relation with *Mauloutchia chapelieri* [AF197594.1]. The results of phylogeny reconstruction also show that Nutmeg from Tahuna has genus-level kinship with *Horsfieldia amygdalina, Staudtia kamerunensis, Virola michelii, Virola kwatae, Myristica fragrans, Compsoneura atopa, Compsoneura capitellata, Virola sebifera, Horsfieldia amygdalina, Coelocaryon preussii, Compsoneura ulei, Compsoneura mutisii, Knema tenuinervia, Myristica cf. Sepicana* (Figure 3).

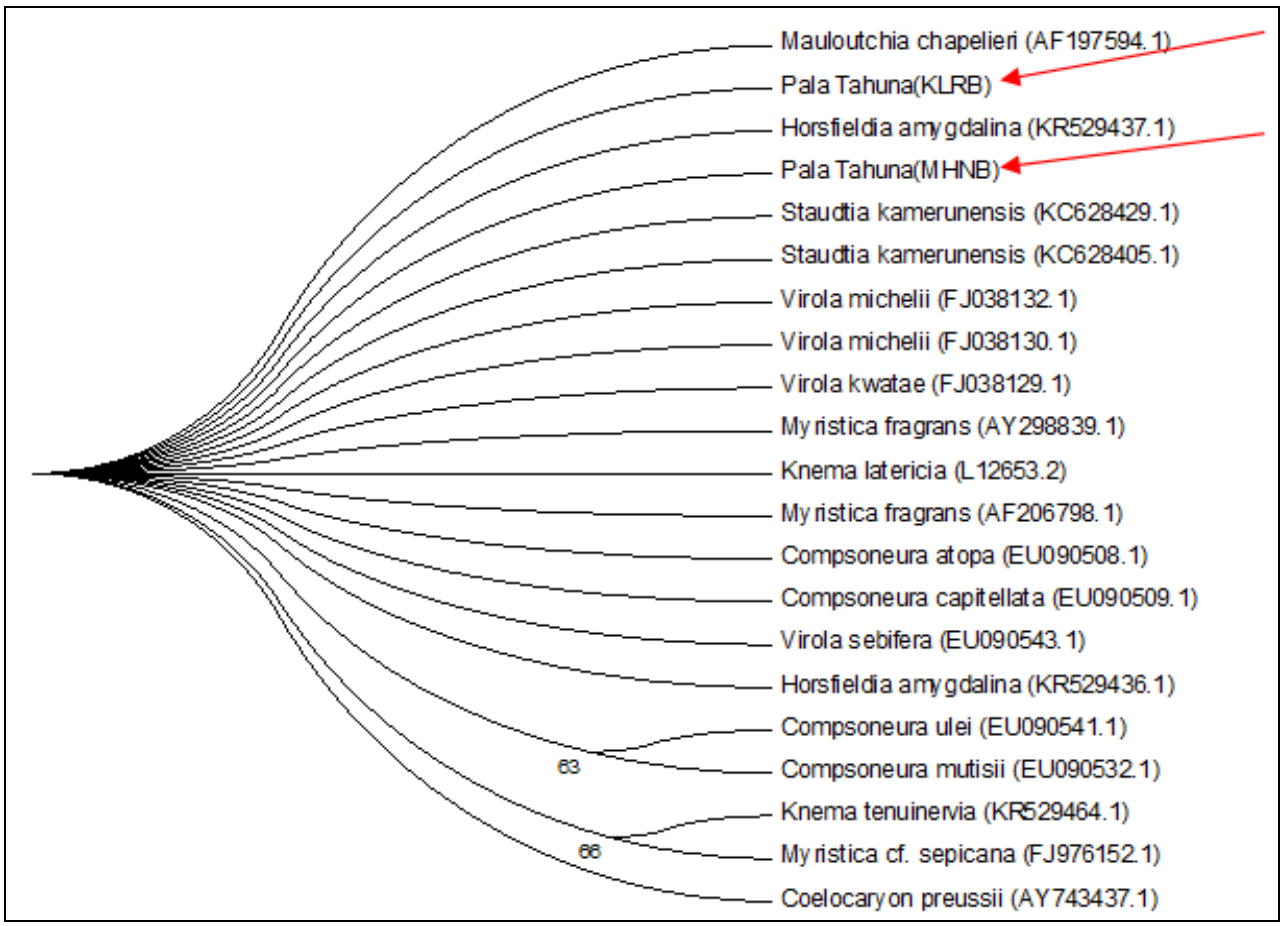

Fig 2: Reconstruction of Phylogeny Tree of Tahuna Nutmeg Based rbcL gene.

The results of this study indicate that the ability of *rbcL* gene discrimination, as a barcode in the Tahuna nutmeg, belongs to the weak category. The *rbcL* gene can only distinguish the genus level, and not the species level. The use of partial *rbcL* gene of chloroplast DNA, has not been able to distinguish the position of the species of Tahuna Nutmeg. Research carried out by Tallei and Kolondam, on Sanger Nutmeg using partial *mat*K gene of cloroplast DNA, also can not distinguish the position of Sanger nutmeg plant in the genus *Myristicaceae* (Tallei and Kolondam, 2014). Partial *rbcL* gene also can not distinguish the position of endemic plant species from Minahasa, *Sygyzium sp* (Walean, 2014); and *Phalus tancarvillae* plants (Kolondam *et al.*, 2012).

5. Conclusion

Partial *rbcL* gene of DNA chloroplasts, can not distinguish Tahuna Nutmeg at the species level. *rbcL* gene can only be used to differentiate the genus level within *Mysristicacecae* family.

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