Biosaintifika 15 (1) (2023): 20-25 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

Taxonomic Confirmation of Rare Fruits in West Kalimantan Using rbcL and matK Sequences

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Submitted: 2022-08-15. Revised: 2022-12-23. Accepted: 2023-02-17

Abstract. Phylogenetic analysis for plants can be very helpful in determining species identification or taxonomic status morphologically. rbcL and matK are widely used as genetic markers in constructing seed plant phylogenies. Different identification and new collection during the re-inventory from the previous study were found in two and four types of rare fruits in Sibohe Forest, West Kalimantan. The six types include pisang karok (*Musa* sp.) and Kandis (*Garcinia* sp.), as well as nubik (*Artocarpus* sp.), tehengan (*Artocarpus* sp.), smallest arok (*Ficus* sp.), and amok (*Alpinia* sp.). This study aimed to analyze the phenetic relationship of six rare fruits from Kalimantan based on rbcL and matK genes. DNA samples were obtained from dried body parts of the previous study and amplified by PCR using both forward and reverse primers for rbcL and matK genes. Amplification was observed on electrophoresis gel for rbcL gene of nubik, tehengan, smallest arok, amok, and pisang karok, while only pisang karok in matK gene. However, matK gene sequence was also obtained for amok even though the band was not seen on the gel. Phylogenetic analysis using the two genes confirmed the morphological identification reported in the previous study; however, using matK as a single gene for taxonomic confirmation must be reconsidered. The information of rbcL and matK sequences of six rare fruits from West Kalimantan could be the first information for building the DNA barcodes of the rare fruits in West Kalimantan.

Keywords: matK, plant phylogeny, rbcL, taxonomic confirmation, West Kalimantan.

How to Cite: Candramila, W., Fajri, H., Mardiyyaningsih, A. N., Firmansyah, R., & Akbar, D. (2023). Taxonomic Confirmation of Rare Fruits in West Kalimantan Using rbcL and matK Sequences. *Biosaintifika: Journal of Biology & Biology Education*, *15* (1), 20-25.

DOI: http://dx.doi.org/10.15294/biosaintifika.v15i1.40284

INTRODUCTION

Two chloroplast genes, rbcL and matK, are widely used as genetic markers in constructing seed plant phylogenies. Maturase K (matK) is a chloroplast gene that encodes an organelle intron maturase protein that cuts Group II introns (Barthet et al., 2020). Compared to other maturase enzymes, this protein has an X domain, and the rest of the reverse transcriptase domain is relatively unchanged (Hausner et al., 2006). Meanwhile, the rbcL gene, about 1400 bp in length, provides many characters for phylogenetic studies (Hollingsworth et al., 2009). However, the percentage of success varies between species and is primarily determined by the geographic scale and complexity of the plants used (Hollingsworth et al., 2016). The role of the rbcL gene, which encodes the RuBisCO protein, is thought to cause this gene sequence to have a low mutation rate compared to other barcode genes in chloroplast DNA so that the level of similarity between species is relatively high. In fact, antigenic associations between proteins in plants and cyanobacteria and the ability to use chloroplast genes to isolate analogous cyanobacteria genes were early indications that rbcL is highly conserved from bacteria to plants, most likely related to its function in the continued adaptation to variations in CO_2 concentrations (Sen et al., 2011). This low mutation rate provides an advantage for in-depth studies of intraspecies genetic and phylogenetic variation.

Phylogenetic analysis for plants can be very helpful in determining species identification or taxonomic status morphologically, as was done by Roslim et al., (2016) for tuntun angin (*Elaeocarpus floribundus*) using matK and ITS sequences. The same conditions can be applied to the case found in (Candramila et al., 2022). Two types of rare fruit experienced different identification during the re-inventory from the study by Daningsih et al., (2019), namely pisang karok (*Musa* sp.) and kandis (*Garcinia* sp.). Candramila et al. (2022) also found four types of fruit that were not found in Entin et al., (2019) even though the inventory was carried out at the same location. The four types are nubik

(*Artocarpus* sp.), tehengan (*Artocarpus* sp.), the smallest arok (*Ficus* sp.), and amok (*Alpinia* sp.).

On the other hand, DNA tagging for land plants has its own challenges compared to animals since many candidate loci show different strengths and weaknesses (Fazekas et al., 2012; Schoch et al., 2012). The CO1 gene commonly used for DNA markers in animals (Dentinger et al., 2011) is not effective when used in plants(Cho et al., 1998; Cho et al., 2004; Lv et al., 2014). In plant species, hybridization and many cases of speciation often occur through mechanisms such as polyploidy and alternation of breeding systems (Kiewnick et al., 2014). In addition, many combinations of life history traits result in fewer and fewer effective markers for tracing the boundaries of plant species (Stuessy et al., 2013; Sheth et al., 2020; Soltis & Soltis, 2021). The common use of rbcL and matK as genetic markers could be the first step toward a more solid type of assignment. In that case, this study aims to confirm the taxonomic identification and the phenetic relationship of six rare fruit plants previously re-inventoried by Candramila et al. (2022) based on phylogenetic analysis in the matK and rbcL gene sequences. Proper taxonomic identification can help with subsequent biological studies, especially if associated with conservation efforts, given its position as a rare, edible fruit. This study could be the basic information to build the specific DNA barcodes for rare fruit from West Kalimantan. Furthermore, information about the effectiveness of using the rbcL and matK genes as genetic markers for the six rare fruits from Kalimantan can provide further research considerations, including for conservation purposes.

METHODS

Plant Samples

In this study, inter-species kinship analysis was carried out on fruits that had been inventoried previously. Identification up to genus level was carried out at the Bogoriense Herbarium, Botany Division, Biology Research Center-LIPI Number B-146/V/DI.05.07/10/2021. The plant identification at the genus level obtained in the previous study was compared with the identification results reported by Daningsih et al. (2021). Therefore, DNA samples were extracted from dried body parts (herbarium) collected in a previous study (see Candramila et al., 2022). The selection of samples focused on fruit types that had not been found in previous research reports (Daningsih et al., 2021), namely nubik (*Artocarpus* sp.), tehengan (*Artocarpus* sp.), the smallest arok (*Ficus* sp.), amok (*Alpinia* sp.), as well as two types of fruit plant with different names from that reported in Candramila et al. (2022), namely pisang karok (*Musa* sp.) and kandis (*Garcinia* sp.).

DNA Isolation

Total genome isolation of each fruit plant using the Promega Wizard® Genomic DNA Purification kit. A total of 100 mg of dried leaf samples were crushed using liquid nitrogen. Then the samples were denatured using 350 µL of Lysis Buffer A solution, 350 µL of lysis buffer B, and purified from RNA with RNAse enzyme. DNA precipitation was carried out by adding 90% ethanol and a gDNA binding solution. Separation of the total genome from the precipitation solution with the help of a microtube purification column and elution buffer. The total genome quantity was measured with a spectrophotometer at 260 nm and 280 nm wavelengths. Total genome quality was verified by gel electrophoresis.

DNA Amplification

Amplification of matK and rbcL genes was done by PCR method. The total genome of each plant was used as a template for the isolation of the matK and rbcL genes by PCR. PCR used specific primers for the matK and rbcL genes designed from the conservative regions of the matK and rbcL genes of several plant species available in gene banks (NCBI, EBI). The PCR composition consisted of 10 ng DNA, 2.5 pmol forward primer, 2.5 pmol reverse primers, 25 µL Taq polymerase mix, and ddH_2O to a final volume of 50 µL. The primers used are:

rbcL Forward : 5’ – ATG TCA CCA CAA ACA GAG ACT AAA GC – 3’
rbcL Reverse : 5’ – GTA AAA TCA AGT CCA CCR CG – 3’
matK Forward : 5’ – CGT ACA GTA CTT TTG TGT TTA CGA G – 3’
matK Reverse : 5’ – ACC CAG TCC ATC TGG AAA TCT TGG TTC – 3’

The PCR conditions consist of a pre-denaturation step for 2 minutes at 95°C, denaturation for 1 minute at 92°C, annealing (primer attachment) for 30 seconds at 55°C, DNA polymerization at 72°C for 1 minute, and post-polymerization at 72°C for 5 minutes. The denaturation, annealing, and DNA polymerization stages were repeated for up to 35 cycles.

DNA Sequencing and Analysis

Sequencing of nitrogenous bases and phylogenetic analysis of the sample plants based on matK and rbcL genes. Sequencing was carried out at the 1st BASE Laboratory, Malaysia. The matK and rbcL gene sequence data from all sequenced samples were aligned with the data in the gene bank with the Basic Local Alignment Search Tool (BLAST) at NCBI. Then, sequence analysis was performed using the W cluster menu in the MEGA 11 software. The phylogenetic tree was built with the Neighbor-Joining Tree.

RESULTS AND DISCUSSION

The results of DNA amplification in the rbcL and matK genes for the six types of fruit can be seen in Figure 1. Not all types of fruit samples can be amplified by the two primers used. For the rbcL gene, only nubik, tehengan, the smallest arok, pisang karok, and amok were amplified, while for the matK gene, only pisang karok was amplified, as seen on the gel.

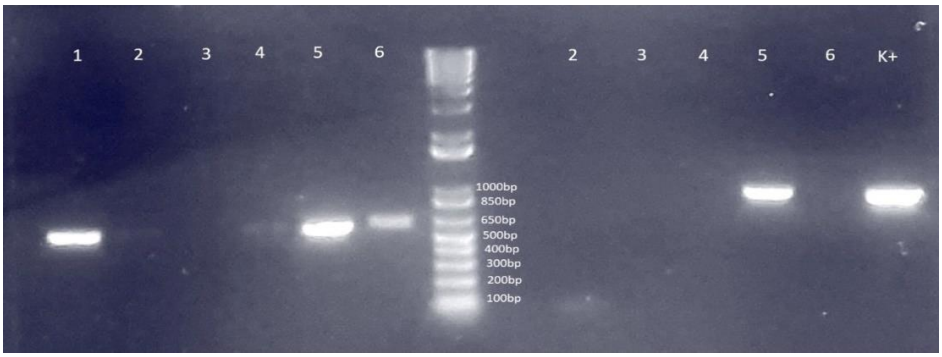

Figure 1. Results of DNA amplification in the rbcL (left) and matK (right) genes. Description: 1. Nubik, 2. Tehengan, 3. Kandis, 4. Smallest Arok, 5. Pisang Karok, 6. Amok, K^+ = positive control

The size of the DNA sequences of the rbcL and matK genes in the five types of fruit are presented in Table 1. The amplified DNA fragments using two pairs of primers for the rbcL and matK genes varied relatively for each species. For the rbcL gene, the size of the DNA fragment ranged from 551 bp in nubik to 690 bp in amok and smallest arok. Meanwhile, for the matK gene, it was only found in pisang karok of 925 bp. However, to anticipate the amplification results that were too low to be observed on gel electrophoresis, all samples were sequenced.

Table 1. The size of the DNA fragments amplified by the rbcL and matK genes from the six types of fruit in this study.

Samples	The DNA size amplified (bp)	
	*rbc*L	*mat*K
1. Nubik	551	-
2. Tehengan	618	-
3. Kandis	-	-
4. Smallest Arok	690	-
5. Pisang Karok	594	925
6. Amok	690	-

Description: - = not amplified according to the visual band on the electrophoresis gel

The results of tracing kinship using the rbcL gene were consistent with identifying species based on previous morphological characteristics (Figure 2). According to the morphological identification conducted by Candramila et al. (2022), nubik and tehengan were in the same family and genus, namely *Artocarpus* in the Family Moraceae. So, the branching line between nubik and tehengan is the closest. Meanwhile, nubik, tehengan, and the smallest arok are grouped in the same family but in a different genus in which arok belongs to the genus *Ficus*. This is in accordance with the existence of a separate branch between nubik and tehengan with arok. This family comprises up to 38 genera and more than 1100 species (Christenhusz & Byng, 2016). At another branch, amok is closer to pisang karok. Referring to the morphological identification results, amok belongs to the genus *Alpinia*, Family Zingiberacea, while pisang karok belongs to the genus *Musa* in the Family Musaceae. However, both families belong to the Order Zingiberales, which consists of kinds of ginger and bananas. Zingiberales is a monophyletic group that has a clear character and consists of eight families (Simpson, 2019). Monophyletic groups are typically characterized by shared derived characteristics or synapomorphies, distinguishing organisms in the clade from other organisms (Mehta et al., 2016).

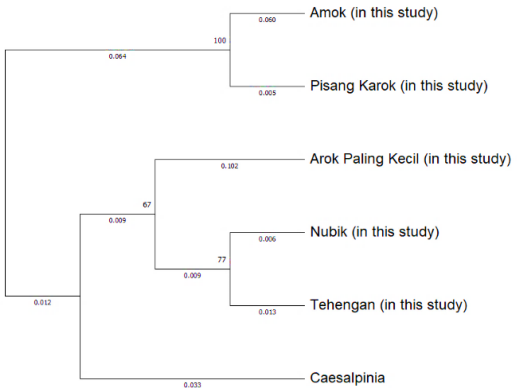

Figure 2. A phylogenetic tree showing the relationship between amok, pisang karok, nubik, tehengan, and the smallest arok using rbcL sequences and *Caesalpinia* as an outgroup.

Similar results were seen in the phylogenetic tree using the matK gene sequence (Figure 3). Amok and pisang karok are in the same branch, although amok is closer to *Cephalantus occidentalis* and *Microtropis biflora*. *Cephalantus occidentalis* is a species of flowering plant in the Family Rubiaceae (Bonner, 1974; Schoch et al., 2012)while *Microtropis biflora* belongs to the Family Celastraceae(Meril & Freeman, 1940; Schoch et al., 2012). The two families also belong to different orders, in which the Family Rubiaceae belongs to the Order Gentianales, while Celastraceae belongs to the Order Celastrales (Simpson, 2019). The same problem was found in using a single matK locus for *Myristica fragrans* because it showed 100% similarity with the other three *Myristica* species (*Myristica fatua*, *M. maingayi*, and *M. globose*). A high degree of similarity was also found for *M. fragrans* with *Rivola sebifora* (identity 99.58%) and *Knema laurina* (identity 99.43%) (Tallei & Kolondam, 2015). On the other hand, Sun et al., (2012) found that using matK provided the highest nucleotide variation, followed by psb-trnH for *Dioscorea* species. From the description of these results, using a single matK gene to confirm morphological taxonomic identification results needs further consideration. This phenomenon may be due to the matK gene having maintained its ancient and generalized function in chloroplasts, as found by Duffy, Duffy et al., (2009), even in organisms that lose their flanking intron.

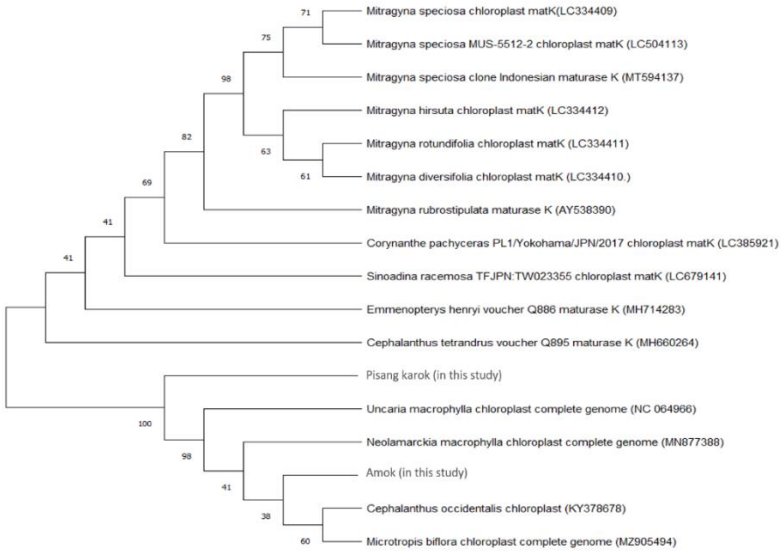

Figure 3. The position of amok and pisang karok relative to other plants from the genebank in the phylogenetic tree based on variations in the nucleotide bases in the matK gene.

According to the results obtained in this study, one of the potential strategies in determining the proper phylogenetic status of rare fruits from Kalimantan is by using more than one genetic marker. Phylogenomic or multi-gene analyses of hundreds of genes are commonplace, not only in

plants but also in other living creatures (Goh et al., 2013; Ma et al., 2022). The datasets may contain more phylogenetic signals, and it is assumed that phylogenetic bias is neutralized. However, precautions must be made when merged genes performed different evolutionary histories (horizontal gene transfers vs. vertical inheritance; organellar vs. nuclear genes; genes with very different evolutionary rates), therefore, could result in phylogenetic artifacts. On the other hand, getting more information on genetic data is a potential factor in analyzing to improve the suitability of biodiversity conservation strategies. In the case of rare fruits from Kalimantan, further research still needs to be done to get a better understanding of their genetic variation to be implemented in conservation activities.

CONCLUSION

Phylogenetic analysis can only be done on the smallest arok, amok, nubik, tehengan, and pisang karok. With the primers designed in this study, using the rbcL gene can potentially confirm taxonomic identification of the smallest arok, amok, nubik, tehengan, and pisang karok, while the primers designed for the matK gene have potential only in amok and pisang karok. Single-use of rbcL or matK genes must be reconsidered to get proper phylogenetic identification for the six rare fruits from Kalimantan.

ACKNOWLEDGMENT

This research is funded by DIPA FKIP Universitas Tanjungpura Year 2022. The authors thank Devinda for her help in the laboratory.

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