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Molecular taxonomy via DNA barcodes for species identification in selected genera of Fabaceae

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Abstract. *Fabaceae is an invaluable plant family with considerable ecological and economic importance, for example, as a food source, bio-fertilizer, and medicinal properties. However, several members of this family have been overexploited in Indonesia, thereby the existence of several species belonging to this family is critically endangered. Therefore, it is essential to support conservation efforts to ensure the overall survival of this plant family. We provided a molecular survey of Fabaceae in converted landscapes of Indonesia through DNA barcoding and aimed to evaluate the effectiveness of core barcoding chloroplast markers matK, rbcL, and their combination (matK+rbcL), as DNA barcodes for species identification in Fabaceae. We generated DNA barcodes of matK and rbcL regions from 51 species belonging to 28 genera and 47 species belonging to 31 genera, respectively. The results showed that the highest accuracy level for species identification was at 90% with matK+rbcL and 82.05% with matK. Additionally, matK had the highest mean of interspecific and intraspecific distances at 0.134 and 0.003, respectively. Furthermore, the most highly resolved phylogenetic tree was generated using the Neighbor-Joining method. Based on the overall performance, matK is superior compared to rbcL, and the use of combined matK+rbcL barcodes is highly recommended for the selected genera in this study.*

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INTRODUCTION

Fabaceae is the third-largest family of flowering plants (angiospermae) after Orchidaceae and Asteraceae with 19 500 species and 750 genera (Christenhusz and Byng, 2016; Willis, 2017). Botanists divided it into 6 sub-families, namely Caesalpinioideae (146 genera and 4 400 species), Cercidoideae (12 genera and 335 species), Detarioideae (84 genera and 760 species), Dialioideae (17 genera and 85 species), Duparquetioideae (1 genus and 1 species), and Faboideae or Papilionoideae (503 genera and 14 000 species) (Gomes *et al.*, 2018). Fabaceae include a large number of cultivated plants with high economic value, such as food crops, animal feed, ornamental plants, medicinal plants, timber and wood products (Graham and Vance, 2003).

Globally, Fabaceae is found to be highly diverse in tropical regions (Yahara *et al.*, 2013). In Indonesia, despite its wide utilization (Purwanto, 2012; Suherman and Herdiawan, 2015; Widodo *et al.*, 2019; Suwardi *et al.*, 2020), reports on Fabaceae distribution and diversity so far are very limited (Hariyati *et al.*, 2018; Putri and Dharmono, 2018; Mountara *et al.*, 2021; Suarna and Wijaya, 2021). Five plants of Fabaceae in Indonesia, namely *Pseudosindora palustris* Symington, *Archidendron royenii* Kosterm., *Sophora rubriflora* P.C.Tsoong, *Pterocarpus indicus* Willd., and *Albizia rufa* (Hassk.) Benth. are listed as endangered species in the IUCN Red List (https://www.iucnredlist.org). According to Budiharja *et al*. (2011), habitat loss is the major cause of plant endangerment in Indonesia.

Conservation efforts are urgently needed to prevent a further decrease of the species diversity within Fabaceae Family. This is influenced by the accuracy of species identification, which is carried out using conventional taxonomic methods and molecular techniques. However, many species are similar in morphological appearance, thereby making it difficult to distinguish between species. According to Elansary *et al.* (2017), morphological identification is not effective, especially for complex taxonomic groups, such as *Argyreia* (Convolvulaceae) (Traiperm *et al.*, 2017), *Cuscuta* (Convolvulaceae) (Park *et al.*, 2019), *Pulsatilla* (Ranunculaceae) (Li *et al.*, 2019), and *Vicia* (Fabaceae) (Han *et al.*, 2021). Moreover, morphological characters are influenced by the environment, as some reproductive traits are only seasonally available, making morphological species identification less specific in the absence of reproductive structures, affecting the accuracy of species identification (Hikmah *et al*., 2016). Therefore, the potential of molecular techniques needs to be explored for the proper identification of specimens belonging to Fabaceae.

DNA barcoding is a molecular technique used to identify species using DNA code-based similarity in combination with morphological characters, which minimizes errors from conventional identification (Liu *et al.*, 2017). It has the basic principle of identification using a short DNA sequence "*barcode*" from a standardized genome part of the specimen being studied (Hebert *et al.*, 2003). The unknown *barcode* sequence is compared with known *barcode* reference sequences and identified as a specimen when the query sequence matches with the target sequence with a high percentage of identity and similarity (Lis *et al.*, 2016). Meanwhile, it may reveal morphological misidentification or even allows for the identification of cryptic species (Hajibabaei *et al*., 2007).

The Consortium for Barcode of Life (CBOL, 2009) stated that plant identification generally uses chloroplast DNA maturase K (*mat*K) and ribulose-1, 5-bisphosphate carboxylase oxygenase (*rbc*L), as well as a combination of *mat*K+*rbc*L (Hollingsworth *et al*., 2011). Amandita *et al.* (2019) reported that the use of two plastid markers, *mat*K and *rbc*L, is efficient in identifying flowering plants from the lowland rainforest of Sumatra to the genus level. Meanwhile, a study carried out by Gao *et al.* (2011) reported that the *mat*K marker correctly identified approximately 80% and 96% of specimens at the species and genus level of Fabaceae. Saadullah *et al.* (2016) stated that the combination of *matK*+*rbcL* markers is the best method for identifying 62 specimens from the Fabaceae Family originating from Pakistan.

In addition to species identification, DNA barcoding is also useful to determine the species genetic relatedness by constructing a phylogenetic tree, a representation of evolutionary relationships in a group of organisms with a common ancestor (Ochieng *et al.*, 2007; Patwardhan *et al*., 2014). Hartvig *et al.* (2015) stated that the *maximum parsimony* and *neighbor-joining* methods were the best approaches for the genus *Dalbergia*. According to Saadullah *et al.* (2016), *neighbor-joining* is an appropriate approach to identify specimens at the Fabaceae Family level. The use of DNA sequences in this study is aimed to investigate the ability of DNA *barcode*s *matK* and *rbcL* in identifying Fabaceae plant species, as well as to evaluate its accuracy level in reconstructing the phylogenetic relationship between the sampled species.

METHODS

DNA Barcode Sequences

A total of 43 *matK* sequences and 106 *rbcL* sequences were derived from the CRC990-EFForTS project in cooperation with IPB University (Bogor, Indonesia), Jambi University (Jambi, Indonesia), Tadulako University (Palu, Indonesia), and University of Göttingen (Göttingen, Germany) as summarized in Table 1 (Amandita *et al*., 2019). Furthermore, 156 sequences of *matK* and 112 sequences of *rbcL* were obtained from the Barcode of Life System (Ratnasingham and Hebert, 2007) database to increase the sample size and enhance species representation. Sequences of *matK* and *rbcL* originating from the same sample, as indicated by the sample ID, were concatenated (Vaidya *et al.*, 2011) to form *matK*+*rbcL*, resulting in total of 35 sequences. The overall data consisted of 123 species from 48 different genera of Fabaceae. Two species, namely *Ceiba speciosa* and *Adansonia digitata* of Malvaceae were selected and added to each *mat*K, *rbc*L and *mat*K+*rbc*L dataset as an *outgroup*. Meanwhile, two species from the Polygalaceae Family, namely *Monnina aestuans* and *Polygala chamaebuxus* were also added as a sister group (Doyle *et al.*, 2000).

Table 1 DNA sequences of *mat*K, *rbc*L, and *mat*K+*rbc*L used in the study

Marker	N° of Species	N° of Genus	N° of Sequences	
			This Study	BOLD
*mat*K	86	45	43	156
*rbc*L	81	48	106	112
*mat*K + *rbc*L	21	18	30	5

Editing and Alignment

Each sample's forward and reverse sequences were aligned using Codon Code Aligner Software (http://www.codoncode.com/) and combined into a consensus sequence (contig). Multiple alignments were performed using MEGA7 Software (Tamura *et al*., 2016) to determine the similarity level and align the bases among the contigs. Gaps (the sign "-") were added when necessary to align the bases and interpreted as deletions (missing nucleotide bases in DNA sequence) (Christinawati *et al.*, 2010). Changes to certain bases were made when differences between paired sequences from the same specimen were found by checking the chromatogram reading of the respective sequence in Codon Code Aligner and comparing to reference sequences of similar species from BOLD.

Data Analysis

The multiple alignment results were used for further analysis, namely identification suitability analysis, barcoding gap analysis, and phylogenetic analysis. The identification suitability analysis was carried out using the sequences obtained from the CRC990-EFForTS project only to compare the morphological identification by the affiliated taxonomist with the molecular identification using the Basic Local Alignment Search Tools (BLAST) in The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/; Porter and Hajibabaei, 2018) database. The top BLAST result was taken as the best match for specimen identification when the similarity percentage was at least 80%. The identification suitability percentage was calculated for species, genus, and family level.

A barcoding gap analysis was carried out after obtaining data of intraspecific and interspecific genetic distances using MEGA7 Software with *Kimura 3 Parameter* (Tamura *et al*., 2016) and ExcaliBAR (Alibadian *et al*., 2014). Barcoding gaps for each marker were visualized by generating distribution bar charts of intraspecific and interspecific distances using Microsoft Excel. ANOVA analysis and t-tests were also carried out using SPSS Software (Brady *et al*., 2015) to determine significant differences between intraspecific/interspecific distances.

The last analysis was conducted to evaluate the resolution of each phylogenetic tree reconstructed with the Maximum Parsimony (MP), Neighbor Joining (NJ), and Maximum Likelihood (ML) algorithms using MEGA7 Software with 1 000 bootstrap replicates. The bootstrap values were categorized as high (85%), moderate (70-85%), weak (50-69%), or very weak (<50%) following Kress *et al.* (2002). The percentage of monophyletic clade formation of each tree was calculated at species and genus level.

RESULTS AND DISCUSSION

Comparison of Morphological and Molecular Identification

The percentage of corresponding molecular and morphological identifications (identification suitability) of the samples obtained from CRC990-EFForTS project is shown in Table 2 for individual and combined markers. These samples were morphologically identified by comparing their herbarium with the LIPI herbarium collection.

Table 2 Identification suitability percentage of each marker used for CRC990-EFForTS project samples

Identification Suitability (%)	*matK*	*rbcL*	*matK+rbcL*
Up to species level	82.05	79.21	90.00
Up to genus level	17.95	10.89	4.29
Up to family level	-	5.94	5.71
Mislabelling	-	3.96	-

The highest percentage was obtained at the species level for all the markers, in contrary to Gao *et al.* (2011), which reported higher identification suitability at the genus level for Fabaceae. Molecular identification of Fabaceae species in this study performed better using *mat*K compared to *rbc*L, and the use of multilocus *mat*K+*rbc*L improved the identification performance. Other similar studies (Kolondam *et al.*, 2012; Amandita *et al.*, 2019; Alasmari, 2020) reported the superiority of *mat*K compared to *rbc*L in terms of plant identification. Meanwhile, 3.96% of molecular identification did not match the morphological identification at all, and was thus determined as mislabeling, meaning that the sample was probably mislabeled during the field collection or laboratory analysis.

Barcoding Gap Analysis

A barcoding gap analysis was performed to evaluate if the investigated markers were sufficiently diverse in order to discriminate between two different species. Table 3 shows that the average interspecific genetic distance of *mat*K and *rbc*L is 0.134 and 0.047, respectively, which is significantly higher than the intraspecific genetic distance (0.003 and 0.001). These figures are in accordance with Saadullah *et al.* (2016), who reported the discriminatory power of *mat*K and *rbc*L on 22 species of Fabaceae, as well as for other families, such as Myristicaceae (Newmaster *et al.*, 2008) and Rosaceae (Pang *et al.*, 2010). Moreover, the low resolution of *rbc*L compared to *matK* might be due to the low mutation rate of this gene, as reported by Frascaria-Lacoste *et al.* (1993) and Stenøien (2008).

Table 3 Average values of intraspecific and interspecific distances of each marker

Marker	Intraspecific Distance		Interspecific Distance		T-test P Value
	Range	Mean (SD)	Range	Mean (SD)	
*mat*K	0.000-0.071	0.003 (0.009)	0.000-0.268	0.134 (0.063)	<0.0001***
*rbc*L	0.000-0.019	0.001 (0.003)	0.000-0.129	0.047 (0.018)	<0.0001***
*mat*K+*rbc*L	0.000-0.006	0.001 (0.002)	0.000-0.144	0.093 (0.031)	<0.0001***

***: significant

The one-way ANOVA shown in Table 4 indicates that the interspecific genetic distances were significantly different for the three markers tested, but this was not the case for intraspecific genetic distances, except for the *mat*K and *rbc*L comparison. Furthermore, the intra- and interspecific genetic distances of *mat*K+*rbc*L were intermediate, as the properties of intra- and interspecific genetic distances acquired from *mat*K and *rbc*L were compromising each other.

Table 4 One-way ANOVA results for each marker

Marker Comparison	Intraspecific Distance		Interspecific Distance	
	Mean Difference	P value	Mean Difference	P value
*mat*K *x rbc*L	0.0016	<0.05	0.0875	<0.05
*mat*K *x mat*K+*rbc*L	0.0018	>0.05ns	0.0416	<0.05
*rbc*L *x mat*K+*rbc*L	0.0003	>0.05ns	0.0459	<0.05

ns: not significant

Despite the significant differences between the intra- and interspecific genetic distances of the investigated markers, Figure 1 shows that none of the markers used in this study revealed a clear barcoding gap. The absence of a barcoding gap due to the overlap of intra- and interspecific genetic distances might indicate that the marker is not a suitable DNA barcode for the taxa in question. However, other factors such as sample size and taxonomical representation also influence the distribution of the intra- and interspecific variation within the dataset (Meyer and Paulay, 2005).

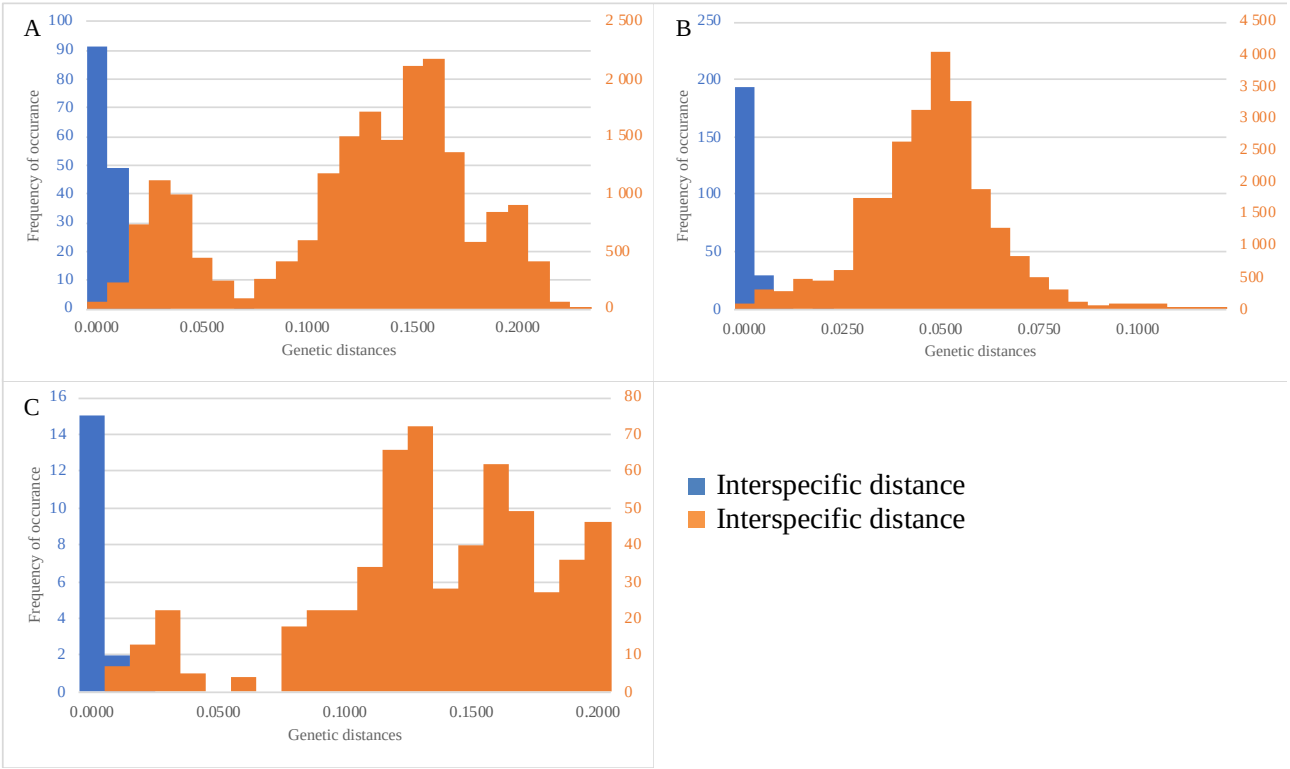

Figure 1 Distribution of interspecific and intraspecific distances for markers (A) *mat*K, (B) *rbc*L, and (C) *mat*K+*rbc*L

Species-Tree Inferences

Phylogenetic Tree Reconstruction

Phylogenetic trees are important tools to acquire information on biodiversity, genetic classification, and to study evolutionary relationships. In this study, nine phylogenetic trees were reconstructed based on the aligned sequences of *mat*K, *rbc*L, and *mat*K+*rbc*L using Neighbor Joining, Maximum Parsimony, and Maximum Likelihood algorithms. Figures 2-4 show phylogenetic trees constructed using the Neighbor Joining approach as the best algorithm to provide highly resolved phylogenetic relationships in the Fabaceae Family, meanwhile the phylogenetic trees reconstructed using Maximum Parsimony and Maximum Likelihood are presented in Supplementary Material (Figures S1-S6).

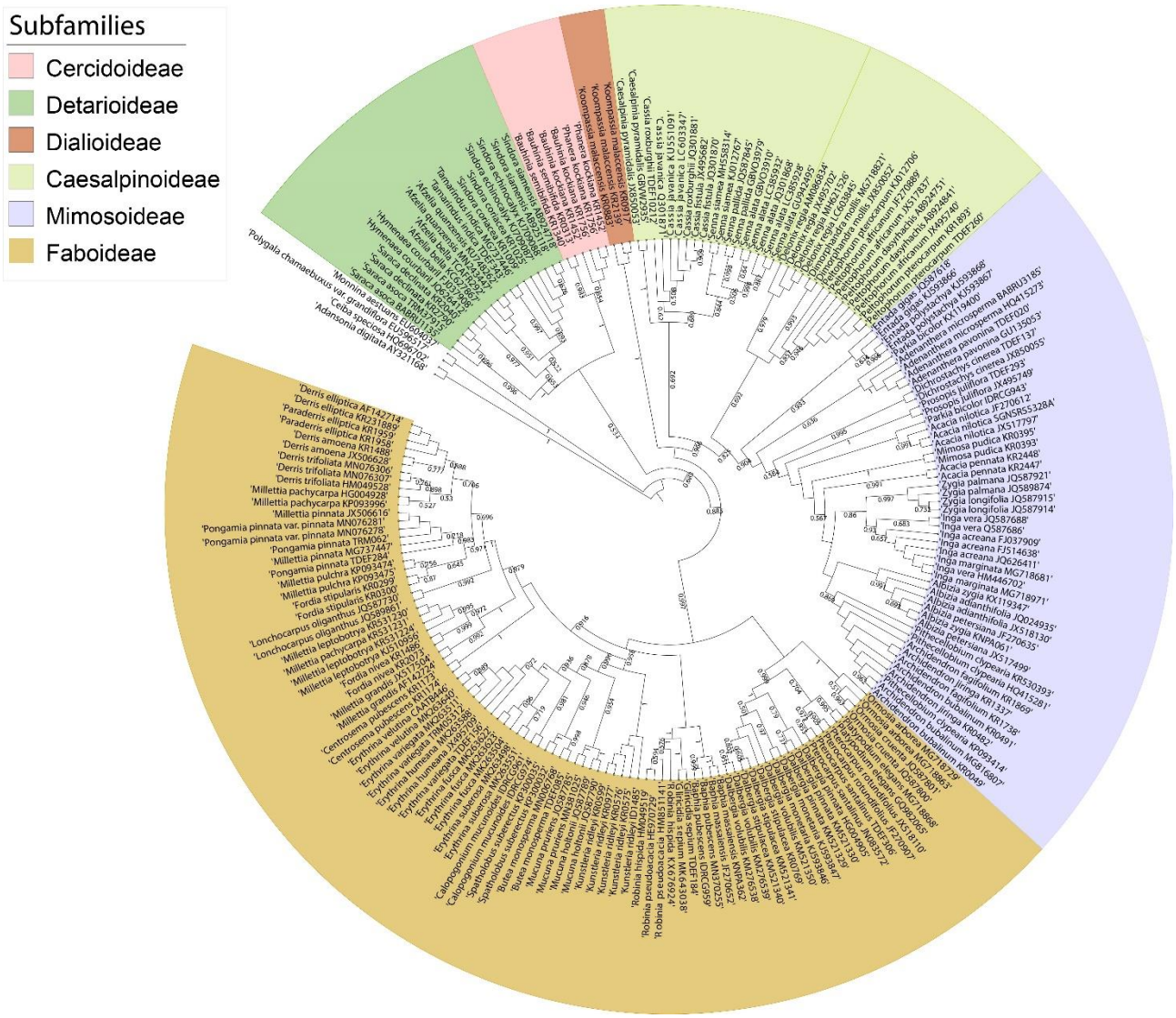

Figure 2 Neighbor Joining tree of selected Fabaceae species based on *mat*K data set, the clades highlighted represent the subfamilies: Cercidoideae; Detarioideae; Dialioideae; Caesalpinoideae; Mimosoideae; and Faboideae (Node values represent bootstrap support)

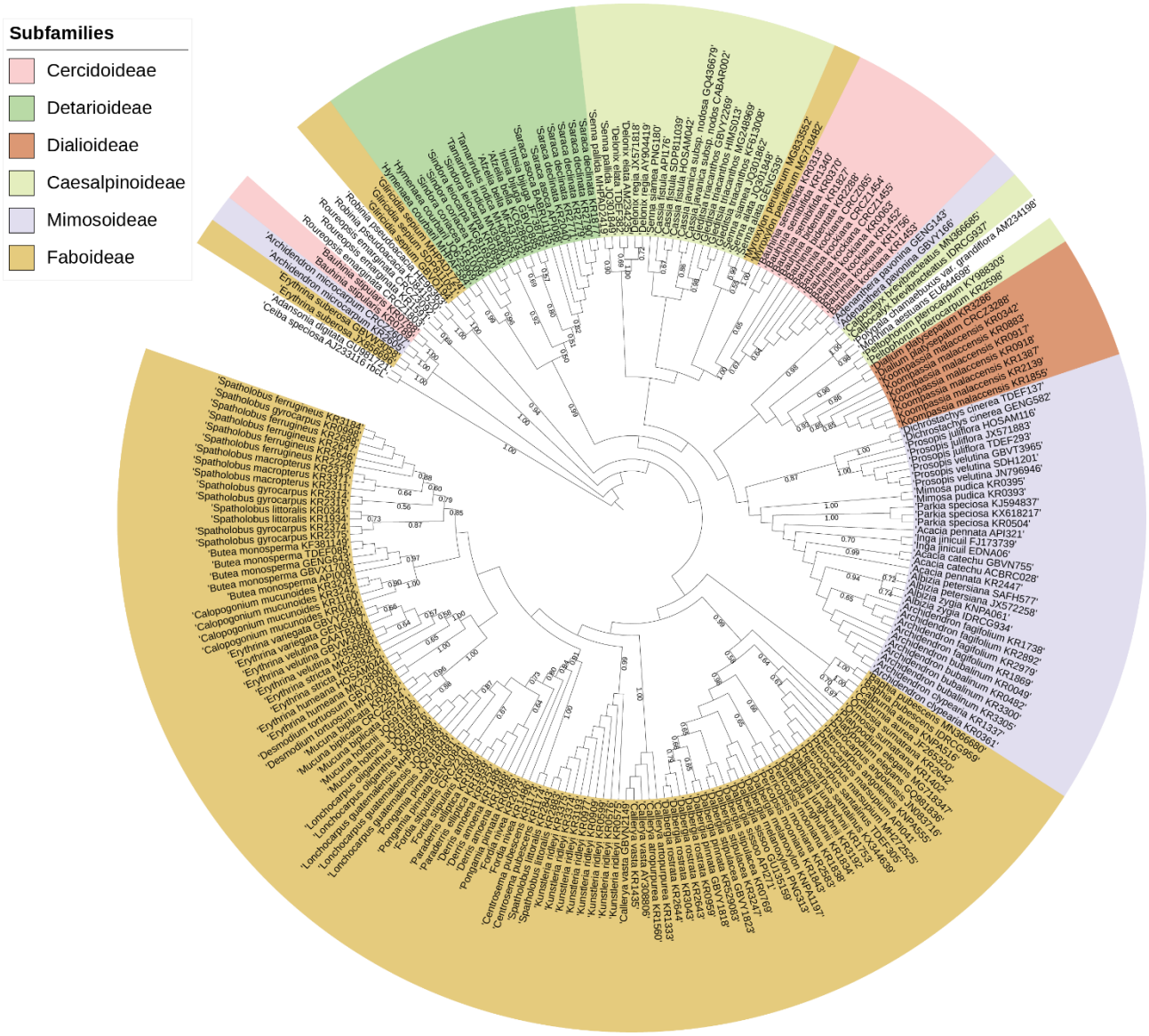

Figure 3 Neighbor Joining tree of selected Fabaceae species based on *rbc*L data set, the clades highlighted represent the subfamilies: Cercidoideae; Detarioideae; Dialioideae; Caesalpinoideae; Mimosoideae; and Faboideae (Node values represent bootstrap support)

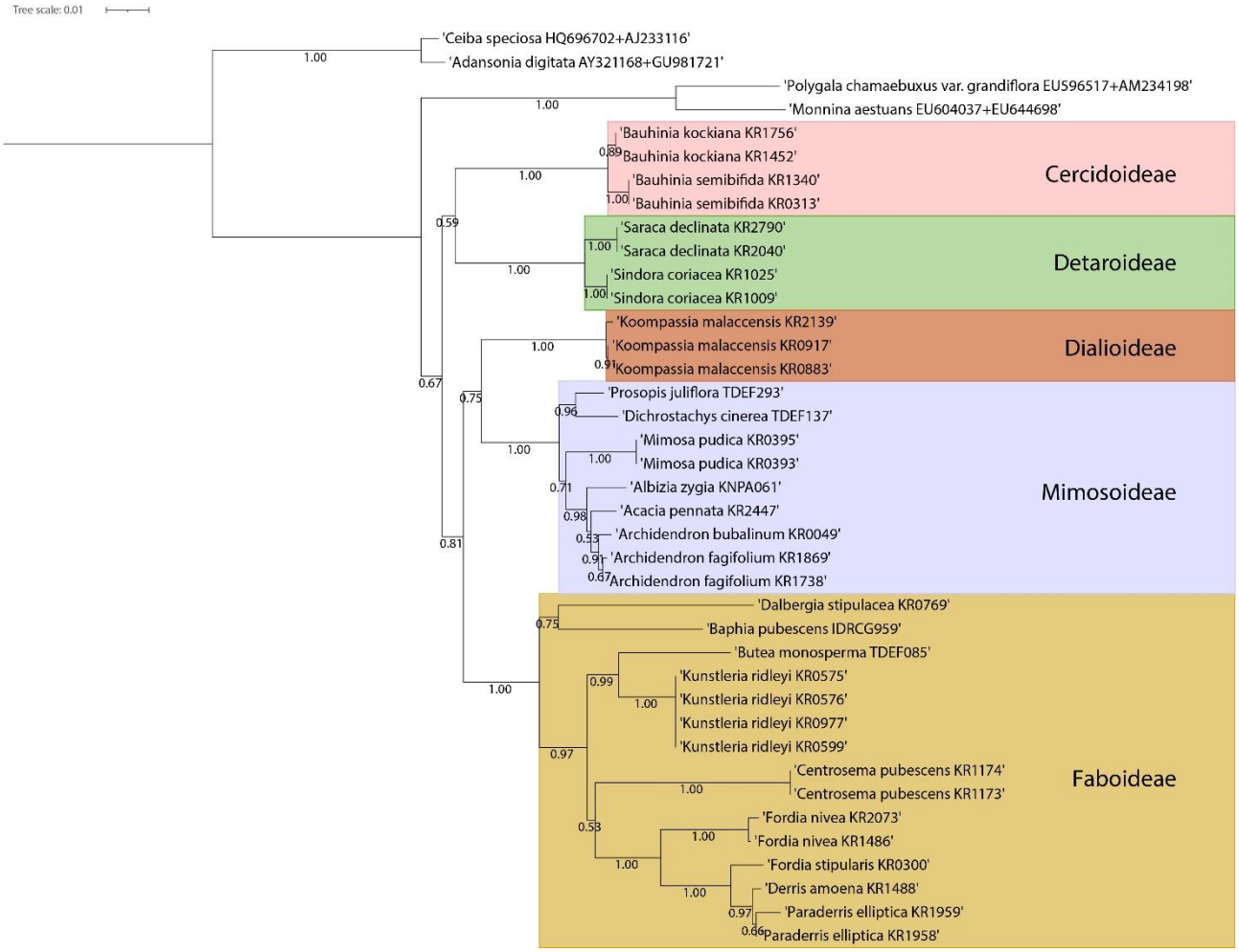

Figure 4 Neighbor Joining tree of selected Fabaceae species based on the *matK+rbcL* data set, the clades highlighted represent the subfamilies: Cercidoideae; Detarioideae; Dialioideae; Mimosoideae; and Faboideae (Subfamily Caesalpinoideae is not represented in the dataset, and Node values represent bootstrap support)

A "good" phylogenetic tree in biosystematics needs to be monophyletic, dichotomous, consistent, with high bootstrap value, shows no polytomies, and forms well-resolved clades. A monophyletic group originates from a single ancestor therefore, their members have similar traits, genetic patterns, and biochemistry (Rahayu and Jannah, 2019). The topologies of the phylogenetic trees reconstructed based on *mat*K and *rbc*L in this study were generally congruent, but there were some differences in the clade positions and bootstrap values. The resolution of the trees was evaluated based on the percentage of the monophyletic clades at species and genus level, as shown in Table 5.

Table 5 Percentage of monophyletic clades in the phylogenetic trees

Algoritm	Species level (%)		Genus level (%)	
	*mat*K	*rbc*L	*mat*K	*rbc*L
Neighbor Joining	95.74	92.00	91.67	86.00
Maximum Parsimony	85.94	85.96	81.63	73.47
Maximum Likelihood	84.38	89.00	78.43	85.71

Monophyletic clades with bootstrap values less than 0.7 were excluded from the estimation as considered unreliable (Hillis and Bull, 1993). Both *matK* and *rbcL* show high species-level resolution (92-95%), meaning most of the species included in the dataset were resolved to be monophyletic clades with bootstrap values

higher than 0.7. The percentage of monophyletic clades in the *mat*K+*rbc*L phylogenetic trees was not calculated as the data set is relatively limited compared to *mat*K and *rbc*L. However, the phylogenetic visualization of this combined marker confirmed the results based on the single markers. As an overview of the effectiveness of *mat*K and *rbc*L as plant barcodes, this study showcased that these two plastid markers worked well in identifying plant species of Fabaceae, at least for the selected genera included, which are particularly important to expand the knowledge of Indonesian floral composition.

CONCLUSION

Molecular identification with DNA *barcodes* is effectively applied to the Fabaceae species with high accuracy by *mat*K and *mat*K+*rbc*L compared to *rbc*L. Recommendations for the phylogenetic approach of Fabaceae Family are *Neighbor Joining* which is more informative in phylogenetic tree reconstruction. Future studies should include supplement markers, such as *psbA-trnH* or ITS/ITS2 in combination with *mat*K and *rbc*L.

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