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ASSESSMENT FOR IDENTIFICATION OF GENUS *Durio* spp. FROM NORTH MALUKU INDONESIA USING THREE BARCODE DNA

SUNDARI[1*], DIDIK WAHYUDI[2], ESTRI LARAS ARUMINGTYAS[3], LUCHMAN HAKIM[3] and RODIYATI AZRIANINGSIH[3]

[1]*Department Biology of Khairun University, Ternate North Maluku Indonesia*
[2]*Faculty of Science and Technology, State Islamic University of Maulana Malik Ibrahim, Malang, Indonesia*
[3]*Biology Department of Brawijaya University, Malang Indonesia*
[*]*E-mail: sundari@unkhair.ac.id*

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ABSTRACT

Durians in North Maluku (*Durio* spp.) have many local names which are taxonomically ambiguous. These ambiguous can be solved with selecting suitable barcodes for identification. It has been identified for first time using molecular technique. DNA barcode is one technique that can be used for species identification using standard DNA sequences. The objectives of this study is to evaluate the effectiveness of three DNA barcodes to identify and discriminate species of local Durian in North Maluku. A total of 36 samples representing Durian with some local names were collected from three islands (Ternate, Tidore, Jailolo) and were identified using three DNA barcode: *matK* gene, ITS regions and *trn*L intron. ITS showed the highest sequencing success, the highest variable site and parsimony informative, most effective for identification using BLASTN NCBI and resulted the most reliable phylogenetic tree. Moreover ITS also has the highest distribution of intraspecific K2P distance (> 0.02) that make ITS can be used as marker to discriminate the Durian. In other hand *matK* showed the lowest sequencing success but more effective for identification than *trnL*. In general the results of this research informs that among the three DNA barcodes, i.e. *matK*, ITS, and *trnL*, ITS region is the most effective for identifying and discriminating *Durio* spp.

Key words: Species identification, DNA barcodes, *Durio*, taxonomy

INTRODUCTION

Indonesia is one of the distribution centres of Durian (*Durio* spp.) in the world (Sastrapradja *et al.*, 1989; Uji, 2005). The diversity data of Durian showed that there are 27 species in the world, and 20 species of them are found in Indonesia (Orwa *et al*., 2009; Uji, 2005). Furthermore the ecological diversity of Durian in Indonesia are in Borneo, Sumatra and some of them spread throughout the islands in Indonesia (Uji, 2005; Santoso, 2012). In the east of Indonesia, North Maluku is one of Durian spots and have diversity of local Durian characterised by morphological and local names variations. In North Maluku Local Durian are distributed on the three islands:Ternate, Tidore and Jailolo.

There are many different variants of Durian in North Maluku in terms of fruit shape, taste and colour of flesh (Figure 1). Some researcher reported that there was a morphological diversity of local Durian at Ternate island (Sundari *et al*., 2015), Jailolo (Sundari *et al*., 2014), and Tidore islands (Sundari *et al.*, 2016). Furthermore, the genetic diversity of local Durian at Tidore and Jailolo) based on RAPD marker was also reported (Sundari *et al.*, 2016).

Based on the phenology, local Durian in North Maluku are mostly Durian that grow in lowlands (coast), and some of them are distributed in the highlands (<1000 m a.s.l.). Many local Durian names in North Maluku which represent morphological variation cause some problems in the identification in taxonomic levels. So far the identification based on morphological characters (Sundari *et al*., 2015) and RAPD profile (Sundari *et al.,* 2017), not yet able to answer and distinguish their inter-species or intra-species diversity of local Durian in North Maluku.

* To whom correspondence should be addressed.

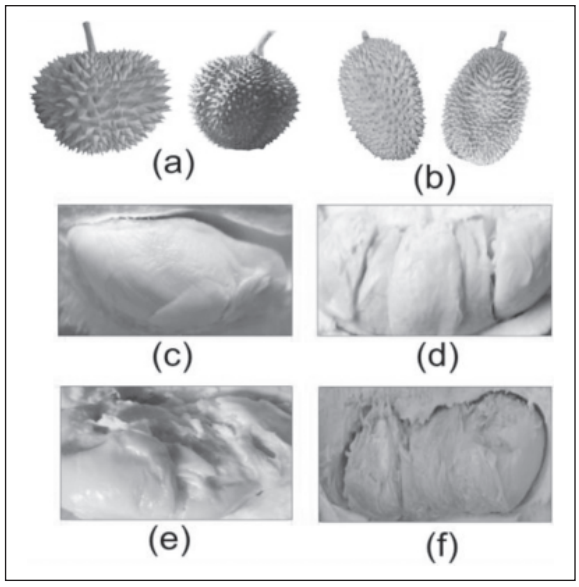

Fig. 1. Variability of Fruit shape, colour and texture flesh in *Durio* spp. from North Maluku. (a) Fruit shape ovoid (b) Fruit shape ellipse (c) Creamy white colour (d) lemon yellow colour (e) Smooth texture (f) Fibrous texture.

DNA barcodes are a new approach and quick molecular technique for taxonomic identification using short DNA region (Hebert, 2003), that have sufficient variation to correctly recognise and identify species (Hebert, 2013; Maideen, 2016). In order to find out the universal DNA Barcodes for plant, various genome source including nuclear genome such as ITS 1 and ITS 2 (Chen *et al*., 2010), mitochondrial genome such as CO1 and chloroplast genome such as *matK*, *rbcL*, *trnL* and *trnH- psbA* has been used (Kress *et al.*, 2007).

Since mitochondrial DNA known have slower nucleotide substitutions (Mower *et al.,* 2007), chloroplast and nuclear genome had become the primary choice for plant DNA barcoding and microsatellite (Hebert, 2013). Plant Working Group of the *Consortium for the Barcode of Life* (CBOL, 2009) recommended *rbcL* and *matK* (chloroplast genome) as major barcode for plant identification. Both barcode locus (*rbcL* + *matK*) was recently approved for identifying ferns (Li *et al*., 2011), Podocarpaceae (Little *et al.,* 2013) and Crasullaceae (Zhang *et al.*, 2015). Furthermore the nuclear region of internal transcribed spacer (ITS) was proposed as candidate barcodes and the best single barcode for *Rhodiola* (Zhang *et al.*, 2015). Consortium Barcode of Life (CBOL, 2009), also revealed that chloroplast region of PSBA-*trnH*, *rpo*B2, *rpo*C1, and intron *trnF*-*trnL* and *trnL* interspacer, were proposed as complementary regions for plant barcode. In this study, we used DNA barcode candidate including *matK*, ITS and intron *trnL* to evaluate their ability in identifying inter or intra-species variation of local Durian in North Maluku.

MATERIALS AND METHODS

Plant materials

Total of 36 local Durians were used in this study. Fifteen local Durians were collected from Ternate island, nine from Tidore islands and 12 from Jailolo as well as two of *Durio kutezensis* and *Ceiba petandra* as outgroup (Table 1).

Table 1. List of local Durian in North Maluku, codes and collection site

Code	Local Name	Site	Code	Local Name	Site
T1	Durian Cinta	Ternate	R15	Durian Gajah	Tidore
T2	Durian Urat	Ternate	R17	Durian Gosi	Tidore
T3	Durian Mentega	Ternate	R25	Durian Kohori	Tidore
T5	Durian Boso	Ternate	R36	Durian Blek	Tidore
T6	Durian Coklat	Ternate	J1	Durian Malal	Jailolo
T7	Durian Gosi	Ternate	J2	Durian Yang	Jailolo
T10	Durian Gajah kuning	Ternate	J3	Durian Lole	Jailolo
T11	Durian Pare	Ternate	J4	Durian Serkaya	Jailolo
T12	Durian Rua 1	Ternate	J6	Durian Boga	Jailolo
T13	Durian Biji mati	Ternate	J8	Durian Pisang	Jailolo
T15	Durian Ratem	Ternate	J16	Durian Tabesang	Jailolo
T16	Durian Luri	Ternate	J21	Durian Ping	Jailolo
T17	Durian Pondak	Ternate	J28	Durian Gumala	Jailolo
T18	Durian Biasa	Ternate	J32	Durian gajah	Jailolo
T19	Durian Sina	Ternate	J34	Durian Sambiki	Jailolo
R6	Durian Baba	Tidore	J35	Durian Pare	Jailolo
R7	Durian Boso	Tidore	R	Ceiba petandara	Malang
R10	Durian Nona	Tidore	K	Durio kutezensis	Kalimantan
R12	Durina Sambiki	Tidore			
R13	Durian Mafu	Tidore			

Table 2. Sequences of PCR primers used in this study

Primer Name	Sequences
matK	F: 5'-ATATCCGCTTATATTTCAGGAGT-3' R: 5'-GAACTAGTCGGATGGAGTAG-3'
ITS	ITS L: 5'-TCGTAACGTTTCCAAGGTAGGTG-3' ITS 4: 5'-TCCTCCGCTTATTGATATGC-3'
trnL	c: 5'-CGACGGAATCTATAGACGCG-3' d: 5'GGGGATAGAGGGACTTGAAC-3'

DNA isolation, amplification and sequencing

Total DNA was extracted from 100-200 mg of dried or fresh leaves of the thirty-eight local Durian in North Maluku using modified CTAB method (Doyle & Doyle, 1987). The quality of extracted DNA was measured using nanodrop method. PCR amplification of partial *matK*, ITS and *trnL* were performed using 50-100 ng of total DNA as a template in 20 µL of reaction mixture. The reaction mixture consisted of 10 µL PCR Mix (Introns); 7 µL ddH2O; 1 µL (10 pmol) each of forward and reverse primer (Table 2), and 1 µL DNA template.

PCR amplification carried out in TaKara PCR thermal cycler with the following cycling: *matK* (Pre-denaturation at 95°C for 5 min.; followed by 35 cycles of 95°C for 45 seconds; 61.3°C for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 7 min), *trnL* (Pre-denaturation at 95°C for 5 min.; followed by 35 cycles of 95°C for 45 seconds; 61.3°C for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 7 min) and ITS (Pre-denaturation at 95°C for 5 min.; followed by 35 cycles of 95°C for 45 seconds; 54.5°C for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 7 min). Furthermore, 5 µL of PCR products was electrophoresed in 1.5% agarose gel and visualized in UV GelDoc. Remaining PCR product were sequenced in the 1stBase Laboratories Sdn Bhd. (Malaysia).

Data analysis

Sequences data were evaluated using sequences scanner v.10. (http://www.appliedbiosystems.com). Potential contamination and effectiveness of identification was identified using queried in GenBank using BLASTN (Altschul *et al*., 1990; Zheng *et al*., 2000; Aleksandr *et al*., 2008). Only with minimal 3 hit, e-value 0.0 and 100% identification are considered as the effectiveness identification. Sequences were aligned using Clustal W in the MEGA 5.03 package (Larkin *et al*., 2007). Sequences were analysed using DnaSP 5.10 (Librado *et al*., 2009) for identifying insertion-deletion, gap site, polymorphic site and constant site. To evaluate the effectiveness of DNA barcode sequences for species discrimination, we also conducted a tree-based analysis. A phylogenetic tree was predicted using neighbour-joining (NJ) tool in MEGA 5.03 (Saitou *et al*., 1987) with bootstrap 1000 replicates (Felsenstein, 1985). Substitution model selected based on Models with the lowest BIC (Bayesian Information Criterion) scores (Nei and Kumar, 2000), is Kimura-2-Parameter model (Saitou *et al*., 1987). Genetic distances analysis (interspecies and intra-species) were calculated using the Kimura-2-parameter (K2P) model in MEGA 5.03 (Kimura, 1980) with bootstrap 1000 replicate (Felsenstein, 1985). All positions containing gaps and missing data were eliminated.

All statistical analysis regarding sequencing success, QV20+, species identification with BLASTn were performed using ANOVA one way and Tukey test at P = 0.05 on SPSS 16.0 software (SPSS Inc, 1989-2007).

RESULTS

PCR/sequencing success and sequences quality

PCR amplification successfully produced 245 bp *matK*, 700 bp ITS and 700 bp *trnL* respectively. Total of 38 samples are used for sequencing and resulted 198-205 bp for *matK* (71.3% SE 0.6), 479-784 bp for ITS (98.9% SE 0.8) and 563-632 bp for *trnL* (85% SE 0.2). Sequencing success was statistically different only for ITS (Duncan test; $P < 0.05$) (Figure 2).

Marker identification power using blast NCBI

Blast analysis using Gen Bank query was performed. ITS showed the effectiveness of identification compared with both *matK* and *trnL* marker. ITS have the highest 100% identification success, followed by *trnL* and *matK*. All of Durian in North Maluku were identified as *G. caribensis* based on *trnL* but identified as *D. zibethinus*, *D. graveolens* and *D. dulcis* based on *matK* and ITS (Table 3). It means that *trnL* is not effective for identifying Durian caused by its misidentification.

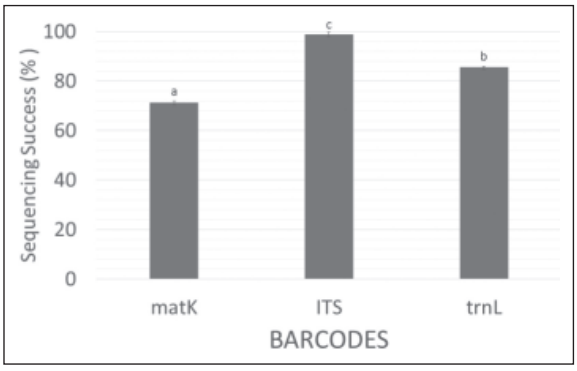

Fig. 2. Sequencing success with one-way ANOVA and Duncan test $P < 0.05$.

Table 3. Success rate of species identification based on BLAST NCBI

Local name	Site	% of identification			Identified		
		matK	*trnL*	ITS	*matK*	*trnL*	ITS
Durian Cinta	Ternate	–	98 (-)	98 (-)	–	*G. caribensis*	*D. zibethinus*
Durian Urat	Ternate	99 (-)	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. graveolens*, *D. dulcis*
Durian Mentega	Ternate	–	98 (-)	–	–	*G. caribensis*	–
Durian Boso	Ternate	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Coklat	Ternate	99 (-)	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Gosi	Ternate	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Gajah kuning	Ternate	–	97 (-)	100	–	*G. caribensis*	*D. zibethinus*
Durian Pare	Ternate	99 (-)	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. graveolens*
Durian Rua 1	Ternate	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Biji mati	Ternate	99 (-)	97 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Ratem	Ternate	–	–	99 (-)	–	–	*D. graveolens*
Durian Luri	Ternate	99	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Pondak	Ternate	99	97 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. graveolens*, *D. zibethinus*, *D. dulcis*
Durian Biasa	Ternate	99	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Sina	Ternate	–	–	99 (-)	–	–	*D. graveolens*, *D. zibethinus*, *D. dulcis*
Durian Baba	Tidore	99	97 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. zibethinus*, *D. dulcis*
Durian Boso	Tidore	99 (-)	97 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Nona	Tidore	99 (-)	97 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durina Sambiki	Tidore	99 (-)	98 (-)	98 (-)	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Mafu	Tidore	99 (-)	97 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Gajah	Tidore	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Gosi	Tidore	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Kohori	Tidore	99 (-)	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Blek	Tidore	–	98 (-)	99 (-)	–	*G. caribensis*	*D. graveolens*, *D. dulcis*
Durian Malal	Jailolo	99 (-)	98 (-)	99 (-)	*D. zibethinus*	*G. caribensis*	*D. graveolens* *D. zibethinus* *D. kutejensis*
Durian Yang	Jailolo	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Lole	Jailolo	99 (-)	98 (-)	–	*D. zibethinus*	*G. caribensis*	–
Durian Serkaya	Jailolo	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Boga	Jailolo	99 (-)	–	99 (-)	*D. zibethinus*	–	*D. zibethinus*, *D. dulcis*
Durian Pisang	Jailolo	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Tabesang	Jailolo	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Ping	Jailolo	99 (-)	98 (-)	100	*D. zibethinus*	*G. caribensis*	*D. zibethinus*
Durian Gumala	Jailolo	99 (-)	–	–	*D. zibethinus*	–	–
Durian Gajah	Jailolo	99 (-)	98 (-)	–	*D. zibethinus*	*G. caribensis*	–
Durian Sambiki	Jailolo	99 (-)	98 (-)	–	*D. zibethinus*	*G. caribensis*	–

Note: Genera have been abbreviated: P= *Pachira*, D= *Durio*, C= *Ceiba*, G= *Gyranthera*. (-) failed identification because of <100% identification.

Sequence characteristics

Quality value 20+ (QV 20+) was analysed using sequence scanner v.10 program. The QV is per-base estimation of the base-caller accuracy. % of QV 20+ is obtained from total QV 20+ : total sequenced DNA. *trnL* obtained the highest QV 20+ with QV20+ vary from 119 to 589 (91.65% SE. 2.13) followed by ITS vary from 351-740 (88.5 % SE. 2.2) and matK vary from 154-191 (86.9 % SE. 0.82). However QV 20+ did not statistically differ (not significant) at Duncan test $P < 0.05$ among 3 DNA barcodes (Figure 3). Samples with sequencing success and QV20+ below of 70% were excluded and remained 27 samples for *matK* 30 for ITS and 34 for *trnL* and used for further analysis (Table 3).

The sequences characteristic of *matK*, ITS and *trnL* are shown in Table 3. Alignment resulted in 181 bp of *matK*, 675 bp ITS and 555 bp for *trnL* intron. *matK* had the highest percentage of conserved region (92.8%) followed by *trnL* (92.1%) and ITS (72%). The highest percentage of variable site was obtained by ITS (22.9%) followed by *trnL* (7%) and *matK* (6.1%). However ITS had the highest gap site (4.7%) followed by *matK* (1.1%) and *trnL* (0.9%). In other hand, ITS had the highest parsimony informative and singleton variable (Table 4).

Distance and phylogenetic analysis

trnL has the highest distribution of intraspecific K2P distance (0-0.005) followed by *matK* and ITS (Figure 4). However ITS had the highest distribution of intraspecific K2P distance (> 0.02). *matK* has the distribution of K2P distance which tends to decrease as distance value increase.

The phylogenetic tree analysis inferred from *matK*, and ITS using NJ method revealed that all individuals of each species generated a monophyletic group (Figure 5 and Figure 7), whereas DNA barcodes using *trnL* showed that local Durian in North Maluku produced paraphyletic with the outgroup (Figure 6). Since monophyletic cladogram was the most accepted cladogram in the systematic, ITS and *matK* were the best marker in this study in terms of the ability to construct cladogram.

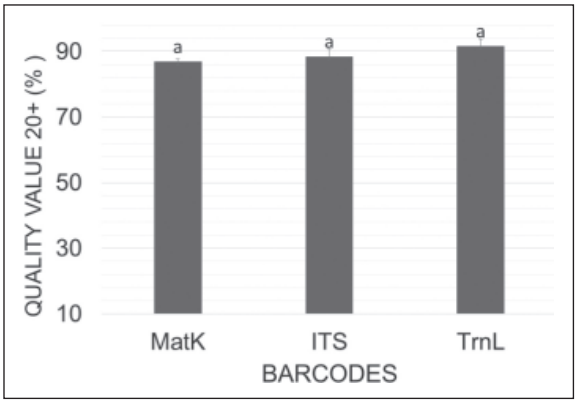

Fig. 3. Quality value 20+ of 3 DNA barcode sequences with one-way ANOVA and Duncan test $P < 0.05$.

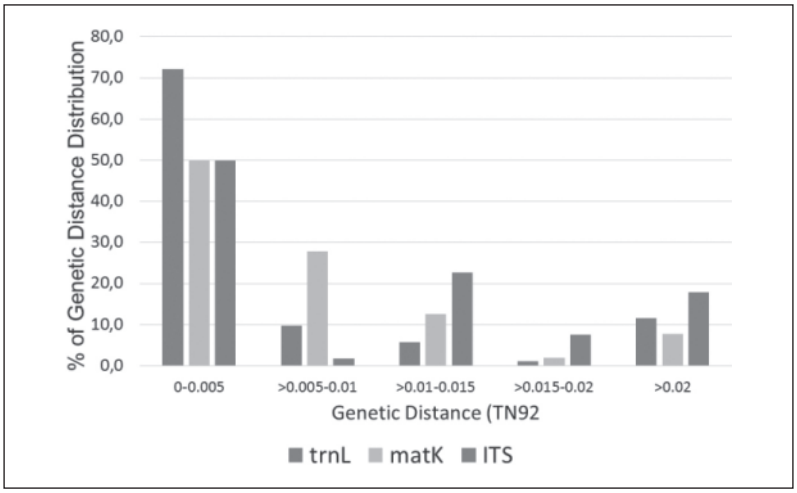

Fig. 4. Relative Distribution of K2P distance for three DNA barcodes in Durian.

Table 4. Sequences characteristic analyzed using Mega 5.0 and DnaSP 5.10

Barcodes	Alligned length (bp)	Conserve	Variable site	Gap site	Parsimony informative	Singgletone	Total individual
matK	181	168 (92.8%)	11 (6.1%)	2 (1.1%)	4 (2.2%)	7 (3.8%)	27
ITS	675	490 (72%)	155 (22.9)	32 (4.7%)	19 (2.8%)	165 (24.4%)	30
trnL	555	511 (92.1%)	39 (7%)	5 (0.9%)	1 (0.1%)	38 (6.8%)	34

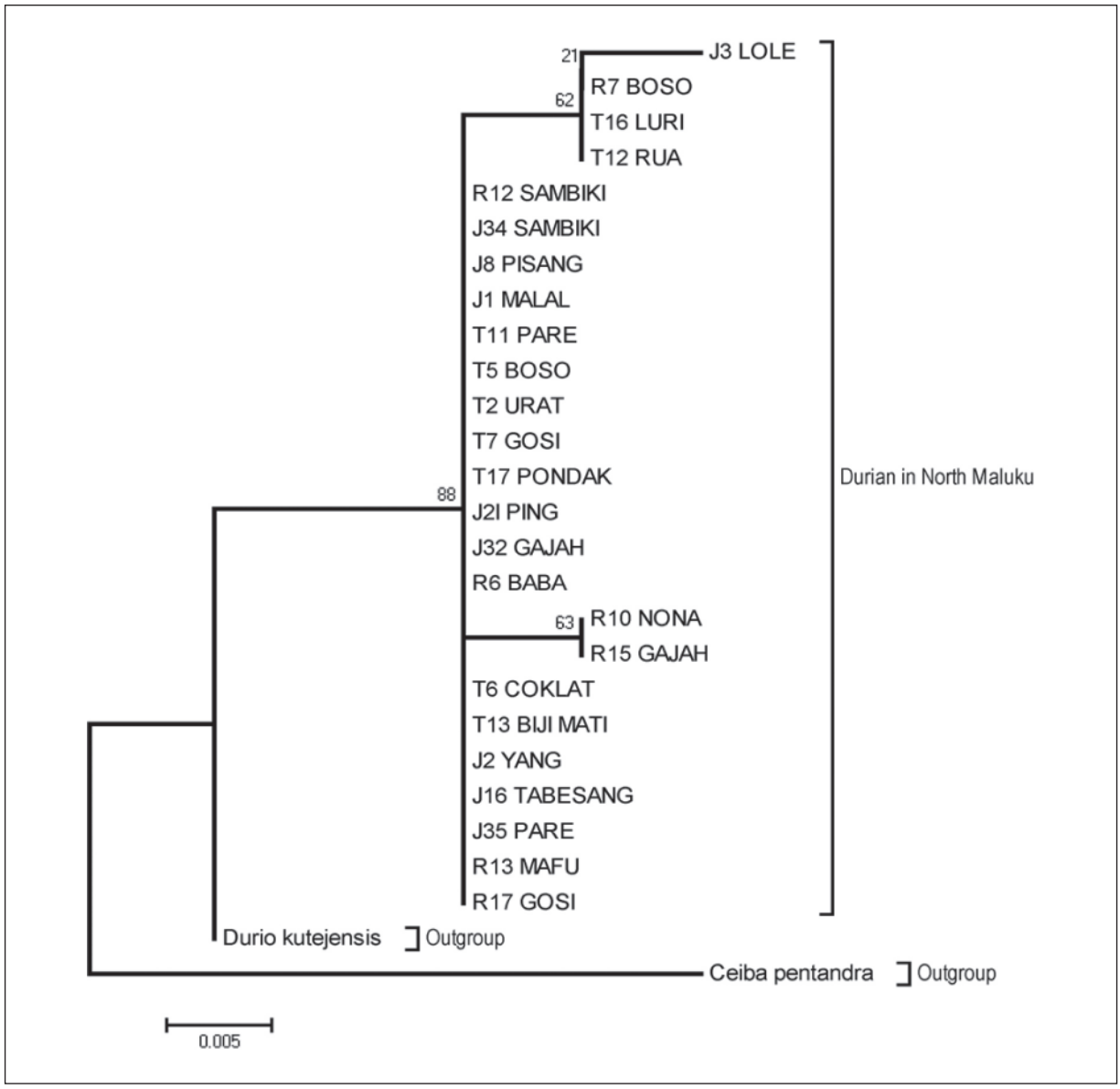

Fig. 5. Neighbour-joining tree of Durian species inferred from *matK* sequences. Bootstrap values are shown above branches.

DISCUSSION

DNA barcode is standardised sequence DNA that consist of 400-800 bp (Kress *et al.*, 2008). One of the main criteria in selecting DNA barcode is the strength in species discrimination both inter and intraspecies (Hebert *et al.*, 2003; Kress *et al*., 2007; CBOL, 2009). DNA barcodes are ideal candidates to provide high discrimination and identification of species (Kress *et al*., 2007; Hollingsworth *et al.*, 2009). Species discrimination are considered successful if the minimum value of interspecific genetic distance is greater than the maximum value of intra-specific genetic distance (CBOL, 2009), or if all the individuals in the species form a monophyletic group in the phylogenetic tree (Hollingsworth *et al.*, 2009).

In order to find out the suitable DNA barcoding for identifying Durian, we attempted three of DNA barcodes in Durian. There are four criteria to be considered and weighted in selecting the best DNA barcodes for plant (Kress *et al.,* 2008) such as universal PCR amplification, range of taxonomic diversity, power of species differentiation, and bioinformatic analysis and application. We found the ITS region as the most powerful of the three tested barcodes in Durian. ITS has the highest sequencing success (Figure 2), the highest distribution of intraspesific K2P (Figure 4) and the best representing phylogenetic tree (Figure 7).

ITS is the most useful barcode in Durian as revealed in other plant barcoding studies such as Rhodiola (Zhang *et al*., 2015), Codonopsis (Wang *et al*., 2017) and seed plant (Li *et al*., 2011).

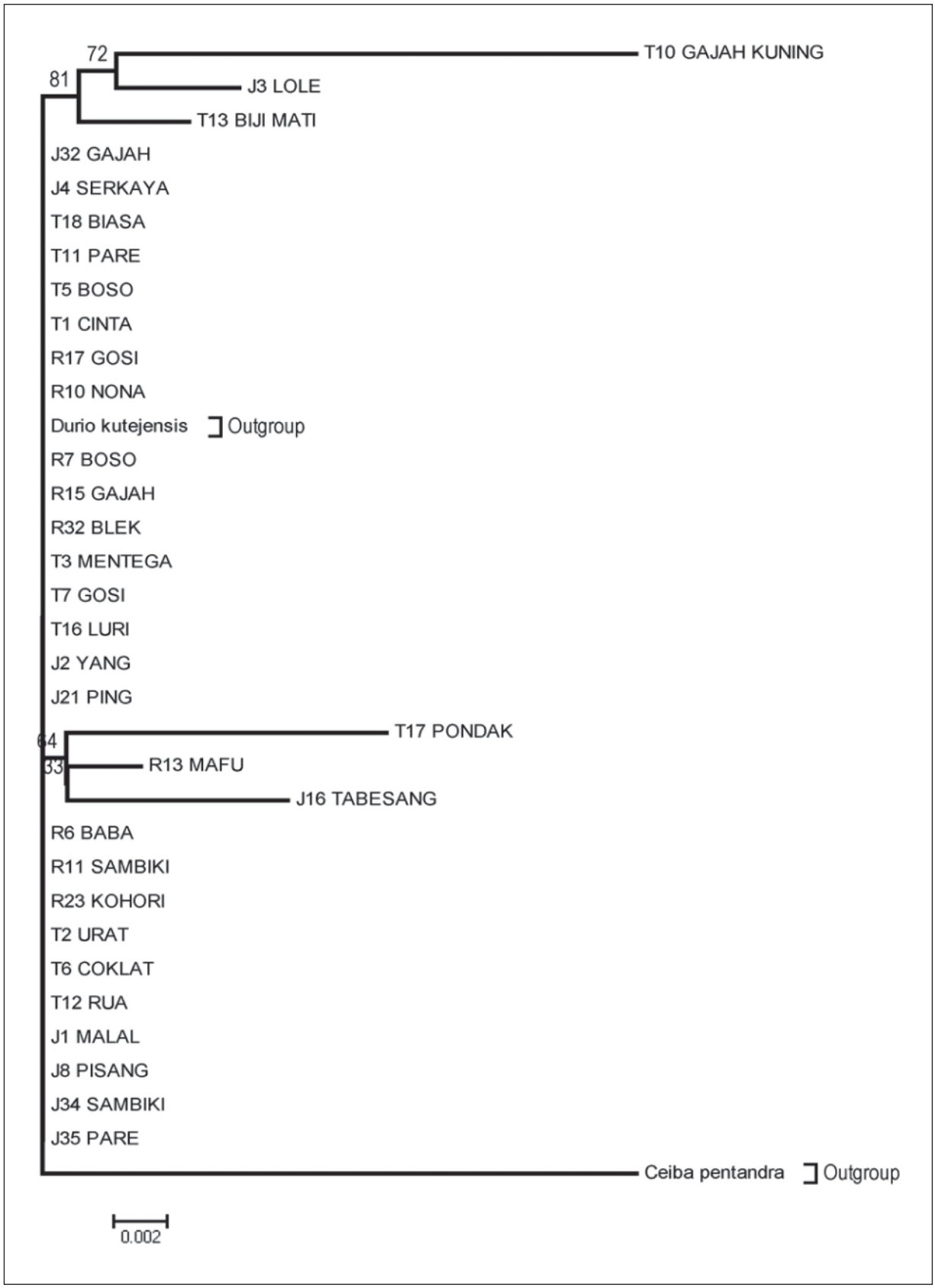

Fig. 6. Neighbour-joining tree of Durian species inferred from *trnL* sequences. Bootstrap values are shown above branches.

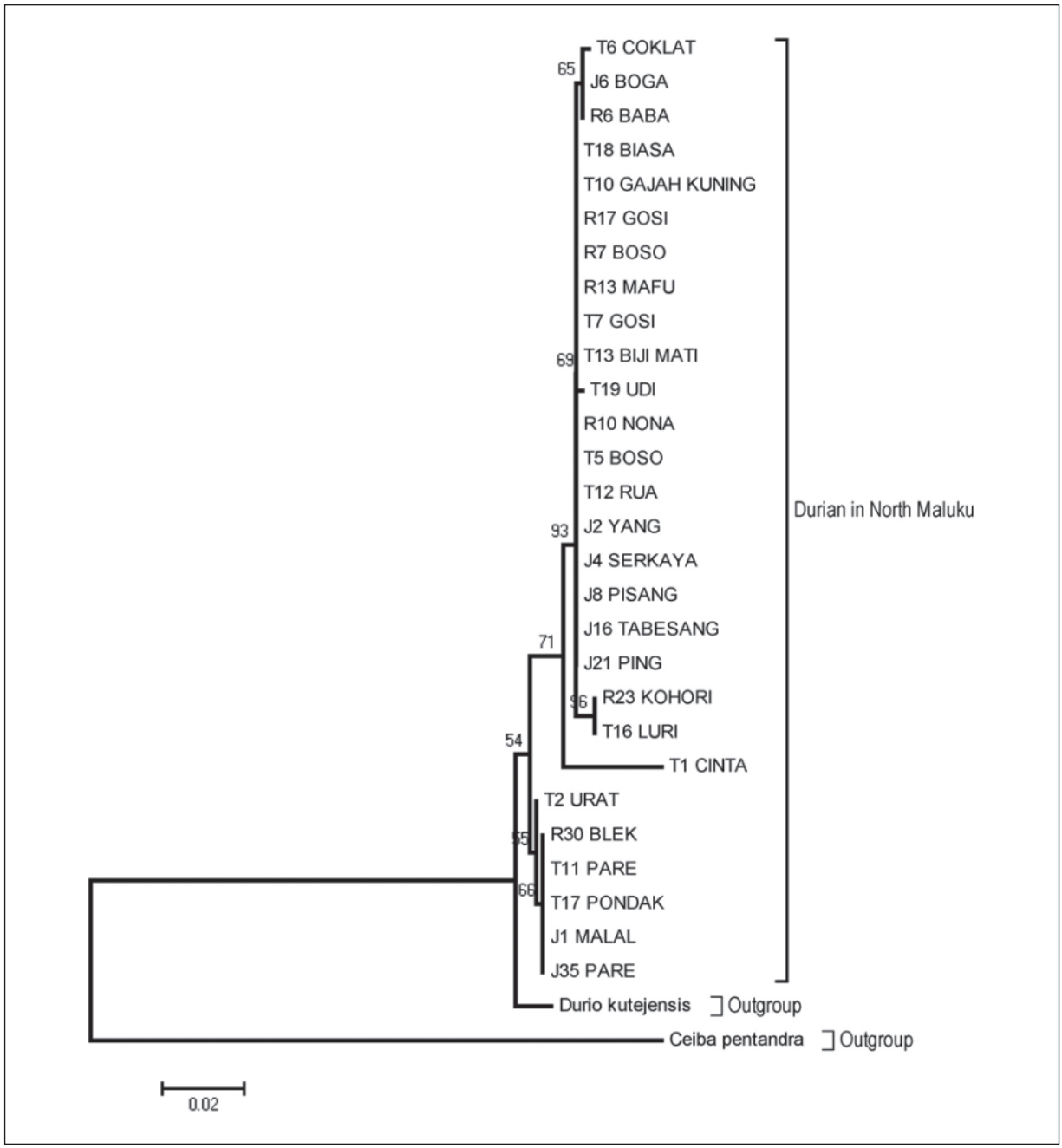

Fig. 7. Neighbour-joining tree of Durian species inferred from ITS sequences. Bootstrap values are shown above branches.

However the main challenge of using ITS as standard barcode is that it is quite difficult to be amplified and sequenced (Sass *et al.*, 2007), due to divergent paralogous copies within same individual (Shaik *et al.,* 2016). Although sequencing success for ITS were high in this study (Figure 2), but more attempt is needed to reach it.

CBOL: Plant Working Group (CBOL, 2009) revealed that the combination of 2-markers, consisting of *trnL* and *matK*, is proposed as the main barcode for land plants. Nevertheless, *trnL* and *matK* denoted significantly lower discriminatory power than ITS in this study. *trnL* second intron has been proposed as robust DNA barcode for plant such as Fabaceae (D'yachenkoa *et al*., 2014). However in this study *trnL* showed lower parsimony informative (Table 3) and the highest relative distribution in 0-0.005 (Figure 4), due to relatively low resolution compared with other noncoding region (Taberlet *et al.*, 2007).

Another chloroplast region belong to coding region used in this study is *matK*. The *matK* showed the highest conserved region, the lowest sequencing success and the lowest QV20+. Moreover, *matK* showed lower success rate of identification using BLASTN NCBI (Table 3) but higher than *trnL.*

However, it is well known that *matK* has problematic in developing universal primer (Kress *et al*., 2008) and we encountered the same problem in this study. The primer pair used in the present study (Table 2) is relatively well although it only has 27 individuals from 38 individuals can be amplified (Table 4) and has the lowest % sequencing success (Figure 1)

Monophyletic clad in all local Durian inferred by *matK* and ITS (Figure 5 & Figure 7), indicated that the member of those taxonomic group (Durian Ternate, Tidore and Jailolo) was derived from one common ancestor. The member of a monophyletic group shared many synapomorphic character. Synapomorphic characters based on nucleotide bases can be used as a candidates DNA barcode from the family to the genus level (Hidayat & Pancoro, 2008).

Based on the ease of PCR amplification, sequencing process, the highest order of variation between species, and species with high resolution, ITS region is recommended for plant DNA barcode but technically need modifications. On the other hand DNA barcode *matK* gene and intron *trnL* is technically relatively easy to amplify, and sequence, but has lower ability to discriminate intra and interspecies.

CONCLUSIONS

DNA barcode is one technique that can be used for identify species with taxonomic problems including cryptic species, using standard DNA sequence. The research showed that the DNA barcode was efficient in discriminating local Durian species in North Maluku. The most effective barcode for discriminating Durian species is using ITS sequence.

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