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Assessment for Identification of *Stelechocarpus burahol* and Sister Species Complex of Annonaceae Family Using trnL Intron Sequences

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Abstract: *Stelechocarpus burahol* (kepel) belongs to the Annonaceae family, and is considered to be a native species to Indonesia which is mainly distributed on the island of Java. However, the plant's existence is currently difficult to find, so it is categorized as rare in Indonesia. A molecular approach using DNA barcoding technique is significant to assist plant identification. The gene that is widely used and proven to be accurate for Annonaceae is *trnL*-F. This study was aimed to evaluate the efficiency of trnL as DNA barcode for the identification of *S. burahol* and its relatives (Annonaceae) from Java Island. In total 10 specimens have been used in this study. Whole genome DNA was isolated by Tiangen kit and amplified by Polymerase Chain Reaction (PCR) technique using a specific primer. Results showed that *trn*L was easily amplified with a DNA fragment length of 500-600 bp. The trnL amplicons have successfully sequenced as indicated by the high QV20+ values. The sequence compositions were high in AT bases (63.9%). BLAST analysis of the sequences showed that S. burahol and sister species have been confirmed its identity according to the reference sequences in NCBI with query cover identities 98%-100%. This research can be concluded that *trnL*-F is suitable and recommended as a DNA barcode for *S. burahol* and its relatives. However, further research is suggested to combine analysis of both *coding* (*rbc*L, *mat*K, etc) and *non-coding* (*trn*L) markers for better identification results.

Keywords: Annonaceae, assessment, DNA barcoding, *Stelechocarpus burahol, trn*L.

Introduction

Annonaceae belongs to the pantropical family which has varying plant habits of trees, shrubs and lianas (Chatrou et al., 2012; Chaowasku et al., 2018). Nowadays, Annonaceae has about 110 genera and 2,430 species (Guo et al., 2018; Xue et al., 2018). Moreover, Annonaceae diversity is very high in Asia, including Indonesia (Lestari & Ningrum, 2021). One of the important species from the Annonaceae is *Stelechocarpus burahol*, which is popularly known as kepel fruit. It is considered as a native species to Indonesia, with native range from Borneo to South Malaya (POWO, 2023). However, currently the plant's existence is increasingly difficult to find in Indonesia due to tradition restriction, hence kepel is categorized as a rare plant (Angio & Firdiana, 2021). Studies related to a rare species are interesting and highly prioritized.

Furthermore, there are many different local names for several species of the Annonaceae in each region of Indonesia. Meanwhile, in general Annonaceae is called as a member of the soursops. In particular of *S. burahol*, it is also known as burahol and turalak (in Sunda); kecindul, simpol, and cindul (in Java) (Angio & Firdiana, 2021). Several species of the Annonaceae family which have some different

names in each region include: *S. burahol*, *Uvaria littoralis*, *Uvaria purpurea*, *Uvaria rufa*, *Cananga odorata*, *Anomianthus auritus*, *Polyathia glauca*, *Saccopetalum horsfieldii*, *Orophea hexandra*, *Annona muricata*, *Annona reticulata*, *Annona squamosa etc*. Therefore, species identification is important to scientifically recognize the Annonaceae species precisely.

Some researchers have reported the high morphological diversity and key characteristics of several species in the Annonaceae family (Lestari et al., 2017; Lestari & Ningrum, 2021). However, there are several problems, including the observed generative characters not appearing completely which affected the identification results at the species level (Meade & Parnell, 2018). Thus, the identity and position of taxa and tribe groupings in the Annonaceae remained unclear. Species identification based on morphological characters has not yet been able to answer and distinguish the inter-species or intra-species diversity of *S. burahol* and sister species in Indonesia.

The genetic character approach using DNA sequences is believed to have a higher level of accuracy and quick molecular technique for taxonomic identification (Probojati et al., 2019; Probojati et al., 2021). Some universal DNA barcodes from various sources have been optimized for family of Annonaceae, including chloroplast genome such as *rbcL*, *mat*K and *trnL* (Chaowasku et al., 2018; Guo et al., 2018; Lestari et al., 2018; Thomas et al., 2017). Especially, the *trn*L gene is part of the *trn*L-F region that is split by group I intron, the intergenic spacer and *trn*F exon (Yulita, 2013). Due to its conservation nature, the *trn*L intron has been widely applied for phylogenetic reconstruction in Angiosperms, including Annonaceae.

Currently, there is limited scientific knowledge of the DNA barcode sequence reference for identification of *S. burahol* which has been stored in the Genbank National Center for Biotechnology Information (NCBI) and Barcode of Life Database Systems (BOLDS). Therefore, it is necessary to conduct research on DNA barcode sequences of *S. burahol* in Indonesia as inventory data molecular identity. This study aimed to evaluate the efficiency of *trn*L intron sequences for identification of *S. burahol* and its sister species complex of Annonaceae. The results of this research are significant as basis reference for species identification and further molecular biosystematic studies.

Materials and Method

DNA Isolation

The plant materials examined in this study were 8 specimens from the Annonaceae family (as in-group) and 2 specimens from the Magnoliaceae family (as out-group) (Table 1). These plant specimens were collected from Purwodadi Botanic Garden (PBG), National Research and Innovation Agency located in Purwodadi, Pasuruan, East Java, Indonesia. Whole genome DNA was isolated from 100 mg of young leaves. Whole genome DNA isolation was carried out using Tiangen kit, following its manufacturer's instructions.

Polymerase Chain Reaction (PCR) Analysis

PCR reactions were conducted in 30 µl volume consisted of 15 µl of PCR Master Mix Nexpro, 3 µL DNA template (100 ng/µL), 6 µL nuclease free water, 3 µL of 10 pmol each of forward and reverse primers. Primer pairs used was consisting of forward primer trnL c 5' CGAAATCGGTAGACGCTACG 3' and reverse primer trnL d 5' GGGGATAGAGGGACTTGAAC 3' (Wahyudi et al., 2013). PCR cycle consists of DNA pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 61.3 °C for 45 s and extension at 72 °C for 45 s. Final extension carried out at 72 °C for 7 min. Furthermore, PCR products were purified and sequenced at 1st BASE Laboratories Sdn Bhd, Malaysia.

Data Analysis

The chromatogram of each DNA sequence of trnL was analyzed using ABI sequence scanner v.10 program and BioEdit software. The analysis menu used was sequencing success and Quality Value 20+ (QV20+). Then, the percentage of sequencing success was calculated by total DNA sequenced/total DNA amplified x 100%. Meanwhile, the percentage of QV20+ was calculated by total nucleotides with QV20+/sequencing success x 100% (Sundari et al., 2022). Furthermore, to determine the

homology of *trn*L sequence with NCBI queries and effectiveness of identification was conducted in using Basic Local Alignment Search Tool (BLAST) in NCBI Genbank website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. List of *S. burahol* and sister species (Annonaceae) used in this study

No	Species name	Collection number	Registration number	Locality
1	*Stelechocarpus burahol* (Blume) Hook.f. & Thomson	I.A.48	P1977040130	Purwodadi, Pasuruan, East Java
2	*Stelechocarpus burahol* (Blume) Hook.f. & Thomson	XII.G.D.4	P1965010005	Malang, East Java
3	*Annona muricata* L.	XVIII.C.18	P1977020046	Yogyakarta
4	*Annona montana* macfad	II.B	P1947060002	Bogor, Jawa Barat
5	*Fissistigma latifolium*	XVIII.C.6	P1982030062	Tuban, East Java
6	*Artabotrys suaveolens* Blume.	XVIII.C	P1979070075	Malang, East Java
7	*Annona glabra* L.	XVIII.C	P1980030009	Cianjur, Jawa Barat
8	*Desmos chinensis* Lour.	XVIII.C	-	Blitar, East Java
9	*Magnolia candollii* (Blume) H.Keng	XVIII.D.II.2	P19821171	Lebakharjo
10	*Michelia champaca* (L.) Baill. Ex Pierre	XIV.G.I.10	P1997110091	Malang, East Java

Results and Discussion

Amplification and DNA sequences of trnL-F genes

Amplification using primers of *trn*L intron was successfully carried out on the whole genome of *S. burahol* and sister species (Annonaceae) examined, resulting in single fragments at approximately 500-600 bp (Figure 1). Direct sequencing of these amplicons produced DNA sequences at 480-579 bp length (Table 2). Some previous studies were reported that *trn*L intron produced amplicons at 600 bp in Annonaceae (Pirie et al., 2007), 500-600 in Dipterocarpaceae (Yulita, 2013), 519 to 528 in Lauraceae (Sevindik & Okan, 2019), and 254–767 bp in mix land plants (Taberlet, 2007).

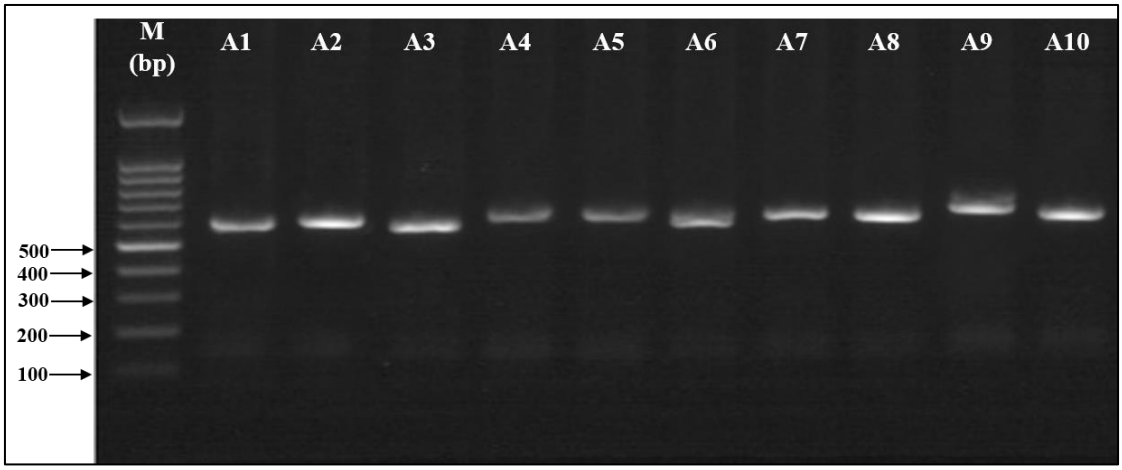

Figure 1. Electropherogram amplicon of trnL intron of S. burahol and sister species (Annonaceae). M= marker, A1-A2= S. burahol, A3= A. muricata, A4= A. montana, A5= F. latifolium, A6= A. suaveolens, A7= A. glabra, A8= D. chinensis, A9= M. candollii, A10= M. Champaca

Nucleotide composition of the *trn*L intron of the samples examined was dominated by AT bases content than GC. The average composition value of each base was 26% T(U) bases, 37.9% in A bases, 18.3% in G bases and 17.8% in C (Table 2). Barcodes from chloroplat region mostly high in AT, related with their functions in transcription and protein translation. A region with high AT and low GC content signified high conservation level, reduced spots of mutation and recombination rates (Sundari et al., 2023).

Table 2. Nucleotide composition of *S. burahol* and *sister species* (Annonaceae) used in this study

No.	Species name	Sequences length (bp)	Nucleotide (%)			
			T(U)	C	A	G
1	*Stelechocarpus burahol*	537	25.9	17.9	37.1	19.2
2	*Stelechocarpus burahol*	539	25.6	17.4	37.8	19.1
3	*Annona muricata*	480	26.5	18.3	38.8	16.5
4	*Annona montana*	534	24.5	18.5	36.7	20.2
5	*Fissistigma latifolium*	523	25.2	18	36.9	19.9
6	*Artabotrys suaveolens*	532	26.1	17.5	39.1	17.3
7	*Annona glabra*	536	25.2	18.5	38.6	17.7
8	*Desmos chinensis*	579	28.2	16.8	39.4	15.7
9	*Magnolia candollii*	537	26.4	18.1	36.9	18.6
10	*Michellia champaca*	528	26.3	17.4	37.3	18.9
	Average	**532.5**	**26**	**17.8**	**37.9**	**18.3**

Note: G= Guanin, A= Adenin, C=Cytosin, T= Thymin, U= Uracil

Furthermore, all ten *trn*L PCR products were successfully sequenced as indicated by the QV20+ values (Figure 2). QV20+ value is the total number of bases in the entire trace that have basecaller quality value higher than or equal to 20. The value is equivalent to the base pair length of the sequencing results. In addition, the higher value of QV20+ indicates the higher the quality of the sequences produced. The trnL intron for *Durio* spp. was obtained the highest QV20+ sequences compared to other barcodes (Sundari et al., 2019). From this study all ten specimens were produced high QV20+ values, with the lowest value was found in specimen A3 (*A. muricata*).

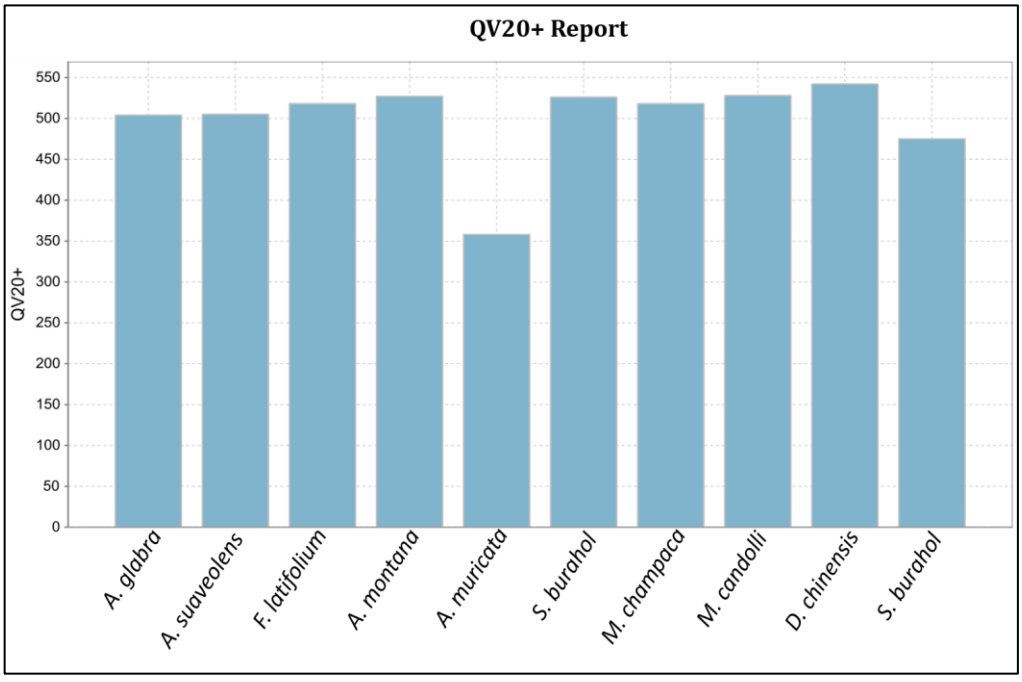

Figure. 2. Quality value 20+ of *trn*L gene analysed with sequence scanner software 2.0

Identification of *S. burahol* and sister species

After evaluating the quality of the sequence with QV20+, then the sequence was BLASTed to match with the reference sequence deposited in the NCBI database. The BLASTN results were confirmed the species identity of each sample, as shown in Table 3. All samples were confirmly identified in accordance to their registered species name in PBG database with query cover 98-100%, except A8 and A9. Sample A8 was identified as *Hiptage benghalensis* with a query cover of 90%. Further, these two species belong to different families; *D. chinensis* belongs to the Annonaceae, while *H. benghalensis* belongs to the Malpighiaceae. Further analysis is needed to ensure the accuracy of the species identification for A8.

Meanwhile, sample A9 was identified as *Magnolia liliifera* with a query cover of 98%. This is a synonym and accepted name for the species *M. candollii* (POWO, 2023). Therefore, the plant species catalogue of PBG suggested to be updated. Moreover, sample A5 was identified as *Fissistigma* sp. because there is no reference sequence of *Fissistigma latifolium* deposited in NCBI. Nevertheless, species identification using trnL based on BLASTN search in NCBI can be accepted as a DNA barcoding requirement because query cover is 100% achieved.

Table 3. Blast analysis result search on NCBI of trnL intron of *S. burahol* and sister species

Code	Samples	Identified	% Query Cover	E Value	Identity
A1	*Stelechocarpus burahol*	*Stelechocarpus burahol*	99%	0.0	99.07%
A2	*Stelechocarpus burahol*	*Stelechocarpus burahol*	99%	0.0	99.63%
A3	*Annona muricata*	*Annona muricata*	100%	6e-132	84.67%
A4	*Annona montana*	*Annona montana*	98%	0.0	96.62%
A5	*Fissistigma latifolium*	*Fissistigma* sp.	99%	0.0	96.78%
A6	*Artabotrys suaveolens*	*Artabotrys suaveolens*	98%	0.0	95.49%
A7	*Annona glabra*	*Annona glabra*	99%	0.0	94.38%
A8	*Desmos chinensis*	*Hiptage benghalensis*	90%	0.0	97.91%
A9	*Magnolia candollii*	*Magnolia liliifera*	98%	0.0	99.43%
A10	*Michelia champaca*	*Michelia champaca*	100%	0.0	99.81%

This study result proved the power of *trn*L intron as DNA barcode therefore recommended for use in plant identification, especially in the Annonaceae family. The *trn*L intron (non coding region) as part of *trn*L-F gene is an informative chloroplast gene and is able to show relationships between species so it is suitable for use in identification at the species level (Lestari et al., 2018). In contrast, due to the high conservation level of trnL intron, it was failed to show variations in their sequence (Siew et al., 2018). To cope this limitation, *trn*L intron (non-coding region) in combination with coding region barcodes from the chloroplast genome such as *rbc*L and *mat*K is suggested. The combined analysis resulted in a better identification and reconstruction of the phylogeny. The phylogenies are commonly used to support data on the evolution of morphological characters, biogeography, and molecular clock (Pirie et al., 2006). Thus, this research still needs further analysis by combining both coding and non-coding sequences.

Conclusions

The *trn*L intron were successfully amplified and sequenced in *S. burahol* and its relative species samples, with high quality value. The average nucleotide length was 532.5 bp and high in AT bases (63.9%). All samples were confirmly identified in accordance to their registered species name in PBG database with query cover 98-100%, except one sample. This study result proved the power of *trn*L intron as DNA barcode therefore recommended for use in plant identification, especially in the Annonaceae family. Further research to use a combination both coding and non-coding regions is suggested to produce a higher sequence length and more effective identification.

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