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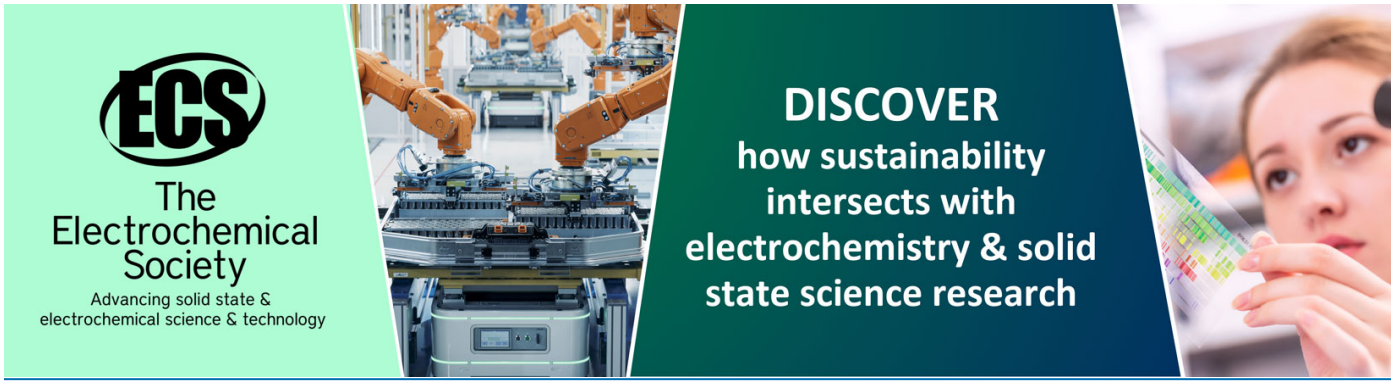

Amplification and Analysis of Rbcl Gene (Ribulose-1,5-Bisphosphate Carboxylase) of Clove in Ternate Island

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Abstract. DNA Barcoding is recommended as a tool for identifying and confirming species within the taxonomy framework. The rbcL gene is the barcode DNA for plant species. Ternate Island is one of clove plantation center in North Maluku. The diversity and productivity of cloves on the Ternate island known since in earlier times. This study has a purpose to amplify the rbcL gene in clove plants collected from clove plantations of communities in Ternate island. Isolation of total DNA carried out with using ZymoBiomic (Zymo Research DNA Extraction) Kit from leaves tissue, then DNA specimen is amplified based on rbcL gene with forwarding sequence rbcLaF 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3' and reverse sequence is rbcLaR 5'-GTA AAA TCA AGT CCA CCR CG-3'. The results showed that the specimen was successfully amplified with an amplicon size of 600 bp. Furthermore, BLASTN analysis results note that the sequence has a similarity of 99% with Syzygium cumini vc. J.R. Abbott 23676 (FLAS) ribulose-1,5-bisphosphate carboxylase large subunit (rbcL) gene, but phylogenetically the precise position of sample could not found because the limitation of rbcL gene.

Keywords: Amplification, rbcL, clove, barcode DNA, Ternate

1. Introduction

North Maluku has the potential for spice plant diversity. Some of the world's spice plants are cloves and nutmeg. Clove commodities in North Maluku occupy the first rank so that the clove is an identity-flora. Ternate is one of the centers of clove plantations in North Maluku besides Tidore, Halmahera, Makian and Moti islands. Clove gardens range from small-scale communities to large-scale clove plantations spread across several of these islands. So far, there is no genetic information about the genetic diversity of clove varieties in Ternate island [1,2].

Genetic diversity in living things occurs through mutation and recombination mechanisms [3]. One approach used to assess genetic diversity is by DNA barcoding [4]. This method can be used to identify species [5] without having expert identification skills in the field [6]. DNA barcoding is usually used for pedigree reconstruction, forensics, and biodiversity surveys [7]. DNA barcoding is one of the methods in molecular level which has an approach to identify new species [8,9]. Last but not least, building a phylogenetic tree is not the main goal of DNA barcoding but to identify the unknown organism [10]. Choroplast DNA (cpDNA) is a region highly conserved and often used in DNA barcodes for plants [11]. Choroplast DNA (cpDNA) has a circular shape with a length between

85-2000 kilobase (kb); this region has a function to control the production of two types of RNA, i.e. tRNA and rRNA, also almost of proteins on chloroplasts organelles. Subunits complex form for photosynthetic protein contain with codes, and one of them is ribulose 1.5-biphosphate carboxylase oxygenase [12]. The cpDNA has several characters that are a stable structure, a small genome with a high conservative region and low substitution of nucleotide [13].

The *rbcL* gene is a part of DNA sequence located in cpDNA and has a chance to be used as a DNA barcode [14–17] because this coding region is given universality and ease in amplifying and analyzing [18]. This gene provides many characters to study phylogenetic because it has full length approximately 1400 bp [19]. This sequence has a low level of mutation compared with other barcodes in cpDNA, and because this sequence has a high level of similarity between species [20]. Low level of mutation is the superiority of the *rbcL* gene. Therefore, in-depth study of intraspecies genetic and phylogenetic variations can be done using this gene. This study aims to amplify the *rbc*L gene in clove plants collected from clove plantations in Ternate island community. Molecular phylogenetic data evidently can solve some taxonomic issues, where with the ways the data are hard to get through [21–26]. Based on the presented background, the research was conducted and the obtained results are expected to be used as a reference to develop cloves plant breeding programs on Ternate Island.

2. Methods

2.1. DNA isolation and amplification of rbcL gene

The leaves and stems of cloves plant were harvested from experimental plants to isolate the total DNA. The total DNA was isolated using DNA Presto TM Mini gDNA kit KIT (Geneid) kit. The PCR was proceeded by MyTag Red Mix (Bioline). Forward primer used in this research was rbcLaF (5'-ATG CCA CAA ACA GAG ACT AAA GC-3') and the reverse primer was rbcLaR (5'-GTA AAA TCA AGT CCA CCR CG-3') with total volume of PCR 30 mL. The PCR program was 95 ^{0}C for denaturation, 55 ^{0}C for annealing, and 72 ^{0}C for extension and 72 ^{0}C for final extension. Zimoclean TM gel DNA recovery KIT (Zimo research) used for purified PCR products. Sequencing was carried out in 1st Base Malaysia services.

2.2. Phylogenetic analysis

Some programs were used to analyze the obtained data. MEGA5 was employed for DNA alignment and also construction of the Phylogenetic tree with Neighbor Joint (NJ) method; BLASTn was utilize to compare the obtained sequence with DNA sequences from GenBank.

3. Results and Discussion

DNA barcoding is one of the ways to fulfill The Barcode of Life Database which aims to collect the reference sequences. This effort used variations of shorts standardized gene regions to identify new species [8]. The first step in DNA barcoding was the isolation of total DNA from the sample. The total DNA of cloves plant was successfully obtained. The next step was measuring the quality and quantity of DNA using DNA spectrophotometer and agarose electrophoresis. The purity and concentration of the obtained DNA are presented in Table 1.

Table 1. The results of DNA isolation

No	Sample name	Conc (ng/mL)	A260/280	A260/230	Volume (µL)
1	MB1	37	1.83	0.17	30
2	SD1	21.7	1.92	0.32	30

The table shows the two samples of DNA isolation of clove plants had 37 ng/µL and 21.7 ng/µL with a purity between 1.8-2.0. The next step was the amplification of DNA using the *rbcL* gene. Genome DNA containing the *rbcL* gene was amplified by polymerase chain reaction (PCR). The

amplification of the *rbcL* gene that had been successfully carried out was tested by electrophoresis (Figure 1).

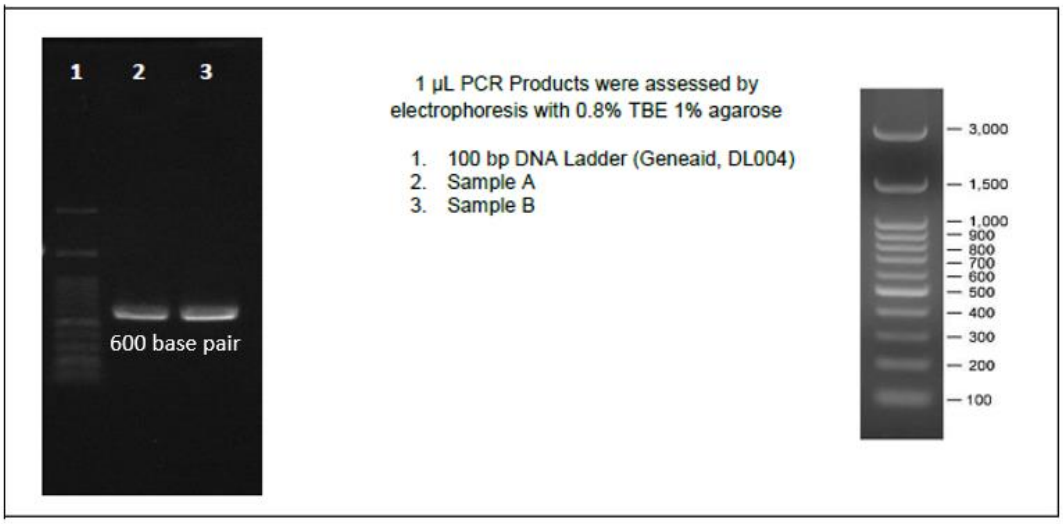

Figure 1. Electropherogram results of the *rbc*L gene amplification in clove plants from Ternate

DNA band obtained from the amplification of the *rbcL* gene was ± 600 bp. The sample of SD1 primer *rbcL* does not proceed to sequence because it cannot be amplified even though PCR repeats have been performed. Furthermore, the BLASTN analysis showed that MB 1 isolates were identical to Syzygium cumini J.R vc. Abbott 23676 (FLAS) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, with an identical value of 99%. The data from the similarity analysis (Blastn) from the MB1 sample are shown in Figure 2 below:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Luma apiculata voucher OMH-A58 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial c	908	908	100%	0.0	99%	KX162972.1
Backhousia citriodora ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast	904	904	100%	0.0	99%	JN676920.1
Acca sellowiana chloroplast, complete genome	902	902	100%	0.0	99%	KX289887.1
Uromyrtus australis ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit (rbcL) gene, partial cds; chloroplast	902	902	100%	0.0	99%	KU761898.1
Campomanesia xanthocarpa voucher UPCB:UFPR Blum10-109 ribulose-1,5-bisphosphate carboxylase/oxygenase large	902	902	100%	0.0	99%	KF561906.1
Campomanesia xanthocarpa voucher UPCB:UFPR Blum10-101 ribulose-1,5-bisphosphate carboxylase/oxygenase large	902	902	100%	0.0	99%	KF561905.1
Campomanesia xanthocarpa voucher UPCB:UFPR Blum10-100 ribulose-1,5-bisphosphate carboxylase/oxygenase large	902	902	100%	0.0	99%	KF561904.1
Syzygium cumini chloroplast, complete genome	902	902	100%	0.0	99%	GQ870669.3
Melaleuca quinquenervia voucher J.R. Abbott 23686 (FLAS) ribulose-1,5-bisphosphate carboxylase/oxygenase large sul	902	902	100%	0.0	99%	GU135164.1
Syzygium cumini voucher J.R. Abbott 23676 (FLAS) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rb	902	902	100%	0.0	99%	GU135161.1

Figure 2. The analysis of BLAST *rbc*L sequence of clove plants

Phylogenetic analysis was carried out to find the species name of the sample. The first step was comparing the sequence of the sample with the data from GenBank using BLASTn method in NCBI. The position in taxon from clove samples could be known from this analysis. The result of the relationship analysis (phylogenetic) of clove samples is presented in Figure 3 below:

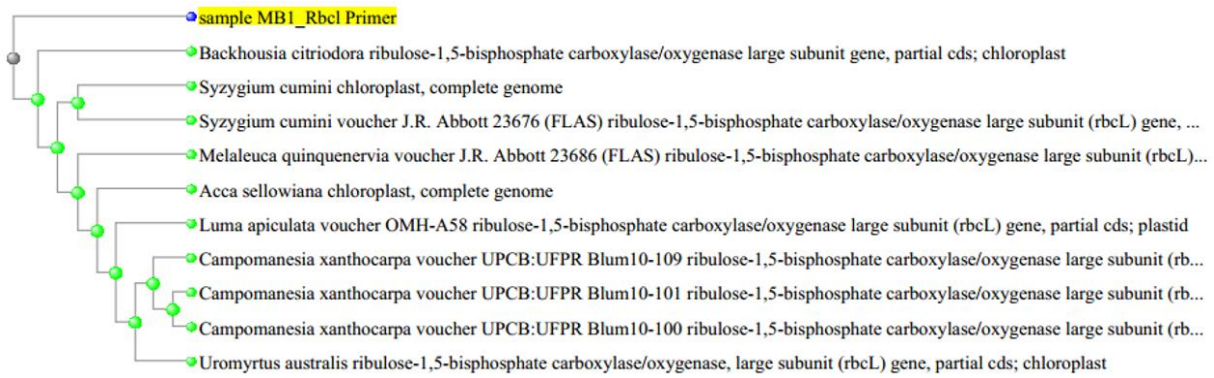

Figure 3: Phylogenetic analysis from a sample of MB 1 Ternate clove plants

Neighbor-Joining (NJ) method in the analysis of phylogenetic can describe the clarity of species identification; the difference is limited by cluster and node. The Sample can be in the same cluster even though they are from different areas [7]. Species relationship based on genetic similarities is shown in the phylogenetic tree. The samples of MB1 clove plants were located in different clusters with *Synzigium cumini* (Figure 3). The results of BLASTn analysis showed the *rbcL* gene clove sample with MB 1 code which had a 99% similarity with *Syzygium cumini* J.R vc. Abbott 23676 showed a distant taxon position. The databases on the *rbc*L gene have been owned by many species, making it easier to compare data analysis [28].

Only MB 1 sample of clove in Ternate that was successful in amplifying using *rbcL* gene. Meanwhile, the DNA band of the *rbcL* gene did not appear on SD 1 sample; this condition might occur because the sample was degraded. The amplification of the *rbcL* gene using one or two universal primer types had a high success rate [19]. Moreover, using the *rbcL* gene rate of success bidirectional (two-way sequencing with forward and reverse primers) sequencing increase higher compared with other barcode gene candidates. The success rate of the *rbc*L gene could reach 100% in 251 plant species with only two primary types [29]. Another researcher recommended using four primers for *rbcL* has a significantly greater length (approximately 1.428 bp) [30]. However, the *rbc*L gene is essential to identify phylogenetic relationship at interfamilial level [31].

cpDNA coded many proteins, and one of them is Ribulose-1,5-biphosphate carboxylase-oxygenase (RuBisCO) which has participated in carbon fixation in photosynthesis [32]. This enzyme contains two kinds of protein subunit called the large chain and the small chain. The large chain gene (*rbcL*) is encoded by cpDNA and has been widely used for analysis of phylogenetic in plant taxonomy [16]. The utilization of the *rbcL* gene to identify in species level was rejected [31,33,34]. The phylogenetic based on the *rbcL* gene is usually used at the generic or higher level or [30,31]. This case was proven in the previous study that used the *rbcL* gene to reveal monophyly in the order of Myrtales [35,36]. It is suggested to use non-coding regions of cpDNA like *tnhpsb5* and *ndITs* to analyze the phylogenetic flowering plants at lower taxonomic levels, not only because this region tends to evolve rapidly rather than coding sequence gene but also this region has a smaller length and thus phylogenetic could be easier to analyze [10,30,31]. Non-coding DNA has a higher number of variable sites when compared with coding DNA, because Non-coding DNA is better to be used in phylogenetic [37] and the single region in the plastid genome do not have variable sequence enough to be used as a barcode gene [38]. Furthermore, the study compared between *ndhF, matK,* and *rbcL* gene showing both *ndhF* and *matK* faster-evolving rather than *rbcL* [39]. Another option besides using non-coding regions in phylogenetic is using combination between *rbcL* + *matK*, because *rbcL* gene is easy in amplifying and analyzing [40] while *matK* is coding sequence located in plastid genome and has a high rate to evolving [41]. This combination performs slightly higher than *rbcL* alone [6].

Comparing the divergence of plastid genomes in *Atropa* and *Nicotina*, resulted in the lowest divergence in plastid genome of *rbcL* gene (0.83%) [30]. Meanwhile, another study compared the species discrimination between 7 leading candidate plastid DNA regions; the result was *rbcL* gene had

58% - 66% of single-locus barcodes range [19]. In addition, another study also compared the use of *rbcL* gene to identify genus and species level, the results showed the percentage of correct identify in genus level was 67.71% and in species level is 16.95% [18]. Therefore, the *rbcL* gene had been knocked off for discrimination in the level of species [30].

4. Conclusion

The *rbcL* gene that had been successfully amplified from one sample of clove plant in this study, had a length of 600 bp. The sequence had a similarity of 99% with JR's voucher *Syzygium cumini*. The exact species could not be found because the *rbcL* gene could be identified at the generic level or higher.

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