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ANALYSIS OF NEW PRIMER PAIR CANDIDATES OF rbcL GENE FOR IDENTIFICATION OF MICROALGAE SCENEDESMACEAE

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Abstract

Scenedesmaceae is one of the microalgae groups that has been widely studied as promising biodiesel feedstock. Its morphological identification is often confused by environmental changes, so it requires molecular identification as well. The current study aimed to obtain primer pair candidates that identify the Scenedesmaceae based on the rbcL gene. The research used bioinformatics tools, which harvested rbcL protein sequence data, performed multiple sequence alignments, and designed primers based on conserved and less-conserved regions. The best left and right primers selected based on sequence length, melting temperature, 3' end stability, number of hairpins, and self-dimers, were paired, and three candidates were obtained. The three pairs were examined based on melting temperature difference, number of hetero-dimers, length of amplified nucleotide product, number of hits, and number of genera captured from the GenBank. Sce-16 (F, 5'-TGGTCGTGCTGTTTATGAATGT-3' and 1_RL, 5'-TGCCAAACATGAATACCACCA-3'), which is back-translated according to Hariotina sp. (AOY36008.1), is the most preferred candidate compared to the other two pairs after discussing their advantages and disadvantages. In the future, the proposed primer candidate needs to be validated through in vitro amplification with some optimizations to eliminate potential weaknesses.

Keywords*: rbcL gene; Scenesdesmaceae; Primer design; Molecular identification*

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INTRODUCTION

Microalgae is a source of biofuel that attracts global attention because it is a renewable fuel source with high-yield prospects. Its production can save land use, and coupling biofuel production with carbondioxide mitigation strategies and wastewater treatment (Shuba & Kifle, 2018; Rukminasari et al, 2021). Scenedesmaceae are among the microalgae with high potential as a source of lipids for biofuels. *Scenedesmus* is one member that contains up to 55% (w/v) lipid (Mata et al., 2010) and produces up to 39 mg L^{-1} d^{-1} (Mandotra et al., 2016).

The microalgae diversity is estimated at 200,000-800,000, which has not been fully described (Cheng & Ogden, 2011). Identification of organisms, including microlgae, is needed to determine their properties based on literature. Identification of microalgae is generally done by observing their cell morphology, and molecular method by comparing their genes. Microalgae morphology is partly determined by environmental conditions. *Scenedesmus* can build coenobia consisting of 1, 2, 4, and 8 cells as a form of adaptation to environmental changes. This polymorphism can be a marker of other properties, such as the number of lipids, proteins, and other substances (Giraldo-Zuluaga et al., 2018). However, the variable morphology makes it difficult to identify the species.

Molecular identification becomes a complement to morphological identification because it is not related to phenotypic changes by environmental conditions. Polymerase chain reaction (PCR) is one of the most useful molecular methods for the identification of microalgae on a small scale (Ebenezer et al., 2012). By PCR-amplifying particular genes, ~~a~~ microalgae can be identified by its genus or species. *rbc*L (ribulose-15-bisphosphate carboxylase/oxygenase large) is a gene in the chloroplast that is often used as a marker in the identification of algae and plants. In addition to producing a perfect match with a sequence from reference libraries associated with a single species (Manoylov, 2014), this gene is deposited a lot (>360,000) in GenBank NCBI so that it can be used as a comparison reference.

PCR requires a pair of primers that start the process of amplifying DNA fragments from the left and right of the fragment. The *rbc*L sequence data which includes non-microalgae may also cause a lack of specificity in detecting this group, especially those from Scenedesmaceae.

Thus, the available primers from existing publications have not been specifically used for Scenedesmaceae. Therefore, by designing primers based on the alignment of conserved regions of available *rbc*L gene sequences from Scenedesmaceae members, the current study aimed to obtain suitable primer pairs to identify as many genera as possible that belong to the family.

RESEARCH METHODS

Materials

The material used in this research is some tools for bioinformatics, namely MEGA11, GeneDoc, Primer3Plus, and OligoAnalyzer. The sequence used was obtained from GenBank, The National Center for Biotechnology Information (NCBI).

Methods

Protein sequences of Scenedesmaceae *rbc*L were harvested in NCBI, and their amino acids were aligned in MEGA11. The alignment result was displayed in GeneDoc, then conserved regions (black to dark gray) and less conserved (light gray), both on the left end (for forward primers) and right end (for reverse primers) were observed. The sequences with a minimum of 6 consecutive amino acids were taken and recorded. The sequence number for the species having the longest sequence was opened in the NCBI protein. The amino acid sequences were back-translated to nucleotide sequences (codons) according to the instruction in NCBI (Table 11 Bacteria, Archaea, Chloroplast) /transl_table=11), using https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/ by selecting *Anabaena* sp. as codon usage table. The translation results were fed to Primer3Plus to obtain possible primers. The back-translation results were re-selected by using Primer3Plus. The default setting of %GC and melting temperature (Tm) was set to narrow down the results. The selection was carried out in stages and explained in the discussion. Each pair of primer candidates was analyzed for %GC, Tm, hairpin potential, self-dimer, heterodimer, etc., using Primer3Plus and OligoAnalyzer https://sg.idtdna.com/calc/analyzer. The new primer candidates were also examined in Primer-BLAST NCBI. In analyzing data, the new primer candidates were compared with each other to select the best primer pair based on the smallest potential weakness and the ability to capture the most Scenedesmaceae.

RESULTS AND DISCUSSION

Results on Retrieving of the Scenedesmaceae *rbc*L Protein Sequence

The search for the protein *rbc*L which belongs to Scenedesmaceae in Protein NCBI as of 9 Agustus 2022 yielded 261 data. About 72 selected sequences were harvested, representing 16 genera (Table1).

Table 1. Genera members of Scenedesmaceae with recorded sequence of *rbc*L (ribulose-15-bisphosphate carboxylase/oxygenase large) subunit partial (chloroplast) in NCBI as of August 9, 2022

No.	Genera with available *rbc*L data	Number of classified species	No.	Genera with unavailable *rbc*L data	Number of classified species
1.	*Acutodesmus*	1	1.	*Coelastrela*	13
2.	*Asterarcys*	2	2.	*Coelastropsis*	1
3.	*Chodatodesmus*	2	3.	*Coelastrum*	8
4.	*Comasiella*	1	4.	*Coronastrum*	1
5.	*Crucigenia*	4	5.	*Didymocystis*	1
6.	*Desmodesmus*	47	6.	*Dimorphococcus*	1
7.	*Enallax*	2	7.	*Hylodesmus*	1
8.	*Flechtneria*	1	8.	*Pectodictyon*	1
9.	*Halochlorella*	1	9.	*Pediludiella*	1
10.	*Hariotina*	6	10.	*Pseudodidymocystis*	2
11.	*Komarekia*	2	11.	*Pseudospongiococcum*	1
12.	*Neodesmus*	3	12.	*Tetranephris*	1
13.	*Pectinodesmus*	3	13.	*Verrucodesmus*	2
14.	*Scenedesmus*	23			
15.	*Tetradesmus*	14			
16.	*Westella*	1			
	Total species	**113**		**Total species**	**34**

Meanwhile, a search with Taxonomy NCBI showed that at least 29 genera were recorded as members of the Scenedesmaceae. Sixteen genera described 55% of the Scenedesmaceae members with known *rbc*L protein sequences, while the other 13 genera (45%) have no available information. However, genera for which protein sequences comprising 113 species are considered representative of other genera, compared to 34 species for which *rbc*L data are not available. Those numbers do not include unclassified species. In addition, 16 genera or 113 species harvested by *rbc*L data represented 37% of genera or 33% of species belonging to Scenedesmaceae recorded in AlgaeBase. According to AlgaeBase as of August 9, 2022, the recorded Scenedesmaceae consisted of 349 species spread across at least 43 genera. Therefore, the harvested data are considered to be fairly

representative of the unavailable *rbc*L sequences from other genera of Scenedesmaceae. These search result confirm that *rbc*L is a promising marker, which has been revealed by several researchers that it can be a barcode for green algae (Hadi et al., 2016). The availability of sequence libraries is used to determine the conserved and variable regions to design universal primers (Manoylov, 2014).

Results on Alignment of the Scenedesmaceae *rbc*L Protein Sequence

The alignment results showed that the *rbc*L protein sequence data of Scenedesmaceae deposited in NCBI were not the same among the species. However, the alignment results showed that the most conserved region was mostly in the middle region marked by dark gray color at amino acid positions number 170s-370s shown using GeneDoc (Figure 1). No regions are completely conserved (dark or black colors are found). However, several variable regions (light gray) were distributed among the conserved regions. The design of universal primers, as in the history of SSU rDNA sequences, is facilitated by the presence of conserved and variable regions that allow phylogenetic studies (Manoylov, 2014). ; Roslim et al., 2020; Agustina & Roslim, 2021).

The center region of the protein is of concern to obtaining candidates for the primary pair. The concentration of the most conserved regions in the middle, not at the left nor right ends of the *rbc*L protein. The length of the PCR product that can be produced is about 600 bases, predicting that the DNA sequence encoding the *rbc*L would be produced was incomplete. But it is not considered as a big problem for identification purposes, as long as the amplified region is targeted, i.e. it can distinguish between species as accurately as possible. The length of this PCR product resembles that of amplification by other *rbc*L primer pair used in other Chlorophyte (Fitriyah et al., 2021; Hadi et al., 2016; Yanuhar et al., 2019), although the complete length of the gene is 1400 bp.

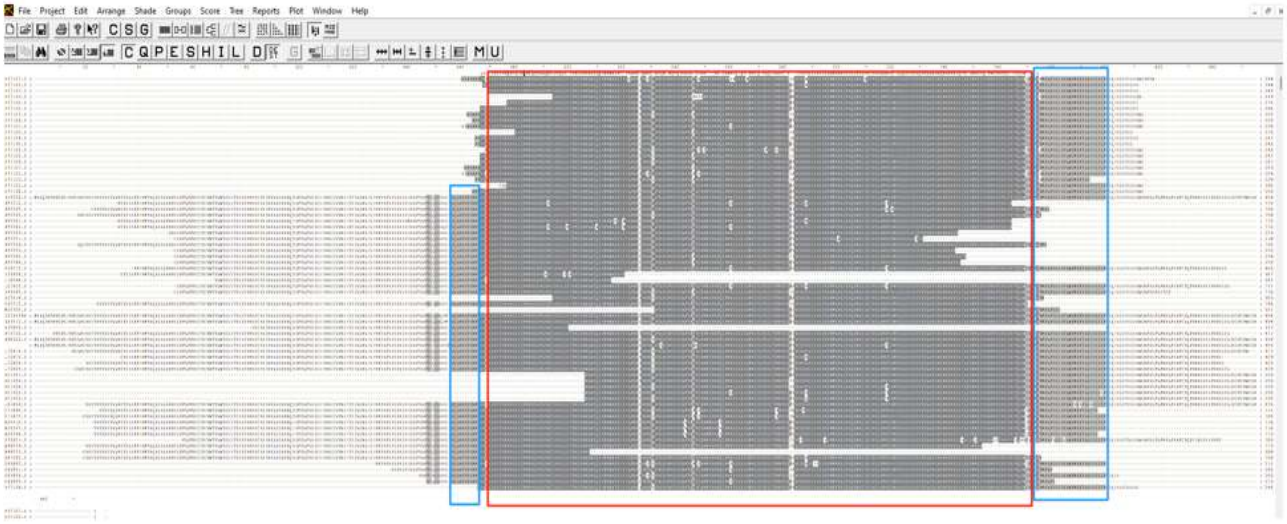

Figure 1. GeneDoc display of the alignment results of Scenedesmaceae *rbc*L protein sequences showing conserved (darker gray, red box) and variable (lighter gray, blue box) regions

Table 2. Amino acid sequences of *rbc*L protein of Scenedesmaceae and their back-translated results targeted for searching candidates of primer pairs

Region		Amino acid sequence	Sequence number*	Back-translated sequences**
Conserved region	Left	LGCTIKPKLGLSAKNY GRAVYECLRGGL	170-198	TTAGGTTGTACTATTAAACCAAAATTAGG TTTATCTGCTAAAAATTATGGTCGTGCTG TTTATGAATGTTTACGTGGTGGTTTA
	Right	EKDRSRGIYFTGDW	355-369	GAAAAAGATCGTTCTCGTGGTATTTATTT TACTGGTGATTGG
Less conserved region	Left	ICVERDKLNKYGRG	155-169	ATTCAAGTTGAACGTGATAAATTAAATAA ATATGGTCGTGGT
	Right	MEVASGGIHVWHMP AIVEIFGDDA	375-399	ATTCAAGTTGAACGTGATAAATTAAATAA ATATGGTCGTGGT

*according to *Hariotina* sp. MMOGRB0030F (AOY36008.1)
**according to *Anabaena* codon

The alignment results also showed that there were less conserved regions at the left (no.160s) and right (no.390s) ends (Figure 1, signed by the blue box). As a precaution not to waste more region, the regions were also targeted to obtain candidates for primer pair. Therefore, there are two regions of concern as candidates, the conserved region and the non-conserved region so that the amino acid sequences were retrieved from them (Table 2). The amino acid numbers follow the longest (most complete) sequence of the aligned species, namely AOY36008.1 ribulose-15-bisphosphate carboxylase/ oxygenase large subunit (chloroplast) *Hariotina* sp. MMOGRB0030F. The rbcL protein has been knew tend to be more variable, but still has the advantage of avoiding contamination by microorganisms that do not have chloroplasts (Krienitz & Bock, 2012).

Analysis Result of the Primer Pairs Candidates

Selecting primers using Primer3Plus on the back-translated sequences resulted in several candidate primers. By setting %GC min.40 and Tm min.55°C, 9 left and 11 right primer candidates were obtained from conserved and less-conserved regions. No right primers were generated from the less-conserved regions with such settings, because the back-translated sequences are mostly AT bases (Table 2). And then to save on selection, the best five primers of each right and left, were taken based on the penalty values. Primer3Plus calculates the penalty value based on the percentage of max.%GC, min. Tm, possible product length, and non-redundancy. The primers were then sorted by penalties, with the lowest penalty defining the best primer (Table 3).

The 3’ stability is the maximum stability for the last five 3' bases of a left or right primer. Bigger numbers mean more stable 3' ends. The value is the maximum ΔG (kcal.mole^{-1}) for duplex disruption for the five 3' bases as calculated using the nearest-neighbor parameter values by Primer3Plus. In the ten primers, the number of GC bases at the 3' end does not exceed 3-5 bases (Sasmito et al., 2014; Utomo et al., 2019), so they are still good candidates for primers.

However, the results of primary selection based on %GC and Tm contain weaknesses in the form of hairpin and self-dimer formation, which are a consequence of variations in the sequence of nitrogen bases (Table 3). Hairpins and self-dimers are difficult to avoid, so the primer selection is taken with the least risk. All of the ten primers had potency in forming hairpins mostly by $\Delta G<2$ kcal.mole^{-1}, which is within tolerable values (Sasmito et al., 2014). Similarly, in the number of self-dimers, mostly only 2 base pairs occurs, although some of them are 4 base pairs. The potential for hairpin and self-dimer must be minimized through PCR mixture as often suggested by various PCR protocols.

Table 3. Analysis result of the primer pairs candidates by Primer3Plus (set: %GC min.40; Tm min.55)* and IDT OligoAnalyzer**. Only the best five primers of each left and right are displayed.

Left (F) and right (R) primers	Sequence (5' to 3')	Length (nt)	Tm (°C)*	GC (%)*	3' stability, ΔG (kcal.mole^{-1})*	Penalty*	No. of hairpins**	No. of self-dimers**
F	TGGTCG TGCTGT TTATGA ATGT	22	58.6	40.9	2.7	3.408	8, mostly ΔG<2 kcal.mole^{-1}	8; 2 bp, ΔG<-4 kcal.mole^{-1}
1_F	TGGTCG TGCTGT TTATGA ATG	21	56.9	42.9	2.7	4.101	8, mostly ΔG<2 kcal.mole^{-1}	8; 2 bp, ΔG<-4 kcal.mole^{-1}
2_F	GGTCGT GCTGTT TATGAA TGT	21	56.9	42.9	2.7	4.103	8, mostly ΔG<2 kcal.mole^{-1}	8; 2 bp, ΔG<-4 kcal.mole^{-1}
3_F	GGTCGT GCTGTT TATGAA TGTT	22	57.6	40.9	2.7	4.420	8, mostly ΔG<2 kcal.mole^{-1}	9; 2 bp, ΔG<-4 kcal.mole^{-1}
4_F	TGAATG TTTACG TGGTGG TT	20	55.5	40.0	3.7	4.500	7, with ΔG<2 kcal.mole^{-1}	8; 1 of them 4 bp, ΔG=-6 kcal.mole^{-1}
R	ACCACG AGAACG ATCTTT TTC	21	56.6	42.9	2.3	4.421	1, ΔG= -1.8 kcal.mole^{-1}	9; 1 of them 4 bp, ΔG=-4 kcal.mole^{-1}
2_R	TACCAC GAGAAC GATCTT TTTC	22	56.5	40.9	2.3	5.503	1, ΔG= -1.8 kcal.mole^{-1}	10; 1 of them 4 bp, ΔG=-4 kcal.mole^{-1}
RL	TGCCAA ACATGA ATACCA CCAG	22	59.2	45.5	4.0	2.825	5, with ΔG<2 kcal.mole^{-1}	11; 1 of them 4 bp, ΔG=-5 kcal.mole^{-1}
1_RL	TGCCAA ACATGA ATACCA CCA	21	58.1	42.9	4.2	2.882	5, with ΔG<2 kcal.mole^{-1}	11; 1 of them 4 bp, ΔG=-5 kcal.mole^{-1}
2_RL	GCCAAA CATGAA TACCAC CAGA	22	58.9	45.5	3.9	3.089	3, with ΔG<2 kcal.mole^{-1}	8; 1 of them 4 bp, ΔG=-5 kcal.mole^{-1}

The pairing of the tenth primer produced 25 primer pairs named Sce-1—Sce-25 which had been analyzed for the Tm difference and the number of hetero-dimers that might occur. However, the Tm difference between the left and right primers varied from 0.3—3.7°C, so they have been selected again by 0.3—0.5°C of Tm difference. The selection presented 3 candidates of primers with lower

differences between primers after excluding those having more heterodimers for the same Tm difference (Table 4). Sce-2 and Sce-3 have the same Tm difference and hetero-dimer, but Sce-2 was chosen because the 3' end of the primer 1_F (Sce-2) is G, not T as in 2_F (Sce-3) (Table 3). The same reason applies to the selected Sce-7 instead of Sce-8. The nucleotide base at the 3' end of the primer should be G or C to avoid a mismatch of A or T (Utomo et al., 2019).

Table 4. Primer Pair Candidates with Tm difference and Hetero-dimer Analyzed by IDT OligoAnalyzer

Name of primer pair	Left (F) and right (R) primers	Tm difference	Hetero-dimer (no., max. ΔG)
Sce-2	1_F and R	0.3	19, -8
Sce-7	1_F and 2_R	0.4	19, -8
Sce-16	F and 1_RL	0.5	25, -7

Results of Scenedesmaceae Trapped Using the Primer Candidates

The three candidates of primer pairs captures 14 genera (Table 5). Thirteen (81.3%) or most of them were part of the 16 genera possessing the *rbc*L gene (Table 1) that were retrieved for primer design. One other genus, *Coelastrela*, which was captured by the primers was not the genus used to design the primer. The genera not trapped by the three primers were *Chodatodesmus, Halochlorella,* and *Neodesmus*. It was presumably because their *rbc*L gene sequences deposited were too short and did not include the conserved regions being observed.

Table 5. Scenedesmaceae trapped using the primer candidates by using Primer-BLAST

Name of primer pair	Product length (bp)	Number of hits trapped	*Number of genera*
Sce-2	529	109	14
Sce-7	529	109	14
Sce-16	603	67	14

Sce-2 and Sce-7, resulted in the same length of PCR product, number of hits, and genera (Table 5), indicating that the addition of base T at the 5' end of the primer 2_R did not affect the yield. However, primer 2_R (in Sce-7) has a higher %GC and less self-dimer, so in this context, Sce-7 is preferred over Sce-2 (Table 3). Meanwhile, Sce-16 produced a longer product (603 bp) than that of Sce-2 and Sce-7, by the length of the partial *rbc*L gene containing conserved regions.

The lower number of hits from Sce-16 does not reduce the quality of gain because the number of genera remains the same as that of the others. The potential for hetero-dimers between the constituent primers of Sce-16 was greater than that of the others (Table 4). However, most of the pairing that occurs is 2 base pairs, and only one cross-dimer occurs at the 3' by ΔG= -1.34 kcal.mole^{-1}. The cross-dimer at the 3' end withΔG= -5 kcal.mole^{-1} was tolerated (Sasmito et al., 2014). Regardless, in applying the primers PCR reaction needs special modification of conditions and mixtures to eliminate the hetero-dimers.

CONCLUSION

The candidate of primer pairs proposed to capture Scenedesmaceae based on the conserved and less-conserved region of *rbc*L genes is Sce-16 (F, 5'-TGGTCGTGCTGTTTATGAATGT-3' and 1_RL, 5'-TGCCAAACATGAATACCACCA-3'). The use of the primers has to be accompanied by observations of cell morphology which led to the initial assumption that the identified specimen belongs to the Scenedesmaceae group. In addition, both require validation in real PCR reactions, by the right conditions and mixtures, as well as product verification by sequencing. The resulting primer pairs were selected according to the conserved and less-conserved regions of Scenedesmaceae, not yet taken from the non-Scenedesmaceae *rbc*L gene. Therefore, in the future ~~in order~~ to obtain primers that are specific for capturing only Scenedesmaceae *rbc*L and as few as possible of non-Scenedesmaceae, it is necessary to align them with broader representatives of *rbc*L at least in the Chlorophyta group.

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