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Optimization of PCR Analysis Based on Start Codon Targeted Markers (SCoT Markers) for Identification of Genetic Variation of Seaweed

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Abstract

Seaweed is a fishery commodity that has high economic value because it contains carrageenan which can be used in the pharmaceutical, food, cosmetics, and industrial fields. Central Sulawesi Province is the second largest seaweed producer in Indonesia after South Sulawesi Province. The genetic diversity of seaweed in Central Sulawesi has not been studied much. Therefore, molecular-based characterization activities to identify the genetic variation of seaweed in the province are urgently needed. The purpose of the study was to optimize PCR techniques based on targeted start codon markers (SCoT markers) to identify the genetic diversity of seaweed accessions from Central Sulawesi Province. The results showed that the parameters for seaweed PCR amplification have been optimized. Of the fifteen SCoT primers, 10 of them can work to amplify seaweed DNA collected from several regions in Central Sulawesi. This is shown by the production of PCR result amplicons. PCR results also indicate polymorphism from the seaweed samples tested, although some seaweed samples have not been successfully amplified. The parameters of the PCR technique still need to be improved, especially the concentration of DNA prints, so that valid PCR results will be obtained for use in identifying seaweed genetic diversity.

Introduction

Seaweed (Kappaphycus alvarezii Doty) is one of the marine products that is widely consumed by the people of Indonesia and seaweed cultivation is quite in demand because of its high economic value. Of the approximately 8,000 thousand species of seaweed in the world, about 555 of them grow well in Indonesian waters. Seaweed is one of the red algae species of the genus Gigartinales (Rhodophyta) and also one of the leading commodities of the Indonesian fisheries and marine industry because it produces carrageenan which has high economic value. Carrageenan has many uses in pharmaceuticals, food, and cosmetics as well as industrial fields as stabilizers, thickeners, gellers, and emulsifiers.

Seaweed with superior properties of the type of Kappaphycus alvarezii, contains carrageenan and has the highest price, this seaweed is threatened with extinction due to mining waste from 2012 until now (Diskanlut, 2022). On the other hand, Gracilaria sp seaweed, contains agar, lives in brackish, and has a relatively cheap price. Central Sulawesi Province, especially Morowali Regency, is ranked second as Indonesia's largest seaweed producer after South Sulawesi Province (Utojo et al., 2016). This is supported by the availability of resources, namely large cultivated land considering the length of the coastline which is 4,013 Km as a

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potential seaweed development area owned by Central Sulawesi Province (Moore &; Ndobe, 2008). The availability of seaweed cultivation land is not comparable to its utilization which has only reached 7%, thus causing the need for utilization efforts to increase Central Sulawesi seaweed production (Ya'la, 2008). In the process of seaweed development in Central Sulawesi where the determination of seaweed as a strategic leading commodity of aquaculture in Central Sulawesi Province is due to the advantages it has compared to other commodities such as a very large amount of production. Currently, seaweed accessions in Central Sulawesi Province are not yet known for genetic diversity. Therefore, molecular-based characterization activities to identify the genetic variation of seaweed in Morowali are urgently needed.

As is known, genetic diversity is one of the important components in seaweed breeding activities. Through the collection of various types of seaweed germplasm, breeders can assemble new superior clones that have better character. Genetic diversity provides information for breeders to develop new high-yielding varieties/clones with desired characteristics (Govindaraj et al., 2015). Seaweed accessions in Morowali need to be characterized and evaluated for genetic diversity to determine the potential of each accession and prevent duplication of collections (Singh et al., 2019). In addition, the genetic diversity information obtained can be used by breeders to determine future plant breeding programs. Analysis of genetic diversity can be performed using morphological or agronomic, biochemical, and molecular markers. Genetic diversity analysis using PCR-based molecular markers has several advantages, including being able to be carried out in early stadia without the need to wait for plants to mature, not being influenced by the environment, being able to distinguish accessions with very close kinship to the nucleotide level, high reproducibility, automated, and having a wider genome coverage area (Nadeem et al., 2018).

Advances in the field of genomics research have resulted in the development of new markers directed at specific genes. Currently, research has applied a new and simple marking system, namely start codon targeted polymorphism (SCoT). The new markers developed from a short, conservative region flanking the early ATG translational codons of the gene. This molecular marker is very likely to be a potential marker in onion plants. This SCoT mark has been applied to several commodities, including coconut (Rajesh et al., 2015), rice (Collard & Mackill, 2009), potato (Gorji et al., 2011), and sugarcane (Que et al., 2014). In this study, we will optimize SCoT markers to identify genetic diversity in seaweed accession collections and it is hoped that specific markers of important genes in seaweed will be obtained for seaweed genetic improvement activities, one of which is genome editing.

The purpose of the study was to optimize PCR analysis based on targeted codon start markers (SCoT markers) for the identification of genetic variation in seaweed accession from Central Sulawesi Province.

Methods

The sampling location was in 2 seaweed-producing districts, namely Donggala Regency and Morowali Regency. A sampling of 9 station points in Donggala Regency and 7 station points in Morowali Regency. A total of 28 seaweed samples collected from several seaweed production areas in Central Sulawesi Province, were used in this study (Table 1).

Table 1. Seaweed Samples Used For Research

Sample Code	Description
1A	*Gracilaria 38isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 1
1B	*Gracilaria 38isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 2
1C	*Gracilaria 38isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 3

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Sample Code	Description
1D	*Gracilaria 39isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 4
1E	*Gracilaria 39isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 5
1F	*Gracilaria 39isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 6
1G	*Gracilaria 39isulfate*, Kab. Morowali Utara, Desa Bungintimbe, Station 7
2A	*Padina* sp., Kab. Donggala, Desa Dondo, Station 1
2B	*Padina* sp., Kab. Donggala, Desa Dondo, Station 2
2C	*Padina* sp., Kab. Donggala, Desa Dondo, Station 3
2D	*Padina* sp., Kab. Donggala, Kelurahan Kabonga Besar, Station 1
2E	*Padina* sp., Kab. Donggala, Kelurahan Kabonga Besar, Station 2
2F	*Padina* sp., Kab. Donggala, Kelurahan Kabonga Besar, Station 3
3A	*Ulva* sp., Kab. Donggala, Desa Labuan Badjo, Station 1
3B	*Ulva* sp., Kab. Donggala, Desa Labuan Badjo, Station 2
3C	*Ulva* sp. Kab. Donggala, Desa Labuan Badjo, Station 3
W1K	
W1	
W2	
W3	
DGLA	
DGLB	
DP	
DK	
G	
E1	
E2	
E3	

Genomic DNA Isolation

Total genomic DNA was isolated using the method developed by (Doyle, 1990) modified by the addition of mercaptoethanol. A total of 0.4 grams of seaweed tissue was crushed in a mortar using 500 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% (w/v) CTAB (cetyltrimethylammonium bromide), 2% (w/v) PVP (polyvinylpyrrolidone), and 0.38% (w/v) sodium 39isulfate). The results of the scouring were then put into a 2 ml micro tube and added back the extraction buffer to a final volume of 1 ml and 2 µl β-mercaptoethanol. The samples were then incubated at 65 oC for 15 minutes in a waterbath. The sample was flipped every 5 minutes to make it homogeneous. The sample was then added 800 µl of chloroform: isoamyl alcohol (24:1) solution, and centrifuged at 12,000 rpm for 10 min at 20 oC. The supernatant formed was transferred into a new micro-tube. This extraction step was repeated once more to eliminate contaminant compounds more optimally. Next, the sample was added with 3M sodium acetate pH 5.2 as much as 1/10 of the supernatant volume and cold isopropanol as much as one time the supernatant volume. The mixture was then incubated at -20 oC for one hour and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet formed was washed using 70% ethanol solution as much as 500 µl. The pellet was then dried and re-dissolved in 100 µl of TE solution (10 mM Tris pH 8.0 and 1 mM EDTA) to which RNAse A enzyme (10 mg/ml) had been added. Furthermore, the stock DNA solution was incubated at 37 oC for 1 hour and then stored at -20 oC until the DNA was ready for use.

DNA Amplification and Agarose Gel Electrophoresis

DNA from each seaweed sample was amplified by PCR technique in a total reaction of 10 µl containing 10 ng µl-1 mold DNA as much as 2 µl; 2x MyTaq HS (Bioline, UK) as much as 5

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µl; forward and reverse primers with a concentration of 10 µM each as much as 0.5 µl, and sterile ddH2O. DNA amplification was performed using 18 pairs of ScoT primers with sequence sequences as presented in Table 2. PCR reactions were performed on a Thermocycler machine (Bio Rad, USA) with the following PCR profile: Initial denaturation was performed at 95 oC for 5 minutes, followed by 35 cycles of denaturation process at 94 oC for 30 seconds, primer attachment stage at 50-55 oC for 1 minute, and base elongation stage at 72 oC for 1 minute. The PCR reaction ends with a final base elongation step at 60 oC for 15 minutes.

PCR products were electrophoresed on a 2% agarose gel in a tank containing 1x TAE buffer at 90 V for 65 min. The agarose gel was then stained with ethidium bromide dye (10 mg ml-1) and visualized under UV light using a UV Transilluminator (Bio Rad, USA).

Tabel 2. ScoT primers are used for PCR optimization (Collard & Mackill, 2009)

Primary Name	Sequence (5' – 3')
SCoT-1	CAACAATGGCTACCACCA
SCoT-2	CAACAATGGCTACCACCC
SCoT-3	CAACAATGGCTACCACCG
SCoT-4	CAACAATGGCTACCACCT
SCoT-5	CAACAATGGCTACCACGA
SCoT-6	CAACAATGGCTACCACGC
SCoT-7	CAACAATGGCTACCACGG
SCoT-8	CAACAATGGCTACCACGT
SCoT-9	CAACAATGGCTACCAGCA
SCoT-13	ACGACATGGCGACCATCG
SCoT-26	ACCATGGCTACCACCGTC
SCoT-28	CCATGGCTACCACCGCCA
SCoT-29	CCATGGCTACCACCGGCC
SCoT-30	CCATGGCTACCACCGGCG
SCoT-31	CCATGGCTACCACCGCCT
SCoT-32	CCATGGCTACCACCGCAC
SCoT-35	CATGGCTACCACCGGCCC
SCoT-36	CATGGCTACCACCGGCCC

Data Analysis

The results of agarose gel visualization were then analyzed for the success of the primers used to produce DNA bands from PCR amplification. Primers that work well will produce DNA bands from amplified samples. In addition, the results of PCR amplification can also be scored as binary data. Each visualized amplified band is considered as one allele. Bands that have the same rate of movement are considered to be the same locus. Each visible band was given a score of 1, invisible bands were given a score of 0, and samples that did not produce amplicons were scored 999 and considered missing data. Furthermore, the scoring data were analyzed using PowerMarker 3.25 software (Liu & Muse 2005) to determine the value of the main allele frequency, genetic diversity, heterozygosity, and PIC (Polymorphic Information Content) generated by microsatellite markers and SCoT used in this study. Principal coordinate analysis (PCoA) and phylogenetic tree construction were performed using the Neighbor-joining program on DARwin software version 6.0 (Perrier & Jacquemoud-Collet 2006).

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Results and Discussion

Identifying the genetic diversity of seaweed accessions collected from several areas in Central Sulawesi Province is useful in determining the level of genetic variation of these samples. This information is very important in the development of superior seaweed in the future. In addition, genetic diversity analysis is also useful for detecting the extinction rate of existing seaweeds. Genetic diversity analysis can be done with molecular techniques through genomic DNA amplification of seaweed samples. The advantages of this technique are that the analysis is not influenced by the environment, can distinguish accessions with very close kinship relationships to the nucleotide level, has high reproducibility, and can read variations in a wider genomic area (Nadeem et al., 2018).

In this study, the genetic diversity of seaweed samples was detected with a molecular marker, the SCoT marker. The use of this marker in seaweed has never been done. Therefore, the use of this marker in seaweed requires optimization so that valid identification results can be obtained. Optimization of PCR amplification techniques also needs to be done to streamline the time and use of chemicals so that the detection process can be done quickly and precisely. The results of PCR technique optimization in this study showed that of the 18 SCoT markers analyzed against 28 seaweed samples, 10 SCoT markers provide DNA amplification results indicated by the formation of DNA bands (Figures 1 - 10, Table 3). SCoT markers that worked well in this optimization activity were SCoT-1, SCoT-8, SCoT-9, SCoT-13, SCoT-26, SCoT-28, SCoT-30, SCoT-31, SCoT-35, and SCoT-36. Meanwhile, 8 other SCoT markers did not produce DNA bands in the seaweed samples tested.

The PCR optimization results also showed that, although the SCoT markers were able to amplify seaweed DNA samples, each SCoT marker did not successfully amplify all 28 seaweed samples tested. No SCoT markers produced DNA bands in all samples (Table 3). In addition, two SCoT markers also showed amplification results that were not so good and clear and only managed to amplify some seaweed samples, namely SCoT-8 and SCoT-9 (Table 3). Information related to the formation or absence of DNA bands from PCR amplification with 10 SCoT primers on 28 seaweed samples is shown in Table 4. Of the 10 SCoT markers used, 2 markers can produce DNA bands in more than 90% of the samples tested, namely markers SCoT-28 and SCoT-30. Using these two markers, of the 28 samples analyzed by PCR, 26 samples were successfully amplified and produced DNA bands and 2 samples did not produce DNA bands (Table 4). In this optimization, we also obtained information that there were samples that produced DNA bands on only 1 or 2 SCoT markers (less than 20% occurrence), namely samples 2A, 2E, and E2.

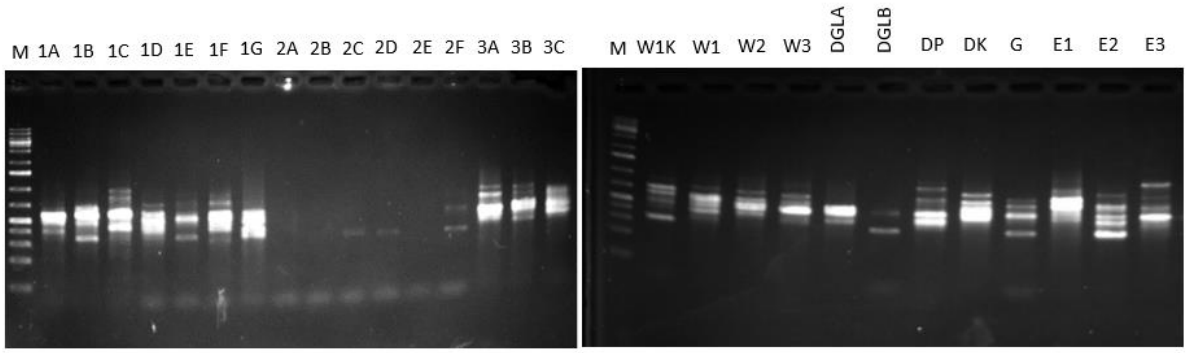

Figure 1. Electrophoregram of PCR amplification results from 28 seaweed samples with primer SCoT-1

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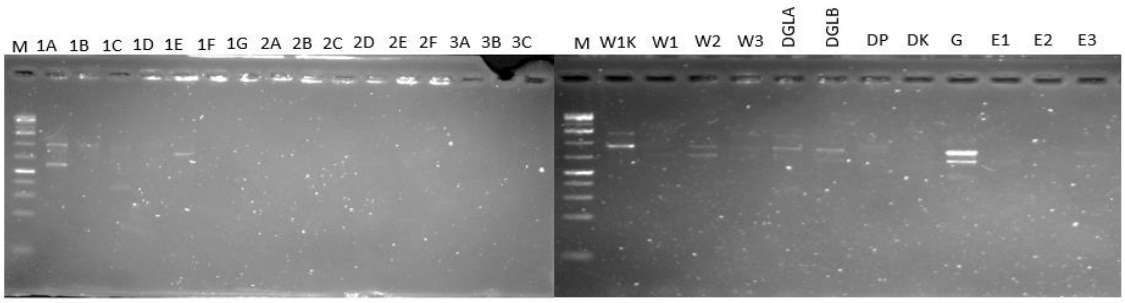

Figure 2. Electrophoregram of PCR amplification results of 28 seaweed samples with SCoT-8 primers

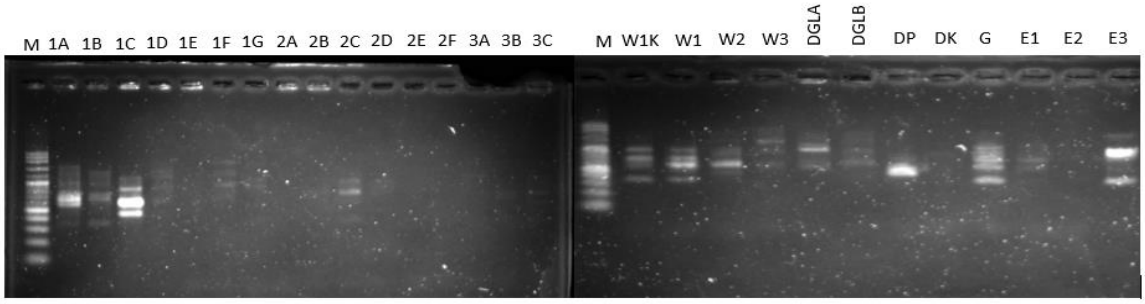

Figure 3. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-9

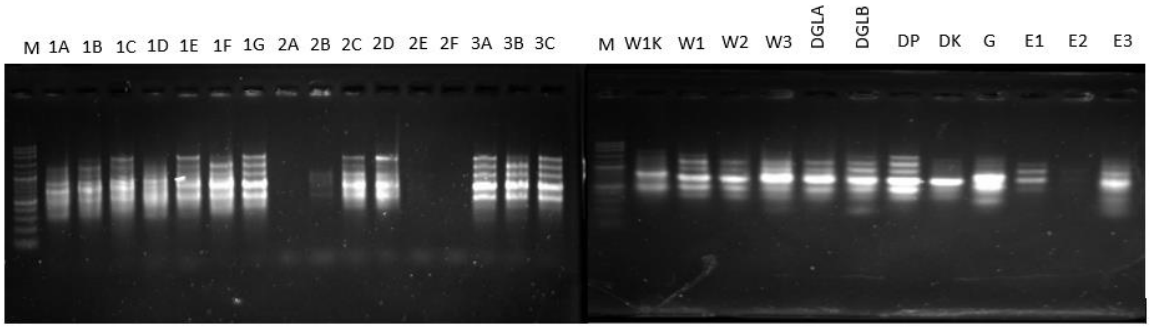

Figure 4. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-13

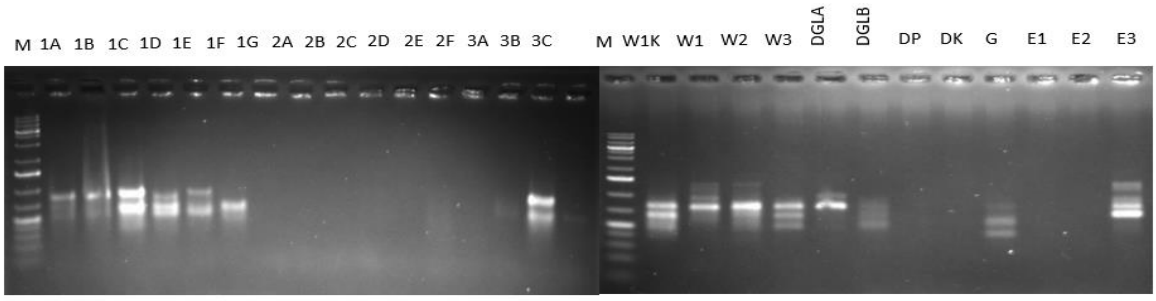

Figure 5. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-26

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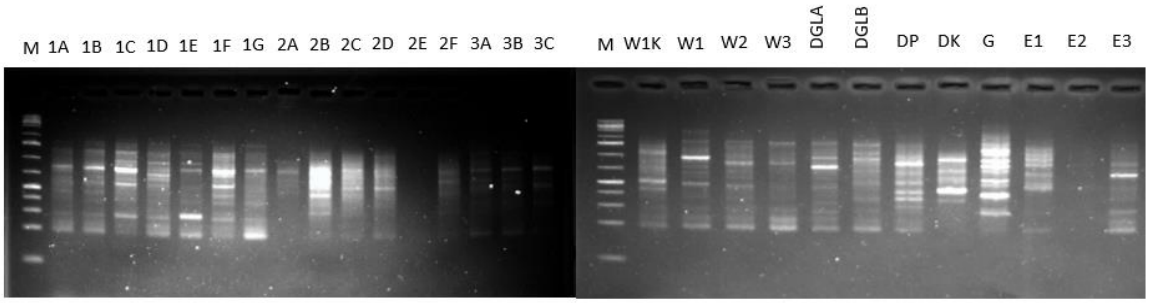

Figure 6. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-28

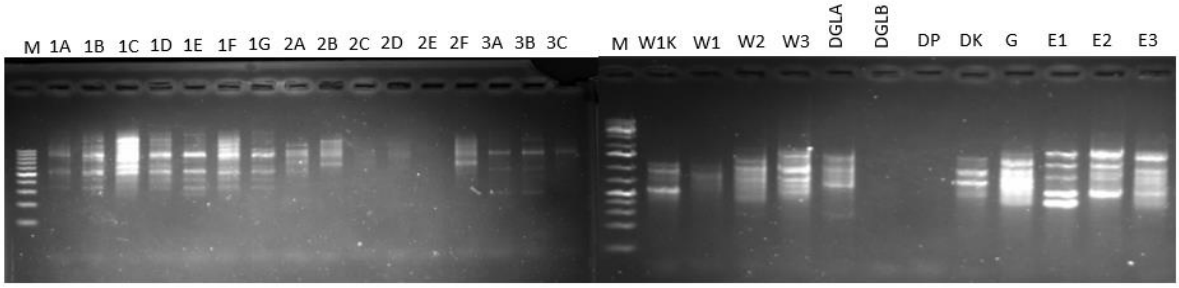

Figure 7. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-30

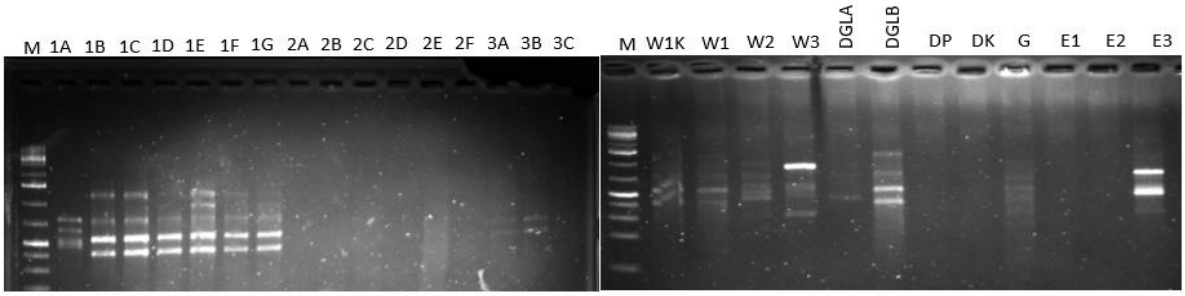

Figure 8. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-31

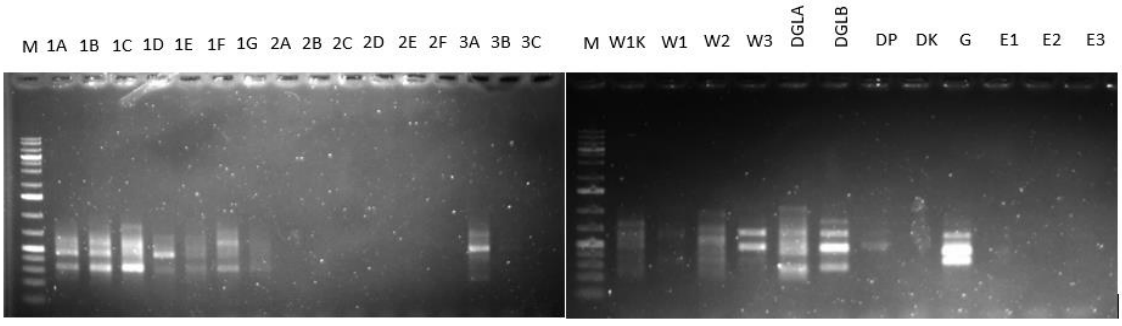

Figure 9. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-35

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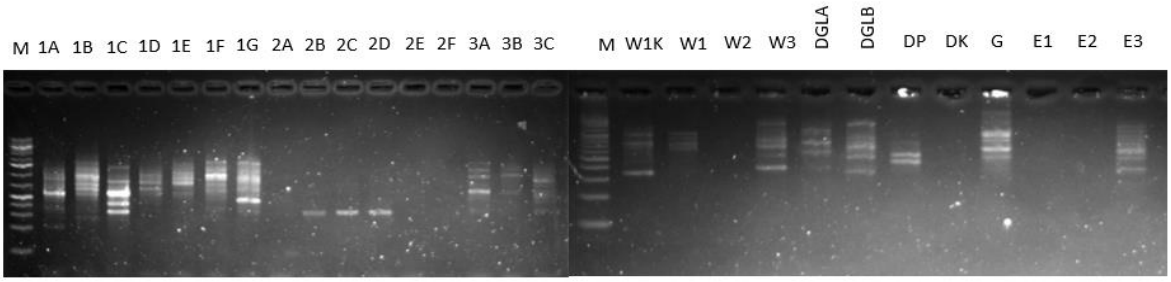

Figure 10. Electropherogram of PCR amplification results of 28 seaweed samples with primers SCoT-36

Optimization of PCR conditions in this study usually involves several stages including the temperature of primer attachment to DNA template (annealing). In this research, the annealing temperature used was 50 oC to attach the SCoT primers to the DNA template. Based on the results of PCR amplification, it was found that with this annealing temperature, only 10 SCoT primers out of 18 primers successfully amplified DNA. Eight primers were not successful in amplifying the sample DNA. This is presumably because the annealing temperature is still too high for these 8 primers. Optimization of the annealing temperature for the 8 primers was carried out by reducing the annealing temperature to 46-48 oC.

In addition, what determines the success of PCR is the concentration and quality of DNA. The results showed that some seaweed samples tested did not produce DNA bands or produce DNA with unclear band quality. The absence of DNA bands of PCR amplification results in some seaweed using SCoT markers indicating the quality and quantity of DNA is low. Therefore, before performing PCR analysis is necessary to check the quality and quantity of DNA from the sample to be analyzed.

Table 3. PCR Amplification Results With 18 Scot Primers On 28 Seaweed DNA Samples Collected from Several Areas In Central Sulawesi

Primary Name	presence of DNA bands	Number of DNA bands	DNA band quality
SCoT-1	Available	Multi	Clear
SCoT-2	No	-	-
SCoT-3	No	-	-
SCoT-4	No	-	-
SCoT-5	No	-	-
SCoT-6	No	-	-
SCoT-7	No	-	-
SCoT-8	Available	Multi	Less clear
SCoT-9	Available	Multi	Clear enough
SCoT-13	Available	Multi	Clear
SCoT-26	Available	Multi	Clear
SCoT-28	Available	Multi	Clear
SCoT-29	No	-	-
SCoT-30	Available	Multi	Clear
SCoT-31	Available	Multi	Clear
SCoT-32	No	-	-
SCoT-35	Available	Multi	Clear
SCoT-36	Available	Multi	Clear

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The identification of genetic diversity of seaweeds collected from several areas in Central Sulawesi Province will show good results depending on the approach used. As previously explained, the use of molecular markers in identification activities is more advantageous than the morphological marker approach. One of the advantages of using molecular markers is that they can distinguish closely related accessions.

In this study, the identification of genetic diversity was carried out using SCoT markers. This was done for several reasons, including that SCoT markers have desirable features and have gained many important advances in the field of genomics and molecular breeding. SCoT markers target the region flanking the start codon, a highly conservative region in plant genes. Therefore, these markers can distinguish genetic variation in specific genes associated with specific traits. It is a simple, novel, cost-effective, highly polymorphic, and reproducible molecular marker that requires no prior sequence information (Rai, 2023). This SCoT marker uses a single 18-mer primer in single primer PCR and the annealing temperature used is 50°C. Therefore, in principle, this technique is similar to RAPD or ISSR or single primer amplification reactions because single primers are used as forward and reverse primers (Figure 11) (Collard & Mackill, 2009; Gupta & Rustgi, 2004). The PCR amplicons obtained are analyzed using standard agarose gel electrophoresis (Collard & Mackill, 2009) making this technique suitable for most research laboratory plants with standard equipment. In previous studies, SCoT markers have been used in many commercially important crop species, including coconut (Rajesh et al., 2015), rice (Collard & Mackill, 2009), potato (Gorji et al., 2011), sweet potato (Nair et al., 2016), common bean (Yeken et al., 2022), sugarcane (Que et al., 2014), and banana (Igwe et al., 2022). However, these markers are still underutilized for various applications, such as interspecific/generic genetic relationship analysis, cultivar/hybrid/species identification, linkage map construction, differential gene expression, and genetic fidelity analysis of plants cultivated through tissue culture (Rai, 2023).

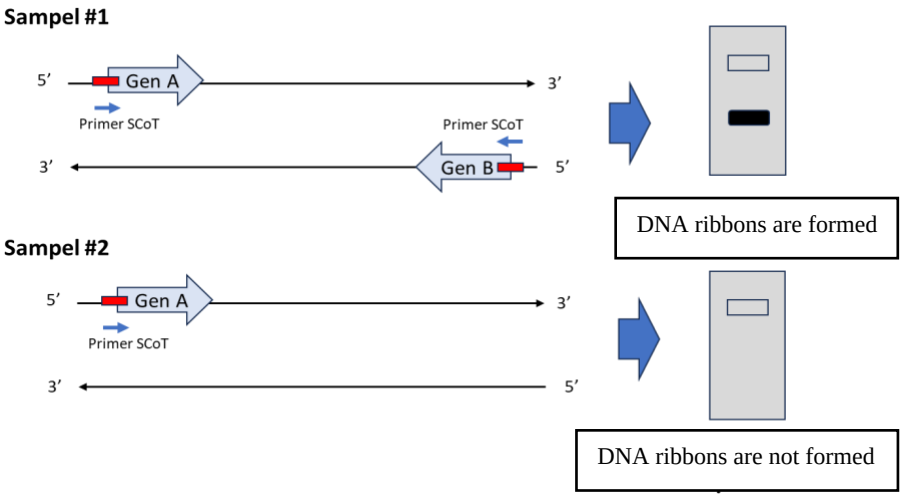

Figure 11. showing the working principle of SCoT markings

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Table 4. Appearance or absence of DNA bands from PCR amplification with SCoT primers from 28 tested seaweed samples

Sample Code	Primer SCoT									
	1	**8**	**9**	**13**	**26**	**28**	**30**	**31**	**35**	**36**
1A	+	+	+	+	+	+	+	+	+	+
1B	+	+	+	+	+	+	+	+	+	+
1C	+	+	+	+	+	+	+	+	+	+
1D	+	-	+	+	+	+	+	+	+	+
1E	+	+	-	+	+	+	+	+	+	+
1F	+	-	+	+	+	+	+	+	+	+
1G	+	-	+	+	-	+	+	+	+	+
2A	-	-	-	-	-	+	+	-	-	-
2B	-	-	+	+	-	+	+	-	-	+
2C	+	-	+	+	-	+	+	-	-	+
2D	+	-	-	+	-	+	+	-	-	+
2E	-	-	-	-	-	-	+	-	-	-
2F	+	-	-	-	-	+	+	-	-	-
3A	+	-	-	+	+	+	+	+	+	+
3B	+	-	-	+	+	+	+	+	-	+
3C	+	-	-	+	-	+	+	-	-	+
W1K	+	+	+	+	+	+	+	+	+	+
W1	+	-	+	+	+	+	+	+	+	+
W2	+	+	+	+	+	+	+	+	+	-
W3	+	+	+	+	+	+	+	+	+	+
DGLA	+	+	+	+	+	+	+	+	+	+
DGLB	+	+	+	+	+	+	-	+	+	+
DP	+	+	+	+	-	+	-	-	+	+
DK	+	-	-	+	-	+	+	-	-	-
G	+	+	+	+	+	+	+	+	+	+
E1	+	+	+	+	-	+	+	-	-	-
E2	+	-	-	-	-	-	+	-	-	-
E3	+	+	+	+	+	+	+	+	-	+
Total (+)	**25**	**13**	**18**	**24**	**16**	**26**	**26**	**17**	**16**	**21**
Total (-)	**3**	**15**	**10**	**4**	**12**	**2**	**2**	**11**	**12**	**7**

Description: + = produces DNA bands; - = does not produce DNA bands

Utilization of SCoT markers on the genetic diversity of seaweed accessions has also never been done. This initial study shows that SCoT markers have a great opportunity to be utilized for genetic studies on seaweed. The results of the initial study also indicated the existence of genetic diversity among the seaweed samples tested. Therefore, the results of the optimization of PCR techniques on 28 seaweed samples using SCoT markers can be a foothold for studying genetic diversity studies in more detail and validity so that information on the genetic background of seaweed in Central Sulawesi will be obtained.

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Conclusion

The findings from this study yield several key conclusions. Firstly, employing a PCR profile with a primer annealing temperature of 50 oC facilitated the amplification of DNA samples extracted from seaweed, resulting in the identification of 10 SCoT primers that successfully generated DNA amplicons. This signifies the effectiveness of the selected primer annealing temperature. Secondly, it is imperative to emphasize the significance of optimizing both the quality and quantity of DNA extracted from seaweed samples for PCR analysis. This optimization process is crucial in ensuring the accuracy and reliability of the obtained results. Lastly, the study affirms that SCoT markers serve as valuable tools for discerning the genetic diversity among seaweed samples sourced from various locations within Central Sulawesi Province. The application of SCoT markers in genetic analysis emerges as a promising avenue for understanding and characterizing the genetic variations within seaweed populations in the region.

To enhance the effectiveness of future research endeavors, it is recommended to fine-tune the PCR conditions, particularly the primer annealing temperature, in order to explore additional SCoT primers for amplifying DNA samples from seaweed. Standardized DNA extraction protocols should be established to ensure consistent and reliable results, emphasizing both the quality and quantity of extracted DNA. Researchers are encouraged to broaden the geographical scope of genetic diversity studies beyond Central Sulawesi Province, utilizing SCoT markers to explore variations in seaweed populations. Additionally, integrating multiple molecular markers alongside SCoT markers and fostering collaborative research initiatives could provide a more comprehensive understanding of seaweed genetics, facilitating knowledge exchange and contributing to the advancement of this field.

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