Original Research Article

Analysis of Genetic Diversity of *Mobula* sp. in Cilacap Using RAPD (Random Amplified Polymorphic DNA) Markers

ABSTRACT

This study aims to determine the genetic diversity of mobula in Cilacap based on DNA bands of RAPD (Random Amplified Polymorphic DNA) polymorphism genetic markers. This research was conducted in September 2021 – June 2022. Sampling was carried out at PPS Cilacap and molecular analysis was carried out at the Biotechnology Laboratory building 3, Faculty of Fisheries and Marine Sciences, Padjadjaran University. The research procedure began with preservation of the stingray (Mobula sp.) sample, isolation of sample DNA, electrophoresis of isolated DNA results, calculating DNA purity, DNA amplification, electrophoresis of DNA amplification results, and data processing and analysis. DNA isolation was carried out using the Promega Kit method. The electrophoretic results of DNA isolation from the three stingray samples showed quite good results. Band samples CIL 10 and CIL 9 looked thick and did not smear, while C13 produced bands and there was a visible smear. The results of calculating the purity of DNA isolated from stingrays CIL 9, CIL 10, CIL 13 have a purity value of 1.94, 1.96, 1.80. Results The highest concentration of DNA was CIL-13 sample with a value of 452.90 ng/µL while the lowest concentration was CIL-10 which was 419.90 OPE-01 resulting in 10 out of 20 visualized polymorphic bands. The similarity index of CIL-9 to CIL-10 in the OPE-01 primer was 40% while the similarity index of CIL-9 and CIL 10 to CIL-13 was 35%. The average similarity index shows that the genetic diversity of the Mobula species is moderate. Morphologically, CIL-09 is a species of Mobula thrustoni, CIL 10 is a species of Mobula mobular, and CIL-13 is a species of Mobula tarapacana. CIL-9 (M. Thrustoni) has a closer similarity index to CIL-10 (M. Mobular) than CIL-13 (M. Tarapacana). OPE-01 primer is good for analyzing diversity in stingrays (Mobula sp.). OPE-01 has 10 out of 20 visualized polymorphic bands.

Keywords: Amplification, M. Mobular, M. thrustoni, M. tarapacana

1. INTRODUCTION

The Mobula genus is currently facing the problem of a high rate of extinction due to overfishing triggered by market demand for mobula meat and skin [1]. Several places in Indonesia have caught stingrays, for example in Central Java in 2019 it reached 5,763.16 tons and for the mobula genus at TPI PPS Cilacap in 2019 it reached 5.83 tons [2]. According to [3], the stingrays that landed at TPI PPS Cilacap were Mobula Japanica, M. tarapacana, M. thursoni, M. cf khulii, and Manta birostris as by-catch from drift gill nets.

Mobula ray from the genus Mobula are animals that come from the Mobulidae family. Based on [4], states that species in the genus Mobula in Cilacap waters are included in the endangered category such as M. thurstoni, M. Mobular and M. Tarapacana [5, 6, 7, 8, 9, 10]. Small populations are more susceptible to several adverse genetic effects so these animals experience a decrease in their ability to evolve or adapt to changing environments [11].

Mobula rays are found in productive coastal waters with regular up-welling processes, groups of small islands, around seamounts, and offshore waters. [12]. The condition of the aquatic environment determines the location of the movement of stingrays. The relationship between populations in one location with another can be known through genetic markers [13].

A genetic marker is a piece of genetic material (usually DNA) that can be easily identified to differentiate between cells, individuals, populations, or species [14]. Nucleotide base sequences that vary between species can be used as specific markers that provide knowledge about phylogenetic relationships to overcome doubts in systematics. Genetic marker Random Amplified Polymorphic DNA (RAPD) is a DNA genome amplification based on the PCR technique [15]. Attachment segments with different DNA fragment lengths are assumed to follow Mendelian inheritance [16]. The RAPD marker has advantages including not requiring nucleotide base sequence information to read [17,18], the primers used are commercially available, do not require information about target DNA sequences or gene organization [19], and is fast to do [20]. The disadvantage of RAPD is that it is not able to distinguish between homozygous and heterozygous loci, so it requires analysis of the nucleotide base sequence using high-resolution techniques [21]. RAPD analysis can be used to see genetic diversity based on the level of polymorphism, inheritance patterns, check the origin of populations, and help define a species [22]. These markers are widely used in identifying genetic diversity at the interspecies and interspecies levels [20].

The diversity of genetic resources of a population has factors that influence the response of a population to natural and artificial selection. Populations with high genetic diversity have a better chance of survival [23]. This is because each gene has a different response to environmental conditions so if an individual has various kinds of genes in a population, there is a chance of response to environmental changes [24].

The existence of stingrays is in endangered status, therefore it is necessary to carry out conservation measures. One of the first steps in supporting conservation efforts is the need to identify the level of genetic diversity. Research on the genetic diversity of stingrays from the genus Mobula in Cilacap using the RAPD technique has not been carried out. Therefore, it is necessary to research the analysis of the genetic diversity of the genus Mobula stingrays in Cilacap waters using the RAPD technique. This study aimed to determine the genetic diversity of stingrays in Cilacap based on DNA bands of RAPD (Random Amplified Polymorphic DNA) polymorphism genetic markers.

2. METHODOLOGY

This research was conducted in September 2021 – June 2022. Sampling was carried out at PPS Cilacap and molecular analysis was carried out at the Biotechnology Laboratory building 3, Faculty of Fisheries and Marine Sciences, Padjadjaran University..

The methods are included in the survey method with quantitative and qualitative descriptive analysis. Quantitative data were obtained from DNA purity calculations while qualitative data were obtained from the appearance of visualized bands from DNA amplification electrophoresis results.

The research procedure began with preservation of the stingray (Mobula sp.) sample, isolation of sample DNA, electrophoresis of isolated DNA results, calculating DNA purity, DNA amplification, electrophoresis of DNA amplification results, and data processing and analysis. DNA isolation was carried out using the Promega Kit method.

2.1 Procedures

The research procedure began with preservation of the stingray (Mobula sp.) sample, isolation of sample DNA, electrophoresis of isolated DNA results, calculating DNA purity, DNA amplification, electrophoresis of DNA amplification results, and data processing and analysis.

2.1.1 DNA Isolation

DNA isolation was carried out in two ways, Wizzard Genomic Purification Kit (Promega) [25].

The purity of isolated DNA was measured with a Multimode Reader Infinite 200 PRO NanoQuant spectrophotometer. The purity of DNA is obtained from the division between A260 and A280. The isolated DNA sample was diluted 50× Nuclease Free Water into a blank. The absorbance was measured at a wavelength (λ) of 260 nm and 280 nm and then the absorbance was recorded for each wave. The results for purity and quantification were recorded.

2.1.2 DNA Amplification

DNA amplification was carried out by [26] method. The primer used was OPE-01 (CCCAAGGTCC).

12.5 µl of MyTaqTM HS Red Mix (Bioline), 9.5 µl of NFW, 1 µl of primer, and 2 µl of DNA sample, put into a 0.2 ml microtube then the solution was centrifuged for 30 seconds at 1,000-speed rpm. Amplification consists of several stages, namely pre-denaturation (94°C, 60s, 3 cycles), pre-annealing (35°C, 60s, 3 cycles), pre-extension (72°C, 120s, 3 cycles), Denaturation (94°C, 10s, 37 cycles), annealing (40°C, 30s, 37 cycles), extension (72°C, 120s, 37 cycles), post extension (72°C, 300s, 1 cycle) and cooling (4°C, 60s, 1 cycle) [27].

2.1.3 Electrophoresis of DNA Isolation and Amplification

Electrophoresis is a separation method that utilizes the electric field generated from the electrodes to separate DNA molecules which will later produce DNA bands that can later be analyzed based on the presence or absence of bands in the sample.

Agarose powder was weighed as much as 0.4 grams and dissolved with 40 ml of TAE 1X. The solution was heated in the microwave for three minutes. When finished, the agarose solution was allowed to stand until warm and 0.6 µl of red gel was added and homogenized. The agarose gel mold was prepared, then the agarose solution was poured into the mold. The agarose gel is left for 15-30 minutes to freeze. The agarose gel was put into the electrophoresis tank which already contained the TAE running buffer solution until it was completely submerged. Each sample was entered successively based on the primer into the agarose gel wells until all samples occupied the wells. The composition of the isolated sample was 4 µl of DNA isolation sample, and 2 µl of loading dye. The composition of the amplified sample is 3 µl. The composition of the marker DNA ladder of 100 bp was 2 µl. The Mupid-Exu electrophoresis machine was set at to voltage of 70 v, for 30 minutes for DNA isolation results, and a voltage of 70 v, for 90 minutes for DNA amplification results. Agarose gel was visualized by placing it on a UV transilluminator. The visualization results are documented for further analysis.

2.1.4 Data Analysis

DNA amplification produces random DNA fragments with RAPD primers. The length of the separated fragments using RAPD primers can be determined from the migration distance of the DNA bands from the wells and compared with the markers used using the CorelDraw application.

Migration distance data is then entered into the excel application and analyzed to obtain band sizes. The bands visualized on the agarose gel are translated into a binary matrix. Bands that appear are defined as one (1) and bands that do not appear are defined as zero (0). Binary data is entered into the excel application with excel format 1997-2003. Binary matrix data that has been processed in the excel application is then processed in the Ntedit application. Data that has been processed in the Ntedit application is entered into the NTSys application to obtain a dendrogram tree. The genetic similarity index is calculated based on the Dice Coefficient formula [27].

3. RESULTS AND DISCUSSION

3.1 DNA Concentration and Purity

The electrophoretic results of DNA isolation from the three stingray samples showed quite good results but there were still smears. The best band results are samples CIL 10 with code C10 and CIL 9 with code C9. Band samples CIL 10 and CIL 9 looked thick and did not smear, while C13 produced bands and there was a visible smear. CIL 13 carried out a different isolation method, namely the CTAB isolation method. The CTAB isolation method was performed because the band on the Promega Kit isolation method did not appear. This is because the storage time of the sample has reached 1 year. According to [29], storage of samples using glycerol alcohol will decrease by around 60-80%.

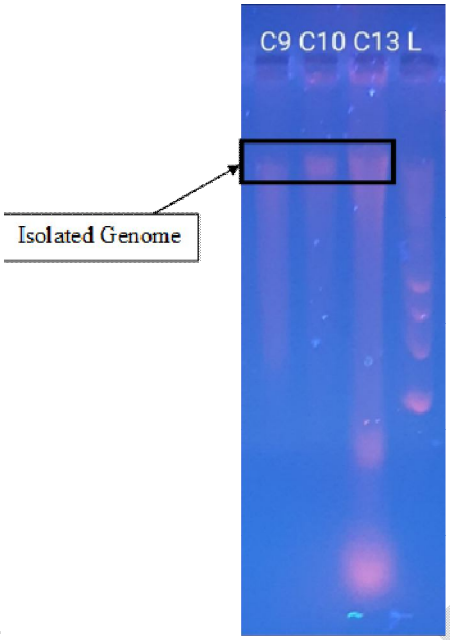

Figure 1. DNA electrophoresis results
L=Ladder 1kb, C9=CIL-9, C10=CIL-10, C13=CIL-13

The results of calculating the purity of DNA isolated from stingrays CIL 9, CIL 10, CIL 13 have purity values of 1.943, 1.958, 1.803. These DNA purity value shows high results and can be used for PCR. Purity results are good if they range from 1.8 to 2.0 [29, 30]. The purity value of DNA isolation above 2 indicates that it still contains RNA, while a purity result of less than 1.8 indicates that the DNA extract still contains protein [31]. UV light with a wavelength of 260 nm can be absorbed by ds-DNA, while light with a wavelength of 280 nm can be absorbed by protein or phenol contaminants [32].

The highest concentration of DNA was CIL-13 sample with a value of 452.8988 ng/µL while the lowest concentration was CIL-10 which was 419.8988 452.8988 ng/µL. This difference in concentration values can be caused by differences in isolation methods. The CTAB isolation method has the advantage of being able to perform strong lysis and facilitate protein precipitation so it can produce high concentrations of DNA [33].

Table 1. Results of Quantification of DNA Concentration and Purity

No	Sample	A260	A280	Consentration (ng/µL)	Purity of DNA
1	CIL-9	0,17256	0,088796	431,3988	1.943
2	CIL-10	0,16796	0,085796	419,8988	1.958
3	CIL-13	0.,18116	0,100496	452,8988	1.803

Isolation using the Wizard Genomic DNA Purification kit Brand Promega and CTAB from samples of Mobula stingrays that had been preserved with glycerol alcohol could be tested to produce good DNA. DNA samples can be tested for the next process, namely DNA amplification because testing the results of isolation qualitatively and quantitatively shows pure DNA results.

3.2 Genetic Diversity of Mobula

Fragments appear in all primers used for amplification of 3 samples. The primer used in this DNA amplification is OPE-01. OPE-01 produces 20 bands. All bands amplified using prunes were analyzed further.

The size of the DNA fragments amplified in the OPC-20 primer was, OPE-01 was 390 - 5078 bp. According to [35], the base length (bp) found in RAPD DNA fragments in fish ranges from 200 - 1500 bp, whereas according to [25], the base length in fish ranges from 300-1500 bp..

Table 2. OPE 01 primer polymorphic and monomorphic bands

Fragment Range (mm)	CIL-9	CIL-10	CIL-13	Band Size (bp)
203		--*		5078
239			--*	2681
252	--	--		2226
274			--*	1692
287	--	--		1467
303			--*	1252
317		--*		1104
333		--	--	970
348			--*	867
362		--	--	788
373		--	--	735
386		--	--	680
391	--*			661
407	--		--	606
412	--	--		591
433	--	--	--	534
448	--		--	499
463		--*		469
477	--*			444
514	--*			390

OPE-01 produced 10 out of 20 visualized polymorphic bands. Polymorphic bands are bands that are not present in all samples [35], while monomorphic bands are bands that exist in other samples so that they do not have variations [36]. According to [35], differences in banding patterns can describe genetic differences in samples so that unique and different banding patterns can indicate genetic variation. The size and number of amplified fragments depend on the nucleotide sequence of the DNA source [37]. The selection of primers in the RAPD method can increase polymorphism [25].

A dendrogram tree is a tree that describes a kinship relationship between species [25]. According to [38], genetic diversity is closely related to the genetic distance where the greater the genetic distance the greater the individual genetic diversity in the population. Conversely, the smaller the genetic distance, the lower the genetic diversity. There was a dendrogram

produced in this study based on the OPE-01 primer. Dendogram tree analysis uses the dice similarity index [27]. The dice similarity index is used to see similarities and compare one sample to another so that the higher the percentage, the more similar the two populations are. From the genetic similarity index can be analyzed the genetic diversity of the individual. There is a dendrogram produced in this study based on the OPE-01 primer.

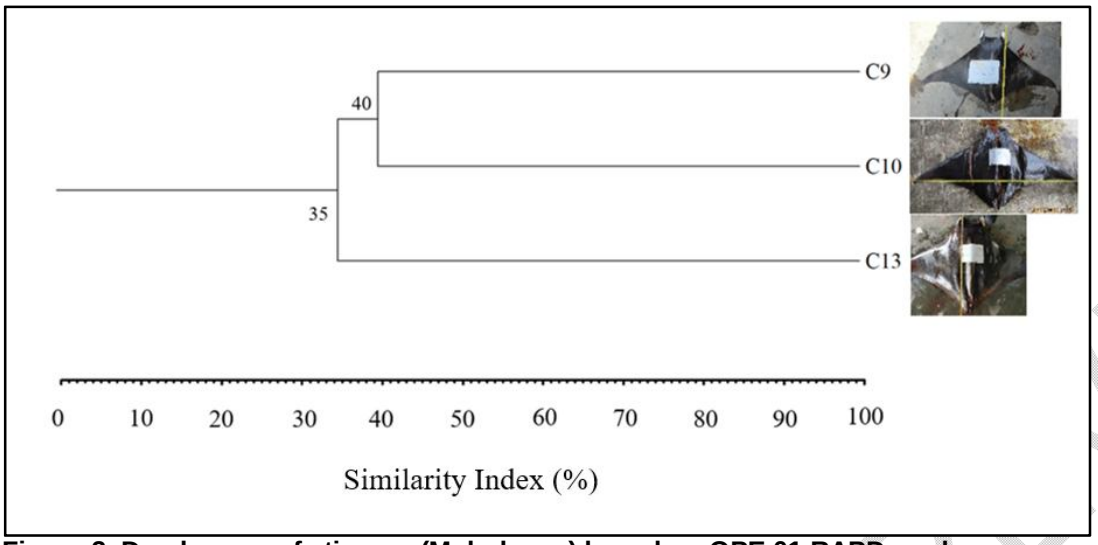

Figure 2. Dendogram of stingray (Mobula sp.) based on OPE 01 RAPD marker

The similarity index of CIL-9 to CIL-10 in the OPE-01 primer is 40%. This genetic distance is far away, therefore the genetic diversity is getting bigger. According to [37], the greater the genetic distance, the greater the individual genetic diversity in the population, conversely, the smaller the genetic distance, the lower the genetic diversity. At CIL 13 both primers have a similarity index of 35%. The closeness of CIL-9 and CIL-10 may be due to the phenotypic similarities in the two samples, namely having white fins.

According to [39], the value of genetic diversity ranges from 0.1 – 0.4 which is included in the low category, while values of 0.5 – 0.7 are classified as in the medium category, and 0.8 – 1.0 is in the high category. The average of the genetic diversity of this mobula ray is 62.5%, therefore the genetic diversity is moderate.

Morphologically, CIL-09 is a species of Mobula *thrustoni,* CIL-10 is a species of Mobula *mobular*, and CIL-13 is a species of Mobula *tarapacana*. According to [40], the morphology of the three species is described in table 3.

Table 3. *Mobula* sp. Morphology

Species	Morphology
Mobula mobular	1. wide body plate and slightly curved sharply 2. the tip of the dorsal fin is white 3. there is a thorn at the base of the tail 4. spiracles shaped like a small slit and rounded elongated, above the end of the body plate
Mobula thrustoni	1. short head shape, less than 16% of body width 2. the tip of the dorsal fin is white 3. flat tail base 4. Slightly rounded spiracles, located under the ends of the body plates

Species	Morphology
Mobula tarapacana	1. the body plate is not too wide and sharply curved 2. The dorsal fin is plain, the tip is not white 3. no spines at the base of the tail 4. spiracles like slits and elongated round, located above the end of the body plate

The principle of RAPD is to randomly attach a single primer to DNA [41]. Most of the sample DNA is present in the nucleus, but a very small amount of DNA is also present in the mitochondria [42]. This grouping is similar to the study [43], where Mobula *mobular* is closer to Mobula *tarapacana* than Mobula *thrustoni* in mitochondria however, in the nucleus Mobula *mobular* is closer to Mobula *thrustoni* than Mobula *tarapacana*.

4. CONCLUSION

Based on the results of the discussion of the research that has been done, the conclusion obtained is that the average similarity index indicates that the genetic diversity of the Mobula species is moderate. Morphologically, CIL-09 is a species of Mobula thrustoni, CIL 10 is a species of Mobula mobular, and CIL-13 is a species of Mobula tarapacana. CIL-9 (M. Thrustoni) has a closer similarity index to CIL-10 (M. Mobular) than CIL-13 (M. Tarapacana). OPE-01 primer is good for analyzing diversity in stingrays (Mobula sp.). OPE-01 has 10 out of 20 visualized polymorphic bands.

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