

Genetic Variance of Infant *Chelonia mydas*'s mtDNA

Rini Puspitaningrum^{1,*}, Dwi Anita Suyandari², Yanti Susiyanti³

Abstract— *Chelonia mydas* were one of 6 turtle species in Indonesia which its population were decrease of illegal trade and hunting. Sea turtle conservation technique must supplied by molecular identification technique. This could be done with TCR Sequence Isolation from *Chelonia mydas*'s mtDNA TCR Sequence is a non-coding control region in tRNA gene those faster in accumulate structural mutation and have sensitifiy for population analysis. Abundant TCR Sequences could supplied by PCR optimization with varying concentration titration of primer (0,25; 0,5; 0,1 μ M) and DNA template (25; 50ng), in 30 and 35 cycles. This research were conducted at Biology Laboratory of FMIPA UNJ and Biochemistry Laboratory of FKIK UIN-SH, from January to July 2006. Infant *Chelonia mydas* has being borned from eggs. Genomic DNA extraction from infant *Chelonia mydas*'s flipper tissue were used as DNA template for TCR sequence isolation. TCR sequence from optimal titration PCR product were being detected at 0,5 μ M primer and 25ng DNA template in 35 cycles, by gel electrophoresis. A 383bp TCR sequence nucleotides were detected by sequencing method.

Keywords: *Chelonia mydas*, mtDNA, TCR Sequence, PCR product, optimal titration, gel electrophoresis, sequencing method.

INTRODUCTION

Green turtles are the most common species in Indonesia, because almost all of the nesting beaches in Indonesia are green turtles. The high level of distribution of green sea turtles in Indonesia, apparently influences the high level of green turtle population. In fact, the green turtle population in Indonesia is shrinking every year. This is due to the illegal hunting and trading of green turtles. Green turtles are much hunted for their meat, while green turtle eggs are widely consumed because t

hey are believed to increase libido. Considering the condition of the green turtle which has been very worrying, the conservation of green turtles must continue to be developed in Indonesia.

So far, quite a lot of research has focused on turtles in order to find the right conservation strategy. Therefore, in order to support the conservation of sea turtles in Indonesia, at this time it is very necessary to identify molecular sea turtles. This can be started from the earliest stages, for example isolating certain sequences of green turtle mitochondrial DNA (mtDNA).¹

MtDNA green turtle contains ideal genetic data that can be used in maternal genetic tracing for various purposes. For example to test kinship, see the level of genetic variation to estimate the rate of occurrence of inbreeding, find out the effective number of populations and so on.¹

MtDNA green turtle contains a sequence of control regions known as the turtle control region (TCR) sequence. Several studies have reported the use of TCR sequences as markers of increasing outcomes in discriminatory studies of green turtle populations.² Therefore, TCR sequences can be used as important genetic markers in the molecular research of *Chelonia mydas*.³ Based on this, *Chelonia mydas*'s MTDNA TCR sequence needs to be further investigated to explore the deeper life of green turtles.

Considering the importance of molecular green turtle identification, TCR sequences are needed that are quite a lot for research purposes. For this reason, a multiplication of TCR sequences was performed with PCR techniques. In order to obtain a good PCR product, it is necessary to optimize the amplification by modifying the template DNA and primary concentrations and the number of PCR cycles. Thus, it is expected to obtain PCR products in the form of optimal *Chelonia mydas* mtDNA TCR sequences. In the end, this sequence can be used as a genetic marker for further analysis.

METHOD

Hatching from *Chelonia mydas* eggs

A total of 50 green turtle eggs (*Chelonia mydas*) from one turtle mother were collected from Pangumbahan beach, Sukabumi, West Java. It takes about 60 days to incubate turtle eggs in the laboratory. Every 3 days the sand temperature is measured in the hatchery box. The newly hatched hatchlings are transferred into a plastic tub filled with sea water. After the green turtle egg hatches, biological evaluation and identification for all hatchlings is immediately carried out. Then one hatchling was used as a test animal for the retrieval of the front flipper muscle tissue.

¹Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jl. Rawamangun Muka no 1, Jakarta Timur, Jakarta 13220. Indonesia. Email : rini_puspitaningrum@unj.ac.id ; rini_puspitaningrum@yahoo.com

²FKUI. Jl. Salemba Raya no 6 Jak Pus 10430. No Telp.021.31930379.

³FK UIN Syarif Hidayatullah Jakarta. Alamat Jl. Kertamukti no 4 Cirendue 15419. Email: umi30573@yahoo.com

Genomic DNA isolation from *Chelonia mydas* flipper tukik network

1.5 cm flipper tukik network was crushed and added with 3ml EDTA 0.5M. The sample was put into a 1.5ml tube and then added 500pl Nucleic Lysis Solution and 10pl Proteinase K then incubated 55 ° C for 3 hours. After incubation, the sample was added to 3pl RNase Solution and 200ul Protein Precipitation Solution then incubated in ice for 5 minutes. Then the sample was centrifuged for 4 minutes at 16,000 rpm (on the surface of the tube a white pellet will be formed), and the supernatant containing the DNA was transferred to a sterile tube and then added 600 pi of isopropanol.

Centrifugation was carried out again for 1 minute at 16,000 rpm, then the supernatant was removed and then 70% ethanol was added after centrifuging again at 16,000 rpm for 1 minute, the tube was dried using a blow dryer for 15 minutes. 100 mic Rehydration Solution is added to the microtube and incubated at 65 ° C for 1 hour. Isolated genomic DNA is stored at 4 ° C.

PCR amplification

To obtain amplicons (amplification products) from *Chelonia mydas* mtDNA sequences, TCR 5 and TCR primers were used. The primers were designed with reference to journal publications.² The sequence of the primary sequence, as follows:

1. TCR Primary 5 (forward): 5' TTG TAC ATC TAC TTA TTT ACC AC 3'
2. TCR 6 Primer (reverse): 5' CAA GTA AAA CTA CCG TAT GCC 3'

First of all, PCR material is mixed into the PCR tube, which consists of:

1. DNA template at 25ng and 50ng concentrations.
2. Primary TCR 5 and TCR 6 at concentrations of 0.25µM, respectively; 0,5µM; 1µM
3. 25µl PCR master mix (consisting of: 50unit/mL Taq DNA Polymerase; 3mM MgCl₂; 400µM dATP, dGTP, dCTP, dTTP, respectively)
4. Nuclease free water reaches 50 µl of reaction volume.

Then the microtube containing the PCR material was put into a thermal cycler machine. The PCR program for TCR sequence amplification² was carried out with an initial denaturation phase at 93°C for 1 minute, the next cycle up to the 30th cycle of the cycle including the denaturation phase at 93 ° C for 40 seconds, phase attachment at 55 ° C for 50 seconds, elongation phase at 72 °C for 40 seconds, and final elongation phase at 72 ° C for

2 minutes. PCR products is stored at 4°C. The optimization of the PCR method was done by titrating the template and primary DNA concentrations and modifying the PCR cycle. The DNA template was titrated at 25ng and 50ng while the primer was titrated at a concentration of 0.25µM; 0,5µM; and 1µM. The PCR cycle is run as many as 30 and 35 cycles.

Primer DNA template	0,25µM	0,5µM	1µM
25ng	0,25µM-25ng	0,5µM-25ng	1µM-25ng
50ng	0,25µM-50ng	0,5µM-50ng	1µM-50ng

Table 1. Titration table for template DNA concentrations and primers for optimization of PCR techniques

TCR Sequencing

The sequencing process is carried out using the ABI 3130x1 Genetic Analyzer machine, for the good services of the Indonesian Eijkman Molecular Biology Institute.

RESULT AND DISCUSSION

Hatching from *Chelonia mydas* eggs

Hatching hatchlings in the laboratory were successful, from 50 turtle eggs hatched into 44 hatchlings. The percentage of hatching is 90%. This percentage of hatching exceeds the average hatching percentage in nature, which is 69% .⁴ Naturally, the percentage of newly hatched hatchlings in nature shows high rates, this is partly due to the many predators and susceptibility of the hatchling body. So it is noted that the percentage of hatchlings that can survive in nature to adulthood is below 5% of each birth.⁵

Chelonia mydas belongs to the endangered animal category and therefore, 1 hatchlings that died during maintenance in the laboratory were then used as test animals.



Figure 1. *Chelonia mydas* eggshells after hatching.

In general, *Chelonia mydas* hatchlings have greenish black karapak. The average value of hatchlings' body weight is 29gr, the average length and width of the turtle is 5.7cm and 4.9cm. In the first week, there were 10 dead green turtle hatchlings.



Figure 2. *Chelonia mydas* has just hatched

DNA Isolation of Genome Tukik *Chelonia mydas*

The genomic DNA values obtained were 1.57 for the DNA purity index and 14.325 μ g/ml for DNA concentration. The isolated genome DNA was then used as a DNA template in PCR techniques.

Optimization of PCR techniques for the cyt gene b mtDNA *Chelonia mydas*

The 1st optimization is a preliminary trial of the PCR program to amplify the cyt gene b. The results of detection on the 1st optimization showed that the band position of the PCR product was located far below the 1000bp DNA marker, while the expected PCR product size was 2300bp for the cyt b gene⁶ then concluded that the cyt b gene is not amplified. In the 2nd optimization, the PCR process is carried out using complete DNA template primary concentration titration. Detection results on the 2nd optimization did not show any signal from PCR products and DNA markers. This is thought to occur because the electrophoresis preparation process is less perfect so that PCR products and DNA markers do not enter the agarose gel column. Furthermore, from the results of the detection on the 3rd optimization it was found that there was no signal from the PCR product, but the DNA marker had migrated in the form of a smear and it was known that the control without DNA in the 3rd optimization did not show a signal, meaning that there was no contamination in the PCR optimization process to -3. In addition, the PCR program and primers used are suitable programs to amplify the cyt b gene for 2300bp.⁶ So it can be concluded that the PCR process on this optimization has gone well.

There is a presumption that the genome DNA used as DNA template is not isolated. However, the results of the detection of genomic DNA through spectrophotometer showed that isolated genomic DNA had a sufficient purity index and could be used as a template DNA in PCR reactions. Thus based on the optimization results of the 1st, 2nd and 3rd, it was concluded that the cyt b gene could not be amplified. To overcome this problem, it is planned to detect the isolated

genome DNA used as DNA template, through agarose gel electrophoresis.

If the cyt b gene has been successfully amplified, data on the cyt b gene nucleotide sequence is needed as a reference material to detect amplification products. Therefore, a search for the nucleotide sequence of the cyt gene is carried out b. The search is carried out by searching for mitochondrial *Chelonia mydas* genpm sequences listed in the genebank on the NCBI site. The results of the search for the nucleotide sequence of the cyt b gene obtained were apparently incomplete. This is because the cyt b gene has a fairly large base size (2300bp), and there is still research on several fragments in the cyt b gene that have not been published. While the publication of research that has been conducted on the cyt b gene⁶ did not publish the results of the cyt gene sequencing b, so that the complete nucleotide sequence of the cyt b gene cannot be known. Attempts to contact the researchers and universities of the location of the Cyt B gene research have also been carried out, but no answers were obtained. Complete data on the nucleotide sequence of the cyt b gene were not obtained, thus it was decided to directly conduct the sequencing process against the cyt b gene with the aim of exploration. if the cyt b gene is successfully amplified.

Several obstacles in the process, sequencing, include sequencing programs with the services of the Eijkman Molecular Biology Institute, which can only be carried out on DNA sequences with a base size of <500bp (less than or equal to 500bp) in each reaction. That is, the process of sequencing the 2300bp cyt b gene must be carried out in 6 reactions. To be able to carry out sequencing in 6 reactions, the cyt gene b needs to be restrained first. Then it is necessary to know the restriction map and restriction enzyme data from the cyt gene b. To map the restriction site and find out the appropriate restriction enzymes for the cyt b gene, the complete cyt b gene nucleotide sequence is needed. Because the cyt b gene sequence is not obtained completely, the restriction map cannot be traced and the Cyt b gene sequencing program cannot be performed.

If there will be an effort to optimize the restriction method for the cyt gene, there are a number of constraints, including inadequate allocation of research time. In addition, the chemicals needed to optimize restriction methods are very limited and to meet them there are limitations in terms of research costs.

Thus it was decided not to continue amplification of the cyt b gene, and further amplification would be carried out on other sequences in *Chelonia mydas* mtDNA which had a size below 500bp and had complete sequence data to support detection. Thus, the amplification of TCR (Turtle Control Region) sequences from *Chelonia mydas* mtDNA is \pm 384bp.

Optimization of PCR techniques for *Chelonia mydas* mtDNA sequences

To obtain optimal PCR results, titration is carried out on the primary concentration of 0.25; 0.5; and 1 μ M and the template DNA concentration is 25ng and 50ng. Titration is carried out on the primary concentration, to obtain the optimal PCR product by using the right amount of primer. In the PCR reaction the primer in the right amount is needed to produce the optimal PCR product, the excess primer will result in mispriming and dimerization.⁷ While titration of DNA template is done to obtain optimal PCR products with the efficient use of materials. In addition, in the PCR reaction, the PCR components that determined the PCR process specifically were interactions between primary and DNA templates⁸ and in this optimization, a PCR Master Mix kit was used so that the concentration of other PCR materials was in accordance with PCR reaction procedures.

The variation of the primary concentration for this optimization was carried out using a range between 0.25-1 pM. This range is determined based on the range recommended by the Master Mix PCR kit, which is 0.1-1 μ M. Besides that based on the standard primary concentration for a PCR reaction is 0.25 μ M. While the variation of template DNA concentration used for this optimization is 25ng and 50ng. This concentration is in accordance with the provisions of the use of template DNA concentrations based on the recommendation of the PCR Master Mix kit, which is below 250pg. In addition, the range of template DNA concentrations in order to produce amplification products that can be detected through agarose and polyacrylamide gel electrophoresis is about 0.01 pg to 100pg.⁹ Even according to¹⁰ DNA templates for PCR reactions can be added up to 1 μ g.

For different amplification process conditions, it is necessary to re-optimize the material and PCR program, because there is no standard PCR program that can guarantee the success of all amplification process conditions.⁸ Therefore, to obtain optimum results with a Thermal Cycler PCR (BioRad), several PCR optimization techniques have been carried out. In this study the PCR program was run by modifying the PCR program² by trying two conditions, namely the number of amplification cycles of 30 and 35 cycles. In general, PCR programs run as many as 25 to 35 cycles are efficient enough to produce up to 1 μ g of PCR products from 50ng DNA templates.

a. Optimization of PCR technique with program A on *Chelonia mydas* mtDNA sequence

The program A PCR technique was carried out by carrying out titration of the primary concentration and DNA template through a 30-cycle PCR program (Fitzsimmons, 1999).

1) Optimization of the 1st PCR technique with program A against *Chelonia mydas* mtDNA sequence

The 1st optimization is a preliminary experiment on PCR program by running 2 variations of DNA template

concentration (25ng and 50ng) and 1 variation of primary concentration (0.5 μ M), and without primary control.

Qualitative detection through 1% agarose gel electrophoresis showed that PCR products were not formed. In addition, markers of DNA and control without primers are also not seen. This can happen because some possibilities, for example, see controls without primers that do not reveal the band, presumably too little template DNA concentration. Given that the detection of genomic DNA as a DNA template is not supported by detection techniques through electrophoresis, there is a possibility that genomic DNA has not been isolated. This can be seen in the PCR results in each kokxn agarose gel which gives negative results. However, when referring to a column of DNA markers that do not show a signal, it is likely that this is caused by a less than perfect staining technique, namely because the concentration of ethidium bromide is too low so it is unable to color DNA. To overcome this, the next optimization is done.

2) Optimization of the 2nd PCR technique with program A against *Chelonia mydas* mtDNA sequence

Before the 2nd PCR optimization, detection of genomic DNA through 0.9% agarose gel electrophoresis showed positive results. In agarose gel results from the 2nd optimization detection, the marker band DNA has migrated, but not well distributed. 500bp DNA marker band is clearer, so it can be used as a reference band.

PCR products on titration of 0.5 μ M primary-25ng DNA template and titration of 1 μ M primary-50ng DNA template showed 2 non-specific bands, each of which was parallel and below 500bp. Non-specific bands can occur due to several causes, including primary dimerization and the formation of other PCR products.⁸ Primary dimerization can occur due to the presence of primers that polymerize to form a band. Inter-primary polymerization can occur because the number of primers used is too much. While the formation of other PCR products is caused by the primary ability to attach to other DNA sequences. This means that the primary used may not be specific.

On titration of 0.5 μ M primary-50ng DNA template and titration of 1 μ M primer 25ng DNA template, there is 1 band below 500bp. It can be seen that PCR products are formed specifically, that is, a single band is formed and has an expected base size, which is below 500bp or around 384bp. Thus the allegation that the primer is not specific is proven to be incorrect. Furthermore, it can be concluded while that the optimal titration of the 2nd optimization is in the titration which shows a strong density band, namely the titration of 1 μ M primary 25ng DNA template. Control without clean DNA indicates that the PCR process is carried out in a sterile state. The position of the entire band of PCR products shows a size that is not the same, so to get a better PCR product, then the PCR program is repeated.

3) Third optimization PCR technique with program A on *Chelonia mydas* mtDNA sequences

In this optimization, the PCR process is repeated with complete titration. Detection of the 3rd optimization PCR results was done by 2% agarose gel electrophoresis.

a) The first detection of the 3rd optimization of PCR techniques with program A against *Chelonia mydas* mtDNA sequence

Detection was carried out on 25µl PCR products, the volume of PCR products detected in the 3rd optimization was more than the volume detected in the second optimization, with the aim that the band density was more apparent.

It is known that PCR products are still near the column, while DNA markers have migrated, so the distribution of marker DNA is not parallel to all PCR products. It is seen that all PCR products are in a parallel position, thus it can be assumed that all PCR products have the same base size, but the size of the PCR product cannot be identified. To obtain better detection results, the next detection is done.

b) Second detection of the 3rd optimization of PCR technique with program A against *Chelonia mydas* mtDNA sequence

To get better results, detection by polyacrylamide electrophoresis 10% was carried out. detection of the second optimization PCR product was carried out simultaneously with the 4th optimization detection, which was carried out on a polyacrylamide gel. Detection results showed that the 3rd optimization PCR product band was parallel to the 4th PCR optimization product band so that it can be seen that the base size of the 3rd optimization PCR product and the 4th optimization are equal and stable. In addition, the detection results show that all PCR products show a single band, so it can be seen that PCR products have been specifically amplified. However, migration of DNA markers is not parallel to PCR products, so the size of PCR products cannot be identified.

As for all of the titrations in the 3rd optimization, it is known that there are two of the clearest signals, namely in the titration of 1µM primary-25ng DNA template and in titration of 1µM primary-50ng DNA template. Titration of a 1µM primary-25ng DNA template yields a band density that is stronger than a titration of 1µM primary-50ng DNA template. So it can be concluded while that the optimal titration in the 3rd optimization is in titration of 1µM primary-25ng DNA template. This is consistent with the results of the 2nd optimization.

In this detection, the migration of DNA markers is not parallel to the migration of PCR products, it is suspected that the quality of DNA markers is not good enough so that for optimization, DNA markers will be replaced with new DNA markers of the same brand and size. The next optimization is done by adding controls without primers and controls without DNA.

4) Optimization of the 4 PCR techniques with program A against *Chelonia mydas* mtDNA sequence

a) First detection of the optimization of the 4 PCR techniques with program A against *Chelonia mydas* mtDNA sequences

It is known from the results of detection through polyacrylamide gel electrophoresis, that the entire band of PCR products are parallel, but the position of the DNA marker lags behind the PCR product, which is still at the top of the column so that DNA markers cannot be used to identify the size of PCR products.

In all the 4th optimization PCR titrations, there were two clearly visible signals, namely in the titration of 1µM primary-25ng DNA template and 1µM primary-50ng DNA template. Titration of 1µM primary-25ng DNA template has produced a band with a strong density, so it can be concluded that the optimal titration of the 4th optimization is in the titration of 1µM primary-25ng DNA. This is consistent with the results of the 2nd optimization and 3rd optimization, ie optimal titration is found in the titration of 1µM primary-25ng DNA template. The control without DNA template does not show a signal, this shows that the PCR process is sterile, but the control without primer which does not show a signal is thought to occur because the template DNA concentration used for the control is too low, ie 25ng. Thus, the base size of PCR products on the first detection of the 4th optimization cannot be identified, to detect them the second detection will be carried out.

b) Second detection of the optimization of the 4 PCR techniques with program A against *Chelonia mydas* mtDNA sequence

Detection of the second PCR optimization product 4 was done through agarose gel electrophoresis 2%. Electrophoresis is run with a power of 95volt for 36 minutes, according to.¹¹ Detection is carried out through agarose gel 2%, with the aim of obtaining a perfect distribution of DNA markers. It is known that DNA markers have migrated well, but all PCR products and 2 controls are not visible (undocumented). Control without blank DNA template shows PCR job sterility, but control without empty primer shows the low DNA template concentration used. The undetectable PCR product was thought to be due to the small amount of amplicon contained in the PCR product, because it had been used for previous detection. In addition, there may be instability in the PCR process preparation technique. So that the PCR product does not enter completely into the gel column. To overcome this, the PCR program will be done again.

c) Test the quality of DNA (Promega) markers Before re-optimization, testing of new DNA markers with the same brand and size as the old DNA marker was carried out. DNA markers were long replaced because the conditions were not good, ie migration was not parallel to PCR products and was not well fragmented. The marker DNA quality test was carried out by running the 4th optimization PCR product (titration of 1 µM primary-25ng DNA template and titration of 1 µM primer-50ng DNA template), as well as

the old DNA marker and new DNA marker through 2% agarose gel electrophoresis. As many as 5 μ L DNA markers are used in accordance with the procedure for using DNA markers.

Detection results in Fig. 15 show that both DNA markers are well distributed, and 500bp size DNA markers are clearly visible and can be used as a reference. The titration band 1 μ M primer-50ng DNA template appears as a very thin smear, so the band tends to be invisible. While the titration band 1 μ M primary-25ng DNA template shows a signal, but it is not well distributed. Optimal PCR products on the 4th optimization through DNA marker quality testing have not been detected. However, it is known that the quality of new DNA markers and old DNA markers is equally good.

Detection of the 4 optimizations that have been done has not given the expected results, namely the band density of PCR products is not strong enough, thus to get the optimal detection of PCR products, further optimization of PCR technique with program B.

b. Optimization of PCR technique with B program on *Chelonia mydas* mtDNA sequence

Program B PCR technique was carried out in 35 cycles, with the same PCR program stages as the PCR program stages A. Adding the number of cycles in the PCR A program was carried out to obtain more amplicons. According to Erlich (1989), the template DNA was duplicated in each PCR process cycle. By running 30 PCR cycles, 230 or one billion amplicons will be obtained, while by adding 35 PCR cycles, 235 or 30 billion amplicons will be obtained. Therefore, an increase in the number of PCR cycles is expected to increase the signal density of PCR products.

1) Optimization of 1 PCR technique with B program on *Chelonia mydas* mtDNA sequence.

a) First detection of the 1st optimization of PCR technique with B program on *Chelonia mydas* mtDNA sequence.

In the optimization of the 1st program B, the electrophoresis time was added to 46 minutes to make PCR product migration more perfect. Considering the optimization of the 4th program, electrophoresis was run with a power of 95volt for 36 minutes according to¹¹, but the optimization results showed that PCR products had not migrated completely. The results of visualization show that DNA markers have been perfectly distributed, which is perfectly distributed per 100bp. However, PCR products and controls do not show a signal at all (not documented). This is thought to occur due to the instability of the technique of inserting the sample material into the agarose gel column. Thus, it will be re-detected until the best visualization is obtained.

b) Second detection of the 1st optimization of PCR technique with B program on *Chelonia mydas* mtDNA sequence

In this electrophoresis, the running process is carried out in stages, which is 4 times running and visualizing successively. This is done so that the migration of PCR products goes perfectly and to avoid the escape of PCR products outside the gel during the running process. After the running process for the first 15 minutes is complete, the results show that the DNA marker has begun to migrate, but the PCR product has not been seen migrating from the agarose gel column, then the next stage of running is carried out. After running is completed for the next 7 minutes, the results that appear are still the same as the results in the first 15 minutes. Then running is run again for 10 minutes. The results of running the third stage indicate that the marker DNA has fully migrated but only appears as a smear. While PCR products are still not visible. Then running is run again for 6 minutes, with the aim that the PCR product fully migrates. It turns out that the results that appear are still the same as the results in previous runs. Running electrophoresis in 4 stages was carried out with a total time of 38 minutes. Thus, it is known that in the electrophoresis process for 38 minutes, PCR products and 2 controls were not detected (undocumented). It is suspected that in the second detection, the technique of inserting ingredients into the agarose gel column is unstable, so that the material does not enter completely. Thus, electrophoresis detection will be carried out again.

c) Third detection of the 1st optimization of PCR technique with B program on *Chelonia mydas* mtDNA sequence

The results of the detection showed that the marker DNA had fully migrated, and appeared as smears, a 500bp DNA marker was clearly visible because it appeared as a thicker band. Whereas PCR products have migrated but only appear as longitudinal smears, so that the size of the base cannot be identified (not documented). When compared to the third detection, it is better than the two previous detections because on the detection of the three bands, PCR products have been seen. This is thought to be caused by a better technique for inserting ingredients into the gel column, namely the tips used in the third detection are the smallest size tips (0.1-10 μ l), so that the sample material can enter perfectly and intact into the column.

In addition the control column without empty DNA showed that there was no contamination in the 1st optimization PCR process with program B., and the smears in the control column without primers showed the positivity of the template DNA used. Detection results have been seen but in the form of smears, so that PCR products cannot be identified for their size. To overcome this problem, the next electrophoresis was carried out with a larger volume of PCR products so that the band density of the PCR product was clearer.

d) The fourth detection of the 1st optimization of PCR technique with B program on *Chelonia mydas* mtDNA sequence

In this detection, to get the best results, the electrophoresis running process was carried out in three stages of time, namely by successively running the three running and visualizing processes. In the first 16 minutes of the running process, the DNA marker has appeared to migrate but the PCR product has not been seen, then the running process is run again. After the next 20 minutes, it was found that DNA markers and PCR products had appeared in the form of smears. Because PCR products have not migrated as a whole, the running process is run again for the next 20 minutes. The results obtained with a total time of 56 minutes is that the marker DNA signal and the entire PCR product signal have been seen (no documentation) but in the form of a smear. In addition, the control column without an empty DNA template showed that there was no contamination in the PCR process and the signal on the control without primer in the form of a smear showed the positivity of the DNA template used. DNA marker signals and all PCR products that appear in the form of smears are thought to occur because the amplicon concentration contained in PCR products has decreased. Thus, to obtain sensitive detection of PCR products sensitively, it will be re-detected by polyacrylamide gel electrophoresis.

e) Detection of the fifth optimization of the 1st PCR technique with B program on *Chelonia mydas* mtDNA sequences

The fifth detection was carried out through 10% polyacrylamide electrophoresis in order to get a clearer PCR product band appearance. It is known that DNA markers have been fragmented well. Two controls did not show a band, the control column without an empty DNA template showed that the PCR process was sterile. But the control column without an empty primer shows the low DNA template concentration used. The PCR product band appears to be a thin smear so it tends to be invisible. Not yet known the primary concentration and DNA template titration that can produce optimal PCR products. When referring to PCR product signals that often appear in the form of smears there is a presumption that the quality of the DNA template stock used is not good. In addition, the inventory of the 1st optimization PCR product has been depleted due to the use of five detection times. To overcome this, PCR optimization will be done again using a new DNA template stock with the same sample material.

2) Optimization of the two PCR techniques with B program on *Chelonia mydas* mtDNA sequences

The second optimization with program B, is done by using a new DNA template stock with the same sample material. In this optimization, the PCR process is run with complete titration and 2 pieces of control.

PCR products were detected by polyacrylamide 10% gel electrophoresis with the aim to obtain a clearer band density. Detection results that the entire band of PCR products is parallel, and is below the 500bp DNA marker. Thus it can be seen that PCR products are about 384bp in size, which is in accordance with the length of the TCR sequence which can be

amplified with primers TCR 5 and TCR 6.² Control without DNA does not indicate a signal, this indicates that the PCR process has been carried out in a sterile state. while controls without primers that do not show signals, this is thought to occur because the DNA concentration of the template used is too low. Detection results showed that the titration of 0.25 μ M primary-25ng DNA template and 0.25 μ M primary-50ng DNA template showed a lower density when compared with a titration of 0.5 μ M primary-25ng DNA template and 0.5 μ M primary-50ng DNA template. Whereas a titration of 0.5 μ M primer-25ng DNA template and 0.5 μ M primary-50ng DNA template showed the same density as titration of 1 μ M primary-25ng DNA template and 1 μ M primary-50ng DNA template.

Based on the consideration of the efficiency of the experimental material, it can be concluded that the optimal titration of the TCR sequence PCR technique is in the titration of 0.5 μ M primary-25ng DNA template, with a PCR cycle of 35 cycles.

5. Detection of nucleotide sequences of TCR sequence PCR products by sequencing method

The reading of the nucleotide sequence on PCR products from the TCR sequence was carried out automatically using ABI 3130x1 Genetic Analyzer. The reading of the nucleotide sequence was carried out on the merits of both the Eijkman Molecular Biology Institute. To obtain all the information on the nucleotide sequence of the TCR sequence of PCR products, the nucleotide sequence reading technique was carried out in two directions. That is from the forward direction using TCR 5 primer and from reverse direction using TCR 6 primer.

The sequence of TCR nucleotide sequences that have been successfully read has a size of 399bp for forward direction and 405bp for reverse direction. The reading in the reverse direction gives pretty good results, while the reading in the forward direction gives unfavorable results, that is, it contains more unidentified nucleotides.

The expected results of this reading are along \pm 384bp for the TCR sequence.² The TCR sequence base size of PCR products is not suitable because in the nucleotide sequence the results of the readings are that there are many unidentified nucleotides which are indicated by the 'N' notation on the readings. Nucleotides that are not identified occur when there is a wave of noise on a site that results in disruption of the computer's color radiation capture process, so that the computer cannot identify the type of nucleotide on the site. Therefore, it must be done manually editing the results of readings that have been obtained using a computer program.

The nucleotides that have not been identified from the results of these readings were identified by referring to 24 TCR *Chelonia mydas* sequences that have been registered in the genebank (<http://www.ncbi.nlm.nih.gov>), Using the BioEdit Sequence Alignment Editor 3.0 program, (Installshield Corporation Inc.). The result of the edited reading for identification is the reading that contains at least 'N', which is the result of reading the reversed direction nucleotide sequence whose nucleotide sequence has been complemented first.

The complete sequence of nucleotide TCR sequences has been obtained through the editing process. In the editing process, it was found that there was a lot of noise that interposed between the TCR sequence of PCR products. After the insertion of 'N' was removed from the nucleotide sequence of the TCR sequence, it was found that the nucleotide sequence matched between the nucleotide of the TCR sequence of the PCR product and the reference TCR sequence. In accordance with Trimarsanto, H.'s explanation, through direct communication (September 14, 2006), it was found that the TCR product base sequence size of PCR products was longer because it received insertion of noise in the form of unidentified nucleotides ('N') which could be derived from contaminants in PCR material products sample sequencing. Contamination is thought to occur because PCR products used as sequencing sample material are PCR products combined from 6 primary concentration titrations and DNA templates. This is done because of the limited volume of PCR products.

The results obtained from the editing process are in the form of nucleotide sequences of TCR sequences which are complete forward directions that have a size along 383bp. This size is still in the range of TCR sequence base lengths that have been used as molecular research markers, ie between 380 to 384bp.¹³ Most of the nucleotide sequences of TCR sequences of optimal PCR products have the same sequence as the nucleotide sequence of TCR sequences of 24 *Chelonia mydas*. In the nucleotide sequence data of the TCR sequence of PCR products, there are several nucleotides that are different from the reference TCR sequence. In accordance with this is an mtDNA genetic variation among individuals of green turtles (*Chelonia mydas*). Thus *Chelonia mydas* mtDNA sequences along 383bp have been isolated successfully.

Detection of nucleotide sequences of TCR sequence PCR products by PCR method

The PCR process is run as much as 35 cycles using complete titration and 2 controls. Furthermore, PCR products were detected by 10% polyacrylamide gel electrophoresis. From the detection results in Figure 2, it is known that the entire band of PCR products is parallel, and is below the 500bp DNA marker. Control without DNA and controls without primers does not indicate a signal. It is known that the titration of 0.25 μ M primary-25ng DNA templates and the titration of 0.25 μ M primary-50ng DNA templates shows a lower density when compared with titration of 0.5 μ M primary-25ng DNA templates and titration of 0.5 μ M primary-50ng DNA template. Whereas a titration of 0.5 μ M primary-25ng DNA template and titration of 0.5 μ M primary-50ng DNA template showed the same density as titration of 1 μ M primary-25ng DNA template and titration of 1 μ M-50ng DNA template.

The reading of the nucleotide sequence on PCR products from the TCR sequence was carried out automatically using ABI 3130x1 Genetic Analyzer. The reading of the nucleotide sequence was carried out on the merits of both the Eijkman Molecular Biology Institute. To obtain all information about the nucleotide sequence of the TCR sequence of PCR

products, the reading technique uses TCR 5 primer and reverse direction using TCR 6 primer.

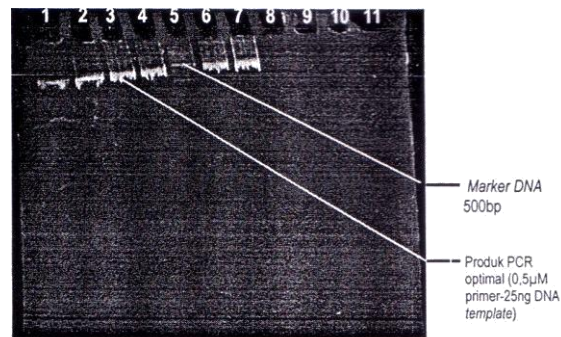


Figure 3. PCR products from PCR sequence detection of TCR..

Description: 1st column titration 0.25 μ M primary-25ng DNA template; The 2nd column titrates 0.25 μ M primary-50ng DNA template; The 3rd column titrates 0.5 μ M primary-25ng DNA template; The 4th column titrates 0.5 μ M primary-50ng DNA template; 5th column DNA marker; The 6th column is the titration of 1 μ M primary-25ng DNA template; The 7th column is the titration of 1 μ M primary-50ng DNA template; The 8th column controls without DNA templates; The 9th column controls without primers. The 10th and 11th columns are not filled.

The sequence of TCR nucleotide sequences that have been successfully read has a size of 399bp for forward direction and 405bp for reverse direction. The reading in the reverse direction gives pretty good results, while the reading in the forward direction gives unfavorable results, that is, it contains more unidentified nucleotides.

The expected results of this reading are along \pm 384bp for the TCR sequence.² The TCR sequence base size of the PCR product is not suitable because in the sequence of nucleotide sequences it is done in two directions of reading. Nucleotides from the readings there are many nucleotides that are not identified as indicated by the notation 'N' in the reading. Nucleotides that are not identified occur when there is a wave of noise on a site that results in disruption of the computer's color radiation capture process, so that the computer cannot identify the type of nucleotide on the site. Therefore, it must be done manually editing the results of readings that have been obtained using a computer program.

The nucleotides that have not been identified from the results of these readings were identified by referring to 24 TCR *Chelonia mydas* sequences that have been registered in the genebank (<http://www.ncbi.nlm.nih.gov>), Using the BioEdit Sequence Alignment Editor 3.0 program, (Installshield Corporation Inc.). The result of the edited reading for identification is the reading that contains at least 'N', which is the result of reading the reversed direction nucleotide sequence whose nucleotide sequence has been complemented first.

The complete sequence of nucleotide TCR sequences has been obtained through the editing process. In the editing process, it was found that there was a lot of noise that interposed between the TCR sequence of PCR products. after the insertion of 'N' was removed from the nucleotide sequence of the TCR sequence, it was found that the nucleotide sequence matched between the nucleotide of the TCR sequence of the PCR product and the reference TCR sequence. In accordance with Trimarsanto, H.'s explanation, through direct communication (September 14, 2006), it was found that the TCR product base sequence size of PCR products was longer because it received insertion of noise in the form of unidentified nucleotides ('N') which could be derived from contaminants in PCR material products sample sequencing. Contamination is thought to occur because PCR products used as sequencing sample material are PCR products combined from 6 primary concentration titrations and DNA templates. This is done because of the limited volume of PCR products.

The results obtained from the editing process are in the form of nucleotide sequences of TCR sequences which are complete forward directions that have a size along 383bp. This size is still in the range of TCR sequence base lengths that have been used as molecular research markers, ie between 380 to 384bp.¹² Most of the nucleotide sequences of TCR sequences of optimal PCR products have the same sequence as the nucleotide sequence of TCR sequences of 24 *Chelonia mydas*. In the nucleotide sequence data of the TCR sequence of PCR products, there are several nucleotides that are different from the reference TCR sequence. In accordance with³, this is an mtDNA genetic variation among individuals of green turtles (*Chelonia mydas*). Thus *Chelonia mydas* mtDNA sequences along 383bp have been isolated successfully.

1	TAGCATATGA	CCAGTAATGT	TAACAGTTGA	TTTGGCCCTA	AACATGAAAA
51	TTATTGAATC	CACATAAATA	TTTTAATAAC	ATGAATATTA	AGCAGAGAAT
101	TAAAAGTGAA	ATGATATAGG	ACATAAAATT	AAACCATTAT	ACTCAACCAT
151	GAATATCGTC	ACAGTAATTG	GTTATTTCTT	AATTAGCTAT	TCACGAGAAA
201	TAAGCAACCC	TTGTTAGTAA	GATACAACAT	TACCAGTTTC	AGGCCCATTT
251	AGTTTATAGC	GTACATAACC	TGATCTATTC	TGGCCTCTGG	TTGTCTTTTC
301	AGGCACATAC	AAATAGTAAC	GTCCATTOGT	TCCTCTTTAA	AAGGCCCTTG
351	GGAATGAGT	TCTTACATF	TAAATTTTAA	CCC	

Figure 4. The results of editing the data of the *Chelonia mydas* mtDNA sequence nucleotide sequences along 383 mer.

REFERENCES

- [1]. Myoglobin expression in *Chelonia mydas* brain, heart and liver tissues R Puspitaningrum, SI Wanandi, RR Soegianto. . . - HAYATI Journal of Biosciences, 2010
- [2] Moritz, Craig., et al..2003. *Genetic Analysis of Marine Turtle Populations*. QIAGEN News. <http://A:/QIAGEN literature.htm..03/06/05> pukul 14.00 WIB.
- [3] Fitzsimmons, Nancy., et al.. 1997. *Philopatry of Male Marine Turtle Inferred from Mitochondrial DNA Markers*. Proc.Natl. Acad. Sci. vol. 94. pp.8912-8917 United States of America
- [4] Moritz, Craig., et al..2002. *Population Genetics of Southeast Asian and Weastern Pasific Green Turtles, Chelonia mydas*. Australia: Final Report of UNEP/CMS.
- [4] Yusuf., Akil., et al /.. 2000. *Mengenal Penyus*. Jakarta: Yayasan Alam Lestari.
- [5] Prambudi, S., et al 2000. *Penggunaan Teknik-teknik Genetika dalam Upaya Konservasi Penyus Hijau (Chelonia mydas)*. Makalah. Depok: Jurusan Biologi FMIPA Universitas Indonesia
- [6] Novak, Richard. 2003. *Efficient Purification of DNA from Turtle QIAGEN News*. <http://A:/QIAGEN literature.htm..03/06/05> pukul 16 WIB.
- [7] Puspitaningrum, R. 1998. *Isolasi Fragmen DNA yang Mengandung Elemen Promotor Gen dan Gen Penyandi Alfa-Fetoprotein (AFP) Tikus dengan Menggunakan Teknik PCR*. Tesis. Program Studi Ilmu Biomedik- Program Pascasarjana Fakultas Kedokteran Universitas Indonesia. Tidak dipublikasikan.
- [8] Erlich, A. Hendry. 1989. *PCR Technology; Principles and Applications for DNA Amplification*, United States of America: Stockton Press.[9] Nuovo, Gerard, J.. 1992. *PCR In Situ Hybridization, Protocols and Applications*. New York: Raven Press.
- [10] Sambrook, J., Fritsch, E.F., and Maniatis, T.. 1989. *Molecular Cloning Laboratory Manual. Second edition, volume 2*. New York: Cold Spring Harbour Laboratory Press.
- [11] Paramita, Reni., *Mutasi Gen Human Voltage Dependent Anion Channel (hVDAC3) Exon 8 pada Sperma Mortalitas Rendah Pasien Astenozoospermia*, Tesis, 2006, Program Studi Ilmu Biomedik - Program Pascasarjana Fakultas Kedokteran Universitas Indonesia, Jakarta..
- [12] Witzell, Wayne, N. et al.. 2000. *Origin of Immature Loggerhead Sea Turtle (Caretta caretta) at hutchinson Island, The Evidence from mtDNA Markers*. http://www.google.q=cache:3v_0va6h6cAJ:fishbull.gov. 11/5/2006 pukul 17.00 WIB.
- [13] PUSPITANINGRUM, R.I.N.I., MANTOLINI, K.N., RUSDI, SADIKIN, M. Comparison of Hemoglobins from Various Subjects Living in Hypoxia.(2013) HAYATI Journal of Biosciences, 20 (3), pp. 99-104
- [14] PUSPITANINGRUM, R.I.N.I., WANANDI, S.I., SOEGIANTO, R.R., SADIKIN, M. Blood Gasses Contents of Green Turtle (*Chelonia mydas*) Hatch Treated by Different Temperatures (2011) HAYATI Journal of Biosciences, 18 (3), pp. 147-150
- [15] SADIKIN, M., PUSPITANINGRUM, R.I.N.I., WANANDI, S.I., SOEGIANTO, R.R..Isolation and Characterization of *Chelonia mydas* Myoglobin. (2012) HAYATI Journal of Biosciences, 19 (2), pp. 60-64
- [16] PUSPITANINGRUM, R.I.N.I., WANANDI, S.I., SOEGIANTO, R.R., SADIKIN, M., WILLIAMS, D.R.,
- [17] COSSINS, A.R. Myoglobin Expression in *Chelonia mydas* Brain, Heart and Liver Tissues