UNIVERSITY OF NAMIBIA

MOLECULAR DELINEATION BETWEEN *MERLUCCIUS CAPENSIS* AND *M. PARADOXUS* USING MITOCHONDRIAL DNA SEQUENCES

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DECLARATION

"I hereby declare that this work is the product of my own research efforts, undertaken under the supervision of Mr. Lineekela Kandjengo and has not been presented elsewhere for the award of a degree or certificate. All sources have been duly and appropriately acknowledged."

Signature…….. Date……

Moses S Kalola

Certification

"This is to certify that this report has been examined and approved for the award of the degree of Bachelor of Science (Honours) in Fisheries and Aquatic Sciences of the University of Namibia"

Signature……

External examiner

Signature……..

internal examiner

Signature…….

Supervisors

Signature…….

Head of Department

Acknowledgement

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Abstract

The use of DNA-based methodologies in identification of hake species belonging to the Merluccius genus proved to be successful. DNA was extracted using the Qiagen kit and an amount of 20 samples for each species were used for this study and as such the extraction was successfully done. DNA Bands of both Merluccius capensis and M.paradoxus were obtained after the extraction with smears of RNA at the bottom of the gel. Furthermore, the hake-specific PCR product, due to its limited size, was obtained in a variety of tissue samples with different levels of DNA concentration and degradation. Analysis includes PCR products that were amplified with primers of 12s rRNA that were also visible from their respective wells. Species identification by phylogenetic analysis of sequences or by PCR methodologies is useful in a variety of scenarios including species determination of individuals whose morphological characters are removed.

CHAPTER 1

1. Introduction

The Hake fishery is one of the most important commercial fish resources in Namibia in terms of revenue earnings and employment creation. Two sympatric species of hake, *Merluccius capensis* and *M. paradoxus,* (-also known as the shallow- and deep-water hake respectively) inhabits the coastal waters of southern Africa and support a large bottom trawl fishery. The former species generally inhabit continental shelf waters to a depth of 440m, whereas the latter species inhabits deeper waters from 140-830m (Kapewongolo 2005).

The geographical distributions of both fishes are closely associated with cold water of the northward flowing Benguela Upwelling System on the west coast (Botha 1980, Shannon 1985). *Merluccius capensis* is distributed between 12˚S latitude on the west coast of Africa to about 27˚ E longitude on the south coast, but *Merluccius paradoxus* has a more restricted geographic distribution between 18˚S latitude on the west coast to about 31.5˚E longitude on the south coast. These two species of southern African hakes are harvested by bottom trawl more or less continuously on the west coast from the Agulhas Bank to Walvis Bay depending upon bottom topography. Due to its greater geographic range, *M. capensis* is also fished in the northern part of Namibia and the geographic distributions of fishing effort also reflect the more or less continuous distributions of the two species (Chakra borty et al 1986).

Hakes are found close to the bottom during the day time but rise to intermediate waters during the night time, probably following their prey. Scientists were only able to differentiate the two species according to differences in the numbers of gill rakers and on the presence and absence of prominent pigment spots on tip of gill tubercles. *Merluccius capensis* have 15-20 gill rakers but

they do not have any prominent pigmentation on the tip of gill tubercles whereas *M. paradoxus* have 18-23 gill rakers but with prominent pigment spots on tip of gill tubercles.

Since it is hard to distinguish the two species phenotypically, mitochondria DNA (mtDNA) was used to precisely and uniquely identifies the individuals beyond doubt by observing the polymorphisms in DNA using relevant techniques.

The following are pictures of the hake species:

Figure 1: *Merluccius capensis*

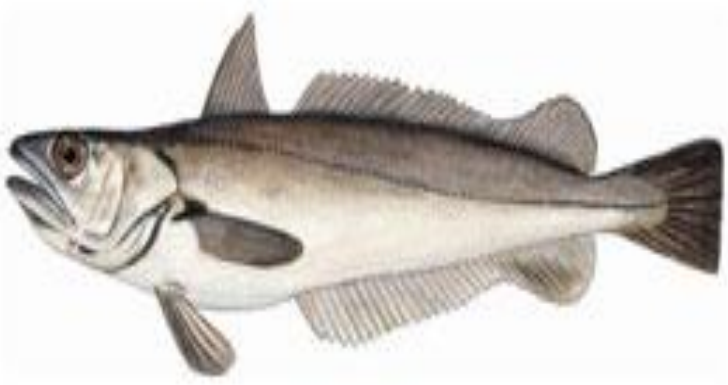

Figure 2: *Merluccius paradoxus*

Literature Review

During the past decade, geneticists have used endonucleases rather than sequencing to examine variation within and between species in specific segments of mitochondrial DNA (Grant et al. 1987). Although the indirect assessment of sequencing variation obtained with the restriction endonucleases method is known to have many drawbacks, sequence data have been difficult to obtain (Livia et al 2006). The construction and screening of clone libraries demanded too much expertise for routine use by those geneticists and taxonomists who must analyze many individual (Grant et al. 1987).

Several methods have been employed to measure the amount of relatedness between stocks. Tagging is one method, which yields direct evidence of migration (Haugen et al. 1969), but cannot be applied to hakes because few fish survive the transit to the surface (Jones 1974). Other approaches are to define stocks (e.g. salmon) by geographic differences in body morphology or meristic characters or by the analyses of otoliths (Ihssen et al. 1981). But these methods depend upon finding pronounced environmental differences between areas, a situation that does not occur in the range of southern African hakes (Ihssen et al. 1981). None of these techniques, however, yields unequivocal data on the genetic relatedness between stocks. There have been few genetic studies of other species of hake in the genus *Merluccius*. One electrophoretic study of the Pacific hake (*M. productus*), however, demonstrated the presence of allele-frequency differences between populations located in Puget Sound, a large marine embayment, and offshore oceanic populations (Utter et al. 1970). Another study of the North Atlantic hake (*M. merluccius*), however, failed to detect any genetic differentiation among populations.

Dependence on restriction analysis has limited the understanding of the dynamics of DNA sequence evolution. The presence and absence of a restriction site reveal little about the kinds of nucleotide substitutions that have occurred. Thus, although restriction analysis of mtDNA from closely related mammals first showed that these genomes have a higher rate of evolutionary substitution than does nuclear DNA. The demonstration is that these acceleration results mainly from an increase in the numbers of transitions relative to transversions come only from conventional cloning and sequencing (Ihssen et al. 1989).

The use of appropriate sampling methods, the type of tissue and the use of feasible protocols for DNA extraction are crucial aspects of studies based on PCR. In fish, the use of fins, scales, buccal cells (Livia *et al*., 2006), ovules and muscle (Chakraborty *et al*., 2006) requires several protocols, micro-wave, liquid nitrogen, phenol-chloroform and salt protocols have been widely used. Extraction protocols using phenol-chloroform extraction have been the most frequently used methods to obtain fish genomic DNA. These protocols produce good results for samples of diverse origins. However, it is a slow, time consuming and contaminant-producing method.

The DNA extraction protocol using salt (NaCl) is a simple, easy, fast and non-contaminant alternative for obtaining good quality DNA in sufficient quantities from fish tissue samples (Nelson et al. 2008).

Because most studies of animal mtDNA have used restrictions analysis, it has been difficult to determine whether a high rate of evolution and a transition bias are characteristic of all animal mtDNA. Since then there has been a need for simple methods of sequencing mtDNA to examine the pattern of evolutionary substitution in other animals groups. A fast alternative of conventional cloning emerged in the form of the Polymerase Chain Reaction (PCR).

2. Problem statement

The problem to be addressed by this research project is that due to difficulties in distinguishing these two species of hake (although showing some differences phenotypically), it could result in depletion of one species while leaving the other unharmed. For the past few years after independence the Ministry of Fisheries and Marine Resources has been allocating quotas to the fishing companies but yet with no in depth understanding of which is abundance and which is not, posting threat and risk of over-exploitation to the species. A detailed mitochondrial DNA will therefore be used to precisely and uniquely identify the individual beyond doubt by observing the polymorphisms in DNA using the relevant techniques. If this problem (generally) remains unsolved, it will result in over-exploitation (of one species or both) and eventually causing the fishery collapse in the long run as is the case with some of the commercially important species which have been over-exploited in the late 1970s and are showing no sign of recovery to-date. As a result this will mean hunger to most people whose livelihood is depended primarily on fishing activities; reducing revenue landing to Gross Domestic Products (GDP) at the state level as well as exacerbating the unemployment situation in the country since the fishing industry employs more people.

3. Research objectives

The main objective of this research is:

- To determine differences in mitochondrial DNA sequences of the two hake species, *M. capensis* and *M. paradoxus* in order to develop a DNA diagnostic tool for detection of the two species.

4. Hypothesis to be tested

Specifically, the following hypothesis will be tested by the research:

- H0: It is not possible to distinguish two species of hake using variations that occur in the mitochondria DNA
- H1: It is possible to distinguish two species of hake using variations that occur in the mitochondria DNA

CHAPTER 2

5. MATERIALS AND METHODS *(Study design/laboratory analysis)*

5.1 DATA COLLECTION

Since the Ministry of Fisheries and Marine resource hold annual hake biomass surveys to estimate the biomass of Hakes and that of other commercially important Namibian fish species, whole fish samples of both Merluccius capensis and M. paradoxus was donated by the National Marine Institute and Research Centre of the Ministry of Fisheries and Marine resources in Swakopmund. These samples are of fish collected from coastal waters extending from Orange River in southern Namibia all the way to the Kunene river open mouth in the northern coast of Namibia.

5.2 DNA EXTRACTION

DNA was extracted using the Qiagen kit. The kit contains Genomic DNA Lysis solution, RNase free water solution, Coral load concentration, Primers, Protein Precipitation Buffer, Top Tag Master Mix, DNeasy mini spin column placed in a 2ml collection tube and DNA Hydration solution. An amount of 20 samples for each species were used for this study and as such the extraction was successfully done in steps as follow:

Things that were done before starting

- Buffer ATL and Buffer AL forms precipitate upon storage and as a result it was warmed to 56ºC until the precipitates have fully dissolved.

- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, an appropriate amount of ethanol (96-100%) as indicated on the bottle was added to obtain a working solution.
- A shaking water bath (with a thermometer attached) was heated to 56ºC for use in step 2.

Step 1

Tissue samples of 25mg were cut from each fish and were transferred to a mortar where liquid nitrogen was added to enable more efficient lysis, and samples were ground to pure powder using a pestle. (Note that all the equipments and some of the reagent used in this study were all completely sterilized prior to DNA extraction). Tissue samples were then placed in a 1.5ml microcentrifuge tube and 180µl of buffer ATL was added.

Step 2

An amount of 20µl proteinase K was added to the samples in step 1 and was mixed thoroughly by vortexing and was incubated at 56ºC until the tissue is completely lysed. The mixture was placed in a shaking water bath and stayed overnight in order to complete the lysis process. The sample was then placed on ice until the next step.

Step 3

From the mixture above on the next day, the samples were vortexed for 15seconds and 200µl Buffer AL was added and again mixed by vortexing. Ethanol (200µl at 96-100%) was also added

and mixed again thoroughly by vortexing. It's essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogenous solution. They were premixed and added together in one step to save time since multiple samples were processed.

Step 4

The mixture from step 3 (including any precipitation formed) was pipeted into the DNeasy Mini spin column placed in a 2ml collection tube. It was then centrifuged at 6000 x g for 1 minute. Discard flow-through and collection tube.

Step 5

The DNeasy Mini spin column from step 4 was placed in a new 2ml collection tube and 500µl Buffer AW1 was added. It was centrifuged for 1 minute at 6000 x g and the flow through as well as the collection tube were discarded

Step 6

DNeasy Mini spin column from the above step was again placed in a new 2ml collection tube and 500µl Buffer AW2 was added and centrifuged for 3 minutes at 20,000 x g in order to dry the DNeasy membrane. The flow through as well as the collection tube were discarded.

NB: During this step it's important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. Further centrifuge for about 2minutes was done to ensure that no residual ethanol will be carried over during the elution process.

Step 7

DNeasy Mini spin column was placed in a clean 1.5ml microcentrifuge tube and 100µl Buffer AE was pipeted directly onto the DNeasy membrane. It was incubated at room temperature for 1 minute and centrifuged for 4 minutes at 6000 x g to elute. Elution with 100µl Buffer AE and not 200µl (as indicated in the protocol) increases the final DNA concentration in elute and this step should be repeated once for maximum DNA yield.

DNA STORAGE

After all the above steps were fully completed, the final DNA concentration was stored at 3℃ in a fridge. This is done to keep the DNA in a cooler place in order to maintain their quality before analysis them on Agarose gel.

Analysis of extracted mtDNA on Agarose gel

Visualizing and analysis was done with the aid of the Agarose gel as follow;

An amount of 1g of Agarose and 100ml of distilled water was added to a 250ml flask and boiled in a microwave until it had completely dissolved. To the flask, 2ml of 50XTAE electrophoresis buffer was also added. The gel solution was then cooled to room temperature by swirling under running water followed by the addition of 5µl of ethidium bromide.

The flask was then swirled to mix the reagent. The warm Agarose gel was then poured in a gel tray with a well former (comb). The comb was gently removed from the gel and the electrophoresis tank was filled with 1xTAE buffer to submerge the gel for at list 1cm. With the use of a pipetman, 5µl of the extracted mtDNA was then transferred into an epi-tube, where 3µl of loading dye was added. The sample was then mixed with the aid of the pipetman and 5µl of DNA ladder and the 8µl mixture of loading dye and DNA template were then pipeted into their respective wells. The gel was then run on a power pack at 90V for 45 minutes. Although the sample from the first four DNA samples did not show positive results, after several extraction the DNA band were visualized on a gel documentation system (microscope) and pictures of the gel were taken.

CONJUGATION PREPARATION FOR PCR

The following are the steps that were followed prior to PRC preparation:

Step 1

The primer solutions and template DNA were mixed well before use. This was done such that 2.5µl of each primer was added to 2.5µl DNA template.

Step 2

The Top Tag Master Mix was mixed well by vortexing and 25µl was dispensed into each PCR tube. It is of great importance to mix the Top Tag Master Mix before use in order to avoid localized concentrations of salt. It is not necessary to keep reaction vessels on ice since Top Tag

Master Mix exhibits significantly reduced polymerase activity at room temperature due to the unique buffer formulation.

Step 3

An appropriate volume of 2.5µl amount of diluted primers was pipetted into each PCR tubes containing the Master Mix.

Step 4

To the mixture above, 5µl of 1x Coral Load concentration and 10µl of RNase-free water was added to the individual PCR tubes.

Step 5

An amount of 2.5µl of each different template DNA was added to the individual PCR tubes. The PCR tubes were placed in the thermal cycler and the cycling program was started as following.

5.3 STATISTIC ANALYSIS

The DNA sequences obtained from this study were supposed to be analyzed using Bioinformatics tools. Unfortunately due to some technical difficulties encountered during this study, it was not carried out as such. In bioinformatics, Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold (Utter et al.1971). To run, BLAST requires a query sequence to search for, and a sequence to search against (also called the target sequence) or a sequence database containing multiple such sequences. BLAST will find subsequences in the database which are similar to subsequences in the query.

POLYMERASE CHAIN REACTION (PCR)

The table below shows the mitochondrial DNA primers that were used for the PCR.

Table 1: Mitochondrial DNA primers.

PRIMERS	MITOCHONDRIAL NUMBERS	ENCODED SEQUENCE	ANNELING Temperature˚C
Cytochrome b	Mit 1	AAAAAGCTTCCATCCAACAT CTCAGCATGATGAAA	65
	Mit 2	AAACTGCAGCCCCTCAGAAT GATATTTGTCCTCA	68
12s rRNA	Mit 3	AAAAAGCTTCAAACTGGAT TAGATACCCCACTA	66
	Mit 4	TGACTGCAGAGGGTGACGGG GTAAACC	73
Control region	Mit 5	TCAAAGCTTACACCAGTCTT GTAAACC	63
	Mit 6	CCCAAAGCTAAAATTCTAA	51
	Mit 7	TAACTGCAGAAGGCTAGHAC CAAACCT	66

The following table shows amplification of DNA fragments using the polymerase chain reaction (PCR) which was performed in a thermal cycler, by adding the following reagents/components to a 0.2 ml thin walled tube.

Table 2: DNA Amplification

Component	Volume
RNase-free water	7.5µl
Primers	2.5µl
TopTaq Master mix, 2x	25µl
mtDNA	2.5µl
CoralLoad Concentrate, 10x	5µl

Table 3: Polymerase chain reaction (PCR) cycling program

	Cytochrome b primer	12rRNA
Mitochondrial number	Mit 1 and Mit 2	Mit 3 and Mit 4
Denaturation	94℃ for 1 minutes	94℃ for 1 minutes
annealing	65℃	66℃
Extension	72℃ for 2 minutes	72℃ for 2 minutes
Number of cycles	30 cycles	30 cycles
Final Extension	5 minutes at 72℃	5 minutes at 72℃

Sequencing

The amplified PCR products were both visualized with the aid of Agarose gel and after amplification; samples were stored overnight at 2-8℃. After storage, the PCR products were sent to South Africa for sequencing which would have given an idea of the polymorphisms that exists between the two species but due to some delay in transportation, payments and other unforeseen circumstances beyond our control, sequencing results were not received at the time of writing up this report and as a result no any sequencing analysis could take place.

CHAPTER 3

6. Results

Figure 1 shows only four samples that were loaded and run on the gel. They were the first four samples that were extracted using the Qiagen kit. As it is visible from the picture above, no bands were observed from the wells.

Ladder 1 2 3 4

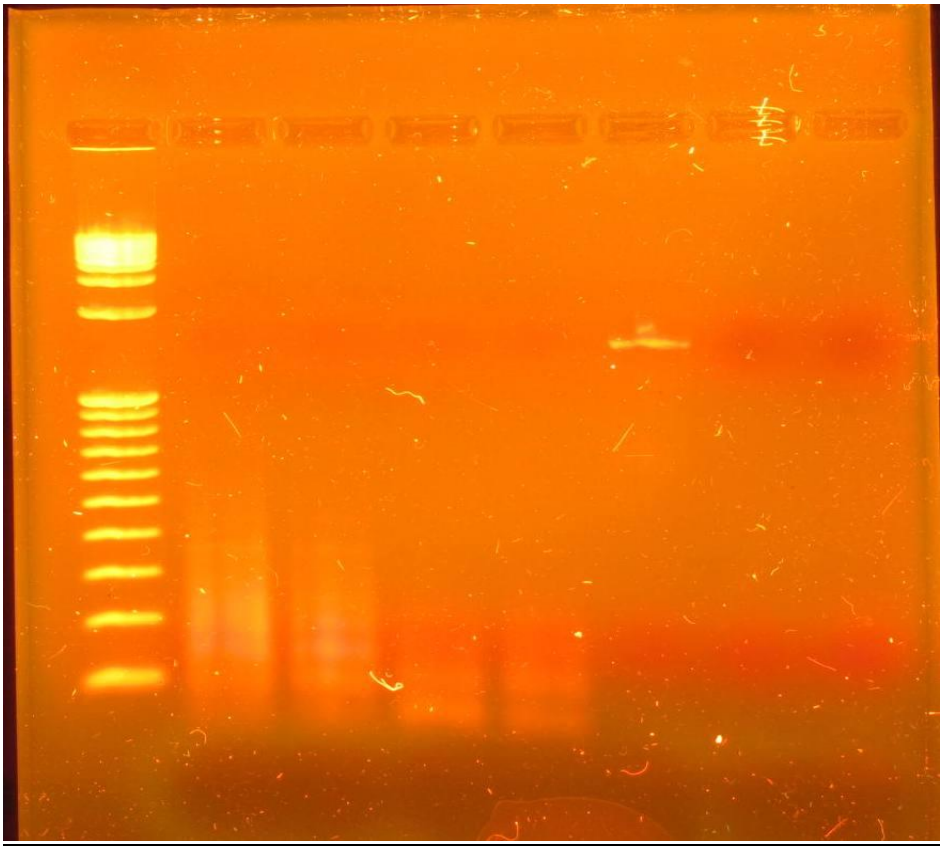

Figure 1: First four samples of DNA loaded.

Figure 2 also shows DNA bands that were observed from 10 samples loaded in the gel, although with a slight difference in the quality of the bands and the reason was being that the running time was probably short.

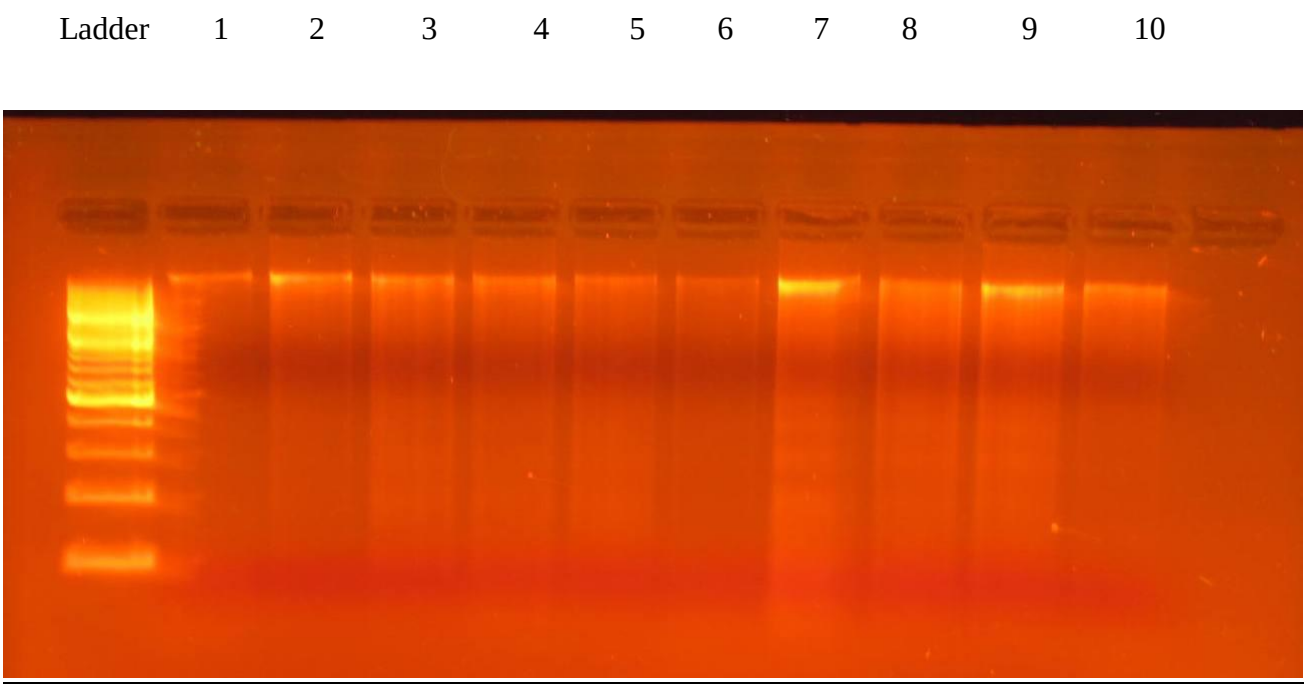

Figure 2: DNA bands that were observed from 10 samples.

Figure 3 below shows the DNA bands on the gel that were obtained after the DNA extraction. The smears visible at the bottom of the gel are for RNA. The presence of the DNA bands for each sample that was run on the gel can be seen in their respective wells.

Ladder 1 2 3 4 5 6 7

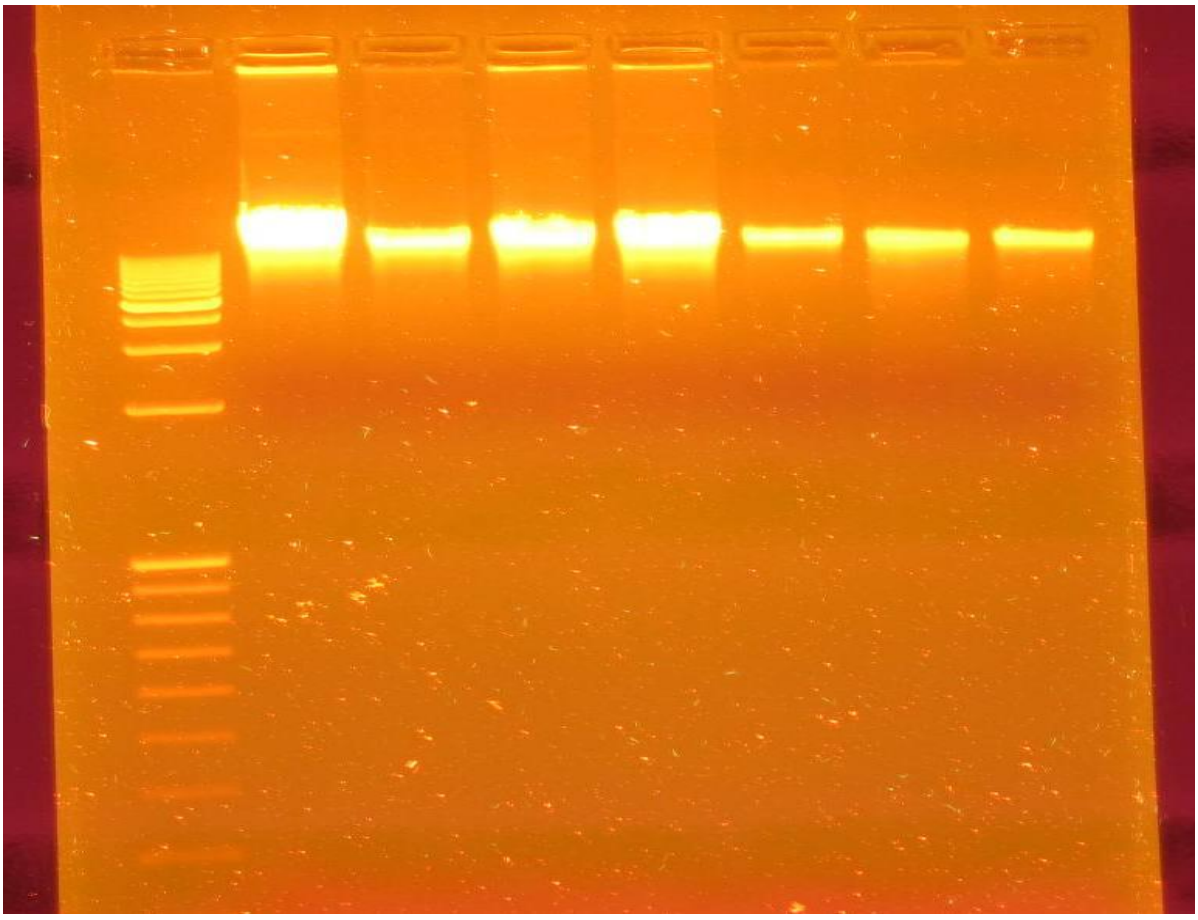

Figure 3: DNA bands visible on the gel after DNA extraction.

Figure 4 shows the gel that was loaded with PCR products of samples1, 2, 3, and 4 which were run with cytochrome b primers in their respective wells. As it can be seen from the picture above, no DNA bands were observed at all. This is to say that the DNA fragments were not amplified.

Ladder 1 2 3 4

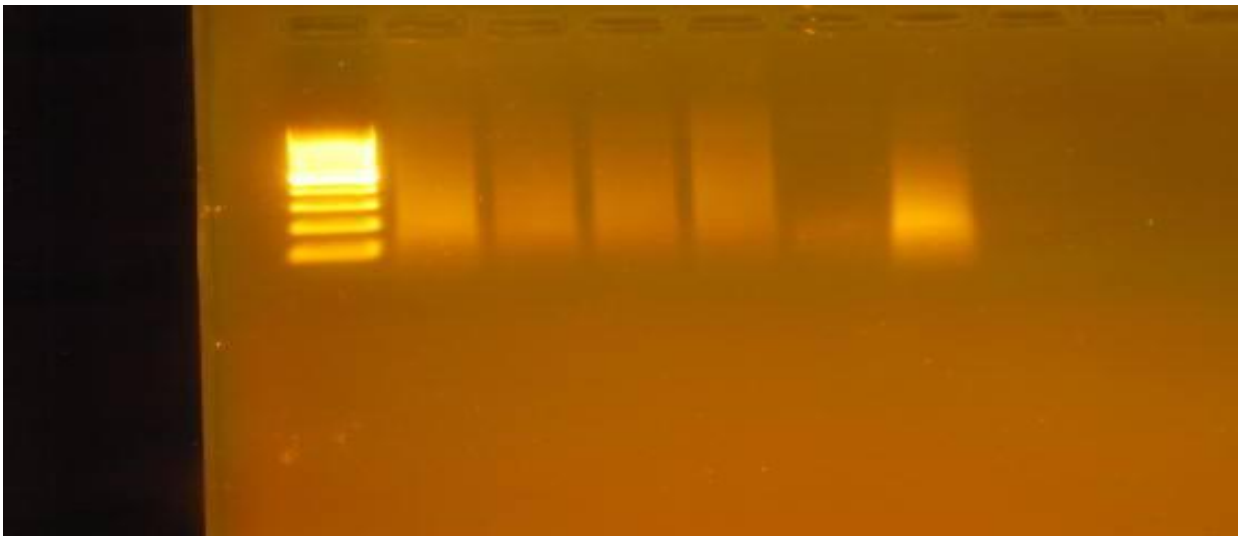

Figure 4: PCR products of samples1, 2, 3 & 4 run with cytochrome b primers.

Figure 5 below shows 19 samples of PRC products that were loaded on the gel seen from their respective wells. The bands are for the PCR products that were amplified with primers of 12s rRNA. As it can be seen from the diagram above, samples 5, 8 and 15 did not show any PCR results due to some undefined factors, but more interesting I would say almost 80% of the samples show positive results.

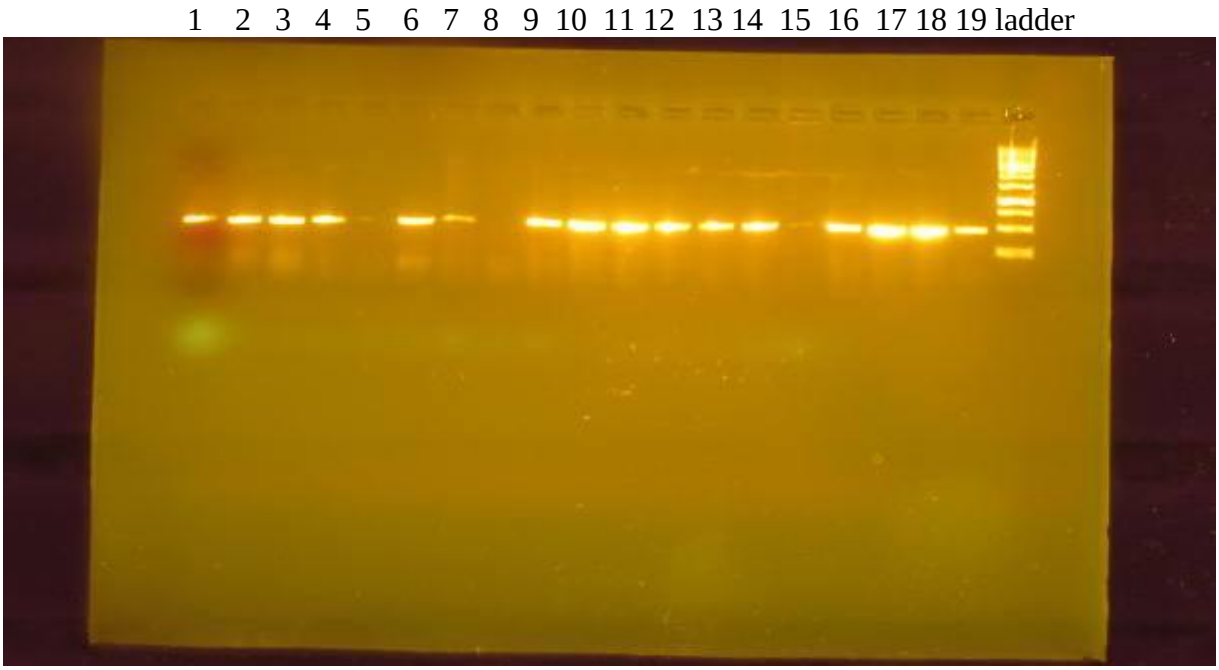

Figure 5: PCR products amplified with primers of 12s rRNA.

No DNA purification or digestion was made for this project due to the unavailability of the necessary equipments needed to carry this out.

CHAPTER 4

7. Discussion

The main aim of this study was the detection of polymorphisms in the mitochondrial DNA sequence of the two hake species but was not concluded due to the failure in getting the sequencing results and analysis. However, further discussion is on the DNA extraction and PCR results that were observed. Figure 1; no DNA band was observed during the first extraction and this was the case because the lysis time was very short (2 hours only). Having identified this, the lysis time was therefore increased to disperse the sample and was placed in shaking water bath and stayed overnight. The next two DNA extractions (figures 2&3) shows positive result and the presence of the DNA bands for each sample that was run on the gel can be clearly seen in their respective wells.

Similarly in figure 4, we are seeing the gel that was loaded with PCR products of samples1, 2, 3, and 4 which were run with cytochrome b primers in their respective wells. As it can be seen from the picture, no DNA bands were observed at all. This reason could be that there was a need to adjust a bit and play around with the PCR conditions such as the pre-denaturation and the denaturation temperature, the annealing temperature (which is depended on primers), and the Elongation time. Due to the fact that a large number of students were doing DNA extractions and all using the same PCR machine, the timing for such adjustment was a nightmare. However, Figure 5 above shows 19 samples of PCR products that were loaded on the gel seen from their respective wells. The bands are for the PCR products that were amplified with primers of 12s rRNA. As it can be seen from the diagram above, although not all the 19 samples did show PCR

results due to some undefined factors, it is more interesting that one would say almost 80% of the samples loaded have shown some positive results.

However since not any enzyme digestion were utilized in this study, or any further sequencing results were obtained, it would therefore be misleading to conclude that no polymorphisms exists in the two hake species understudy without having the sequence at hand and either testing each and every restriction enzyme. Should there be the sequencing, the analysis in today would have involved some computer programs such as BLAST which are used daily to search sequences from more than 260 000 organisms, containing over 190 billion nucleotides (Nelson et al. 2008). These programs can compensate for mutations (exchanged, deleted or inserted bases) in the DNA sequence, to identify sequences that are related, but not identical (Utter et al. 1971).

Sequences would then be aligned and phylogenetic methods will be applied to permit identification of problem samples by clustering. The unknown sequences will have to be included in a monophyletic clade with those closely related sequences belonging to the same species. Genetic distance would then have been calculated using the Tamura-Nei model and phylogenetic trees would have been constructed by the neighbor-joining method, rooted at midpoint and evaluated by bootstrapping analysis with the MEGA program. Polymorphism information will then be obtained with DNAsp program (Javier et al 2001).

8. Conclusion

In conclusion, although there was a constraint for this study, the DNA bands and PCR results obtained tells much how successful and possible is the use of mitochondrial DNA sequencing in the study to detect the polymorphisms that exists between different animals species. It is therefore clearly desirable to conduct a more comprehensive survey and also possible to imagine numerous applications using this method. Should the sequencing process carried out be successful, the sequences would have been assembled, aligned and eventually specific computer programmes used to draw the phylogenetic trees which would then provide an answer about the polymorphisms of the two hake species.

9. Recommendations

Based on the difficulties that I encountered during the research period, I would recommend that students who would consider doing genetic studies in the future should start as early as possible with their projects. This is because the process is too complex and requires time. This should be done because currently the University lacks the necessary equipment for DNA sequencing which as a result require some external support from sister Universities especially in South Africa and if not carefully monitored will result in delay in completion of projects and students becoming victims in most instances.

10. References

Botha, L. (2005). *Growth and otolith morphology of the Cape hakes, Merluccius capensis and M. paradoxus* Franca. Invest1 Rep. Div. Sea Fish. Un. S. Afr. 97: 1-32.

Chakraborty R. Haag M. Ryman N. Stahl G. (1982). *Hierarchical gene diversity analysis and its application to brown trout population data*. Academic press: New York.

Grant W.S, Leslie R.W and Becker I.I (1987). *Genetic stock structure of the southern African hakes M. capensis and M. paradoxus, Department of Microbiology*. University of Cape Town, Cape Town.

Haugen, C. W., Messersmith, J. D., Wickwire, R. H. (1969). *Progress report on anchovy tagging off California and Baja California*, March 1966 through May 1969: California

Ihssen, P. E., Booke, H. E., Casselman, J. M., McGlade, J. M., Payne, N. R., Utter, F. M. (1981). *Stock identification: materials and methods*. Fish Aquatic Science. 38: 1838-1855

Jones, B. W. (1974). *World resources of hakes of the genus Merluccius.* Sea fisheries research: New York

Javier Quinteiro, Rodrigo Vidal, Mo´nica Izquierdo, Carmen G. Sotelo, Marı´a Jose´ Chapela, Ricardo I. Pe´rez-Martı´n, Harmut Rehbein (2001*). Identification of Hake Species (Merluccius Genus) Using Sequencing and PCR-RFLP Analysis of Mitochondrial DNA Control Region Sequences*. Portugal

Kapewongolo H.P. (2005). *Dynamics of mitochondrial DNA evolution in Namibia, Merluccius capensis and M.paradoxus) Department of Biology.* University of Namibia, Windhoek

Livia, L., P. Antonella, L. Hovirag, N. Mauro, and F. Panara. (2006). *A nondestructive, rapid, reliable and inexpensive method to sample, store and extract high-quality DNA from fish body mucus and buccal cells.* Molecular Ecology Notes 6:257-260.

Nelson M. Lopera-Barrero1, Jayme A. Povh, Ricardo P. Ribeiro, Patricia C. Gomes (2008) *Comparison of DNA extraction protocols of fish fin and larvae samples, modified salt (NaCl) extraction*. Maringa, Brazil.

Shannon, L. V. (1985). *The Benguela ecosystem, Part I. Evolution of the Benguela physical features and processes.* Oceanography. Page 23: 105-182

Utter, F. M. (1971). *Biochemical polymorphisms in the Pacific hake (Merluccius paradoxus*). Int. Counc. Explore. Page 87-89.