Molecular phylogenetic structure between geographically distant marine fish *Macrourus holotrachys* and *M. berglax*

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Campus Westville, South Africa. The research was financially supported by National Research Foundation.

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ABSTRACT

The taxonomic status of *Macrourus* species has been in disarray due to their morphological similarities and overlapping distribution. Given the number of research that have been conducted in an attempt to resolve the taxonomic confusion in this group, there are still some species with unconfirmed taxonomy. *Macrourus holotrachys* found in the Southern Ocean and *M. berglax* from the North Atlantic Ocean, are among the species that need taxonomic review. These species differ morphologically, and the minor differences have been used as a basis to separate them into different species. DNA molecular studies that have reviewed the taxonomy of these species using the cytochrome c oxidase subunit I (COI) gene found low sequence divergence between *M. holotrachys* and *M. berglax*. The findings of these studies provide motivation for population genetic studies to compare genetic structure between these distantly distributed species. The present study aimed to close this gap by assessing the genetic structure between *M. holotrachys* and *M. berglax* using the COI and displacement loop (D-loop) gene regions. Aligned sequences of these genes were 532 base pairs (bp) and 703 bp long, respectively. The COI gene revealed seven haplotypes from 26 sequences, while D-loop region had eight haplotypes from 55 sequences. The mismatch distribution curves were unimodal and the haplotype network trees had a star shape pattern for both datasets, which is consistent with populations undergoing demographic expansion. Although neutrality test values were not significant, negative values were observed for Tajima's D, supporting the populations undergoing demographic expansion. The F_{ST} pairwise distance method revealed no substantial differentiation between the populations of *M. holotrachys* and *M. berglax*. These results suggest that a recent dispersal of populations may have occurred such that there was not enough time for the separated populations to develop different genetic traits. In conclusion, this study demonstrated no significant genetic differentiation between *M. holotrachys* and *M. berglax* and confirms that they are one species consisting of North and South Pole populations, respectively. Additionally, this study has added more literature on the population genetic structure of these species.

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CHAPTER 1: INTRODUCTION

1.1. Classification

The family Macrouridae commonly known as the grenadiers or rattails, is very genetically and phenotypically diverse and belongs to the order Gadiformes (Smith et al., 2011). The Macrouridae is one of the largest families consisting of more than 300 species classified into 36 genera, with most of these species being benthopelagic (Smith et al., 2011; Gorny and Hernandez, 2018). There are four subfamilies in this family; namely, Macrourinae, Macrouroidinae, Trachyrincinae and Bathygadinae (Figure 1.1), with distribution ranging from the Arctic to the Antarctic (Munster et al., 2016; Shi et al., 2016; Varon and Orti, 2009). The morphology of the Macrouridae is characterised by a large head, a short trunk, a long tapering tail (hence the name rattails), and usually the lack of a caudal fin (Gorny and Hernandez, 2018). The Macrourinae is the most diverse, with more species than all gadiforms put together (Phleger, 1971). The Macrouroidinae has two species only and is recognisable by an inflated head and a single dorsal fin (Phleger, 1971). The Trachyrincinae has five species; these are distinguished by minuscule rays in the first dorsal fin, an extended flattened snout and rough scales (Phleger, 1971). The Bathygadinae has 25 species easily identifiable by their large terminal mouth (Phleger, 1971).

The genus *Macrourus* belongs to the Macrourinae and this genus has five known species; namely, *M. holotrachys* (commonly known as bigeye grenadier), *M. whitsoni* (circumpolar Whitson's grenadier), *M. carinatus* (ridge-scaled grenadier), *M. caml* (caml grenadier) and *M. berglax* (rough-head/onion-eye grenadier) (McLellan, 1997; Smith et al., 2011). The first four species are found in the Southern Ocean (Figure 1.2), while *M. berglax* is found in the North Atlantic Ocean (Smith et al., 2011). *Macrourus* species feed on a multitude of invertebrates and fishes found in the waters they inhabit, and can be found in depths ranging from 200 m to 3000 m (Cohen, 1990). The most distinguishing morphological features of these species include, a large head, rounded snout with modified tricuspid scale at tip, suborbital ridge that extends posteriorly onto preopercle ending in a sharp point, jaws extend beyond vertical through midorbit and the outer gill rakers on the first arch are absent (Cohen, 1990).

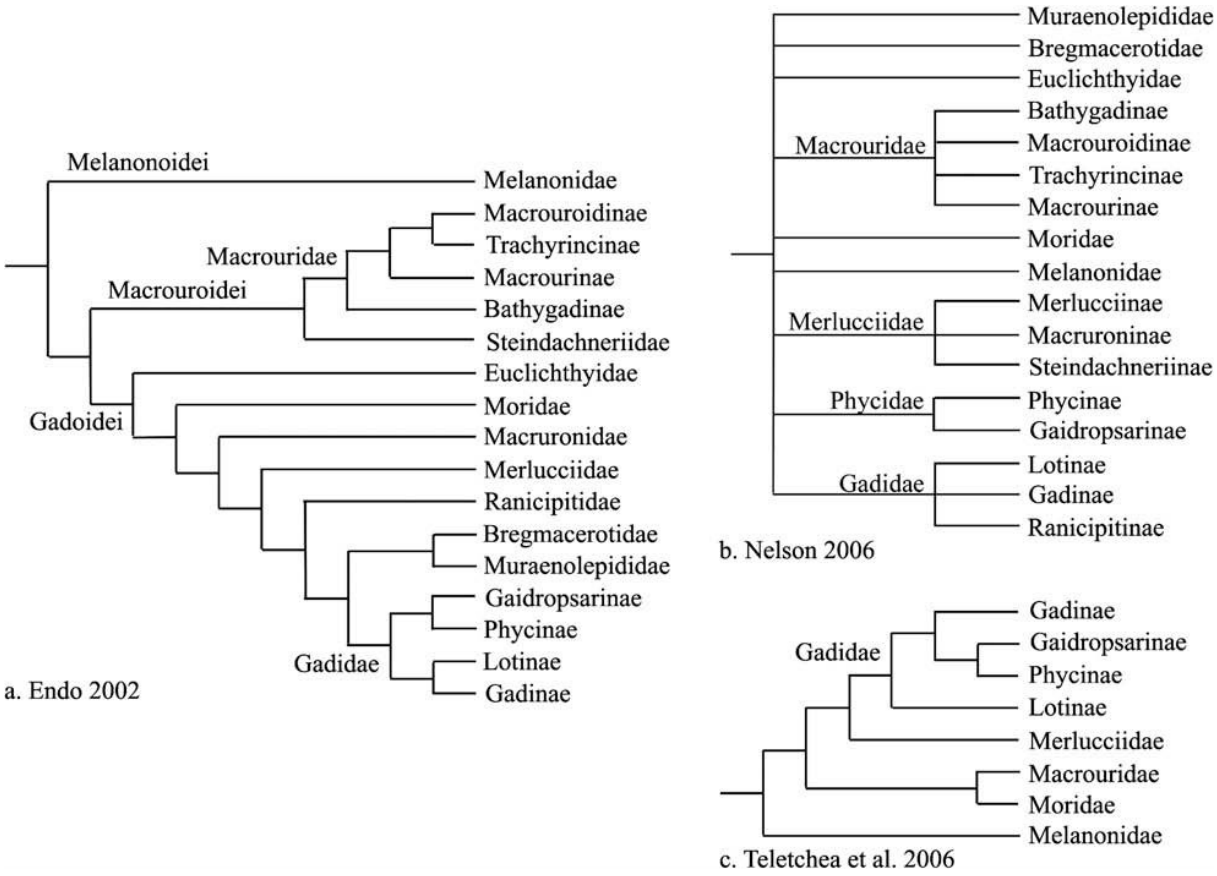

Figure 1.1. Morphology and molecular based phylogenetic trees hypothesis for Gadiformes (Varon and Orti, 2009).

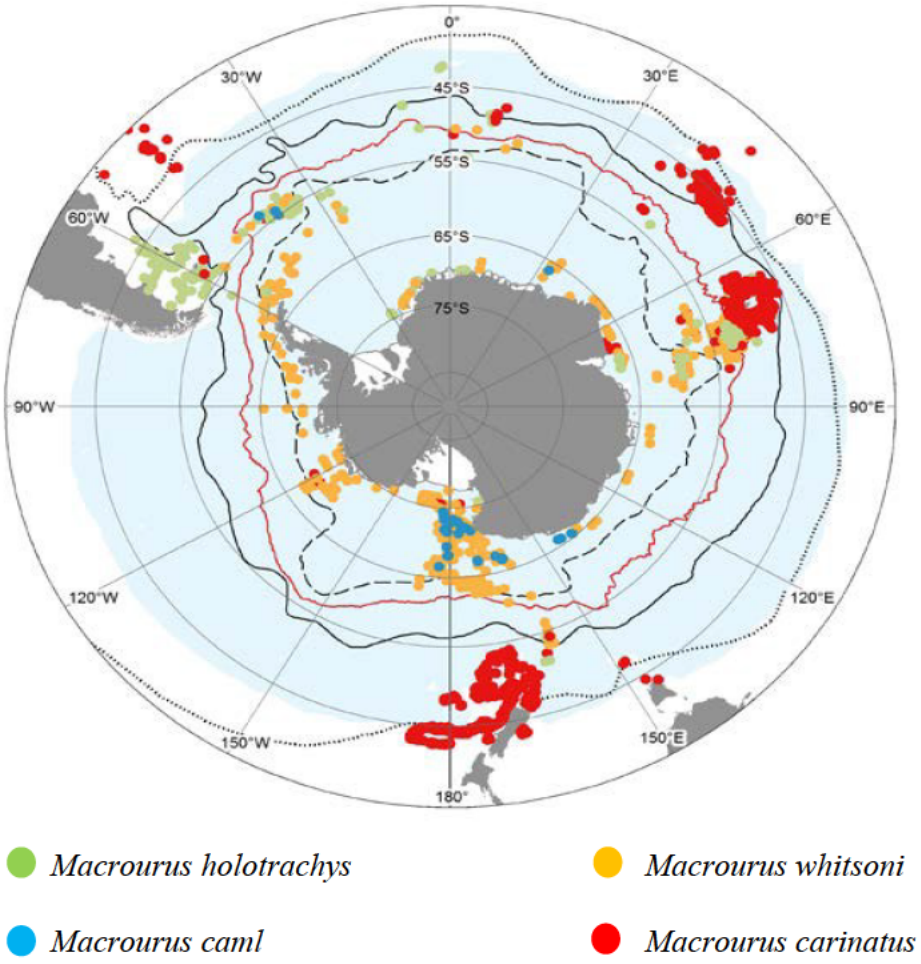

Figure 1.2. The distribution of the four *Macrourus* species of the Southern Ocean (De Broyer et al., 2014).

The first species of the *Macrourus* genus to be described was *Macrourus berglax* caught in the North Atlantic specifically around the Greenland and Icelandic region (McMillan et al., 2012). It was reported as having five rows of teeth on the upper jaw and three on the lower jaw, with scales all around the body and a dorsal fin ending with a spine (COSEWIC, 2007). The second species to be described was *M. holotrachys* during an expedition of the Her Majesty's Ship challenger, which took place in the years 1872-1876 (Fitzcharles, 2014). The distinguishing traits were gleaned from a sole sample collected from the coast of Argentina and it was reported to have a head covered with scales except on the underside, a large eye and short snout as well as five scales between the first dorsal spine and lateral line (Fitzcharles, 2014). The third species to be described was *M. carinatus* at Prince Edward Island the species has a single row of teeth on the lower jaw, a very pronounced snout, six

scales between the first dorsal spine and lateral line (Fitzcharles, 2014). The fourth species to be described was *M. whitsoni* caught during the Antarctic Fishes of the Scottish National Antarctic Expedition, which took place in the years 1902-1904 (Fitzcharles, 2014). It was chronicled from two specimens from Coats Land in the Weddell Sea and detailed as having a large eye, scaled body, scales on the side of the head, pronounced snout, a protruding orbital ridge and seven scales between the dorsal fin and lateral line (McMillan et al., 2012). *Macrourus calm*'s distinguishing characters are eight pelvic fins, 30-40 scales in a diagonal row from anal fin origin to lateral line this species was described recently by McMillan et al. (2012), following a molecular study by Smith et al. (2011) where they discovered that Ross Sea specimens consist of *M. whitsoni* and an unrecognised species.

When comparing *M. holotrachys* (Figure 1.3) and *M. berglax* (Figure 1.4), it is apparent that there are some similarities and differences in terms of their morphology, distribution and spawning patterns. Both these species lack scales on the underside of the head behind the mouth, which is a trait that is present in the other three *Macrourus* species (Figure 1.5) (McMillan et al., 2012). *Macrourus holotrachys* and *M. berglax* share the same spawning characteristics relating to season and duration (Murua and Motos, 2000). These species also have a similar life cycle and occurrence of sexual dimorphism where the females grow larger than the males (Murua, 2003). Morphological differences include the number of pyloric caeca ranging from 9 to 16 for *M. holotrachys* and 14 to 23 for *M. berglax* (McMillan et al., 2012), the scales in diagonal rows from the anus to lateral lines range from 18 to 26 for *M. holotrachys* and 13 to 17 for *M. berglax* (McMillan et al., 2012).

Macrourus holotrachys has a second dorsal fin positioned anteriorly to the anus (Fitzcharles, 2014) while in *M. berglax* the second dorsal fin begins further forward of the origin of the anal fin (Cohen et al., 1990); and *M. holotrachys* is characterised by a brownish to grey colour, with darker fins and sometimes a light to medium brown colour while *M. berglax* has an overall grey colour with darker fins (Fitzcharles, 2014). There are also differences in their distribution, with *M. holotrachys* being found in the Southern Ocean and *M. berglax* in the North Atlantic Ocean (Smith et al., 2011).

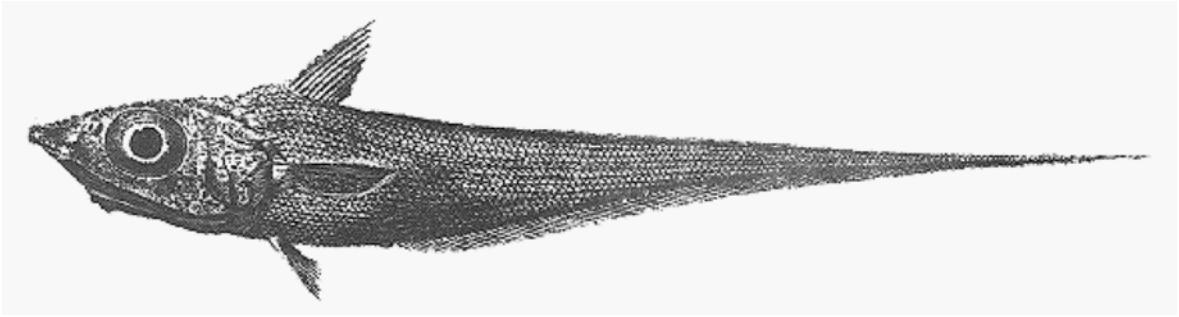

Figure 1.3. Illustration of *Macrourus holotrachys* (Fitzcharles, 2014).

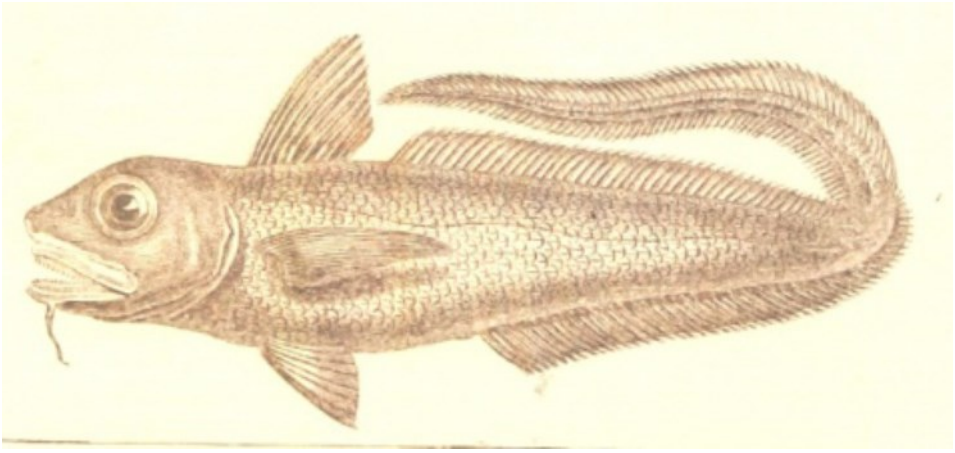

Figure 1.4. Illustration of *Macrourus berglax* (Fitzcharles, 2014).

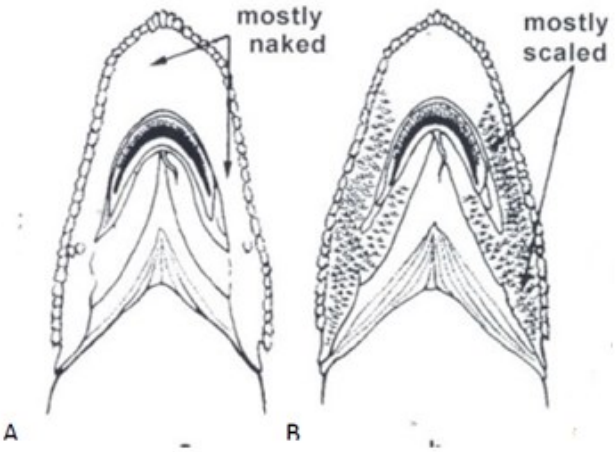

Figure 1.5. Illustration showing the variation in scaling on the underside of the head used to differentiate between *Macrourus* species. A. Represents *M. holotrachys* and *M. berglax*; B. Represents other *Macrourus* species (Cohen, 1990).

1.2. Distribution of study species

1.2.1 *Macrourus holotrachys*

To get a comprehensive understanding of the two species it is important to be cognizant of the environment in which they occur, and this enables for a better understanding of their evolution (Melbinger and Vergassola, 2015). *Macrourus holotrachys* is found in the Southern Ocean which is also known as the Antarctic Ocean (Fitzcharles, 2014). The Southern Ocean was formed approximately 34 million years ago due to the separation of South America and Antarctica which enabled the formation of the Antarctic Circumpolar Current also known as the West Wind Drift to flow freely (Figure 1.6) (Barker et al., 2007). This ocean current flows clockwise from west to east around Antarctica, keeping warm waters away from Antarctica (Barker et al., 2007). Most of the Southern Ocean has depths ranging between 3000 and 4000, with shallow waters found along the islands, coasts and sub-sea ridges (DeVries and Steffensen, 2005). The Southern Ocean is demarcated as southern parts of the Indian, Atlantic and Pacific Ocean connecting to a southern boundary at Antarctica with the Antarctic Polar Front as a northern boundary (McMillan et al., 2012). *Macrourus holotrachys* is found 37 º S southward more specifically South Georgia, Prince Edward Island, Lena Seamount, Ob Seamount, and the east coast of South America (McMillan et al., 2012). The records of *Macrourus holotrachys* localities are mostly just south of the Antarctic Polar Front with a few extending north of the Sub-Antarctic Front (Gon et al., 2021). *Macrourus holotrachys* is a benthopelagic species that occurs at a depth range of 300 to 1200 meters, its hospitable temperature can range from -2 to -10 ºC (Cohen et al., 1990).

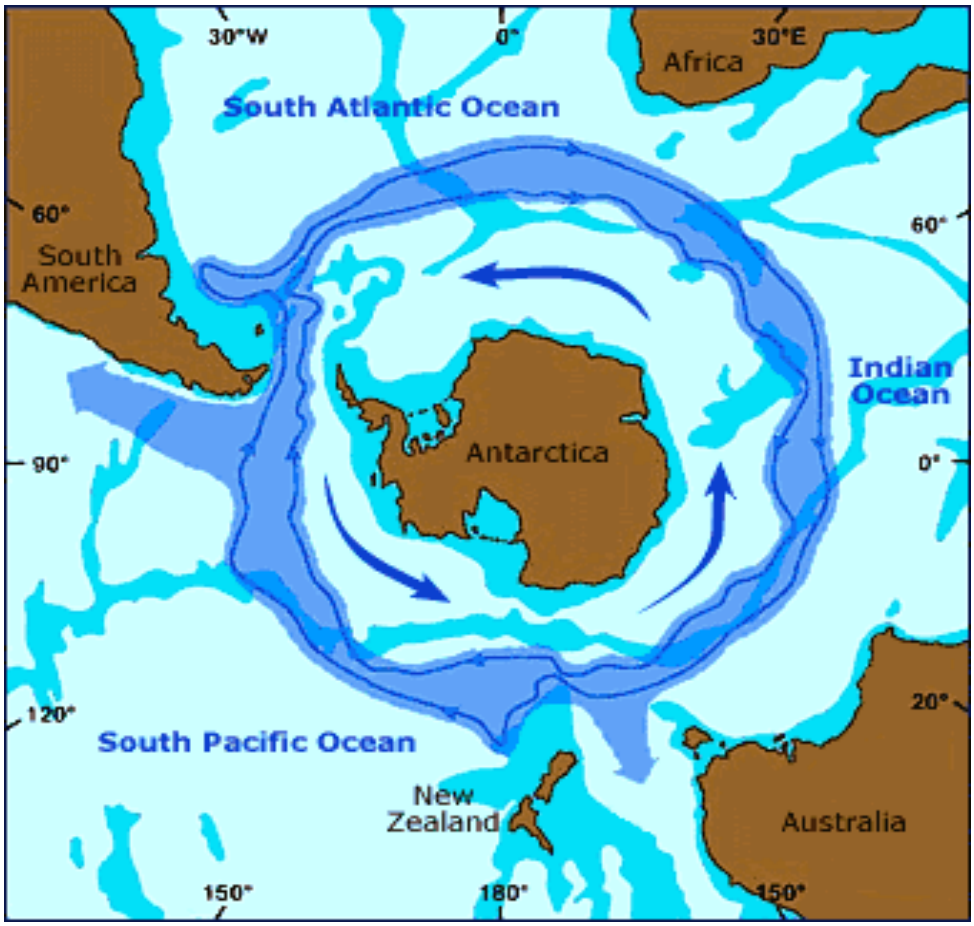

Figure 1.6. Antarctic Circumpolar current (three large arrows) and the Antarctic Front is indicated by the darker shaded area.

(http://msuinantarctica.blogspot.com/2010/01/antarctic-circumpolar-current.html) last accessed 08/05/2020

1.2.2 *Macrourus berglax*

Macrourus berglax is found in the North Atlantic Ocean, which forms part of the Atlantic Ocean, the second largest ocean in the world (Smith et al., 2011; Rice et al., 2016). The North Atlantic Ocean originated from in the opening of the Central Atlantic around 200 to 170 million years ago during the initial break up of Pangea (Seton et al., 2012). This is attributed to the volcanic eruptions of the Central Atlantic magnetic province (CAMP) (Seton et al., 2012). McMillan et al. (2012) indicated that the distribution of *M. berglax* is widespread from Greenland, Iceland to North Atlantic (figure 1.7). More specifically, the east coast of Greenland,

the north and east in the eastern Atlantic to Spitzbergen as well as the British Isles, Norwegian and Barents Seas; North American coasts 37 º N northward (McMillan et al., 2012). *Macrourus berglax* occurs at a temperature range of zero to 4.5 ºC and a depth range of about 100 to 1000 metres (Cohen et al., 1990).

Figure 1.7. The distribution of *M. berglax* demarcated by the darkened regions.

(http://www.fao.org/figis/geoserver/factsheets/species.html?species=RHG-m&prj=4326) last accessed 05/07/2020

1.3. Genetics and conservation

It has been demonstrated in most molecular studies that the mitochondrial Cytochrome C Oxidase Subunit I (COI) gene (685 bp in length) can differentiate up to 95 % of species in many taxa, this is possible due to the gradual accumulation of genetic differences in evolving lineages (Herbet et al., 2003; Bingpeng et al., 2018). DNA barcoding using the COI gene generally leads to correct taxonomic conclusions as it is the most commonly used molecular marker for species identification (Pentinsaari et al., 2016). Another commonly used marker is

the mtDNA displacement loop (D-loop) because it has a high mutation rate, an abundance of polymorphisms, and very little recombination therefore, it can be utilised in species recognition and lineage tracing (Goncalves *et al.,* 2011). These markers are the more comprehensive in the sequence database (Barcode of Life) when compared to nuclear markers (Pentinsaari et al., 2016). Nuclear markers such as microsatellites and SNPs are also highly polymorphic among populations but are rarely used in phylogenetic studies (Jian et al., 2018). The utility of genetic markers in species identification and differentiation depends greatly on the extent of sequence variation of said marker (Zurita amd Cutillas, 2021). The comparison of mtDNA markers and nuclear markers reveals a higher degree of sequence variation in mtDNA markers than most nuclear markers (Zurita amd Cutillas, 2021). The nuclear internal transcribed spacer (ITS) regions show a high degree of sequence variation and have been successfully used for species differentiation (Zurita amd Cutillas, 2021). However, mtDNA genes have been shown to be more successful in interspecific discrimination among species (Zurita amd Cutillas, 2021). Overall, using the mitochondrial genome markers is more advantageous than the nuclear genome markers because of a smaller sequence, maternal inheritance and non-recombination loci (Jiang et al., 2016). This makes it more efficient at reconstructing phylogenetic relationships and due to the mutation rate being much higher it is more suitable at resolving relationships among closely related species (Jiang et al., 2016). Rathipriya et al. (2019) conducted a study on molecular identification and phylogenetic relationship of flying fishes using the COI gene since the morphological traits of the three fish species are very similar. It was reported that COI data provided sufficient genetic differentiation to distinguish among *Cheilopogon cyanopterus*, *Cheilopogon furcatus* and *Hirundichthys coromandelensis* (Rathipryia et al., 2019). A study by Zhang et al. (2011) demonstrated a high efficiency of species identification when using the COI gene. The average genetic distance was found to be 50-fold higher between species than within species and the genetic distances averaged 15.74 % among congeners and only 0.31 % for intraspecific individuals (Zhang et al., 2011). Molecular analyses are also used to describe and identify species that are weakly differentiated by morphological characters, such as cryptic species which are extremely difficult to reveal by analysis of morphological makers alone (Lukhtanov, 2019).

Uncovering and resolving cryptic species is contingent on pre-existing data (morphological analysis) and understanding of the species in a genus (Rubinoff et al., 2006). The main taxonomic goal of DNA barcoding is the identification of species initially described by other

criteria and allowing for quicker identifications, which had previously been made on morphological grounds alone (Rubinoff et al., 2006). In addition to this there is linking specimens unidentifiable by other methods to established species identification, for various purposes such as forensics and detailing of life cycles (Rubinoff et al., 2006). Other species identification techniques/analyses include phylogenetic analyses where a variety of mitochondrion (mtDNA) and nuclear genes can be sequenced (Bingpeng et al., 2018). These can then be used in phylogenetic analysis to determine genetic similarity or/and differences by construction of maximum likelihood, maximum parsimony or Bayesian inference trees (Yang and Rannala, 2012). These genes can also be used in population genetic studies, Smith et al. (2011) conducted the first species identification study of the *Macrourus* species in the Southern Ocean using mtDNA COI gene. This study discovered a "cryptic" species which was identified as the new species, *M. calm*. A study by Coscia et al. (2017) investigated the population structure of *M. berglax* in different geographic regions of the North Atlantic Ocean using mtDNA control region (D-loop) and species-specific microsatellites. The study found no evidence of significant structure with both sets of molecular markers producing the same results of overall homogeneity in *M. berglax*.

Additionally, genetic techniques are used to determine the genetic history, population structure and phylogenetics of species (Carvalho and Beheregaray, 2018). This information is essential in conservation efforts especially in the management of shrinking populations due to the occurrence of inbreeding and genetic drift (Neaves et al., 2015). These phenomena decrease the genetic diversity and thus the ability of a particular species to adapt to changing environments (Neaves et al., 2015). *Macrourus holotrachys* and *M. berglax* are caught as by-catch just like other *Macrourus* species (Smith et al., 2011) and this poses a great danger to their population numbers, considering that they have long life cycles, low fecundity which makes them more susceptible to overfishing (Fitzcharles, 2014). Therefore, being aware of the genetic diversity of a population is fundamental to ensuring stable numbers of species (Neaves et al., 2015). Furthermore, identifying and classifying species correctly is also important for conservation and genetics fulfils this role well especially with understudied species as is the case with *M. holotrachys* and *M. berglax* (Fitzcharles, 2014).

With regards to conservation there is a great need for some regulation to maintain stable numbers of grenadiers, because many fisheries for example toothfish *Dissostichus* longline and trawl fisheries of the Southern Ocean have reported catches of all grenadiers (Smith et al.,

2011). For instance, across the region managed by the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR), the catches of grenadiers amounted to more than 1500 tons from 2008-2009 (Smith et al., 2011). The Kerguelen Islands fishery catch of *Macrourus* species increased from 6 tons in 1998 to 537 tons 2007 (Palomares and Pauly, 2011). The Ross Sea catch increased from 9 tons in 1998 to 462 tons in 2005 (Fitzcharles, 2014). The advent of new fishing techniques particularly longline fishing in the late 1990s, has allowed for greater exploitation of the ocean enabling access to deeper waters (Palomares and Pauly, 2011). This is further compounded by illegal, unregulated and unreported catches with fish being processed on the ships and traded using different names usually to the northern hemisphere (Fitzcharles, 2014).

Macrourus berglax is fished commercially in the North Atlantic but is caught mainly as by-catch in Greenland halibut fishery and deep-water shrimp trawl (Healey and Mahe, 2005). It has been estimated that the stocks of *M. berglax* in the North Atlantic Ocean have declined by 93.3 % because of overfishing (Devine et al., 2012). It has been suggested by Baker et al. (2009) that the recovery of *M. berglax* in Canadian waters is approximately 18 to 125 years and 19 to 248 years if 5 % by-catch is included. According to Murua (2001) *M. berglax* is an unregulated species, not protected by any legislation or regulation and is not under any species protection convention. Therefore, successful management of fisheries such as having a cut-off in the number of *Macrourus* species that can be caught as by-catch is required and tougher penalties such as fines for fisheries that partake in illegal trade of this species must be implemented. The species must also be included in species protection conventions, these strategies used effectively can ensure stable numbers of the *Macrourus* species thus nullifying the risk of these species becoming endangered. Without genetics, conflicting management strategies can arise from conservationists, fishing industries and governmental agencies; thus, genetics allows for prioritisation of management strategies therefore improving conservation success (Carvalho and Beheregaray, 2018).

1.4. Study Rationale

There is a literature dearth and absence of consensus on whether *M. holotrachys* and *M. berglax* are of the same species or not. Some studies have indicated that they are two separate species while others have indicated that these two fish species are possibly one species differing only in geographic location (Cohen et al., 1990; Fitzcharles, 2014). The two species differ slightly

morphologically when looking at their pigmentation, the pyloric caeca number, the diagonal rows of scales from the anus to lateral lines and the position of the second dorsal fin (McMillan et al., 2012). These minor differences have been used as a base to separate them into different species.

Species misidentification is a common phenomenon for *Macrourus* species. Smith et al. (2011) showed the existence of a "cryptic" species within *Macrourus* in the Southern Ocean. Using DNA bar-coding of cytochrome c oxidase subunit 1 gene (COI gene), this study discovered an additional clade to the clades of the three known Southern Ocean *Macrourus* species (*M. holotrachys*, *M. whitsoni* and *M. carinatus*). This additional clade consisted of species that were morphologically identified as *M. whitsoni* and the other unrecognised (Smith et al., 2011). McMillan et al. (2012) aimed to describe this unrecognised species utilising meristic evidence and it was named *M. caml*.

A molecular study that reviewed *M. holotrachys* and *M. berglax* using the COI gene and ribosomal RNA subunit 16S found that they are genetically identical (Fitzcharles, 2014). However, further work needs to be done to verify this as there is a lack of population studies to compare genetic structures within these distant species. Therefore, the present study aims to close this gap by assessing the genetic structure between *M. holotrachys* and *M. berglax,* using mtDNA COI and D-loop dataset and determine whether they are the same species or not. These genes were selected for this study because of their ability to differentiate between closely related species (Goncalves *et al.,* 2011; Hebert et al., 2003).

The objectives are:

- To assess the phylogenetic relationship between *M. holotrachys* and *M. berglax*. This was achieved by reconstructing the phylogenetic tree and estimating evolutionary distances, including all valid *Macrourus* species. The questions that may be resolved are as follows:

    - Are *M, holotrachys* and *M. berglax* different species or geographically distant populations of the same species?

- To assess genetic structure between *M. holotrachys* and *M. berglax* populations. This was achieved by constructing a haplotype network, performing an AMOVA analysis and calculating the population pairwise F_{ST} values.
    - Is there genetic structuring between the two populations or is there no structuring between the two populations?
- To assess the demographic history among the study populations of *M. holotrachys* and *M, berglax*. This was achieved by constructing mismatch distribution curves.
    - Are the populations of *M. holotrachys* and *M. berglax* undergoing demographic expansion or contraction?

1.5. Dissertation structure

This dissertation consists of four chapters, the first chapter is a literature review and gives information regarding classification of *M. holotrachys* and *M. berglax*, distribution of the two species, genetics and conservation and the study rationale. The second chapter is the methods and materials which provides information on the study areas and analyses methods used for this study. The third chapter contains the results of the study. The fourth chapter contains the discussion and interpretation of the results found. Chapter five is the final chapter which presents the conclusions drawn from the study and further recommendations for future studies.

CHAPTER 2: Methods and Materials

2.1. Study Area

2.1.1 *Macrourus holotrachys*

Macrourus holotrachys samples were collected near Prince Edward Islands, Lena Seamount and Ob Seamount (Figure 2.1). The islands are located between Antarctica and Africa, approximately 2000 km southeast of South Africa (Pakhomov et al., 2003). The Prince Edward Islands in particular are 450 000 years old and have an area of 45 km^2. These islands are under the derestriction of South Africa and were used for research into the Southern Ocean marine and terrestrial systems until the islands were proclaimed as marine protected areas (MPA) in 2013 (Pakhomov et al., 2003, Brooks et al., 2020). The islands experience cool and strong winds that generally flow in the north easterly direction and have an annual rainfall average that ranges from 2400 mm up to over 3000 mm (Pakhomov et al., 2003). The temperature in these regions is usually less than 5 ºC with cloudy conditions resulting in very little radiation reaching the islands (Pakhomov et al., 2003). The marine climate of sub-Antarctic islands is influenced by the Antarctic Circumpolar Current (ACC), the Sub-Antarctic Front (SAF) in the north and the Antarctic Polar Front (APF) in the south (Pakhomov et al., 2003).

2.1.2 *Macrourus berglax*

Macrourus berglax samples are from the North Atlantic Ocean, and were downloaded sequences from (https://www.ncbi.nlm.nih.gov/nucleotide/) National Center for Biotechnology Information (NCBI) GenBank. Specific localities for COI samples were not specified, while D-loop samples were from Svalbard and Norway (Appendix A). The Atlantic Ocean is the second largest of the world's oceans (Rice et al., 2016). It is located in between Europe and Africa and extends to the North connecting to the Arctic Ocean called the North Atlantic Ocean. The North Atlantic Ocean is separated by the South Atlantic Ocean, which is part of the Southern Ocean, by the Equatorial Counter Current (Wyrtki, 1974). This current flows from west to east in the Atlantic, Indian and Pacific basins (Wyrtki, 1974). The temperature in the Atlantic Ocean can range from as low as -2 ºC to over 30 ºC, high temperatures are usually recorded north of the equator and low temperature near the Polar Regions (Rice et al., 2016).

2.2 Sample collection

The vessels involved in the sampling of *Macrourus holotrachys* were the El Shadai (ZAF), Koryu Maru (ZAF) and Shinsei Maru (JPN). The specimens were captured during the summer of 2015/16 at depths of 640-1800 m, as by-catch of the Patagonian toothfish (*Dissostichus eleginoides*) fishery in the Southern Ocean. A total of 45 *M. holotrachys* individuals were captured using long line fishing methods thereafter muscle tissue samples were collected from each individual and stored in absolute ethanol. A total of 37 *Macrourus berglax* mitochondrial DNA (mtDNA) sequences were obtained from a GenBank database. Eight of these sequences were representatives of cytochrome c oxidase subunit I (CO1) gene, while 29 were representatives of the displacement loop (D-loop) control region.

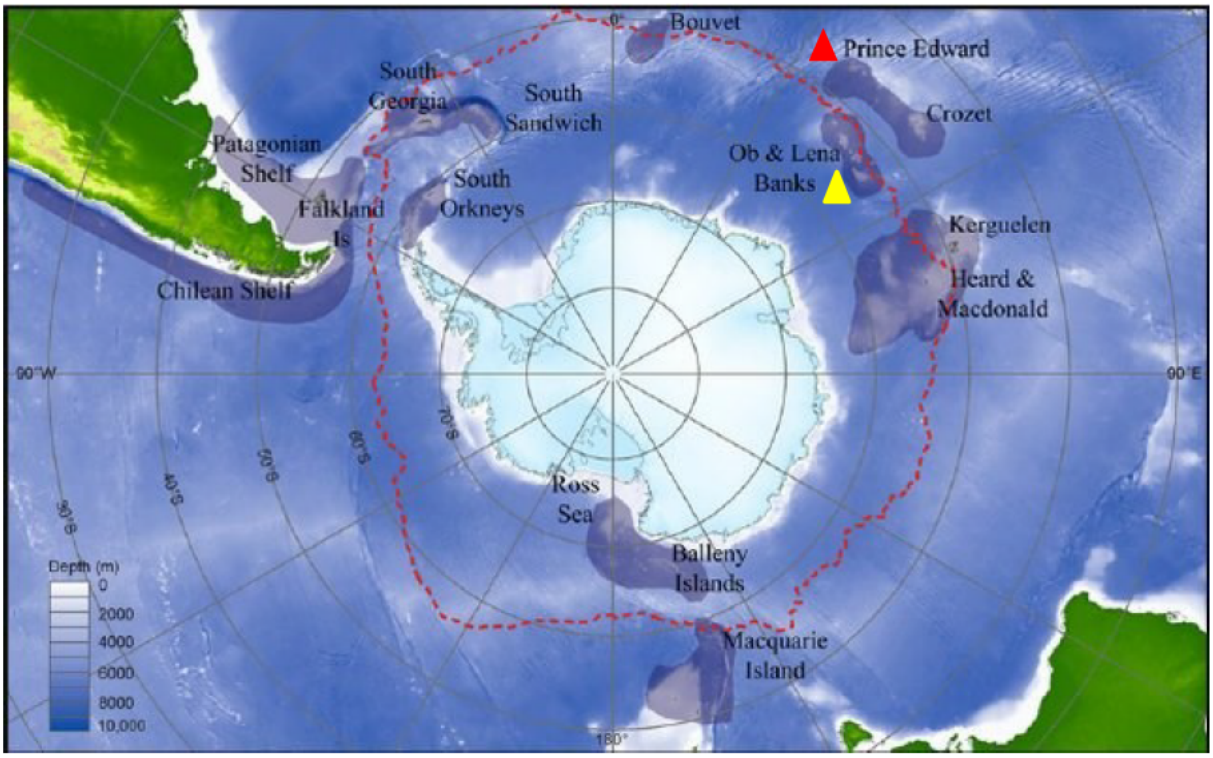

Figure 2.1. Collection sites Prince Edward Islands, Lena Seamount and Ob Seamount in the Southern Ocean for *Macrourus holotrachys* samples (Collins et al., 2010).

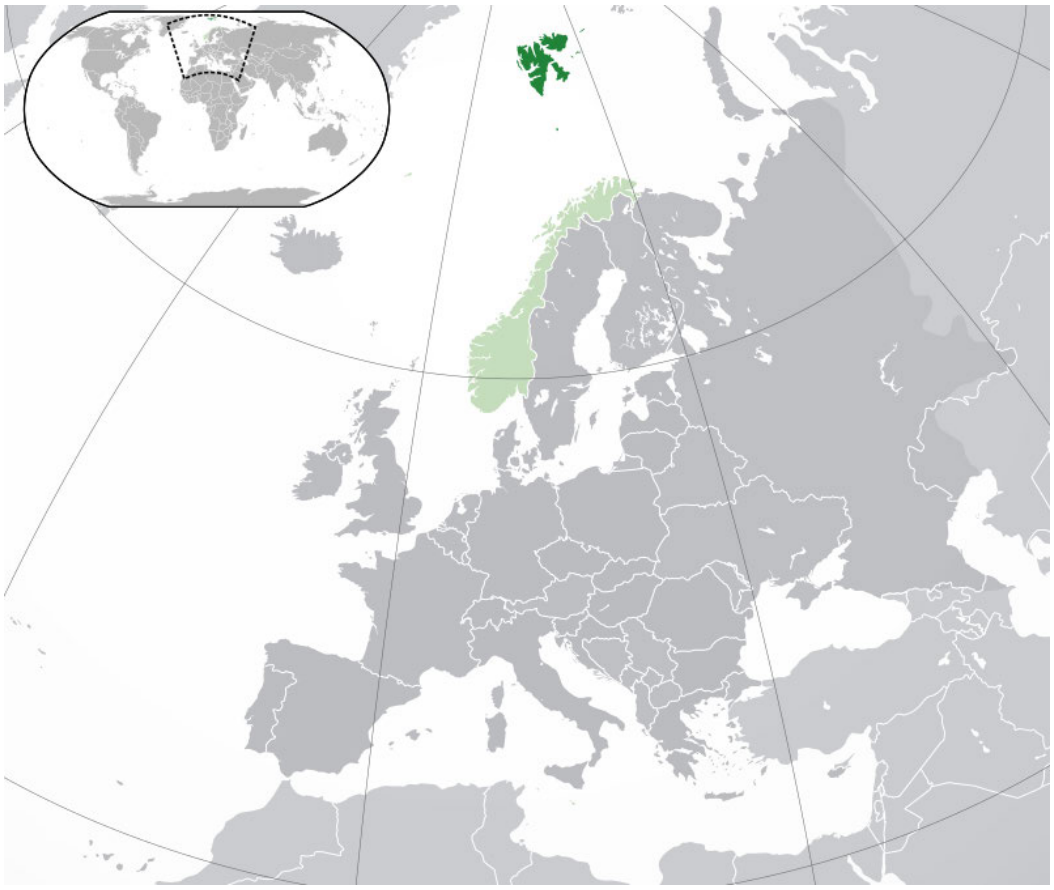

Figure 2.2. The collection sites Svalbard and Norway in the North Atlantic Ocean for *Macrourus berglax* samples.

(http://axj.dk/bh/BH/Norge.php) last accessed 29/09/2021

2.3 DNA extraction, primer design and PCR amplification

Tissue samples from 45 *M. holotrachys* specimens were cut into small pieces of 25 mg and placed into 1.5 mL microcentrifuge tubes. Genomic DNA was extracted using the Quick-DNA Miniprep Plus kit (Zymo Research Inc., 2016) following the manufacturers specification. DNA extracts were stored in a refrigerator overnight (Stored overnight due to low concentration readings being obtained when samples were measured right after extraction) at 3 ^{o}C before measuring their concentration using a Nanodrop spectrophotometer (ND-100) (Bio Rad), and being used for polymerase chain reaction (PCR) amplifications.

Prior the PCR amplification of the D-loop region, specific primers for *Macrourus* species were designed using Primer3 tool embedded in the NCBI by selecting *Macrourus* sequences with accession number MG702488.1. To determine which primer pair is closest to the desired

product size, a low self-complementarity and an appropriate melting temperature must be selected. The selected primer pair consisted of forward primer (MB-F) sequence of 5'-TCA ACT GTC TCC CGT TT-3' and reverse primer (MB-R) sequences 5'-TGG AGT TCG GAC TAT TCC TTG T -3' which were then used to amplify the D-loop region. The final volume for PCR reaction was 25 µL containing 12.5 µL One Taq 2X Master Mix, 0.5 µL forward and reverse primer (10µM each), 9.5 µL nuclease free water and 2 µL genomic DNA (25-485 ng/µl). The PCR thermocycling conditions for D-loop region were as follows; an initial denaturation of 4 minutes at 94 ºC followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing of 30 seconds at 51 ºC, extension of 1 minute and 30 seconds at 72 ºC, and a final extension at 72 ºC for 5 minutes.

The amplification of COI gene used the universal primer dLCO1490 (forward) 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and dgHCO (reverse) 5'-TAA ACT TCA GGG TGA CCA AAR AAY CA-3', published by Folmer et al. (1994). The PCR reaction was similar to that of the D-loop region with the following PCR conditions; an initial denaturation of 5 minutes at 95 ºC followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing of 30 seconds at 52 ºC, extension of 50 seconds at 72 ºC, and a final extension at 72 ºC for 5 minutes. The PCR products for both D-loop and COI gene were electrophoresed on 1 % agarose and viewed using a ChemiDoc system (Bio Rad) and photographed by UV transillumination, to check if the amplified DNA is of the desired size of the required DNA region. The amplified DNA that showed bands of an appropriate size on the gel were sent to Inqaba Biotechnical Industries for sequencing.

2.4 Sequence analyses

Sequences were edited using ChromasPro version 2.6.6 (Technelysium) and aligned using MEGA X version 10.0.2 (Kumar et al., 2018). These were analysed using DnaSP version 5.10.01(Rozas et al., 2017) to calculate the number of haplotypes (nh), haplotype diversity (hd) and nucleotide diversity (π). Parameters that contain information on population history such as neutrality test values (Tajima's D and Fu's Fs values), mismatch distribution graphs and raggedness index (r) were used to explore the demographic history of the species (Rozas et al., 2017). The haplotype network was generated by using the median-joining method in Network version 10.0 (Fluxus Technology), to represent the mutational relationships present in *M. holotrachys* and *M. berglax*. Arlequin version 3.5.1.2 (Excoffier et al., 2005) was used to

calculate the Analysis of molecular variance (AMOVA) and other statistical tests such as the neutrality test (Tajima's D and Fu's Fs test) and pairwise F_{ST} values to determine the extent of differentiation between samples (Ceballos et al., 2012). A phylogenetic tree was constructed using the maximum likelihood (ML) method in MEGA X version 10.0.2 (Kumar et al., 2018), with the Hasegawa-Kishino-Yano model. The clade credibility was tested by bootstrapping, where 1000 repeated sampling tests were done to produce the support values of each node on the tree (Kumar et al., 2018). Two macrourid species, *Coryphaenoides carminifer* and *Coryphaenoides mediterrraneus* were used as outgroups and the sequences were obtained from GenBank. An additional phylogenetic analysis using Bayesian inference was performed using MRBAYES v.3.2.7 (Huelsenback and Ronquist, 2001). The (HKY+G) model was utilised and MRBAYES was run using 20 000 generations and four concurrent Markov chains and four hot chains sampled at two intervals, and tree sampling every 100 generations. The posterior probabilities were included next to the bootstrap values of the maximum likelihood tree.

CHAPTER 3: RESULTS

3.1.1 COI results

A total of 26 sequences were used for the COI gene analyses, of which, 18 were *M. holotrachys* produced by this study and eight sequences were *M. berglax* obtained from GenBank database. The final alignment of these sequences consisted of 538 base pairs long sequences. An analysis of these sequences revealed a mismatch distribution with a unimodal curve (Figure 3.1 A). This indicates that populations of *M. holotrachys* and *M. berglax* are undergoing demographic expansion and this observation is supported by the low raggedness index (*r*) of 0.0991. Furthermore, the haplotype network tree for the COI gene indicated a star-like shape topology (Figure 3.2 A) that is consistent with a population undergoing demographic expansion.

Further analyses of this dataset showed an overall high haplotype diversity (Hd) of 0.551 and a low nucleotide diversity (π) of 0.002 (Table 3.1). The same trend of high haplotype and low nucleotide diversities was observed when analysing the two species separately. The network tree showed six haplotypes, of which three (H2, H3 and H4) consisted of specimens identified as *M. holotrachys*, two (H5 and H6) had specimens of *M. berglax*, and only one haplotype, H1, was shared between the two study species (Figure 3.2 A). Haplotype H1 constitutes the majority of the study samples (65 %), meaning only a few specimens are not in this haplotype (Figure 3.2 A).

The AMOVA results showed little genetic structuring among the two species for the COI dataset. The variance among *M. holotrachys* and *M. berglax* was found to be very low 22.62 % (Table 3.2). The lowest pairwise F_{ST} value of 0.108 was found between *M. holotrachys* and *M. berglax* specimens in comparison with the higher values (0.478-0.961) observed between other *Macrourus* species (Table 3.3). Indicating that *M. holotrachys* and *M. berglax* are genetically different from the other three species. This is consistent with the findings in the maximum likelihood and Bayesian inference phylogenetic analyses where, *M. holotrachys* and *M. berglax* did tend to cluster together on the same clade forming a highly supported monophyletic clade with a bootstrap value of 100 % (Figure 3.3). Additionally, the analysis also recovered three distinct clades which correspond well with the other three *Macrourus* species (*M. carinatus*, *M. whitsoni* and *M. caml*). These results and those from the population

demography analyses, genetic diversity and haplotype relationships indicate that *M. holotrachys* and *M. berglax* are the same species (conspecific).

3.1.2 D-loop results

A total of 55 sequences were used for the D-loop region, with 26 sequences of *M. holotrachys* being produced by this study and 29 sequences for *M. berglax* were sourced from the GenBank database. The final alignment of these sequences was 701 base pairs in length.

Similar to the COI results the mismatch distribution had a unimodal curve (Figure 3.1 B), a low raggedness index of 0.0731 and a star-like topology for the haplotype network (Figure 3.2 B). Thus, supporting the indication that the populations of *M. holotrachys* and *M. berglax* are undergoing demographic expansion. There was a total of eight haplotypes and seven polymorphic sites with, high Hd of 0.715 and low π of 0.002 (Table 3.1). When analysing the species separately, *M. holotrachys* showed the same trend of high Hd and low π while *M. berglax* had both parameters at the lower end. The study species were grouped into four separate haplotypes with no shared haplotype (Figure 3.2 B).

The AMOVA results showed little genetic structuring among the two species for D-loop region dataset. The variance among *M. holotrachys* and *M. berglax* was found to be very low 4.15 %, (Table 3.2). These results are consistent with those of the COI results therefore indicating that *M. holotrachys* and *M. berglax* are the same species.

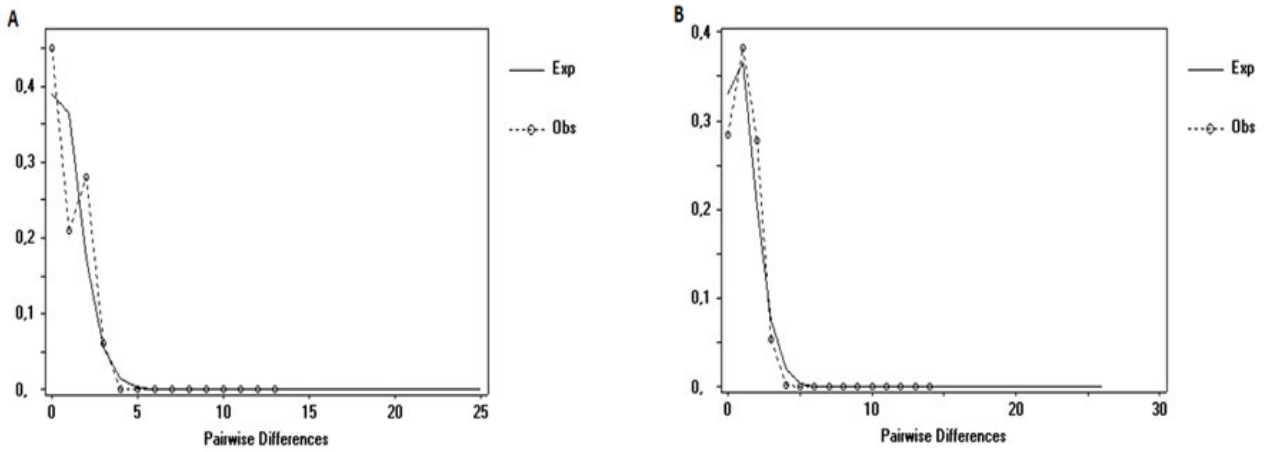

Figure 3.1. Mismatch distribution curves under a population expansion model based on (A) the COI gene dataset and (B) the D-loop region dataset for *M. holotrachys* and *M. berglax* populations.

Table 3.1. Diversity and neutrality indices resulted from analyses of COI gene and D-loop region.

Gene		n	S	hd	π	Neutrality test		*r*
						Fu's Fs	Tajima's D	
COI	*M. holotrachys*	18	4	0.575	0.002	-	-	-
	M. berglax	8	2	0.464	0.001	-	-	-
	Overall	26	6	0.551	0.002	-1.992	-1.164	0.0991
D-loop	*M. holotrachys*	26	3	0.625	0.001	-	-	-
	M. berglax	29	3	0.259	0.0003	-	-	-
	Overall	55	7	0.715	0.002	-2.445	-0.718	0.0731

n: number of sequences, S: segregating sites, hd: haplotype diversity, π: nucleotide diversity, *r*: raggedness index.

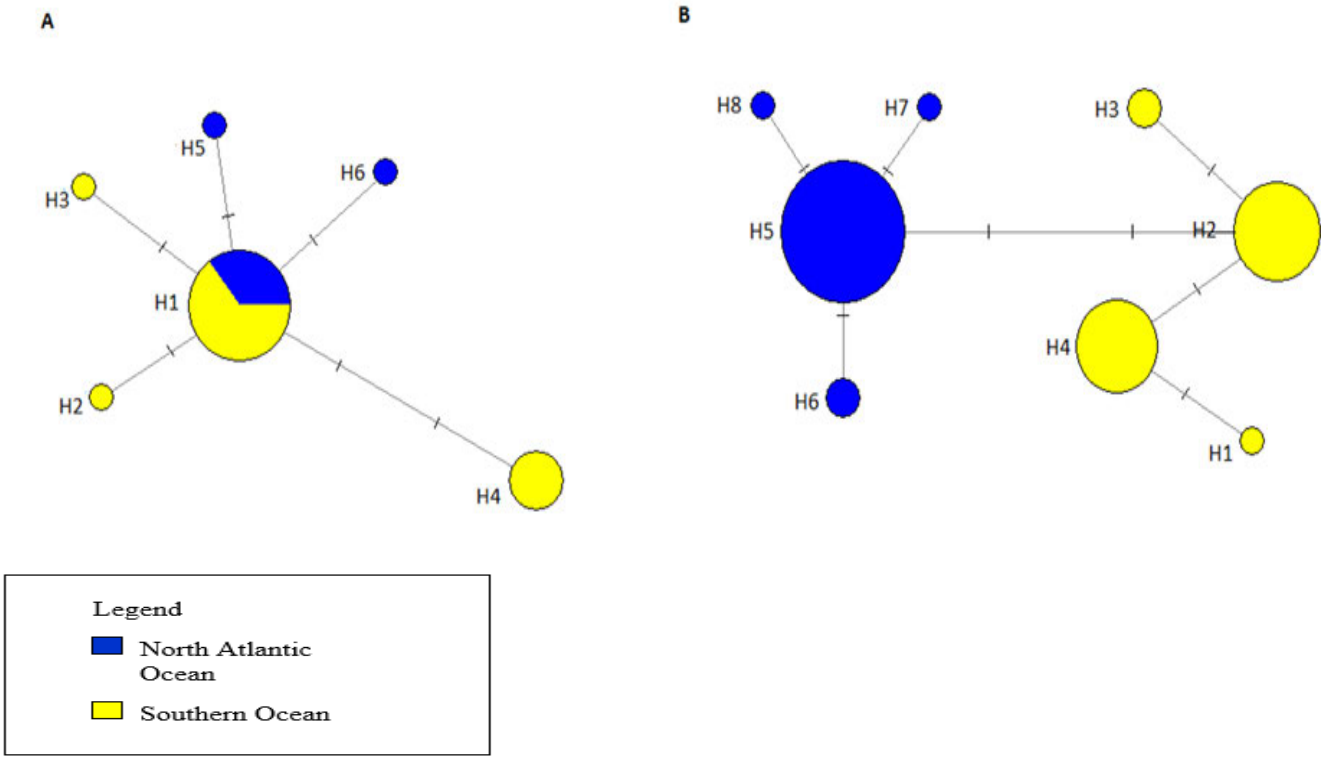

Figure 3.2. Haplotype network trees constructed using (A) COI and (B) D-loop datasets. Each haplotype is represented by a circle and shared haplotypes are indicated by multi-coloured circles. The size of each circle is relative to the number of samples sharing a specific haplotype. The number of strokes on the lines indicate the number of mutations separating the two adjacent haplotypes.

Table 3.2. Analysis of molecular variance (AMOVA) for COI gene and D-loop region.

COI gene				
Source of variation	df	Sum of squares	Percentage of variation	P-values
Among oceans	1	1.062	0	0.329
Among *M. holotrachys* and *M. berglax* populations within oceans	1	1.234	22.62	0.068
Within *M. holotrachys* and *M. berglax* populations	23	9.627	**83.83**	**0.005**
D-loop				
Source of variation	df	Sum of squares	Percentage of variation	P-values
Among oceans	1	16.829	67.78	0.316

Among *M. holotrachys* and *M. berglax* populations within oceans	1	0.630	**4.15**	**0.014**
Within *M. holotrachys* and *M. berglax* populations	52	12.432	**28.07**	**0.000**

df = degrees of freedom

Table 3.3. Population pairwise F_{ST} distance method for *Macrourus sp*. Using COI gene dataset.

	M. holotrachys	*M. berglax*	*M. whitsoni*	*M. caml*	*M. carinatus*
M. holotrachys	0.000				
M. berglax	0.108	0.000			
M. whitsoni	**0.736**	**0.803**	0.000		
M. caml	**0.702**	**0.681**	**0.478**	0.000	
M. carinatus	**0.921**	**0.961**	**0.938**	**0.825**	0.000

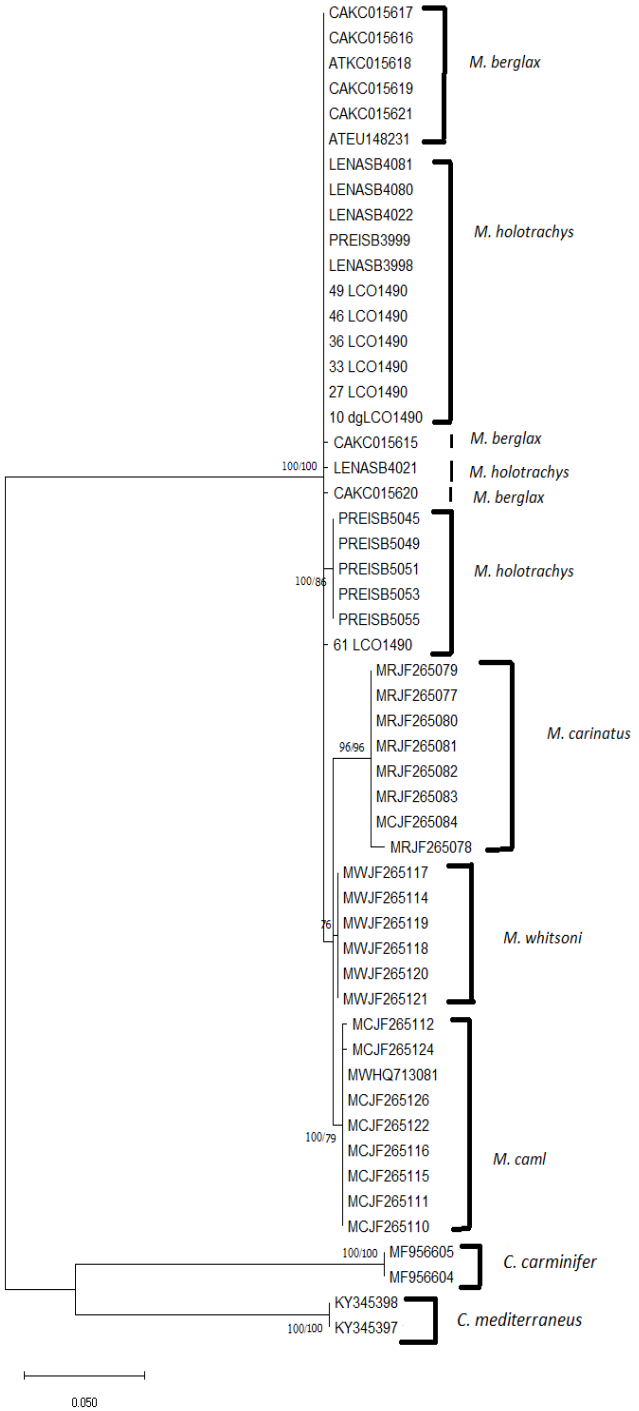

Figure 3.3. Maximum Likelihood (ML) tree resulted from the COI gene for all *Macrourus* species. Numbers at nodes represent Bayesian inference (BI) (posterior probabilities ≥ 95) and bootstrap values of ML (≥ 50) analyses. The tree is based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985).

CHAPTER 4: DISCUSSION

The present study investigated whether *Macrourus holotrachys* and *M. berglax* are the same species or not by assessing genetic structure between them. *Macrourus holotrachys* found in the Southern Ocean and *M. berglax* in the North Atlantic Ocean are among the species that need taxonomic review. These species differ slightly morphologically and these minor differences have been used as a base to separate them into different species (Fitzcharles, 2014). For the COI gene seven haplotypes were found from 26 sequences and in the d-loop region eight haplotypes were found from 55 sequences.

A high haplotype diversity and a low nucleotide diversity was obtained from the overall datasets of both the COI gene and the D-loop region for *M. holotrachys* and *M. berglax*, Similar to these findings Aboim et al. (2005) observed a high haplotype diversity and a low nucleotide diversity, in populations of an Atlantic benthopelagic fish *Helicolenus dactylopterus* using the mtDNA control region (D-loop). This pattern of genetic diversity is common to populations that have undergone a recent population expansion/growth, after a low effective population size due to bottlenecks or founder events (Grant and Bowen, 1998). During population growth there is a more rapid accumulation of mutations in the mtDNA because of a higher rate of sequence evolution (Grant and Bowen, 1998). Low genetic diversity is also consistent with populations that have experienced historical long distance dispersal, which plays an imperative role in shaping the genetic structure and distribution of marine fish populations (Aboim et al., 2005). This study revealed that *M. holotrachys* and *M. berglax* are genetically identical and are likely to be the same species despite the geographic separation and slightly different morphological characteristics. The diversity indices served as a base to determine the genetic diversity present in this species.

Low levels of genetic variance was observed among *M. holotrachys* and *M. berglax* populations within the Southern Ocean and North Atlantic Ocean, indicating little genetic structuring (Table 3.2). It is not uncommon to observe little genetic structuring even in populations that are separated by thousands of kilometres as is the case with *M. holotrachys* and *M. berglax* (Longmore et al., 2014). The results of little genetic structuring are analogous to the findings of Coscia et al. (2017) which found no evidence of structuring in *M. berglax*. The same conclusion was reached in Mediterranean grenadier and Roundnose grenadier, deep sea fish which are from the same family as the *M. holotrachys* and *M. berglax species* (White

et al., 2009; Catarino et al., 2013). The low level of genetic variance in *M. holotrachys* and *M. berglax* may be attributed to a recent evolution and expansion of *M. holotrachys* into the North Atlantic Ocean (Yodsiri et al., 2017; Fitzcharles et al., 2014).

The low level of genetic variation between *M. holotrachys* and *M. berglax* is also substantiated by the observations gleaned from the two haplotype networks. Each network had a star-like shape which is consistent with populations that are undergoing demographic expansion (Ceballos et al., 2011). The sharing of haplotypes in the haplotype network (Figure 3.2 A) for *M. holotrachys* and *M. berglax* using the COI gene suggests that there is some gene flow between the two species (Yodsiri et al., 2017). This may be due to deep-water currents acting as a transport mechanism for larval dispersal (Barker et al., 2007; Coscia et al., 2017). The gene flow/ genetic connectivity between the two species despite the geographic separation might also be maintained by an overlapping spawning season and maybe a similar spawning ground (Niu et al., 2019). The non-sharing of haplotypes in haplotype network (Figure 3.2 B) for *M. holotrachys* and *M. berglax* using the D-loop region is not sufficient to conclude that there is no gene flow as the haplotypes of *M. holotrachys* and *M. berglax* (Figure 3.2 B) are only separated by two mutations, which is consistent with species that have gene flow between them (Lord et al., 2015). A possible reason for the non-sharing of haplotypes observed on haplotype network (Figure 3.2 B) may be the faster evolutionary rate of the D-loop region and a faster divergence time due to a higher mutation rate, and no recombination, leading to a higher genetic diversity compared to the COI gene (Joshi et al., 2019; Kenechukwu et al., 2019; France and Hoover, 2002).

The extent of genetic differentiation was determined by producing the population pairwise fixation index using the COI gene in all *Macrourus* species (*M. holotrachys*, *M. berglax*, *M. whitsoni*, *M. caml* and *M. carinatus*) and the D-loop region in *M. holotrachys* and *M. berglax* only because of the lack of D-loop region data pertaining to the other three *Macrourus* species used in this section of the analyses. The values for differentiation can range from 0.0 which indicates no differentiation to 1.0 which indicates complete differentiation (Bird et al., 2011). When comparing *M. holotrachys* and, *M. berglax* to the other three *Macrourus* species (*M. whitsoni*, *M. caml* and *M. carinatus*) the results revealed substantial differentiation (Table 3.3), indicating that *M. holotrachys* and, *M. berglax* are genetically different from the other three species. The *M. holotrachys* and *M. berglax* populations revealed the lowest pairwise F_{ST} value (Table 3.3). These results indicate no substantial differentiation between the two

populations. A recent dispersal of populations may have occurred resulting in no substantial genetic differentiation between *M. holotrachys* and *M. berglax*, due to genetic exchange between species or insufficient evolutionary time (Niu et al., 2019). Similar to the findings in this study, the *M. berglax* populations analysed by Coscia et al. (2017) were found to have a low level of genetic differentiation. Using the D-loop dataset for *M. holotrachys* and *M. berglax* a high and significant pairwise F_{ST} value was found between the species, indicating a substantial differentiation. This may also have been caused by the faster evolutionary rate of the D-loop region, a faster divergence time due to its higher mutation rate, and no recombination as opposed to the low mutation rate, slower divergence time and slower evolutionary rate of the COI gene (Joshi et al., 2019; Kenechukwu et al., 2019; France and Hoover, 2002). This also suggests that there is genetic sub-structuring for the D-loop region of *M. holotrachys* and *M. berglax*, not that they are conclusively genetically different. There may be speciation that is occurring in the species but due to their recent expansion, there has not been enough separation time for them to be considered substantially different. Therefore, they are likely to be the same species but there is genetic sub-structuring, with enough time of separation maybe they can be considered as different species (complete speciation has occurred). The population genetic structure results and some similarities in morphology and spawning indicates that *M. holotrachys* and *M. berglax* are the same species rather than two different species.

The relationship between the species was further investigated using the phylogenetic analyses. *Macrourus holotrachys* and *M. berglax* formed a monophyletic lineage with a bootstrap of 100 % and posterior probability of 100, indicating that they share a common ancestor and are one species (Figure 3.3). *Macrourus carinatus*, *M. whitsoni* and *M. caml* are all positioned as distantly related lineages, each separate and genetically distinct (Figure 3.3). Congruently Smith (2011) also found that *M. holotrachys* formed a separate clade from the other three *Macrourus* species analysed in the study. Gon et al. (2021) showed in a maximum likelihood tree that the four *Macrourus* species from the Southern Ocean formed at monophyletic group although they were clearly separated. Furthermore a specimen morphologically identified as *M. carinatus* grouped with *M. holotrachys* in the genetic tree and another specimen identified as *M. caml* grouped genetically with *M. whitsoni* (Gon et al., 2021). Identification using only morphological traits can lead to misidentification and affect taxonomic status (Rubinoff et al., 2006).

As shown in this study, the taxonomic status of *M. holotrachys* and *M. berglax* was in question, was re-evaluated and found to be one species. The absence of scales on the underside of the head behind the mouth in *M. holotrachys* and *M. berglax*, is a trait that is present in the other three *Macrourus* species and not in *M. holotrachys* and *M. berglax* (McMillan et al., 2012). This observation additionally supports the species in this study being one species, positioned in the same clade instead of two species in different clades, as is the case with the three *Macrourus* species which were positioned in the same clade.

Analyses of the demographic history of these species (*M. holotrachys* and *M. berglax)* indicated that these species are undergoing demographic expansion (Figure 3.1 A and B). Negative values observed for Tajima's D in the neutrality tests also supported the findings that the populations are undergoing demographic expansion (Table 3.1) (Aboim et al., 2005). Similar to these findings Coscia et al. (2017) also observed Atlantic populations of *M. berglax* conforming to the demographic expansion model. These results suggest a recent population expansion possibly due to diversification into new habitats (Coscia et al., 2017).

The life histories that contributed to the observed demographic history of this species (*M. holotrachys* and *M. berglax*) is the prolonged life cycle which is characteristic of deep-water grenadiers (Morley et al., 2004). The prolonged spawning pattern which occurs in winter and early spring may extend throughout the year (Murua and Motos, 2000). The fecundity of this species (*M. holotrachys* and *M. berglax*) which can range from 14 400 to 260 000, correlating to body size the larger the body size the higher the fecundity (Morley et al., 2004; Murua, 2003). The hexagonal pattern membrane of the eggs is hypothesised to aid in slowing down the rise of the eggs after release into the water (Eliassen and Peterson, 1985; Fitzcharles, 2014). The eggs are usually released at the adult depth range, but if they are not they can move through the thermocline hatch and larvae would move to the appropriate depth (Fitzcharles, 2014). This is substantiated by the lack of larvae caught near surface waters (Merrett and Barnes, 1996). The populations of this species (formerly identified as *M. holotrachys* and *M. berglax*) is suspected to represent an anti-tropical species occurring in the Southern Ocean and North Atlantic Ocean (Smith et al., 2011). The larvae of this species may have been dispersed into the North Atlantic Ocean from the warmer Southern Ocean by oceanographic features such as the Atlantic meridional overturning circulation (AMOC) possibly in conjunction with the South Atlantic Current and the Benguela Current pushing the larvae northward. The AMOC is characterised by the carrying of warm waters into the upper

layers of the Atlantic Ocean and returns cold deep waters southward into the Southern Ocean (Bryden et al., 2005). While the South Atlantic current flows eastward coming from the Brazil Current and feeds into the Benguela Current, which then flows northward up to the Angola Benguela front (Garzoli and Gordon 1996). The observed demographic history is consistent with populations that originate from an expansion, due to the populations being affected by the founder effect and genetic drift (Coscia et al., 2017). Furthermore according to the findings of Coscia et al. (2017) the populations found in the North Atlantic Ocean started expanding demographically 8 000 years ago and then spatially 4 000 years ago, which is quite recent in evolutionary terms. This coincides with a postglacial range expansion which occurred in the last Younger Dryas glaciation, resulting in the dispersal of larvae into the Atlantic (Coscia et al., 2017). This is consistent with the findings of Garcia et al. (2011) that the phenomenon of demographic expansion or contraction is largely attributed to climatic changes such as glacial changes. Which is what seems to have occurred with this particular species thus, it is possible that this species may have come from populations in the Southern Ocean.

This species (formerly identified as *Macrourus holotrachys* and *M. berglax*) shows the potential of contradiction that can occur between the population genetic structure (genetic homogeneity) and the geographic and morphological characteristics that have been used to classify them as different species (Niu et al., 2019). This conveys the need to carefully identify and manage these species, as Healey and Mahe (2005) have showed that *M. berglax* is caught as by-catch in Greenland halibut fishery and deep-water shrimp trawl. The population of this species in the North Atlantic Ocean has declined by 93.3 % because of overfishing and it has been observed that it takes a long time for population numbers to recover (Devine et al., 2012; Baker et al., 2009). This species is not protected by any legislation or regulation and is not under any species protection convention making it more susceptible to population decline (Murua, 2000).

Hence, the results on the population genetic structure presented in this study also have the following biological implications that can be used to maintain stable numbers of this species. This may lead to better management strategies being used by fisheries to limit the number of individuals caught as by-catch for example, by having a cut-off in the number of *Macrourus* species that can be caught as by-catch. An additional measure may include promoting tougher penalties such as fines for fisheries that go beyond this cut-off number and those that partake

in illegal trade of this species. It can be added in species protection conventions to further ensure stable numbers of the *Macrourus* species thus nullifying the risk of these species becoming endangered. Using the results in this study, it can be lobbied that these *Macrourus* species (*M. holotrachys* and *M. berglax*) should have the same protections and fisheries should apply the same management strategies to them. Therefore, preventing conflicting management strategies from conservationists, fishing industries and governmental agencies from arising.

In conclusion this study investigated the population genetic structure of *M. holotrachys* and *M. berglax*, given the confusion and absence of consensus on whether they are the same species or not particularly due to genetic homogeneity in the species and the morphological characteristics they possess. This study revealed an absence of significant genetic differentiation between populations of *M. holotrachys* in the Southern Ocean and *M. berglax* in the North Atlantic Ocean. The outcomes of this study, suggest that *M. holotrachys* and *M. berglax* are the same species due to the great genetic similarity between them. This study has added more data on the population genetic structure of the species, which is especially lacking in the area of assessing the genetic structure of *M. holotrachys*. This study also has the potential of aiding in the conservation of this species since it is being excessively caught as by-catch in the North Atlantic Ocean. For future studies more variable markers can be used such as microsatellites to elucidate fine scale population dynamics of *Macrourus* populations in the Southern Ocean and North Atlantic Ocean.

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APPENDIX A: INFORMATION ON APPENDICES

Table 1. NCBI accession numbers retrieved from GenBank database with collection sites for *M. berglax* COI and D-loop sequences.

NCBI GenBank Acc.no.			
COI	Collection site	D-loop	Collection site
EU148231.1	North Atlantic Ocean	MG702488.1	Svalbard
KC015621.1	North Atlantic Ocean	MG702487.1	Svalbard
KC015620.1	North Atlantic Ocean	MG702486.1	Svalbard
KC015619.1	North Atlantic Ocean	MG702485.1	Svalbard
KC015618.1	North Atlantic Ocean	MG702484.1	Svalbard
KC015617.1	North Atlantic Ocean	MG702483.1	Svalbard
KC015616.1	North Atlantic Ocean	MG702482.1	Svalbard
KC015615.1	North Atlantic Ocean	MG702481.1	Svalbard
		MG702480.1	Svalbard
		MG702479.1	Svalbard
		MG702478.1	Svalbard
		MG702477.1	Svalbard
		MG702476.1	Svalbard
		MG702475.1	Svalbard
		MG702474.1	Svalbard
		MG702473.1	Svalbard
		MG702472.1	Svalbard
		MG702471.1	Svalbard
		MG702470.1	Svalbard
		MG702469.1	Svalbard
		MG702468.1	Norway
		MG702467.1	Norway
		MG702466.1	Norway
		MG702465.1	Norway
		MG702464.1	Norway
		MG702463.1	Norway
		MG702462.1	Norway

	MG702461.1	Norway
	MG702460.1	Norway