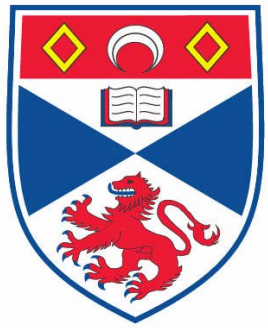
Genetic Diversity of Antarctic Fish

Elaine M. Fitzcharles



This thesis is submitted in partial fulfilment for the degree of PhD

at the

University of St Andrews

May 2014

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Acknowledgements

This part-time PhD was funded by the British Antarctic Survey. First and foremost I have to thank my supervisors, Alex Rogers and Melody Clark, for having enough faith in me as a scientist to let me begin this journey, and Andy Brierley for having enough faith in Alex to agree to take me on. It has been a longer journey than any of us intended so thank you, especially to Melody, for your continued support when life, work and unplanned distractions got in the way.

Special thanks go to Martin Collins and Mark Belchier for the fish samples, advice and support over the years. Your interest in the results has helped to keep me going. Also, to the number of fisheries scientists who have kindly collected me samples over the years; Sue Gregory, Judith Brown and Katie Brigden to name just a few. Thanks to Peter Smith, Steve Parker and Peter McMillan for the samples from the Far Side (Ross Sea), and Sarah Helyar for the ones from the North and the microsatellite collaboration.

I have to thank Will Goodall-Copestake for the technical help and training in phylogenetic analysis, as well as general support and encouragement, Huw Griffiths for the assistance with the GIS maps and Michelle Taylor for her last minute advice and reassurance. My BAS management has changed over the year but particular thanks go to Tim Moffat for recognising the importance of this work and giving me the time to finish it. I also need to thank my colleagues in the biology lab management team, Guy Hillyard, Paul Geissler and Michelle King, for continued support over the years but particularly these last few months.

Taking on a part-time PhD and a full-time job is not an easy task and would not have been possible without the support of my work colleagues in the Angel lab and fellow PhD students (too many to mention). Particular thanks go to Katrin Linse and Sandra

McInnes for their advice, support and friendship, and to Natalie Ensor and Mags Wallace for being there and understanding. On a personal note I have to thank my brother, Colin Mitchell, for helping to keep me sane, Ria (Bob) Kishen for the shared frustrations over molecular biology (we never had these problems with McGregor's cakes) and Leanne Hulse for giving me a home from home on my visits to St Andrews.

Most of all I have to thank my husband, Scott Fitzcharles, for agreeing to let me do this and being there for me throughout. There have been some tough times during the course of this PhD but also the happiest times. Thank you for Frewin and Caelan. Having two boys while trying to juggle the job and PhD probably wasn't the most sensible idea but it was the best one. They may add to the frustrations, exhaustion and general chaos but they make everything worthwhile.

Finally, thank you to my mother for the continued support in everything I decide to do in life (even the bad ideas) and always being there when I need her. To my father, I know how proud you would have been.

Abstract

Correct species identification is fundamental to all areas of biology, but particularly the policy related areas of conservation and fisheries management. To enable guidelines to be developed for environmental management and conservation, such identifications need links to studies of the evolutionary history, biological factors and environmental influences driving species divergence and population dynamics for the target species.

This study concerns two genera of gadiform fish, *Muraenolepis* and *Macrourus*, found in southern temperate and Antarctic waters, with a single species, *Macrourus berglax*, present in the North Atlantic. With similar distribution patterns to toothfish species, *Dissostichus eleginoides* and *D. mawsoni*, they are a major food source and by-catch of the toothfish fishery. Both are slow growing and long lived, with different evolutionary histories, life expectancies and strategies for reproduction. For both genera, the accuracy of morphological keys, number of described species and their distribution is under debate.

This study has identified specimens to species level using both morphological and genetic techniques, redefining the range for morphological features and taxonomic keys. For *Muraenolepis*, this has clarified confusion over *Mu. marmoratus* and *Mu. microps* being a single species, confirmed some mis-identification from sexual dimorphism and provided genetic evidence for the recently described species *Mu. evseenkoi*. For *Macrourus*, this work has identified a new species, now named *Ma. caml*, and found that *Ma. holotrachys* and *Ma. berglax* are genetically identical, raising the question of bipolar distribution or recent divergence.

The low level of genetic variation within both species suggests a recent evolution and expansion into Antarctic waters. Similar geographic species limits imply common processes influencing divergence, with the oceanographic fronts as potential barriers. Further investigation of niche overlap and fine scale population structure are required to fully understand the processes driving speciation and provide the underlying data required for fisheries management.

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CHAPTER 1

General introduction

1.1 Introduction

Accurate identification of species and defining population structure are fundamental to understanding ecosystem functioning. Without this information, it is difficult to accurately predict how populations will evolve and also respond to exploitation (e.g. fisheries) or environmental perturbation (e.g. global climate change or pollution). The Southern Ocean provides a unique environment, isolated by a continuous circumpolar current that also serves to link the Atlantic, Indian and Pacific Oceans, with warming of Antarctic waters is thought to affect global circulation and climate change (Gille, 2002). It is possibly the last ocean not fully exploited by commercial fisheries (Dodds, 2000).

1.2 Antarctica and the Southern Ocean

The evolutionary history of an environment is important to the understanding of the evolution of its fauna. In the early Tertiary period (65-55 MYA), South America, South Australia and Antarctica were part of a single faunal region, a remnant of Gondwanan supercontinent (Miller, 1993a). The deep water isolation of Antarctica was due to the opening of the Tasman Gateway, between Australia and Antarctica, ~33 MYA (Kuhnt et al., 2004), and the beginning of the Drake Passage opening, between South America and Antarctica, ~41-37 MYA (Scher and Martin, 2004), although precise timings of these events have not been fully resolved. Continued plate tectonics and volcanic activity have resulted in a series of basins, ridges, seamounts and islands (Figure 1-1), which form the bathymetry of the Southern Ocean (Tomczac and Godfrey, 2001).

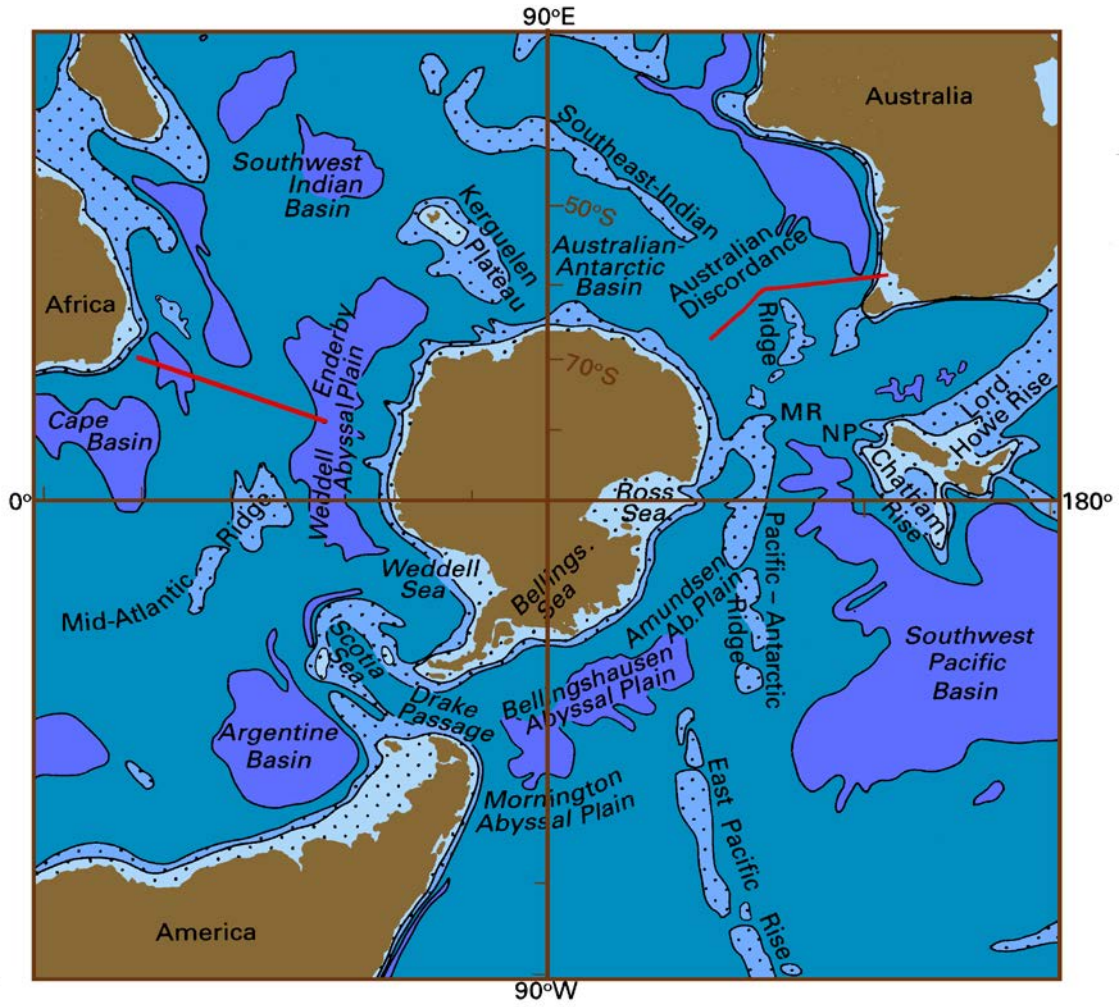


Figure 1-1 Bottom topography of the Southern Ocean. Shaded areas are less than 3000m (Tomczac and Godfrey, 2001).

The Scotia Arc is thought to have been a link between the Antarctica Peninsula (West Antarctica) and South America, and is composed of continental fragments, uplift of oceanic sediments and volcanic activity. Formed over the last 40 MY, the youngest of these islands are the volcanic South Sandwich Island chain, formed as recently as 5 MYA (Thomson, 2004).

Ocean circulation

The opening of deep seaways 33-41 MYA between Australia (Tasman Rise) and East Antarctica, and South America and Antarctic Peninsula (Drake Passage) resulted in the

formation of the Antarctic Circumpolar Current (ACC), an eastward flowing deep current driven by the West Wind Drift (Lawver and Gahagan, 2003, McGonigal and Woodworth, 2004). At the continent, the currents are driven by the East Wind Drift and flow in the opposing direction (Figure 1-2) causing formation of cyclonic gyres in the Weddell and Ross seas.

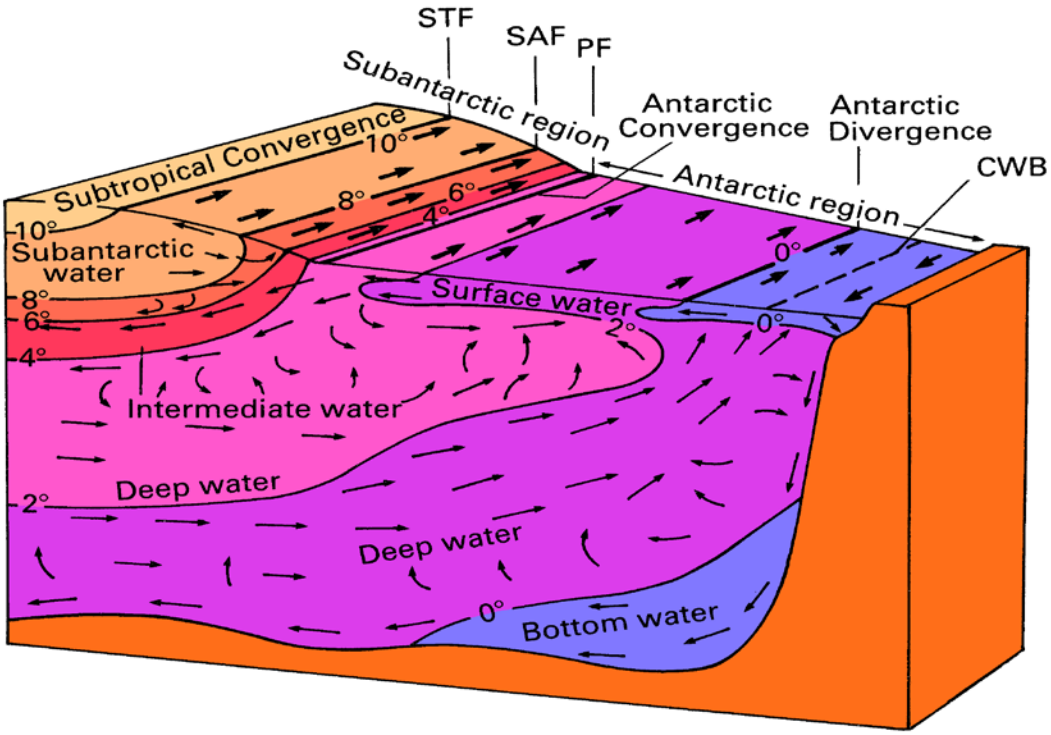


Figure 1-2 Block diagram of the circulation of the Southern Ocean (STF: Subtropical Front; SAF: SubAntarctic Front; PF: Polar Front; CWB: Continental Water Boundary) (Tomczac and Godfrey, 2001).

This ACC is linked to a series of fronts (sharp changes in temperature and salinity) known as the Polar Frontal Zone (PFZ), found between 40˚S and 60˚S (McGonigal and Woodworth, 2004) (Figures 1-2 & 1-3). These fronts may act to transport fauna and larvae or act as barriers to dispersal (Rogers et al., 2006, Rogers, 2012). The position of these fronts has varied with glaciations, other oceanic influences (El Niño) and with bathymetry (Tomczac and Godfrey, 2001, Becquey and Gersonde, 2002).

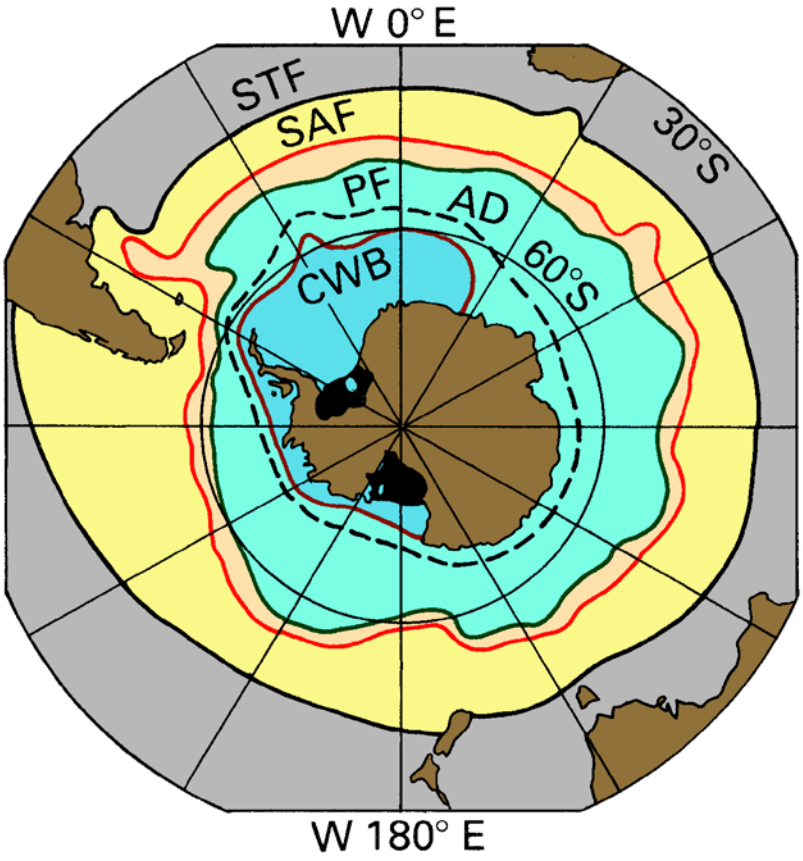


Figure 1-3 Schematic representation of the convergences and divergences of the Southern Ocean. STF: Subtropical Front; SAF: SubAntarctic Front; PF: Polar Front; AD: Antarctic Divergence; and CWB: Continental Water Boundary (Tomczac and Godfrey, 2001).

Movement of the PFZ northwards may have lead to colonisation of new coastal habitats (South America and South Africa) followed by isolation when the fronts moved south during periods of global temperature change (Tomczac and Godfrey, 2001). Certainly in the past, periods of cooling and glaciations have lead to the loss of coastal habitats and extinction of fauna, but have also lead to the formation of new habitats offering the potential for evolution of new species or new populations (Clarke and Johnston, 1996). Localised currents around islands may act to produce microhabitats, retaining nutrients and larvae in coastal regions (Tomczac and Godfrey, 2001).

Recent studies of marine invertebrate and fish species in the Southern Ocean have found that populations once thought to be homogenous are genetically distinct sub-populations or cryptic species, and gene flow in the Southern Ocean may be restricted (Appleyard et al., 2002, Parker et al., 2002, Rogers, 2012). This genetic diversity has been partially attributed to the Antarctic Polar Front acting as a barrier to larval dispersal (Parker et al., 2002, Rogers et al., 2006). Deep-water troughs, coastal currents, distance between sites and sedentary lifestyles are also thought to contribute to the genetic structure (Shaw et al., 2004, Rogers et al., 2006).

1.3 Evolution of Antarctic Fish

The fossil record for Antarctica extends back 400 MYA and includes both freshwater and marine fish. The fossil record is limited and with no clear connection to the modern fish fauna (Eastman, 1993a). Therefore, it will not be discussed further here and only the modern fauna will be considered. Despite the size and age of Antarctica, only approximately 1% of the World's fish fauna species can be found there (Eastman, 1993b).

The evolution of modern fish fauna has been governed by a number of factors; geographic movements of tectonic plates and land masses, changes in water temperature, effects of ice cover, changes in oceanographic currents and habitat availability (particularly the lack of shallow continental shelf habitats, see Figure 1-1). The most predominant order of fish in the modern Antarctic fauna is the perciform suborder Notothenioidei. This suborder is endemic to the Antarctic and comprises 45% of the total fish fauna (Eastman and McCune, 2000). It is thought that the original coastal fauna began to disappear in the late Eocene (38-25 mya), being replaced by these cold-tolerant fish. Notothenioids are most abundant in coastal waters, both benthic

and pelagic, and are thought to have evolved in this area, associated with the continental shelf (Eastman, 1993c).

There are a number of theories behind the distribution of non-notothenioid fish. Some are thought to have evolved in the Southern Ocean but been constrained in their distribution by the formation of the Antarctic Convergence. Others are thought to have evolved in the Atlantic, the most probable route into the Antarctic being the Scotia Sea (Eastman, 1993c). A few are thought to have originated in the Pacific. Many also have sister taxa present in the Arctic, suggesting a possible Arctic origin (Anderson, 1990).

1.4 Study Fish Species

The fish chosen for this study belong to the order Gadiformes (or Anacanthini). There are over 500 species distributed globally throughout the World's oceans and many species are of ecological importance. There is much disagreement on the division of this order into families and subfamilies and morphological distinction is unclear. They have a preference for cooler benthopelagic habitats of tropical and temperate seas, with only a few species present in the Antarctic (Cohen, 1990). These Antarctic species comprises *c*. 7.6% of the Antarctic fish fauna in terms of number of species (Eastman, 1993b).

The two genera under consideration belong to families in the order Gadiformes and have species ranges that include temperate, subAntarctic and high Antarctic waters. They have been chosen for this study for two main reasons. Firstly, they are by-catches of the *Dissostichus eleginoides* fishery and secondly, they are both long-lived deep-sea species, thus enabling more generalised comparisons to be made of gene flow between deep-sea Southern Ocean fish species.

The first is the genus *Muraenolepis*, belonging to the family Muraenolepididae (suborder Muraenolepididei). There are nine species described within this genus: *Muraenolepis marmoratus* Gunther, 1880; *Mu. orangiensis* Vaillant, 1888, *Mu. microps* Lönnberg, 1905; *Mu. microcephalus* Norman, 1937; and the recently described *Mu. andriashevi* Balushkin and Prirodina, 2005; *Mu. trunovi* Balushkin and Prirodina, 2006; *Mu. kuderskii* Balushkin and Prirodina, 2007; *Mu. pacifica* Balushkin and Prirodina, 2007; *Mu. evseenkoi* Balushkin and Prirodina, 2010. It has also been proposed that *Mu. microcephalus* should be considered as a separate genus, *Notomuraenobathys microcephalus* Balushkin and Prirodina, 2010. Further information on each species is given in Chapter 3.

The second is the genus *Macrourus*, belonging to the family Macrouridae (subfamily Macrourinae). There are five described species for this genus: *Ma. berglax* Lacepede 1801; *Ma. carinatus* (Günther, 1878); *Ma. Holotrachys* Günther, 1878; *Ma. whitsoni* (Regan, 1913) and the recently described *Ma. caml* Smith, 2011. Further information on each species is given in Chapter 4.

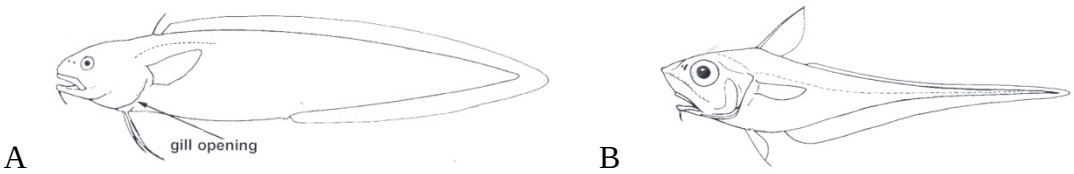


Figure 1-4 Typical morphology of the families (A) Muraneolepididae and (B) Macrouridae (Cohen, 1990) p13 &14.

The typical morphology of these families is shown in Figure 1-4. Very little is known about the life cycle and biology of these species and the systematics of both groups is open to question, making them ideal candidates for molecular investigation.

1.5 Antarctic Fisheries

Both *Muraenolepis* and *Macrourus* species are caught as by-catch of Antarctic fisheries and, like other deep-sea fish, tend to have low fecundity and long life cycles making them particularly susceptible to over-fishing. In recent years the exploitation of the Southern Ocean's marine resources has become a cause for concern with the depletion of fish stocks as a result of over-fishing, and among these species are the Patagonian toothfish (*Dissostichus eleginoides*) and Antarctic toothfish (*Dissostichus mawsoni*). Fishery prospecting in the 1960s established a fishery on the Kerguelen Plateau in 1970 and expansion to include toothfish trawls in 1984 (Palomares and Pauly, 2011). Similar fishing efforts have been in place around South Georgia during this time (Agnew, 2000). The introduction of new fishing techniques, such as longline in late 1990s, has allowed access to new fishing areas and deeper waters, which has lead to the rapid growth of the toothfish industry (Constable et al., 2000, Lack and Sant, 2001, Palomares and Pauly, 2011). This situation is further complicated by illegal, unregulated and unreported (IUU) catches. Fish are processed onboard ships, traded under a variety of names and sold mainly in the northern hemisphere (Ferguson et al., 1995).

Successful management of these fisheries is essential for sustainability. Many of these fisheries are within the Convention area of the Commission for the Conservation of Marine Living Resources (CCAMLR) and are governed by strict quotas per annum of allowable catch (Croxall and Nicol, 2004, Constable et al., 2000). Catch limits are also set for ecologically important by-catch species (CCAMLR Conservation measures 33-

03) and reaching these limits results in the closure of the fishery even if the catch of target species is well within limits; as was the case for *Macrourus* species for the Ross Sea (88.2) toothfish fishery in 2006 (http://fs.fish.gov.nz/Doc/23465/021_TOT_November_2013.pdf.ashx).

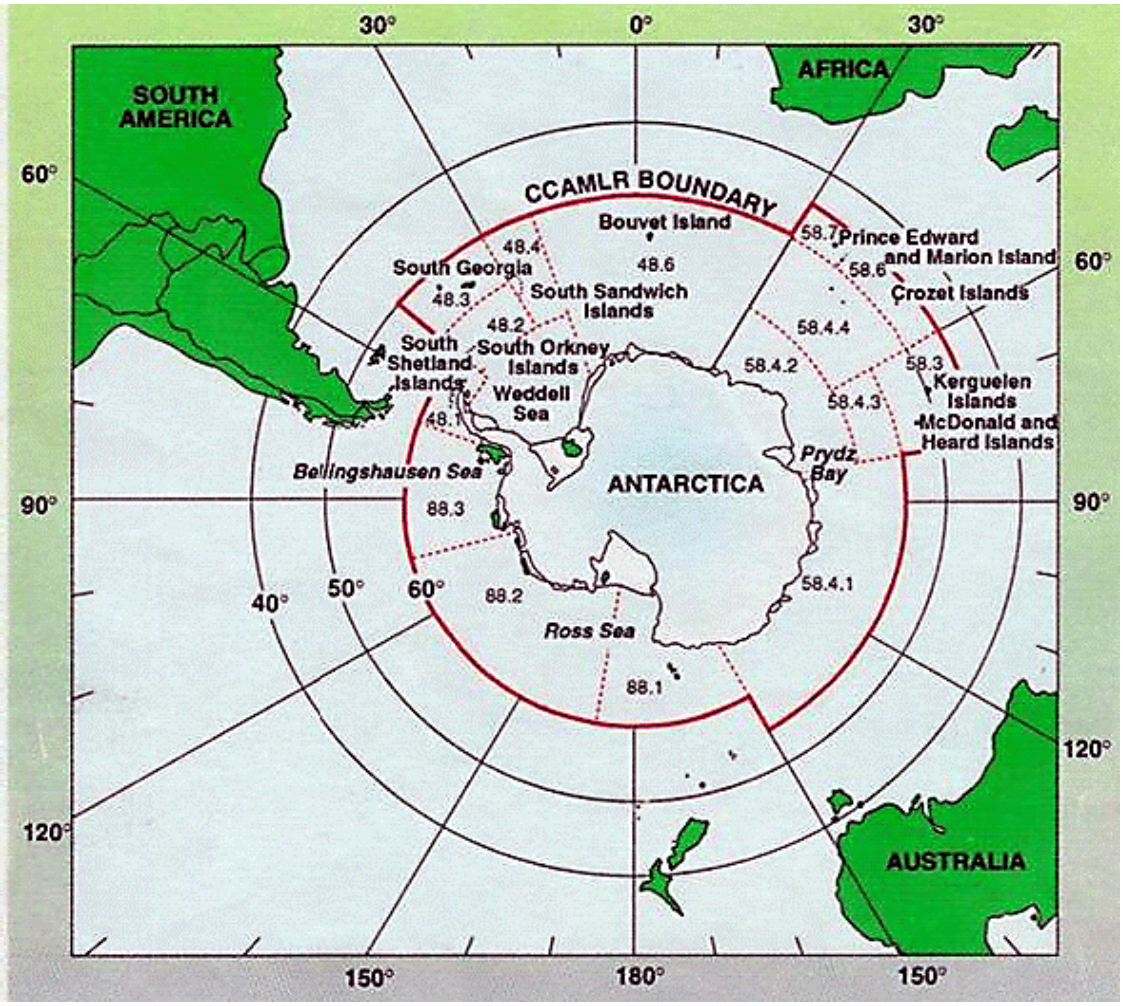


Figure 1-5 Map of CCAMLR zones for the Antarctic and subAntarctic islands showing the division into fishing areas (Fallon and Stratford, 2003).

The fish under study in this thesis are caught as by-catch of the toothfish fishery. The level of by-catch has increased in line with increased efforts by the fishing industry. For the Kerguelen Islands (area 58.5.1) fishery the *Macrourus* species catch has increased from 6 tons in 1998 to 537 tons in 2007 (Palomares and Pauly, 2011). For the Ross Sea

(area 88.1) it has increased from 9 tons in 1998 and peaked at 462 tons in 2005; and 4 tons in 2002 to 92 tons in 2006 for area 88.2 (http://fs.fish.gov.nz/Doc/23465/021_TOT_November_2013.pdf.ashx).

The North Atlantic *Macrourus* species, *Ma. berglax*, is among the grenadier species that has been fished commercially in the North Atlantic, but is also a by-catch species of Greenland halibut and deep-water shrimp trawls. The stocks of *Ma. berglax* in the north Atlantic are estimated to have declined by 93.3% as a result of overfishing (Devine et al., 2006, Devine et al., 2012). Recovery time for *Ma. berglax* in Canadian waters has been estimated at 18 to 125 years, or 19 to 248 years if 5% by-catch is included (Baker et al., 2009).

The Southern Ocean species are of little commercial importance in their own right, with the exception of *Macrourus* species in the Patagonian region (Cohen, 1990, Coggan et al., 1996). Assessment of the stocks of *Ma. carinatus* in Falkland Islands waters estimated that a 2-4% removal of stock would yield 1000-2000 mt while maintaining a sustainable fishery. From 1988 to1990, 100,365 mt of *Macrourus* species were caught in this area by Soviet squid and finfish fisheries (Laptikhovsky et al., 2008).

All of the fish species targeted in this study have similar distribution patterns and life cycles associated with the continental shelves. They are all slow-growing, long-lived species with low overall recruitment rates, making them vulnerable to over fishing and at risk of becoming endangered (Devine et al., 2006, Morley et al., 2004).

1.5 The role of genetics in conservation

Successful conservation and good fisheries management rely on a good understanding of population (stock) structures. Molecular markers can be used for management and

conservation to identify and classify species, identify fish products and determine biologically meaningful stocks. They can provide information on population structure, phylogenetics, dispersal and evolutionary history that remain hidden to traditional alpha taxonomic techniques (Avise, 1998). Therefore, genetic studies have become a very useful tool in conservation and fisheries management.

Maintenance of genetic diversity is also essential for population survival. Declines in population size can lead to a reduction in genetic diversity through inbreeding. This potentially reduces the ability of the population to adapt to changing circumstances, such as increased environmental temperatures in line with global climate change predictions (Frankham, 2003).

The fundamental role genetics can offer to conservation is the correct identification of species. The mitochondrial gene region, cytochrome oxidase I, has been shown to be very useful for species identification and is the molecule of choice for DNA barcoding (Morita, 1999, Ward et al., 2005), including in the barcode of life project (Savolainen et al., 2005) and the Census of Antarctic Marine Life (Dettai et al., 2011).

However, barcoding cannot define fine-scale population dynamics, and for this a more variable marker is required. Determining effective stocks for fisheries management and conservation measures can be achieved by the use of microsatellite markers. These are highly variable, non-coding regions of nuclear DNA, and have become a popular choice for studies in fisheries genetics (Liu and Cordes, 2004). For example, where mitochondrial markers revealed no significant population heterogeneity for the New Zealand snapper (*Pagrus auratus*), microsatellites (and previously allozymes) revealed differentiation between north-east and southern populations with the Tasman Bay population isolated from both; possibly because coastal currents were acting as barriers

to dispersal (Bernal-Ramirez, *et al*, 2003). In contrast, for the mesopelagic Antarctic fish, *Electrona antarctica*, microsatellite analysis revealed a high level of genetic variability but lack of population structure within the Southern Ocean (Van de Putte et al., 2012). Genetic studies such as these help to understand the role life-cycle and environment in governing population dynamics and lead to a better understanding of the ecosystem as a whole.

1.6 Aims and objectives

The aim of this thesis is to investigate the systematics and population structure of two Southern Ocean deep-sea fish genera, *Muraenolepis* and *Macrourus*. Currently very little is known of the relationships between *Macrourus* and *Muraenolepsis* species. Using phylogenetic analysis I will supply molecular tags and provide clear species definition. By comparing the gene flow of *Macrourus* and *Muraenolepsis* species I will provide an important insight into the factors that structure the genetic variation of deep-water species in Antarctic waters, with associated implications for understanding dispersal in Antarctic species and the influence that historical factors had on evolution of the Southern Ocean Biota.

The specific objectives are:

- Species Identification and phylogenetic characterisation of *Muraenolepsis,* species using mitochondrial DNA sequences, to confirm current systematics and resolve the evolutionary history of these species.
- Species Identification and phylogenetic characterisation of *Macrourus* species, using mitochondrial DNA sequences, to confirm current systematics and resolve the evolutionary history of these species.

- Population genetic analysis of *Macrourus* species using a suite of microsatellite markers developed for *Macrourus berglax* (north Atlantic species).
- Synthesis of data to compare species with respect to geographical history and ocean currents.

1.7 This thesis

My project originated from the observations made, and difficulties experienced, by fisheries scientists working around South Georgia regarding the accurate morphological identification of the species present in the region. Originally, my study was to focus on the South Georgia area with samples collected during CCAMLR ground fish survey (2003). However, the outcome of these initial results led to further collaborative and sampling opportunities, which expanded the geographic area to include the South Sandwich Islands, Falkland Islands and Ross Sea.

The samples for this thesis were collected on scientific and fisheries vessels over an eleven year period, from 2002 to 2013, form part of the CCAMLR ground fish survey, and are stored at the British Antarctic Survey for genetic analysis**.** Additional samples were also obtained opportunistically from fishing vessels or scientific cruises. The morphological methods used for identification and molecular techniques employed are detailed in Chapter 2.

The results of my study are presented in four chapters. The first, Chapter 3, concerns the morphological and molecular species identification, of the species in the genus *Muraenolepis* (Family: Muraenolepididae) and examines the phylogenetic relationship between species and any evidence for population structure within the Southern Ocean. Chapter 4 describes the morphological and genetics of the Southern hemisphere species

whitin the genus *Macrourus* (Family: Macrouridae), with an aim to identify phylogenetic relationships between species and population structures.

Chapter 5 will look at the relationship of both of these genera in comparison to other families within the order Gadiformes. Chapter 6 tests the cross-amplification of nuclear DNA markers, known as microsatellites, isolated and developed for *Ma. berglax* on the four Southern Ocean species, and evaluates their suitability for use on population genetic analysis.

In the final section, Chapter 7, I will draw together the results of the previous chapters, comparing the combined results in light of the evolutionary history and population dynamics of other Antarctic species.

CHAPTER 2

Materials and Methods

2.1 Sample collection and Morphological Identification

This project was designed to utilise samples collected during CCAMLR ground fish surveys, which were already available in the specimen collection at BAS, Cambridge. There had been no scope within this project's funding for planned sampling via dedicated cruises, however, further samples were collected by CCAMLR Fisheries Observers and scientists, BAS scientists, or were kindly donated by researchers from other institutes. For the *Macrourus* study, a small number of *Ma. berglax* (from the North Atlantic) tissue samples were obtained so that all species belonging to the genus could be included in the phylogenetic analysis.

Samples were labelled, at source, to genus or species level by morphological identification carried out by the scientific observers onboard the vessels using standard taxonomic keys for species identification or by assumptions from catch depth or geographic location.

After species identification was completed, a small tissue sample was collected for genetic analysis. This varied in size from 5 mm^3 to 5 cm^3 and was muscle tissue, liver tissue or fin clippings. Samples were placed in individual tubes and preserved in 96% ethanol or frozen at -80°C. Whole specimens were then discarded except in a small number of cases where the whole specimen was frozen for further identification.

Muraenolepis Species Identification

The genus *Muraenolepis* is a poorly studied group and there is some controversy over the number of species described and the morphological characteristics used to discriminate between species. A range of standard measurements used for full morphological identification are shown in Figure 2-1. This work predates the description of the additional species in this genus (Balushkin and Prirodina, 2013) and so they were not considered in the morphological identification.

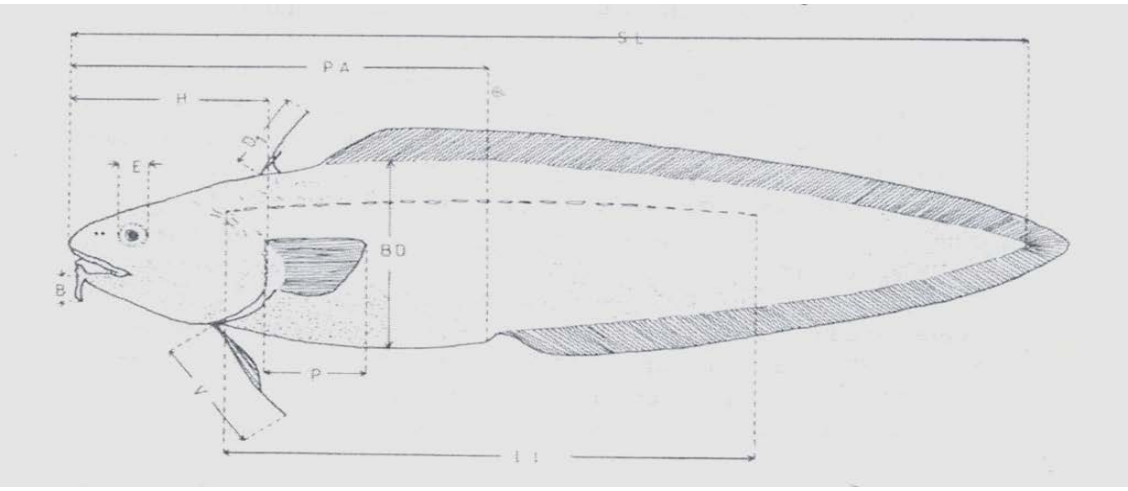


Figure 2-1 Diagram showing the measurements for species identification. SL – standard length; PA – preanal distance; H – head length; D1 - length of dorsal filament; E – eye diameter; B – length of mental barbell; DB – body depth; P – length of pectoral fin; V – length of pelvic fin; 1.1 – chord length of lateral line. (Figure taken from Kompowski and Rojas, 1993; p90).

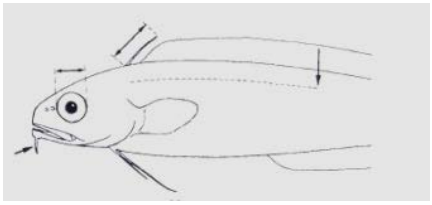
Where x-rays are available, the following key can be applied (Chiu and Markle, 1990):

Species	Lateral Line	Vertebrae	2nd Dorsal Fin	Anal Fin
Mu. marmoratus	Regular, middle of D2	67-71	128-147	89-108
Mu. orangiensis	Regular, middle of D2	74-76	161-175	124-131
Mu. microcephalus	Irregular, short	83-85	160-176	122-135
Mu. microps	Irregular, short	70-74	133-146	99-112

Table 2-1 Morphological key for *Muraenolepis* as proposed by Chiu and Markle (1990).

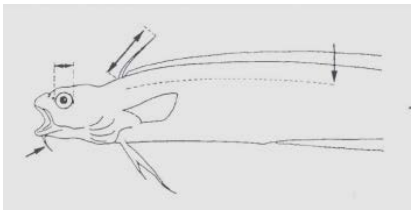
The most simplistic key for identifying the four species, and therefore more practical for identification at capture, is based on four morphological features: length of lateral line, eye diameter, length of mental barbell and length of dorsal filament (Cohen, 1990).

1. Lateral line extends past middle of second dorsal fin.
    a. First dorsal fin ≥ eye diameter; mental barbel < eye diameter.



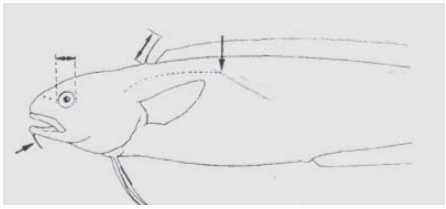
Mu. marmoratus

    b. Dorsal fin > 3x eye diameter; mental barbel = eye diameter



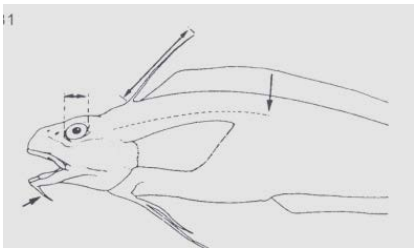
Mu. orangiensis

2. Lateral line extends slightly past pectoral fins
    a. First dorsal fin ≤ 2x eye diameter; mental barbel > eye diameter.



Mu. microps

    b. Dorsal fin > 4x eye diameter; mental barbel = eye diameter.



Mu. microcephalus

Figure 2-2 Key to identification of *Muraenolepis* species (Cohen, 1990).

Samples were identified to species level using this key or designated only to genus level. Where possible, further measurements were recorded by fisheries scientists.

Macrourus Species Identification

For the genus *Macrourus*, all samples were identified, at source, to species level using a number of morphological features, although overlapping ranges for some of the characteristics often leads to problems distinguishing between species. One of the principle taxanomic features used is the presence or absence of scales on the underside of the head (as shown in Figure 2-3). However, in some specimens, particularly juvenile fish, this scale coverage is reduced or are removed during capture and can lead to misidentification.

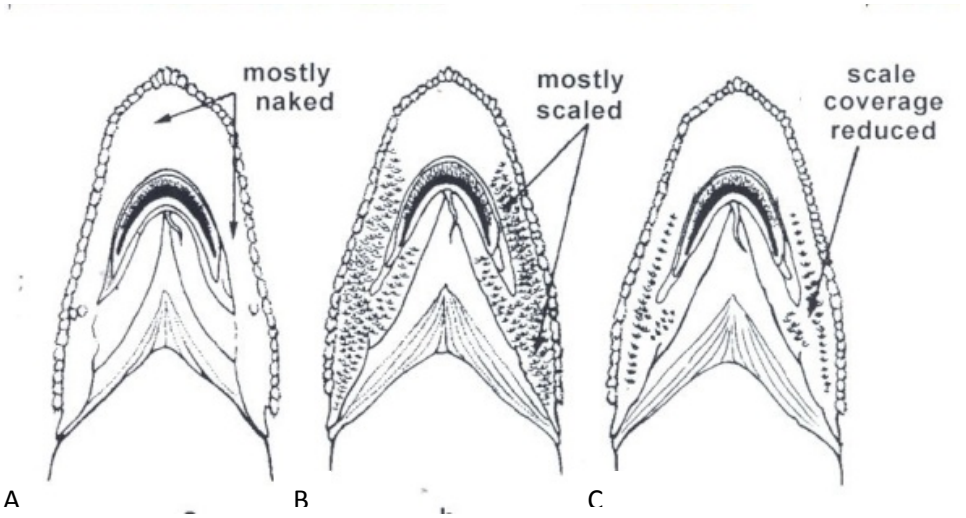


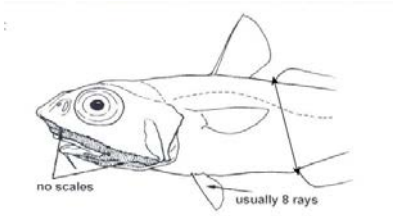
Figure 2-3 Drawing showing the difference in scaling to the underside of the head as used to distinguish between *Macrourus* species; (A) *Ma. holotrachys*, (B) *Ma. carinatus* and (C) *Ma. carinatus* where scales are reduced (Cohen, 1990).

Additional morphological features used to assist identification were the number of rays in the pelvic fin, the number of pyloric caeca (finger-like structures of the intestine), position of the dorsal fin relative to the anal fin, and number of scales between anal fin and lateral line (Cohen, 1990, Morley et al., 2002). The key for species identification is

shown in Figure 2-4. Geographic location and depth were also taken into consideration as these species are considered to have distinct ranges (Table 2-1).

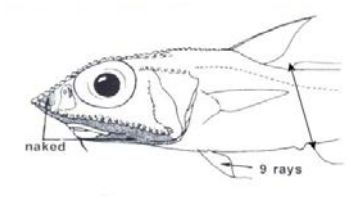
1. Underside of head without scales, or only 1-3 above corner of mouth.

   Pelvic rays = 8, pyloric caeca ≈ 20



Ma. berglax

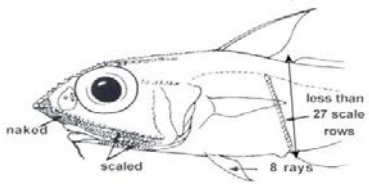
   Pelvic rays = 9, pyloric caeca = 8 to 16, start of second dorsal fin anterior to start of anal fin.



Ma. holotrachys

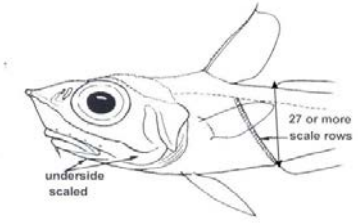
2. Scales between suborbital ridge and jaw, and underside of lower jaw.

   a. Scales in oblique row between anal fin and lateral line <27. Start of second dorsal fin slightly anterior to start of anal fin.



Ma. carinatus

   b. Scales in oblique row between anal fin and lateral line >27. Start of second dorsal fin directly above start of anal fin.



Ma. whitsoni

Figure 2-4 Key to identify *Macrourus* species (modified from (Cohen, 1990, Morley et al., 2002).

Species	Distribution	Depth range (m)	Pelvic fin ray	Colour	Pyloric caeca	Underside head
Ma. berglax	Temperate to Arctic waters of North Atlantic	100-1000 (300-500 greatest conc)	8 (7-9)	overall grey, darker ventrally on trunk, fins darker	19-20	entirely naked or 1-3 scales
Ma. holotrachys	Patagonian slope, South Georgia	300-1200	9 (8)	light to medium brown, grayish brown; fins darker	8 - 16	entirely naked or 1-3 scales
Ma. carinatus	SubAntarctic, temperate waters South America, South Africa, New Zealand	300-1100	8 (9)	medium brown to straw; fins darker	13 - 21	scaled
Ma. whitsoni	Circumpolar Antarctic waters, Malvinas shelf	400-3185	8 (7-9)	dark brown to swarthy; some much paler	18 - 28	scaled

Table 2-2 Geographic distribution and morphological identification of *Macrourus* species (Iwamoto, 1990, Cohen, 1990).

General comments on identification

For a limited number of samples, detailed measurements were recorded and further species identification carried out by fisheries scientists. For *Muraenolepis*, most specimens measured for Total Length (TL). For specimens where only Standard Length (SL) was recorded a comparable conversion factor for the relevant species was used was calculated using specimens where both measurements had been recorded. For *Macrourus*, length was generally measured as SL. Where only TL was recorded, a conversion factor was used to convert to SL. Length was plotted against weight using

SigmaPlot10 (Systat Software, San Jose, CA) to determine whether there were any trends indicating differences between species or between male and female specimens.

Full details of sample collection and morphological identification are given in Chapter 3 (*Muraenolepis*) and Chapter 4 (*Macrourus*).

2.2 Molecular Methods

For the molecular identification, the species identifications were carried out using either PCR amplification and sequencing of regions of the mitochondrial genome (barcoding), or High Resolution Melt (HRM) analysis.

DNA extraction

Tissue samples were removed from ethanol and a small subsample weighing approximately 25 mg was taken for DNA extraction, which was extracted using DNeasy tissue kits (Qiagen, UK) according to manufacturer's instructions. The DNA was resuspended in elution buffer and the DNA yield checked using a Nanodrop ND1000 spectrophotometer (Labcare International, UK), and the concentrations used ranged from 25 ng/µl to 170 ng/µl.

Mitochondrial DNA PCR amplification

The regions of the mitochondrial genome selected for use in this study were the 16S large ribosomal RNA subunit and the Cytochrome Oxidase I subunit. The 16S region mutates at a slower rate than the other regions of the mitochondrial genome (Palumbi, 1996) and is therefore relatively conserved with some regions of high sequence substitutions, providing a longer fragment compared to the small ribosomal subunit, 12S.

The Cytochrome Oxidase I gene is protein coding and is highly conserved across phyla, therefore has low variability, with few intra-species substitutions. This makes it ideal for use in phylogenetic studies. Since beginning this project the COI region has become the standard gene used for species identification and DNA barcoding in initiatives such as the Barcode of Life (BOLD) (www.boldsystems.org) and Fish Barcode of Life (www.fishbol.org)(Ward et al., 2009). However, for some species, the level of interspecific variation may not be sufficient to resolve species identity (Dettai et al., 2011)

16S rRNA amplification

The partial 16S rRNA region was sequenced using the universal primers 16S AR (5'-CGC CTG TTT ATC AAA AAC AT- 3') and 16S BR (5'-CCG GTC TGA ACT CAG ATC ACG-3') (Palumbi et al., 1991). The following reaction conditions were used for a 50 µl reaction: 5 µl 10X PCR buffer (Tris-HCl, KCl, pH 7.8, containing 1.5mM $MgCl_2$), 1 µl $MgCl_2$, 1 µl dNTP (10mM each dNTP), 0.5 µl each primer (10nM), 0.25 µl Taq (2.5 U), between 0.5 µl and 2 µl DNA and between 41.25 µl and 39.25 µl dH_2O; all reagents from Qiagen. Reaction conditions were: 94˚C for 4 minutes, 30 cycles of 94˚C for 30 seconds, 50˚C for 1 minute, 72˚C for 1 minute, then 72˚C for 10 minutes. All reactions were carried out on an MJ Research Peltier Thermal Cycler 225 DNA Engine Tetrad-Gradient. PCR products were cleaned using Qiaquick (Qiagen, UK) PCR spin columns following manufacturer's instructions.

COI amplification

The partial CO1 mRNA region was amplified using the universal primers LCO 1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO 2198 (5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3') (Folmer et al., 1994). The reaction

conditions were as follows in a 50 µl reaction: 5 µl 10X PCR buffer (Tris-HCl, KCl, pH 7.8, containing 1.5mM $MgCl_2$), 3 µl $MgCl_2$, 1 µl dNTP (10mM each dNTP), 0.5 µl each primer (10nM), 0.25 µl Taq (2.5 U), between 1 µl and 10 µl DNA and between 39.25 µl and 30.25 µl dH_2O; all reagents from Qiagen. The reaction was carried out on a MJ Research Peltier Thermal Cycler 225 DNA Engine Tetrad-Gradient using the following thermal profile: 95˚C for 4 minutes followed by 5 cycles of 95˚C for 1 minute, 45˚C for 90 seconds and 72˚C for 90 seconds, then 35 cycles of 94˚C for 1 minute, 50˚C for 90 seconds and 72˚C for 1 minute, and finally 72˚C for 5 minutes. PCR products were cleaned using Qiaquick PCR spin columns following manufacturer's instructions.

2.3 Species' Specific COI Primers for *Macrourus* Species

A high number of *Macrourus* samples produced double banded PCR products when using the LCO 1490 and HCO 2198 primers (Folmer et al., 1994). A number of attempts to optimise the reaction, such as varying the magnesium concentration and altering the annealing temperature, failed to overcome the problem. Therefore species-specific primers were designed. Sequence data from samples that had been successfully amplified were aligned and examined for conserved regions which could provide suitable primer binding sites. Primers were designed using Primer3 (Rozen, 2000), checked for self-compatibility using Operon Oligo Analysis Tool (http://www.operon.com/tools/oligo-analysis-tool.aspx) and visually inspected for suitability.

Three primer pairs were selected for testing and all combinations produced a single band product of the expected size with high efficiency.

1. Product size 542bp; Tm 57°C (annealing 52°C)

   Mac_F1 5'- AGCCCGGAGCACTTTTAG -3'

   Mac_R1 5'- GGCAGGATCAAAGAAAGAAG -3'

2. Product size 543bp; Tm 57°C (annealing 52°C)

   Mac_F2 5'- GCCCTAAGCCTTCTCATTC -3'

   Mac_R2 5'- CGGCAGGATCAAAGAAAG -3'

3. Product size 557bp; Tm 56°C (annealing 61°C)

   Mac_F2 5'- GCCCTAAGCCTTCTCATTC -3'

   Mac_R3 5'- GTATTAAGGTTTCGATCTGTGAG -3'

Primer pair Mac_F1 and Mac_R1 was chosen for use and the reaction conditions used were 5µl 10X Buffer, 1.5µl $MgCl_2$, 1µl dNTPs, 1µl each primer (10nM), 0.25µl Taq (500U) (all reagents Qiagen, UK), 34.25µl H_2O and 2µl template DNA with a thermal profile of 94°C for 10 minutes followed by 30 cycles of 94°C for 30 seconds, 50°C for 60 seconds and 72°C for 60 seconds, then a final annealing step of 72°C for 10 minutes. PCR product was cleaned as before.

DNA sequencing

Initially, sequencing was carried out in-house using BigDye Dye Terminator Mix (Applied Biosystems, UK) according to manufacturer's instructions and using the following thermal profile: 25 cycles of 96˚C for 10 seconds followed by 50˚C for 10 seconds, with a final extension step of 60˚C for 4 minutes. Products were cleaned by ethanol precipitation (30µl of 1:25 3M sodium acetate:ethanol added to each sample and

centrifuged at 4˚C for 50 minutes then washed twice with 50µl 70% ethanol) and re-suspended in 10µl MegaBACE loading solution (Amersham Bioscience, GE Healthcare, UK). Sequences were visualised using a MegaBACE 1000 capillary DNA sequencer and Sequence Analyser version 3.0 software (Amersham Bioscience, GE Healthcare, UK).

Following the loss of in-house sequencing facilities at the British Antarctic Survey the PCR product was sent to a commercial sequencing facility, either LGC Genomics (Berlin, Germany) or Source Bioscience (Cambridge, UK), for product clean-up and Sanger sequencing. All samples were sequenced using the forward and reverse primers to maximise read length.

2.5 Molecular Analysis of Mitochondrial Sequences

All sequences were manually edited and aligned using CodonCode Aligner Version 2.0.6 (CodonCode Corporation, USA), which allowed direct reference to, and visualisation of, the raw chromatogram data. Each data set was edited to remove characters with more than 50% gaps (Doolittle, 1987). The sequence alignments produced were viewed in Geneious Version 6.1.6 (www.geneious.com). The COI data were translated to amino acid codons using the first reading frame in CodonCode Aligner to ensure that the sequences were mitochondrial DNA and not nuclear pseudogenes (numpts) (Bensasson et al., 2001, Zhang and Hewitt, 1996). The haplotypes were identified for 16S, COI datasets by identifying sequences with identical residues and extracting the unique sequences.

***Muraenolepis* species**						
Dataset	**Unique Haplotypes**	**Outgroups**	**Characters**	**A**	**B**	**C**
16S	9	2	508	416	92	36
COI	25	2	600	420	180	78
***Macrourus* species**						
Dataset	**Unique Haplotypes**	**Outgroups**	**Characters**	**A**	**B**	**C**
16S	4	2	500	408	92	36
COI	11	2	420	291	129	49

A=constant characters; B=variable characters; C=parsimony informative characters

Table 2-3 Summary of characters for 16S and COI phylogenetic analysis, including outgroups.

Sequences generated within this study for the *Muraenolepis* species were used the outgroup for the *Macrourus* species, and vice versa, alongside sequence data for *Merluccius paradoxus* (voucher DAAPV F8) obtained from Genebank. *Merluccius paradoxus* was selected as it is a similar genetic distance from both of the genera under investigation in this study and sequence data were available for 16S and COI regions from the same individual.

Standard phylogenetic models make the assumption that evolution is under stationary, reversible, homogenous conditions and data which do not fit these conditions increase the risk of phylogenetic errors (Ho et al., 2006). The haplotype alignments were tested for nucleotide frequency compositional heterogeneity by performing a Chi-squared test of homogeneity across all taxa in PAUP v4.0b10 (Swofford, 2002). The results are shown in table 2-4.

***Muraenolepis* species**		
Dataset	**Chi-square**	**P**
16S	5.374645 (df=30)	0.99999983
COI	26.322695 (df=78)	0.99999999
***Macrourus* species**		
Dataset	**Chi-square**	**P**
16S	4.932397 (df=15)	0.99926835
COI	13.266982 (df=36)	0.99980721

Table 2-4 Results from Chi-square test of homogeneity.

The two genes were analysed separately using standard analysis methods of heuristic searches using unweighted maximum parsimony (optimal trees produced on assumption of simplest chain of events for given characteristics), heuristic searches using Maximum Likelihood with DNA substitutions models (trees obtained are best fit for the data and model chosen), and Bayesian inference using substitution models (Markov chain Monte Carlo algorithm and posterior probability to define the tree which best represent the phylogeny) were applied. The maximum parsimony analyses were conducted using PAUP v4.0b10 (Swofford, 2002), all characters equally weighted with each search starting from a random tree, with 50 random addition replicates, one tree held at each step, tree bisection-reconnection branch-swapping, steepest decent on and a maximum of 10,000 saved trees. Default values were used for all other setting.

The maximum likelihood (ML) analysis were conducted using GARLI v2.0 (Zwickl, 2003) on default settings, with the exception of the nucleotide models. The nucleotide models used were the best fitting models inferred by jModelTest version 2.1.2 (Guindon and Gascuel, 2003, Posada, 2008) using the Bayesian information criteria selection procedure and ML optimised topologies.

Dataset	BIC best fitting model
Muraenolepis 16S	TMP2+I or TMP2+G (TMP2+G chosen)
Muraenolepis COI	TPM2uf+G
Macrourus 16S	TMP2+I or TMP2+G (TMP2+G chosen)
Macrourus COI	HKY+G or TMP2+G (both chosen)

Table 2-5 Nucleotide substitution models fron JModelTest, selected for use in ML and MP analysis

Phylogeny confidence values were generated for both analyses by non-parametric bootstrapping (Felsenstein, 1985), with 10,000 replicates for each heuristic tree for the parsimony analysis and 1000 replicates for the Maximum Likelihood analysis.

The data were also analysed using MrBayes (Huelsenbeck and Ronquist, 2001). The models suggested by jModelTest were not available in MrBayes so the nearest equivalent models were selected: For 16S for both species and COI for *Macrourus*, this was GTR+G (Nst=6) instead of TMP2+G and for *Muraenolepis* COI it HKY instead of TMP2uf+G. Two runs were conducted, each with two chains of 10,000,000 generations sampled every 500 generations, first 7,500 trees as burnin. Convergence statistics produced by MrBayes (PSRF), effective samples sizes (ESS) and posterior distributions of parameters from Tracer v1.5 (Rambaut et al., 2007) suggested this level of burnin was sufficient for mcmcMarkov chains to reach stationarity. All analyses were repeated to ensure consistency of results.

A second method of analysis was carried out to test for nucleotide frequency compositional heterogeneity using match-based pairs tests for symmetry (Ababneh et al., 2006), with the software SeqVis V1.3 (Ho et al., 2006). Significant compositional

heterogeneity is inferred where x% of the matched-pairs tests of symmetry produced P-values greater than or equal to x (Goodall-Copestake et al., 2009). For both 16S data sets, no significant heterogeneity was shown. For COI however, significant heterogeneity was inferred for both datasets:

Dataset	**x% at p-value<0.05**	**x% at p-value<0.01**
Muraenolepis 16S	0.0000	0.0000
Muraenolepis COI	0.1254	0.0826
Macrourus 16S	0.0000	0.0000
Macrourus COI	0.1538	0.1282

Table 2-6 Results for matched-pairs test for nucleotide frequency compositional heterogeneity.

Therefore, additional analysis was required using phylogenetic methods developed to accommodate compositional heterogeneity, to account for any possible negative impact of the heterogeneity.

There are two approaches available for phylogenetic analysis which account for this level of heterogeneity. One option is to use parameter rich models (Gowri-Shankar and Rattray, 2007). However, the COI data sets were not sufficiently suited to the requirements of these models. The other option is to simplify the data using RY coding (Phillips et al., 2004, Ishikawa et al., 2012), where the base characters are recoded, A or G into purine (R) and T or C into pyrimidine (Y). This approach reduces the degree of compositional heterogeneity but can also result in the loss of variation, with the loss of information on transition substitutions (A-G or T-C).

The COI data sets were converted to RY-coding and this successfully reduced the level of compositional heterogeneity. However, the level of variation was also reduced.

RY Coded Dataset						
Species	**Unique Haplotypes**	**Outgroups**	**Characters**	**A**	**B**	**C**
Muraenolepis	25	2	539	61	18	
Macrourus	11	2	373	47	23	
AGY Coded Dataset						
Dataset	**Unique Haplotypes**	**Outgroups**	**Characters**	**A**	**B**	**C**
Muraenolepis	25	2	507	93	36	
Macrourus	11	2	351	69	32	

A=constant characters; B=variable characters; C=parsimony informative characters

Table 2-7 Comparison of statistics for RY coded and AGY coded COI sequence data.

The approach to reduce the heterogeneity whilst maintaining maximum variation was to partially recode the data, changing the C and T to Y, and keeping the A and G characters unchanged (AGY coding). This approach proved to be effective, maintaining a higher signal of variation than for the RY coded data.

The level of heterogeneity was also shown to be reduced to within acceptable levels when analysed using both the Chi-squared test in PAUP and matched-pairs test for symmetry in SeqVis.

Chi-square Test		
Dataset	**Chi-squared**	**P**
Muraenolepis	3.305876 (df=52)	1.00000000
Macrourus	2.087536 (df=24)	1.00000000

Mismatched-pairs Test		
Dataset	**x% at p-value<0.05**	**x% at p-value<0.01**
Muraenolepis	0.0057	0.0000
Macrourus	0.0000	0.0000

Table 2-8 Results for matched-pairs test for nucleotide frequency compositional heterogeneity for AGY coded COI data.

This AGY data set was analysed using maximum parsimony as described above. However, for maximum likelihood and Bayesian analysis, special nucleotide models are required and these were not available within the more routinely used phylogenetic software. The software package PHASE (http://www.bioinf.man.ac.uk/resources/phase) offered models suitable for AGY coded analysis. The Bayesian program mcmcPHASE was applied to the original COI datasets and the THREESTATE (general time-reversible AGY) model selected. The same parameters were set as for MrBayes, with 10,000,000 generations sampled every 500 generations, the first 7,500 trees discarded as burnin. The analyses were repeated three times to ensure consistency of results.

For consistency and to compare the results obtained with the output from MrBayes, the 16S data were also analysed using mcmcPHASE, with a standard GTR+G model. The results from mcmcPHASE were processed using mcmcSUMMARISE to obtain majority-rule trees and the trees then edited to display species names and support values using MEGA (http://www.megasoftware.net).

Gadiform comparison

Additionally, gadiform species present in the same fishing area were identified, as listed in FAO Species Catalogue (Cohen, 1990) and, where available, sequence data for 16S and COI obtained from Genbank (Appendix II). Sequences were aligned and unique sequences identified, as before. Each of the genera were analysed individually and then the data set as a whole, including both *Macrourus* and *Muraenolepis* data from this study. *Dissostichus eleginoides* and *Zeus faber* were selected as outgroups.

Muraenolepis species						
Dataset	**Taxa**	**Outgroups**	**Characters**	**A**	**B**	**C**
16S	24	2	234	129	105	84
COI	54	2	359	189	170	162
COI (AGY)	54	2	359	238	121	108
***Macrourus* species**						
Dataset	**Taxa**	**Outgroups**	**Characters**	**A**	**B**	**C**
16S	19	2	234	129	105	83
COI	41	2	420	233	187	180
COI (AGY)	41	2	420	288	132	124
All data						
Dataset	**Taxa**	**Outgroups**	**Characters**	**A**	**B**	**C**
16S	28	2	234	127	107	85
COI	61	2	420	231	189	183
COI (AGY)	61	2	420	285	135	126

A=constant caracters; B=variable characters; C=parsimony informative characters

Table 2-9 Summary of statistics for gadiform 16S, COI and AGY coded COI sequence data.

The data were tested for nucleotide frequency heterogeneity using the Chi-square test and matched pairs test, as before.

	Chi-square Test	
Dataset	**Chi-square**	**P**
Gadiform 16S	24.533460 (df=87)	1.00000000
Gadiform COI	130.693938 (df=186)	0.99926030
Gadiform (AGY)	59.351682 (df=186)	1.00000000

Table 2-10 Results from Chi-square test for nucleotide frequency heterogeneity for gadiform sequence data.

	Mismatched-pairs Test	
Dataset	**x% at p-value<0.05**	**x% at p-value<0.01**
Gadiform 16S	0.1034	0.0000
Gadiform COI	0.2227	0.0917
Gadiform COI (AGY)	0.0420	0.0077

Table 2-11 Results from matched-pairs test for nucleotide frequency heterogeneity for gadiform sequence data.

Both tests show the same issue with heterogeneity for the COI data sets, as was observed for the previous analyses for each genus, and this was resolved by AGY coding the data sets. For the *Macrourus* 16S data set, a possible problem with heterogeneity was indicated from the mismatched-pairs tests; however, there was no problem evident from the Chi-squared test.

The maximum parsimony analyses were conducted using PAUP, un-weighted parsimony search with 10,000 non-parametric bootstrap replicates of each heuristic tree search. The Bayesian analysis was carried out in PHASE, using the GTR (REV) model for the 16S data sets and AGY THREESTATE model for the COI datasets. Extended 50% majority rule consensus trees and cladograms were produced from both outputs, using both bootstrap support values and Baysian posterior probability support values, where support values were greater than 50%.

Population structure

The geographic location for each of the species identified in this study were mapped using ArcGIS 9.3 (ESRI, 2011) for visual clarification of the species distribution patterns. The haplotype data sets were partitioned by geographic location and year and tested for population structure in Arlequin V3.5 (Excoffier and Lischer, 2010) using a standard AMOVA test to calculate pairwise distances and Fixation Index (Fst). Samples where there were less than five individuals per site were not included in this analysis due to the lack of statistical significance. This population structure analysis was repeated to test for temporal variation where samples collections were available from the same geographic location for more than one year.

2.5 High Resolution Melt Analysis (HRMA) for *Macrourus* Species

Due to degradation of the DNA from poor sample preservation, a relatively high number of *Macrourus* samples were still problematic, even with the species' specific primers. This was particularly the case with the Ross Sea 2006 samples, where 76 of the 96 samples generated sequencing data of low quality. Correct species identification was required for accurate analysis of the data in the subsequent microsatellite study, hence it was decided to test HRMA as a potential alternative technique to mitochondrial barcoding.

High Resolution Melt Analysis (HRMA) is a closed-tube post-PCR analysis which can distinguish between samples with as little as a single base change in DNA sequence. Following conventional quantitative real-time PCR incorporating a DNA intercalating fluorescent dye, the product is denatured and the changes in sample fluorescence with temperature are monitored. Changes in base composition alter the temperature profile produced as the double stranded DNA separates and the fluorescent dye is activated. These differences in profile can be related to known sequences and utilised to discriminate between species. The technique was successfully applied to gene regions containing a single base difference between species.

Primer design and development of protocol

After aligning *Macrourus* COI sequence data using Geneious V5.3 (Drummond and Moir, 2010), the sequence data were examined to identify a fragment of less than 400bp in length (Wittwer, 2009), which incorporated a minimum of one polymorphism specific to each of the four identified species (figure 2-5).

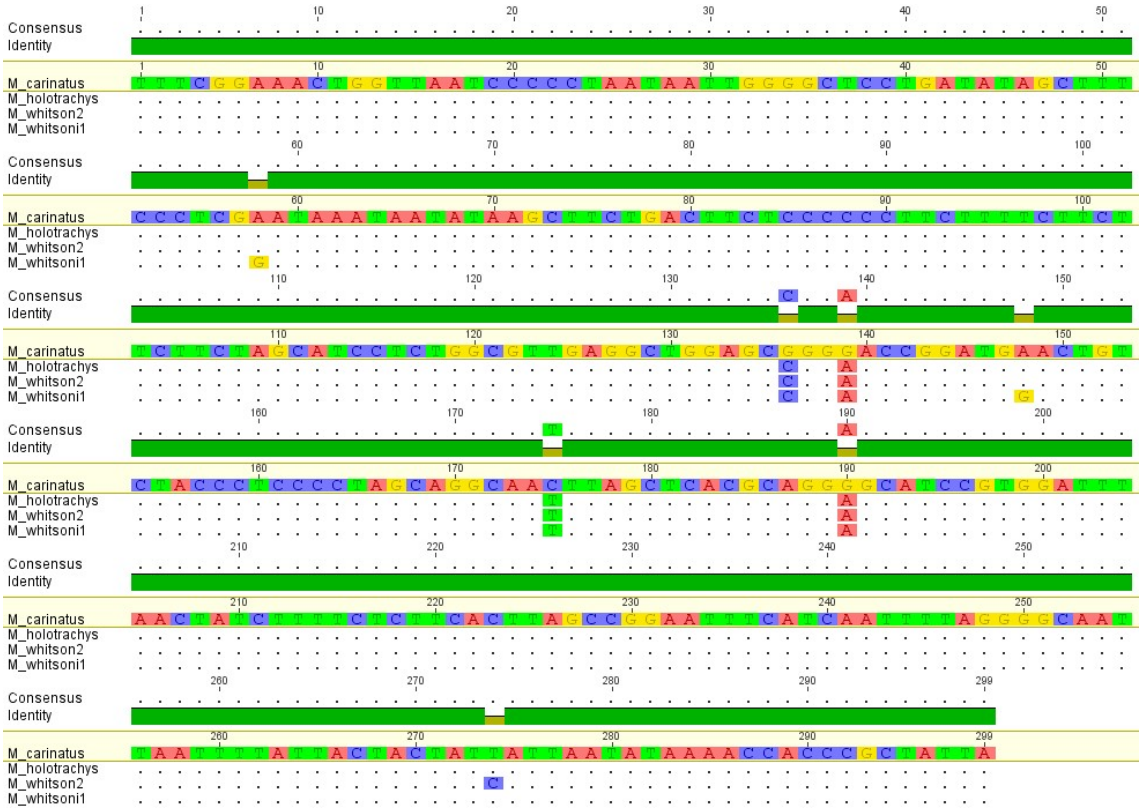


Figure 2-5 COI sequence alignment for the four species showing polymorphic sites.

Initially, four sets of primers were designed for testing, using Primer3 (Rozen, 2000).

1. Product size 344bp; Tm 52°C (Annealing 51°C)

   HRM_Mac_F1 5'– CTAATAATCGGGGGTTC -3'

   HRM_Mac_R1 5'- GCTCAGACAAATAAAGGTG -3'

2. Product size 388bp; Tm 55°C (Annealing 55°C)

   HRM_Mac_F2 5'- CTCTAATAATCGGGGGTTC -3'

   HRM_Mac_R2 5'- ACAGGAAGCGACAGTAAAAG -3'

3. Product size 212bp; Tm 53°C (annealing 53°C)

HRM_Mac_F3 5'- TAATAATTGGGGCTCCTG -3'

HRM_Mac_R3 5'- AATTCCGGCTAAGTGAAG -3'

4. Product size 249bp; Tm 54°C (annealing 54°C)

HRM_Mac_F4 5'- TCTAATAATCGGGGGTTC -3'

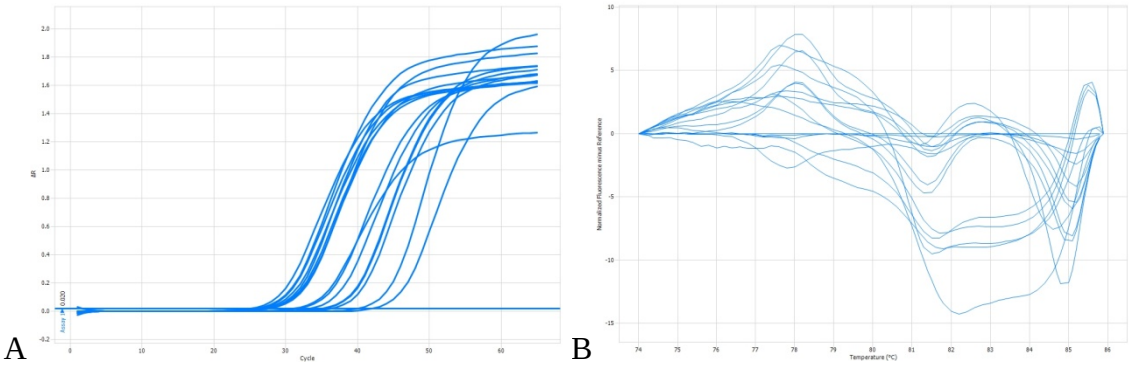
HRM_Mac_R4 5'- ATTCCGGCTAAGTGAAGAG -3'

Six samples where COI sequences had been successfully obtained were selected for each species and the DNA extract diluted to a standard concentration of 20 ng/µl. Standard PCR was carried out with 1 µl 10X Buffer, 0.2 µl $MgCl_2$, 1µl dNTPs, 0.2 µl each primer, 0.05 µl Taq (all reagents Qiagen, UK), 6.5 µl H2O and 0.5µl DNA. The reaction was performed on a DNA Engine2 (Genetic Research Instrumentation Ltd, UK) with a thermal profile of 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, a gradient of 47°C to 55°C for 60 seconds and 72°C for 60 seconds, then a final elongation step of 72 for 10 minutes. The PCR product was then visualised on a 1.5% gel (1.5g agarose/100ml TBE). Primer pairs HRM_Mac_F1/R1, HRM_Mac_F3/R3 and HRM_Mac_F4/R4 produced PCR product for all 24 of the selected samples. Unfortunately, primer pair HRM_Mac_F2/R2 failed to produce PCR product for all samples and showed strong primer self-bonding so was not included in further testing.

For the HRMA, a total reaction volume of 25 µl was made up of 12.5 µl Reaction Buffer (Type-It HRM Kit, Qiagen, UK), 1.75 µl of primers, 7 µl H_2O and 2 µl of DNA. The reaction was carried out on an ECO Real-Time PCR system (Illumina Inc., USA) with a thermal profile of 50°C for 2 minutes and 95°C for 10 minutes, then 35 cycles of

95°C for 15 seconds and 50°C for 1 minute. The samples were then heated to 95°C for 15 seconds, 50°C for 15 seconds and 95°C for 15 seconds. The changes in fluorescence were measured during the final heating cycle and analysed using Eco software (Version 3.1).

The number of cycles had to be increased by incorporating additional cycles during the program. The Ct values were in the region of 25 to 42 cycles and the number of cycles was increased to 65 cycles for subsequent tests to ensure that all of the samples reached plateau. Analysis of the initial results showed distinct melt profiles for each of the four species (figure 2-6).



A B

Figure 2-6 Graph of Ct values (A) and Difference Plot for Melt Curve (B) from initial test.

The results indicated that while the three primers sets were successful for conventional PCR they were not performing optimally on real-time PCR. The primers were redesigned specifically for real-time PCR using QuantiProbe Design Software (Qiagen, UK) and two sets of primers were selected for testing:

5. Product size 299bp

HRM_Mac_F5 5’- TTTCGGAAACTGGTTAATC -3’

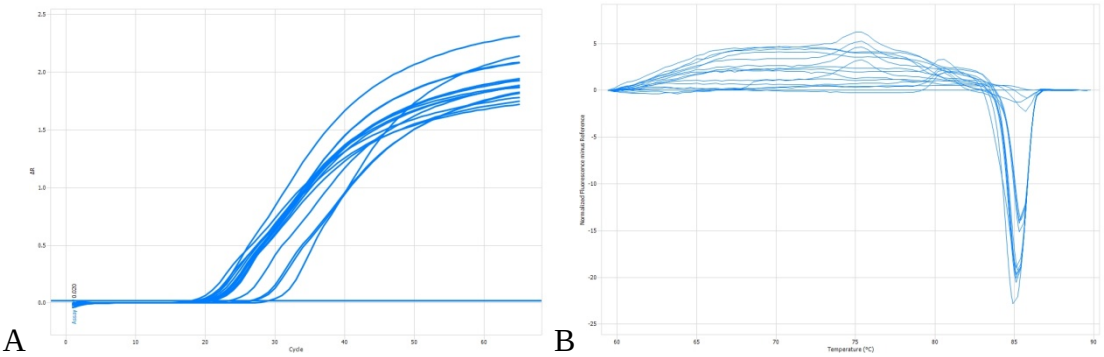
HRM_Mac_R5 5’- TAATAGCGGGTGGTTTTA -3’

6. Product size 300bp

HRM_Mac_F6 5’- TTTCGGAAACTGGTTAATC -3’

HRM_Mac_R6 5’- GTAATAGCGGGTGGTTTT -3’

The reactions conditions were as before with the annealing temperature increased to 55°C and the number of cycles reduced to 55 cycles. As a result, the range of Ct values produced was reduced to 16 to 25 cycles, which was within the acceptable level (Figure 2-7). The higher number of cycles was maintained in the program to allow for any samples where the DNA degradation had lowered the concentration, to reach plateau.



A B

Figure 2-7 Graph of Ct values (A) and Difference Plot for Melt Curve (B) generated using the redesigned primers.

Both selected primers pairs produced similar results and four clear melt profiles could be seen which corresponded with the four target species (Figure 2-8).

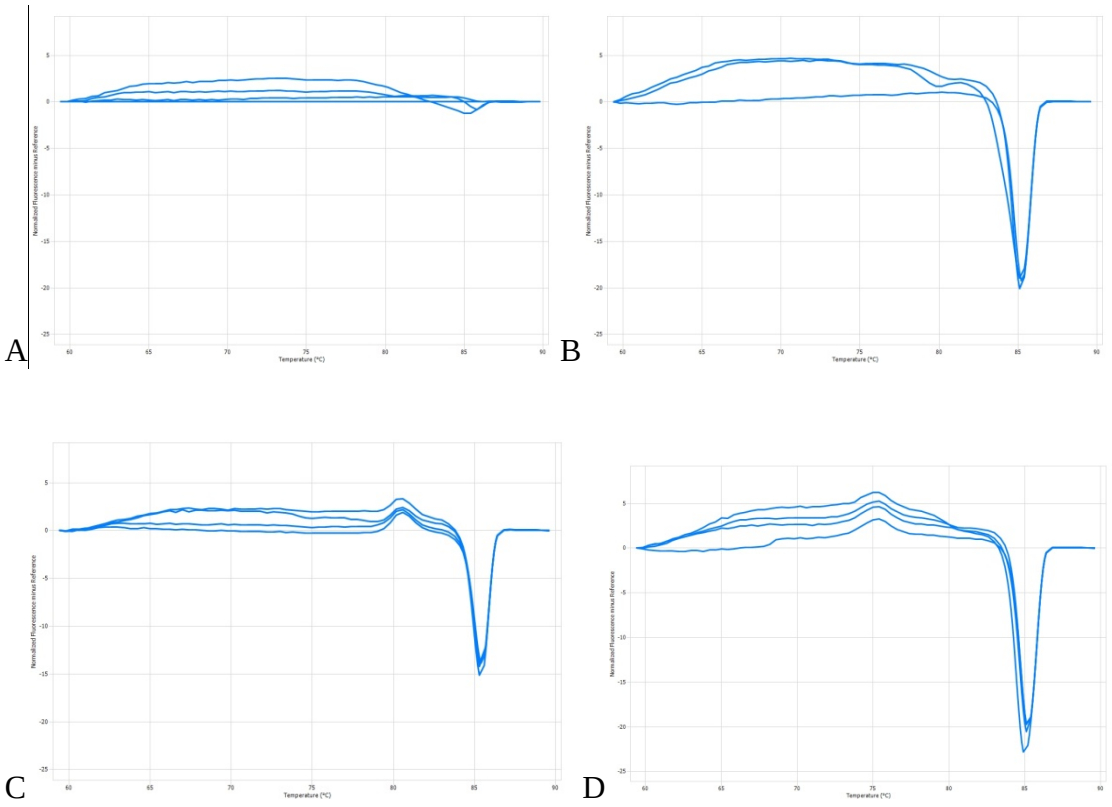


Figure 2-8 HRM difference profiles for (A) *Ma. carinatus* (reference species), (B) *Ma. holotrachys*, (C) *Ma. whitsoni* 1 and (D) *Ma. whitsoni* 2.

Validation and application of method

Having established the protocol, a sample of each species was selected for use as the positive reference sample. In each case, the species identity had been confirmed by high quality sequence data. The technique was validated using samples of known COI species identity (28 *Ma. carinatus*, 28 *Ma. holotrachys*, 30 *Ma. whitsoni* type 1 and 20 *Ma. whitsoni* type 2), a blind test of 43 random samples where sequence data had been obtained, and 24 degraded samples (Ross Sea 2006 collection). The technique was then applied to all of the Ross Sea 2006 samples which had failed to generate informative sequence data for COI to enable their incorporation into the microsatellite study.

This HRMA work has been published as Fitzcharles, E. M. (2010) Rapid discrimination between four Antarctic fish species, genus *Macrourus*, using HRM analysis. Fisheries Research **127-128**, 166-170.

2.6 Microsatellite Markers

The original outline of this project involved the in-house isolation of microsatellites for each species, followed by the design and testing of primers for these potential microsatellite markers. The final aim was to develop a suite of microsatellite markers for both genera, ideally allowing for multiplex reactions to be set up. Potential microsatellites were isolated for *Muraenolepis* sp., *Macrourus carinatus*, *Ma. holotrachys* and *Ma. whitsoni* (samples from South Georgia; believed at that time to be the only *Ma. whitsoni* species at that time). The full details of this procedure are given in Appendix I.

Despite extensive searching and requests to international collaborators, sufficient sample numbers for *Muraenolepis* could not be obtained from different geographic locations to generate data of statistical significance. Thus the decision was made not to continue with this work for *Muraenolepis*. It is hoped this work can be resumed at a later date if sufficient samples are obtained or via a collaborative opportunity.

The COI sequence data for the Southern Ocean *Macrourus* species, carried out in this thesis, suggest very little variation between these species and *Ma. berglax*. During the course of the in-house microsatellite development the Marine Biodiversity Ecology and Evolution research group based at University College Dublin published a suite of microsatellite markers for *Ma. berglax* (Helyar, 2010) and were in the process of using these markers to carry out a genetics study for *Ma. berglax* populations in the North Atlantic. To maximise the potential of global comparisons it was decided to use the *Ma.*

berglax microsatellite primers on the Southern Ocean species. A Memorandum of Understanding was agreed with this research group to collaborate on this work and they provided details on the markers and experimental conditions used in order for the work to be replicated using the Southern Ocean species. This work will be described in detail in Chapter 6.

CHAPTER 3

The phylogenetics and phylogeography of the genus *Muraenolepis*

3.1 Introduction

The Gadiform family Muraenolepididae (suborder Muraenolepidoidei) was a single genus, *Muraenolepis*, with a second genus, *Notomuraenobathys*, recently proposed (Balushkin and Prirodina, 2010c). It is the only gadiform family restricted to southern temperate, subAntarctic and Antarctic waters. Their morphology with a small head and long tapered body has given them the common name of eel cods. The evolution of this family is disputed and its ancestry and position within the order is the subject of much debate.

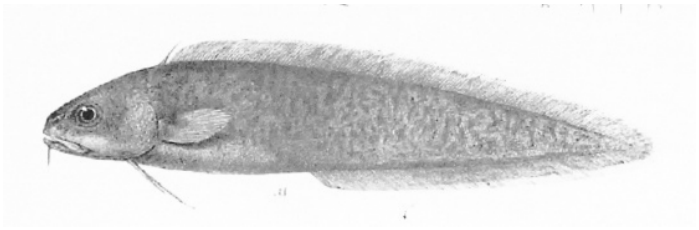
Original Species

The first mention of this genus was in the *H.M.S. Challenger* (1872-1876) report, published in 1880, describing the species *Muraenolepis marmoratus* (marbled moray cod) from a specimen caught off Kerguelen Island (Günther, 1880). A second specimen was identified by Vaillant in 1888 from the Mission Scientifique du cap Horn expedition, 1882-1883. This differed from the previous description in having a longer body, eye larger than the inter-orbital space, four rays in the ventral fin (*Mu. marmoratus* described as having five rays) and well defined teeth. This species was named *Muraenolepis orangiensis* (Patagonian moray cod).

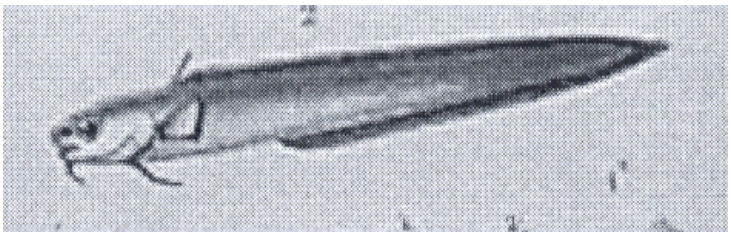
Specimens collected during the Schwedische Südpolar-Expedition 1901-1903 are described by Lönnberg (1905) as differing from *Mu. marmoratus* in that the eye

diameter, inter-orbital width and snout length were smaller. Lönnberg also describes a specimen matching this morphology from Tierra del Fuego and designated this morphotype to be a geographic sub species, *Mu. marmoratus microps* (later to be regarded as *Mu. microps* (Regan, 1914), smalleye moray cod). The fourth species was identified during the B.A.N.Z. Antarctic Research Expedition, 1929-1931 and published by Norman (1937). The specimen was caught off Enderby Land and described as being more like to *Mu. orangiensis* in morphology and named *Mu. microcephalus* (smallhead moray cod). Illustrations of the four species are shown below in figure 3-1.

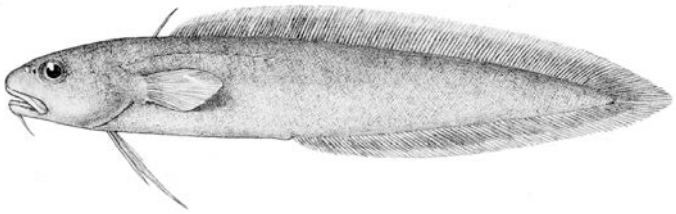
A



B



C



D

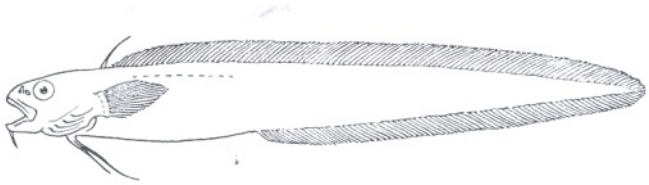


Figure 3-1 Illustrations showing the morphology of the described species, (A) *M. marmorata* (Günther, 1880), (B) *Mu. orangiensis* (Vaillant, 1888), (C) *Mu. microps* (Regan, 1914), (D) *Mu. microcephalus* (Norman, 1937).

There is some debate over the designation of these four species with *Mu. microps* and *Mu. marmoratus* thought to be very closely related, possibly the same species (Norman, 1937, Kompowski and Rojas, 1993, Balushkin and Prirodina, 2010b).

Recent Species

While this research was in progress an additional five new species were described, and a new genus, Muraenolepididae, proposed in a series of publications from Soviet and Russian Antarctic cruises (from 1957 to 2007). Illustrations of all the species are shown in figure 3-2.

Muraenolepis andriashevi (Balushkin and Prirodina, 2005) was discovered at Agulhas Bank off the coast of South Africa, at a depth of 980 to 1010m (single specimen). It is described as having an elongate body with a humped appearance, most similar to *Mu. orangiensis*, the other temperate water species. Specimens have also been described from the Discovery Sea Mounts (Balushkin and Prirodina, 2010a).

Muraenolepis trunovi (Balushkin and Prirodina, 2006) was described from a single specimen found in the Lazarev Sea at a depth of 730-860m. It has a small head, and more elongated pectoral and dorsal fins than the previously described species.

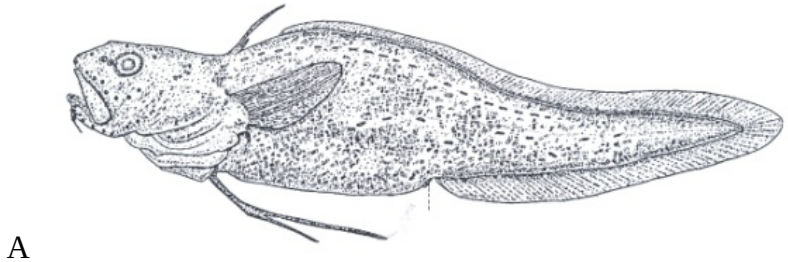
Muraenolepis pacifica (Prirodina and Balushkin, 2007) was collected off the Ridge of Hercules (Pacific-Antarctic Rise) at 200-500m depth. As with *Mu. andriashevi*, this species is described as being most similar to *Mu. orangiensis* in terms of vertebrae number and ray ratio of fins, but with a differing body proportions.

Muraenolepis kuderskii (Balushkin and Prirodina, 2007) was caught in the Scotia Sea, off South Georgia, at a depth of 440-605m. The new species is described from 6

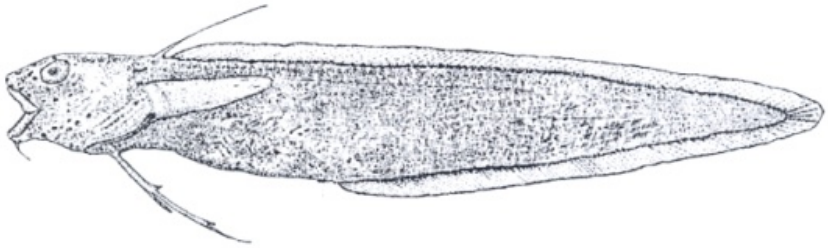
specimens caught across 3 stations and is described as being most similar to *Mu. trunovi* but differing in several measurements from all previously described species.

Muraenolepis evseenkoi (Balushkin and Prirodina, 2010b) has been described from specimens caught in the continental marginal Antarctic seas (Commonwealth, Riiser-Larsen, Amundsen and Ross) at depths of 500-2010m. This species was found in the high Antarctic, as is *Mu. trunovi*, but differed from *Mu. trunovi* in several body proportions.

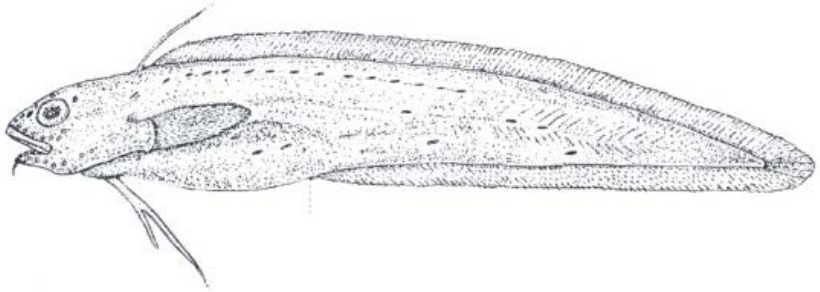
In addition, Balushkin and Prirodina have suggested that *Mu. microcephalus* should form a separate genus, and propose the name *Notomuraenobathys* (Balushkin and Prirodina, 2010c). This genus would consist of the single species, renamed *Notomuraenobathys microcephalus* (Norman, 1937). This designation of a new genus has not been taken into consideration at this point but will be discussed, in view of the molecular results obtained in this study.



A

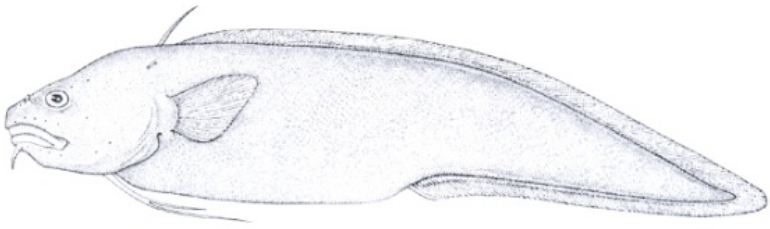


B



C

D



E

Figure 3-2 Illustrations showing the morphology of the described species; (A) *Mu. andriashevi* (Balushkin and Prirodina, 2005), (B) *Mu. trunovi* (Balushkin and Prirodina, 2006), (C) *Mu. kuderski* (Balushkin and Prirodina, 2007), (D) *Mu. pacifica* (Prirodina and Balushkin, 2007), (E) *Mu. evseenkoi* (Balushkin and Prirodina, 2010b).

To summarise, there are nine species to be considered in the context of this study. The holotypes for each of these species are listed in table 3-1 and the geographic location shown in figure 3-3.

Species	Location	Co-ordinates	Depth (m)	TL (cm)	Reference
Mu. marmoratus BMNH 1879.5.14.640	Kerguelen Islands	49°28' S 70°13' E	50	23.5	Gunther, 1880
Mu. orangiensis MNHM 1884-0819	Cape Horn, Orange Bay	55°58' S 67°16' W		63	Vaillant, 1888
Mu. microps NRM 11213	Cumberland Bay, South Georgia	54°17' S 36°30' W	100	32.1 (F)	Lonnberg, 1905
Mu. microcephalus BMHN 1937.9.21.96	Off Enderby Land	63°51' S 54°16' E	3000	12.5	Norman, 1937
Mu. andriashevi ZIN 53487	Agulhas Bank, southern coast of Africa.	35°20'S 18°40'E	980 - 1010	47.4 (F)	Balushkin & Prirodina, 2005
Mu. trunovi ZIN53780	Lazarev Sea.	69°15'S 11°49'E	730 - 860	25.5 (M)	Balushkin & Prirodina, 2006
Mu. kuderskii ZIN 50618	South Georgia	53°36'S 36°43'°W	500 - 600	22.4 (M)	Balushkin & Prirodina, 2007
Mu. pacifica ZIN 53543	Pacific-Antarctic Ridge, Ridge of Hercules.	53°34'S 140°39'W	200 - 450	39 (F)	Balushkin & Prirodina, 2007
Mu. evseenkoi ZIN 54651	Commonweath Sea, Kemp Coast	66°30'S 59°30'E	500-1180	35.1 (F)	Balushkin & Prirodina, 2010

Table 3-1 Type locality for all described species of *Muraenolepis.*

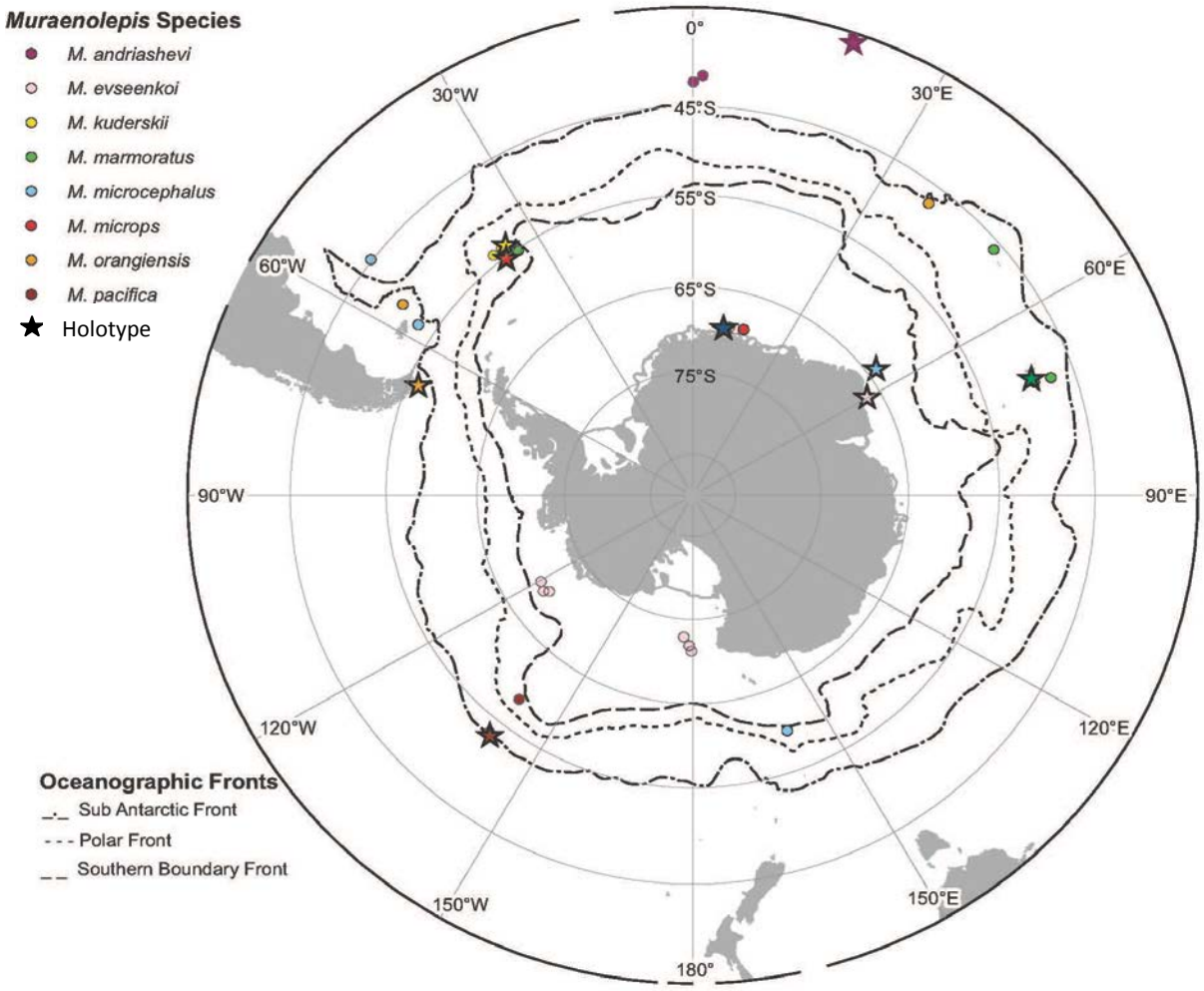


Figure 3-3 Map of the holotype locality for each species (see Table 3-1) and additional recordings for species from recent literature where new species are recognised (Balushkin and Prirodina, 2005, Balushkin and Prirodina, 2006, Balushkin and Prirodina, 2007, Prirodina and Balushkin, 2007, Balushkin and Prirodina, 2010b, Balushkin and Prirodina, 2013).

Geographic Distribution

Norman (1937) suggested that despite the fact these fish can be caught mid-water they should be regarded as coastal fish. There is considerable disagreement over the range of

the original four described species, resulting in part from the difficulties in accurate species identification.

The temperate species *Mu. orangiensis* has been described from the Patagonia region and Falkland Islands in the South Atlantic, seamounts of the South Atlantic and the subAntarctic Islands of the Indian Ocean (Crozet, Kerguelen and Heard). The depth range extends from 135 m to 860 m (Cohen, 1990, Chiu and Markle, 1990, Miller, 1993c).

For the subAntarctic species *Muraenolepis marmoratus* there is some disagreement over its distribution range. Cohen (1990) restricts the distribution to the subAntarctic Islands of the Indian Ocean (Crozet, Kerguelen and Heard) from depths of 30 m to 1600 m. However it has also been reported from the Scotia Sea (Chiu and Markle, 1990, Miller, 1993c).

From literature, *Mu. microps* has the widest distribution range of the four species; spanning the Scotia Sea islands of South Georgia, South Sandwich, South Orkney and South Shetland to the continental shelves of Victoria Land and the Ross Sea, with depths from 10 m to 1600 m (Chiu and Markle, 1990). This range has also been extended to include the continental shelf around Tierra del Fuego and Burdwood Bank (Cohen, 1990, Miller, 1993c).

For *Muraenolepis microcephalus,* specimens have been recorded from the Scotia Sea islands of South Georgia, South Sandwich, South Orkney and South Shetland, the northern Antarctic Peninsula and the continental slope of the East Antarctic coast (Enderby coast) (Chiu and Markle, 1990, Cohen, 1990, Miller, 1993c). This is the deepest dwelling of the species with a range of 1976 m to 3040 m (Chiu and Markle, 1990).

At present, the recordings for the new species are too limited to define species ranges. The location of specimens collected to date and any additional recordings for the original four species from samples collections by Balushkin and Prirodina have been mapped to demonstrate the distributions (Figure 3-3).

The locations of specimens for the five newly proposed species overlap with the ranges of the original four species. In an extension to the previously defined range, *Mu. microcephalus* was recorded in the Patagonian-Falkland region (Balushkin and Prirodina, 2013). Two of the new species, *Mu. andriashevi* and *Mu. pacifica*, have been recorded outside the areas previously described for *Muraenolepis* distribution (Balushkin and Prirodina, 2005, Balushkin and Prirodina, 2010a, Prirodina and Balushkin, 2007). Further resolution is required for both the species identification and geographic distribution of this Family.

Ecology

The Muraenolepididae family has been poorly studied in term of morphological and ecological aspects. Pre-juvenile specimens of *Mu. marmoratus* have been observed in the water column off Kerguelen Island and it was speculated that this unusual ichthyoplanktonic, rather than benthic, phase may aid dispersal (Duhamel et al., 2000). In the South Georgia region the abundance of *Mu. marmoratus* was found to increase with depth. Males are thought to mature at 3-5 years (21-27 cm length) and reach a maximum length of 46 cm (12 years) (Kompowski and Rojas, 1996).

For *Mu. evseenkoi* off the Antarctic continental shelf, the highest abundance was at depths of 1000-1500 m. A fecundity of 81,000-441,000 oocytes was found in mature females (over 38 cm in length). It is proposed that this fish has a single winter spawning season as there was no evidence of previtellogenic oocytes in ovaries of mature fish.

The eggs are 1.3-1.35 mm in diameter and pelagic (Prut'ko and Chimilevskii, 2011). Age estimates for *Muraenolepis* species in the Ross Sea suggest a maximum age of 9.5 years and total length 45 cm, with growth rates slowing after four years (Prut'ko and Chimilevskii, 2011).

All species are benthopelagic and thought to feed on zooplankton. The main diet of *Mu. microps* has been shown to be invertebrates such as gammarids, isopods, benthic shrimp, decapods, mysids, polychaete worms, fish and krill (Permitin and Tarvediyeva (1972) in Miller, 2003). Diet appears to change with size, small fish feeding on benthic animals, preferentially amphipods, and the proportion of fish in the diet increasing with size. *Notocrangon antarcticus* (Antarctic shrimp) and *Euphausia superba* (Antarctic krill) have both been identified as important food sources, for average size and large fish respectively, with krill comprising up to 95% of food weight in some regions. (Kompowski, 1993).

Muraenolepids play an important role in the Antarctic ecosystem and are an important food source for other fish, in particular the Patagonian toothfish (*Dissostichus eleginoides*). Studies of the diet of juvenile toothfish around South Georgia classed *Muraenolepis* species as a minor component of the diet (Barrera-Oro et al., 2005) and it has been identified in 1.4% of the fish stomachs studied (Pilling et al., 2001). It has also been shown to vary with predator size and was found in 3.8% of stomachs for 40-49cm long fish, but no other size classes, on the Argentinean slope (García de la Rosa et al., 1997). For the Antarctic toothfish (*Dissostichus mawsoni*) in the Ross Sea, *Muraenolepis microps* was a more notable prey item, occurring in 7.3% of fish stomachs (Fenaughty et al., 2003). Any conservation or fisheries management measures are reliant on correct species identification and geographic distribution.

3.2 Morphological Identification

As the molecular results for the work, which are presented in this chapter, started to come through preliminary analysis indicated that these would not necessarily correlate with the species previously designated using morphological characteristics. Therefore, retrospectively, it was decided that it was essential to gain a fuller understanding of the morphological species identification. Hence, access to the type specimens (holotypes) were obtained and examined (Figure 3-4). For the aid of clarity these morphological results are presented first. The type specimens for *Mu. marmoratus* and *Mu. microcephalus* are held at the British Museum of Natural History, London. The type specimen for *Mu. orangiensis* is held at the Muséum National d'Histoire Naturelle, Paris. Unfortunately this latter specimen could not be provided on loan; however, photographs and x-rays were supplied on request. The type specimen for *Mu. microps* is held at the Natural History Museum, Sweden and was obtained on loan to the British Museum of Natural History.

The condition of the *Mu. orangiensis* and *Mu. microcephalus* specimens was very poor and made morphological examination difficult. Type specimens for the five newly proposed species could not be obtained from the Museum of the Zoological Institute of the Russian Academy of Science for examination but measurements for these species (and any accompanying data recorded for the original species) were taken from literature and summarised in table 3-2.

A

B

C

D

Figure 3-4 Type specimen for (A) *Mu. marmoratus*, (B) *Mu. orangiensis*, (C) *Mu. microps* and (D) *Mu. microcephalus*. Images A, C & D courtesy of the British Museum of Natural History, London. Image B courtesy of the Muséum National d'Histoire Naturelle, Paris.

Characteristic (% of Standard Length)		*Muraenolpis marmoratus* *n*=17	*Muraenolpis orangiensis* *n*=4	*Muraenolpis microcephalus* *n*=5	*Muraenolpis andriashevi* *n*=5	*Muraenolpis trunovi* *n*=1	*Muraenolpis kuderskii* *n*=6	*Muraenolpis pacifica* *n*=7	*Muraenolpis evseenkoi* *n*=12
SL	Standard Length (mm)	144-341	63-286	72-304	351 - 445	243	166-214	250-414	214-415
Lmx	% Upper Jaw Length	8.8-10.8	5.0-7.0	4.9-6.6	10.4 - 11	7	7.0-7.8	7.3-9.2	8-9.8
H(A)	% Body depth	17.2-19.9	11.1-15.3	8.4-10.9	15 - 17.9	14.8	12.9-15.6	12.5-16.4	14.9-18.5
c	% Head Length	19.2-24.1	13.7-16.9	13.7-15.9	20.7 - 25.4	17.5	15.7-18.9	16.7-20.3	18.3-23.1
aA	% Pre Anal Length	47.1-51.3	34.3-46.2	36.4-43.1	49.6 - 55.6	43.2	39.0-41.6	46.6-53.4	47.2-54
postA	% Post Anal Length	50.4-54.4	58.4-59.8	58.7-63.6	44.9 - 50.4	56.8	58.4-61.0	50.0-51.7	47.7-52.8
o	% Eye Diameter	3.6-5.5	2.8-5.1	2.6-3.5	3.6 - 4.1	4.1	4.2-5.3	3.0-4.0	3.1-4.3
io	% Inter-orbit Distance	3.5-5	2-3.4	1.2-2.8	2 - 3.1	2.1	1.8-3.5	1.9-2.7	1.9-3.8
ao	% Snout Length	5.5-6.7	3.8-5.6	3.4-4.9	5.9 - 7	4.3	4.1-4.9	5.0-6.7	5.1-6.6
Lbarb	% Barbel Length	2.8-4.1	2.6-4.1	2.8-3.8	4 - 4.8	5.1	1.5-2.2	3.2-3.9	2.4-5
HD1	% 1st Dorsal Fin Length	6.5-9.0		6.4-9.9	8.3 - 15	16.9	11.0-14.8	6.2	6-10.3
LV	% Pelvic Fin Length	14.4-16.8	10.8-14.2	12.1-15.5	23.4 - 28.2	23.4	17.7-22.9	13.7-16.9	17.8-21.5
LP	% Pectoral Fin Length	12.5-14.3	9.6-10.8	9.7-11.2	11.7 - 13.5	17.1	14.3-16.9	8.5-10.5	11.7-15

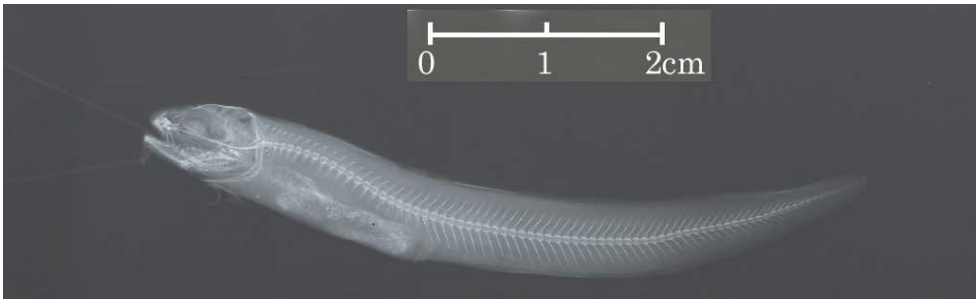
Table 3-2 Morphometric measurement ranges for all described species, taken from literature (Balushkin and Prirodina, 2005, Balushkin and Prirodina, 2006, Balushkin and Prirodina, 2007, Prirodina and Balushkin, 2007, Balushkin and Prirodina, 2010b).

Vertebrae Counts

A further method used to distinguish between *Muraenolepis* species is the number of vertebrae. For this purpose, x-rays were obtained for each of the type specimens and the number of vertebrae examined (Figure 3-5.; Table 3-3). X-rays were also available for three additional museum specimens (Table 3-4).

A

B



C

D

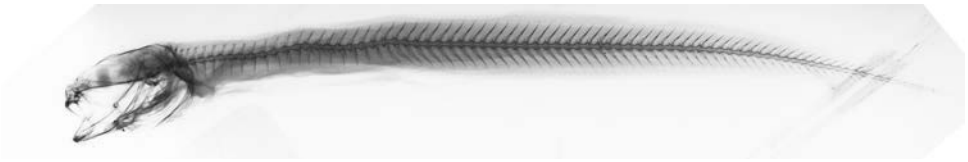


Figure 3-5 X-rays of the type specimen for (A) *Mu. marmoratus*, (B) *Mu. orangiensis*, (C) *Mu. microps* and (D) *Mu. microcephalus* (Norman, 1937). X-rays A, C & D courtesy of the British Museum of Natural History, London. X-ray B courtesy of the Muséum National d'Histoire Naturelle, Paris.

Species	Vertebrae Range for Species	Holotype Vertebrae Count
Mu. marmoratus	67 - 71	68
Mu. orangiensis	74 - 76	75
Mu. microps	70 - 74	70
Mu. microcephalus	83 - 85	83

Table 3-3 Expected vertebrae ranges for each species (Chiu and Markle, 1990) and vertebrae count for the holotype of the species.

In addition, further museum specimens were available.

Species	Museum Code	Count	Location	Co-ordinates	Depth	Date
Mu. microps	NRM9364	68	Cumberland Bay, South Georgia	45°14'0"S; 36°28'0"W	20m	1902
Mu. microps	NRM11106	73	Tierra del Fuego, Argentina	55°10'S; 66°15'W	125m	1902
Mu. marmoratus	MNHN 1985-0447	67	Kerguelen Islands	50°37' S ; 71°34'E		1974

Table 3-4 Vertebrae counts for additional museum specimens where the x-ray was available.

It should be noted that, in spite of species designation, the vertebrae counts of one of these specimens (*Mu. microps* NRM9364) is outside the expected species range.

For the further five species, the vertebrae count for the holotypes and all other samples given in this literature was collated and presented in Table 3-5.

Species	Museum Code	Vertebrae Count	Location	Depth	Date	Vertebrae Range for Species
Mu. andriashevi	ZIN 53487	73	Africa 35 20'S 18 40'E	980-1010m	1974	72-73 (n=5)
Mu. trunovi	ZIN 53780	73	Lazarev Sea 69 15'S 11 49'E	730-860m	1983	73 (n=1)
Mu. kuderskii	ZIN 50618	69	South Georgia 53 36'S 36 43'W	500-600m	1981	68-69 (n=6)
Mu. pacifica	ZIN 53543	74	Hercules Ridge 53 34'S 140 39'W	240-290m	1977	73-75 (n=7)
Mu. evseenkoi	ZIN 54651	70	Commonwealth Sea 66 30'S 59 30’E	1180-500m	1957	70 (n=12)

Table 3-5 Vertebrae counts for the holotypes of the newly described species, taken from literature (Balushkin and Prirodina, 2005, Balushkin and Prirodina, 2006, Balushkin and Prirodina, 2007, Prirodina and Balushkin, 2007, Balushkin and Prirodina, 2010b).

Sample Collection for Molecular Analysis

The samples collected for use in this study (*n*=355) were identified by the scientific observer onboard vessels, using the key given in Figure 2-2 (see also Table 3-6). Accurate identification was made more difficult by the overlapping ranges of the features used and time constraints on-board collection vessels precluding accurate measurements. For these reasons most of the specimens were identified to genus-level only, with only 9% designated as *Mu. marmoratus* and 3% as *Mu. microps*. *Mu. microcephalus* and *Mu. orangiensis* were not specifically identified.

			Species identified at collection				
Location	**Year**	**Collection**	**Genus only**	***Mu. marmoratus***	***Mu. microps***	***Mu. microcephalus***	***Mu. orangiensis***
South Georgia	2004/5	Dorada	44	18	9		
South Georgia	2008	Dorada		8			
Scotia Arc	2006	JR145	16	3			
Burdwood Bank	2006	MUO	4				
Kerguelen Island	2006	MNHN	5				
South Sandwich	2010	ZMGO	5	3	3		
South Sandwich	2010	Argos Foyannes	20				
Ross Sea	2005-8	NIWA	24				
Ross Sea	2013	NIWA	194				
Total			**312**	**32**	**11**	**0**	**0**

Table 3-6 Collections of *Muraenolepis* specimens and species identification at collection.

3.3 Molecular Identification and Phylogenetics

Molecular methods were used to investigate the specimens collected (Table 3-5) to confirm the species identity. Two mitochondrial gene regions, ribosomal RNA subunit 16S and Cytochrome Oxidase I (COI) were targeted. In the analysis, the results from each of these markers was analysed separately. Two outgroups were selected for use in the analysis; *Macrourus holotrachys* or *Macrourus whitsoni* (from this study) and *Merluccius paradoxus* (voucher DAAPV F8) sequence obtained from GenBank (GU324141; GU324176).

Species names have been assigned to the phylogenetic trees. These were applied post analysis for clarity of the results. The method used to determine the correct species

name to assign to each branch, based on morphology and geographic location, is detailed after the phylogenetic results.

16S Data

The 16S gene region was sequenced for 161 samples (the Ross Sea 2013 sample collection were not available when 16S processing occurred. Later work on these samples using COI identified a single species, so the decision was taken not to retrospectively sequence the 16S region from the Ross Sea samples). The sequences were edited to remove poor quality data and a 510 bp read length was successfully obtained for 149 of the samples. These sequences were aligned using Geneious and nine unique haplotypes (individuals with identical sequence grouped together) were identified.

These nine haplotypes were checked to ensure there was no evidence for compositional heterogeneity using Chi-square test and mismatched-pairs test for symmetry. The sequences were analysed using standard methods of heuristic searches using unweighted maximum parsimony (PAUP), heuristic searches using maximum likelihood with DNA substitution models, as identified by jModelTest (GARLI; substitution model TPM2+G) and Bayesian inference substitution models (MrBayes; substitution model HKY).

The data were also analysed for Bayesian inference using PHASE with the program mcmcPHASE, GTR+G substitution model, and the results processed in SUMMARIZE to obtain the majority rule tree (Figure 3-6). The PHASE Bayesian inference posterior probability support values are also shown. The full details of the molecular analysis methods are given in Chapter 2.

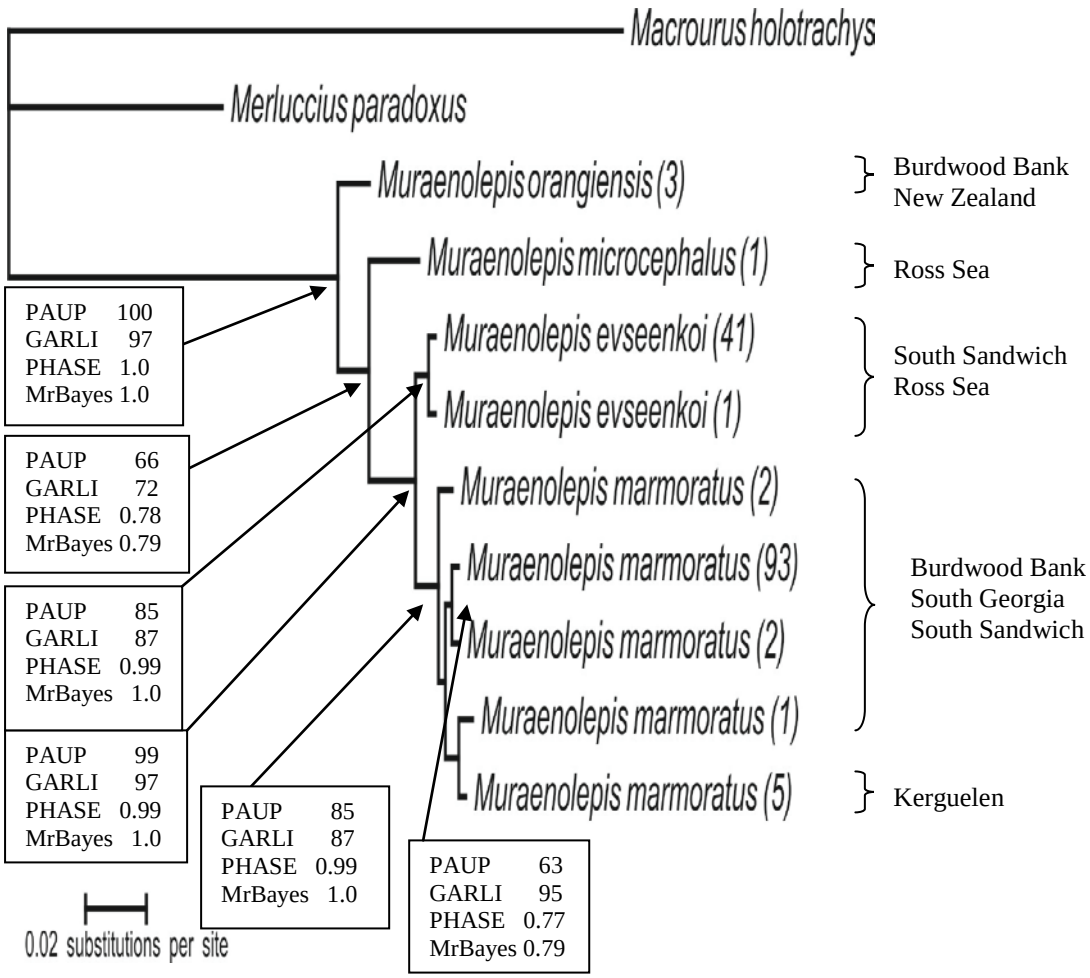


Figure 3-6 Majority rule phylogenetic tree for *Muraenolepis* 16S data showing bootstrap support values and branch posterior probability support values from the different analyses performed. Haplotype frequencies are shown in parentheses. Geographic locations of samples are given on right.

Essentially, this phylogenetic tree (Figure 3-6) shows four main clades (one based on a single sample). The main branch nodes between species were well supported with a bootstrap support value of greater than 80% for maximum parsimony and maximum likelihood analysis, and greater than 0.95 for the posterior probability support. Two branches receive poor support; *Mu. microcephalus* and *Mu. evseenkoi*/*Mu. marmoratus*, and within the *Mu. marmoratus* clade. The only branch shown with less than 50% (0.5) support is within the main *Mu. marmoratus* group (Burdwood Bank, South Georgia and

South Sandwich Islands). This branch is still shown due to its non-conflicting relationship within the extended majority rule tree.

There were only 24 variable sites for this gene region (5%) so it was decided to examine another region of the mitochondrial genome to determine whether the same tree topology was to be obtained and improve statistical support.

COI Data

The COI region is more variable than 16S and therefore is a more informative marker for molecular phylogenetics (This data set also included the additional 194 Ross Sea 2013 samples). After editing to remove poor quality data, a 600bp sequence for the COI gene region was obtained for 297 (95%) of the samples. As expected, the COI data were more informative, with 72 variable sites (12%) for this gene region. Alignment of the sequence data in Geneious and identification of identical sequences resulted in 25 unique haplotypes.

As for the 16S data, these haplotypes were checked for evidence of compositional heterogeneity. The values obtained for the Chi-square tests were within acceptable limits ($P>0.999$), however, the mismatched-pairs test for symmetry revealed a level of heterogeneity ($x\%=0.1254$ at p-value <0.05; $x\%=0.08526$ at p-value <0.01). The data were analysed as above but the resultant phylogenetic trees gave poor support values for both bootstrap percentage and posterior probability for a number of the branches, and these values varied between the different analysis methods.

The initial attempt to resolve this issue using RY-coding (Phillips et al., 2004) succeeded in removing the heterogeneity but also resulted in an unacceptable loss of variation. Converting the data to AGY-coded proved more successful, reducing the

heterogeneity to within accepted limits (P=1.0000; x%=0.0057; x%=0.0000) for phylogenetic analysis.

The original data were analysed in PHASE using the program mcmcPHASE and THREESTATE substitution model, a general time-reversible AGY model. A majority rule tree with Bayesian posterior probability support values was produced using SUMMARIZE (Figure 3-7).

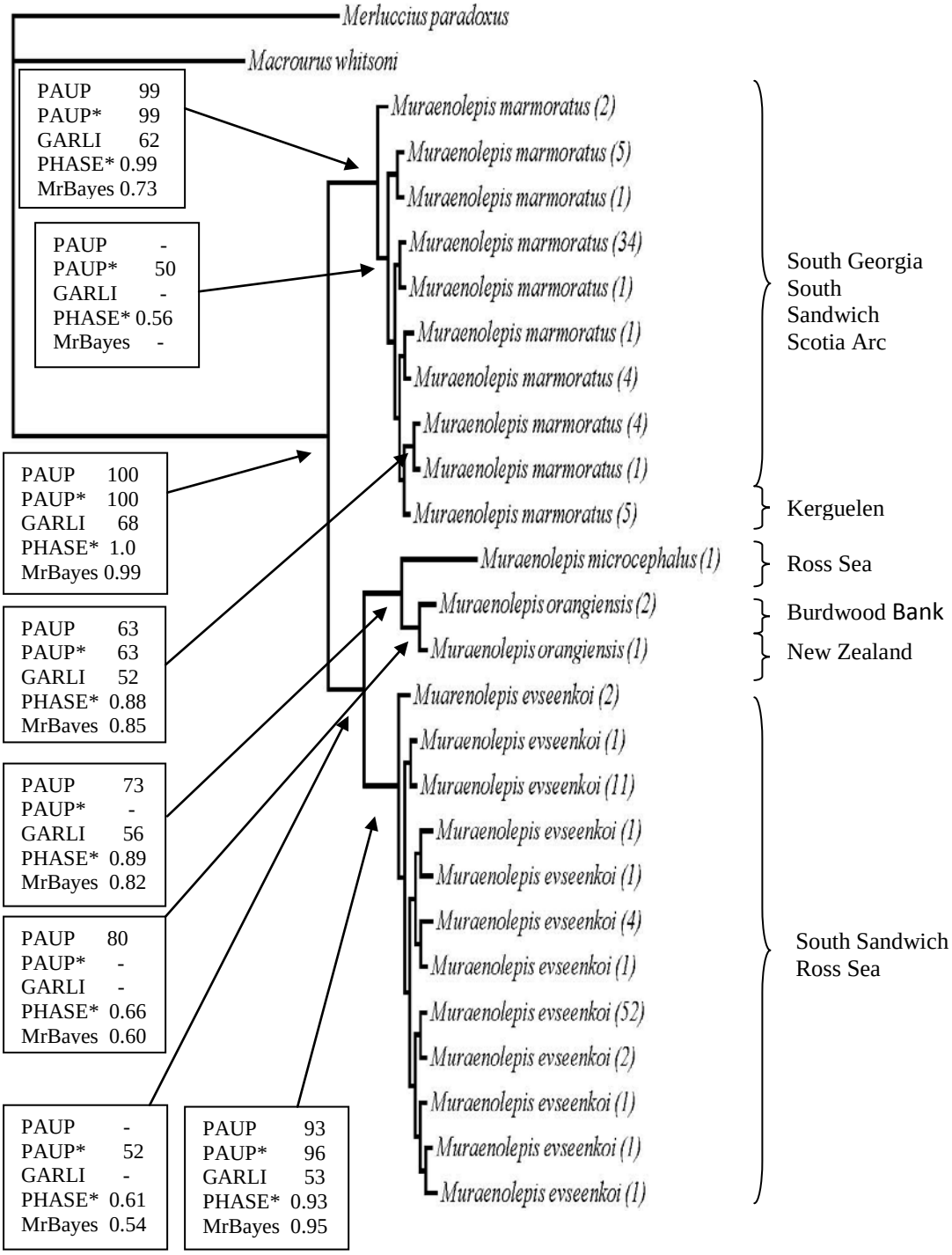


Figure 3-7 Majority rule phylogenetic tree for *Muraenolepis* COI data showing bootstrap percentage and posterior probability branch support values for each analyses method (*AGY coded dataset). Haplotype frequencies are given in parentheses. Geographic locations of sample collections are given on right.

This phylogenetic tree (Figure 3-7) differs from the 16S data (Figure 3-6), showing the same four clades but structured differently. The initial branch separates *Mu. marmoratus* whereas the initial branching separated *Mu. orangiensis* for 16S. The branch support values vary between the different analysis methods, which can be attributed to the high level of nucleotide frequency heterogeneity causing the COI data set to be unsuitable for use with standard models. Higher posterior probability values are obtained for the AGY coded data set. Many of the branches are poorly supported for all analysis methods, which may be attributed to the weak statistical power of low level genetic variation.

Assigning species names

Both phylogenetic trees (Figures 3-7 and 3-8) show four clades which can be attributed to the presence of four species. Accurately assigning names for each of these species required consideration of all factors: i.e. catch location and morphology of original type specimens; comparison with the catch location of the samples used in this study; and any morphological measurements or descriptions recorded for the studied specimens.

Only one species, *Mu. marmoratus*, was present at the main study area of South Georgia and this was genetically identical to the samples from Kerguelen Island, Burdwood Bank and South Sandwich Islands. As mentioned in the introduction (Chapter 1 and this chapter), there has been some debate over the presence of a second species at South Georgia and the designation of *Mu. microps* as a separate species. The results obtained in this study support the hypothesis of a single species present at both South Georgia and Kerguelen Islands. Re-examination of the type specimen for *Mu. microps* revealed that the lateral line extended past the middle of the body. According to the key developed by Chiu and Markle (1990) this is consistent with the taxonomy for *Mu.*

marmoratus and not *Mu. microps*; suggesting that *Mu. microps* was not a separate species. *Mu. marmoratus* was first described from Kerguelen Island in 1880 and *Mu. microps* from South Georgia in 1905. Therefore, according to the International Code of Zoological Nomenclature (ICZN) (2000), *Mu. marmoratus* takes seniority and *Mu. microps* becomes the junior synonym.

The second major grouping consisted mainly of the Ross Sea 2013 samples (initially identified as *Muraenolepis* sp) and also the majority of the South Sandwich Island samples (initially identified as *Muraenolepis* sp., *Mu. marmoratus* and *Mu. microps*). The holotypes described from the high Antarctic are *Mu. microcephalus* (Norman, 1937), *Mu. trunovi* (Balushkin & Prirodina) and *Mu. evseenkoi* (Balushkin & Prirodina). The Ross Sea 2013 samples were identified as *Mu. evseenkoi* after careful examination of their morphological features (Peter McMillan, NIWA, personal communication).

There were no morphological data available for the samples that composed the two smaller clades (*Mu. microcephalus* and *Mu. orangiensis*), with the exception of a description of “elongate form” for the single sample from the NIWA mixed geographic location sample set, caught in the Ross Sea.

The samples originating from Burdwood Bank and New Zealand had no accompanying morphological data. They were given the designation of *Mu. orangiensis*. This is a speculative designation based on the location for the holotype of *Mu. orangiensis* which was caught off Cape Horn, Orange Bay (1888), and the circumpolar temperate distribution of the specimens identified in this study.

The final clade, from the Ross Sea, also lacked associated morphological data. It has been given the designation of *Mu. microcephalus* based on the catch location, depth 208-1621 m and the description of “elongate form”. There were three potential holotype candidates to consider: *Mu. microcephalus*, *Mu. trunovi*, and *Mu. evseenkoi*. All three

were found in East Antarctic waters but only *Mu. microcephalus* has an elongate body morphology. Again this designation is tentative and additional specimens with accompanying morphological data are required for both of these genotypes to confirm the association with the phenotypes.

3.4 Morphological Data

Combining all of these morphological and molecular results the species designation on collection were corrected following molecular identification (table 3-7). Indentifications based on the COI data set were used for morphological comparison as data were available for a greater number of specimens.

			Species identified by COI Sequence				
Location	**Year**	**Collection**	***Muraenolepis marmoratus***	***Muraenolepis orangiensis***	***Muraenolepis evseenkoi***	***Muraenolepis microcephalus***	**Fail**
South Georgia	2004/5	Dorada	71				
South Georgia	2008	Dorada	8				
Scotia Arc	2006	JR145	19				
Burdwood Bank	2006	MUO	2	2			
Kerguelen Island	2006	MNHN	5				
South Sandwich	2010	ZMGO			11		
South Sandwich	2010	Argos Foyannes			20		
Various	2005-8	NIWA		1	20	1	2
Ross Sea	2013	NIWA			178		16
Total			**105**	**3**	**229**	**1**	

Table 3-7 *Muraenolepis* collections with species as identified by COI sequence data.

Since there was a considerable disparity between the molecular results and the original species characterisation by morphology, it was decided to re-examine the morphology of specimens used in this study. This was only possible for the specimens identified as *Mu. marmoratus* and *Mu. evseenkoi*, where morphological data were recorded. The collections with sufficient numbers and availability of morphological data were: South Georgia 2005 (*Mu. marmoratus*), South Sandwich Islands 2010 (*Mu. evseenkoi*) and Ross Sea 2013 (*Mu. evseenkoi*).

Characteristic		***Mu. marmoratus*** *(n=71)*	***Mu. evseenkoi*** *(n=215)*
SL	**Standard Length**	118 – 385 (144-341)	173 – 537 (214-415); **332**
Lmx	**Upper Jaw %**	7.9 - 10.5 (8.8-10.8)	**9.3**
H(A)	**% Body depth**	14.5 - 25.2 (17.2-19.2)	**16.9**
c	**% Head Length**	15 - 20.7 (19.2-24.1)	15 – 23 (18.3-23.1); **20.1**
aA	**%Pre Anal Length**	40 - 52.3 (47.1-51.3)	42.6 - 57.5 (47.2-54); **53.3**
postA	**%Post Anal Length**		49 - 67.9 (47.7-52.8); **47.9**
o	**%Eye Diameter**	1.9 - 5.3 (3.6-5.5)	**3.6**
io	**%Inter-orbit Width**	3.3 - 5.9 (3.5-5)	**2.4**
ao	**%Snout Length**	4.9- 7.6 (5.5-6.7)	5.4 - 8.5 (5.1-6.6); **5.7**
Lbarb	**%Barbel Length**	1.7 - 4.3 (2.8-4.1)	1.8 - 4.4 (2.4-5); **2.4**
HD1	**%1st Dorsal Fin Length**	2.5 - 12.4 (6.5-9)	5.3 - 14.7 (6-10.3); **8.1**
LV	**% Pelvic Fin Ray**		12.3 - 28.2 (17.8-21.5); **21.1**
LP	**% Pectoral Fin Ray**		9.6 - 15.7 (11.7-15); **14.8**

Table 3-8 Summary of morphometric measurements taken for *Mu. marmoratus* and *Mu. evseenkoi* (sample collections South Georgia 2005, South Sandwich Islands 2010, Ross Sea 2012 and Ross Sea 2013). The range expected from literature (from Table 3-2) is given in parenthesis and data for holotype (where available) given in bold.

Previously no size difference between male and female specimens have been found for *Muraenolepis* species (Horn and Sutton, 2010, Parker et al., 2012). To determine whether this was also the case for the current data set, the standard length was plotted

against the weight for male and female specimens of both species. This also allowed for comparison of growth rates between species.

	South Georgia	South Sandwich Is	Ross Sea
	Mu. marmoratus (n=71)	*Mu. evseenkoi* (n=20)	*Mu. evseenkoi* (n=195)
Total length (mm)	178-365	370-540	255-562
Weight (g)	42-629	490-861	104-1620

Table 3-9 Length and weight ranges for *Mu. marmoratus* (South Georgia 2005) and *Mu. evseenkoi* (South Sandwich Islands 2010; Ross Sea 2012, 2013).

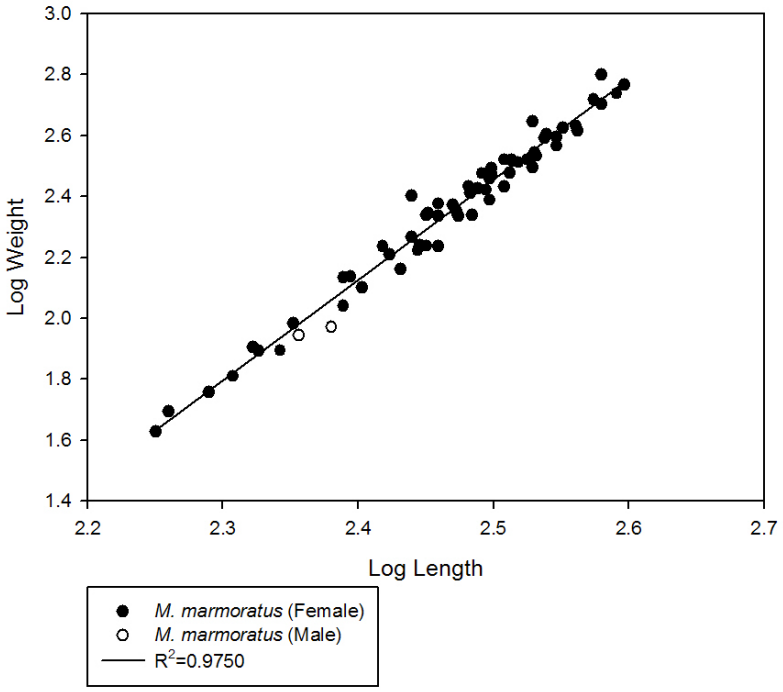


Figure 3-8 Graph showing standard length to weight ratio for *Mu. marmoratus*.

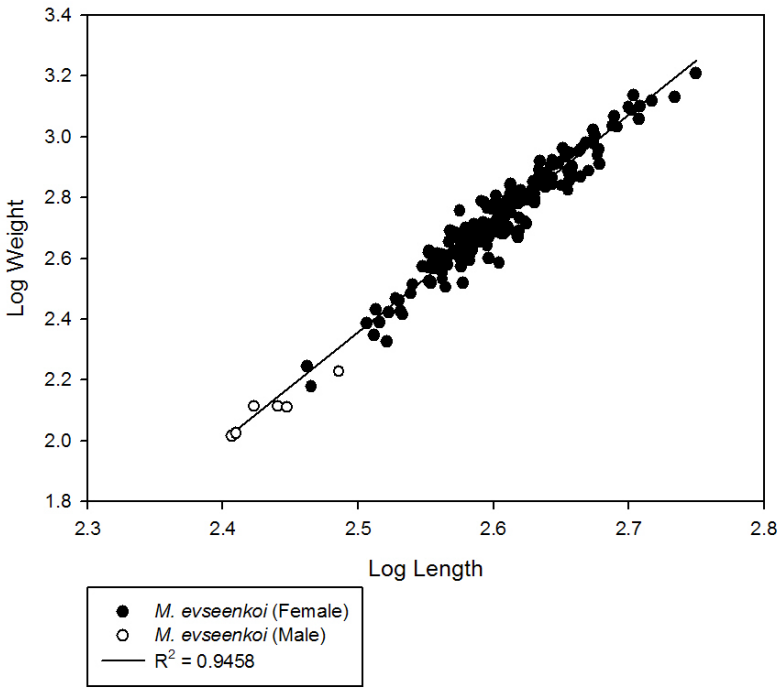


Figure 3-9 Graph showing standard length to weight ratio for *Mu. evseenkoi*.

The graphs (Figure 3-8 and Figure 3-9) show that the catch was predominantly female for both species and where males were present they were smaller in size than the females. This consistent size difference can be interpreted as sexual dimorphism. It is possible that some of the confusion over species identification has originated from sexual dimorphism. The overall trend shown is that the *Mu. evseenkoi* are larger than *Mu. marmoratus*.

3.5 Geographic Distribution

Having clearly defined the identity of the *Muraenolepis* species, using molecular barcoding, the different species distributions were plotted to visualise any potential special separation/habitat preference. The geographic range for each of these species was examined by plotting the species identity by geographic catch location.

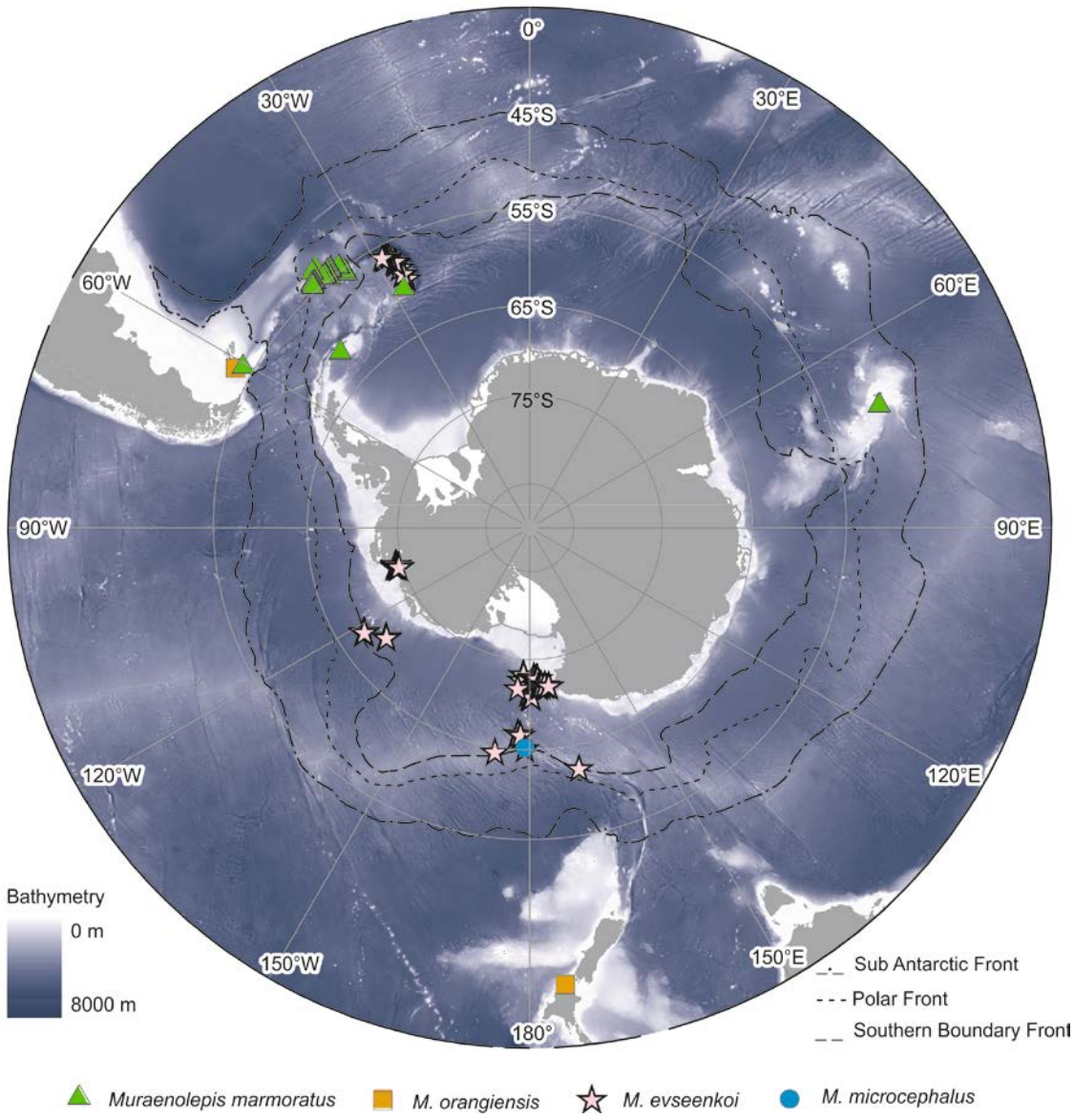


Figure 3.10 Circum-Antarctic geographic distributions of *Muraenolepis* species, as identified by COI sequence data.

Muraenolepis orangiensis was found in two locations, Falkland Islands and New Zealand, both north of the Polar Front. The single specimen for *Mu. microcephalus* was found within the Polar Front, close to the Southern Boundary Front, in the Ross Sea region (depth 1850 m). The distribution of *Mu. marmoratus* ranged from the Falkland Islands and along the Scotia Arc to South Orkney Island, crossing the Polar Front. The subAntarctic range also extended to Kerguelen Island in the Indian Ocean Sector (no

depth data available). *Mu. evseenkoi* was found within the Southern Boundary Front at the South Sandwich Islands and the Ross Sea region (depth range 1560 m to 771 m).

The main study area of the Scotia Arc was examined in closer detail.

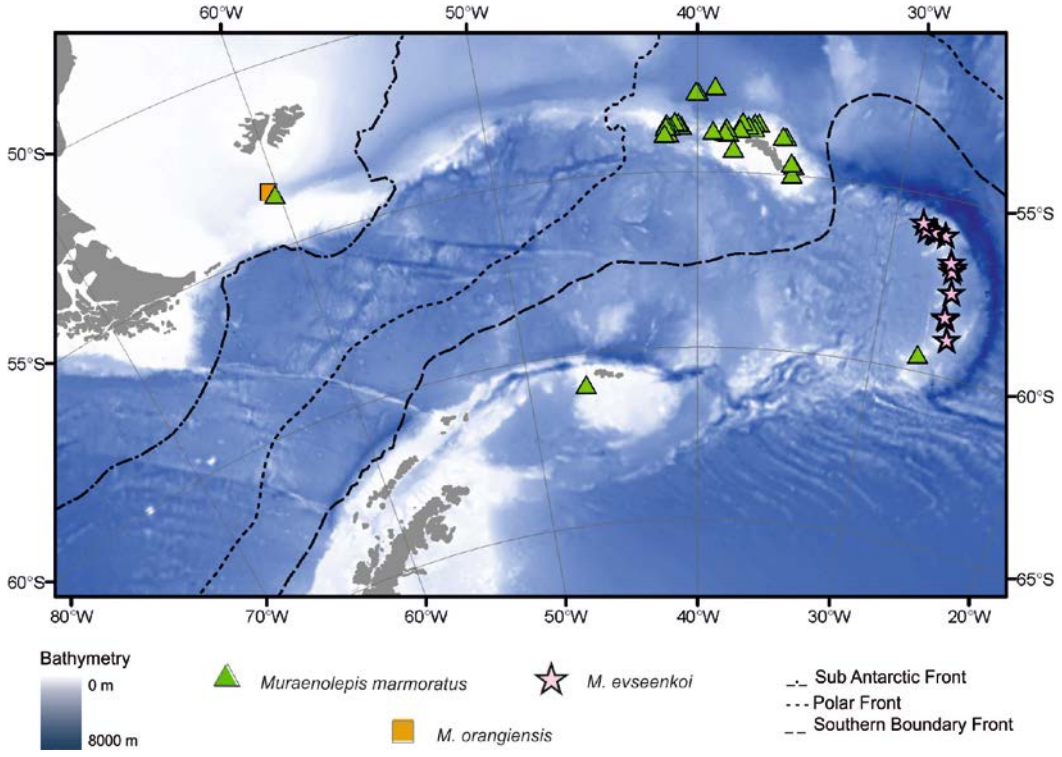


Figure 3.11 Geographic distributions in the Scotia Arc region for *Muraenolepis* species, as identified by COI sequence data.

There is a clear difference between South Georgia and the South Sandwich Islands in terms of species composition, with only *Mu. marmoratus* present around South Georgia and *Mu. evseenkoi* as the predominant species at the South Sandwich Islands.

The *Mu. marmoratus* samples around South Georgia were collected close to the Continental Shelf with a depth range of 1000 m to 117 m. The two samples of *Mu. marmoratus* found south of the Southern Boundary Front were collected by 200 m Agassi trawl whereas the *Mu. evseenkoi* in this region were collected by 1000 m Agassi

trawl (1502 m to 1238 m). Both *Mu. marmoratus* and *Mu. orangiensis* specimens from the Falkland Islands were collected at depths of 974 m to 950 m.

3.6 Population Genetics

With the more accurate species identification and revised distribution patterns, where sufficient numbers of samples were available, a more detailed population structure was tested in AMOVA using the haplotype frequencies. Data were discounted where insufficient numbers of samples were present for analysis using Arlequin (n<5). As there were insufficient samples for *Mu. orangiensis* and *Mu. microcephalus* they have been excluded from this analysis. The haplotype data for *Mu. marmoratus* and *Mu. evseenkoi* were partitioned by species to avoid any erroneous statistical significance between populations as a result species composition within populations. Each species was then partitioned into groups according to catch location and year.

Mu. marmoratus

To test for geographic variation the populations from South Georgia 2004 (n=18), South Georgia 2005 (n=51), Scotia Arc 2006 (n=19), South Georgia 2008 (n=7), Kerguelen Islands 2006 (n=5) were compared (Figure 3-10). It was found that 64.83% of the variation was within the groups and 35.13% of variation within populations. There was no statistical significance between populations from South Georgia and the Scotia Arc (F_{ST} (Φ_{ST})=0.02008; p=0.87598). There was however a significance (F_{ST} (Φ_{ST})=0.85253;p=0.00098+/-0.0010) between the genetic population at Kerguelen compared to the Scotia Arc and South Georgia.

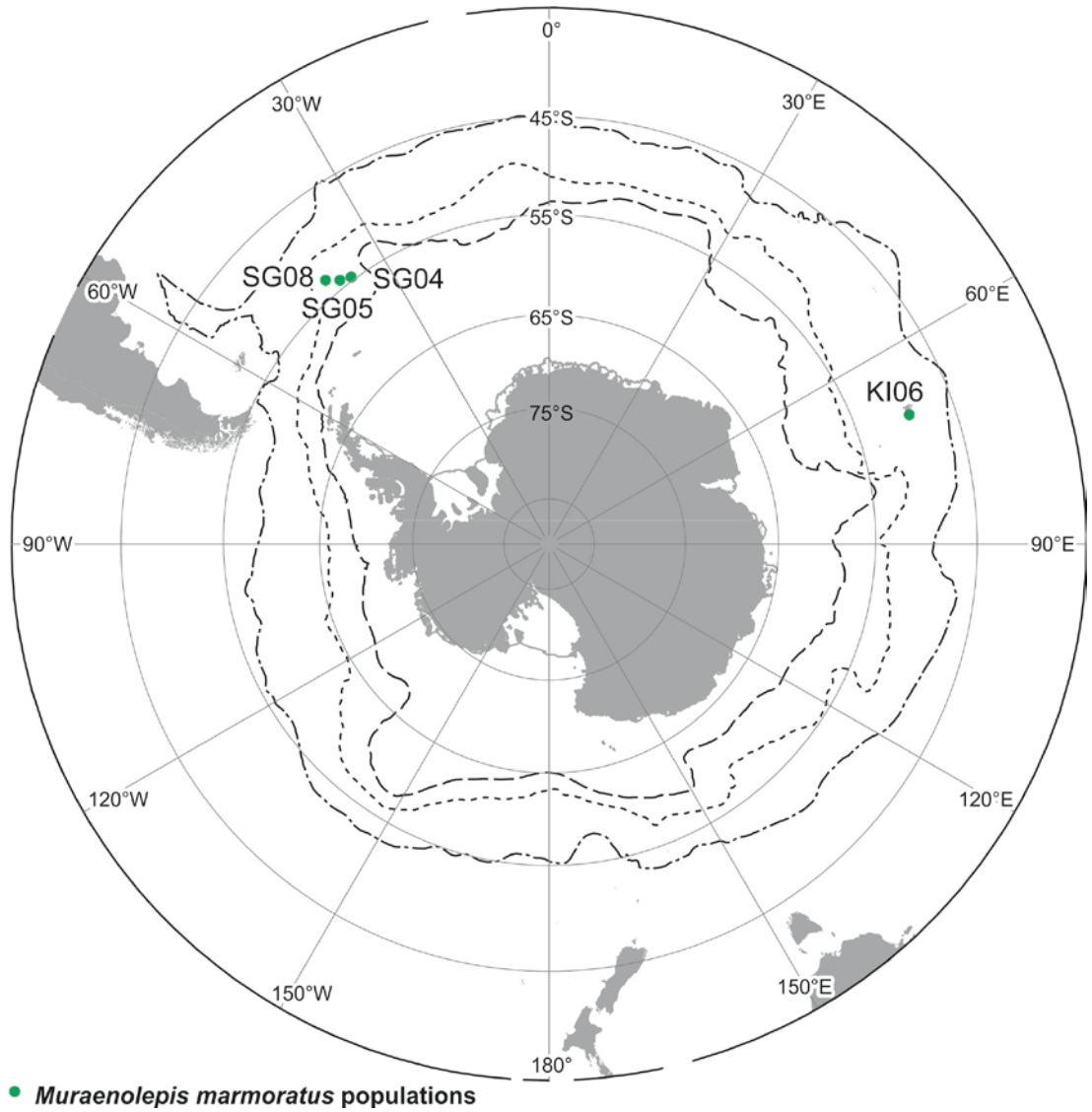


Figure 3-12 Map showing the location of *Mu. marmoratus* populations analysed for population structure based on haplotype frequencies.

To test for temporal variation the populations from South Georgia 2004 (*n*=18), South Georgia 2005 (*n*=51), South Georgia 2008 (*n*=7) were compared. For these data, 99.80% of variation was within populations and F_{ST} (Φ_{ST})=0.01811; *p*=0.19531+/-0.0096 between years, suggesting temporal stability of populations at South Georgia over this time period.

Mu. evseenkoi

Geographic variation was tested by comparing the of populations from Mawson Bank (Ross Sea subarea 88.1H) 2008 (*n*=5), South Sandwich Islands 2010 (*n*=30) and Ross Sea 2013 (subdivided by subarea; 88.2G (*n*=14), 88.1K (*n*=19), 88.1J (*n*=10), 88.1H (*n*=32), 88.1I (*n*=11), 88.1H (*n*=11), 88.1I (*n*=39), 88.2H (*n*=32), 88.1C (*n*=12)) (Figure 3-13). It was shown that 98.71% of the variation was within populations. There was no statistical significance between South Sandwich Island and Ross Sea populations.

Statistically significant results were obtained for Ross Sea subarea 88.2H compared with Mawson Bank 2008 (F_{ST} (Φ_{ST})=0.30364 ; *p*=0.04980+-0.0070) and Ross Sea subarea 88.1J (F_{ST} (Φ_{ST})=0.20218; *p*=0.04688+-0.0084). However, when the data for this region was partitioned into two areas (longitude 122˚/123˚W and longitude 127˚/128˚W) there was no significant difference between any geographic locations.

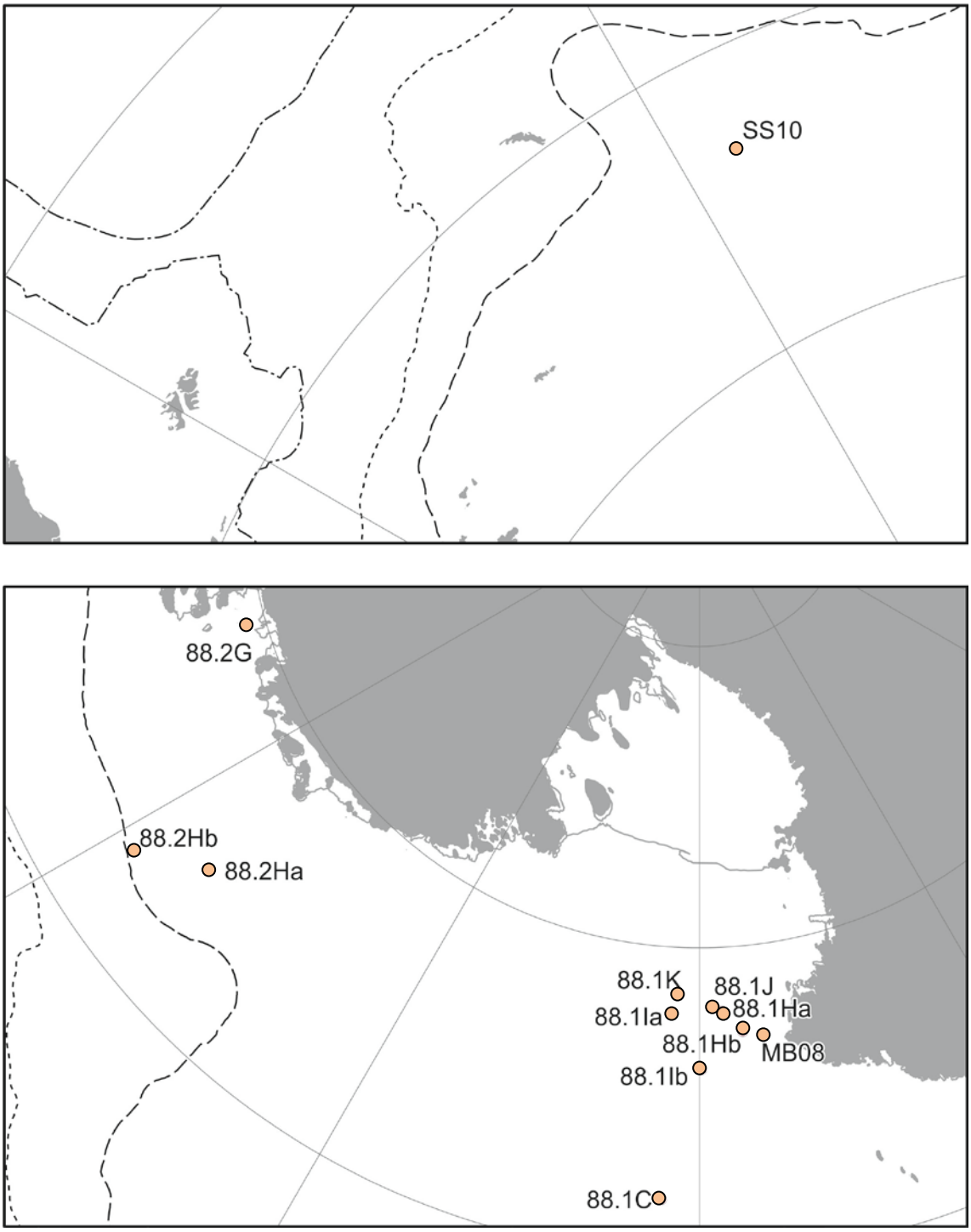


Figure 3-13 Map showing the location of *Mu. evseenkoi* populations within the Ross Sea analysed for population structure based on haplotype frequency.

3.7 Discussion

Muraenolepis is the monotypic genus of the family Muraenolepididae with, at the start of this study in 2005, four described species: *Mu. marmoratus*, *Mu. orangiensis*, *Mu. microps* and *Mu. microcephalus*. The genus was poorly understood and there has been much debate over its origin and the number of species present. Recently, five new species (*Mu. andriashevi*, *Mu. trunovi*, *Mu. kuderski*, *Mu. pacifica* and *Mu. evseenkoi*) have been described (Balushkin and Prirodina, 2013), adding to the confusion over correct identification of specimens.

In the process of this study it became evident that some of the species identification confusion has been caused by taxonomically important morphological features having greater intraspecific variability than had previously described. Much of this was due to low specimen numbers studied for morphology and, for some specimens, incorrect identification of the original examples. It was also found that some taxonomic features, used to distinguish species, may be attributed to sexual dimorphism. For example, based on measurements of *Mu. marmoratus* specimens collected at South Georgia, the length of the male's first dorsal fin is longer than the female's (Martin Collins, personal communication). Using the standard taxonomic key (Cohen, 1990) leads to misidentification of males as *Mu. orangiensis*. Similarly, there is speculation that the recently described single specimen of *Mu. trunovi*, for which a key distinguishing feature is also a long dorsal fin, may infact be a male specimen of *Mu. evseenkoi*. This is based on comparison with the larger morphological data set now available for *Mu. evseenkoi* (Peter McMillan, personal communication). Thus, accurate species identification is the foundation of any further study, be it morphological, ecological or genetic.

Species Identification and Geographic Range

The first aim of this study was to identify the species using molecular methods and compare these results to morphological identification (Fitzcharles et al., 2012). Two mitochondrial DNA markers, 16S and COI, were selected for this as both have been used previously for phylogenetic analysis, and COI is now generally regarded as the DNA barcoding gene for fish species identification (Ward et al., 2009).

Both markers were found to be informative for species identification, with COI providing a higher level of variation between species. Within the sample collection, four species were indicated to be present but these were initially designated by haplotype number rather than species identity. Re-examination of the type specimens and careful consideration of both morphological data and geographic information were required before species designation could be concluded.

There were two main samples groups within the genetic data set. The first included samples from the Falkland Islands, South Georgia and the Scotia Arc, and Kerguelen Island. Where morphological identification to species had been carried out (when originally caught), these samples had been identified as a mix of *Mu. marmoratus* and *Mu. microps*. The second group concerned samples from the Ross Sea and South Sandwich Islands. The Ross Sea samples were identified to genus only (when originally caught), while the South Sandwich Island samples had been identified either to genus or as a mix of *Mu. marmoratus* and *Mu. microps* (Table 3-2).

There has been considerable debate over the existence of *Mu. marmoratus* and *Mu. microps*, particularly around the South Georgia region (Norman, 1937, Kompowski and Rojas, 1993, Balushkin and Prirodina, 2010b, Balushkin and Prirodina, 2007). The Kerguelen Islands is the type locality for *Mu. marmoratus* and is therefore the accepted

species in this region, with a range extending to South Georgia (Chiu and Markle, 1990). *Muraenolepis microps* was thought to have a wider distribution, from South America to the Antarctic continental shelf (Cohen, 1990, Chiu and Markle, 1990) (see Figure 3-14).

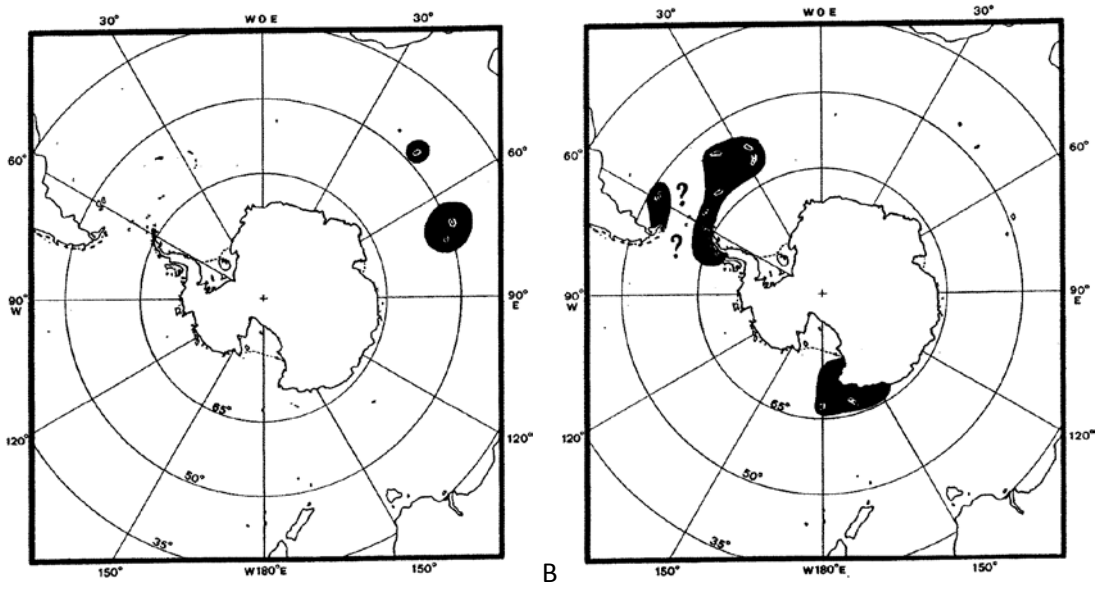


Figure 3-14 Distribution maps for (A) *Mu. marmoratus* and (B) *Mu. microps* taken from FAO Species Catalogue (Cohen, 1990).

These species are morphologically similar and a key taxonomic feature used to distinguish between them is the length of the lateral line (extending past the start of the second dorsal fin for *Mu. marmoratus*, reduced or absent for *Mu. microps*) (Chiu and Markle, 1990). The re-examination of the type specimen for *Mu. microps* showed that the lateral line extended past the origin of the second dorsal fin and was thus in-keeping with that expected for *Mu. marmoratus* (in agreement with Balushkin and Prirodina, 2010b). Similarly the vertebrae count also fitted with the description of *Mu. marmoratus*.

Combining this with the genetic data provided by this study, which identified a single species with a subAntarctic distribution that extended from the Falkland Islands through the Scotia Arc to Kerguelen Islands, indicates probability of the South Georgian species being the same as that at Kerguelen Islands. This would place *Mu. microps* as the junior synonym of *Mu. marmoratus*.

The second species identified from genetics was restricted to south of the Southern Boundary Front. Further morphological research identified these samples as *Mu. evseenkoi* (Peter McMillan, personal communication). The sample distribution from this study concurs with the high Antarctic distribution described for *Mu. evseenkoi* (Balushkin and Prirodina, 2010b).

The original account for *Mu. microps* was from South Georgia (as *Mu. marmoratus microps* – Lönnberg, 1905), which was elevated to species by Regan (1914) based on examples from the Ross Sea. This means that *Mu. marmoratus microps* would have been elevated based on an example of *Mu. evseenkoi*. Add to this that the taxonomic key proposed by Chiu and markle (1990) was based on their own specimen collection (not type material) with samples identified as *Mu. microps* collected from the Ross Sea region and those identified as *Mu. marmoratus* from the Scotia Sea. Judging from the overlap in the geographic ranges and information provided in this study, it would suggest that the Chiu and Markle (1990) classification of *Mu. microps* was in fact an early description of what has now been described as *Mu. evseenkoi.* These errors have lead to much of the confusion surrounding these species.

The genetic analysis identified two other species which were more difficult to resolve due to the small sample numbers and lack of accompanying morphological data. One of the two species was collected from two samples at Burdwood Bank and a third sample

from New Zealand. Based on the circumpolar distribution north of the Polar Front, it would suggest this species is *Mu. orangiensis*. The second of the two species was from a single sample found in the Ross Sea and was described at collection as 'elongate form'. This high Antarctic location and morphological description would suggest that the sample is *Mu. microcephalus*. The recognised ranges for both of these species are illustrated in Figure 3-15. As with the previous two species, the full range for these species is still undetermined.

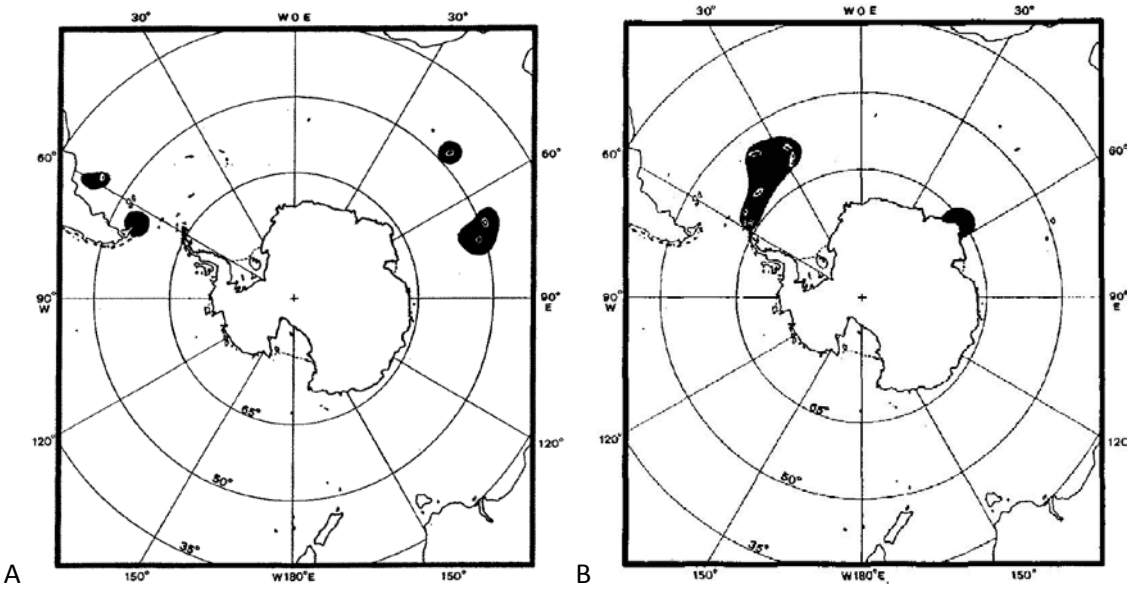


Figure 3-15 Distribution maps for (A) *Mu. orangiensis* and (B) *Mu. microcephalus* taken from FAO Species Catalogue (Cohen, 1990).

The evidence of this study has shown that the taxonomic identification of *Muraenolepis* species based on morphology was prone to error in comparison with the clearly distinct molecular results. Indeed, many scientists and observers were only able to identify to genus. Much of this problem can be generally attributed to the standard morphological identification keys and the limited knowledge of species presence and distribution in the different geographic regions. The combination of genetics and re-examination of the morphology, which has also highlighted where past errors had been made, has allowed

clarification of the species identification key with a redefined *Mu. marmoratus* and *Mu. evseenkoi,* and to demonstrate that within these species the range of measurements for taxonomical morphological features extends further than had previously been thought.

With regards to the other recently described species, no examples were collected from the geographic areas proposed for *Mu. andriashevi* (Balushkin and Prirodina, 2005, Balushkin and Prirodina, 2010a), *Mu. trunovi* (Balushkin and Prirodina, 2006), or *Mu. pacifica* (Prirodina and Balushkin, 2007). At South Georgia, there was no evidence to support a second species, as suggested for *Mu. kuderskii* (Balushkin and Prirodina, 2007). *Muraenolepis kuderskii* was collected at a depth of 440-605 m, which is within the depth range of this study, and morphologically, the length of lateral line and vertebrae count are within the expected range for *Mu. marmoratus.* However, the morphological measurements of head features (eye diameter, inter-orbital width, snout length and barbel length) do fall below the expected range. Genetic identification of this species is required to determine its relationship to *Mu. marmoratus* and the other described species.

Finding of specimens of *Mu. microcephalus* in the Magellanean Region (1000-750 m) and Macquarie Ridge (2109-2030 m) extends both the geographic and depth range previously described for this species (Balushkin and Prirodina, 2013). The morphological features, most noticeably the vertebrae count, absence of an anal lateral line and reduced upper lateral line all suggest a correct identification based on the current species descriptions. It is possible, given the extensive range now proposed for this species, that this represents a previously undescribed sister species to *Mu. microcephalus*, which has a shallower, subAntarctic distribution. Currently there are too few specimens of this species in collections to provide a clear view of the morphology or distribution and so further work is needed.

The geographic ranges shown here for *Mu. marmoratus* and *Mu. evseenkoi* clearly show a separation between subAntarctic and high Antarctic distributions for these two species. The occurrence of *Mu. marmoratus* in shallow water trawls but not deep water trawls south of the Southern Boundary front at the Scotia Arc support the depth structuring proposed for distribution of these species and may be related to oceanographic currents.

Currently, very little is known about the life cycle and ecology of these fish. Measurement of size with depth distribution for *Mu. marmoratus* at South Georgia showed a large range of sizes (9-39 cm) present above 250 m and a range from 15cm to 39 cm below 250 m, with a prevalence of fish between 19-25 cm total length (Kompowski and Rojas, 1996). Age of maturity was estimated at 24 cm for this population and a maximum age of 12 years (45 cm). A study investigationg the stomach contents of fish from this region revealed their diet to be mainly shrimp (*Notograngon antarcticus*), krill (*Euphausia superb*) and other fish (*Nototheniops larseni*), and to a lesser extent, benthic invertebrates. It was found that smaller, juvenile fish (<20 cm) fed mainly on small invertebrates, average sized (sub-adult to adult fish; 20-30 cm) on shrimp and larger, mature adult fish (>30 cm) on fish and Eupasiacea (Kompowski, 1993).

In the Ross Sea, *Muraenolepis* sp. has been found in greatest abundance between 1000 to 1500m on the Ross Sea continental slope. Age estimations from otoliths and growth rate place the majority of fish within the 4-8 years age range and a maximum age of 9.5 years (total length 45cm), with growth rates slowing after 4 years (Horn and Sutton, 2010). Males were absent from this study (Horn and Sutton, 2010) possibly due to their smaller size. *Muraenolepis* species are thought to be semelparous (one-time spawners) with a relatively high fecundity (81,000-441,000 eggs). *Mureanolepis. evseenkoi* in the

Ross Sea region are thought to spawn during the austral winter, earlier than *Mu. marmoratus* at South Georgia (Prut'ko and Chimilevskii, 2011, Parker et al., 2012).

In this study, it was found that the catches were predominantly female and male specimens were generally of smaller size, but this may be an effect of sampling methods. Previous studies on length-weight relationship for *Muraenolepis* species in the Ross Sea did not show any sexual dimorphism (female n= 2297; male n=116), catches were also predominantly female and growth rates for *Mu. evseenkoi* were shown to be higher than for *Mu. marmoratus* (Parker et al., 2012). Comparison with data for *Mu. marmoratus* and *Mu. evseenkoi* from this study agreed with this length-weight distribution, and *Mu. evseenkoi* were generally larger in size than *Mu. marmoratus.*

The predominance of benthic invertebrates in the diet of small, juvenile fish implies a requirement on the availability of continental shelf habitats for this stage of development. Earlier spawning of the high latitude species may be to allow pelagic larvae and juvenile fish optimal time for feeding in the austral summer where sea ice cover is reduced and availability of prey species is increased.

Phylogenetic Evolution

The second aim of this study was to examine the phylogenetic relationship of all four species (*Mu. marmoratus*, *Mu. orangiensis*, *Mu. evseenkoi* and *Mu. microcephalus*) and the population dynamics of the two main species (*Mu. marmoratus* and *Mu. evseenkoi*). The level of genetic variation between and within species was relatively low, with only 5% variation within the mitochondrial gene 16S region and 12% for the COI gene region. Both phylogenetic trees (Figure 3-6 and Figure 3-7) display four clades relating to the four expected species. The evolutionary history implied between clades differed

for the two gene regions, however, both place the two elongate forms, *Mu. orangiensis* and *Mu. microcephalus,* as sister species.

It has been suggested that *Mu. microcephalus* should be regarded as a separate genus from the other species (Balushkin and Prirodina, 2005) due to morphological differences and deep-sea distribution. During the course of this study this newly proposed genus, *Notomuraenobathys* Baluskin et Prirodina 2010, for the deep-sea eel cods, and containing the single species "*N. Microcephala* (Norman 1937)" was published (Balushkin and Prirodina, 2010c). The key diagnostic feature used to differentiate this genus is the larger number of vertebrae (83-85). Neither of the phylogenetic trees produced for this study supported this new genus. However, the allocation of *Mu. microcephalus* to the single sample analysed, for which there were no morphological data beyond the description of "elongate form", was tentative and may represent a yet unreported *Muraenolepis* species. Again, further integrative taxonomy, combining morphology and molecular studies, are required on a greater number of example specimens to confirm the genus and potential new species.

Population Structure

This analysis of the COI haplotype data for evidence of population structuring showed statistical significance between the *Mu. marmoratus* populations around the Scotia Arc and the population at Kerguelen Islands. This suggests that these are separate populations and that gene flow between these populations is limited. In contrast, there was no evidence for population structuring for *Mu. evseenkoi*, despite the equally large geographic range.

Both these species are distributed along the continental shelves and this may be a key factor in their distribution patterns. For the subAntarctic populations, shelf areas around

islands are separated by areas of deep water where there is no habitat available for larvae distributed by the oceanographic currents. This lends itself to isolation of populations within limited gene flow. In contrast, for the high Antarctic populations, there is a continuous continental shelf which offers a potential route for larval dispersal and associated gene flow. This will be discussed further along with the results for *Macrourus* species in Chapter 7.

3.8 Summary of Results

This study has examined both morphological and molecular descriptions for the genus *Muraenolepis* and conclusively demonstrated that four species exist, thus resolving the confusion and sometimes contradictory results of previous investigations.

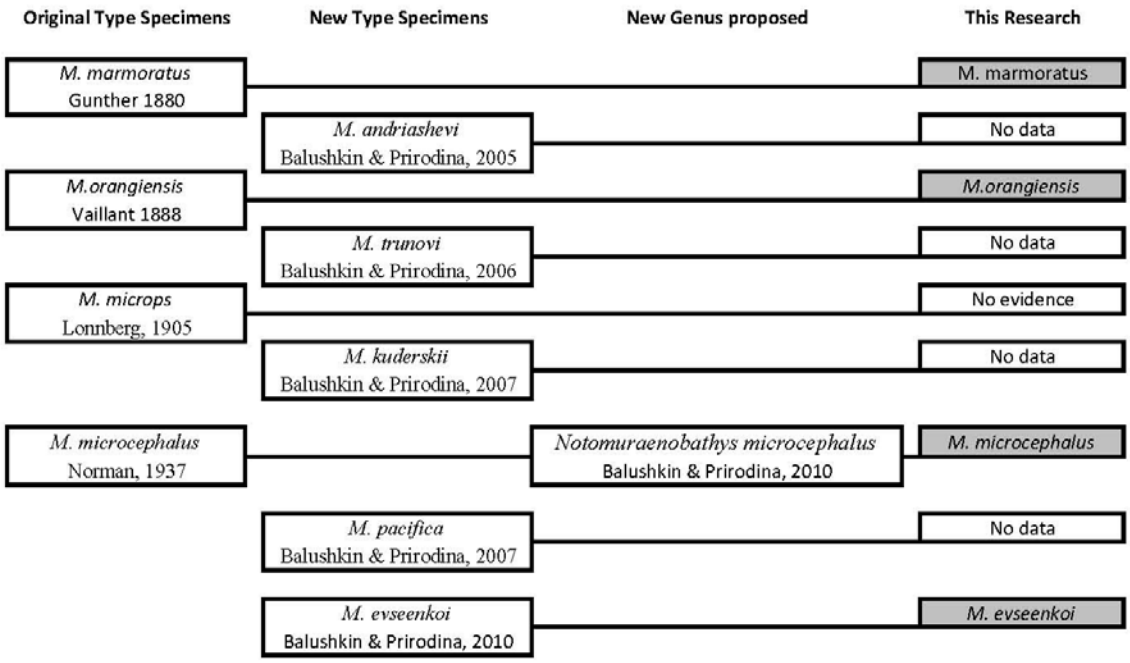


Figure 3-16 Summary of the species proposed for the genus *Muraenolepis* and the outcome of this research.

Species	Lateral Line	Vertebrae	2nd Dorsal Fin	Anal Fin
Mu. marmoratus	Regular, middle of D2	67-71	128-147	89-108
Mu. orangiensis	Regular, middle of D2	74-76	161-175	124-131
Mu. microcephalus	Irregular, short	83-85	160-176	122-135
Mu. evseenkoi	Irregular, short	70-74	133-146	99-112

Table 3.10 Key proposed by Chiu and Markle (1990) edited in respect of the findings from this research.

CHAPTER 4

The phylogenetics and phylogeography of the genus, *Macrourus*

4.1 Introduction

The Macrouridae is a predominant benthopelagic family throughout the World's deep seas. The common name is grenadiers or rattails, morphologically defined by a short body with a long, tapered tail and lack of caudal fin. The family consists of 35 genera with over 300, mostly tropical, species. Only nine species are known in the Southern Ocean (*Coelorinchus faciatus, Coelorinchus marinii, Coryphaenoides ferrier, Coryphaenoides filicauda, Coryphaenoides lecontei, Cynomacrurus piriei, Macrourus carinatus, Macrourus holotrachys, Macrourus whitsoni*) and four of which (*C. ferrier, C. lecontei, C.piriei, Ma. whitsoni*) are endemic. Macrouridae are divided into four subfamilies - Bathygadinae, Trachyrincinae, Macrouroidinae, and Macrourinae – with about 270 of the species belonging to Macrourinae (Iwamoto, 1990, McLellan, 1997), including the species in the genus *Macrourus,* which is the subject of this study.

At the start of this study, the genus *Macrourus* had 4 described species. *Macrourus berglax* Lacepede, 1801 is found in the North Atlantic; *Macrourus carinatus* (Günther, 1878) and *Macrourus holotrachys* Günther, 1878 are both found in temperate to subAntarctic waters; and *Macrourus whitsoni* (Regan, 1913) is found exclusively in Antarctic waters. A fifth species, *Macrourus caml* Smith, 2011, was discovered during the course of this research and in parallel research (Smith et al., 2011). At the time of morphological identification only the four species had been described. The fifth species will be discussed in context of the results from this study.

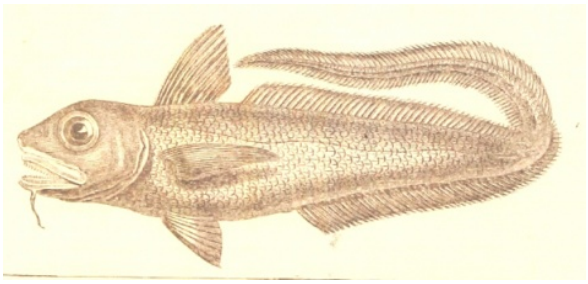
Recognised Species

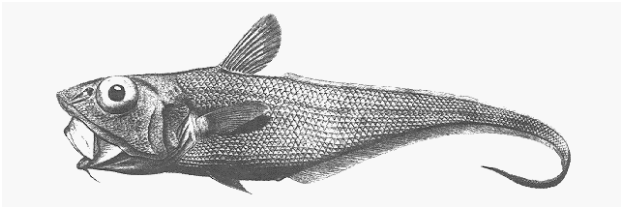
The first *Macrourus* species to be described (in Histoire Naturelle de Poissons (Lacepede, 1801)) for this genus was *Macrourus berglax*, caught off the coast of Greenland and Iceland, in the North Atlantic. Lacepede (1801) concurred with the suggestion of Bloch (1786) to place this fish in a separate genus from *Coryphaenoides*. It was described as being covered in scales with a dorsal fin ending with a spine, which has given rise to the common name of 'rattail'. There were five rows of teeth in the upper jaw and three in the lower jaw. It was also noted that this species is an important prey item for other species.

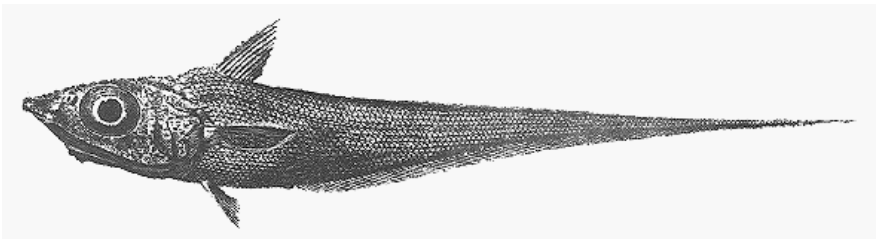
Two further species were then described in the preliminary notices of deep-sea fish from the *H.M.S. Challenger* (1872-1876) expedition (Günther, 1878). *Macrourus holotrachys* was described, from a single specimen collected off the coast of Argentina, as having a short snout and large eye. The head is covered with rough scales on the top and sides but naked on the underside, and there are five scales between the first dorsal spine and lateral line. *Macrourus carinatus* (named *Coryphaenoides carinatus* at collection and later to be renamed in Günther, 1878) was described from a station at Prince Edward Island. The snout is more pronounced compared with the other described species, with a single row of teeth in the lower jaw. The scales were pronounced with six scales between the first dorsal spine and lateral line.

The fourth species to be described was *Macrourus whitsoni* (Regan, 1913) in the Antarctic Fishes of the Scottish National Antarctic Expedition, 1902-1904. It was described from two specimens, found off Coats Land in the Weddell Sea, as having a pronounced snout with a prominent orbital ridge, large eye and scaled body, including side of head, with seven scales between the dorsal fin and lateral line. The illustrations

taken from these texts are shown in figure 4-1 and the catch locations given in table 4-1 and illustrated in figure 4-2 (with the exception of the North Atlantic *Ma. berglax*).

A 

B 

C 

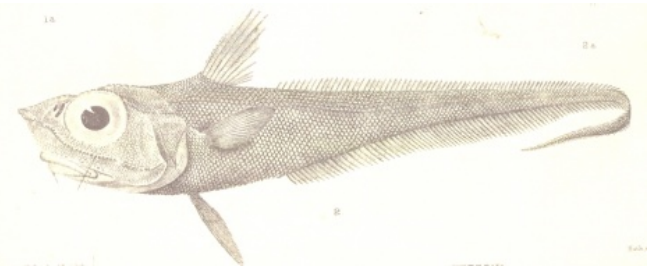
D 

Figure 4-1 Illustrations showing the morphology of the described species; (A) *Ma. berglax* (Lacepede 1801), (B) *Ma. carinatus* (FAO, www.fishbas.org), (C) *Ma. holotrachys* (FAO, www.fishbas.org), and (D) *Ma. whitsoni* (Regan, 1913).

Species	Distribution	Depth	Reference
Ma. carinatus	near Prince Edward Island, 46˚ 41'0" S 38˚ 10'0" E	500 fathoms	Gunther, 1878
Ma. holotrachys	east of Rio de la Plata, 37˚ 17'0" S 53˚ 52'0" W	600 fathoms	Gunther, 1878
Ma. whitsoni	off Coats Land, 71°22' S, 16°34' W	1410 fathoms	Regan, 1913

Table 4-1 Type locality for all described species of *Macrourus* from the southern hemisphere.

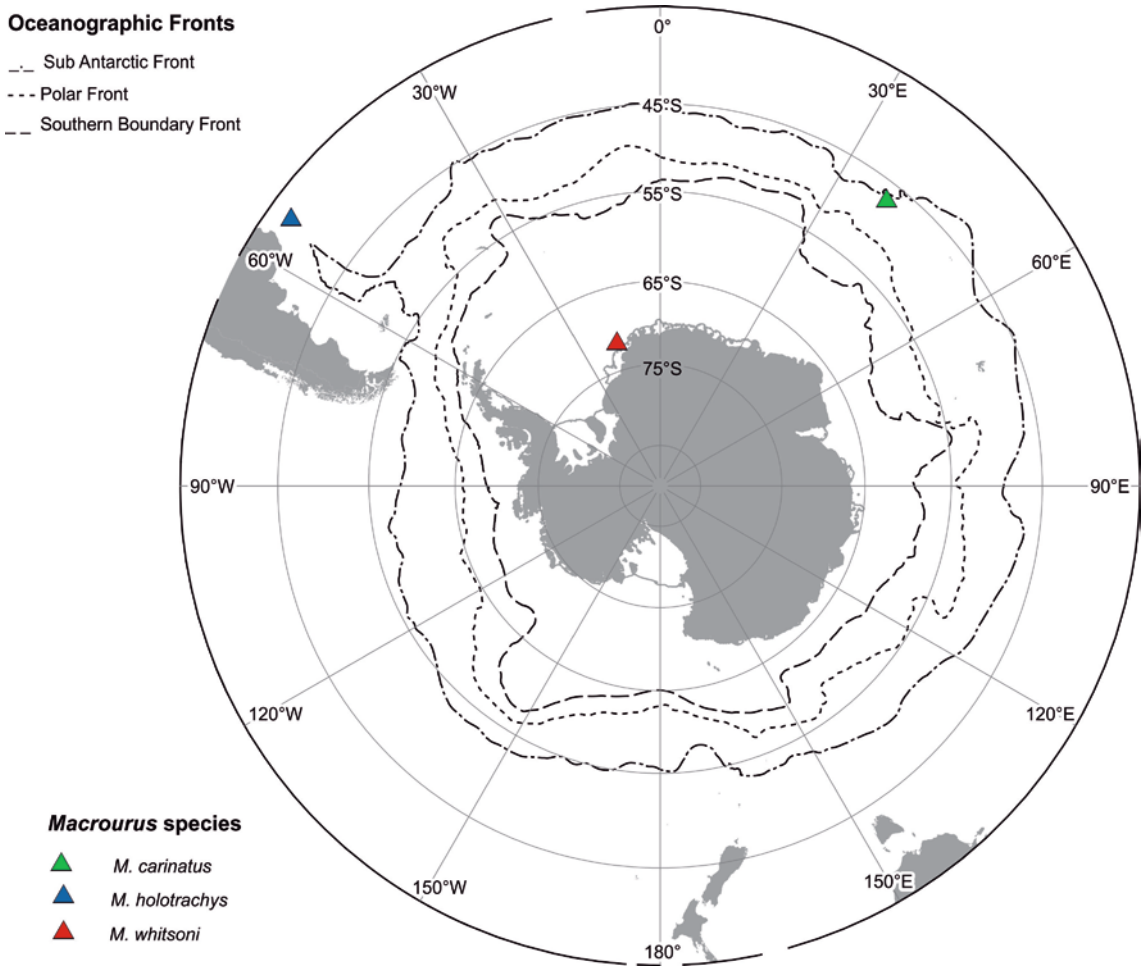


Figure 4-2 Map showing the type localities of southern hemisphere *Macrourus* species.

Geographic Distribution

Although this study is primarily concerned with the distribution of species within the Antarctic and subAntarctic, for completeness, *Macrourus berglax* has been included in the species descriptions of the genus and will also be considered here. This North Atlantic counterpart has a distribution range that extends across the North Atlantic from the northern states of the U.S.A and Canada to Greenland, Iceland and Norway. It is a benthopelagic species with a depth range of 100 m to 1000 m (Cohen, 1990)

For the Antarctic species there are conflicting definitions of the species ranges. According to Cohen (1990) the distribution of *Macrourus holotrachys* is restricted to the Patagonian slope (300 m to 1200 m) and Shag Rocks (South Georgia) but Iwamaoto 1990) extended this to include the continental shelf of southern Chile. Miller (1993) included East and West Antarctica and Laptikhovsky (2005) extended the depths down to 1750 m. The distribution of *Macrourus carinatus* also ranges from the continental shelves of South America but extends to include the subAntarctic islands (Crozet, Prince Edward, Macquarie), seamounts (Discovery, Meteor) and off the coast of New Zealand. It has also been described off Africa (Laptikhovsky, 2005). The depth range is similar to that of *Ma. holotrachys*, with the greatest overlap at about 1000 m. Below 1100 m, *Ma. holotrachys* is the dominant species (98.7% of macrourid catch) (Laptikhovsky, 2005).

The fourth species, *Macrourus whitsoni*, is the true Antarctic species with a circumAntarctic distribution extending from the continental shelf to the Antarctic Convergence and Scotia Arc, at depths of 400 m to 3185 m (Cohen, 1990, Iwamoto, 1990, Miller, 1993b).

Ecology

Studies analysing age structure on *Macrourus* species based on otoliths have found the species to be slow growing and long-lived, and a preference of descending to greater depths with age (Marriot et al., 2003). The maximum age for *Ma. whitsoni* in the Ross Sea has been estimated at over 50 years for both males and females, with females found to grow faster than males; (L_{50} = 12 years for males and L_{50} = 14 years for females), (Marriot et al., 2003). For *Ma. holotrachys* at South Georgia the maximum age was thought to be in excess of 30 years, with maturity reached at 9 years and females growing larger than males (Morley et al., 2004), and indication of sexual dimorphism.

Macrourus carinatus is of similar size to *Ma. holotrachys*, but as with *Ma. holotrachys*, displays sexual dimorphism with females being larger than males. This size difference is possibly a strategy to increase fecundity in nutrient poor deep water (Laptikhovsky, 2005). In contrast, *Ma. carinatus* from Heard and MacDonald Islands showed slower growth rates with no significant size difference between males and females (van Wijke et al., 2003); however this may be attributed to a bias towards smaller sizes caught by trawl fishing methods and seasonal variations.

Examination of the ovaries suggested a complex reproductive cycle with a major, prolonged spawning season. Fecundity has been estimated at 22,000 to 260,000 oocytes, increasing with body size (Morley et al., 2004). The eggs are ornamented with a hexagonal honeycomb structure which may slow the ascent of the eggs in the water column after release. Eggs released by adults at the lower depths of the range would rise to the thermocline, hatch and then the larvae would sink, remaining within the adult depth range. This possibility is supported by the large numbers of juveniles caught at depth and absence of larvae caught in surface waters (Merrett and Barnes, 1996).

Similar egg development at adult depths is also thought to occur in macrourid species in the Ionian Sea (Mediterranean) (D'Onghia et al., 2000).

The parasite fauna comprising of all four macrourid species was found to be very similar, suggesting an overlap in prey and distribution patterns, and a close phylogenetic relationship. *Macrourus whitsoni* revealed a range of exo- and endoparasites, serving as both an intermediate and end host. This is a common find in generalistic, predatory feeding Antarctic fish (Walter et al., 2002).

The rostrum and jaws of grenadiers are the most variable features between the species and indicative of feeding behaviour. A large mouth and blunt snout suggests mostly pelagic and benthopelagic prey. A small mouth and pointed snout is more indicative of benthic prey. Macrourids do not show any great degree of specialisation in the shape.

Macrouridae are generalist feeders with their diet varying with size, depth and season. Analysis of the stomach contents of *Ma. berglax* were shown to alter with fish size (McLellan, 1997). Small fish preyed on benthic prey, large fish on benthopelagic prey. *Macrourus carinatus* shows a preference for pelagic prey such as salps, amphipods, euphausids and bathypelagic fish. The deeper living *Ma. holotrachys* prefers benthic prey such as amphipods, decapods, isopods and polychaetes (Laptikhovsky, 2005, McLellan, 1997).. At the overlap between the two species (1000 m), prey biomass is high and competition for prey is minimal (Laptikhovsky et al., 2008)

As well as being identified as a top predator within the fish assemblages of the Antarctic (Pakhomov et al., 2006), *Macrourus* species are major prey items for both Patagonian toothfish (*Disosstichus eleginoides*) and Antarctic toothfish (*D. mawsoni*). *Dissostichus eleginoides* in the Maquarie Island fishery has been shown to consume 3401t Macrouridea per annum (Goldsworthy et al., 2001) and identified in the diets of fish on

the Argentinean slope (García de la Rosa et al., 1997). For *D. mawsoni* in the Ross Sea *Macrourus* species have been identified in 37% of stomachs (Fenaughty et al., 2003), making it a significant component of the Antarctic ecosystem.

4.2 Morphological Identification

The initial molecular results raised a question over the widely accepted morphological identification and distribution of these species. Therefore, a better understanding of the morphological distinction was required to gain a fuller understanding of the genus. The type specimens (holotypes) for each of the four species described for this genus were obtained for examination. *Macrourus carinatus*, *Ma. holotrachys* and *Ma. whitsoni* are held at the British Museum of Natural History, London. The type specimen for *Ma. berglax* is held at the Muséum National d'Histoire Naturelle, Paris and could not be provided on loan but photographs were supplied on request.

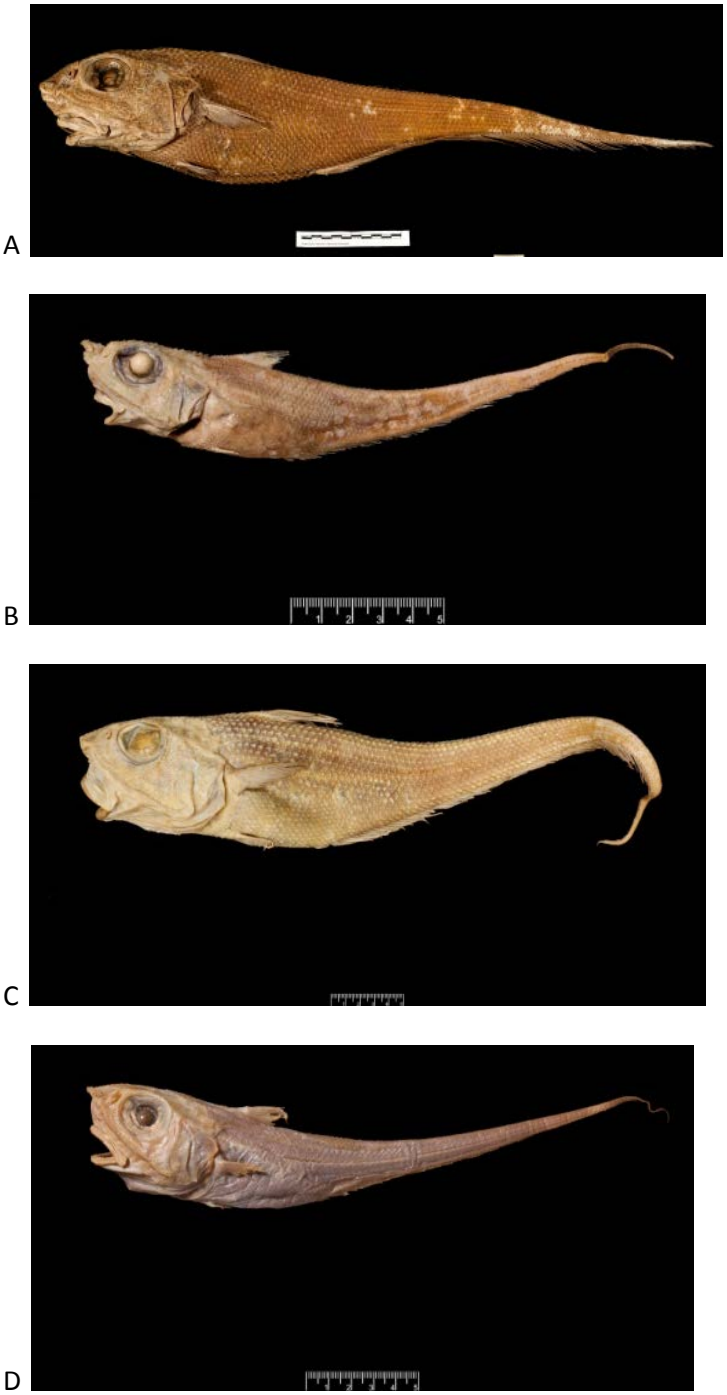


Fig 4-3 Type specimen for (A) *Ma. berglax* (B) *Ma. holotrachys,* (C) *Ma. carinatus* and (D) *Ma. whitsoni*. Images B, C & D courtesy of the British Museum of Natural History, London. Image A courtesy of the Muséum National d'Histoire Naturelle, Paris.

Sample Collection for Molecular Analysis

The samples collected for use in this study (682 samples, see Table 4-2) were identified by scientific observers onboard vessels, using the key given in Figure 2-4. There was no predominant species found within the collection, with 27% identified as *Ma. carinatus*, 29% as *Ma. holotrachys* and 33% as *Ma. whitsoni*. Accurate identification is made difficult, particularly for juvenile specimens, by the overlapping ranges of taxonomic characters used and therefore, some of the specimens (11%) were initially only identified to genus level.

			Species identified at collection			
Location	**Year**	**Collection**	***Ma. carinatus***	***Ma. holotrachys***	***Ma. whitsoni***	**Genus only**
South Georgia	2002	Dorada				7
South Georgia	2003	Dorada	29	29	36	3
South Georgia	2003	Commercial			6	55
South Georgia	2003	Viking Bay	7	57		
South Georgia	2003	Dorada	1			7
South Georgia	2003	Cisne Verde		67		
South Georgia	2003	Isla Alegranza		10		
South Georgia	2003	Isla Alegranza			15	
South Georgia	2003	Isla Alegranza	10			
South Georgia	2006	JCR BioPearl			1	
South Georgia	2009	San Aspiring			29	
South Georgia	2011	CCAMLR	17	17	16	2
South Sandwich Islands	2009	San Aspiring			50	
South Sandwich Islands	2010	Argos Froyannes			15	
Ross Sea	2006	Argos Georgia	85		11	
Ross Sea	2009	Argos Georgia			10	
Ross Sea	2010	Argos Froyannes			34	
Falkland Islands	2010	ZDLTI	36			
Falkland Islands	2012	ZDLC2		20		
TOTAL			**185**	**200**	**223**	**74**

Table 4-2 Collections of *Macrourus* specimens and species as identified by morphology at time of collection.

4.3 Molecular Identification and Phylogenetics

Molecular methods were used to examine and confirm the species identity of these specimens. Two mitochondrial gene regions were selected for this purpose. The first was the ribosomal RNA subunit16S, which is commonly used for phylogenetic studies. The second was the Cytochrome Oxidase I, the standard gene used for species identification and DNA barcoding in fish (Ward et al., 2009). The COI gene is more variable and therefore offers better resolution at the species level. In the analysis, the results from each of these markers were analysed separately. Two outgroups were selected for use in the analysis; *Muraenolepis orangiensis* or *Muraenolepis marmoratus* (from this study) and *Merluccius paradoxus* (voucher DAAPV F8) sequence obtained from GenBank (GU324141; GU324176).

Species names have been assigned to the phylogenetic trees. These were applied post-analysis for clarity of the results. The method used to determine the correct species name to assign to each branch, based on morphology and geographic location, is detailed after the phylogenetic results.

16S Data

The 16S gene region was sequenced for 417 samples. The sequences were edited to remove poor quality data and a 500bp read length was successfully obtained for 392 (94%) of the samples. These sequences were aligned using Geneious and four unique haplotypes (individuals with identical sequence grouped together) were identified.

These four haplotypes were tested to ensure there was no evidence for compositional heterogeneity using Chi-square test (PAUP; $P>0.999$) and mismatched-pairs test for symmetry (SeqVis; x% at p-value<0.05 and 0.01), making them suitable for use with standard phylogenetics programs. The sequences were analysed using standard methods

of heuristic searches using unweighted maximum parsimony (PAUP), heuristic searches using maximum likelihood with DNA substitution models (GARLI; substitution model TMP2+G) and Bayesian inference substitution models (MrBayes; substitution model HKY).

The haplotype was also analysed for Bayesian inference using PHASE with the program mcmcPHASE, GTR+G substitution model, and the results processed in SUMMARIZE to obtain the majority rule tree (Figure 4-4). Full details of the analysis methods are given in Chapter 2.

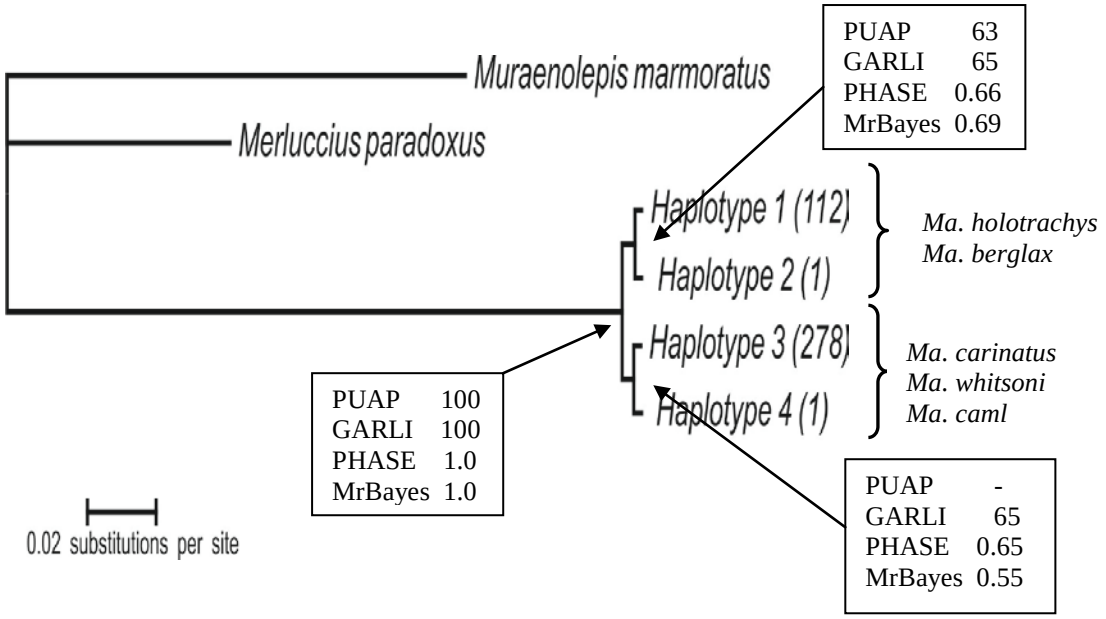


Figure 4-4 Majority rule phylogenetic tree for *Macrourus* 16S data showing PHASE branch posterior probability support values, haplotype frequencies (in parenthesis) and species included within each clade.

This phylogenetic tree shows two main branches (29% of samples and 71% of samples), each with a single sample branch. The posterior probabilities showed the node between branches was well supported (1.0) and the nodes within branches were poorly supported (0.69 and 0.55) There were three variable sites (0.6%), two of which were single nucleotide polymorphisms relating to the two outliers. Obviously, this variation was not

sufficient to resolve the species identification; therefore a more variable gene region was required.

COI Data

The COI region is more variable than 16S and a more informative marker for species identification and phylogenetics. After editing to remove poor-quality data, a 420bp sequence for the COI gene region was obtained for 596 individuals (unfortunately, the 'Ross Sea 2006' samples failed to produce quality sequence data). Alignment of the sequence data in Geneious and identification of identical sequences resulted in 11 unique haplotypes being identified.

As for the 16S data, the haplotypes were checked for evidence of compositional heterogeneity. The values obtained for the Chi-square test were within acceptable limits ($P>0.999$), however, the mismatched-pairs test for symmetry revealed a level of heterogeneity (x%=0.1538 at p-value <0.05; x% 0.1282 at p-value<0.01). The data were analysed as above but the resultant phylogenetic trees gave poor support values for both bootstrap percentage and posterior probabilities for a number of branches, and these results varied between analysis methods.

The initial attempt the resolve this issue using RY-coding (Phillips et al., 2004) resulted in reduced heterogeneity to within acceptable levels but also resulted in a significant loss in variation. Converting the data to AGY coded format proved to be more successful, reducing the heterogeneity to within accepted limits ($P=1.000$; x%=0.0000; x%=0.0000) while maintaining a higher level of variability.

The original haplotype data were analysed in PHASE using the program mcmcPHASE and THREESTATE substitution model, a general time-reversal AGY model. A majority

rule tree with Bayesian posterior probability support values was produced using SUMMARIZE (Figure 4-5).

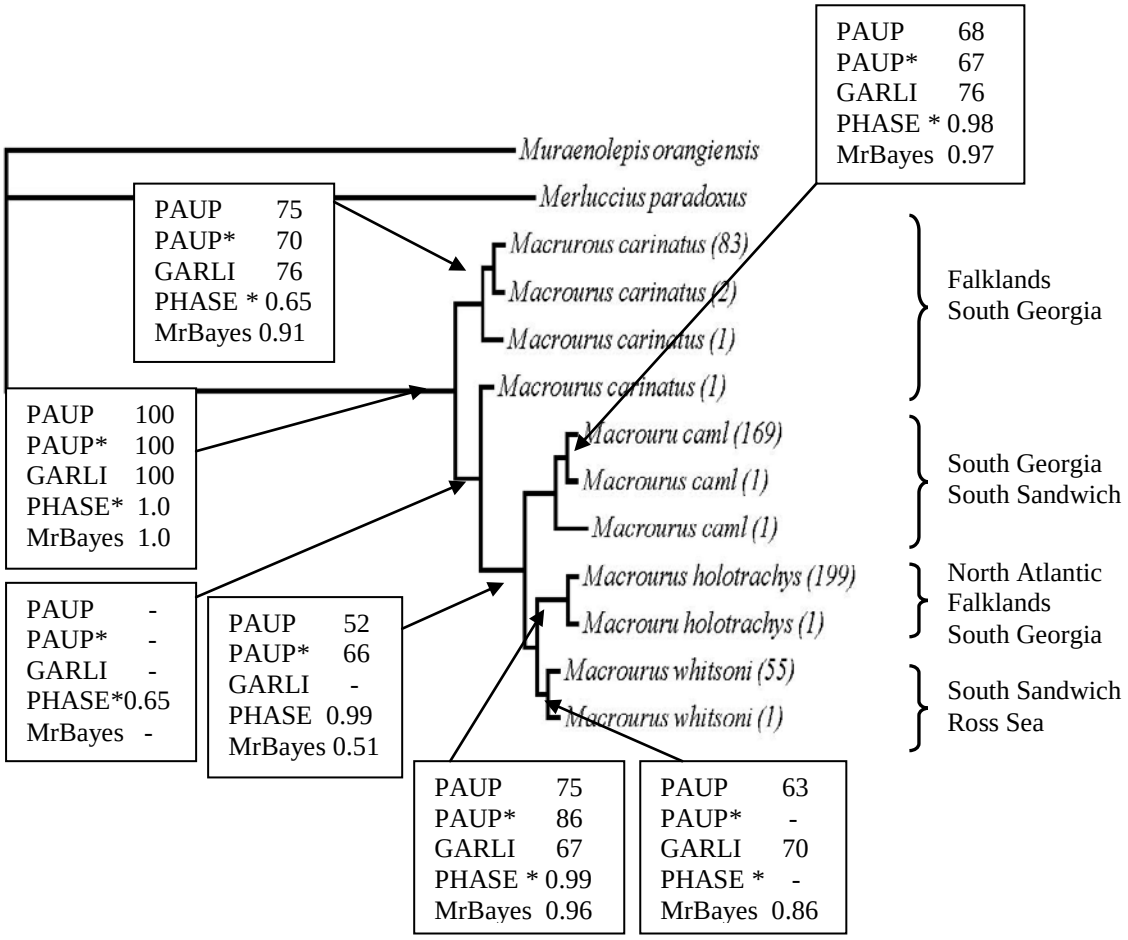


Figure 4-5 Phylogenetic tree for *Macrourus* COI data showing bootstrap percentages and branch posterior probabilities for all analysis methods (*AGY coded). Species names as identified by COI data with haplotype frequencies in parenthesis. Geographic sample locations for each clade are displayed on the right.

As expected, the COI region was more informative than 16S, with 16 variable sites along the read region (4%). In contrast to the 16S tree topography, COI provided four main branch groups, which was sufficient variation to resolve the species identification. The lower branches were well supported by PHASE posterior probabilities (<0.95). The variation between some higher branches was insufficient to obtain support values

greater than 0.5. The support values are low for analysis methods that do not account for the level of heterogeneity.

Assigning species names

Although there are four described species for this genus, the molecular and phylogenetic results did not agree with the expected result:

- North Atlantic species *Ma. berglax* was genetically identical to Southern Ocean species *Ma. holotrachys* for both 16S and COI, suggesting that they are the same species. However, further work is required to verify this.
- *Ma. whitsoni* did not form a single group but two separate branches, suggesting the presence of a new species, which has now been formally identified as *Ma. caml* (McMillan et al., 2012).

Designation of species names to the main branches required careful consideration of both fish morphology and catch location, including data observed from each of the four species Type specimens, and catch location (Figure 4-5).

The first clade, from Falkland Islands and South Georgia, contains samples of which 90% were identified by morphology as *Ma. carinatus* and so all samples within this clade are designated as *Ma. carinatus* by COI data.

Specimens of both *Ma. berglax* from the North Atlantic and *Ma. holotrachys* from Falkland Islands and South Georgia, identified by morphology, formed a single clade for both 16S and COI data. As this study is concerned with species present in the Southern Ocean, morphological data were not available to accompany the genetic samples for the North Atlantic *Ma. berglax* so there could be no further consideration on the species designations from this data. However, if North Atlantic *Ma. berglax* and

Southern Ocean *Ma. holotrachys* are confirmed as one species *Ma. holotrachys* would be the junior synonym. For simplicity of representation of the results, this branch has been designated *Ma. holotrachys*.

The final two clades, one from South Georgia and the South Sandwich Islands, the other from the South Sandwich Islands and Ross Sea, were both identified as *Ma. whitsoni* by morphology. The two clades are sufficiently distinct to indicate the presence of two species. To be able to designate one of these as a new species required re-examination of the type specimen and consideration of where specimens were collected. The type specimen was collected off Coats Land, on the continental shelf of the Weddell Sea (Regan, 1913). The two groups identified in this study showed a distinct geographic separation. One group comprised mainly of samples from South Georgia, with fewer numbers from the South Sandwich Islands and Ross Sea. The second group were the predominant species in the Ross Sea and southern part of the South Sandwich Island chain. The latter samples were shown to be a closer match, both geographically and morphologically, so were given the designation *Ma. whitsoni*, and the other branch designated a new species.

During the course of this study, *Macrourus* species were also examined genetically as part of an initiative to DNA barcode all species from the Southern Ocean during the International Polar Year 2008 (Smith et al., 2011). The results of this independent study concurred with the findings presented here and the name *Ma. caml* was assigned to this new species in recognition of the Census of Antarctic Marine Life (CAML) project (McMillan et al., 2012). This nomenclature will be used for the presentation of the results.

4.4 Morphological Data

The species collections were corrected for molecular identification. Species identification was by COI sequence data. Ross Sea 2006 samples which failed to produce sequence data of sufficient quality and were identified by HRMA technique as described in Chapter 2.

			Species identified by COI sequence				
Location	**Year**	**Collection**	***Ma. carinatus***	***Ma. holotrachys***	***Ma. whitsoni***	***Ma. caml***	**Failed**
South Georgia	2002	Dorada	5	1		1	
South Georgia	2003	Dorada	26 (1H)	30 (2C)		41 (2C;2H)	
South Georgia	2003	Commercial*		54 (1W)		7	
South Georgia	2003	Viking Bay		62 (5C)		2 (2C)	
South Georgia	2003	Dorada	4			4	
South Georgia	2003	Cisne Verde		55		12 (12H)	
South Georgia	2003	Isla Alegranza		10			
South Georgia	2003	Isla Alegranza				15	
South Georgia	2003	Isla Alegranza	8	1 (1C)		1 (1C)	
South Georgia	2006	JCR BioPearl				1	
South Georgia	2009	San Aspiring				29	
South Georgia	2011	CCAMLR	11 (1H)	16 (1C)		23 (6C)	2
South Sandwich Islands	2009	San Aspiring			17 (17Wc)	33	
South Sandwich Islands	2010	Argos Froyannes				15	
Ross Sea	2006	Argos Georgia			4 (2C;2Wc)	84 (76C)	9
Ross Sea	2009	Argos Georgia				10	
Ross Sea	2009	Argos Froyannes			15 (15Wc)	15	4
Falkland Islands	2010	ZDLTI	35	1 (1C)			
Falkland Islands	2012	ZDLC2		20			
TOTAL			**89**	**250**	**36**	**293**	**15**

Table 4-3 *Macrourus* collections with species identification by COI sequence. Information in parenthesis show misidentification by morphology at collection (C - *Ma. carinatus*; H – *Ma. holotrachys*; Wc – *Ma. caml*).

Since the molecular results revealed complexities in species identification, with the discovery of a new species, it was decided to re-examine the morphology. This was only possible for specimens where morphological measurements were recorded: South Georgia 2003 *Dorada* collection and South Sandwich Islands 2010, *Argos Froyannes*. This incorporated the three species: *Ma. carinatus*, *Ma. holotrachys* and *Ma. caml*. Unfortunately there was no morphological data available for *Ma. whitsoni*. The results for the key morphological features are given in Table 4-4.

	Ma. carinatus (*n*=24)	*Ma. holotrachys* (*n*=30)	*Ma. caml* (*n*=37)
Pelvic fin rays	**8-9** (8-9)	**8-9** (8-9)	**7-9** (7-9)
Pyloric caeca count	**14-21** (13-21)	**9-16** (8-16)	**20-36** (18-28)
Scales from anus to lateral line	**18-27** (<27)	**20-28**	**28-37** (>27)
Scales on underside of snout	**Y** (Y)	**N** (N)	**Y** (Y)
Position of second dorsal fin to anus	**in line, some slightly anterior** (slightly anterior)	**Anterior, some in line** (anterior)	**In line** (in line)

Table 4-4 Summary of key morphological features used for identification updated using data from South Georgia 2003 *Dorada* collection and South Sandwich Islands 2010, *Argos Froyannes*. Information in parenthesis shows the range expected for the species (Iwamoto, 1990, Cohen, 1990).

The morphological data were shown to be within the expected range for the species with the exception of pyloric caeca count for *Ma. caml*. This extended beyond the expected range (Iwamoto, 1990, Cohen, 1990).

A common trend found among grenadiers is for the females to grow to a larger size then males (Laptikhovsky, 2005). For the South Georgia (*Dorada*) 2003 collection the total length and weights were recorded (Table 4-5). The log length was plotted against the

log weight for both male and female specimens of all species to examine any trends in size distribution (Figure 4-6).

	Ma. carinatus		*Ma. holotrachys*		*Ma. caml*	
	Male (*n*=13)	Female (*n*=11)	Male (*n*=12)	Female (*n*=9)	Male (*n*=13)	Female (*n*=24)
Total length (mm)	225-623	377-751	217-450	470-630	350-638	410-700
Weight (g)	34-1228	224-2309	42-495	94-1422	210-1204	380-2072

Table 4-5 Length and weight ranges for males and females for each species, from South Georgia 2003 *Dorada* collection.

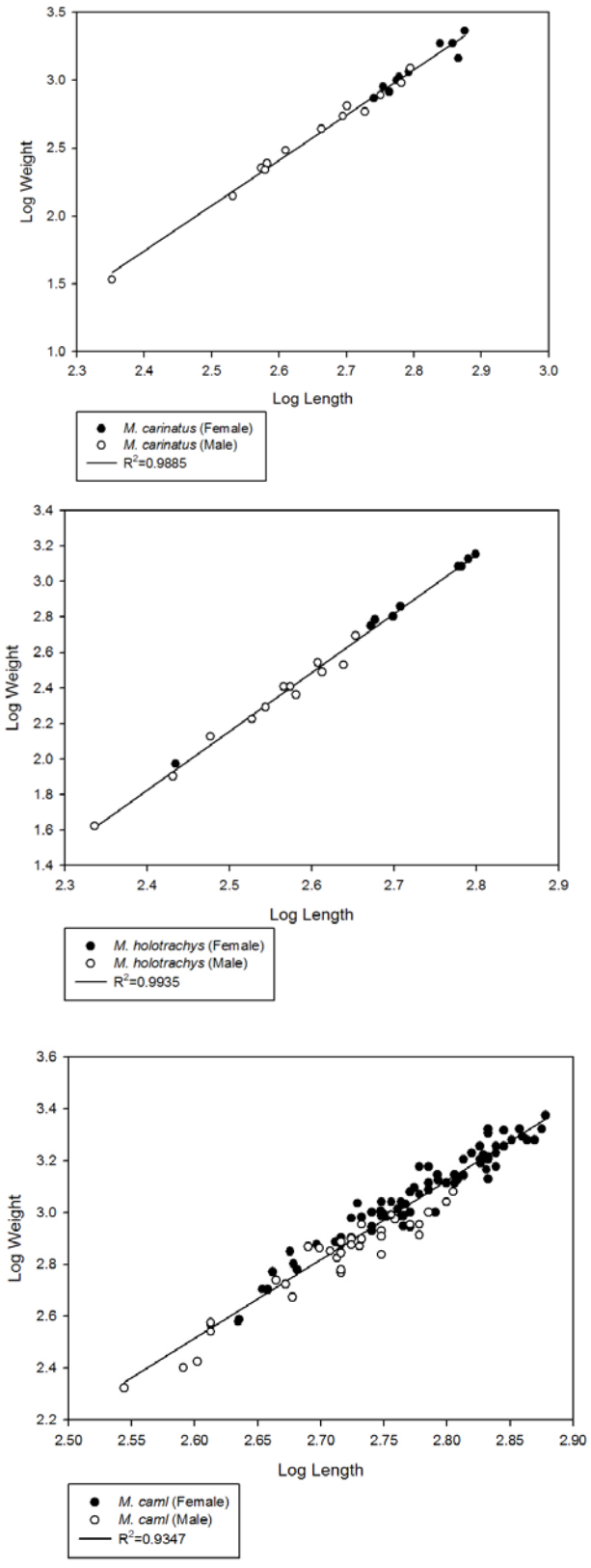


Figure 4-6 Graphs showing the relationship between length and weight for the three species of *Macrourus* from South Georgia 2003 (*Dorada*) collection.

For all three species, the distribution was for females to be larger than males; however there was considerable overlap between ranges. The length to weight distribution was similar for all three species.

It has also been proposed that in the Ross Sea, *Ma. caml* grows slower but to a larger size than *Ma. whitsoni* (Pinkerton et al., 2013). The total length and weight were recorded for these specimens from South Georgia 2003 (*Dorada*), South Sandwich Islands 2009, South Georgia 2009 and Ross Sea 2010. This data are summarised in Table 4.6.

	Ma. caml		*Ma. whitsoni*	
	Male (*n*=32)	Female (*n*=79)	Male (*n*=10)	Female (*n*=40)
Total length (mm)	350-638	135-755	416-554	288-721
Weight (g)	210-1204	369-2364	400-1100	290-1400

Table 4-6 Length and weight ranges for males and females for each species, from South Sandwich Islands 2009 and South Georgia 2009 *San Aspiring* collections.

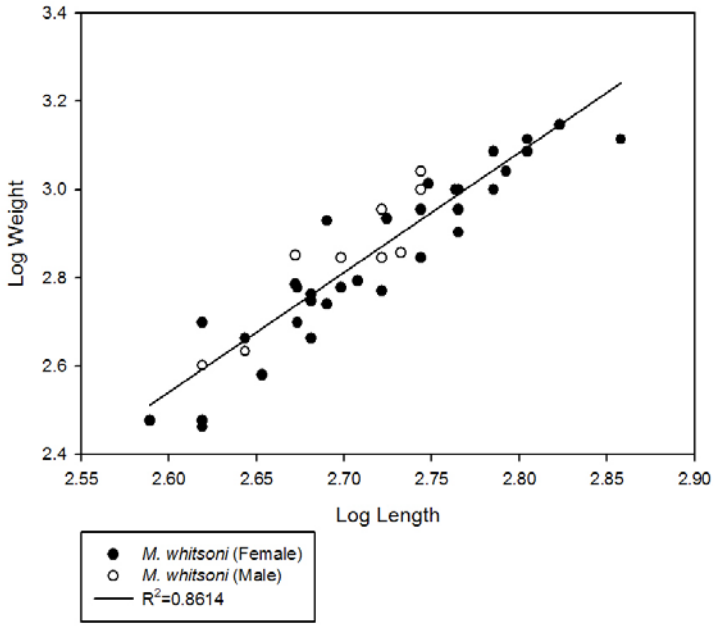


Figure 4-7 Graph showing the relationship between length and weight for *Ma. whitsoni* from South Sandwich Islands 2009 and Ross Sea 2010 collections.

The graph in Figure 4-7 shows similar length to weight distributions for both species. In general, females are larger than males for both species and the size range for *Ma. caml* does extends further than for *Ma. whitsoni*.

In summary, all graphs show a trend for smaller males and larger females. There is no difference in the length-weight relationship between species at South Georgia (*Ma. carinatus*, *Ma. holotrachys* and *Ma. caml*). However the data suggest a difference in length-weight relationship between *Ma. caml* (R^2=0.9347) and *Ma. whitsoni* (R^2=0.8614) with *Ma. caml* growing to a larger size.

4.5 Geographic Distribution

Having clearly defined the identity of the *Macrourus* species, using molecular barcoding, the different species distributions were plotted to visualise any potential spatial separation/habitat preference.

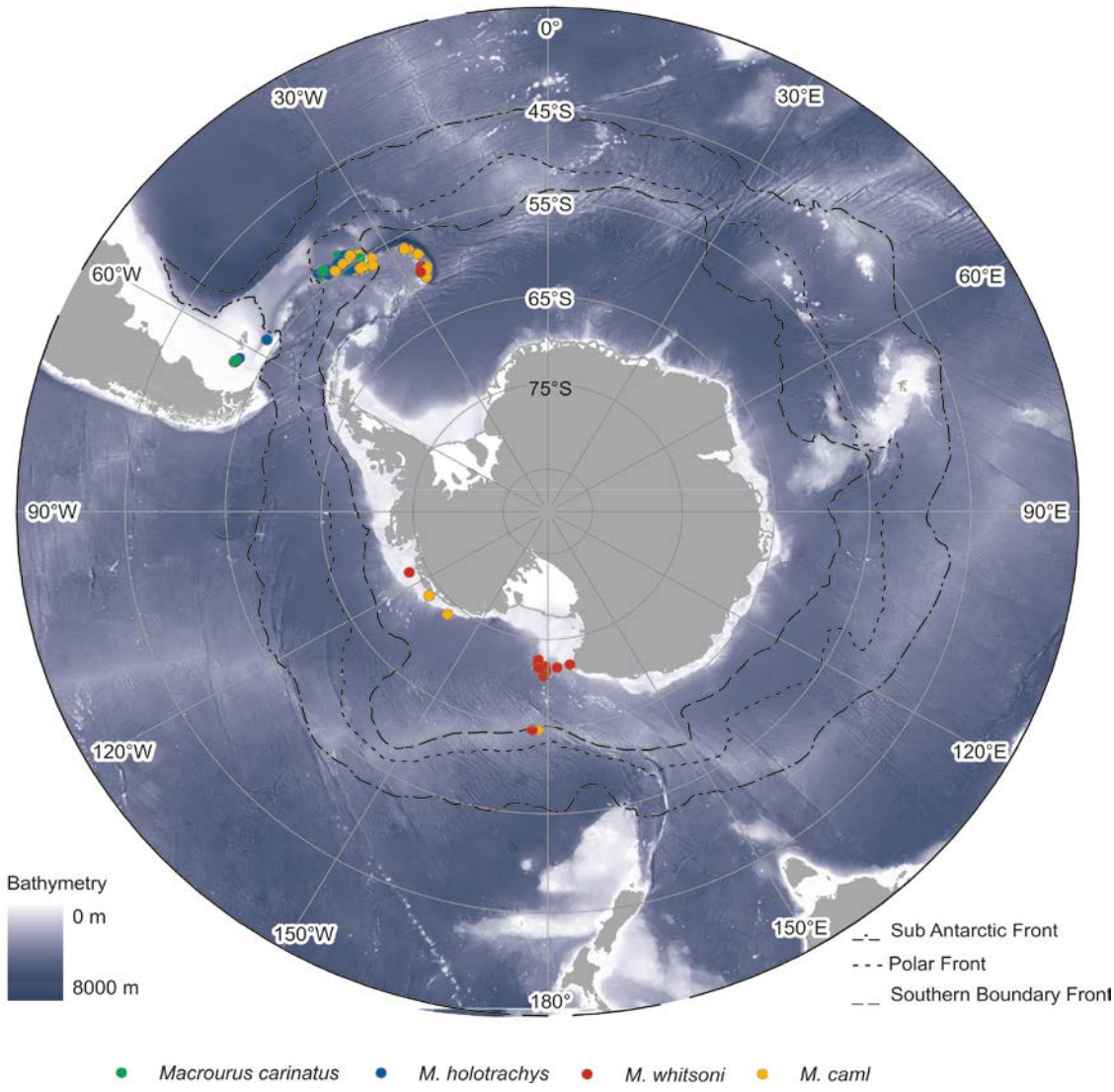


Figure 4-8 Map showing geographic distribution of the four *Macrourus* species, as identified by mtDNA COI sequence data.

It can be seen in Figure 4-8 that both *Ma. carinatus* and *Ma. holotrachys* are distributed from the Falklands to the South Georgia region of the Scotia Arc but not south of the Southern Boundary Front. By contrast, *Ma. whitsoni* has a distribution from the South Sandwich Islands to the Ross Sea but appears to be confined within the Southern Boundary Front. *Ma. caml* also ranges from the Scotia Arc to the continental shelf of the Ross Sea but extends north across the Southern Boundary Front on the Scotia Arc.

The main study area of the Scotia Arc was examined in closer detail.

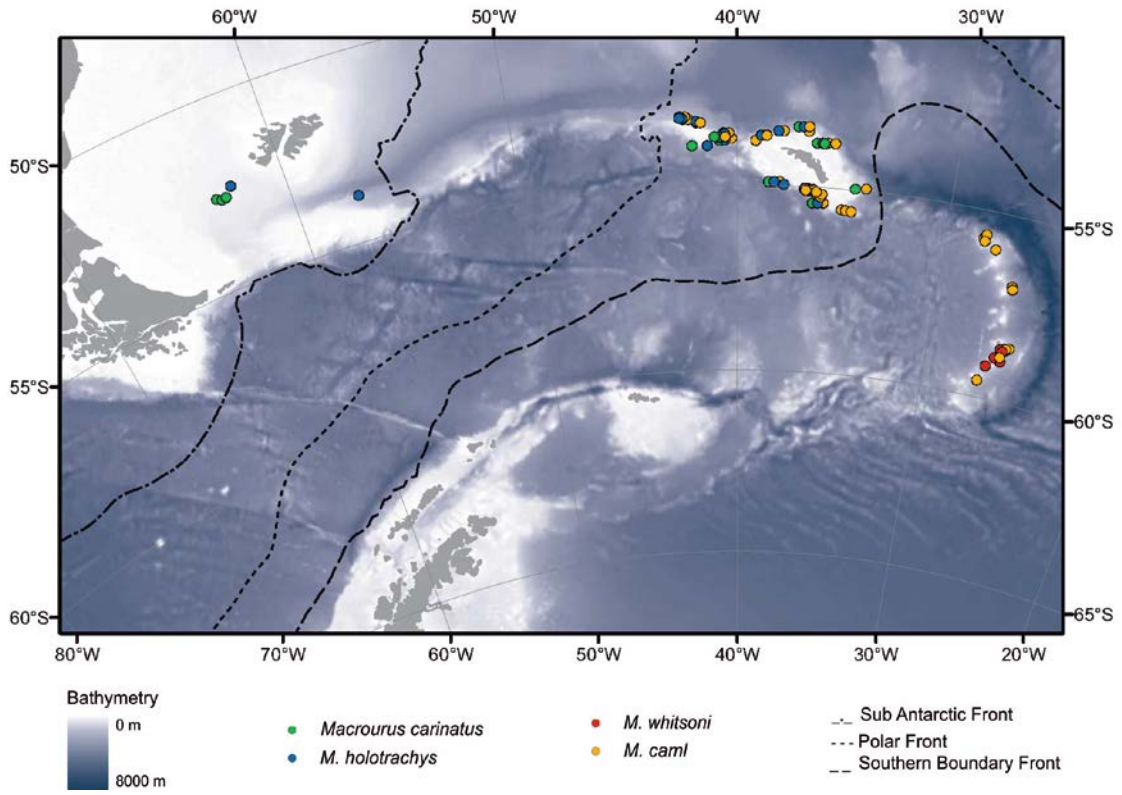


Figure 4-9 Map showing the distribution of the four *Macrourus* species, as identified by mtDNA COI sequence, in the Scotia Arc region.

On the Scotia Arc, Figure 4-9, the distribution of species is shown to change with latitude. In the north the species found were limited to *Ma. carinatus* and *Ma. holotrachys*. *Ma. caml* is present to the north of South Georgia (Shag Rocks) but the samples from this area are still shown to be predominantly *Ma. carinatus* and *Ma. holotrachys*. Further south, *Ma. caml* becomes the predominant species. Neither *Ma. carinatus* nor *Ma. holotrachys* were caught south of the Southern Boundary Front. *Macrourus caml* was the only species found around the northern islands of the South Sandwich Island chain. It was also found in the south but in smaller numbers in comparison to *Ma. whitsoni*.

4.6 Population Genetics

Following molecular and morphological integrated species identification (including the new species) and revised distribution patterns the population structure of the studied *Macrourus* species was tested in AMOVA using the haplotype frequencies. The data were first partitioned by species to remove the effect of species composition within populations from the statistical analysis. The data set was then further partitioned by geographic location and year. Data were discounted where insufficient numbers of samples were present (n<5) as governed by Arlequin requirements.

For *Ma. carinatus* this was South Georgia 2003 (three populations, depth 900-600m), Falkland Islands 2010 (depth 330-300m), and South Georgia 2011 (depth 1400-730m). There was no differentiation between populations (exact *p* value =0.00000+/-0.00000).

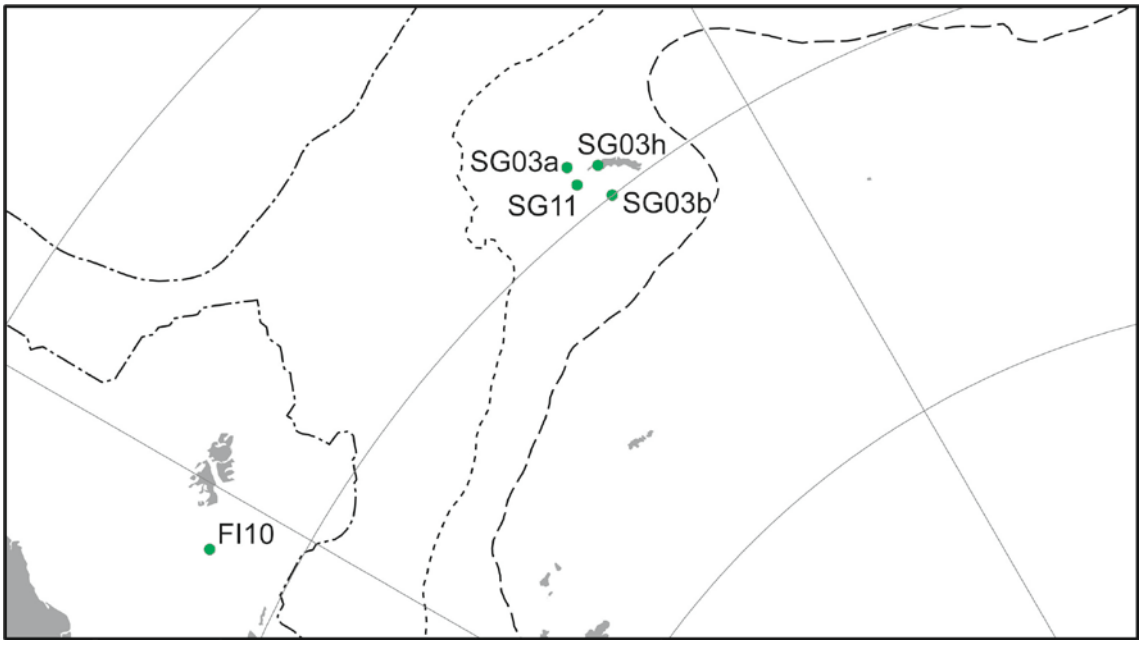
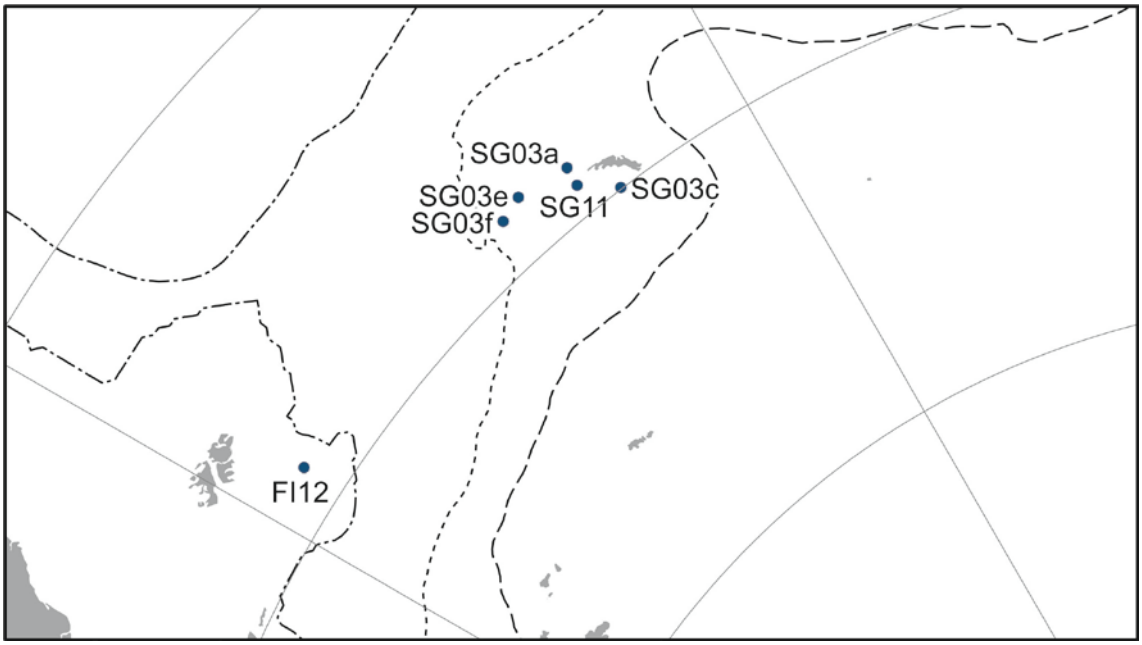


Figure 4-10 Maps showing *Ma. carinatus* population locations for haplotype frequency comparisons.

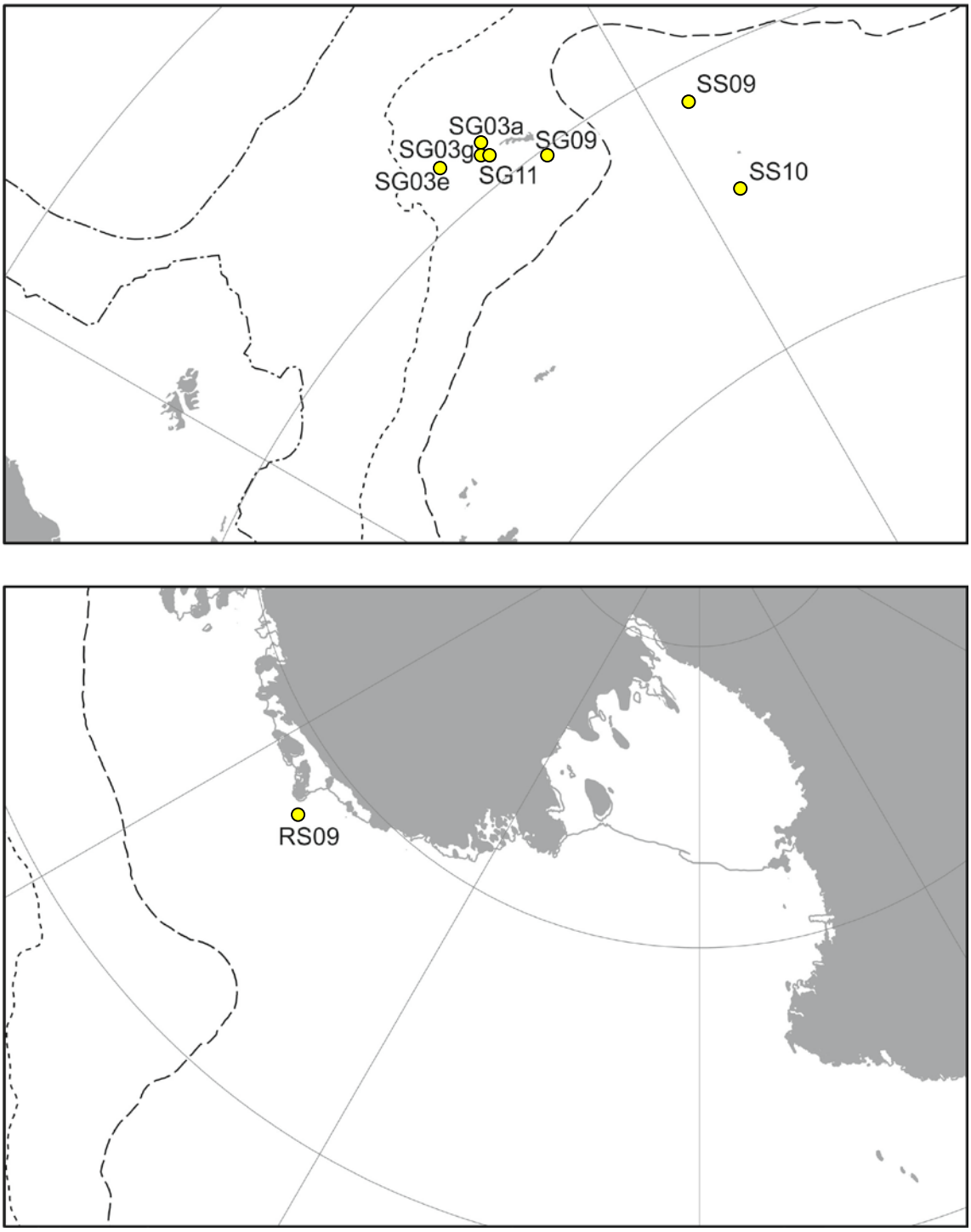
For *Ma. holotrachys* the populations were South Georgia 2003 (three populations, depth 900-600m) and Falkland Islands 2012 (depth 1550m). There was no differentiation between populations (exact *p* value =0.00000+/-0.00000).



● *Macrourus holotrachys* populations

Figure 4-11 Maps showing *Ma. holotrachys* population locations for haplotype frequency comparisons.

For *Ma. caml*, it was South Georgia 2003 (four populations, depth 900-600m), South Georgia 2009 (depth 1450-800m), South Sandwich Islands 2009 (depth 1700-1125m), South Sandwich Islands 2010 (depth 1220m), Ross Sea 2010 (1430-790m), and South Georgia 2011 (two populations, depth 1400-730m). There was no differentiation between populations (exact *p* value =0.00000+/-0.00000).



○ *Macrourus caml* populations

Figure 4-12 Maps showing *Ma. caml* population locations for haplotype frequency comparisons.

For *Ma. whitsoni* the populations considered were South Sandwich Islands 2009 (depth 1450-1280m) and Ross Sea 2010 (1430-790m). There was no differentiation between populations (exact *p* value =0.00000+/-0.00000).

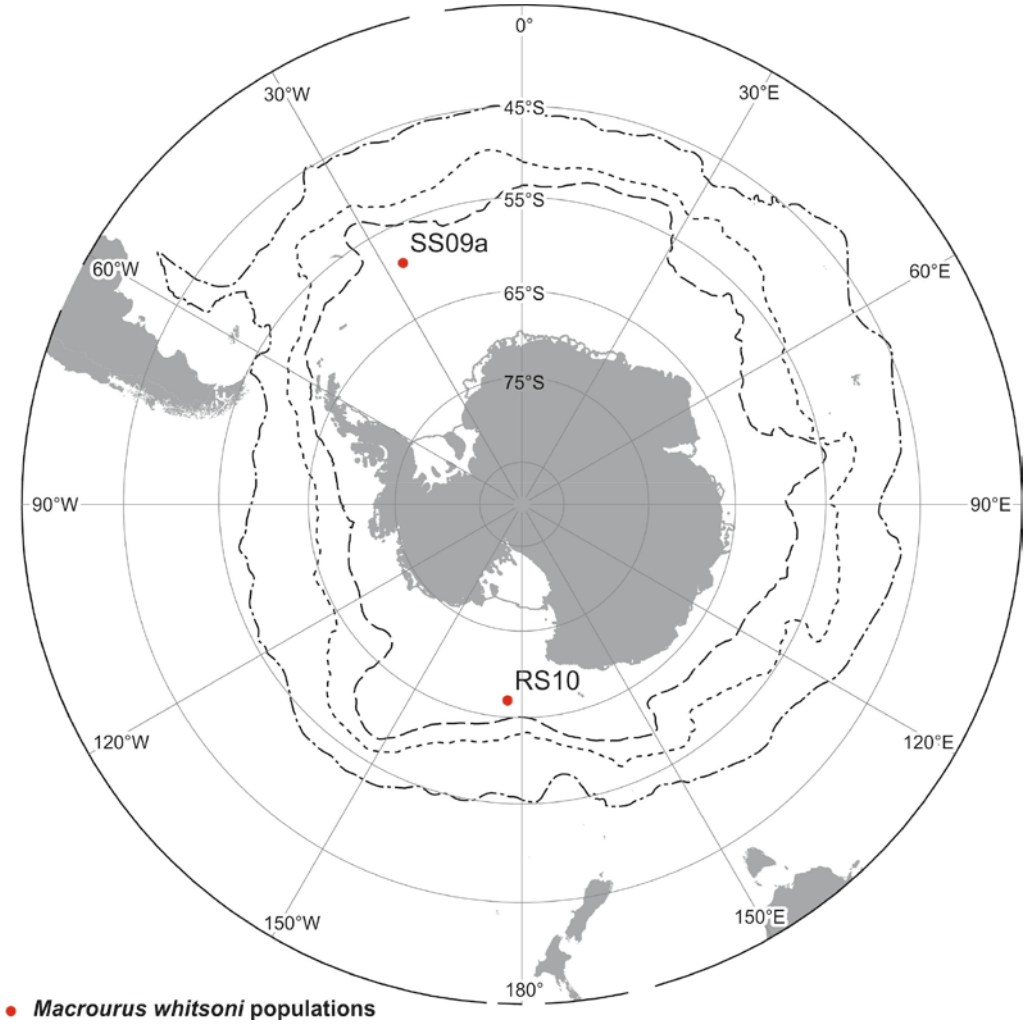


Figure 4-13 Maps showing *Ma. whitsoni* population locations for haplotype frequency comparisons.

For all four species there was no evidence of population structuring inferred from the haplotype, however, this is due to the limited number of haplotypes present and the low level of variability between haplotypes. Analysis with highly variable markers will be required to determine any structuring of populations.

4.7 Discussion

At the beginning of this study it was generally accepted that there were globally four species of *Macrourus*, with *Ma. berglax* in the North Atlantic and, *Ma. carinatus*, *Ma. holotrachys* and *Ma. whitsoni* in the Southern Ocean. All three Southern Ocean species were reported around South Georgia and samples were available for this study. Six samples of *Ma. berglax* were also included to enable phylogenetic analysis of the complete genus. Additional sampling opportunities in the Southern Ocean, occurring during the progress of this study, allowed the inclusion of material from the South Sandwich Islands and the Ross Sea. The initial aim of the study was to use molecular methods to clarify the key used for distinguishing the three Southern Ocean species by morphology. Morphological similarity between species and overlapping ranges made it difficult to accurately distinguish between the species. This was further complicated by specimen damage caused during capture (particularly for trawled fish), which removed key taxonomic features such as scales under the snout and distorted features from expanding swim bladders. Correct species identification was essential before any phylogenetic analysis or microsatellite studies can be carried out and therefore detailed morphological analysis were conducted in parallel to the molecular work.

During the course of this study and confirmed by a separate parallel study (Smith et al., 2011), this problem with what had been the standard taxonomic key was emphasised by the discovery of that *Ma. whitsoni* was actually two species, now named *Ma. whitsoni* (high Antarctic species) and *Ma. caml* (subAntarctic species). This research has shown that *Ma. berglax* (a North Atlantic species) and *Ma. holotrachys* (a Southern Ocean species) are genetically identical for the COI gene region, usually sufficient for DNA barcoding and species identification purposes, which raises the question of whether or not they are the same species.

Initially, this study was concerned only with the species present around South Georgia (CCAMLR area 48.4 ground fish survey). However, as a result of discovering unexpected species complexities and hearing about parallel research being carried out, the geographic range of this study was extended to include the South Sandwich Islands and the Ross Sea, to determine the geographic ranges for these species.

Species Identification and Geographic Range

The first aim of this study was to establish whether using molecular methods to identify the species could be used to clarify the morphological key for this genus. This involved DNA sequencing of two regions of the mitochondrial genome commonly used for phylogenetic analysis, 16S and cytochrome oxidase I (COI). The 16S region is usually informative at the generic level but can be limited for species level differentiation. The COI region has a higher rate of mutation and is commonly used for species identification (Dettai et al., 2011, Grant et al., 2011).

For the *Macrourus* species, the level of interspecies 16S variation was insufficient to resolve all the species present. The COI marker was shown to be more informative and identified four species with low intraspecific variation. However, the four species identified did not concur with the results expected from morphological analysis and thus required further investigation of the morphological features and geographic locations.

Macrourus carinarus

The only species that could be easily matched for both morphological taxonomy and molecular identification was *Ma. carinatus*, with, at collections, 98% of the samples being correctly identified by morphology. Where samples had been misidentified, they had been attributed to *Ma. holotrachys*, but these specimens were juvenile fish. In

Macrourus, juveniles, the taxonomic features are not fully developed, which creates a particular problem for species identification (Laptikhovsky, 2005, Morley et al., 2002).

Macrourus carinatus is thought to have a subAntarctic distribution, shown in figure 4-14. This was confirmed here in this study, with samples identified from Burdwood Bank and South Georgia, but was not present in any of the samples collected south of the Southern Boundary Front. Genetically identified specimens have also been recorded from Prince Edward Island, southern Australia, South Tasman Rise, South Pacific Ocean (New Zealand, Pacific-Antarctic ridge) and a single specimen from the Ross Sea (McMillan et al., 2012).

On the Falklands shelf, the geographic distribution of *Ma. carinatus* varies with age, with juvenile fish dominant in the north (41˚S to 50˚S) and adults in the south (50˚S to 54˚S), with seasonal migration related to reproduction (Laptikhovsky, 2011). The eggs and larvae are pelagic, remaining in the epipelagic and mesopelagic zones. Distribution potential is dependent on oceanographic currents, utilising the Falkland Current (500-1000m depth) for transport of eggs and larvae north to the nursery grounds (Laptikhovsky, 2011). Adults have been observed to spawn all year round, with an autumn peak and lesser spring peak, with both spawning animals and juveniles found over the Falkland slope and Burdwood Bank, from 257 m to 1097 m (Laptikhovsky et al., 2008)

Females have been found to dominate shallow waters (above 400m) and males deeper waters (below 800m), with the maximum age for fish collected at 37 years (Laptikhovsky et al., 2008). *Macrourus carinatus* is thought to grow more slowly and to a smaller size than *Ma. whitsoni* (van Wijke et al., 2003). As with other macrourid species, size has been shown to increase with depth (Coggan et al., 1996).

The length-weight relationship has been found to be statistically significant for individuals from Macquarie Island, and Heard and MacDonald Islands; however, this may be attributed to seasonal and reproductive variation (van Wijke et al., 2003). The length-weight relationship, examined here for this study, also displays the trend for females to be larger than males. Data for this analysis were only available from the South Georgia 2003 Dorada collection so it was not possible to do a comparison between geographic regions. However, there was no significant difference in length-weight relationship between any of the three species in this area.

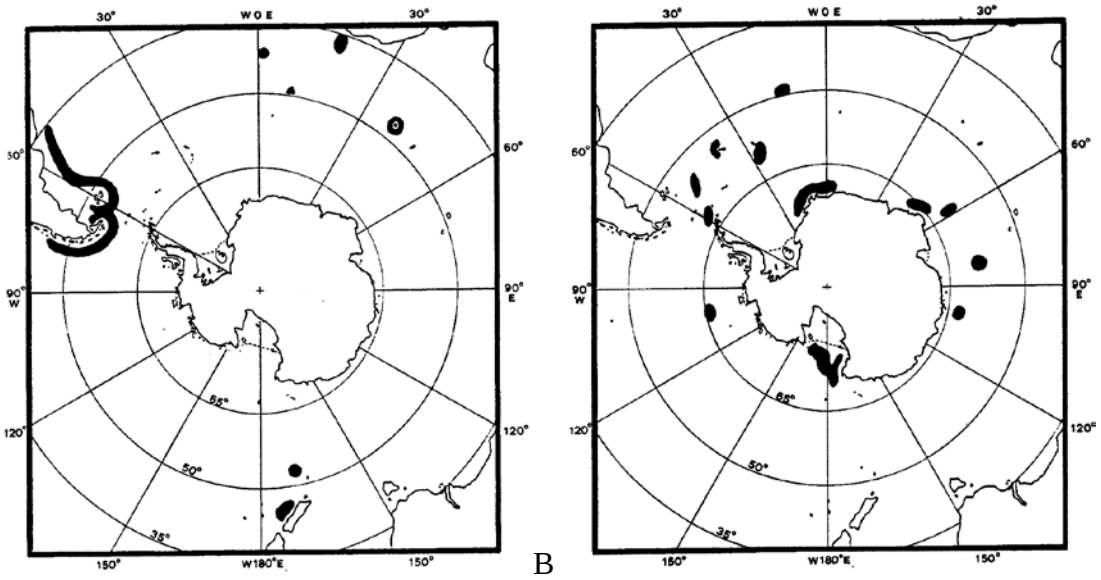


Figure 4-14 Distribution maps for (A) *Ma. carinatus* and (B) *Ma. whitsoni* taken from FAO Species Catalogue (Cohen, 1990).

Macrourus whitsoni and *Macrourus caml*

The original data set for *Ma. whitsoni* (as was the classification at the time) was from the South Georgia 2003 collections. From these data, 77% of the samples had been correctly identified by morphology. The main error was from a single collection where the majority of samples were *Ma. holotrachys* and all samples within the collection had

been designated as the same species. With the removal of that data set, the correct identification increases to 91%, suggesting that as with *Ma. carinatus*, the species morphological keys are sufficient.

The results of the South Sandwich Islands 2009 data set revealed a fourth haplotype for COI, suggesting the presence of a previously un-described species. Obtaining further sub-samples from specimens caught in the Ross Sea confirmed the presence of this new species within the High Antarctic waters. Unfortunately data relating to the morphology of these specimens, i.e. number of pyloric caeca, pelvic fin rays and scales was not available so morphological comparisons with the other species could not be made.

In parallel, the discovery of this fourth Antarctic species was also made by Smith et al (2011) and their examination of whole specimens revealed morphological taxonomic differences to distinguish between the previously cryptic species and *Ma. whitsoni*. The taxonomic features are based on colour, number of pyloric ceaca, pelvic fin rays and dentition. One morphotype was found be pale in colour, with 9 (rarely 10) pelvic fin rays and a single row of long teeth, while the other morphotype was a dark in colour, with 8 (rarely 7) pelvic fin rays and two or more rows of short teeth. The morphological data from specimens used in this study and re-examination of the *Ma. whitsoni* type specimen in this study allows the pale morph to concur with *Ma. whitsoni* and the dark morph as the new species. This new species has now been formally described as *Ma. caml* (McMillan et al., 2012).

The current documented species distribution for *Ma. whitsoni* (figure 4-14) comprises the combined distribution for both *Ma. whitsoni* and *Ma. caml*. Both species have a circum-Antarctic distributions and are found within the same depth range in the Ross Sea, with no observed differences in distributional patterns (McMillan et al., 2012). In

contrast, this study found distinct distribution patterns. In the Scotia Arc differences were observed between the two species, with *Ma. whitsoni* restricted to the southern islands of the South Sandwich Islands, where it was the predominant species. *Macrourus caml* was identified from all areas of the South Sandwich Islands, but in greater numbers in the north where *Ma. whitsoni* was not recorded. *Macrourus caml* was also recorded around South Georgia and Shag Rocks, being the predominant species in collections off the south of South Georgia. Both species were recorded from the Ross Sea, where the main species found was *Ma. whitsoni*. Where both species were recorded, they were present at the same depth. Pinkerton et al. (2013) found some evidence to suggest that the proportion of *Ma. whitsoni* increases with depth.

Differences in size have been reported between the two species, with *Ma. caml* growing more slowly and to greater lengths, but reaching sexual maturity at a shorter length than *Ma. whitsoni* (Pinkerton et al., 2013). Age estimates were 27 years for *Ma. whitsoni* and 35-39 years for *Ma. caml* (Marriott et al., 2006, Pinkerton et al., 2013). The growth rate appears to be similar for males and females in juvenile fish (Marriott et al., 2006), but faster for adult females, growing to a larger size than males, in adult fish (Marriott et al., 2003). Comparison of length-weight relationships for the two species for the data collected in this study found *Ma. caml* to have a greater size distribution. For both species there was a trend for females to be more abundant within catches and be larger in size than males.

Reproductive strategies are similar for both species, *Ma. whitsoni* and *Ma. caml* females with *Ma. caml* reaching maturity at 46cm and *Ma. whitsoni* females at 52cm (14-16 years for both species), with spawning thought to take place over the summer period, from December to January (Pinkerton et al., 2013, Marriott et al., 2006, Prut'ko and Chimilevskii, 2011, Prut'ko, 2012). They are thought to be batch spawners, with one to

four batches, and a relatively low fecundity of 9000-41,000 eggs (Alekseev et al., 1992). The eggs are pelagic, possibly bathypelagic, approximately 4 cm in diameter (Prut'ko, 2012).

Macrourus holotrachys and *Macrourus berglax*

During this research, results which required further investigation was the discovery that *Ma. holotrachys* from the Southern Ocean and *Ma. berglax* from the North Atlantic were genetically identical for both the 16S and COI mitochondrial gene regions. This result has also been confirmed by Smith et al. (2011). According to literature sources (Iwamoto, 1990, Cohen, 1990), the morphological data for *Ma. holotrachys* specimens used in this study were within the expected species ranges. The *Ma. berglax* samples supplied for this study were not accompanied by morphological data which prevented a direct comparison of the two species.

The geographic range for the two species is shown in figure 4-15. The range for *Ma. berglax* extends from approximately 37˚ north to 82˚ north along the continental shelf of North America and northern Europe (Cohen, 1990). *Macrourus holotrachys*, has a range of approximately 40˚ south to 60˚ south along the continental shelf of South America and along islands of the Scotia Arc. This southern distribution overlaps with that of *Ma. carinatus*, but the two species are separated by depth distribution, with *Ma. holotrachys* being the dominant species below 1000 m (Iwamoto, 1990, Laptikhovsky, 2005).

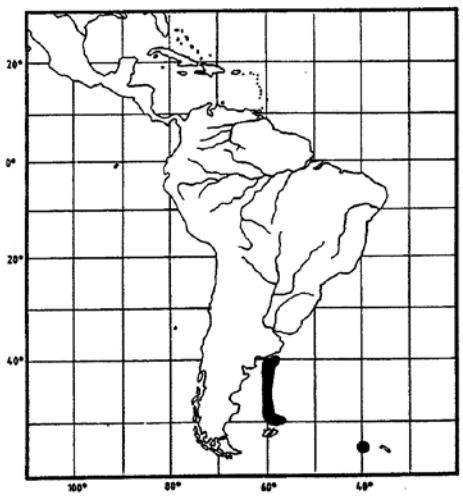
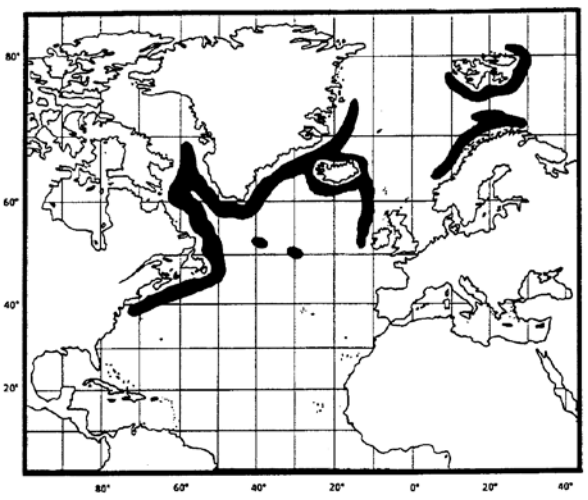
A  B 

Figure 4-15 Distribution maps for (A) *Ma. holotrachys* and (B) *Ma. berglax* taken from FAO Species Catalogue (Cohen, 1990).

A complete review of the genus re-examined the morphology of both *Ma. berglax* and *Ma. holotrachys* specimens (McMillan et al., 2012) and concluded that, although morphologically similar, *Ma. berglax* has a lower number of scales in a diagonal row between the anus and lateral line, and the range for the number of pyloric caeca overlapped but extended higher. These key diagnostic features used for morphological identification, do suggest that these two species should still be regarded as distinct species despite the genetic similarity for 16S and COI gene regions. This appears to be an interesting anomaly where standard barcoding genes do not work and therefore further work is required. By using a more variable gene regions, such as the control region of the mitochondrial genome or nuclear microsatellite markers, it should be possible to determine the extent of the genetic similarity between these two species.

(Laptikhovsky, 2005). (Morley et al., 2004).

Macrourus holotrachys demonstrates the trend for females to be larger than males, with females larger and males smaller than found for *Ma. carinatus*, and this is thought to be a strategy for maximising fecundity (Laptikhovsky, 2005). Spawning females have been

found at depths of 1200m (Laptikhovsky, 2011) and they are thought to be either batch spawners or have a prolonged annual spawning period, with a fecundity of 22,000 to 260,000 oocytes (Morley et al., 2004). *Macrourus holotrachys* is thought to live in excess of 30 years, reaching maturity at about 9 years (Morley et al., 2004).

For comparison, *Ma. berglax*, found in the North Atlantic, occupies habitats along the shelf from depths of 100m to 1000m, with the main concentration at 300m to 500m (Cohen, 1990), although the specimens have been recorded as deep as 1638m (Baker et al., 2012). The growth of males and females has been found for be similar for juvenile fish (up to 9 years), with male growth rates then slower and larger size classes predominantly female (Murua, 2003). Maturity is estimated at 15 to16 years, with a low fecundity of 8,522 to 61,844 oocytes (Murua, 2003) This would suggest a delayed maturity and lower fecundity that has been found for *Ma. holotrachys*, but with similar patterns of growth rates for males and females, as is common for macrourid species (D'Onghia et al., 2000).

Diet

All five species are generalist feeders altering feeding strategies in accordance with prey abundance, but show a preference for benthic prey such as Amphipoda and Decapoda, as well as pelagic prey such as euphausiids and myctophid fish (Morley et al., 2004, Laptikhovsky, 2005, Pinkerton et al., 2012). Prey preference was also shown to vary with depth and seasonality, in response to prey availability and abundance. *Macrourus carinatus* has been shown to have a preference for pelagic prey, whereas *Ma. holotrachys* was found to have a preference for benthic prey, thereby reducing interspecific competition where depth ranges overlap (1000m) (Laptikhovsky, 2005).

A comparison of the differing dentition and mouth shapes for *Ma. caml* and *Ma. whitsoni* suggested that the smaller, more subterminal mouth of *Ma. caml* is more likely to be a benthic feeder (McMillan et al., 2012, McLellan, 1997) as did a study of the intestine shape and a small number of stomach contents (Pinkerton et al., 2013).

A larger study of prey preference for all species would be informative to examine the role diet and interspecific competition has in determining geographic distribution of the four Southern Ocean species, particularly in areas where the species ranges overlap. Combined with a better knowledge of life cycles, spawning grounds, distribution of eggs and larvae in the water column and the role of oceanographic currents as both means of distribution and barriers to distribution, will allow us to understand the roles of both physical and ecological factors in determining species distribution.

Phylogenetic Evolution

The second aim of this study was to examine the phylogenetic relationships of the species and test for any evidence of population structuring. The level of genetic variation was very low, with only 0.6% variation for the 16S gene region and 4% for the COI gene region. The 16S region contained insufficient variation to discriminate between species and displayed only two clades; one incorporating *Ma. berglax* and *Ma. holotrachys*, the other *Ma. carinatus*, *Ma. caml* and *Ma. whitsoni* (Figures 4-2). The higher level of variation for the COI region identified four clades which related to *Ma. carinatus*, *Ma. holotrachys/Ma. berglax*, *Ma. caml* and *Ma. whitsoni* (Figure 4-3). The first node of the general consensus phylogenetic tree separated *Ma. carinatus* from the other species and was well supported by all analysis methods used. All other branches received poor support values, suggesting this phylogeny is not well resolved. This is a result of the low level of variability for the COI region and a more variable region such

as the control region of the mitochondrial genome would strengthen this data and help to clarify the phylogeny.

Population Structure

Analysis of the haplotype data did not show any statistical evidence for population structure either over the geographical range or temporal range of the sample collection. However, this was expected given the low level of intraspecific variation in the COI region for all four species. Analysis using highly variable markers is required to define structuring within populations of recently evolved species and will be essential for fisheries monitoring studies.

4.8 Summary of Results

This study has examined both morphological and molecular descriptions for the genus *Macrourus* and conclusively demonstrated that four species exist to date. This has included the discovery that two previously described species are genetically identical and what was previously thought to be a single species comprises two separate species with differing geographical ranges.

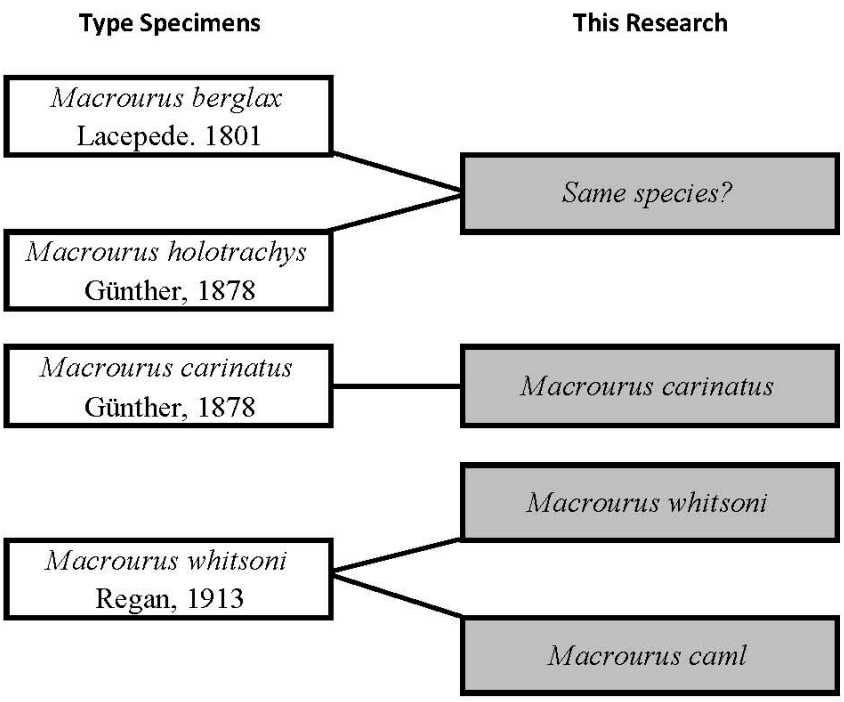


Figure 4-16 Summary of the species proposed for the genus *Macrourus* and the outcome of this research.

	Ma. carinatus	*Ma. holotrachys*	*Ma. caml*
Pelvic fin rays	**8-9**	**8-9**	**7-9**
Pyloric caeca count	**14-21**	**9-16**	**20-36**
Scales from anus to lateral line	**18-27**	**20-28**	**28-37**
Scales on underside of snout	**Y**	**N**	**Y**
Position of second dorsal fin to anus	**in line, some slightly anterior**	**Anterior, some in line**	**In line**

Table 4-7 Key proposed by Iwamoto (1990) with edited in respect of the findings from this research. *Ma. whitsoni* is not included due to lack of morphological data.

CHAPTER 5

Phylogenetic Inference for the Order Gadifomes

5.1 Introduction

The order Gadiformes (Anacanthini) belongs to the Teleost fishes, with species found throughout the World's oceans, predominantly in temperate and cold marine waters (Cohen, 1990). The order comprises ten families; Bregmacerotidae (codlets), Euclichthyidae (Eucla cods), Gadidae (true cods), Lotidae (rocklings), Macrouridae (grenadiers), Melanonidae (pelagic cods), Merlucciidae (merluccid hakes), Moridae (morid cods), Muraenolepididae (eel cods) and Phycidae (phycid hakes) (Cohen, 1990).

Gadiformes are thought to have originated in the Late Cretaceous period, moving into deeper water in the Early Eocene (Nolf and Steurbaut, 1989). Antarctic fossil evidence has been found to suggest Gadiformes were present on the Gondwanan shelf in the Late Eocene (Eastman and Grande, 1991, Kriwet and Hecht, 2008), an exception amongst the modern Antarctic fauna, which are not represented in the Antarctic fossil record (Eastman, 1993c).

The two genera considered in this study, *Muraenolepis* (Muraenolepididae) and *Macrourus* (Macrouridae: Macrourinae) are thought to have very different evolutionary histories to each other.

Muraenolepididae is regarded as a distinct monophyletic group of nine described species but its relationship to other gadiform families is disputed. Based on morphology it has been classed as either a 'higher gadoid' with the families of Macruronidae, Bregmacerotidae, Phycidae, Lotidae, Merlucciidae and Gadidae, and placed in the

suborder Gadoidei (Howes, 1990) or a sister group to Bregmacerotidae (Markle, 1989). Genetic markers (12S and 16S mitochondrial ribosomal RNA and RAG1 nuclear genes) did not support this placement in the Gadoidei and maintained Muraenolepidoidei as a separate suborder and sister group to all other Gadiformes, although the positioning was weakly supported (Roa-Varón and Orti, 2009).

Similarly, the evolutionary origin is unclear. Andriashev (1965, in Chui et al, 1990) suggested the species are typically Antarctic, where as DeWitt (1971, in Chui et al, 1990) proposed they had recently invaded Antarctic waters, with the possible exception of *Mu. microps* having Antarctic shelf origins.

In contrast to the Muraenolepididae, the family Macrouridae is the largest family of Gadiformes with over 300 species divided into 35 genera. The genera can be grouped into four subfamilies; Bathygadinae, Macrouroidinae, Trachyrincinae and the largest subfamily, Macrourinae. (Iwamoto, 1990, McLellan, 1997). Fossil records suggest the origin of the macrourids is in the high southern latitudes in the middle to late Palaeogene (40-30 mya) with migration into the South Atlantic (Kriwet and Hecht, 2008).

Following on from the results of Chapter 3 and Chapter 4, it was decided to examine the implied phylogenetic relationship of *Muraenolepis* (Muraenolepididae) and *Macrourus* (Macrouridae: Macrourinae) with other gadiform species from the data obtained in this study.

5.1 Methods

The 16S and COI sequences available for other gadiform fish present in the same geographic regions were downloaded from Genbank (listed in Appendix II) and aligned

with the species identified from this data. The data from this study identified only four of the possible nine described species of *Muraenolepis*, therefore the 16S data for five *Muraenolepis* specimens and COI data for 12 *Muraenolepis* specimens which were available on Genbank were also included (Table 5-1).

16S Genbank Data				
Accession Number	**Species name at collection**	**Location**	**Collection**	**Reference**
FJ215174	*Muraenolepis marmoratus*	Not recorded on publication	NIWA	Roa-Varon and Orti,. 2009
FJ215175	*Muraenolepis microps*	Not recorded on publication	NIWA	Roa-Varon and Orti., 2009
JX974419	*Muraenolepis microps*	Unknown	Unknown	Rehbein, Unpublished
FJ215176	*Muraenolepis orangiensis*	Not recorded on publication	NIWA	Roa-Varon and Orti., 2009
FJ215177	*Muraenolepis sp.*	Not recorded on publication	Chiba University, Japan	Roa-Varon and Orti., 2009
COI Genbank Data				
EU326374	*Muraenolepis sp.*	Scotia Sea	BOLD	Rock et al., 2008
EU326375	*Muraenolepis sp.*	Scotia Sea	BOLD	Rock et al., 2008
EU326376	*Muraenolepis sp.*	Scotia Sea	BOLD	Rock et al., 2008
EU326377	*Muraenolepis sp.*	Scotia Sea	BOLD	Rock et al., 2008
EU326378	*Muraenolepis marmoratus*	Scotia Sea	BOLD	Rock et al., 2008
EU326379	*Muraenolepis marmoratus*	Scotia Sea	BOLD	Rock et al., 2008
EU326380	*Muraenolepis marmoratus*	Scotia Sea	BOLD	Rock et al., 2008
JN640702	*Muraenolepis marmoratus*	Not recorded on publication	Australian National Fish Collection	Smith et al., 2012
HQ713082	*Muraenolepis sp.*	Dumont d'Urville Sea	CEMARC Campaign	Dettai et al., 2011
HQ713083	*Muraenolepis sp.*	Dumont d'Urville Sea	CEMARC Campaign	Dettai et al., 2011
HQ713084	*Muraenolepis sp.*	Dumont d'Urville Sea	CEMARC Campaign	Dettai et al., 2011
HQ713085	*Muraenolepis sp.*	Dumont d'Urville Sea	CEMARC Campaign	Dettai et al., 2011

Table 5-1 Accession numbers and collection details for *Muraenolepis* data obtained from Genbank.

For 16S, the *Macrourus* specimens from this study could not be resolved to species level and are identified to genus only (see Chapter 4). *Dissostichus eleginoides* (Order

Perciformes; Family Notothieniidae) and *Zeus faber* (Order Zeiformes; Family Zeidae) were selected as out-groups. Data were analysed as for the previous data sets and full details are given in Chapter 2.

5.2 Results

For 16S, 15 Gadiform sequences were downloaded, with an overlapping region of 285bp. For COI, 84 gadiform sequences (38 of them unique) were downloaded, with an overlapping region of 359bp. The results are presented as majority rule phylogenetic trees to display the inferred relationship between species with an indication of evolutionary time frames, and as cladograms to clarify the relationship between families inferred from the data sets.

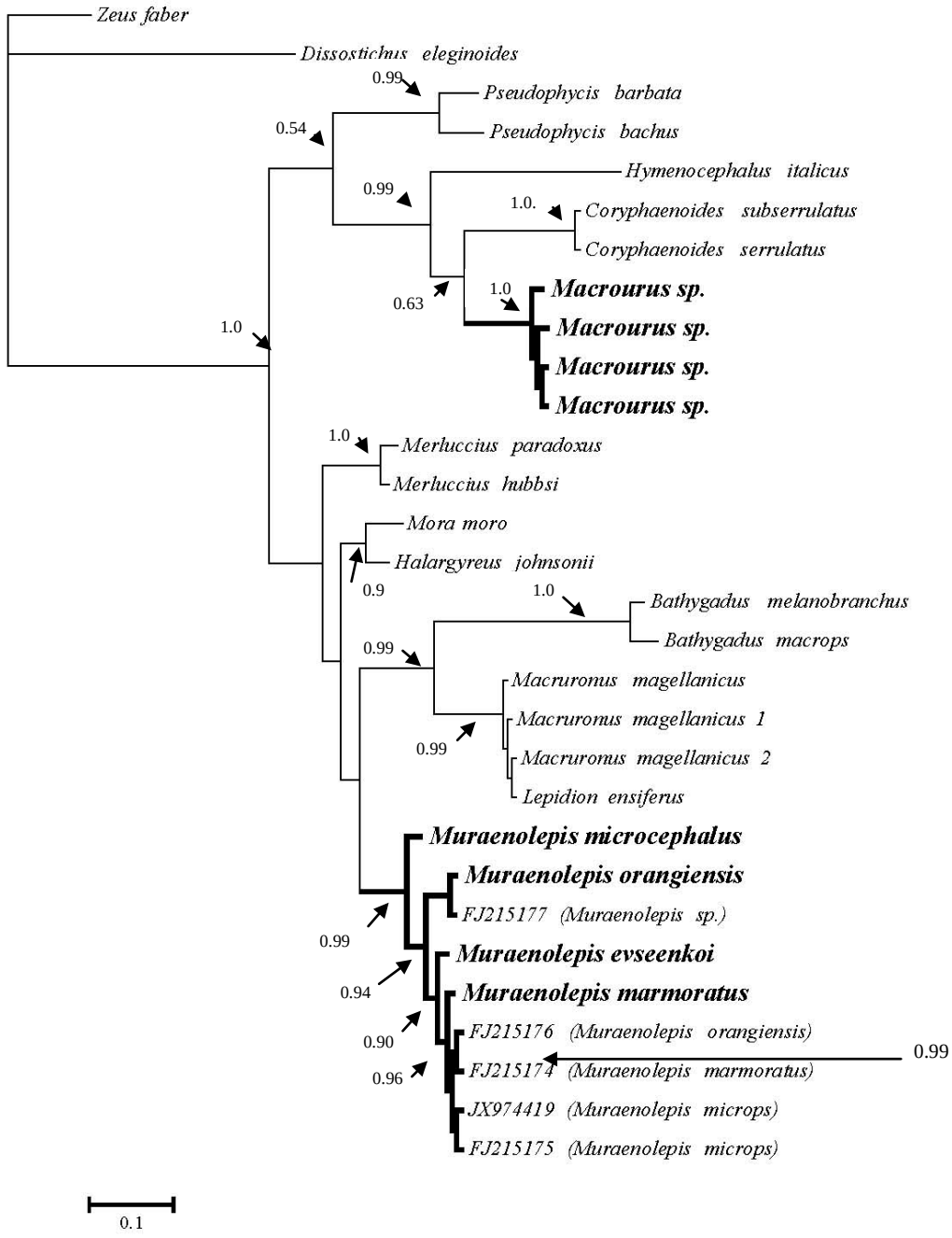


Figure 5-1 Majority rule phylogenetic tree showing the relationship between gadiform species as inferred by COI data. Branch lengths are to scale with posterior probability support values from PHASE displayed. Data from this study are highlighted in bold and have been allocated the full species name, or to genus where species resolution was not possible. For *Muraenolepis* Genbank data, the accession numbers are given with the species identification at collection in parenthesis.

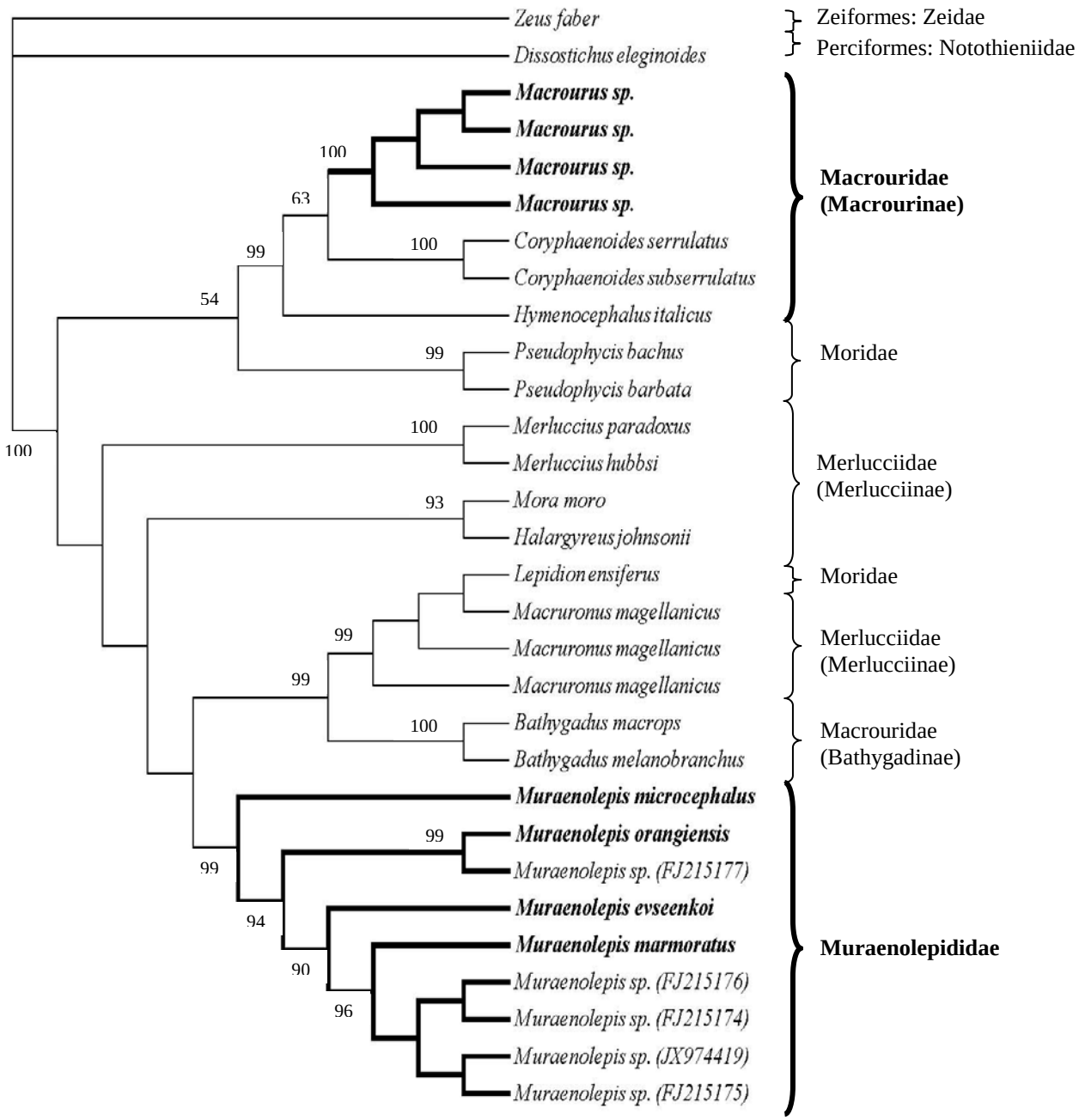


Figure 5-2 Cladogram showing the relationships between gadiform families as implied by 16S data, with posterior probability values from PHASE. Data from this study are highlighted in bold and have been allocated the full species name, or to genus where species resolution was not possible. For *Muraenolepis* Genbank data, the accession numbers are given in parenthesis.

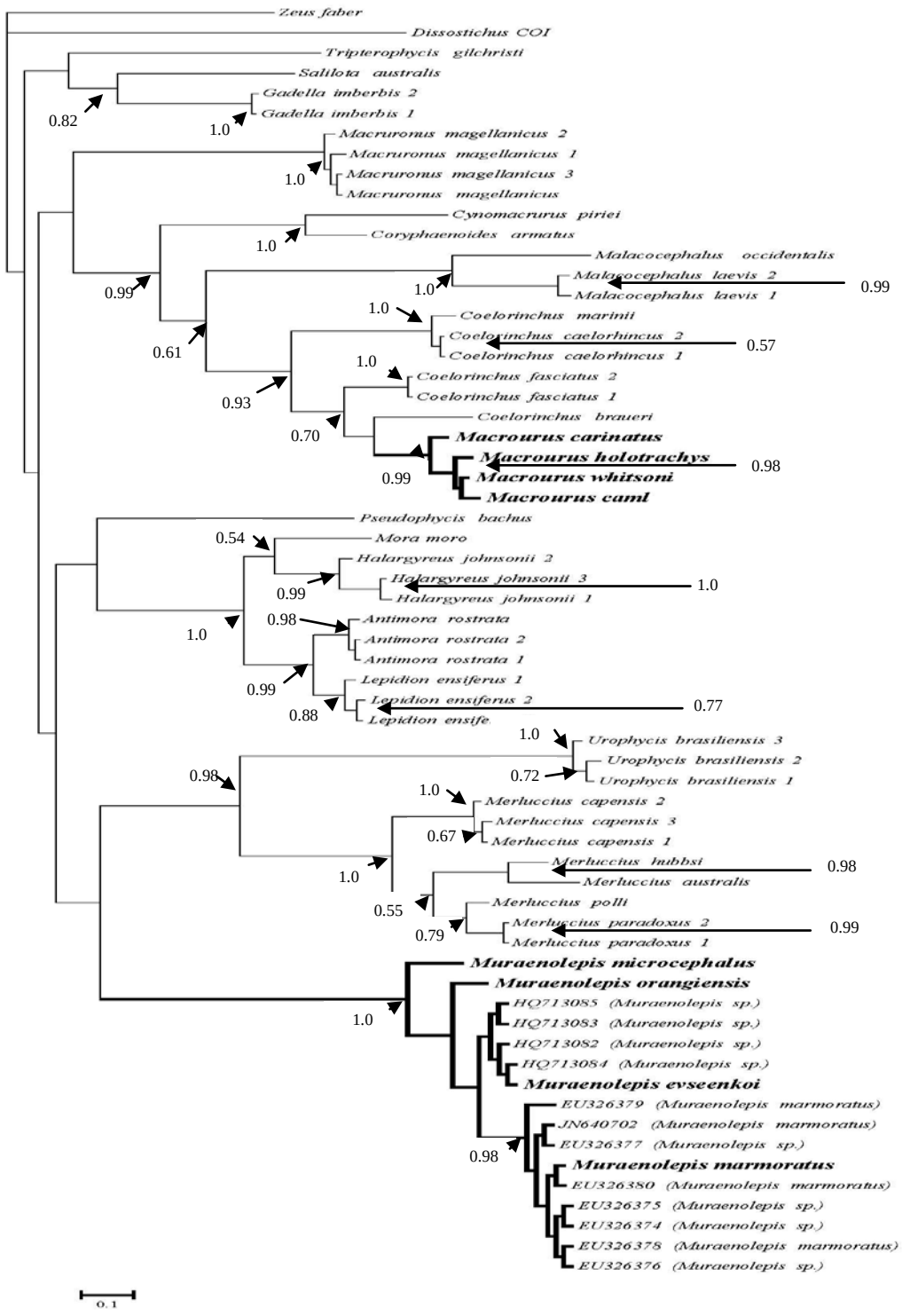


Figure 5-3 Majority rule phylogenetic tree showing the relationship between gadiform species as inferred by COI data. Branch lengths are to scale with posterior probability support values from PHASE displayed. Data from this study are highlighted in bold. For *Muraenolepis* Genbank data, the accession numbers are given with the species identification in parenthesis.

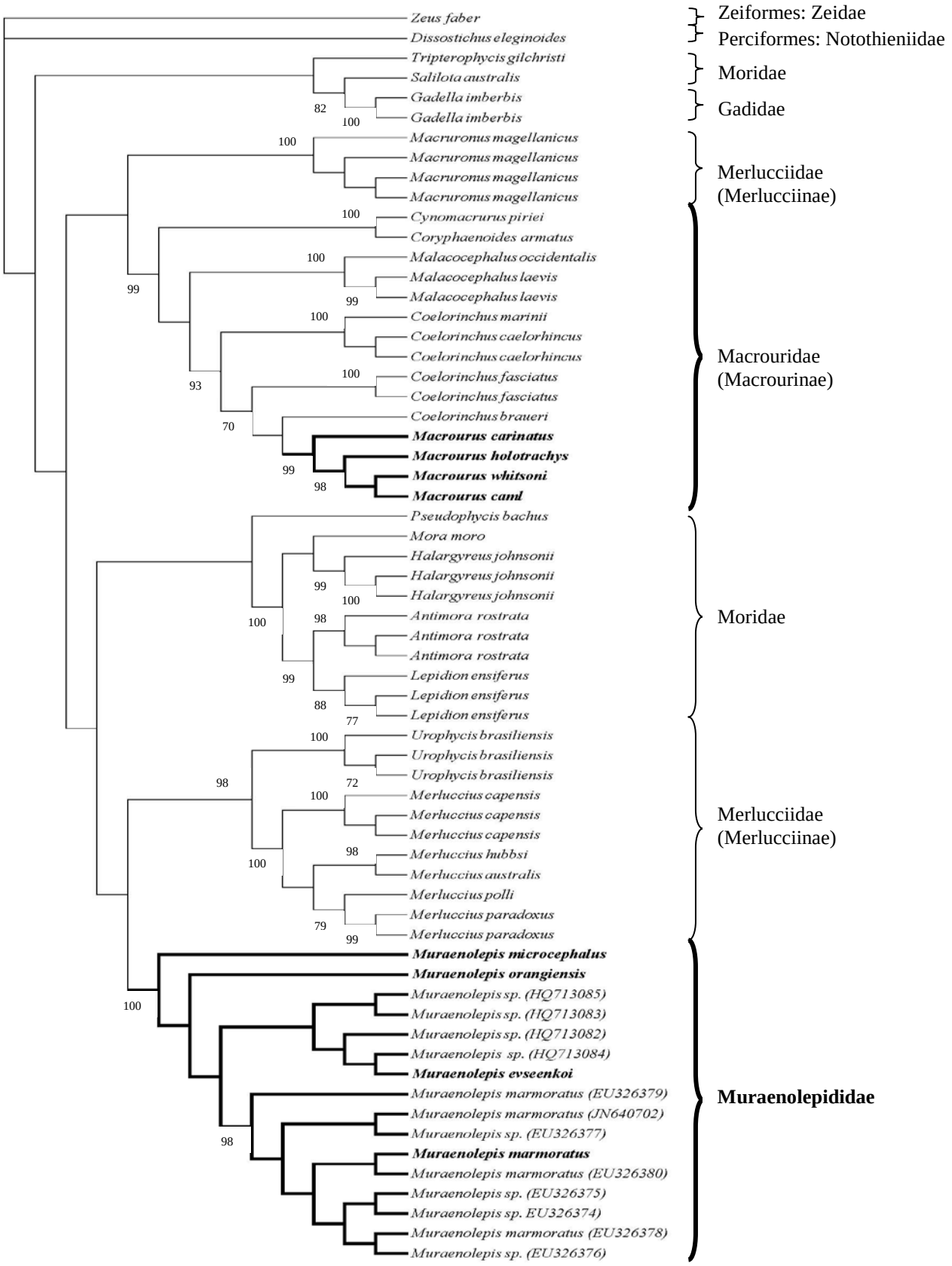

Zeus faber
Dissostichus eleginoides
Tripterophycis gilchristi
Salilota australis
Gadella imberbis
Gadella imberbis
Macruronus magellanicus
Macruronus magellanicus
Macruronus magellanicus
Macruronus magellanicus
Cynomacrurus piriei
Coryphaenoides armatus
Malacocephalus occidentalis
Malacocephalus laevis
Malacocephalus laevis
Coelorinchus marinii
Coelorinchus caelorhincus
Coelorinchus caelorhincus
Coelorinchus fasciatus
Coelorinchus fasciatus
Coelorinchus braueri
Macrourus carinatus
Macrourus holotrachys
Macrourus whitsoni
Macrourus caml
Pseudophycis bachus
Mora moro
Halargyreus johnsonii
Halargyreus johnsonii
Halargyreus johnsonii
Antimora rostrata
Antimora rostrata
Antimora rostrata
Lepidion ensiferus
Lepidion ensiferus
Lepidion ensiferus
Urophycis brasiliensis
Urophycis brasiliensis
Urophycis brasiliensis
Merluccius capensis
Merluccius capensis
Merluccius capensis
Merluccius hubbsi
Merluccius australis
Merluccius polli
Merluccius paradoxus
Merluccius paradoxus
Muraenolepis microcephalus
Muraenolepis orangiensis
Muraenolepis sp. (HQ713085)
Muraenolepis sp. (HQ713083)
Muraenolepis sp. (HQ713082)
Muraenolepis sp. (HQ713084)
Muraenolepis evseenkoi
Muraenolepis marmoratus (EU326379)
Muraenolepis marmoratus (JN640702)
Muraenolepis sp. (EU326377)
Muraenolepis marmoratus
Muraenolepis marmoratus (EU326380)
Muraenolepis sp. (EU326375)
Muraenolepis sp. EU326374)
Muraenolepis marmoratus (EU326378)
Muraenolepis sp. (EU326376)
Zeiformes: Zeidae
Perciformes: Notothieniidae
Moridae
Gadidae
Merlucciidae (Merlucciinae)
Macrouridae (Macrourinae)
Moridae
Merlucciidae (Merlucciinae)
Muraenolepididae

Figure 5-4 Cladogram showing the relationship between gadiform families as inferred by COI data, with posterior probability values from PHASE. Data from this study are highlighted in bold and have been allocated the full species name, or to genus where species resolution was not possible. For *Muraenolepis* Genbank data, the accession numbers are given in parenthesis.

The difficulty in correctly identifying the species from morphology is evident from the sample data downloaded from Genbank. Samples identified and submitted to Genbank as *Mu. microps*, *Mu. marmoratus* and *M. orangiesis* were shown to be genetically identical using 16S sequence data.

5.4 Discussion

Muraenolepis

The results of this analysis using two mitochondrial genes placed the four *Muraenolepis* species as a monophyletic group within Gadiformes, rather than a sister group or ancient branch (Figure 5-1 to Figure 5-4). Both the 16S and COI gene regions infer that the closest family is Merlucciidae, with Moridae also being shown as more closely related than the other gadiform families. The longer branch length for this family with shorter branches between species would suggest an early separation of the family from the other Gadiformes followed by more recent speciation events. Combined with the evidence of Gadiformes in the Antarctic fauna fossil record (Eastman and Grande, 1991), this lends support to the hypothesis that *Muraenolepis* may have been an early inhabitant in Antarctic waters.

Within the Muraenolepididae, these tree topologies (Figure 5-1 and Figure 5-3) would suggest that if the Family Muraenolepididae could be separated into two genera that would comprise *Mu. orangiensis* and *Mu. microcephalus* as one genus and *Mu. marmoratus* and *Mu. evseenkoi* as the other. This would coincide with general body shape (elongate or short) and number of vertebrae and rays in the dorsal and anal fins, which would separate the groups into "few-rayed" moray cods (*Mu. marmoratus, Mu. kuderskii, Mu. trunovi and Mu. evseenkoi*) and "multi-rayed" moray cods (*Mu. orangiensis, Mu. microcephalus, Mu. andriashevi, Mu. pacifica*) (Balushkin and Prirodina, 2010b).

It has been proposed that the "multi-rayed" cods originate from the Pacific sector, west of the Antarctic Peninsula, with *Mu. microcephalus* (Notomuraenobathys) as the basal group, and the "few-rayed" cods evolved in the Atlantic sector, east of the Antarctic Peninsula, after isolation of the two oceanographic regions by the formation of the ACC (Howes, 1990, Balushkin and Prirodina, 2013). Unfortunately, due to the absence of morphological data for the two species tentatively identified as *Mu. microcephalus* and *Mu. orangiensis*, the data from this study cannot provided any further clarification on the origins of these species.

Data for the additional described species and further work with nuclear genes and the more variable regions of the mitochondrial genome is required to fully resolve the phylogenetic relationships and clarify the origins of this family.

Macrourus

The low level of genetic variation for both 16S and COI suggests that this is a recently evolved genus. This is supported by comparative data available for other gadiform fish from the same geographic regions, which, as expected, place the *Macrourus* as a

monophyletic group within the family Macrouridae, sub-family Macrourinae. Both 16S and COI place *Macrourus* as a recently evolved sister genus to *Coelorinchus*, within the *Coelorinchus* clade (Figure 5-1 to Figure 5-4). Analysis of gadiform taxa using concatenated mitochondrial and RAG 1 genes also places *Ma. carinatus* and *Ma. berglax* within the Coelorinchus clade, suggesting these morphologically similar species are very closely related (Roa-Varón and Orti, 2009).

Interestingly, by analysing this larger data set of Gadiformes, the topology of the *Macrourus* clade was altered, with the second branch separating *Ma. holotrachys* from *Ma. whitsoni* and *Ma. caml* (Figure 5-3). These branches were better supported than the topology achieved with just the COI data set, possibly as a result of higher variability between genera and species across the Order providing higher weight to statistical analysis. This also concurred with phylogenetic results using a 650bp region of the COI gene (Smith et al., 2011). The separation of *Ma. carinatus* and *Ma. holotrachys* from *Ma. whitsoni* and *Ma. caml* is also supported by the differing morphology of the species, with *Ma. whitsoni* and *Ma. caml* morphologically very similar and having overlapping geographic distributions.

The bipolarity of the genus, with an absence of genetic variation between *Ma. holotrachys* and *Ma. berglax*, and greatest variation between the subAntarctic species in the phylogenetic trees, supports the theory of origin in South Atlantic waters followed by radiation into the North Atlantic and recent divergence in Antarctic waters (Eastman, 1993c, Kriwet and Hecht, 2008).

Evolutionary studies for other Macrourinae species also suggest a recent evolution of this family. For *Coryphaenoides* species, the radiation time between abyssal and non-abyssal species has been estimated at 3.2-7.6 mya, between the Miocene and Pliocene

(Morita, 1999). Within the Southern Ocean, the coastal nototheniod subfamily Trematominae, species divergence has occurred over the last 10 my, when Antarctic icesheets were re-established, with the main divergence dated ay 3.4 mya (based on mitochondrial DNA mutation rates) (Lautrédou et al., 2012). Species divergence linked with glaciation events can also be shown for other Antarctic fauna, such as the limpet *Nacella concinna* (Gonzalez-Weaver et al., 2013), and octopus *Pareldone turnqueti* (Strugnell et al., 2012), where availability of refugia during the during the Pliocene and Pleistocene glacial maxima played a crucial role in species radiation. Comparison of phylogeographic patterns for a number species, both vertebrate and invertebrate, combined with an understanding of the geographic and climatic history of the environments, evolutionary origins and life histories will provide a greater insight into the forces which drive speciation and adaptation to climate change.

CHAPTER 6

Macrourus microsatellite markers

6.1 Introduction

The grenadier genus *Macrourus* (Family: Macrouridae) was thought to comprise four species; one in the North Atlantic (*Ma. berglax*) and three in the South Atlantic and Southern Ocean (*Ma. carinatus, Ma. holotrachys and Ma. whitsoni*). This research, carried out towards this Ph.D. (Chapter 4), and confirmed by other groups (Smith et al., 2011, McMillan et al., 2012), has highlighted a new species, *Ma. caml*, previously identified as *Ma. whitsoni*, which is present in the Southern Ocean. These two species were found to be genetically distinct for both 16S and COI mitochondrial gene regions. Re-examination of the morphology has also identified that, although similar, there are clear taxonomic differences between the two species.

This research on the genetics of *Ma. berglax* and *Ma. holotrachys* has also shown that they are identical for both the 16S and COI gene regions. These two species have been regarded as distinct species based on their geographic location. *Ma. holotrachys* is found around the sub Antarctic islands of the Scotia Arc and along the continental shelf of southern Argentina (Iwamoto, 1990). By contrast, *Ma. berglax* is found across the North Atlantic, from the continental shelf of Canada to Norway and Greenland (Cohen, 1990). The mitochondrial genes usually found to be sufficient to determine species identity and phylogenetics in fish (Ward et al., 2009) are, in this case, not sufficiently variable to provide information regarding population structuring within the Southern Ocean or a definitive answer to whether or not *Ma. berglax* and *Ma. holotrachys* are the same species. This is a problem that is gradually being highlighted on other taxa (Dettai

et al., 2011, Van de Putte et al., 2009). Further work using more variable markers is required to resolve these issues.

Microsatellite markers were selected for use because of their highly variable nature with a non-coding, neutral mode of inheritance. They are found in nuclear DNA and are particularly abundant in teleost fish, having a higher level of genetic diversity than other loci. (Carvalho, 1995, Ferguson et al., 1995, Frankham, 2002, O'Reilly and Wright, 1995). Regions suitable for use as microsatellite markers have to be isolated for each new species and complementary primers designed. Markers can be used successfully across closely related species (Carvalho, 1998, Gaffney, 2000).

Initially the intention for this study was to isolate potential microsatellites for each of the three Southern Ocean species that have been described in the chapters above (*Ma. carinatus, Ma. holotrachys and Ma. whitsoni*) and develop a suite of markers that could be applied to all three species. This work is detailed in Appendix I. The discovery that *Ma. berglax* and *Ma. holotrachys* were genetically identical for both mitochondrial regions used led to a change of approach. Microsatellite markers had been isolated and designed for *Ma. berglax* as part of an ongoing population genetics study of *Ma. berglax* across the North Atlantic (Helyar, 2010). These markers had been successfully cross-amplified on eight other macrourid species found in the North Atlantic and so were expected to be successful for all the Southern Ocean species. Rather than 're-inventing the wheel' these were applied in preference to continuing the microsatellite development work. This also provided an opportunity to produce a data set for a bi-polar population genetics study (ouside of the scope of thos Ph.D.), comparing the data for the Southern Ocean species with *Ma. berglax* in the North Atlantic. This study is only concerned with testing the suitability of these primers on the four *Macrourus* species found along the Scotia Arc and Falkland Islands.

6.2 Methods

The DNA used for this project had previously been extracted for species identification and phylogenetic analysis (detailed in Chapter 2) and diluted to a standard concentration of 20ng/µl. All samples had been screened by COI sequencing or HRM analysis to verify the species identity (details available in Table 6-1).

			Species identified by COI sequence			
Location	**Year**	**Collection**	***Ma. carinatus***	***Ma. holotrachys***	***Ma. whitsoni***	***Ma. caml***
South Georgia	2002	Dorada	5	1		1
South Georgia	2003	Dorada	26	30		41
South Georgia	2003	Commercial*		54		7
South Georgia	2003	Viking Bay		62		2
South Georgia	2003	Dorada	4			4
South Georgia	2003	Cisne Verde		55		12
South Georgia	2003	Isla Alegranza		10		
South Georgia	2003	Isla Alegranza				15
South Georgia	2003	Isla Alegranza	8	1		1
South Georgia	2009	San Aspiring				29
South Sandwich Islands	2009	San Aspiring			17	33
South Sandwich Islands	2010	Argos Froyannes				15
Ross Sea	2006	Argos Georgia			4	84
Falkland Islands	2010	ZDLTI	35	1		
TOTAL			**78**	**214**	**21**	**247**

Table 6-1 Sample collections used for testing of microsatellite primers and species identified by COI sequence or HRM analysis.

Eight of the published microsatellite markers were found to be polymorphic for *Ma. berglax* and were selected for testing compatibility with the Southern Ocean species (Table 6-2).

Locus name	Repeat Motif	Number of alleles	Range (bp)	Primer Name
Mbe01	$(TG)_{18}$	6	325-337	B12a
Mbe02	$(CA)_{15}$	9	111-135	C04
Mbe03	$(TG)_{34}$	10	218-242	C08
Mbe04	$(ATGG)_{7+}$	4	175-197	D01
Mbe05	$(GT)_{55}$	9	427-443	E09
Mbe06	$(GA)_{19}$	4	138-162	E10a
Mbe08	$(GT)_{18}$	8	408-434	G12a
Mbe10	$(GGT)_9(GGA)_2(GGT)_{13}$	11	475-507	H11

Table 6-2 Microsatellite markers developed for *Ma. berglax*.

The microsatellite markers were initially tested individually for compatibility with the Southern Ocean species using conventional PCR and shown to have a high success rate for all four species. Three samples of *Ma. berglax*, previously scored (Helyar, 2010), were included as positive controls.

To obtain data for all of the microsatellite primers sets, three reactions were required; two multiplex reactions and a single reaction. Each of these had been optimised for the combination of primer concentrations and annealing temperatures. The forward primer of each primer pair was fluorescently labelled (Applied Biosystems, UK) and primers were diluted to a concentration of 10nmol before use. Primer sequences are labelled as given in (Helyar, 2010), which also contains the full details of the primer sequences.

Multiplex 1: 57˚C annealing temperature.

0.1µl each of E10A F & E10A R (NED labelled)

0.3µl each of C04 F & C04 R (VIC labelled)

0.2µl each of C08 F & C08 R (NED labelled)

0.2µl each of D01 F & D01 R (PET labelled)

Multiplex 2: 60˚C annealing temperature.

0.2µl each of B12A F & B12A R (6FAM labelled)

0.15µl each of G12a F & G12a R (NED labelled)

0.2µl each of H11 F & H11 R (VIC labelled)

Single-plex: 60˚C annealing temperature.

0.4µl each of E09 F & E09 R (6FAM labelled)

The conditions for all three reactions were 5µl of buffer with primer volumes as above, and made up to 10µl total volume with deionised H_2O. Reactions were carried out on a DNA Engine2 (Genetic Research Instrumentation Ltd, UK) with a ramp speed of 2˚C per second and the thermal profile of 95˚C for 15 minutes followed by 39 cycles of 95˚C for 45 seconds, 57˚C or 60˚C for 1 minute and 72˚C for 45 seconds, then a final extension step of 72˚C for 45 minutes. The PCR product was sent to the sequencing facility at the University of Oxford, either Liz500 or Liz600 (Applied Biosystems, UK) size standards were added and the product run on an ABI 3130 sequencer.

The microsatellite data obtained was screened using Peak Scanner Software Version 1.0 (Applied Biosystems, UK). The size ranges obtained for each locus exceeded the range

expected for *Macrourus berglax* and quality varied between both species and loci so automatic sizing was not possible. Therefore the data were scored by eye to determine the optimal scoring for each locus and each species. The data were partitioned by species and checked for scoring errors resulting from stuttering and problems such as null alleles and short allele dominance using MicroChecker (Van Oosterhout et al., 2004).

6.3 Results

The size ranges obtained for each locus exceeded the range expected for *Macrourus berglax* and stutter peaks were a particular problem for loci Mbe02, Mbe03 and Mbe06. There was also an issue with excess fluorescence in Multiplex2 for loci Mbe10, compensated for by diluting the reactions, which potentially resulted in drop out of weaker alleles for other loci in the multiplex.

Markers were successfully amplified for all species and were polymorphic, with the exception of loci Mbe04 for *Ma. carinatus* (Table 6-3).

	Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
Ma. carinatus	P	P	P	M	P	P	P	P
Ma. holotrachys	P	P	P	P	P	P	P	P
Ma. caml	P	P	P	P	P	P	P	P
Ma. whitsoni	P	P	P	P	P	P	P	P

Table 6-3 Amplification of *Ma. berglax* primers across other *Macrourus* species, P=polymorphic, M=monomorphic.

The size ranges obtained for each loci were compared to the expected result from the *Ma. berglax* data (Table 6-4). Additional scoring problems were caused by overlapping ranges of alleles in the multiplex reaction and weak alleles being masked by baseline pull-up from stronger adjacent alleles.

Ma. berglax (*n*=48)

Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
6	9	10	4	9	4	8	11
325-337	111-135	218-242	175-197	427-443	138-162	408-434	475-507

Ma. carinatus (*n*=77)

Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
5	35	9	1	4	8	6	18
306-322	118-201	244-260	201	434-450	160-178	396-410	428-542

Ma. holotrachys (*n*=145)

Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
8	13	19	3	12	5	10	11
314-336	114-142	220-258	193-201	412-450	150-164	410-436	456-501

Ma. caml (*n*=146)

Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
16	29	20	4	10	7	11	26
314-370	120-180	288-270	173-201	400-428	160-174	398-436	423-528

Ma. whitsoni (*n*=17)

Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
4	9	7	3	4	4	6	6
324-352	126-158	234-248	185-201	404-432	138-164	418-438	468-492

Table 6-4 Table showing the number of alleles and range of sizes (bp) obtained for each loci and each species (*n*= total number of individuals). *Ma. berglax* data from literature (Helyar, 2010).

Analysis of the data in MicroChecker found no evidence for large allele drop out in any of the data sets. For *Ma. carinatus*, loci Mbe01, Mbe03 and Mbe06 showed evidence of possible mis-scoring caused by to stutter peaks, due to a lower than expected number of heterozygotes with a single repeat unit separation between alleles. A similar problem was evident for *Ma. holotrachys* with loci Mbe05 and Mbe06. There was no evidence of scoring errors because of stutter peaks within the *Ma. caml* and *Ma. whitsoni* data sets.

The data sets were also analysed for the level of homozygosity for each loci to check for the likelihood of null alleles. An excess of homozygotes was evident for *Ma. carinatus* loci Mbe01, Mbe02, Mbe03 and Mbe06, *Ma. holotrachys* loci Mbe03, Mbe05, Mbe06 and Mbe10, and *Ma. caml* loci Mbe02, Mbe03 and Mbe04, which suggested null alleles may be a problem for these loci. The only evidence of homozygote excess for *Ma. whitsoni* was for Mbe05. The expected and observed values of homozygosity are shown in Table 6-5. There was no evidence for significant deviations from Harvey-Weinberg equilibrium for any of the data sets.

		Mbe01	Mbe02	Mbe03	Mbe04	Mbe05	Mbe06	Mbe08	Mbe10
Ma. carinatus									
	n	**46**	**69**	**76**	76	72	**76**	49	49
	He	**0.505**	**0.043**	**0.206**	1	0.349	**0.243**	0.316	0.048
	Ho	**0.695**	**0.174**	**0.355**	1	0.372	**0.315**	0.347	0.102
Ma. holotrachys									
	n	104	135	**134**	135	**130**	**135**	103	**99**
	He	0.235	0.268	**0.138**	0.544	**0.187**	**0.511**	0.177	**0.129**
	Ho	0.24	0.296	**0.201**	0.57	**0.379**	**0.74**	0.194	**0.212**
Ma. caml									
	n	83	**142**	**144**	**141**	106	144	84	82
	He	0.18	**0.05**	**0.072**	**0.773**	0.146	0.634	0.634	0.048
	Ho	0.253	**0.127**	**0.139**	**0.851**	0.151	0.673	0.643	0.085
Ma. whitsoni									
	n	14	15	15	15	**15**	15	14	14
	He	0.173	0.075	0.113	0.873	**0.4**	0.813	0.135	0.109
	Ho	0.071	0.067	0.067	0.867	**0.6**	0.8	0	0.071

Table 6-5 Expected homozygosity (*He*) and observed homozygosity (*Ho*) for each loci (*n*=number of individuals). Loci where *Ho* significantly exceeded *He* are highlighted in bold.

Null alleles were confirmed for loci Mbe10 in one of the populations analysed for *Ma. berglax* (Helyar, 2010). These data suggest a similar problem with that locus for *Ma. holotrachys* but not the other three species. Investigation of null alleles for Mb05 found a possible second allele within the 534bp to 558bp ranges. This is outside the maximum

expected range of 450bp for this locus and requires further investigation to establish if it is a true allele. For Mbe02, preferential amplification of the first peak occurred, particularly for *Ma. carinatus* samples, and the second peak was difficult to distinguish from raised baseline. This was further compounded by an overlap in range with Mbe03 and raised baseline due to pull-up from Mbe03 fluorescence. Preferential amplification of the first allele was also shown in loci Mbe03 and Mbe05.

Although the microsatellites were successfully amplified for all samples, there was varying success in the number of loci amplified and the quality of results. For samples that had been problematic to sequence due to degraded DNA, there was a higher failure rate for loci and a greater problem with stutter peaks that for high quality DNA samples.

6.4 Discussion

The microsatellite markers tested in this study have previously been applied to samples from eight other genera belonging to the family Macrouridae, found in the North Atlantic, where they were found to produce at least one polymorphic locus for each species. However, no maker was successful for all species and many loci were monomorphic (Helyar, 2010). This study obtained a considerably higher success rate for the closely related Southern Ocean species from the same genus as *Ma. berglax*, with all loci being successfully amplified and all polymorphic, with the exception of Mbe04 for *Ma. carinatus*.

However, despite successful amplification, there were a number of issues with the data obtained. Poor peak morphology and multiple stutter peaks with di-nucleotide repeats caused by slippage during PCR (Shinde et al., 2003) made scoring difficult, particularly for homozygous alleles. Preferential amplification of one allele, extended size ranges than those expected from *Ma. berglax* results, and overlapping size ranges resulting in

difficulty identifying both alleles and created a potential problem with null alleles or short allele dominance (Wattier et al., 1998, Van Oosterhout et al., 2004).

Any analysis of genetic variation relies on accurate, representative data which can be reliably scored (Queller et al., 1993, Selkoe and Toonen, 2006). Microsatellites have been commonly used in population genetic studies for fish species, with the high levels of polymorphic alleles making them ideal markers (O'Reilly et al., 1996, DeLeon et al., 1997, O'Reilly and Wright, 1995, Rico et al., 1996). The high level of variability is able to resolve population structure where populations are large and there thought to be few barriers to gene flow, such as the marine environment (O'Reilly et al., 2004).

One of the main problems with the use of microsatellites is the requirement to isolate markers for the target species. Conserved flanking regions can allow them to be used successfully across closely related species or taxa (Moore et al., 1991, Jarne and Lagoda, 1996). However, they should be used with caution as null alleles are a particular concern when amplifying across taxa and can bias further analysis (Roques et al., 1999, Shaw et al., 1999).

The high incidence of null alleles for this data set can generally be attributed to the poor data quality and difficulties with accurate scoring. Null alleles were confirmed for Mbe10 in one population of *Ma. berglax* (Helyar, 2010) and for Mbe02 in this data set. It is also possible that the homozygote excess and any deviation from Hardy-Weinberg equilibrium may result from subpopulation structure (the Wahlund effect) (O'Reilly and Wright, 1995). This may be the case for locus Mbe04 in *Ma. caml* as there is no evidence for excess homozygosity for any of the other species and this locus is a tetranucleotide repeat free of artefacts such as stutter bands. There was no evidence for preferential amplification of one allele in *Ma. caml* or any of the other species for locus

Mbe04. By partitioning the data set by geographic location, the excess homozygosity was revealed to be restricted to the South Georgia populations. This was not attributable to depth or temporal structuring and may be an effect of finer scale population structure around South Georgia. Though unfortunately, the sample numbers for each catch location were insufficient to investigate this further.

Some of the issues can be attributed to the larger sample numbers and variable DNA quality increasing the likelihood of erroneous results. For example, the individuals from the Ross Sea 2006 collection that had failed to sequence successfully for COI did produce scorable microsatellites. However, the data were of poor quality and many loci were deselected for morphology or low peak height. Also, the increase in number of possible alleles within a locus (particularly for *Ma. caml*) may make some markers sub-optimal for future population genetic analyses where population size is limited by sample availability (O'Reilly and Wright, 1995, Selkoe and Toonen, 2006, Carvalho and Hauser, 1994).

Further optimisation of the reactions to reduce the incidence of stutter bands and preferential amplification of alleles is required to produce a data set which is robust enough for reliable population genetic analysis. The microsatellites isolated for the Southern Ocean species (Appendix I) can also be revisited and development continued if the *Ma. berglax* markers are cannot be optimised. Data can then be used to provide insights into population structuring and other valuable information such as migration rates, evolutionary history of the genus, species and populations, population dynamics and dispersal potential. Of particular interest will be the comparison of the Antarctic data set with the North Atlantic data set (a collaborative project, outside this research) as this should reveal divergence times and any present day connectivity between the two geographic regions. This information can then add to the increasing knowledge on

evolution and population structuring of Antarctic fish – a valuable tool for fisheries and conservation management.

CHAPTER 7

Discussion and conclusions

Species Identification

This study has demonstrated the value of comparing of morphological data and molecular analysis for species identification. For *Muraenolepis*, the data from this combined approach has been able to resolve conflicts over the identity and distribution of the species described to date. It supports the presence of *Mu. marmoratus* at both South Georgia and Kerguelen Islands, and the absence of *Mu. microps* in both of these regions base on both morphological and genetic data. The species detected in the Ross Sea based on COI sequence data was matched to the recently described species, *Mu. evseenkoi* (Balushkin and Prirodina, 2010b), using the morphological data recorded. Similarly, the *Macrourus* molecular data has allowed a new species, *Ma. caml*, to be identified from South Georgia and the Ross Sea, which has subsequently been fully described in terms of morphology (McMillan et al., 2012).

In both of these cases, the genetic confirmation of species identification has allowed the range of measurements for morphological characters to be redefined and the morphological keys used for species identification to be refined and adopted by fisheries observers. Correct morphological identification is essential for the validity of any further study, be it ecological, biogeographical or genetic, as this is the main method of identification on fisheries surveys and underpins all fisheries quota calculations and conservation measures. New molecular techniques, such as HRM analysis (Fitzcharles, 2012), are providing simplified methods of genetic screening to confirm species identification. Whilst these approaches can ensure accurate

identification, they will not replace the more traditional methods of identification by morphological features, particularly in field and ship-borne research where genetic methods are not readily available, but could easily be implemented in basic fisheries laboratories in the more remote stations.

Caution should always be taken however in interpreting the molecular results as stand-alone data, which is where the combined approach with traditional taxonomy (integrative taxonomy) has its value (Will et al., 2005, Dayrat, 2005, Riedel et al., 2013, DeSalle et al., 2005). The COI gene region, has become regarded as the barcoding gene for species identification (Dettai et al., 2011, Grant et al., 2011) and has been used extensively in campaigns such as FISH-BOL (Ward et al., 2005, Ward et al., 2009).

The discovery that *Ma. holotrachys* and *Ma. berglax* were genetically identical for the COI gene region has led to the speculation that they may be the same species with a bi-polar distribution. Bipolarity has been recorded for a number of taxa (Kuklinski and Barnes, 2010, Darling et al., 2000), including grenadier, or rattail, species such as *Coryphaenoides armatus*, *Antimora rostrata* (Cohen, 1990), and *Merluccius australis* (Arkhipkin et al., 2010), and a record of the Patagonina toothfish, *Disosstichus eleginoides*, off the coast of Greenland (Moller et al., 2003). This has raised questions regarding the connectivity, both past and present, of the World's oceans.

However, in the case of *Ma. holotrachys* and *Ma. berglax* there is additional anecdotal evidence to suggest that in spite of identical COI genes, these are still two separate species (McMillan et al., 2012). Indeed, the COI region has been found to be unable to discriminate between some morphologically similar fish species, such as two species pairs of *Trematomus* species (Dettai et al., 2011, Van de Putte et al., 2009) and four species of the North Atlantic redfish, *Sebastes* (Bentzen et al., 1998). The differences in

morphology between *Ma. holotrachys* and *Ma. berglax*, combined with the low level of intraspecific variation in the COI region for all *Macrourus* species, supports the theory that these are recently evolved species which cannot be resolved by the COI gene rather than being the same species and therefore more work is required to identify unique molecular markers for conservation identification in these species.

On a more academic level, this still raises the questions regarding gene flow and the timing of speciation events. Divergence times can be estimated using the molecular clock, where assumptions are made that the rate of amino acid substitutions are constant and accumulation of mutations is proportional to time (Thorpe, 1982). However, studies of Antarctic fish are limited. The only fossil record available for Antarctic fish for calibration of the molecular clock is for the notothenioid *Proeleginops grandeastmanorum*, dated at 40 million years ago, which dates the clade of antifreeze glycoprotein bearing Nototheniodes at 24.1+/-0.5 Ma, at the time of the formation of the Antarctic Circumpolar Current and prior to the establishment of polar conditions (Near, 2004). The most recent common ancestor of the two *Dissostichus* species has been estimated at 14.5+/-0.5 Ma (Near, 2004). However, phylogenetic analysis of the Macrouridae genus, *Coryphaenoides*, has suggested a much more recent radiation time between the abyssal and non-abyssal species at 3.2 to 7.6 Ma, with the abyssal species as the basal group (Morita, 1999) and indicates that this species re-colonised the Antarctic after the opening of the Drake Passage and the formation of the ACC. For the limpet, *Naccella concinna*, it has been proposed that recolonisation of maritime Antarctic has occurred from refugia at South Georgia following the Last Glacial maxima (Gonzalez-Weaver et al., 2013).

Evolution and Biogeography

There are a number of factors that influence the evolution and distribution of species. The availability of suitable habitats for colonisation, changes in environmental conditions, availability of prey species, life history and reproductive strategies, as well as dispersal mechanisms and the dynamics of the environment all play a part. Understanding how species have evolved and the population dynamics requires consideration of all of these factors.

Both the genera considered in this study are bathypelagic deep sea fish with geographic species ranges that include the temperate regions off South America and New Zealand, the sub Antarctic islands and the seas off the Antarctic continental shelf. Both demonstrate the separation of species by geographic location as well as depth and useful insights into the evolution and distribution of Antarctic fish can be gained by examining the similarities and differences between these two genera and other Antarctic fauna.

The low level of intraspecific genetic variation for both genera would suggest that there have been relatively recent speciation events. Low levels of variation have also been found for other Antarctic fish, including the Antarctic toothfish, *Dissostichus mawsoni* (Smith and Gaffney, 2005). The phylogenetic analysis infers that *Macrourus*, as a genus, has recently evolved within the family Macrouridae, where as the genus *Muraenolepis* (family Muraenolepididae) has an earlier separation from the other gadiform families, with the subsequent evolution of the four species identified in this study. These inferred relationships should be treated with caution as they are both from mitochondrial DNA sequence data and not reflective of the nuclear genome, particularly for *Muraenolepis* where only half of the described species are represented. However, similar patterns in geographic distribution and similar levels of intraspecific and

interspecific genetic variation suggest there may be commonalities between the factors governing the speciation and distribution patterns.

Both genera are slow growing, long lived species with growth rates slowing after maturity, particularly for male fish, leading to the trend of adult females being larger than males. They also demonstrate the deeper-larger trend common among deep sea fish (Coggan et al., 1996). *Muraenolepis* species are thought to reach maturity at 4 years and reach a maximum age of 9 to 12 years (Kompowski and Rojas, 1996, Kompowski, 1993). In contrast, *Macrourus* species are thought to reach maturity at 9 to 15 years (Morley et al., 2004, Pinkerton et al., 2013) and reach a maximum age of 27 to 39 years (Laptikhovsky et al., 2008). While *Muraenolepis* species produce a greater number of eggs, the overall fecundity is reduced by *Muraenolepis* being a semelparous spawner, with differing spawning periods for the different species (Prut'ko and Chimilevskii, 2011). *Macrourus* is a batch spawner with a low oocyte numbers but a prolonged, possibly continuous, spawning period (Alekseev et al., 1992). Both have pelagic eggs and larvae which remain at depth in the water column rather than rising to the surface waters (Merrett and Barnes, 1996).

The species distribution patterns for both genera are very similar. The high Antarctic species have no identifiable geographic structure within the Ross Sea but there is a clear boundary to the distribution along the Scotia Arc at the South Sandwich Islands. This suggests there is a boundary to distribution between the South Sandwich Islands and South Georgia for this true Antarctic species. This species limitation at the South Sandwich Islands has also been shown (Figure 7-1) for *D. mawsoni* and *D. eleginoides*, the Antarctic and Patagonian toothfish, where the Weddell Front divides the two water masses of the Weddell-Scotia Confluence and the Weddell Gyre (Roberts et al., 2011).

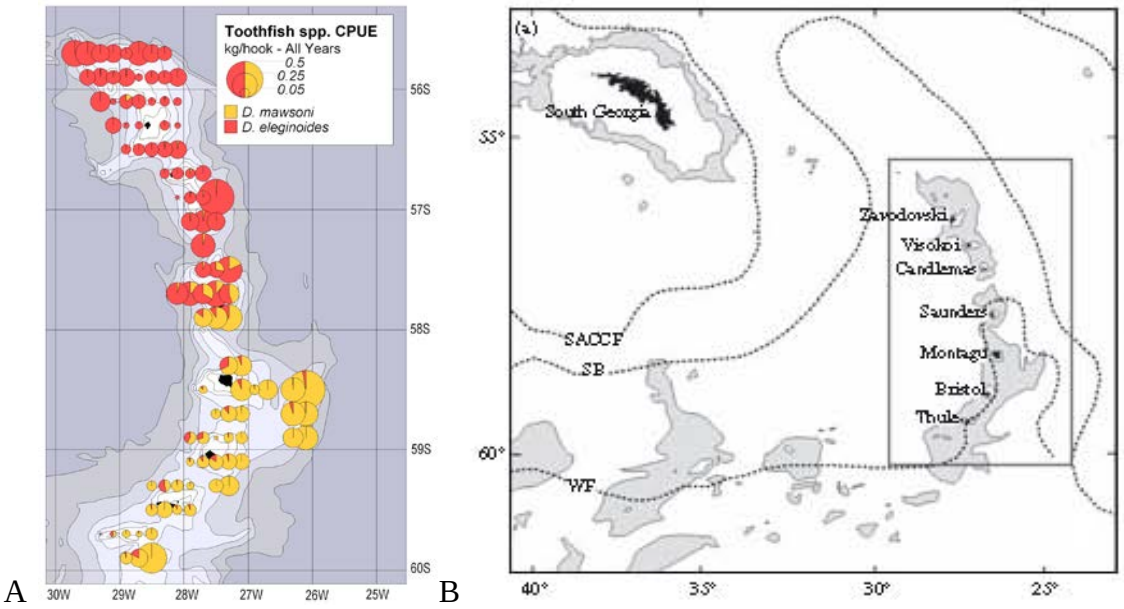


Figure 7-1 (A) Distribution and abundance (catch per unit effort) for *D. mawsoni* and *D. eleginoides* around the South Sandwich Islands, and (B) position of oceanographic fronts (WF-Weddell Front, SB-Southern Boundary of Antarctic Circumpolar Current, SACCF-.Southern Antarctic Circumpolar Current Front) (Roberts et al., 2011).

Oceanographic currents and fronts play a pivotal role in the dispersal of pelagic eggs and larvae. Fronts with a steep hydrological gradient, such as the Sub Antarctic Front act as a barrier to larval dispersal (Koubbi et al., 2009). Gyres and retention zones around islands and coastal currents can limit the dispersal potential, retaining the larvae on the shelf where food sources are more abundant (Koubbi et al., 2009). For some species, oceanographic currents transport the larvae from adult spawning areas to nursery grounds, as can be seen for *Ma. carinatus* on the Falkland's shelf (Laptikhovsky, 2011). Larval distribution of *D. eleginoides* around South Georgia, Shag Rocks and Burdwood Bank provides evidence of juveniles migrating inshore (North, 2002). Oceanographic models capable of the fine scale resolution required for these localised currents can help to understand the mechanisms for dispersal and connectivity

between regions (Young et al., 2011) and can utilise known information live-cycle variables and genetics to predict larval dispersal patterns (Galindo et al., 2010).

Competition for prey is another factor that can influence species ranges. Where the toothfish species overlap, diet studies revealed no difference in trophic niches, with the main prey being fish, the *Macrourus* and *Muraenolepis* species in particular (Roberts et al., 2011). In contrast, where the range overlaps for *Macrourus* species, *Ma. carinatus* was shown to switch to predominantly pelagic prey while for *Ma. holotrachys* the prey items were benthic species, reducing the level of competition between the species and allowing for geographic overlap (Laptikhovsky, 2005). A difference in prey preference has also been indicated where *Ma. caml* and *Ma. whitsoni* distributions overlap in the Ross Sea (McMillan et al., 2012). Diet studies where the three species, *Ma. carinatus*, *Ma. holotrachys* and *Ma. caml* overlap would be a valuable addition to the understanding of the species dynamics, as would a comparison of *Ma. caml* and *Ma. whitsoni* from the South Sandwich Islands where the species ranges appear to be limited.

The level of intraspecific variation obtained from mitochondrial genes sequenced from both genera was insufficient to provide detailed genetic analysis such as population structure. For *Muraenolepis*, haplotype frequencies suggested population structuring between South Georgia and Kerguelen populations, however it was not highly significant due to the low level of variation and small population size for Kerguelen. Further investigation with the more variable control region of the mitochondrial genome or highly variable nuclear microsatellite markers (as isolated for this study, Appendix I) would provide further evidence for any structuring that may be present. There was no evidence for population structure for any of the *Macrourus* species due to the low level of variation in the COI gene region. It is hoped that further optimisation of the

microsatellite markers tested (Chapter 6) or isolated (Appendix I) in this study will provide a means for examining any fine scale population structure and help understand the divergence of these species in the Southern Ocean and provide valuable data for CCAMLR and fisheries quotas.

7.3 Conclusions

The integrative taxonomic approach adopted here to examine both morphological and molecular identification has provided resolution to a number of issues regarding confusions over the correct identification of both *Muraenolepis* and *Macrourus* species.

- *Muraenolepis marmoratus* has been identified and is present at Kerguelen Island and South Georgia, as well as Burdwood Bank.
- *Muraenolepis microps* is a synonym of *Mu. marmoratus* and confusion over its presence at South Georgia has resulted from subsequent literature describing a different species.
- The species mistakenly identified as *Mu. microps* in the Ross Sea region is now identified as *Mu. evseenkoi*.
- The range for *Mu. marmoratus* and *Mu. evseenkoi* was only found to overlap at the South Sandwich Islands, where it was caught at different depths, suggesting depth distribution patterns.
- *Muraenolepis marmoratus* showed some evidence for population structuring between South Georgia and Kerguelen Islands.
- Two other species were identified from the molecular markers: *Mu. orangiensis*, with a temperate distribution from Burdwood Bank and New Zealand; and a fourth species tentatively assigned as *Mu. microcephalus*, from the Ross Sea.

- *Macrourus whitsoni* was discovered to be two separate species differing in both molecular and morphological identification. These have now been identified as *Ma. whitsoni* and *Ma. caml*.
- *Macrourus holotrachys* (Southern Ocean) and *Ma. berglax* (North Atlantic) are genetically identical for both 16S and COI mitochondrial gene regions. Morphological characteristics do not support the hypothesis that they are the same species.
- Both *Muraenolepis* and *Macrourus* species show similar species distribution patterns, which concur with the distribution patterns for toothfish species, *Dissostichus eleginoides* and *D. mawsoni*, This suggests commonalities in factors, such as bathymetry and oceanography, governing the geographic distribution of these species.
- Oceanographic fronts as barriers to dispersal, availability of shelf areas as nursing grounds and oceanographic currents as modes of larval transport are all possible influencing factors.

Further work is required for both genera to fully resolve the issues under investigation here. It is hoped that continuation of the microsatellite work for *Macrourus* will provide clarification of *Ma. holotrachys* and *Ma. berglax* species question. The microsatellite markers are also expected to provide insight into the process of speciation and population dynamics for all *Macrourus* species.

Further sampling for *Muraenolepis* is required, particularly to target the geographic regions for the species that were not included in this study. It is hoped that this will be achieved in collaboration with CCAMLR and NIWA. If sufficient samples can

be collected, there is the option of continuing the work on microsatellite development for these species.

For both genera, additional investigation on their life-cycle, such as identifying spawning and nursery grounds, establishing the period of pelagic egg dispersal and larval development, and migration patterns of developing adults, both with depth and geography, will help understand the dynamics of these fish within the Antarctic ecosystem. Of particular interest would be the trophic ecology and identifying changes in prey species with seasonal variability and niche overlap of species.

Previous physiological and ecological studies on these species have unfortunately been based on species identification which may be incorrect in light of the new findings, particularly for *Macrourus whitsoni* and *Ma. caml*, and all of the *Muraenolepis* species. Molecular identification can play an essential role to confirm the correct identification at the basis of any study, and quick, easy techniques such as HRM Analysis offer a practical solution.

All of these factors are essential for successful fisheries management and effective conservation management. Understanding the ecology of species, population dynamics and identifying any environmental common factors between taxa will provide reliable input to any policy decisions such as locations of Marine Protected Areas (MPAs) and allowable catch quotas for both target and by-catch species for fisheries. The biodiversity of the Southern Ocean, as with all of the World's seas and oceans, must be protected and any part scientific research can play in that process is of great importance.

CHAPTER 8

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APPENDIX I

In-house Microsatellite Isolation for *Macrourus* and *Muraenolepis* Species

This appendix describes the in-house isolation of microsatellite sequences from Macrourus and Muraenolepis

Introduction

Microsatellites are tandem repeats of DNA sequence motifs of 1 to 6 base pairs in length, and up to 300bp in total length. They are non-coding and usually have a neutral, co-dominant, Mendelian mode of inheritance making them suitable for use as genetic markers. Mutation is thought to be a result of slipped-strand mispairing or unequal crossover during replication (Carvalho 1995; O'Reilly 1995).

Microsatellites are particularly abundant in teleost fish and they have a higher level of genetic diversity per locus than other DNA markers. Mutation rates are dependent on the size and type of repeat unit. Di-nucleotide repeats are more common and more variable than tetra-nucleotide repeats and longer repeats are more variable (Carvalho 1995; Ferguson, Taggart et al. 1995; Frankham 2002). In general, microsatellites need to be isolated and new primers designed for each species, although they may be used across closely related species (Carvalho 1998; Gaffney 2000).

Methods

The isolation method used was based on the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) protocol developed by (Zane, Bargelloni et al. 2002)). The 250 ng of DNA required for this reaction was obtained by combining DNA

extractions from three individuals for each of the three species, which was then ethanol precipitated and re-suspended in 17 µl deionised water to concentrate the DNA solution.

The resulting FIASCO-generated PCR products were ligated into the pGEM-T Easy cloning vector using T_4 DNA ligase (Promega, UK) following manufacturer's instructions. This ligation mix was then transformed into bacterial cells using Stratagene Gold ultra competent cells, following manufacturer's instructionsand transformed clones selected on TYE agar plates containing ampicillin. Individual clones were picked into 100µl TB media in 96-well microtitre plates. Two plates of clones were isolated for each species.

DNA was transferred from the clones into PCR medium using 69-pin replicators. The selected clones were amplified using limited dilutions of dNTPs and primers with M13 short primers (5'-GTA AAA CGA CGG CCA G-3' and 5'-AAC AGC TAT GAC CAT GAT-3') under the following conditions: 20µl reaction, 0.28 µl dNTPs (2mM each dNTP), 2 µl 10X PCR buffer, 0.6 µl MgCl2 (50mM), 0.25 µl each primer (10mM), 0.01 µl Taq and 16.5 µl dH_2O (all reagents, Bioline, UK). The reaction was carried out on an MJ Research DNA Engine 2 tetrad thermal cycler: 96˚C for 2 minutes, 35 cycles of 96˚C for 20 seconds, 49˚C for 20 seconds and 72˚C for 45 seconds, followed by 72˚C for 5 minutes. PCR product was diluted by adding 30 µl dH_2O and products run on a 1.5% agarose gel to ensure for variability in the size of inserts.

The diluted PCR product was used directly in the sequencing reaction. Sequencing was carried out using M13F long (5'-GTA AAA CGA CGG CCA GTG AAT-3') and M13R long (5'-AAC AGC TAT GAC CAT GAT TAC G-3') primers. Reaction conditions were 6 µl PCR product, 4 µl BigDye terminator mix (Applied Biosystems) and 0.5 µl 10 µM primer. Thermal cycling conditions were 25 cycles of 95˚C for 20 seconds

followed by 60˚C for 2 minutes 20 seconds. Products were cleaned by ethanol precipitation and re-suspended in 10µl MegaBACE loading solution (Amersham Bioscience, GE Healthcare, UK). Sequences were visualised using a MegaBACE 1000 capillary DNA sequencer and Sequence Analyser version 3.0 software (Amersham Bioscience, GE Healthcare, UK). Sequences were checked visually for microsatellites using Chromas LITE version 2.01 (Technelysium Pty Ltd).

Results

For the genus *Macrourus*, 94 potential microsatellites were identified: 21 *M. carinatus*, 46 *M. holotrachys* and 27 for *M. whitsoni*. These were predominantly dinucleotide repeats, both pure and interrupted, and a small number for 3 to 7 base repeat motifs. Of these, 19 for M. carinatus, 38 for *M. holotrachys* and 23 for *M. whitsoni* contained flanking regions suitable for primer design. Primers were designed using Primer3 (Rozen 2000), checked for self-complimentarity using Operon Oligo Analysis Tool (http://www.operon.com/tools/oligo-analysis-tool.aspx) and examined by eye to determine the best primer options. Consideration was given to designing primers with similar annealing temperatures and varying product length to allow for potential multiplex reactions after primer selection. Where possible, multiple primer pairs were designed for a single microsatellite marker to generate products of differing sizes. Details on the microsatellites selected and primers designed are shown in Table 1 (*M. carinatus*), Table 2 (*M. holotrachys*) and Table 3 (*M. whitsoni*).

For the genus *Muraenolepis*, 52 potential microsatellites were identified, and 44 contained flanking regions suitable for primer design. These were also predominately dinucleotide repeats, both pure and interrupted, with a small number of three to seven base motifs. Details on the microsatellites selected and primers designed are shown in Table 4.

It was decided not to continue this work within the scope of this PhD after obtaining the species identification and phylogenetic analysis results. For *Macrourus* species an opportunity became available to collaborate with a research group at University College Dublin who were working on *Macrourus berglax* from the North Atlantic. This work is detailed in Chapter 2 and Chapter 6. For the genus Muraenolepis, further work is required to resolve this family before the microsatellite analysis can be carried out. It is intended to secure grant funding in collaboration with NIWA to increase the samples numbers and geographic range for all possible species before continuing this work.

Table 1: Microsatellites identified and primers designed for *Macrourus carinatus.*

Name	Microsatellite Repeat	Fragment Length	Primer Size (F)	Primer Size (R)	Primer Tm
Mcar01	17CA-G-8CA	161	22	24	61
Mcar02	10CCT	347	20	25	60
	10CCT	207	21	26	60
Mcar03	21GT	144	22	24	61
Mcar04	2GT-C-6TG-G-7GT-GAGATATC-12GT-G-2GT-GAGATATC-11GT	203	22	19	59
Mcar05	11TG-C-12GT	296	23	26	58
	11TG-C-12GT	152	22	24	58
Mcar06	7CA	585	21	21	59
	7CA	520	22	21	61
	7CA	247	22	21	59
Mcar07	5CAA	156	21	18	60
	5CAA	129	23	19	60
	5CAA	228	24	18	60
Mcar08	9GA-TA-9GA	130	20	22	60
	9GA-TA-9GA	210	19	23	59
Mcar09	15GT-CT-9GT	102	20	18	60
	15GT-CT-9GT	187	20	18	61
	15GT-CT-9GT	367	20	23	61
Mcar10	11CA-C-4CA-CGACAGTATATAAA-6CA	No suitable primers found			
Mcar11	3GT-ATAT-5GT-AT-3GT-AGGTTGACTGTTTGTAT-6GT	158	24	24	61
Mcar12	20GT	No suitable primers found			
Mcar13	8GT-CG-4GT	130	20	20	59
Mcar14	9GT-ATAATAT-11GT	159	23	18	58
Mcar15	9CA	177	18	21	62
	9CA	232	18	23	58
	9CA	344	19	23	59
	9CA	394	23	19	59
Mcar16	12AC	177	25	20	60
	12AC	202	25	18	59
	12AC	296	21	20	60
	12AC	338	23	21	61
	12AC	383	23	18	60
Mcar17	10CA-TACATA-7CA	109	26	24	58
	10CA-TACATA-7CA	199	18	20	60
Mcar18	12CA	139	27	19	59
	12CA	113	20	19	60
	12CA	162	18	19	60
Mcar19	8GA	305	19	21	61
Mcar20	8GT	156	27	21	58
Mcar21	41GT	179	21	20	58

Table 2: Microsatellites identified and primers designed for *Macrourus holotrachys.*

Name	Microsatellite Repeat	Fragment Length	Primer Size (F)	Primer Size (R)	Primer Tm
Mhol01	6GT	156	24	21	59
	6GT	272	24	21	59
	6GT	366	20	21	59
Mhol02	9AT	159	22	26	58
Mhol03	18GT-TT-7GT	156	25	19	59
Mhol04	3GA-TA-4GT-GAGACA-4GT-CA-4GA-CA-4GA-TA-3GA-CG-4GA-CAGACA-4GA-TA-4GA-CAGACA-6GA-CA-6GA	470	24	24	60
Mhol05	11GT-A-12TG	135	21	20	60
	11GT-A-12TG	220	19	20	61
Mhol06	15GT-T-3TG-CA-9TG	133	24	25	60
Mhol07	9GT	113	23	18	62
Mhol08	17GT	141	23	18	60
Mhol09	8GT	103	24	20	59
	8GT	182	25	18	61
Mhol10	32GT	190	23	20	60
	32GT	243	23	20	60
	32GT	314	25	19	60
Mhol11	5TAA	No suitable primer found			
Mhol12	4GT-A-24GT-CGTGCACACAC-5GT-GC-2GT-C-5TG	224	24	23	57
Mhol13	9GT-G-6GT	306	23	19	58
	9GT-G-6GT	364	25	18	60
Mhol14	9GT	Too close to vector site for R primer			
Mhol15	30GT	204	23	19	59
Mhol16	7GT-TT-17TG-C-10TG	Too close to vector site for R primer			
Mhol17	5GT-A-45TG	250	24	22	60
	5GT-A-45TG	374	23	22	61
	5GT-A-45TG	495	22	22	60
Mhol18	7CA	116	22	18	61
Mhol19	6CA	102	18	20	63
	6CA	238	23	21	60
	6CA	185	18	22	60
Mhol20	5AGG-5GAA	263	20	20	60
Mhol21	9CT	100	20	18	58
Mhol22	55GT	212	25	22	59
Mhol23	6GT-AT-9GT-AT-6GT-G-10GT	271	22	22	58
	6GT-AT-9GT-AT-6GT-G-10GT	182	22	22	61
Mhol24	10GT	255	21	21	58
Mhol25	48GT-T-9GT	528	25	24	59
Mhol26	6AC	146	23	22	60

	6AC	267	23	22	60
Mhol27	8CT-GA-6GT	157	18	21	61
	8CT-GA-6GT	199	18	20	57
	8CT-GA-6GT	350	23	20	59
	8CT-GA-6GT	394	19	20	60
Mhol28	15AC	112	18	24	59
Mhol29	12CA	117	21	21	58
Mhol30	14GT-AT-4GT	217	20	20	61
Mhol31	13TG	246	23	23	60
	13TG	198	23	19	59
	13TG	143	24	18	58
Mhol32	17CA	208	20	28	59
Mhol33	9CCT	114	21	21	59
Mhol34	7CA	119	24	24	61
Mhol35	12GT	144	22	18	61
Mhol36	9GT	No suitable primer found			
Mhol37	5TAAAA	Too close to vector site for F primer			
Mhol38	13TG	No suitable primer found			
Mhol39	9GT	No suitable primer found			
Mhol40	8CAA	150	24	18	63
Mhol41	33GT-t-9gt	299	22	21	59
Mhol42	10CA	159	21	20	59
Mhol43	6AGC	Too close to vector site for R primer			
Mhol44	23GT	310	23	23	58
Mhol45	19GT	100	20	21	60
	19GT	291	23	22	60
Mhol46	10GT	195	20	22	59

Table 3: Microsatellites identified and primers designed for *Macrourus whitsoni.*

Name	Microsatellite Repeat	Fragment Length	Primer Size (F)	Primer Size (R)	Primer Tm
Mwhit01	6CA	110	20	21	58
Mwhit02	9GT-G-38GT	203	24	18	59
Mwhit03	9CTG	116	20	25	59
Mwhi04	13GT	No suitable primers found			
Mwhit05	6CA	149	24	24	61
Mwhit06	3GT-AT-23GT	Too close to vector site for R primer			
Mwhit07	6GT-A-6GT	180	22	20	58
	6GT-A-6GT	128	24	18	60
Mwhit08	31GT	129	24	24	60
Mwhit09	25GT	190	22	18	61
Mwhit10	12GT-CGTGTTTTATGC-18GT	271	25	22	58
Mwhit11	24GT	150	22	26	58
	24GT	203	21	26	57
Mwhit12	11CA	130	24	22	59
Mwhit13	6TA	Too close to vector site for R primer			
Mwhit14	7GA	109	21	22	60
Mwhit15	7GT	170	24	23	57
	7GT	129	24	24	59
Mwhit16	35AC	323	20	24	59
	35AC	273	20	24	59
Mwhit17	26TG-T-12TG	189	25	18	59
Mwhit18	28GT	Too close to vector site for F primer			
Mwhit19	7GT-GA-6GT	173	24	18	61
Mwhit20	11CA-C-6CA	Too close to vector site for F primer			
Mwhit21	18GT	120	23	22	60
Mwhit22	12AC	112	22	20	58
Mwhit23	8GT-CTA-39GT	214	22	20	59
Mwhit24	6AGG	177	20	22	61
Mwhit25	13GT-CTGTCTGTCGTA-13TG	273	26	21	60
Mwhit26	17TG-G-20TG-T-16TG	195	20	19	59
Mwhit27	7GT-GC-10GT	131	18	26	61
	7GT-GC-10GT	132	18	24	58
	7GT-GC-10GT	209	21	24	58
	7GT-GC-10GT	307	22	24	59

Table 4: Microsatellites identified and primers designed for *Muraenolepis sp.*

Name	Microsatellite Repeat	Fragment Size	Primer Size (F)	Primer Size (R)	Primer Tm
Mur 1	12GA	No suitable primer sites			
Mur 2	11GA-A-12AG-T-4AG-T-18AG	202	21	27	62
Mur 3	5TG-C-7GT	No suitable primer sites			
Mur 4	33CT-T-6CT	160	19	27	61
	33CT-T-6CT	262	21	21	58
Mur 5	49GA	211	22	22	59
Mur 6	22CT-G-CT-GT-3CT-G-CT-GT-10CT-T-2CT-2GT-15CT-CC-9CT-CC-6CT	254	25	20	59
Mur 7	15CA	108	21	20	61
Mur 8	6CA-ACA-25AC	282	22	21	60
Mur 9	6GT-C-13GT	182	21	19	59
Mur 10	5GTT	168	18	22	61
Mur 11	20GT	152	18	23	58
Mur 12	10AC-G-4CA	156	24	21	58
	10AC-G-4CA	226	27	21	58
Mur 13	5TAA	Too close to vector site for reverse primer			
Mur 14	6CA	100	23	22	60
Mur 15	9CA	327	23	20	59
	9CA	171	22	23	61
Mur 16	11GT	No suitable primer sites			
Mur 17	8GT	155	22	20	58
Mur 18	7GT	105	20	23	58
	7GT	176	20	23	59
Mur 19	7GT	109	20	24	62
	7GT	190	19	25	58
Mur 20	11GT	185	23	21	59
	11GT	261	23	21	62
Mur 21	10AG	291	22	21	58
Mur 22	7CA	210	20	20	61
	7CA	154	20	22	61
Mur 23	5TAA	Too close to vector site for reverse primer			
Mur 24	7AC	Same microsatellite as 22			
Mur 25	54GT	401	20	21	60
	54GT	329	20	18	60
Mur 26	7GT-CTTA-8GT	100	23	23	62
Mur 27	46GT-TTGCGTGCGTG-10GT	Too close to vector site for reverse primer			
Mur 28	6CA	229	24	20	60
	6CA	132	24	19	60
Mur 29	7AAC	244	21	20	60
	7AAC	152	21	22	60
Mur 30	6CCA	182	22	19	61
Mur 31	11AC-TC-8AC-G-7AC	301	18	26	58
Mur 32	11GT	135	21	18	60
	11GT	161	18	21	61
Mur 33	8AC-G-2CA-CG-2CA-CG-7CA	No suitable primer sites			
Mur 34	5CTGT	114	20	19	60
Mur 35a	5AAC	169	21	23	58

Mur 35b	11GT	110	24	20	61
Mur 35c	6GGAT	126	18	24	61
Mur 36	6TTG	135	20	19	62
	6TTG	181	23	21	60
Mur 37	7GT-A-19TG-C-3GT-CT-2GT-CT-11GT	296	25	27	58
Mur 38	8AC	226	20	21	60
	8AC	334	18	21	59
Mur 39	25CA	107	20	22	58
	25CA	210	20	23	61
Mur 40a	7GTCCTCT	170	21	22	60
Mur 40b	16GT	157	26	19	61
Mur 41	16CT-G-9CT	176	20	24	61
Mur 42	7TG-C-9GT	311	19	26	58
	7TG-C-9GT	198	19	18	58
Mur 43	6GT	107	21	25	58
	6GT	262	20	28	58
Mur 44	36CT	201	22	18	60
Mur 45	7CA	153	20	21	61
	7CA	209	20	19	61
Mur 46	17CA	113	23	23	61
	17CA	287	24	22	62
Mur 47	5GGT	133	18	20	59
Mur 48a	4TGAGAGA	267	20	21	61
	4TGAGAGA	338	23	23	60
Mur 48b	4GT-TC-12GT-GA-5GT-TT-15GT	149	24	28	61
	4GT-TC-12GT-GA-5GT-TT-15GT	235	26	28	59
	4GT-TC-12GT-GA-5GT-TT-15GT	298	23	28	60
	4GT-TC-12GT-GA-5GT-TT-15GT	347	24	28	60

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APPENDIX II

Accession numbers for sequences from Genbank with outgroups highlighted in bold.

Species Name	Genbank Accession Number	
	16S	**COI**
Antimora rostrata		JF265150; KC015196; EU148075
Bathygadus macrops	FJ215101	
Bathygadus melanobranchus	FJ215102	
Coelorinchus braueri		JF493233; JF493234; JF493235
Coelorinchus caelorhincus		JQ774515; JQ774516
Coelorinchus fasciatus		EU074373; EU074374
Coelorinchus marinii		EU074382; EU074383; EU074384; EU074385
Coryphaenoides subserrulatus		
Coryphaenoides serrulatus	FJ215124; FJ215125	
Coryphaenoides armatus		FJ164497; EU148117
Cynomacrurus piriei		JN640619; HQ712959; JN640888
Dissostichus eleginoides	**AM180545**	**EF609344**
Gadella imberbis		KC015367; KC015368
Halargyreus johnsonii	AY947851	KC015433; JQ354111; GU806155; JF265170; EU869815; FJ164639; EU148182
Hymenocephalus italicus	FJ215147	
Lepidion ensiferus	FJ215152	JX437968; JX437969; JX437970
Macruronus magellanicus	GU324139; EU348300; DQ274028	EU074460
Malacocephalus laevis		HQ945850; JQ774539; JF493860
Malacocephalus occidentalis		KC015622
Merluccius australis		EU074468
Merluccius capensis		JF493884; GQ988405; JF268620; HQ611082; HM007692
Merluccius hubbsi	EU348299	GU702480; JQ365421; GQ988401; GU324174; EU074478
Merluccius paradoxus	EU348302; **GU324141**	JF493889; GQ988407; JF268613; HM007689; **GU324176**
Merluccius polli		GQ988410
Mora moro	AY392148	EF609410
Muraenolepis marmoratus	FJ215174	EU326378; EU326379; EU326380; JN40702
Muraenolepis microps	FJ215175; JX974419	
Muraenolepis orangiensis	FJ215176	
Muraenolepis species	FJ215177	HQ713082; HQ713083; HQ713084; HQ713085; EU326374; EU326375; EU326376; EU326377
Pseudophycis bachus	EU848440	EF609444
Pseudophycis barbata	EU848441	
Salilota australis		EU074580
Tripterophycis gilchristi		JF494750; JF494751; JF494752; JF494753; JF494754
Urophycis brasiliensis		GU702419; JQ365617; JQ365618; JQ365619
Zeus faber	**AF221896**	**EU869851**