MOLECULAR SYSTEMATICS AND ANTIFREEZE BIOLOGY OF SUB-ANTARCTIC NOTOTHENIOID FISHES

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by

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Abstract

Fishes of the perciform suborder Notothenioidei are found in Antarctic and sub-Antarctic waters that are separated by the Antarctic Polar Front (APF), with some species being distributed on both sides of this front. In this wide latitudinal range, these fishes are exposed to different temperatures ranging from -2 °C in the High Antarctic regions to 12 °C in the sub-Antarctic regions. To survive in icy Antarctic waters, the Antarctic notothenioid species have evolved antifreeze glycoproteins (AFGPs) that prevent their body fluids from freezing. The findings of past research on the AFGP attributes of several notothenioid species inhabiting ice-free sub-Antarctic environments have presented a complex picture. Furthermore, previous taxonomic studies split widely distributed notothenioids into different species and/or subspecies, with other studies disagreeing with these splits. To understand the response of the sub-Antarctic notothenioids to warmer, ice-free environments, it is necessary to have a good understanding of their antifreeze biology and systematics. Therefore, this study aimed to determine the association, if any, between the antifreeze attributes of sub-Antarctic notothenioid fishes and their taxonomic status.

Analyses were done on blood serum and tissue samples of notothenioids species that were collected around the Southern Ocean, and from various museum collections. Serum AFGP activities were determined in terms of thermal hysteresis (a difference between the melting and non-equilibrium freezing points). Several sub-Antarctic species possessed no or lower levels of AFGP activities in their body fluid, compared to their Antarctic taxa. Nototheniids including *Paranotothenia magellanica*, *Patagonotothen guntheri*, *P. ramsayi* and *Lepidonotothen*

squamifrons had thermal hystereses values that are near the margin of error (>0.1 °C) for hysteresis measurements, suggesting that they either have too little or no serum AFGP activities. The *Notothenia rossii* specimen from the warmer ice-free waters of the Ob' Seamount possessed the lowest serum AFGP activity (thermal hysteresis of 0.44 °C) compared with specimens from the ice-cold waters of the Antarctic (1.08-1.43 °C). However, resolution of serum AFGP size isoforms on a polyacrylamide gel electrophoresis (PAGE) revealed that all *N. rossii* populations possess extensive complements of AFGP proteins. The magnitude of AFGP gene dosage in genomic DNA of notothenioids was assessed using Southern blot analysis. The species with high levels of serum AFGP activities possessed large AFGP gene families, while species with low serum AFGP activity have no or small AFGP gene dosages. The small AFGP gene dosage detected in DNA of *L. squamifrons*, consistent with unmeasurable serum AFGP activity, was found to be non-functional as assessed by their expression in mRNA using Northern blot.

The AFGP characteristics were mapped onto notothenioid phylogenetic trees constructed from two mitochondrial (ND2 and COI) and one nuclear (S7 intron 1) genes. The family Nototheniidae, which has the highest number of widespread species, was paraphyletic and consisted of four main clades with species possessing different amounts of AFGP gene dosage in each clade. However, species with similar levels of AFGP grouped together in distinct sub-clades with the exception of *Dissostichus eleginoides*. *Lepidonotothen squamifrons* with non-functional AFGP was more closely related to the AFGP-null *Patagonotothen* species than it is to its congeners *L. larseni* and *L. nudifrons* that have functional AFGP, making the genus *Lepidonotothen* paraphyletic. Therefore, the placement of *L. squamifrons* in this genus should be re-evaluated. The results of this study also disagreed with splitting of *L. squamifrons* and *L.*

larseni, respectively, proposed by past authors and supported the inclusion of *Gobionotothen marionensis* and *G. acuta* in a ‘marionensis’ species group. This study contributed in closing the research gap between the notothenioid species of the Antarctic and sub-Antarctic regions of the Southern Ocean with regard to their AFGP attributes and systematics

Declaration

I, the undersigned, declare that to the best of my knowledge the thesis hereby submitted for the Doctor of Philosophy in Ichthyology at the Department of Ichthyology and Fisheries Science, Rhodes University, is the original work. This thesis has not been previously submitted in any form to another university.

Signature…… Date……

Dedication

This thesis is dedicated to my late mother Ms Ntaoleng Mokoena (may her soul rest in peace), my little sister Ms Boitumelo Mokoena, my husband and friend Mr Ngongoma Miya and my beautiful daughter Nkanyezi Miya.

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Tuuli Makinen and Albert Chakona; Messrs Mpho Ramoejana and Thabo Maake; Mrs Nosiphiwo Springbok-Njokweni and Mss Sisanda Mayekiso, Kerry van der Walt and Taryn Bodill are thanked for the fruitful discussions regarding research in general. Messrs Willem Coetzer and Nkosinathe Mazungula of SAIAB kindly assisted with the preparation of maps.

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Chapter One

General introduction

1.1. Background

The Southern Ocean surrounding Antarctica is the body of water encompassing the southern parts of the Pacific, Atlantic and Indian oceans, and occupies about 10% of the world's ocean (Figure 1.1). The typical depths of the Southern Ocean range between 3 000 and 5 000 m over most of its extent with isolated plateaus and ridges, with the greatest depth being 7 300 m (Lutjeharms 1990; Knox 2007; Shotton and Tandstad 2011). The Southern Ocean is characterised by year-round frigid water and abundance of ice in the southern extremes (DeVries and Steffensen 2005). The dominant feature of this ocean is the Antarctic Circumpolar Current (ACC), which flows around the continent in a clockwise direction driven by persistent westerly winds. It is located between 47°S and 60°S and its width varies from 200 to 1 200 km in various parts of the Southern Ocean (Foster 1984; Lutjeharms 1990; DeVries and Steffensen 2005). Some recent studies have estimated the ACC to have evolved in the Eocene to Oligocene period, a timescale congruent with the onset of global cooling and Antarctic glaciation (Barker and Thomas 2004; Katz *et al*. 2011).

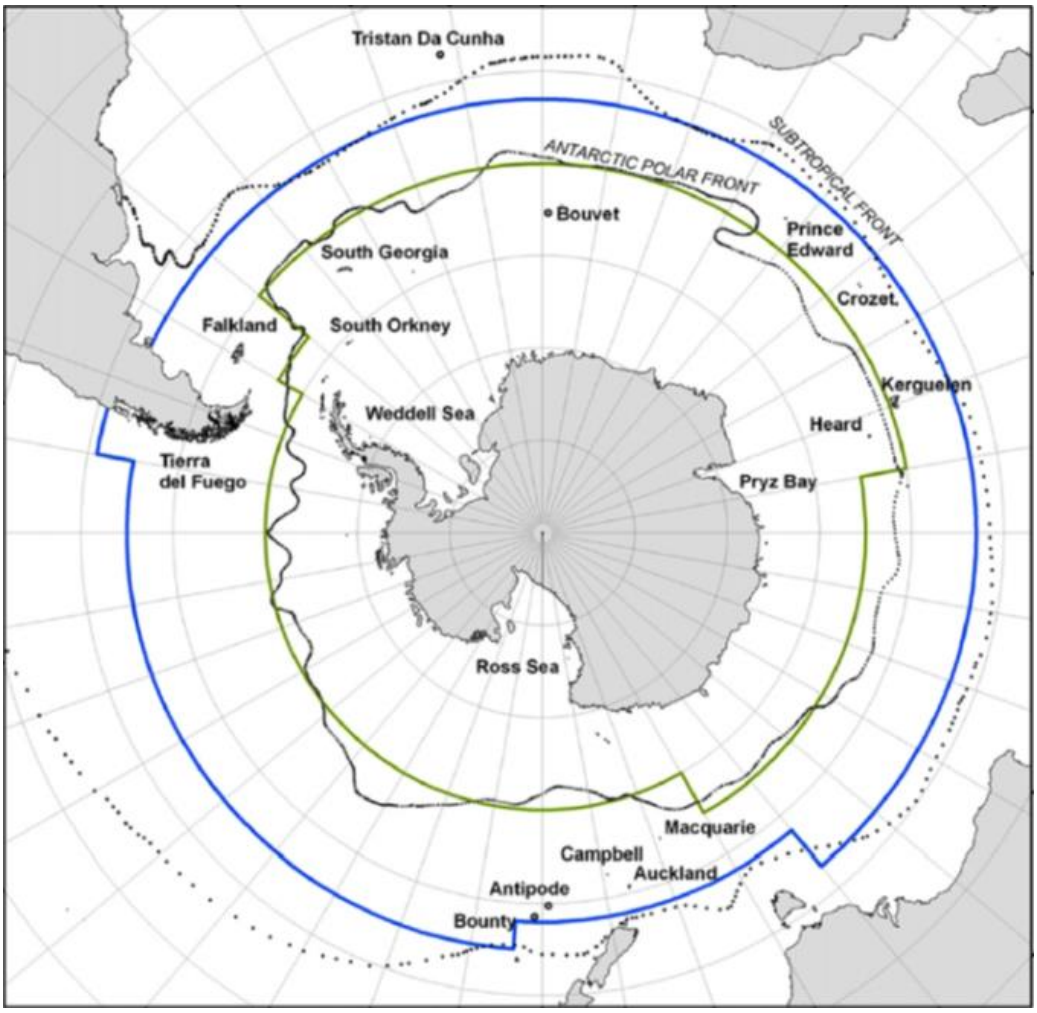

Figure 1.1: The Southern Ocean region and the Antarctic Polar Front. The green line indicates the average northern limit of the Antarctic region, while the blue line is the northern limit of the sub-Antarctic region (Map obtained from de Broyer *et al.* 2011).

The Southern Ocean consists of sub-Antarctic and Antarctic regions that are separated by the Antarctic Polar Front (APF) (Figure 1.1), the northern boundary of the ACC. This front is a transition zone between the cold Antarctic surface water and the warmer sub-Antarctic water (Foster 1984). The APF is not a stationary front as it forms twists and loops that extend as much as 150 km north and south, and the temperature of the surface water around this front varies at different latitudes of the Southern Ocean (Kennet 1982; Foster 1984). The High Antarctic region, the area near the Antarctic continent, is the coldest with ice present throughout the year and a

water temperature range from -1.0 to -1.9 °C. The area just south of the APF is called the Low Antarctic region and has a temperature range of -1.0 to 5 °C with seasonal pack-ice in the colder areas (Knox 2007). The sub-Antarctic region (north of APF) is warmer and ice-free year-round with water temperatures ranging from 1 to 12 °C. This region includes the waters around the southern part of the South Island of New Zealand and the southern tip of South America (Eastman 1993; DeVries and Steffensen 2005; Knox 2007). Although the APF and ACC serve as a physical barrier preventing migration of fish in north/south direction, the ACC acts as a mode of transport circulating eggs and larvae around the Antarctic continent (Knox 1970, Foster 1984). Nevertheless, several Southern Ocean organisms, such as notothenioid fish species, made use of the ACC conveyor belt and have dispersed across the ACC barrier to colonise waters around sub-Antarctic islands as well as around the southern tip of South America and New Zealand (DeVries and Steffensen 2005).

A number of new species have been described in the Southern Ocean, since the publication of '*Fishes of the Southern Ocean*' (Gon and Heemstra 1990), due to taxonomic revisions and new collections from continuing fisheries and scientific expeditions (Eastman and Eakin 2000; Prirodina 2000, 2002, 2004; Cziko and Cheng 2006; Balushkin and Korolkova 2013; Shadikov *et al.* 2013). There are therefore currently over 320 valid fish species in 50 families known in this ocean making up about 2% of all known marine fishes (Eastman 2005). About 50% of these species are in the suborder Notothenioidei (Eastman 1993; 2005). The notothenioids are dominant in terms of species diversity, abundance and biomass, as well as dominating the benthic groups (Eastman and McCune 2000; DeVries and Steffensen 2005). They fill ecological niches occupied by taxonomically diverse groups of fishes in temperate and tropical oceans, including groups that are not represented in the Antarctic waters (Eastman 1993; Eastman and McCune 2000). The notothenioid species studied so far appeared to be both stenothermal (Somero and DeVries 1967) and stenohaline (O'Grady and DeVries 1982). Some of these fishes can survive undercooling to -2.5°C, as long as there is no contact with ice, and most (especially species inhabiting the High Antarctic region) will not survive temperatures higher than 6°C (DeVries and Cheng 2005; DeVries and Steffensen 2005).

Notothenioids are generally distributed on both sides of the APF, with the majority found to the south of it (Gon and Heemstra 1990), and they are exposed to different temperatures ranging from -2 to 12°C (DeVries and Steffensen 2005; Knox 2007). The Antarctic notothenioids have diverged in the sub-zero waters of the Antarctic continental shelf since the Oligocene-Miocene transition, about 24 million years ago (mya) (Eastman 2005; Matshiner *et al.* 2011). To survive in these extreme environments some species possess a wide range of adaptations, such as antifreeze glycoproteins (AFGPs) (Chen *et al.* 1997) and its associate antifreeze potentiating protein (AFPP) (Jin 2003), retinal reorganisation (Pointer *et al.* 2005) and loss of heat shock response (Hofmann *et al.* 2005; Clark *et al.* 2008). Furthermore, the oxygen rich Antarctic waters compensate for the loss of the oxygen binding pigments haemoglobin and myoglobin in the icefish family Channichthyidae (Montgomery and Clements 2000). The sub-Antarctic notothenioid species established themselves in the warmer environments after diverging from their Antarctic sister in the mid-Miocene to Pleistocene (12 to 1 mya) (Stankovic *et al*. 2002; Cheng *et al.* 2003). In these non-freezing sub-Antarctic environments, notothenioid species have no need for freezing avoidance mechanisms. However, previous studies have demonstrated that some sub-Antarctic notothenioids do have characteristics suggesting the presence of antifreeze proteins or genes (Gon *et al.* 1994; Cheng *et al.* 2003; Cheng and Detrich III 2007). The present study is therefore aimed at providing a broader understanding on the antifreeze biology of the sub-Antarctic notothenioids as well as their taxonomic status.

1.2. Biology of notothenioid species

Notothenioid species are the members of Acanthomorpha, a group that comprises all teleost fishes possessing spines in their dorsal and anal fins (Rosen 1973; Johnson and Patterson 1993; Nelson 2006). Although the suborder Notothenioidei is monophyletic based on genetic analyses (Chen *et al.* 2003; Matshiner *et al.* 2011), there is no unique morphological character distinguishing this suborder. It is therefore diagnosed by a presumably unique combination of morphological characters (Eastman 1993). In the absence of such synapomorphic characters, it is very difficult to recognise notothenioid fossils (Grande and Eastman 1986; Eastman 1993).

However, Balukshin (1994) regarded the fossil fish *Proeleginops grandeastmanorum*, originally described as a gadiform by Eastman and Grande (1991), as a notothenioid assignable to the family Eleginopsidae, based on evidence from comparative skull anatomy. Although Balushkin's (1994) argument was convincing, the taxonomic assignment of this fossil is still debatable (Near *et al.* 2004; Matshiner *et al.* 2011). Therefore, hypotheses pertaining to the time and geographic origin of the group are speculative due to scarcity of notothenioids in the fossil record (Bargelloni *et al.* 2000; Matshiner *et al.* 2011; Near *et al*. 2012).

Notothenioid species lack a swim-bladder, with the majority of species being benthic and inhabiting water less than 1000 m deep, with the depth range of some species being considerable (DeWitt 1971; Eastman and DeVries 1982; Eastman 1993). In the absence of the swim bladder, notothenioids have evolved several mechanisms for reducing density and/or increasing buoyancy. These mechanisms include a reduction in the extent of bone and scale mineralisation, substitution of bone with cartilage, and the presence of subcutaneous and intramuscular lipid sacs composed of modified adipocytes surrounding large lipid droplets. This lipid storage structure is found in species such as *Pleuragramma antarctica* (Eastman and DeVries 1982; Clarke *et al.* 1984; Eastman 1993). Species of the family Artedidraconidae are the most benthic and sedentary in this suborder and spend almost all their time motionless on the substrate (Eakin 1990; Eastman 1993). Generally, notothenioids spawn on the bottom, but the larvae and juveniles are frequently pelagic (Kock and Kellermann 1991).

Notothenioids are red-blooded fishes and have lower concentrations of haemoglobin (Hb) compared to other teleosts, with the exception of the Channichthyidae, the only known vertebrate group whose adult's blood is devoid of Hb (Figure 1.2) (di Prisco *et al*. 1991a, di Prisco 2000). The reduced concentration of Hb has been linked to the environmental conditions such as the high stability and constancy of physico-chemical properties, and the high oxygen content in cold Antarctic water (Verde *et al.* 2006). Low temperatures also reduce the overall metabolic demand for oxygen while increasing its solubility in the blood plasma, so that more oxygen can be carried in physical solution and with less need to be bound to Hb (di Prisco 2000; di Prisco *et al.*

2007). The Channichthyidae have also developed other physiological adaptations that maintain adequate tissue oxygen such as enhanced gas exchange using highly vascularised gills and skin, as well as increased cardiac output, circulatory volume and heart size (di Prisco 2000).

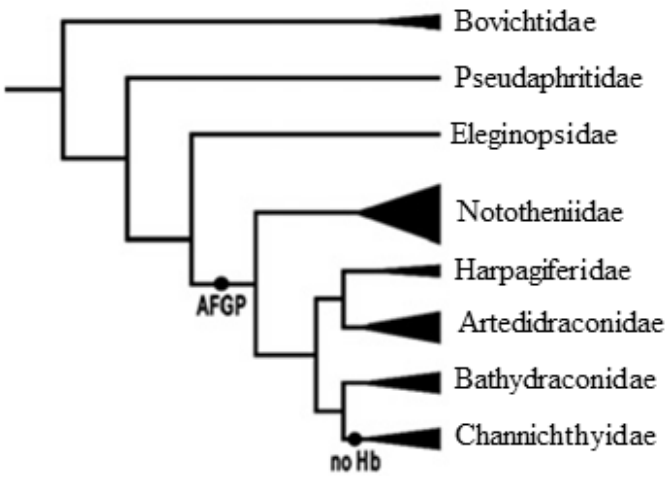

Figure 1.2: Phylogenetic relationships within the families of the suborder Notothenioidei resulting from parsimony analysis of the complete 16 rRNA gene (Near *et al.* 2004). Antifreeze glycoproteins (AFGPs) characteristics and the loss of haemoglobin (no Hb) in channichthyids are mapped; modified from di Prisco *et al.* (2007).

A key physiological and biochemical adaptation of Antarctic notothenioids is the evolution of antifreeze glycoproteins (AFGPs) (Figure 1.2) in their blood and body fluids that enable them to survive in freezing ice-laden waters (DeVries 1971). These proteins prevent freezing of the blood and body fluids of these fishes by a mechanism known as adsorption-inhibition, where the AFGP adsorbs to small ice crystals in the body thereby inhibiting their growth (Raymond and Devries 1977; DeVries 1988; Harding *et al.* 2003; DeVries and Cheng 2005). AFGPs therefore lower the freezing point of the blood and body fluids below that of their surrounding waters (DeVries 1988; DeVries and Cheng 2005). However, the three non-Antarctic notothenioid families (Bovichtidae, Pseudaphritidae and Eleginopsidae) possess no AFGP in their blood and body fluids (Cheng *et al.* 2003). These families presumably diverged and established themselves in

coastal waters of the southern continental blocks before the cooling of the Southern Ocean and the evolutionary gain of AFGP (Eastman 1993; Cheng *et al.* 2003).

1.3. Antifreeze biology of notothenioid fishes

Biological antifreeze molecules are proteins with ice-binding properties, which enable organisms to survive and successfully colonise their respective icy and freezing habitats (DeVries 1971, 1982; Cheng 1998a). These proteins were first discovered in the Antarctic notothenioids by DeVries (1971) as a family of glycosylated proteins he named antifreeze glycoproteins (AFGPs). Studies of the origin of these AFGPs suggested that their evolution in Antarctic notothenioid fishes was directly driven by the geological and thermal evolution of the Antarctic freezing environment (Chen *et al.* 1997; Cheng and Chen 1999). Raymond *et al.* (1975) later discovered similar AFGPs in two northern cod fishes; the polar cod *Boreogadus saida* and the Atlantic cod *Gadus morhua*. The AFGP gene of these two phyletically unrelated groups of fish, i.e. Antarctic notothenioids (order Perciformes) and northern cods (order Gadiformes), have evolved independently and is synthesised by the exocrine pancreas in both groups (it is also synthesised by liver in Arctic cods) (Chen *et al.* 1997; Cheng *et al.* 2006; Evans *et al.* 2012). This was followed by discoveries of four other types of antifreeze proteins (AFPs) which were identified in various fish taxa that are found in the Arctic Ocean (Duman and DeVries 1976; Slaughter *et al.* 1981; Hew *et al.* 1984; Deng *et al.* 1997). The AFPs were also found in a wide range of overwintering terrestrial insects (Duman *et al.* 1998; Jai and Davies 2002; Nickell *et al*. 2013), plants (Duman 1994; Griffith and Yaish 2004; Middleton *et al*. 2012), fungi (Raymond and Janech 2009; Kondo *et al*. 2012), yeast (Lee *et al*. 2010; Park *et al*. 2012) and bacteria (Raymond *et al*. 2008; Garnham *et al*. 2011).

Antifreeze glycoproteins of the Antarctic notothenioids are encoded as distinct molecules linked in a series by conserved three-residue spacers (Leu/Phe-Ile/Asn-Phe) within large polyprotein precursors (Figure 1.3) (Chen *et al.* 1997). The AFGP gene comprises two exons and a single intron, which evolved from a functionally unrelated pancreatic trypsinogen-like serine protease gene. This occurred through the recruitment of the front (exon 1 and intron 1) and the tail

segments (exon 6 and 3′ flanking DNA sequence) of the ancestral protease gene, and de novo amplification of a 9-nt ThrAlaAla coding element that linked the intron 1 and exon 2 to create the repetitive ice-binding AFGP coding region (Chen *et al*. 1997; Cheng 1998b; Cheng and Chen 1999). The AFGPs exist as a family of at least eight isoforms (AFGPs 1-8) (Figure 1.4), all composed of repeats of a simple glycotripeptide monomer (Thr-Ala/Pro-Ala) with the disaccharide galactose-N-acetylgalactosamine attached to each threonine residue (DeVries 1988; O'Grady *et al.* 1982; Cheng and DeVries 1991; DeVries and Cheng 2005). These isoforms are commonly subcategorised into two size groups, i.e. AFGPs 1-5 which consist of the larger molecules [10 500-33700 daltons (Da)] and AFGPs 6-8 consisting of smaller molecules (2 600-7 900 Da) (DeVries *et al*. 1970; DeVries and Cheng 2005). These groups have a peptide backbone of Ala-Ala-Thr repeats (Figure 1.3) with a Pro occasionally replacing the first Ala of the tripeptides (Cheng and Chen 1999). The larger AFGPs 1-5 isoforms are more effective in depressing freezing point in notothenioid fishes, while the AFGPs 7-8 isoforms are abundant and comprise about two-thirds of circulating AFGP in most species (Raymond and DeVries 1977; DeVries and Cheng 2005).

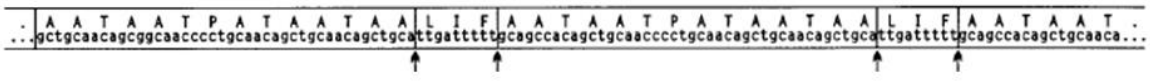

Figure 1.3: AFGP polyprotein gene partial sequence from the Antarctic notothenioid fish, *Dissostichus mawsoni*. The translated amino acid sequence shows repeats of the tripeptide backbones and the conserved three-residue spacers (indicated by arrows); modified from Chen *et al.* (1997).

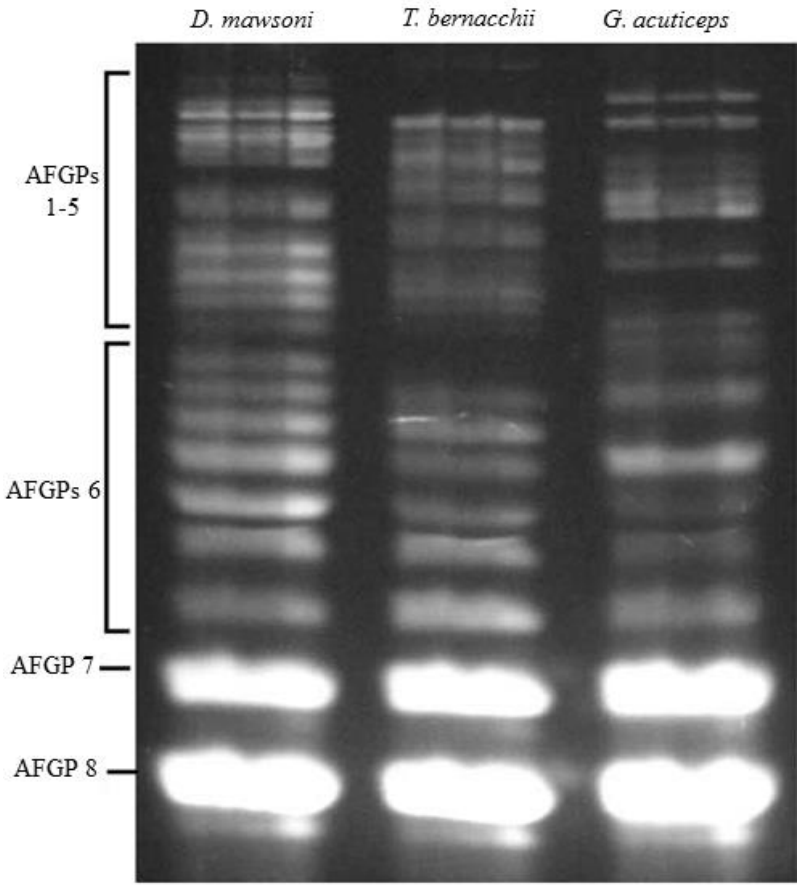

Figure 1.4: Antifreeze glycoproteins (AFGPs) from the High Antarctic notothenioid fishes, *Dissostichus mawsoni*, *Trematomus bernacchii* and *Gymnodraco acuticeps*, showing the size isoforms on gradient polyacrylamide gel electrophoresis; modified from DeVries and Cheng (2005).

Antarctic notothenioid species from high latitude freezing environments have large AFGP gene families that correlate with high levels of blood-borne antifreeze protein necessary to avoid freezing (Cheng 1996; Nicodemus-Johnson *et al.* 2011). For example, *Pagothenia borchgrevinki* of the icy waters (-1.93 °C) of the High Antarctic McMurdo Sound have a blood freezing point of -2.7 °C, which is lower than the ambient temperature (DeVries and Cheng 2005). The difference between equilibrium melting point and the non-equilibrium freezing point of this species is 1.6 °C. This difference is termed thermal hysteresis, which measures the relative levels of AFGP in a fish's blood and can also be affected by the relative abundance of the size isoform groups (DeVries 1982; DeVries and Cheng 2005). Recent studies discovered that these High

Antarctic notothenioid species also possess an antifreeze potentiating protein (AFPP), which enhances AFGP activity by potentiating the activity of the large size AFGPs 1-5. The AFPP is however responsible for approximately 30% or less of the serum antifreeze activity in these fishes and thus AFGP is largely responsible for the high thermal hysteresis of these species (Jin 2003; Yang *et al.* 2013). However, not all Antarctic notothenioid species have high amounts of AFPP, they are found only species inhabiting severe Antarctic habitats such as *P. borchgrevinki* (Yang *et al.* 2013).

There are several notothenioid species, belonging to the five AFGP-bearing families (Figure 1.2) that occur in the less severe environments of the lower Antarctic latitudes and in the non-freezing sub-Antarctic waters where antifreeze protection becomes less critical or unessential (Ahlgren and DeVries 1984; Cheng and Detrich III 2007). Studies of the status of the AFGP trait in some of these species have revealed that they either have much lower AFGP activity than High Antarctic fishes, or no AFGP in their body fluid. For example, Cheng *et al.* (2003) found minute levels of serum AFGP (±0.23 μg/ml) in *N. angustata* of the ice-free water near the Otago Harbour of South Island New Zealand, which were quantified by using enzyme-linked immunosorbent assay (ELISA). On the other hand, *Patagonotothen* species studied so far from cool temperate South America had no detectable AFGP coding sequences based on the genomic Southern blot analyses (Cheng *et al.* 2003; Cheng and Detrich III 2007). This is in conflict with the hypothesis on the origin of AFGP, which suggests that AFGP evolved once at the base of the diversification of the five Antarctic notothenioid families (Figure 1.2) (Chen *et al.* 1997; Cheng 1998a; Cheng and Chen 1999), hence one would expect all species belonging to these families to still possess some AFGP sequences.

1.4. Systematics of the suborder Notothenioidei

Although notothenioids lack a unique character or synapomorphy, most molecular phylogenetic studies have indicated this group to be monophyletic (Chen *et al*. 2003; Near and Cheng 2008; Near *et al.* 2012). This suborder is a relatively recent clade with estimated divergence time in the late Cretaceous (80 to 70 mya) (Matshiner *et al.* 2011; Near *et al.* 2012). Notothenioidei consist

of eight families (Figure 1.2) and the number of species within this suborder ranges between 136 (Eastman and Eakin 2014) and 159 (Eschmeyer and Fong 2014). This study followed the Eastman and Eakin (2004) species count, as this is the most recent. Ten of these species (of 136) belong to the three phyletically basal families, Bovichtidae, Pseudaphritidae and Eleginopsidae. With the exception of one species (*Bovichtus elongatus*), the species of these three families are found in the warmer coastal waters of New Zealand, Australia and South America (Gon and Heemstra 1990; Eastman and Eakin 2000). The derived clade of Antarctic notothenioids is the largest group in the suborder comprising 126 species, of which 16 species are found along the cool-temperate southern coast of South America and New Zealand. The species in the Antarctic clade belong to five families, including the Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae and Nototheniidae (Gon and Heemstra 1990; Eastman 2005). Several species belonging to this Antarctic clade have latitudinal wide-ranging distribution around waters of the Southern Ocean where they encounter different environmental conditions (Gon and Heemstra 1990; Eastman 2005). The Antarctic families have taxonomically diverse groups of fishes (Gon and Heemstra 1990; Eastman and Eakin 2000), and their systematics are discussed in more detail in the following family accounts.

1.4.1. Artedidraconidae

Artedidraconids are the most sedentary notothenioids spending most of their time motionless on the substrate (Eakin 1990). They can be distinguished by the presence of a hook-shaped spine on the opercle and are the only notothenioid family with a mental barbel that probably functions as a sensory organ that detects the presence and position of prey (Figure 1.5) (Hureau 1985a; Eakin 1990; Iwami *et al.* 1996). Since Eakin's (1990) review, a number of new species have been described and there are currently 33 valid species in four genera (Balushkin and Eakin 1998; Eakin *et al.* 2008, 2009; Shandikov and Eakin 2013; Shandikov *et al.* 2013; Balushkin *et al.* 2010; Eastman and Eukin 2014). With the exception of *Artedidraco mirus*, a species endemic to the waters of South Georgia Island, the artedidraconids are confined to the cold waters around the Antarctic shelf and slopes (Eakin 1990). The artedidraconids inhabiting the cold waters studied so far had substantial AFGP levels in their body fluids (Wohrmann 1996; Cheng *et al.* 2003; Ghigliotti *et al.* 2013), while the antifreeze attributes of the low Antarctic *A. mirus* are

unknown. Molecular phylogenetic analyses of this group found it to be monophyletic (Ritchie *et al.* 1997; Near and Cheng 2008).

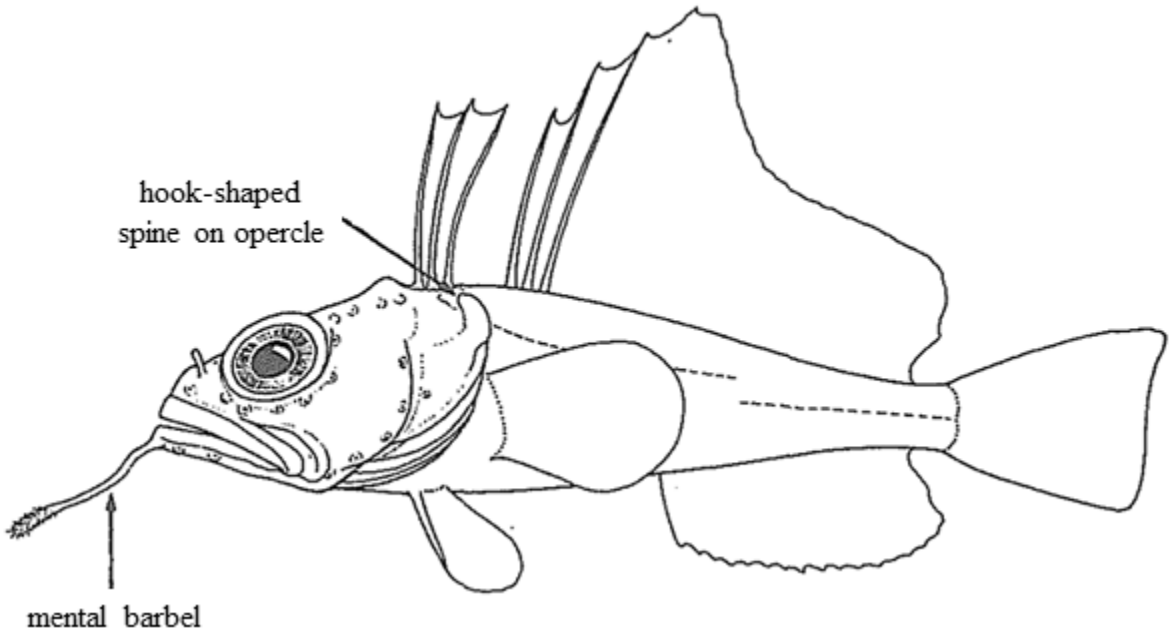

Figure 1.5: Distinguishing characters of species of Artedidraconidae; modified from Hureau (1985a).

1.4.2. Bathydraconidae

Bathydraconids can be distinguished from other notothenioids in that they have a single dorsal fin without spinous fin rays (Figure 1.6) (Hureau 1985b; Gon 1990; Balushkin 1992). Morphological analyses (Iwami 1985) suggest that this group is closer to channichthyids than any other notothenioid family, because they share a non-protrusible jaw (Figure 1.6 and 1.7) and an I-shaped junction between the epihyal and ceratohyal. Currently, there are 11 genera that are recognised and 16 valid species in the Bathydraconidae (Eastman and Eakin 2000 2014). They are found in the coldest, deepest shelf waters at the high latitudes, with *Bathydraco joannae* and *B. antarcticus* also found in the sub-Antarctic waters around Falkland and Kerguelen (Figure 1.1), respectively (Gon 1990; Voskoboinikova and Balushkin 1998). AFGP attributes of

bathydraconid species studied so far, indicated that these species possess substantial amount of AFGP in their body fluids (Ahlgren and DeVries 1984; Wohrmann 1996; Cheng *et al.* 2003). Molecular phylogenetic analyses of this group indicate it as paraphyletic with three possible subfamilies, i.e. Cygnodraconinae, Bathydraconinae and Gymnodraconinae (Derome *et al.* 2002; Near *et al.* 2012).

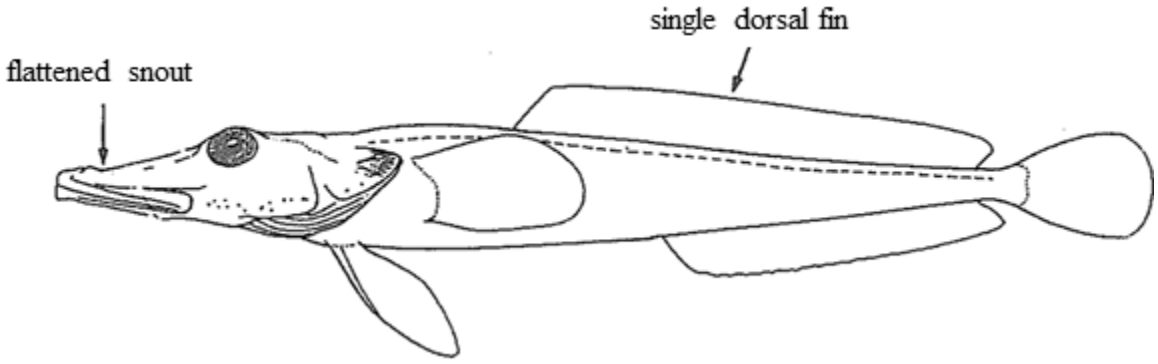

Figure 1.6: General morphological feature of species of Bathydraconidae; comes from Hureau 1985b work.

1.4.3. Channichthyidae

Channichthyids are the only vertebrate animals that lack the circulating oxygen-binding protein haemoglobin in their blood (Ruud 1954; Iwami and Kock 1990). Although they resemble bathydraconids (Figure 1.6), they are easily distinguished by the presence of a spiny first dorsal fin and branched spines on the opercle (Figure 1.7) (Hureau 1985c; Iwami and Kock 1990; Kock 2005). Although the species of *Parachaenichthys*, a genus of Bathydraconidae, also have branched spines on their opercle, they can be differentiated from the channichthyids by their single dorsal fin (Gon 1990). The channichthyids are among the largest of the Antarctic fishes, with *Chaenocephalus aceratus* growing up to 75 cm, and spend a considerable part of their time at or close to the sea floor perching on their pelvic fins and waiting for prey (Kock 2005). Channichthyids include 16 species in 11 genera that are distributed in both High and Low

Antarctic regions, with *Champsocephalus esox* occurring north of Antarctic Polar Front (AFP) and *Channichthys rhinoceratus* on both sides of AFP (Iwami and Kock 1990; Kock 2005; Shandikov 2008, 2011; Eastman and Eakin 2014). A southern blot analysis of *C. esox* from South American waters detected the presence of AFGP sequences in their genomic DNA (Cheng and Detrich III 2007). Molecular phylogenetic studies of this group have found it to be monophyletic (Chen *et al.* 1998; Derome *et al.* 2002; Near and Cheng 2008; O'Brien and Mueller 2010).

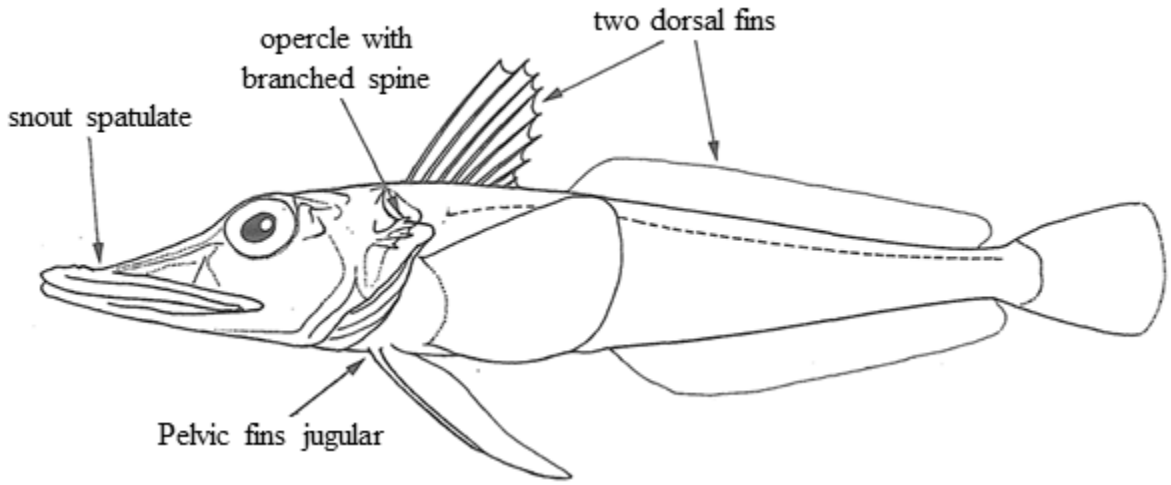

Figure 1.7: General morphological feature of species of Channichthyidae; modified from Hureau (1985c).

1.4.4. Harpagiferidae

Early studies of notothenioid systematics placed harpagiferids and artedidraconids in one group (Norman 1938). However, Andriashev (1965) and Eakin (1981) later separated them into two families. Morphological and molecular phylogenetic analyses of harpagiferids demonstrated that they are monophyletic (Balushkin 1992; Bargelloni *et al.* 1994). This family can be distinguished by the presence of two spines on the opercle and a scale-less body with the upper lateral line forming tubules resembling scales (Figure 1.8) (Hureau 1985d, 1990). Harpagiferids contains 11

species in one genus, *Harpagifer*, that are small bottom dwelling fishes (DeWitt 1971; Hureau 1990; Prirodina 2002, 2004, Eastman and Eakin 2014). They are found in sub-Antarctic waters, with the exception of one species, AFGP-bearing *H. antarcticus*, which is distributed along Antarctic Peninsula waters (Hureau 1990). There is a lack of information regarding the AFGP attributes of sub-Antarctic harpagiferid species.

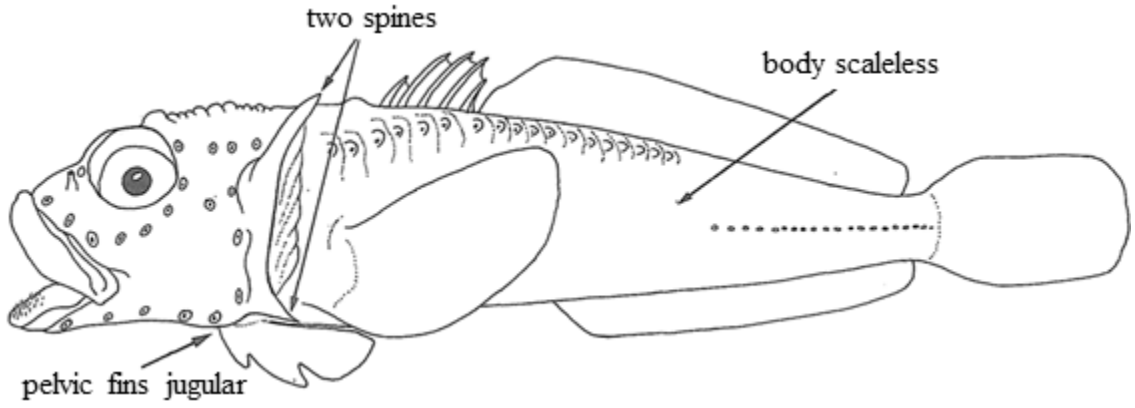

Figure 1.8: Distinguishing characters of species of Harpagiferidae; modified from Hureau (1985d).

1.4.5. Nototheniidae

Nototheniidae is the richest family in terms of both number of species and ecological diversification (Eastman 1993, 2005).There are currently 50 valid species and 12 genera in this family (Eastman and Eakin 2014). Nototheniids can be distinguished by a combination of characters that includes the absence of spines on the opercular bones, the presence of two or three lateral lines, two dorsal fins and scales on the body (Figure 1.9) (Hureau 1985e; DeWitt *et al.* 1990). Over 65% of species of this family occupy benthic habitats, while the others are semipelagic, epibenthic, cryopelagic or pelagic (Eastman 1993). The nototheniids are found throughout the Antarctic and sub-Antarctic regions, as well as in the temperate coastal waters of New Zealand and South America (Figure 1.1) (DeWitt *et al.* 1990). The antifreeze traits of the sub-Antarctic nototheniids studied so far present a complex picture (see section 1.3). The taxonomy of the latitudinally widely distributed species of the Nototheniidae is inconclusive,

with some species previously being divided into two or more species and/or subspecies (Nybelin 1947, 1951; DeWitt 1966; Permitin and Sazonov 1974; Balushkin 1976). Near and Cheng (2008) found the Nototheniidae to be monophyletic with a mitochondrial DNA dataset and paraphyletic with the ribosomal protein S7 intron 1 nuclear gene. However, recent studies with much larger datasets have consistently found this family to be paraphyletic with both mitochondrial and nuclear DNA gene analyses (Rustchmann *et al.* 2011; Dettai *et al.* 2012; Near *et al.* 2012). These studies have also suggested that the *Notothenia-Paranotothenia* clade is sister to the four High Antarctic families, with the sister taxon of this clade being the genus *Gobionotothen*. Dettai *et al*. (2012) further suggested splitting of Nototheniidae into a number of new families which would be mostly represented by a single genus. Alternatively, to avoid creating multiple small families, the latter study suggested integrating the other four Antarctic families into this family and creating subfamilies to conserve the four Antarctic families.

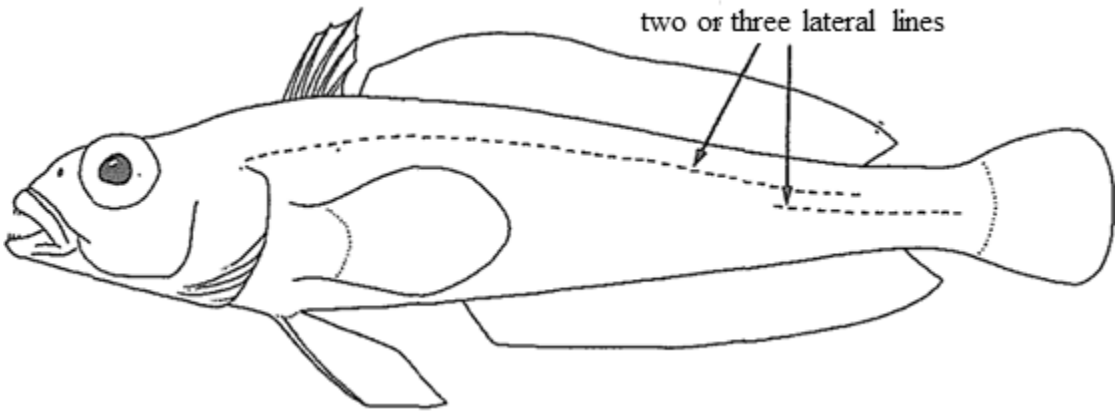

Figure 1.9: Distinguishing character of species of Nototheniidae; modified from Hureau (1985e).

1.5. Study rationale and objectives

Although *Fishes of the Southern Ocean* (Gon and Heemstra 1990) and the research associated with it made significant contributions to the understanding and conservation of Antarctic fishes, more recent studies associated with species of this region have exposed gaps in our knowledge of this unique fauna. The more apparent gap since 1990 has been lack of knowledge of the sub-Antarctic component of the Southern Ocean fish fauna, particularly with regard to the systematics and adaptations of species of the suborder Notothenioidei (di Prisco *et al*. 1991b, 1998, 2000; Eastman 1993; Gerday 1997; Hureau 1997). Much of the research efforts have concentrated on the understanding of systematics and mechanisms of adaptation of notothenioid species inhabiting cold to icy waters of the Antarctic, as well as the areas where commercial exploitation of fish resources had taken place (Hureau and Slosarczyk 1990; Chen *et al.* 1997; Eastman 1993, 2005; Pointer *et al.* 2005; Hofmann *et al.* 2005; Bilyk *et al.* 2012). Relatively little research effort was devoted to studying the physiology of the notothenioid species of the warmer waters of the sub-Antarctic region (Gon *et al.* 1994; Cheng *et al.* 2003) and how this could affect the taxonomic classification of the species of this group in this area.

The sub-Antarctic is the area of the Southern Ocean between the Antarctic Polar Front (APF) and the Subtropical Convergence. It includes the waters around the southern part of the South Island of New Zealand and the southern tip of South America and is warmer than the Antarctic region (Figure 1.1) (DeVries and Steffensen 2005; Knox 2007). The ancestor of some notothenioid species, such as species belonging to the genus *Patagonotothen*, apparently migrated from the cold waters of the Antarctic region and established themselves in the warmer sub-Antarctic waters (DeWitt *et al.* 1990). These also include several species, such as *Champsocephalus esox* that is only found in the warmer South America waters (Iwami and Kock 1990). On the other hand, a number of notothenioid species, for example *Notothenia coriiceps*, are distributed on both sides of the APF (DeWitt *et al.* 1990). As evident from the history of these species, there is disagreement on their taxonomic status such that it caused uncertainty in our knowledge of these latitudinally widespread species. Some authors split them into separate species or subspecies in their different habitats, while others disagreed with the splitting (Nybelin 1947, 1951; DeWitt 1966; Permitin and Sazonov 1974; Balushkin 1976; Gon and Klages 1988;

DeWitt *et al*. 1990; Schneppenheim *et al*. 1994). In their wide geographical range, these species are exposed to water temperatures ranging from -2 to 12 °C (Knox 2007). Given that the AFGP function is to prevent the fish's body fluid from freezing when getting in contact with ice, this adaptation is not needed in the absence of ice. Therefore, the widespread species would be expected to have AFGP when occupying ice-laden waters in the high latitudes and not in the ice-free waters of the sub-Antarctic. However, Gon *et al.* (1994) found freezing avoidance characteristics indicating the presence AFPG in blood of *N. coriiceps* found in the ice-free waters of Marion Island. Determining the antifreeze trait of notothenioid species occupying ice-free waters may enable an inference on the time of their dispersal from freezing environments. In the absence of selection pressure from the thermal environment a secondary loss of the AFGP gene may occur. As a consequence of such loss populations south and north of the APF could become reproductively isolated and, over time, lead to emergence of new species in different isolated areas. Furthermore, such development would validate the hypothesis of the origin of AFGP gene at the base of diversification of the five Antarctic families and also clarify taxonomic issues of the species found on both sides of APF.

Given that there has been disagreement on the taxonomic status and little information concerning the status of antifreeze attributes of latitudinally widespread notothenioids, this study therefore aimed to verify and determine the association, if any, between the antifreeze attributes and the taxonomic status of these fishes. This was addressed in two research objectives. The first objective was to characterise the freezing avoidance attributes of the sub-Antarctic notothenioid species from non-freezing environments, by determining the functional status of their antifreeze systems at protein and gene levels. The second objective of this study was to re-evaluate the taxonomic status of wide spread notothenioid species distributed on both sides of the Antarctic Polar Front, using molecular genetic analyses together with the data gained from the first objective. Previous splitting of the latitudinally widespread notothenioids was based on morphological characters; this study addressed this problem at the DNA level. It is hoped that the information gained from this research will narrow the present gap in the knowledge of the sub-Antarctic notothenioid species with regard to their adaptations and systematics.

1.6. Thesis structure and format

This thesis consists of five chapters, including an introduction, three research chapters addressing the study objectives, and a final discussion. The first objective is addressed in chapter two and chapter three. Chapter two focuses on a comparison of the effect of thermal habitats on the AFGP trait among populations of latitudinally widely distributed species. *Notothenia rossii* is used as the primary species in this research (Miya *et al.* 2014). Chapter three focuses on the characterisation of antifreeze attributes of sub-Antarctic notothenioid species belonging to the five Antarctic AFGP-bearing families. To determine the evolutionary origin of the AFGPs ancestor of the sub-Antarctic notothenioids, the AFGP characters of these species were mapped onto a phylogenetic framework, inclusive of their Antarctic sister taxa.

The specific questions arising from these chapters were:

1. Does temperature have an effect on the AFGP levels among populations of latitudinally widespread species in their respective habitats?
2. Which sub-Antarctic species possess AFGP sequences in their DNA and AFGPs in their blood?
3. Do sub-Antarctic notothenioid species belonging to the five AFGP-bearing Antarctic families possess similar levels of AFGP to their Antarctic relatives?
4. Do sub-Antarctic notothenioid species share a common AFGP-bearing ancestor with Antarctic notothenioid species?
5. Based on AFGP distribution on a phylogenetic tree of notothenioid species, will the hypothesis of origin of the ancestral AFGP gene of these species need to be re-evaluated?

The second objective of this research study is addressed in chapter four by re-evaluating the taxonomic status of three nototheniid species, *Lepidonotothen squamifrons*, *L. larseni* and *Gobionotothen marionensis*, that are found on both sides of the Antarctic Polar Front. These species were selected because they are among the species which previous studies separated into different species or subspecies.

The specific questions arising from this chapter were:

1. Are the notothenioid populations in the Atlantic Ocean sector of the Southern Ocean genetically different from populations in the Indian Ocean sector?
    - What is the level of genetic difference between the Atlantic and Indian oceans populations?
2. If there are documented genetic distances between the two populations, are there also coincidental morphological differences?

The final chapter is a synthesis and summary of the implications from the findings of this research. This chapter also attempts to present a link between the antifreeze attributes of notothenioid species and their taxonomy. Furthermore, recommendations for future research are stated in this chapter.

Chapter Two

The effect of habitat temperature on serum antifreeze glycoprotein (AFGP) activity in *Notothenia rossii* (Pisces: Nototheniidae) in the Southern Ocean.*

Abstract

The marble notothen, *Notothenia rossii*, is widely distributed around the waters of sub-Antarctic islands in the Southern Ocean and is exposed to different temperatures that range from -1.5 to 8 ^{O}C. This study investigates whether the different environmental conditions experienced by *N. rossii* at different latitudes in the Southern Ocean affect the levels of its blood serum antifreeze glycoprotein (AFGP). *Notothenia rossii* specimens were collected from four localities, including the Ob' Seamount in the Indian Ocean sector, and South Georgia Island, South Shetland Islands and Dallman Bay in the Atlantic Ocean sector. Serum AFGP activity was determined in terms of thermal hysteresis, i.e. the difference between the equilibrium melting and non-equilibrium freezing points (f.p.s.). Among the four populations, the Ob' Seamount specimen had the lowest serum AFGP activity (0.44 °C) and concentration (4.88 mg/mL), and the highest non-equilibrium f.p. (-1.39 °C). These results are consistent with the warmer, ice-free waters around the Ob' Seamount. The other three higher latitude populations have 2-3 times greater serum AFGP activity and concentration, and much lower non-equilibrium f.p.s. In contrast, the physiological profiles of serum AFGP size isoforms revealed that all *N. rossii* populations, including the Ob' Seamount specimen, possess an extensive complements of AFGP proteins. Isoform variation was observed, especially in the large size isoforms (AFGPs 1-5), when compared to AFGP of the high Antarctic *Dissostichus mawsoni*. The lower levels of AFGP and the absence of some of the large isoforms are likely responsible for higher non-equilibrium f.p.s. of the Ob' seamount specimen.

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2.1. Introduction

The family Nototheniidae is the most speciose, abundant and ecologically diverse family of the suborder Notothenioidei (Eastman 1993, 2005; Rustchmann *et al.* 2011). Within this family, the marble notothen *Notothenia rossii* is widely distributed around the waters of the sub-Antarctic islands of the Southern Ocean and thus is exposed to different temperature regimes (Buchett and Ricketts 1984; DeWitt *et al.* 1990; Barnes *et al.* 2006). This species is semi pelagic and changes habitat during different stages of its life cycle, moving through a wide depth range from 5 to at least 1000 m. It can grow up to a total length (TL) of 92 cm, with commonly reported lengths of about 50 cm TL (DeWitt *et al*. 1990). Previous studies divided *N. rossii* into two sub-species; *N. rossii rossii,* which was distributed around the warmer Iles Kerguelen, Iles Crozet, Prince Edward islands and Macquarie island, and *N. rossii marmorata* which was found around the colder South Georgia and the Scotia Arc Islands (Nybelin 1947, 1951). However, Gon and Klages (1988) and DeWitt *et al.* (1990) observed that the division of this species was based mainly on differences in coloration and lacked support from meristic and morphometric data. They therefore concluded that there was insufficient evidence for the splitting of this species.

The water temperature near the Antarctic continent of the Southern Ocean (where most notothenioid fishes are distributed) varies between -1.0 to -2.0 °C, with a year-round average of -1.87 °C in the high latitude waters (Knox 2007). Marine teleostean fishes residing in these harsh environments should freeze and die because they have body fluids that have a higher freezing point (-0.8 °C) than the seawater (Black 1951; DeVries 1988). Under selection, Antarctic notothenioids have evolved antifreeze glycoprotein (AFGP) that can lower the freezing point of their body fluids below ambient temperature (DeVries 1971; DeVries and Cheng 2005). Water temperatures in the Low and sub-Antarctic regions are higher, ranging between -1.0 and 12 °C with an annual average of 5 °C (Knox 2007). Notothenioid species residing in these regions generally have higher blood freezing points compared to their Antarctic relatives (Ahlgren and DeVries 1984). For example, *Notothenia rossii* of the lower latitude South Georgia (water temperature of -1 to 2 °C) has a higher serum freezing point of -1.58 °C as compared to its congener *N. coriiceps* from the higher latitude Antarctic Peninsula (-1.4 to 0.6 °C), which has a lower serum freezing point of -2.21 °C (Ahlgren and DeVries 1984; Jin and DeVries 2006).

Studies of the origin of the sub-Antarctic notothenioids belonging to the Antarctic clade suggest that they diverged from their Antarctic sister group after the evolution of AFGP in the late to mid-Miocene (Chen *et al.* 1997; Stankovic *et al.* 2002; Near *et al.* 2012). It has been postulated that the Antarctic ancestor of some of these species migrated to the cool-temperate waters with the shifting Antarctic Polar Front in the late Miocene (Kennett 1982; Petricorena and Somero 2007). These species include *N. angustata* and *N. microlepidota* of the southern coast of New Zealand's South Island (maximum seawater temperature of 14 °C), which possess very low AFGP activity in their blood (Cheng *et al.* 2003). The diminished levels of AFGP activity found in notothenioid species of the sub-Antarctic and cool-temperate environments indicate that selection pressure for freezing avoidance is reduced or removed in these environments. Accordingly, AFGP activity is known to differ among notothenioid species residing in different environmental conditions. However, little is known about the effect of thermal habitats on the AFGP trait among populations of a latitudinally widely distributed species.

In this study, we characterize and compare blood serum AFGPs in *Notothenia rossii* from four Southern Ocean localities where they are exposed to different thermal regimes. These were: (1) the Ob' Seamount, located southeast of the Prince Edward Islands in the Indian Ocean sector, which is ice-free and non-freezing throughout the year (Ansorge *et al.* 2008); (2) South Georgia Island, located in the Scotia Arc Islands of the South Atlantic Ocean, which has seawater temperatures that get below freezing point and infrequent sea-ice in winter (Morley *et al.* 2010; Murphy *et al.* 2013); (3) South Shetland Islands, located southwest of the Scotia Arc, north of the Antarctic Peninsula, which have seasonal pack ice (Naganobu *et al.* 1993); and (4) Dallman Bay, which lies between Brabant and Anvers Islands of the Palmer Archipelago in the western Antarctic Peninsula and have pack ice during most of the year (Morley *et al.* 2010; Murphy *et al.* 2013). This study investigates the AFGP trait among *N. rossii* populations associated with their respective thermal habitats and distribution at the different latitudes of the Southern Ocean. Since antifreeze function in the non-freezing environment becomes non-essential and degeneration occurs, a process of mutational changes in the gene that leads to reduction and ultimately disappearance of function (Cheng *et al.* 2003), determining the levels of AFGP in *N. rossii*,

especially of the most northerly Ob' Seamount specimens, may allow us to infer their geographic evolutionary origin.

2.2. Materials and Methods

2.2.1. Specimen and serum sample collection

Notothenia rossii specimens were collected from waters near the Ob' Seamount (53°30′S, 48°31.5′E) southeast of the Prince Edward Islands, South Georgia Island (54°16′S, 36°30′W), near Elephant Island (61°7.29′S, 55°46.5′W) of the South Shetland Islands and in Dallman Bay near Brabant Island (64°14′S, 62°32′W). The single Ob' Seamount specimen was sent frozen to the South African Institute for Aquatic Biodiversity (SAIAB) where blood samples were taken from the heart after it was partially thawed. Specimens from Ob' Seamount and South Georgia Island were collected using hook and line. *Notothenia rossii* and *N. coriiceps* from the Antarctic Peninsula locations were collected by trawl on board of the United States NSF R/V Laurence M. Gould. Blood samples were drawn from the caudal vein after capture, allowed to coagulate, and then centrifuged at 14,000 g for 10 minutes to separate the serum. Serum samples were stored at -80 °C until analysis.

2.2.2. Determination of blood serum osmolality and thermal hysteresis

The osmolalities of blood serum samples were determined using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Lagon, UT).The Opti-Mole (Wescor Inc., Lagon, UT) 1,000 and 290 mOsm standards were used to calibrate the osmometer before use. The calibration of the osmometer was rechecked after every tenth serum measurement, as notothenioid sera contain a volatile that accumulates on the thermocouple of the osmometer with repeat exposure (Bilyk and DeVries 2010a). The serum osmolality was determined by pipetting 10 µl of serum samples onto sample disc paper on the sample chamber. The chamber was closed immediately after pipetting, to prevent evaporation, and then run for 75 seconds to provide sample osmolality measurements. Serum osmolality was measured in duplicate for each sample and the average taken as the sample's osmolality. A third measurement was performed when the measurements between any

two samples differed by more than 10 mOsm with the two closest osmolalities being averaged. The equilibrium freezing point (same as the equilibrium melting point) was then determined by multiplying the osmolality value by -0.001858 °C $mOsm^{-1}$.

Serum antifreeze activity was determined in terms of thermal hysteresis using a Clifton nanolitre freezing point osmometer (Clifton Technical Physics, Hartford, NY) equipped with a cryoscope following published procedures (Bilyk and DeVries 2010a). A single drop of serum sample was injected, using mineral oil filled microcapillary pipette that was attached to a micrometer syringe, into the six wells of the osmometer's sample holder that contained type-B immersion oil. The samples were then frozen by rapid cooling to -40 °C and then melted by slowly increasing the temperature until only a single small ice crystal (5-10 µm diameter) remained. The melting point was measured as the temperature at which the seed ice crystal begins to slowly melt, and approximates the equilibrium freezing point determined with the Wescor vapour pressure osmometer. The non-equilibrium freezing point was the temperature at which a seed ice crystal was observed to burst into unrestricted ice growth. The seed ice crystal was cooled at the rate of 0.074 °C min^{-1} for all serum samples, as variable cooling rates could result in variable non-equilibrium freezing points within and between samples. Thermal hysteresis was calculated as the difference between the observed melting point and the non-equilibrium freezing point temperatures (DeVries and Cheng 2005). Measurements of thermal hysteresis were repeated at least three times for each serum sample.

2.2.3. Characterisation of serum AFGP concentrations and size isoforms

Purification and characterisation of serum AFGP size isoforms using polyacrylamide gel electrophoresis (PAGE) followed Bilyk and DeVries (2010b) with modification. To precipitate the non-AFGP serum proteins, 0.5 mL of the serum samples were treated with equal volumes of 5% trichloroacetic acid (TCA), vortexed and centrifuged at 14, 000g for 15 minutes. The supernatant, containing the TCA-soluble AFGPs, was dialyzed in Spectro/Pro®3 dialysis tubing (MWCO 3, 000 daltons; Spectrum Labs) against distilled water extensively at 4°C, and then lyophilized in pre-weighed microfuge tubes. The final AFGP dry weight from the starting 0.5mL

serum was obtained by subtracting the tube weight, and the physiological AFGP concentration per millilitre of serum was calculated. For PAGE visualisation of isoforms, the lyophilised AFGPs were dissolved in AFGP sample buffer (450 mM boric acid, 30% glycerol, pH8.6) to a final concentration of 50 mg/mL. From this, 300 µg (6 µl) was fluorescently labelled with 2 µl of 4 mg/mL fluorescamine (Fluram: Roche Diagnostic). The labelled proteins were electrophoresed on a discontinuous tris-borate gel of 4% stacker gel and 10-20% gradient resolving gel at 25 mA constant current. The gel image was obtained using a Kodak EDAS 1D gel documentation system.

2.3. Results

2.3.1. AFGP activity and concentration in the blood serum

The estimated values for serum osmolality, equilibrium freezing point, antifreeze activity (measured as thermal hysteresis) and AFGP concentration of *Notothenia rossii* from the four different Southern Ocean locations are given in table 2.1. Serum osmolalities of all specimens ranged between about 420 -500 mOsm, which translate into equilibrium f.p.s. (freezing points) of about -0.8 to -0.9 °C, typical of Antarctic notothenioid fishes of the lower Southern Ocean latitudes (Bilyk and DeVries 2010a, b). The lowest latitude Ob' Seamount *N. rossii* specimen has the lowest antifreeze activity, reflected in the lowest thermal hysteresis (0.44 °C), lowest serum AFGP concentration of 4.88 mg/mL and the highest non-equilibrium f.p. (-1.39 °C) as opposed to the specimens from the three higher latitudes; South Georgia Island, South Shetland Islands and Dallman Bay. This non-equilibrium f.p. is higher than the freezing point of seawater (-1.9 °C), and thus the Ob' Seamount *N. rossii* would not be able to avoid freezing should it encounter icy seawater. On the other hand, the specimens from the three higher latitudes have 2-3 times greater thermal hysteresis and serum AFGP concentrations, resulting in much lower non-equilibrium f.p.s, below the freezing point of seawater (Table 2.1). These results show an apparent trend of organismal antifreeze capacity correlating with environmental severity, being greatest in the higher latitude population of Dallman Bay, followed by the more southerly South Shetland and South Georgia populations , and the lowest latitude Ob' Seamount individual . Additionally, the limitations due a single *N. rossii* individual from Ob' Seamount are noted, and

its thermal hysteresis may not be representative of the mean of the population. This can only become definitive with capturing more specimens in the future. However, if mean thermal hysteresis of Ob' Seamount *N. rossii* population has a narrow range of within-population variations as seen in the two Antarctic localities, then the thermal hysteresis of the individual in this study is quite clearly much smaller.

Table 2.1: Osmolality, AFGP activity and concentration in blood serum of *Notothenia rossii* from four different localities in the Southern Ocean.

Location	**n**	**Osmolality (mOsm)**	**Equilibrium Freezing Point[a] (°C)**	**Melting Point[b] (°C)**	**Non-equilibrium Freezing Point[b](°C)**	**Thermal Hysteresis[d] (°C)**	**Serum AFGP concentration (mg/mL)**
Ob' Seamount	1	482±1	-0.90	-0.94±0.03[c]	-1.39±0.05[c]	0.44±0.03[c]	4.88
South Georgia	5	421±15	-0.78±0.03	-0.82±0.03	-2.08±0.18	1.26±0.18	11.63±1.28
South Shetland	5	502±49	-0.93±0.09	-1.00±0.08	-2.08±0.10	1.08±0.12	10.97±1.95
Dallman Bay	2	416±6	-0.77±0.01	-0.80±0.03	-2.22±0.16	1.43±0.16	14.56-15.22[e]

Analyses were performed using blood serum; where appropriate values are listed as average and standard deviation of technical replicates.

[a]Equilibrium freezing point calculated by multiplying serum osmolality determined using the Wescor vapour pressure osmometer by -0.001858 °C $mOsm^{-1}$.

[b]Melting point and non-equilibrium freezing point determined using Clifton nanolitre freezing point osmometer. The melting point approximates the equilibrium freezing point.

[c]The value was the average of three separate determinations of the single sample.

[d]Thermal hysteresis calculated as the difference between the melting point and non-equilibrium freezing point.

[e]Range of the two Dallman Bay specimens.

2.3.2. Serum AFGP size isoforms

The physiological profiles of serum AFGP size isoforms of *Notothenia rossii*, with *N. coriiceps* and *Dissostichus mawsoni* as comparisons, are shown in Figure 2.1. Regardless of latitudinal locations, all *N. rossii* populations, including the Ob' Seamount individual, possess a similar, extensive complement of AFGP proteins. All species show the conserved isoforms of AFGPs 6, 7 and 8, typical of Antarctic notothenioid fishes. Isoform variations typically occur in the larger sizes, AFGPs 1-5, which is also evident here. Compared to AFGPs of the high Antarctic *D. mawsoni*, *N. rossii* has AFGP isoforms equivalent to the AFGP 2-4 range, but only one isoform or none in the AFGPs 5 range. Interestingly, its sister species *N. coriiceps*, has a different pattern, with no isoforms in the AFGP 3-4 range, and only a few in the AFGPs 5 range.

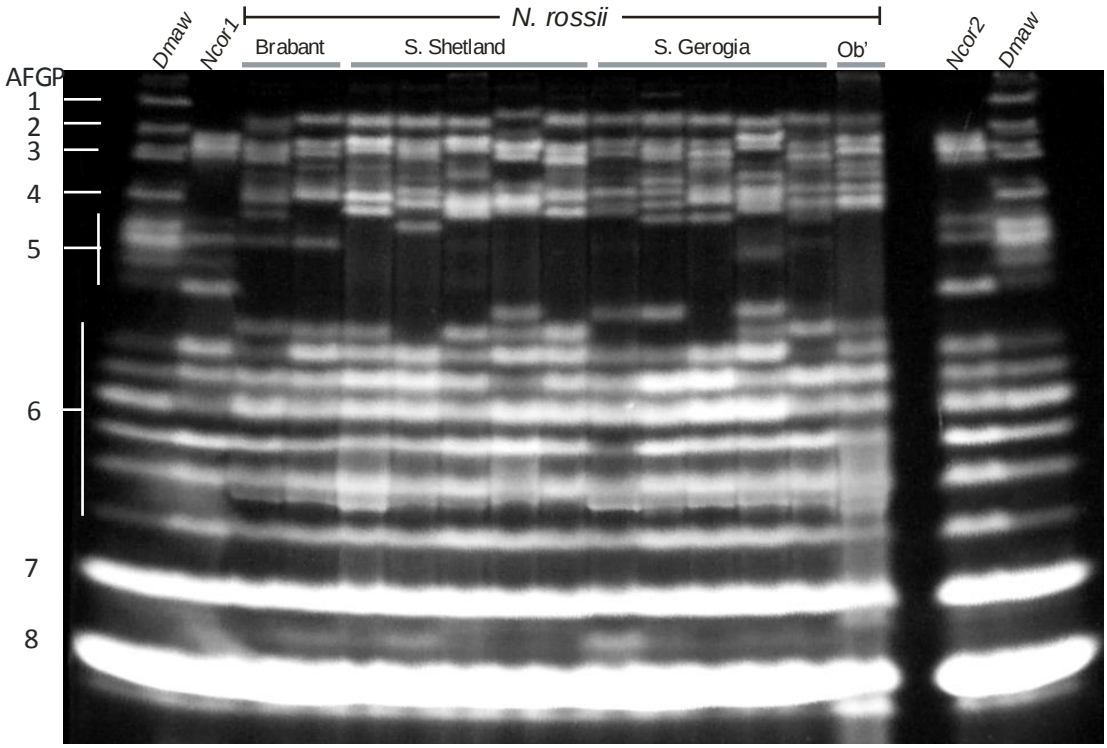


Figure 2.1 Polyacrylamide electrophoretic resolution of physiological composition of AFGP size isoforms (AFGPs 1-8) purified from serum samples of *N. rossii* from Dallman Bay (near Brabant Island), South Shetland Islands (near Elephant Island), South Georgia Island, and Ob' Seamount in the Indian Sector of the Southern Ocean. *Dissostichus mawsoni* (*Dmaw*) and *Notothenia coriiceps* (*Ncor1* and *Ncor2*) AFGPs were used as comparison samples. Each lane represents AFGPs from one individual.

2.4. Discussion

The serum thermal hysteresis of notothenioid fishes has been observed to vary depending on the environmental conditions a species experiences in its habitat. For example, the cryopelagic bald notothen, *Pagothenia borchgrevinki,* which occupies the coldest and iciest habitats associated with the sub-ice platelet layer in high latitude McMurdo Sound, has a much greater serum hysteresis (1.6 °C), compared to those of the blackfin icefish, *Chaenocephalus aceratus* (0.6 °C), which resides in seasonal pack-ice environments of the west Antarctic Peninsula (DeVries and Cheng 2005). Jin and DeVries (2006) found the highest concentration of blood AFGPs in the high-latitude, shallow-water nototheniid fishes of McMurdo Sound (Antarctica) where temperatures are near or at the freezing point of seawater (-1.9 °C) most of the year. The varying magnitudes of antifreeze phenotype from the *Notothenia rossii* specimens in this study are consistent with these reported observations. The Ob' Seamount specimen, collected from the water that is cold-temperate and ice-free throughout the year (Ansorge *et al.* 2008), had one-half to one-third the serum AFGP activity and concentration of the specimens from South Georgia Island, South Shetland Island and Dallman Bay. The Dallman Bay specimens had the greatest serum AFGP activity and concentration, consistent with their higher latitude and colder environment compared to the specimens from the other three localities. On the other hand, Ahlgren and DeVries (1984) observed a non-equilibrium f.p. of -1.5 °C in specimens of *N. rossii* from South Georgia acclimated at 2 °C for several months, substantially higher than that (-2.08±0.18 °C) of the South Georgia Island specimen reported here (Table 1). These findings of AFGP levels, either as a function of varying thermal habitats in nature or of temperature acclimation, indicate that they can be modulated in response to natural environmental or experimental thermal change.

Osmolality measures the concentration of dissolved solutes that depresses the equilibrium f.p. of a solution (DeVries and Cheng 2005). Cold water marine teleost fishes have higher serum osmolalities than temperate or tropical fishes, with the highest being recorded in notothenioid fishes living in the coldest water environments of the High Antarctic. The higher serum osmolality of high latitude Antarctic fishes depresses the equilibrium f.p. to between -1.0 and -1.1 °C. Specimens collected more northerly, in less severe waters of the Antarctic, often have

smaller increases in serum osmolality compared to temperate fishes (DeVries and Cheng 2005; Jin and DeVries 2006). The observed osmolalities of the *Notothenia rossii* specimens collected from South Georgia Island and Dallman Bay were consistent with the lower levels recorded in the more northerly Antarctic species. However, the Ob' Seamount and South Shetland Islands *N. rossii* had blood serum osmolality values similar to those high latitude Antarctic notothenioid fishes with equilibrium f.p.s. near -1.0 °C, which are unexpected values in these waters (Bilyk and DeVries 2010a). Since the Ob' Seamount specimen was frozen before blood was taken, its serum osmolality likely includes intracellular osmolytes from lysed red blood cells (Ramer *et al*. 1995). Therefore, the serum osmolality and equilibrium f.p. of this specimen cannot be used to reliably compare with the specimens from the other three localities.

Antifreeze glycoprotein in notothenioid species is characterised by the eight major size isoforms, which are classified into a large size group (AFGPs 1-5) and small size group (AFGPs 6-8) (DeVries *et al.* 1970). The small size isoforms are only about two-thirds as effective as the large size isoforms in depressing the freezing point in notothenioids (Raymond and DeVries 1977). *Notothenia rossii* and it congener *N. coriiceps* are missing some of the large size isoforms equivalent to those of the high latitude *Dissostichus mawsoni* (Figure 2.1). *Notothenia rossii* is missing isoforms in the AFGPs 5 range, while *N. coriiceps* lack isoforms in AFGPs 3-4 range, which are more potent in depressing the freezing point. Non-equilibrium f.p.s. of *D. mawsoni* from McMurdo Sound are as low as -2.68 °C (Ahlgren and DeVries 1984), substantially lower than those of the two *Notothenia* species from less severe waters of the Antarctic Peninsula and Ob' Seamount (Table 2.1). It has been proposed that the antifreeze activity in the blood serum is influenced by both the total AFGP concentration and the relative proportions of the large (AFGPs 1-5) to the small (AFGPs 6-8) isoforms (Jin and DeVries 2006). The lower abundance of serum AFGPs, and the absence of some of the large isoforms are likely responsible for higher non-equilibrium serum f.p.s. observed for *N. coriiceps* (Jin and DeVries 2006) and *N. rossii* (Table 2.1).

The *Notothenia rossii* specimens from South Georgia Island, South Shetland Islands and Dallman Bay had much higher levels of AFGP in their blood serum than the Ob' Seamount specimen. The annual water temperatures around these South Atlantic Ocean localities are clearly much colder than Ob' Seamount and are at freezing during winter (Morley *et al.* 2010; Murphy *et al.* 2013). The need for adequate antifreeze to avoid freezing is obvious, and thus their sera achieve non-equilibrium f.p.s. lower than the freezing point of the seawater. In contrast, the Ob' Seamount specimen, at 67 cm, is a mature adult (DeWitt *et al.* 1990) that was collected at 1000 m depth in the sub-Antarctic area of the Indian Ocean sector of the Southern Ocean, where antifreeze protection is not needed. To date only adults and no juvenile *N. rossii* have been collected or observed at this seamount and the neighbouring Prince Edward Islands (Gon and Klages 1988; Tankevich 1994). The same situation has been observed with *N. coriiceps* at the latter islands (Gon and Mostert 1992). Therefore, it is possible that *N. rossii* is an occasional migrant at the Ob' Seamount, however there is no available data to support this.

Despite the much lower serum AFGP concentrations, and the presumed absence of a need for AFGP protection in the Ob' Seamount *Notothenia rossii*, it possesses a full complement of intact AFGP isoforms similar to *N. rossii* from South Georgia and the two Antarctic Peninsular locations, as seen when the same amount of the purified physiological AFGP mix from all individuals was resolved on PAGE (Figure 2.1). Apart from the typical among-individual variations of size isoforms in the AFGPs 1-5 range, there is no diminishment in the number or qualitative abundance of the isoforms. Thus one can infer that the encoding genes for this family of isoforms are functional and expressed in the Ob' Seamount *N. rossii*. The reduced serum AFGP abundance therefore may either reflect an across the board reduction in expression of the members of the AFGP gene family, or a much faster turnover rate of the proteins in the temperate water temperatures. Regardless of the scenario, the considerable circulating levels of active AFGPs (4.88 mg/mL) in Ob' Seamount *N. rossii*, which are in fact comparable to some of the lower latitude Antarctic Peninsula notothenioids species (Jin and DeVries 2 006), may be informative of its evolutionary history. In non-freezing environments, antifreeze function is not essential, and will eventually degenerate given enough evolutionary time. For example, the temperate water New Zealand nototheniid *Notothenia angustata* had an Antarctic origin, but

now, after an estimated divergence time of 11 my from its Antarctic ancestor, it exhibits a large number of mutations in its AFGP genes, and possesses barely detectable AFGP levels (0.12-0.46 μg/mL range) (Cheng *et al.* 2003). The presence of a sizable amount of serum AFGPs in the Ob' Seamount *N. rossii* specimen indicates that this species is likely to have extended its distribution into this environment very recently, such that there has been insufficient time for degeneration of the AFGP genes and their expression. In sum, our findings on *N. rossii* provide further support that the magnitude of AFGP phenotype varies in space and time. It is governed by the thermal environment a species experiences in relation to geography, as well as by evolutionary time.

Chapter Three

Characterisation of the freezing avoidance attributes of selected sub-Antarctic notothenioid fishes and inferring the geographic origin of their ancestors.

Abstract

Sub-Antarctic notothenioids are found in warmer environments where antifreeze glycoprotein (AFGP) protection is considered to be unessential. However, studies of antifreeze attributes of sub-Antarctic species belonging to the five Antarctic families present a contrasting picture among species. For example, no AFGP gene was detected in Patagonian toothfish *Dissostichus eleginoides* while small AFGP gene dosage was detected in *Notothenia angustata* of New Zealand. This study therefore aimed to characterise freezing avoidance attributes of sub-Antarctic species and estimate their evolutionary origin using phylogenetic analyses. Antifreeze glycoproteins were characterised in terms of serum thermal hysteresis (AFGP activity) and osmolality, while magnitude of the AFGP gene dosage in genomic DNA was assessed using Southern blot analysis. The two non-Antarctic species and four sub-Antarctic species had thermal hysteresis values (less than 0.1 °C) which were within the margin of error of the equipment used. *Parachaenichthys georgianus*, *Lepidonotothen nudifrons* and *Notothenia rossii* on the other hand showed higher thermal hysteresis ranging from 0.44 to 1.23 °C. Furthermore, these species possessed large AFGP gene families in their genome indicating that they have sufficient levels of circulating AFGPs to survive icy seawaters. *Lepidonotothen squamifrons*, unlike its congeners *L. nudifrons* and *L. larseni* (also with large AFGP gene family), possess a small AFGP gene family, which were non-functinal as detected by Northern blot analysis for AFGP messenger RNA expression. Phylogenetic analyses based on mitochondrial and nuclear NDA genes suggested that sub-Antarctic species shared a most recent common AFGP-bearing ancestor with the Antarctic species. The implication is that the sub-Antarctic species may have diverged after the evolutionary gain of the AFGP gene and that this gene was lost subsequently after they had arrived at their current warmer environments.

3.1. Introduction

There are at least 28 (of 136) notothenioid species that are found in waters around sub-Antarctic islands, as well as in the cool-temperate waters around New Zealand, South America and Australia (Eastman 2005; Eastman and Eakin 2014). These include species belonging to the three non-Antarctic families; Bovichtidae (except *Bovichtus chilensis*), Pseudaphritidae and Eleginopsidae, that are considered to be phyletically basal (Balushkin 1992, 2000; Near *et al.* 2004; di Prisco *et al*. 2007; Matshiner *et al.* 2011), and several species representing the five Antarctic families (Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae and Nototheniidae) (Eastman 2005; di Prisco *et al*. 2007; Matshiner *et al.* 2011). These sub-Antarctic notothenioids are separated from their Antarctic sister taxa by the Antarctic Polar Front (APF), with some species such as the nototheniid *Notothenia coriiceps* being distributed on both sides of the APF (DeWitt *et al.* 1990; Bargelloni *et al*. 2000). Circulating between 50° S and 60° S, the APF was formed 20-25 million years ago (mya) and comprises both a thermal and hydrographic barrier that limits the migration of fish to or from the waters north of it (Kennett 1982; Gon and Heemstra 1990). Molecular studies of the origin of the sub-Antarctic notothenioid species have suggested that several species diverged from their Antarctic taxa after the formation of the APF (Stankovic *et al*. 2002). Since larvae of most notothenioids are planktonic, cold core meso-scale eddies could be responsible for dispersing them across the APF (Clarke *et al*. 2005). The recent origin (1.7 mya) of the channichthyid *Champsocephalus esox* in the sub-Antarctic may be an example of this transport phenomenon (Stankovic *et al*. 2002).

Antarctic notothenioid species have evolved antifreeze glycoproteins (AFGPs) in their body fluid due to selection pressure from their sub-zero freezing environments. These proteins bind with ice crystals to inhibit ice-growth and thus prevent the blood and body fluids of fishes from freezing (DeVries 1971; DeVries and Cheng 2005). Sub-Antarctic notothenioid species on the other hand occur in a less severe environment and ice-free waters, where antifreeze protection becomes unessential (Knox 2007; Eastman 1993). However, the AFGP attributes of sub-Antarctic notothenioids species belonging to five Antarctic families that have been previously studied indicate a more complex picture. For example, Gon *et al*. (1994) found that the blood serum and kidneys of *Notothenia coriiceps* from Marion Island in the south Indian Ocean have

characteristics congruent with the presence of antifreeze proteins. In more recent studies, Cheng *et al.* (2003) detected lower levels of AFGPs in blood serum of the New Zealand *N. angustata*, while Cheng and Detrich III (2007) detected AFGP coding sequences in *Champsocephalus esox* of South America's cool-temperate coastal waters. In contrast, Cheng *et al.* (2003) and Cheng and Detrich III (2007) found no detectable AFGP sequence in the genomic DNA of the notothenиid species belonging to the genus *Patagonotothen* in the same area, and in the Patagonian toothfish *Dissostichus eleginoides*, a sister species to an AFGP-fortified Antarctic toothfish *D. mawsoni*. Furthermore, a phylogenetic analysis indicated that these species share a AFGP bearing common ancestor with their Antarctic sisters (Cheng *et al.* 2003).

The AFGP activity in the blood serum of notothenioid species has been measured as thermal hysteresis, which is the difference between the melting point and non-equilibrium freezing point of a single small ice crystal (DeVries 1971). There is a correlation between environmental temperature, ice abundance and the magnitude of the serum thermal hysteresis. For example, the channichthyid *Chaenocephalus aceratus* residing in 30-300 m of seasonal pack ice environments have a substantially lower thermal hysteresis of about 0.6 ºC (DeVries and Cheng 2005; Bilyk and DeVries 2010a, b), compared to 1.6 °C in the notothenиid *Pagothenia borchgrevinki* that live in 3-30 m depth in High Antarctic waters where ice is present during most of the year (DeVries and Cheng 2005). *Notothenia angustata* living in the ice-free waters of the southern coast of New Zealand's South Island, have minute levels of serum AFGP that can be measured by enzyme-linked immunosorbent assay (ELISA) (Cheng *et al.* 2003). On the other hand, *N. coriiceps* of the Antarctic Peninsula have thermal hysteresis of 1.1 ºC which is similar to that of the notothenиid *Trematomus leonnbergii* that inhabit ice-free waters at 200 m depth in the High Antarctic McMurdo Sound area (DeVries and Cheng 2005; Jin and DeVries 2006). This brief review supports the suggestion that the magnitude of the AFGP phenotype varies with the thermal environment experienced by fish (Miya *et al.* 2014).

Molecular studies on the origin of the AFGP gene indicated that this gene is a synapomorphic character shared by a monophyletic clade of the five predominantly Antarctic notothenioid

families. They estimated that this gene has evolved in the late to mid-Miocene, which coincided with the thermal evolution of the Antarctic freezing environment, and the time of speciation and radiation of notothenioid fishes (Chen *et al*. 1997; Cheng 1998b; Matschiner *et al.* 2011). The species in the three basal notothenioid families possess no AFGP gene in their genome indicating that these families may have diverged before the tectonic isolation and cooling of Antarctica about 38 mya (Cheng *et al.* 2003). Given the complexity of the AFGP gene attributes of the sub-Antarctic species belonging to the five Antarctic families, there is a need for a verification of the status of the antifreeze phenotype and genotype of these species in a manner that can infer their evolutionary history and origin. This chapter attempts to address these issues by mapping AFGP gene characteristics onto notothenioid phylogenetic relationships. This study may also contribute to a better understanding of the response of these species to the warmer sub-Antarctic environment. These analyses should provide information for the re-evaluation of the hypothesis on a single evolutionary origin of the ancestral AFGP gene of notothenioid species.

3.2. Materials and methods

3.2.1. Sample collection

Specimens of notothenioid fishes were collected in the Atlantic Sector waters around South Georgia and South Sandwich Islands, Falkland Islands, Bouvet Island and Tristan da Cunha during the American ICEFISH 2004 cruise (collection period from May to July 2004) aboard the RVIB Nathaniel B. Palmer. Additional Atlantic Sector specimens were collected in the Palmer Archipelago sites including Dallman Bay (July 2008), Hugo Island (August 2008) and Arthur Harbour (July 2008). In the Indian Ocean sector, the specimens were collected from Marion Island during the 2009 and 2011 M.V.S.A *Agulhas* relief cruises (April to May), and from the Ob' Seamount (Chapter Two) during the 2010 *Shinsei Maru 3* fishing trip around the Southern Ocean (Figure 3.1). All these specimens were collected using various collecting methods, such as otter trawl, longline and hook. These specimens include 26 notothenioid species in 14 genera representing both non-Antarctic and Antarctic families (Table 3.1). The tissue samples, including muscle, liver, spleen and gills were harvested from captured specimens and preserved in 99% ethanol. Blood samples were drawn from the caudal vein of fish and allowed to coagulate then centrifuged at 14 000g for 10 minutes to separate serum from red blood cells. Tissue samples,

harvested serum and clotted red blood cells were stored at -80 °C until analyses. Additional DNA sequence data representing 54 Antarctic and two non-Antarctic species were obtained from the GenBank database for the phylogenetic analysis of the notothenioids (Appendix I).

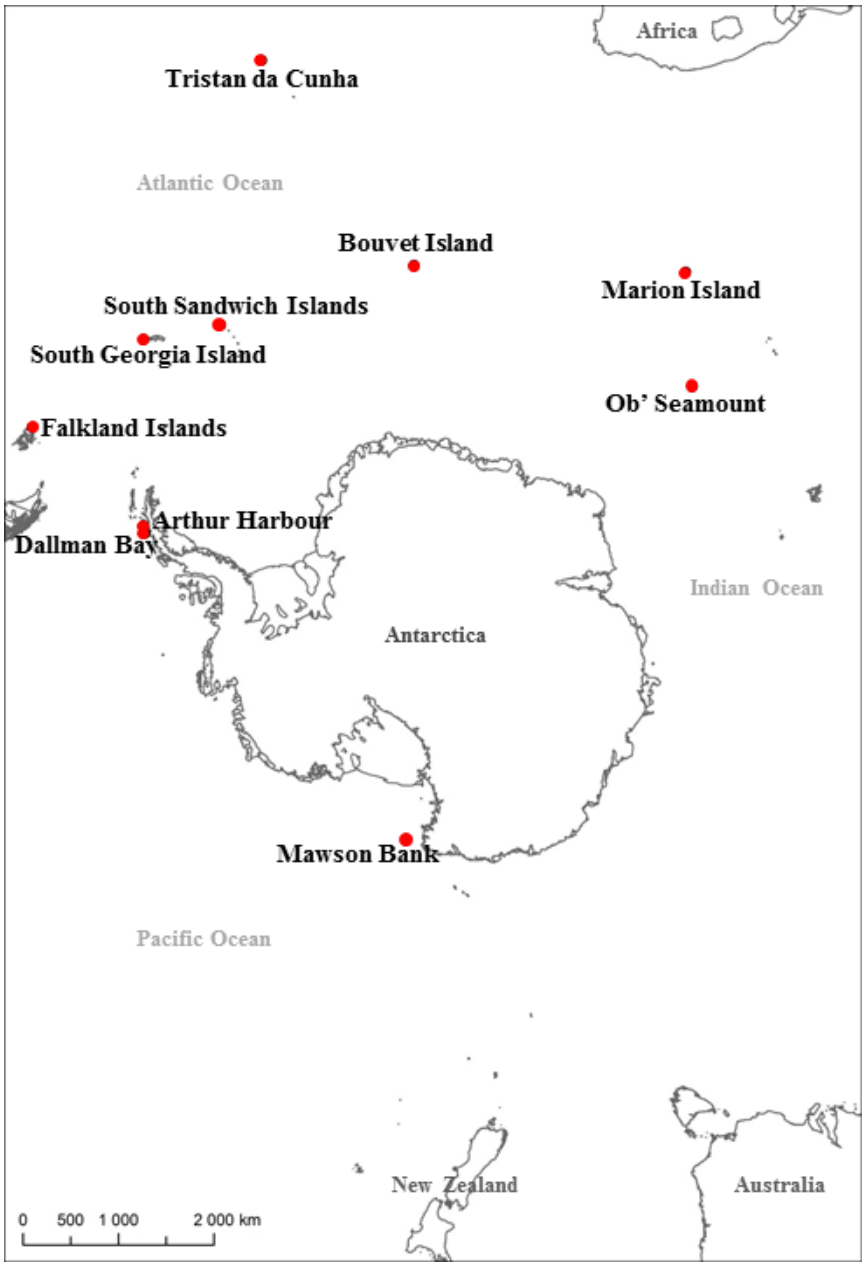


Figure 3.1: Sampling localities of all the specimens used in the study of antifreeze atrributes of notothenioid species.

Table 3.1: Notothenioid species sampled, their locallities and the GenBank accession numbers for each gene sequence.

Species/Family	Locality	GenBank accession numeber		
		ND2	**COI**	**S7**
Bovichtidae				
Bovichtus diacanthus	Tristan da Cunha	KF412875	KF412850	KF412829
Cottoperca trigloides	Falkland Islands	-	KF412851	KF412830
Eleginopsidae				
Eleginops maclovinus[a]	Falkland Islands	KF412874	KF412852	KF412828
Artedidraconidae				
Artedidraco mirus	South Georgia Island	KF412876	KF412853	KF412834
Bathydraconidae				
*Bathydraco sp.**		KF412877	KF412854	-
Parachaenichthys georgianus	South Georgia Island	-	-	-
Channichthyidae				
Champsocephalus esox	Falkland Islands	KF412879	-	-
Champsocephalus gunnari	Bouvet Island	KF412880	KF412856	KF412831
Pseudochaenichthys georgianus	South Georgia Island	KF412878	KF412855	KF412832
Harpagiferidae				
Harpagifer georgianus	Marion Island	KF412881	KF412857	KF412833
Nototheniidae				
Dissostichus eleginoides	Falkland Islands	KF412882	KF412858	KF412835
Gobionotothen acuta[a]	Marion Island	KF412883	KF412861	KF412848
Gobionotothen gibberifrons	Dallman Bay	KF412884	KF412859	KF412847
Gobionotothen marionensis	South Georgia and South Sandwhich Islands	KF412885	KF412860	KF412849
Lepidonotothen kempi[a]	Mawson Bank	KF412886	KF412862	KF412844
Lepidonotothen larseni	Dallman Bay, South Georgia and South Sandwhich Islands	KF412887	KF412863	KF412842

Lepidonotothen nudifrons	South Georgia and Sandwich Islands, Low Island	KF412888	KF412864	KF412841
Lepidonotothen squamifrons	Hugo, Bouvet and South Sandwich Islands	KF412889	KF412865	KF412843
Notothenia coriiceps	Arthur Harbor and Bouvet Island	KF412890	KF412867	KF412845
Notothenia rossii	South Georgia Island and Ob' Seamount	KF412891	KF412866	KF412846
Patagonotothen guntheri	Falkland Islands	KF412892	KF412870	KF412836
Patagonotothen ramsayi	Falkland Islands	KF412894	KF412869	-
Patagonotothen squamiceps[a]	Falkland Islands	KF412897	KF412871	KF412837
Patagonotothen tessellata[a]	Falkland Islands	KF412896	KF412872	KF412838
Patagonotothen wiltoni[a]	Falkland Islands	KF412895	KF412873	KF412839
Paranotothenia magellanica	Marion Island	KF412893	KF412868	KF412840

**Bathydraco* sp was found in the stomach of *Dissostichus mawsoni* collected in the waters of Lazarev Sea (70°04′ S, 09°36′).

[a]Used in the notothenioid phylogenetic relationship construction only.

3.2.2. Determination of blood serum thermal hysterisis and osmolality

Serum antifreeze activity following the procedure described in chapter two using the Clifton Nanolitre Freezing point Osmometer (Clifton Technical Physics). A single drop of serum sample was frozen by rapid cooling to -40 °C and then melted by slowly increasing the temperature until only a single small ice crystal (5-10 µm diameter) remained. The melting point was determined by allowing the ice crystal to set just below its melting point for 3-5 minutes and then cooled slowly by decreasing the temperature until the ice rapidly grows to determine the non-equilibrium freezing point. These processes were examined under a compound light microscope (Zeiss Microscopy) at 250× magnification. Thermal hysteresis (serum antifreeze activity) was calculated as the difference between the observed melting point and non-equilibrium freezing point temperatures. The entire procedure was repeated at least two times for each sample and the average was taken as the final value. Osmolality of blood serum samples was determined using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Lagon, Utah). Serum osmolality was measured in duplicate for each sample and averaged unless the two readings differed by more than 10 mOsm in which case a third measurement was performed.

3.2.3. Southern blot analysis of genomic DNA

Representatives of the five Antarctic families that are also distributed in the sub-Antarctic were included in the search for AFGP gene sequence in genomic DNA. High molecular weight genomic DNA was isolated from 200 to 300 mg of spleen, liver, gills or clotted red blood cells by a standard phenol-chloroform extraction method (Sambrook and Russell 2001). Amounts of 15 to 30 µg of isolated genomic DNA was digested with *EcoRI* enzyme at 37 ºC overnight, and electrophoresed on a 0.9% agarose gel for 14 hours. The digested DNA was vacuum transferred to a Hybond-N nylon membrane (Amersham) and pre-hybridised in QuickHyb solution (Sigma) containing 0.1 mg/mL sheared salmon sperm at 55 ºC for five hours. The membrane was then hybridised to a ^{32}P-labeled probe derived from a 2.2 kb fragment of an AFGP 8 polyprotein gene (clone *Nc*GP8, *Notothenia coriiceps*) that contains only the coding sequence of the highly repetitive tripeptides of AFGP (Hsiao *et al*. 1990). The hybridised membrane was first washed in 1× SSC (15 mM NaCl, 1.5 mM Na citrate)/0.1% SDS at 45ºC and then 0.5% SDS/0.1× SSC at 50 ºC and then the membrane was allowed to dry of the excess liquid for about 5 minutes. The dried membrane (not bone dry)

was scanned with a phosphor-imager (STORM®, Molecular Dynamics) for about 5 hours to examine the hybridisation status of the genomic DNA.

3.2.4. Northern blot analysis of messenger RNA (mRNA)

The functional status of AFGP sequences detected in the genomic DNA of *Lepidonotothen squamifrons*, *L. larseni* and *L. nudifrons* was examined by Northern blot analysis. RNA was isolated from the esophagus-stomach junction, pancreatic tissue and liver using Ultraspec RNA isolation reagent (Biotecx Laboratories, Houston), and then resuspended in 0.5 × TE. The liver was used as a negative control as it is known to express no AFGP in notothenioid fishes (DeVries and Cheng 2005; Cheng *et al.* 2006). Amounts of 4 μg for *Dissostichus mawsoni* (standard), 8 μg for *L. nudifrons* and 16 μg for *L. larseni* and *L. squamifrons* RNA were electrophoresed on a 1.2 % agarose/2.2M formaldehyde gel and vacuum transferred to a nylon membrane. The membrane was then hybridised to the same radioactive antifreeze gene probe as in the Southern blot analysis, and scanned with a phosphor-imager.

3.2.5. Phylogenetic analyses of notothenioid species

The phylogenetic relationships among available notothenioid species was constructed using three genes, including two mitochondrial DNA (mtDNA) protein coding genes [NADH dehydrogenase subunit 2 (ND2) and cytochrome oxidase subunit 1 (COI)], and the first intron of the S7 ribosomal protein nuclear DNA (S7 intron 1) gene.

The complete protein-coding region of the ND2 gene was amplified with the Gln/Asn primer pair (Table 3.2) published by Kocher *et al*. (1995) in a 50 μl polymerase chain reaction (PCR) reaction. Thermal cycling conditions for the ND2 gene consisted of an initial denaturation step at 94 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 45-55 °C for 45 sec and 72 °C for 1 min 10 sec. This was followed by a final extension at 72 °C for 4 min. Prior to DNA sequencing, the ND2 gene PCR products were treated with 1.0 unit of Exonuclease I and shrimp alkaline phosphatase for 60 min at 37 °C to degrade single stranded DNA and primers. This was followed by incubation for 15 min in 85 °C to inactivate the enzymes. The treated PCR products were used as templates for Big Dye V.3

(Applied Biosystems) dideoxy chain termination sequencing and were sequenced in both directions to obtain complete ND2 gene sequences. Sequences were read with an AB 3730xl automated sequencer at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois Urbana-Champaign. The COI gene was sequenced using the DNA barcode (Ivanova *et al.* 2007) C_FishF1t1/C_FishR1t1 cocktail primers (Table 3.2) at the Canadian Center for DNA Barcoding (CCDB). The first intron of the S7 ribosomal protein nuclear gene was amplified with S7RPEX1F/S7RPEX2R primer pair (Table 3.2) published by Chow and Hazama (1998) in a 25 µl PCR reaction. Thermal cycling conditions that amplified this gene consisted of an initial denaturation step at 94 °C for 8 min followed by 35 cycles at 94 °C for 1 min, 57.5 °C for 1 min and 72 °C for 1 min 50 sec, then followed a final extension at 72 °C for 4 min. PCR products of the S7 intron 1 gene were purified and sequenced at Macrogen, South Korea.

Table 3.2: List of primers used for PCR amplification

Primer name	**Primer sequence 5'-3'**
NADH dehydrogenase subunit 2 (ND2)[a]	
Gln	CTACCTGAAGAGATCAAAAC
Asn	CGCGTTTAGCTGTTAACTAA
Cytochrome oxidase subunit 1 (COI)[b]	
VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC
FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC
FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA
FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA
S7 ribosomal nuclear intron 1(S7 intron 1)[c]	
S7RPEX1F	TGGCCTCTTCCTTGGCCGTC
S7RPEX2R	AACTCGTCTGGCTTTTCGCC

[a]Kocher *et al*. (1995); [b]Ivanova *et al.* (2007); [c]Chow and Hazama (1998)

The amplified sequences of all three genes were edited manually using ChromasPro version (v) 1.5 software (Technelysium) and aligned with Lasergene v10 (DNASTAR Inc., Madison) and Mega v5.05 (Tamura *et al*. 2011). The gaps in the alignment of the partial S7 intron 1

sequences were treated with the simple insertion/deletion (indel) coding method incorporated in DnaSP v5.10.01 computer program (Librado and Rozas 2009). The indels with the same 5′ and 3′ termini were considered homologous and indels of different length were treated as different events. Maximum likelihood (ML), parsimony and Bayesian inference analyses were used to construct a phylogenetic tree from all available DNA sequences. The most appropriate models of DNA substitution were tested using Modeltest v3.7 (Posada and Crandall 1998), to determine the optimal molecular evolution for the ML phylogenetic analyses, for each dataset, in PAUP* 4.0b10 (Swofford 2002). The estimated likelihood scores and parameters were selected under the Akaike information criterion (AIC) (Akaike 1973), as this criterion allows non-nested models to be ranked and compared, and facilitates the identification of groups of models that have similar fits to the dataset (Burnham and Anderson 2002; Buckley and Cunningham 2004). The best-fit model selected for both ND2 and COI mtDNA datasets was the general time-reversible model with invariable sites and gamma distribution (GTR+I+G), while for the nuclear S7 intron 1 gene was the transversional model (TVM) (Posada 2003). These models were run in PAUP* 4.0b10 using heuristic searches algorithm with tree-bisection-reconnection (TBR) branch swapping method and 10 random addition replicates to find best ML trees, respectively.

Parsimony analyses were also performed using PAUP* 4.0b10 with equally weighted characters via a heuristic search algorithm with TBR branch swapping method and 100 random addition replicate data sets. Support for nodes was assessed with non-parametric bootstrap analysis using 1000 pseudoreplicates. Bayesian inference analyses were performed using MrBayes v3.0B (Ronquist and Huelesenbeck 2003) to determine posterior probability values with a run of 1×10^6 generations. Four Monte Carlo Markov chains (MCMC) were run simultaneously in each analysis and the analyses were repeated four separate times. This was to ensure that the algorithm was appropriate to provide convergence estimations of the tree topology with the best posterior probability, branch lengths, the parameter values of substitution models and posterior probability estimates of the node support (Near *et al*. 2004). All the posterior probabilities that were greater than 95% were considered to be significant branch support. The non-Antarctic families have been shown previously as consistent sister lineages of the Antarctic clade (Bargelloni *et al.* 1994; Near *et al.* 2004; Near and Cheng 2008), therefore species belonging to these families were used as outgroups in all analyses.

Finally the AFGP characters and geographic distribution of species in family Nototheniidae were mapped onto these phylogenetic trees.

3.3. Results

3.3.1. Serum osmolality and AFGP activity in sub-Antarctic notothenioids

Osmolality, thermal hysteresis and non-equilibrium freezing point levels were tested on available blood serum from the two non-Antarctic bovichtid species, six nototheniids and one bathydraconid (Table 3.3). The thermal hysteresis values of the two non-Antarctic species *Bovichtus diacanthus* and *Cottoperca trigloides*, and the nototheniids *Paranotothenia magellanica*, *Patagonotothen guntheri*, *P. ramsayi* and *Lepidonotothen squamifrons* are near the margin of error (less than 0.1 °C) for hysteresis measurements with the nanoliter osmometer. This suggests that these species have either too little or no AFGPs in their blood. However, a hexagonally faceted ice crystal was observed in blood serum of the four nototheniid species (Figure 3.2), while a round ice crystal was observed in two non-Antarctic species. The hexagonal shape observed in the ice crystal of the nototheniids indicate the adsorption of antifreeze molecules to the prism faces of ice and retardation of a-axes growth (Knight *et al.* 1993). Consquentely, these indicate the presence of antifreeze proteins in the blood of these nototheniids. The other two nototheniids *L. nudifrons* and *Notothenia rossii*, on the other hand, had resolving hysteresis measurements, with thermal hysteresis of 1.05 °C and 0.44–1.23 °C, and serum osmolalities from 536 mOsm and 482-454 mOsm, respectively. Similar to *N. rossii* specimens from South Georgia Island, South Shetland Islands and Dallman Bay (see Chapter Two), *L nudifrons* from South Georgia and South Sandwich Islands had non-equilibrium freezing point (-2.06 °C) below the freezing point of seawater (-1.9 °C) (Table 3.3). This suggests that *L. nudifrons* possesses sufficient AFGPs in their blood that would prevent them from freezing should they encounter icy seawaters. The bathydraconid *Parachaenichthys georgianus* of South Georgia Island also had higher thermal hystersis with lower non-equilibruim freezing points indicating that they possess substantial levels of serum AFGP acitvity.

Table 3.3: Serum osmolality and AFGP activity of selected sub-Antarctic notothenioids.

Species	***n***	**Osmolality (mOsm)**	**Melting Point (°C)**	**Non-equilibrium Freezing Point (°C)**	**Thermal Hysteresis[a] (°C)**
Bovichtus diacanthus	1	362	-0.80	-0.82	0.02[b]
Cottoperca triglides	1	400	-1.04±0.01[b]	-1.07±0.02[b]	0.03±0.01[b]
Paranotothenia magellanica	13	498±26	-1.00±0.14	-1.02±0.14	0.03±0.01[b]
Patagonotothen guntheri	1	434	-0.94±0.02[b]	-0.98±0.02[b]	0.04[b]
Patagonotothen ramsayi	2	441±6	-0.99±0.12	-1.03±0.13	0.05±0.01[b]
Lepidonotothen squamifrons	3	472±30	-0.82±0.08	-0.85±0.08	0.03±0.01[b]
Lepidonotothen nudifrons	3	536±17	-1.02±0.04	-2.06±0.17	1.05±0.15
Notothenia rossii[c]	12	454±52	-0.88±0.11	-2.12±0.16	1.23±0.20[*]
Notothenia rossii[c]	1	482±1	-0.94±0.03	-0.94±0.03	0.44±0.03[†]
Parachaenichthys georgianus	2	555±103	-1.26±0.18	-1.94±0.05	0.67±0.14

Analyses were performed using blood serum from each species; where appropriate values are listed as species average and standard deviation of technical replicates.

[a]Thermal hysteresis calculated as the difference between the melting point and non-equilibrium freezing point.

[b]Thermal hysteresis values (less than 0.1 °C) are within the margin of error for hysteresis measurements and they have no resolving power.

[c]*Notothenia rossii* results from Chapter Two (Miya *et al.* 2014); [*]average of Atlantic sector specimens and [†]the Ob' Seamount specimen in the Indian sector.

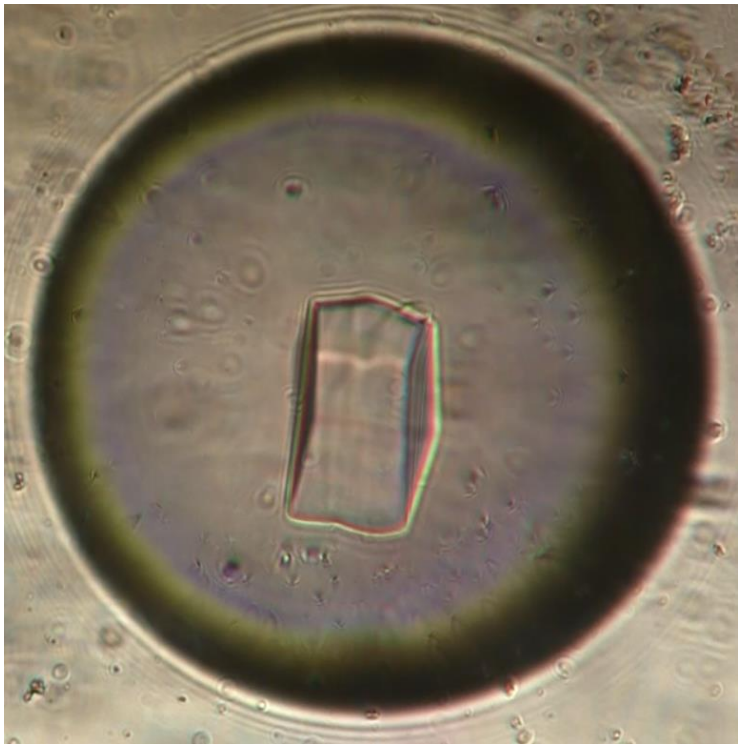


Figure 3.2: Faceted hexagonal ice crystal of *Lepidonotothen squamifrons* at the hysteresis freezing point.

3.3.2. AFGP gene sequence in genomic DNA of sub-Antarctic notothenioids

Results of the Southern blot analyses of *Eco*RI digested genomic DNA from 16 notothenioid species representing the five Antarctic families (Harpagiferidae, Artedidraconidae, Bathydraconidae, Channichthyidae and Nototheniidae) are shown in figure 3.3. The DNA of sub-Antarctic species belonging to the four Antarctic families, except Nototheniidae, had weaker hybridisation bands compared to those of other species in figure 3.3A. However, some species like *Parachaenichthys georgianus* of South Georgia Island had multiple AFGP-positive bands (<6 bands), indicating that this species possesses a large AFGP gene family. This is also consistent with the high levels of circulating AFGPs in the blood of *P. georgianus* (Table 3.3). *Harpagifer georgianus* of Marion Island had three weak hybridisation bands suggesting that this species possesses a small dosage of AFGP genes. On the other hand, the family Nototheniidae had species with varying AFGP genes dosage in their genome as indicated by strong, weak or no hybridisation bands. *Dissostichus eleginoides* from Falkland Islands, a sister species of the Antarctic AFGP-fortified *D. mawsoni* (Figure 3.2A), and the two *Patagonotothen* species (Figure 3.3B), *P. ramsayi* and *P.*

guntheri, had no detectable hybridisation bands indicating the absence of a functional AFGP gene in their genome. These results are consistent with the warmer environments at the Falkland Islands. *Patagonotothen guntheri*, however, is also found in the cold waters at the tip of Antarctic Peninsula, but it had no functional AFGP gene (Cheng and Detrich III 2007). The Marion Island's *Paranotothenia magellanica* (Figure 3.3B) and *Lepidonotothen squamifrons* of Bouvet and South Sandwich Islands (Figure 3.3C) had very weak hybridisation bands, consistent with their non-measurable AFGP phenotype (Table 3.3). This suggests that AFGP function in the genomic DNA of these two species is reduced at the localities where they were collected.

The two *Lepidonotothen* species, *L. larseni* and *L. nudifrons*, unlike their congener *L. squamifrons*, had stronger hybridisation bands indicating that these species possess a large AFGP gene family. Although these two species have sub-Antarctic distribution, they are also found in the freezing Antarctic waters, where antifreeze protection is essential. The presence of large AFGP gene family in the genome of *L. larseni* and *L. nudifrons* (Figure 3.2C) and high levels of circulating AFGPs in blood of *L. nudifrons* (Table 3.3) indicate that these species can survive the ice-laden Antarctic waters. Furthermore, the other nototheniids that are both Antarctic and sub-Antarctic, including *Notothenia rossii*, *N. coriiceps* and *Gobionotothen gibberifrons*, also had multiple AFGP-positive bands indicating the presence of multiple colocalised AFGP gene-bearing DNA fragments. Interestingly, *Gobionotothen marionensis*, which is restricted to the warmer waters of the Southern Ocean had stronger hybridisation band, indicating that this species possesses a large AFGP gene dosage and may be able to survive in ice-laden seawater.

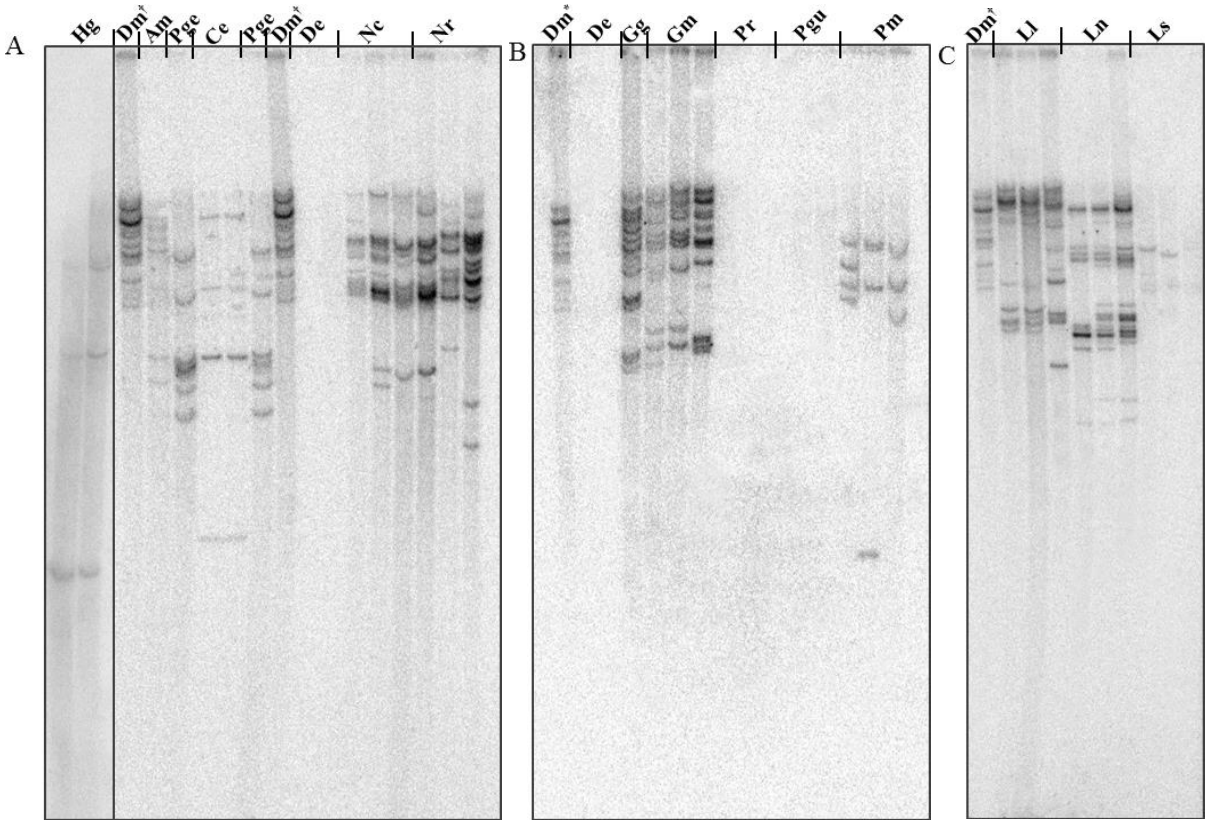


Figure 3.3: *EcoR*I digested genomic DNA blots of notothenioids belonging to five Antarctic Notothenioidei families, hybridised to a P^{32}-labeled AFGP-specific probe. Figure 3.3A consists of two spliced blots separated by a border. *Dissostichus mawsoni* (Dm) was used as standard species in all three blots. Species representing five Antarctic families are Harpagiferidae: Hg, *Harpagifer georgianus* (DNA concentration of 30 µg); Artedidraconidae: Am, *Artedidraco mirus* (30 µg); Bathydraconidae: Pge, *Parachaenichthys georgianus* (20 µg); Channichthyidae: Ce, *Champsocephalus esox* (20 µg); Nototheniidae: Dm, *Dissostichus mawsoni* (*12 µg and †15 ug); De, *D. eleginoides* (*25 µg and †40 µg); Nc, *Notothenia coriiceps* (20 µg); Nr, *N. rossii* (20 µg); Gg, *Gobionotothen gibberifrons* (20 µg); Gm, *G. marionensis* (25 µg); Pr, *Patagonotothen ramsayi* (20 µg); Pgu, *P. guntheri* (15 µg); Pm, *Paranotothenia magellanica* (15 µg); Ll, *Lepidonotothen larseni* (30 µg); Ln, *L. nudifrons* (20 µg); Ls, *L. squamifrons* (30 µg).

3.3.3. AFGP messenger RNA gene expression of *Lepidonotothen* species

Northern blot analysis detected no hybridisation signal in the liver of *Lepidonotothen squamifrons, L. larseni and L. nudifrons.* This was consistent with the fact that the liver is known to express no AFGP (Cheng *et al.* 2006). Although the RNA blot is smeary, it still shows that the stomach-esophagus junction of *L. larseni* and *L. nudifrons* hybridised to the AFGP-specific probe (Figure 3.4). The pancreatic tissue of *L. nudifrons*, like that of *Dissostichus mawsoni* (used as a standard), also hybridised to the AFGP-specific probe. These hybridisations indicate that the AFGP genes in this species are functional and are transcribed into mRNA in these tissues. Furthermore, this is consistent with the stronger hybridisation bands in their genomic Southern blot analyses (Figure 3.3C) and the higher levels of serum AFGPs of *L. nudifrons* (Table 3.3). On the other hand, *L. squamifrons* tissues had no detectable hybridisation signal in all tissue samples, which indicates the absence of the AFGP mRNA. These results suggest that the two weak AFGP-positive bands in the genomic Southern blot analysis (Figure 3.3C) of this species are non-functional, hence AFGP phenotype was unmeasuarable (Table 3.3).

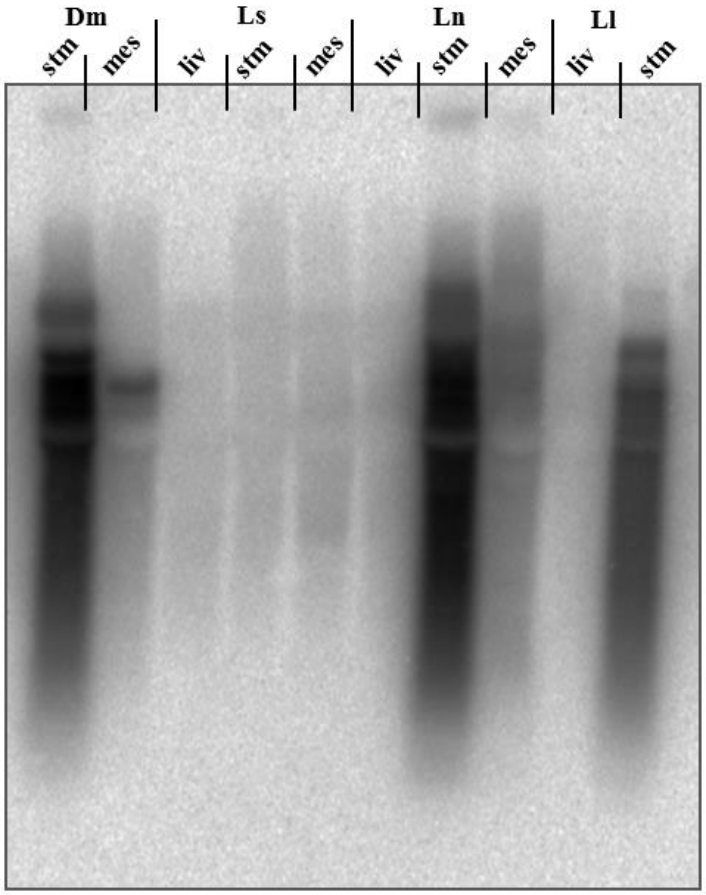


Figure 3.4: Antifreeze glycoprotein gene expression of messenger RNA in the stomach-esophagus junction, pancreatic tissue and liver of *Lepidonotothen* species, hybridised to a P^{32}-labeled AFGP-specific probe. The smears may be due to excess salt in RNA samples probably from the 0.5×TE used to resuspend RNA. Species are Dm, *Dissostichus mawsoni* (RNA concentration of 4 µg); Ls, *L. squamifrons* (16 µg); Ln, *L. nudifrons* (8 µg); Ll, *Lepidonotothen larseni* (16 µg).

3.3.4. Distribution of the AFGP gene on the notothenioid phylogenetic tree

The aligned data set of a complete ND2 and partial COI mtDNA genes, together with GenBank sequences published by other authors (Appendix I), contained 1047 and 608 base pairs (bp), respectively. The partial S7 intron 1nuclear gene alignment contained 614 bp with gaps. These sequences were deposited into the GenBank database with the accession numbers KF412828-KF412897 (Table 3.1). The two mtDNA genes (COI and ND2) produced comparable phylogenetic tree topologies in both parsimony and Bayesian analyses (Appendix II and III), with slight differences to the S7 intron qnuclear trees (Appendix IV), and between the parsimony and Bayesian trees. Two of notothenioid families the Nototheniidae and

Bathydraconidae were paraphyletic. The species belonging to the latter family studied so far, including the Low Antarctic *Parachaenichthys georgianus* (Table 3.3 and Figure 3.3A), possess a large AFGP gene dosage (Ahlgren and DeVries 1984; Wohrmann 1996; Cheng *et al.* 2003). The Nototheniidae have four major clades (A, B, C and D) that differed from each other in the magnitude of the AFGP gene dosage in their taxa. These four clades were moderate to strongly supported in all analyses with parsimony bootstrap scores of ≥ 73% and significant Bayesian posterior probabilities (≥ 96%) (Figures 3.5 and 3.6). Geographic distributions of nototheniid species around different latitudes of the Southern Ocean were characterised following the three Antarctic regions defined in Chapter One, i.e. High Antarctic, Low Antarctic and sub-Antarctic regions.

Clade A consisted of nototheniid species which are found in all three Antarctic regions and have varied AFGP gene dosage in their genome. This clade was strongly supported with 100% bootstrap score and 100 % significant Bayesian posterior probability. The AFGP bearing *Trematomus* species and *Pagothenia borchgrevinki* that are mostly found in the High Antarctic region were in monophyletic sub-clade with bootstrap support of 57%. The mitochondrial DNA trees suggested that *Lepidonotothen squamifrons*, with a non-functional AFGP gene, is more closely related to the AFGP-null *Patagonotothen* species from the sub-Antarctic region, than it is to the other *Lepidonotothen* species with functional AFGP gene that are found in both Antarctic and sub-Antarctic waters (Figure 3.5). This relationship was also observed by Dettai *et al.* (2012) from combined datasets of nuclear and mitochondrial genes. Clade B consisted of *Gobionotothen* species which possess large AFGP gene family in their genome, except for the sub-Antarctic *G. acuta* antifreeze attributes of which are unknown. The S7 intron 1 nuclear gene analyses indicated that clade B is sister to the other four Antarctic families (Figure 3.6), and have bootstrap support of 77% and significant Bayesian posterior probability of 97%. Clade C contained the High Antarctic nototheniid species *Aethotaxis mitopteryx* and *Dissostichus mawsoni* with a large AFGP gene family and its sister *D. eleginoides* found in the Low Antarctic and sub-Antarctic that had no AFGP gene in its genome (Figure 3.3). The S7 intron 1 nuclear gene dataset further revealed that clade C is sister to a group composed of all remaining nototheniid species and the other four Antarctic families (Figure 3.6). Lastly, Clade D represents nototheniid species in the genera *Paranotothenia* and *Notothenia* with bootstrap support of 100% and significant Bayesian posterior probability of 100%. The ND2 analyses (Figure 3.5) of this clade indicated two

monophyletic sub-clades; the first sub-clade contained AFGP bearing *N. rossii* and *N. coriiceps* that are found in both sides of the APF with bootstrap support of 64%. The second group consisted of the sub-Antarctic *N. angustata*, *N. microlepidota* and *P. magellanica* that have small AFGP gene families in their genome (Cheng *et al.* 2003). Unlike the S7 intron 1 nuclear gene dataset, the mitochondrial DNA dataset indicated that this clade was sister to the other four Antarctic families (Figure 3.5). Although there were some differences between the S7 intron 1 nuclear and mitochondrial genes analyses, these four nototheniid clades were congruent in all analyses.

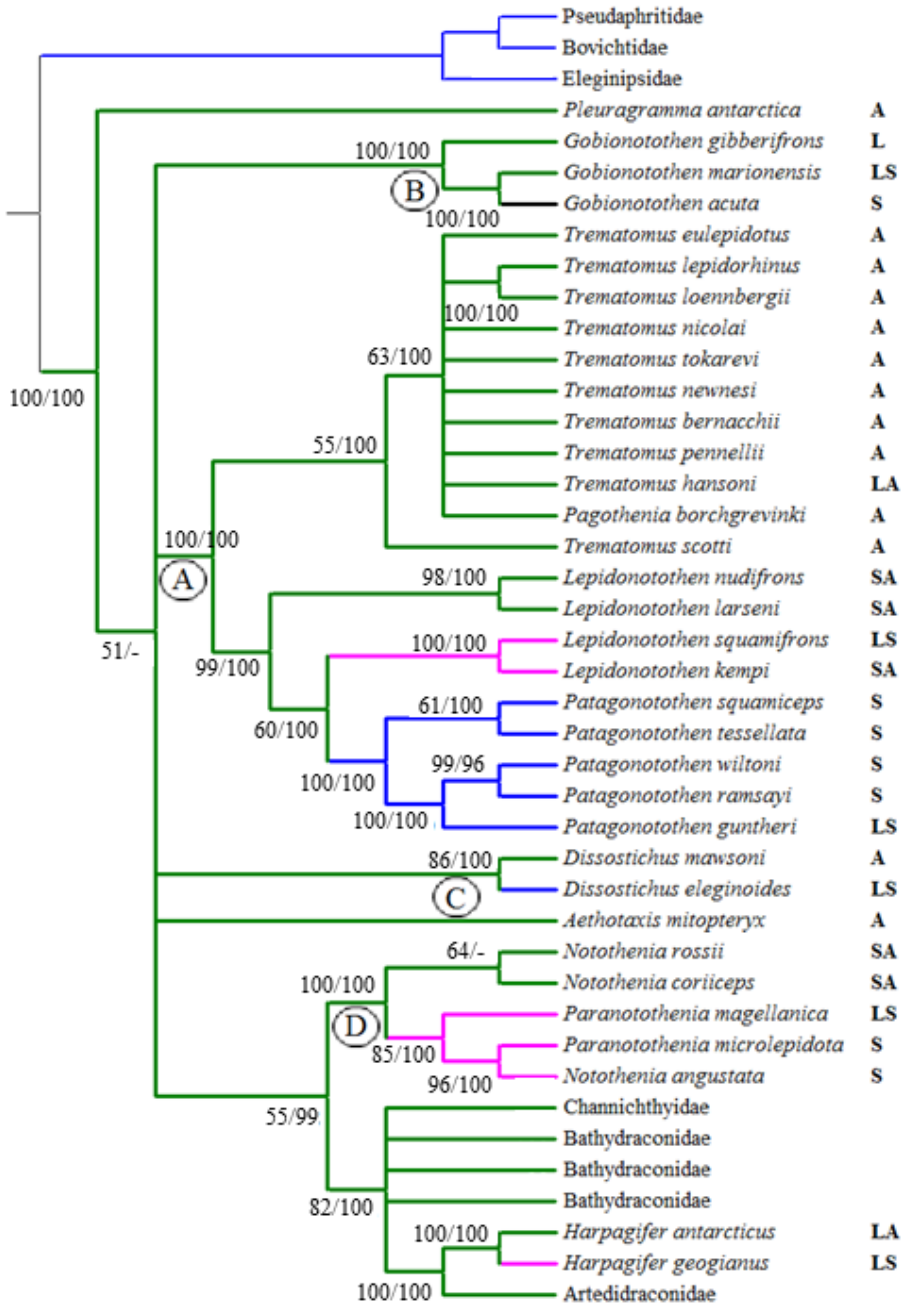


Figure 3.5: Phylogenetic tree resulting from parsimony analysis of the mtDNA ND2 gene dataset; numbers at nodes represent parsimony bootstrap values (≥ 50%) followed by Bayesian posterior probabilities (≥ 95%). Branch lengths are shown on a maximum likelihood tree (Appendix IIIB). The encircled letters refer to specific nototheniid clades discussed in the text. Different colours represent the AFGP gene dosage in each species; Green: large AFGP gene, Pink: reduced or small AFGP gene, Blue: absence of AFGP gene, Black: unknown. Letters after taxa represent geographic distribution of the specie; S: sub-Antarctic and/or cool-temperate, A: Antarctic, L: Low Antarctic, SA: sub-Antarctic and Antarctic, LA: Low and High Antarctic, LS: Low Antarctic and sub-Antarctic. The geographic distribution of notothenioid species followed that of DeWitt *et al.* (1990) and Bargelloni *et al.* (2000).

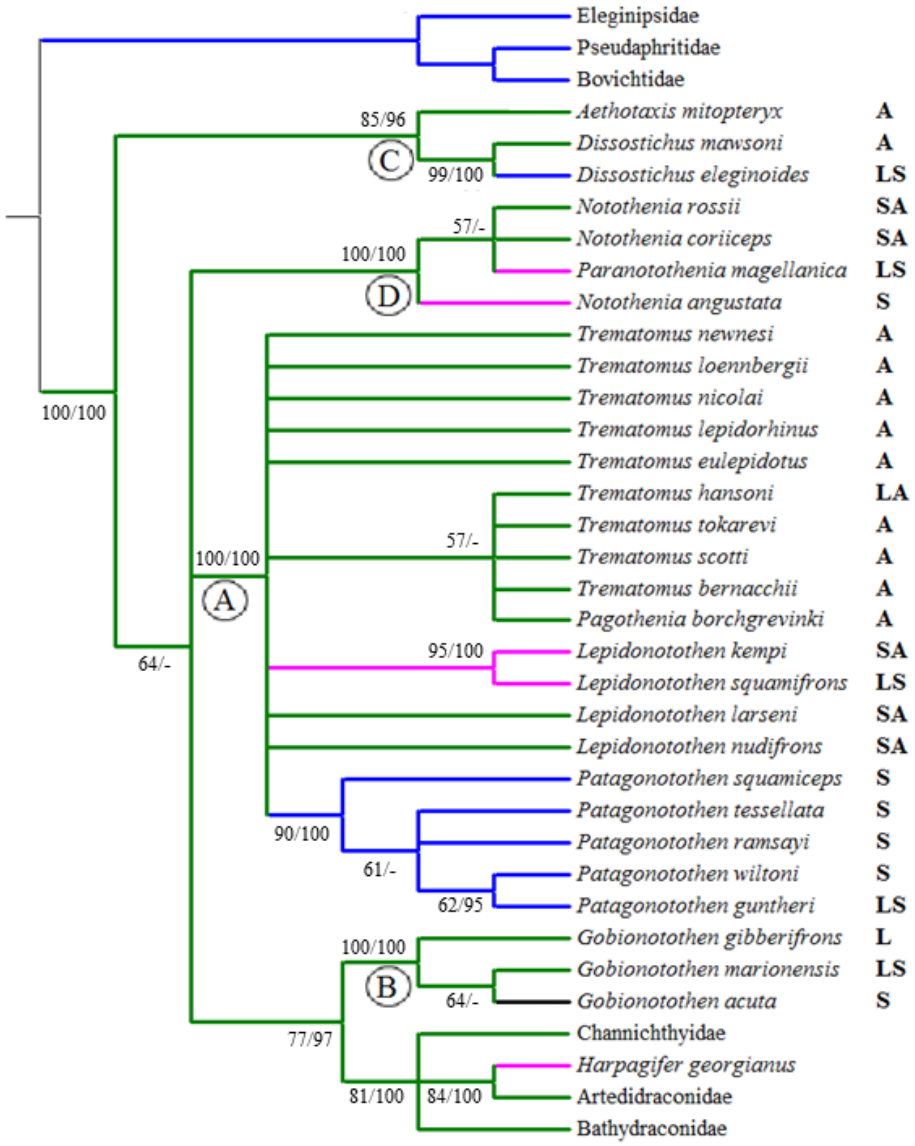


Figure 3.6: Phylogenetic tree resulting from parsimony analysis of ribosomal protein S7 intron 1 nuclear gene dataset. Descriptions of the tree are similar to that of mitochondrial DNA dataset (Figure 3.4). Branch lengths are shown on a maximum likelihood tree (Appendix IVB)

3.4. Discussion

3.4.1. AFGP attributes of sub-Antarctic notothenioid species

Studies of the origin of the AFGP gene in Antarctic notothenioid fishes have estimated that its evolution time correlates with the geological and thermal evolution of the Antarctic freezing environment (Cheng 1998b; Chen *et al.* 1997; Near *et al*. 2012). Phylogenetic analyses supported the monophyly of the five Antarctic notothenioid families [Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae (Appendix II, III and IV)], and previous studies indicated that this clade shares a most recent common AFGP ancestor (Chen *et al*. 1997; Cheng 1998b; Matschiner *et al.* 2011). The Antarctic notothenioid species inhabiting the freezing environments of the high latitudes maintain the high levels of AFGPs in their blood that are necessary to avoid freezing (DeVries 1988; Cheng 1996; DeVries and Cheng 2005). However, a number of notothenioid species belonging to these five Antarctic families are found in the less severe climes in the Lower Antarctic latitudes and in the non-freezing sub-Antarctic waters (Eastman 2005). Of these, species belonging to families Artedidraconidae, Bathydraconidae and Channichthyidae possess large AFGP gene family suggesting that these species would not freeze should they encounter icy seawater (Figure 3.2A). Furthermore, the bathydraconid *Parachaenichthys georgianus* of South Georgia Island had a non-equilibrium freezing point of -1.94 °C (Table 3.3), which is slightly lower than the freezing point of the seawater (-1.9 °C). The harpagiferid *Harpagifer georgianus* of Marion Island, on the other hand, had weak hybridisation bands (Figure 3.2A) suggesting that the AFGP genotype in this species is greatly reduced and has mutated from AFGP coding sequence. However, there is a need to assess the AFGP phenotype data of this species to infer the real AFGP gene dosage in this species.

The family Nototheniidae had species with varying levels of AFGP gene dosage (Table 3.3; Figure 3.2). The Southern blot analysis of genomic DNA demonstrated that nototheniids, namely *Lepidonotothen nudifrons, L. larseni, Gobionotothen gibberifrons, G. marionensis, Notothenia rossii* and *N. coriiceps*, possess large AFGP gene family sizes (about 4-10 positive bands) as detected by their higher hybridisation signal (Figure 3.2). The present study has demonstrated that there is a correlation between the magnitude of AFGP phenotype and genotype, for example *L. nudifrons* and *N. rossii* also have substantial levels of AFGP in their blood (Table 3.3). A similar trend was observed in the bathydraconid *P. georgianus* of

the South Georgia Island. These results therefore constitute evidence that suggest that species with large AFGP gene dosage produce high levels of blood circulatory AFGPs. In contrast, *Paranotothenia magellanica* and *L. squamifrons*, from the warmer waters of the Southern Ocean, had much smaller AFGP gene family size with about two to four AFGP-positive bands of a weak hybridisation signal (Figure 3.2B and C). Since AFGP protection is not needed in the warmer and ice-free waters, the reduced number of AFGP-positive bands of *P. magellanica* and *L. squamifrons* indicate that the AFGP gene in their genomic DNA may be in the process of degeneration. Moreover, the amount of active AFGP proteins in the blood serum (as determined by thermal hysteresis) of these species was unmeasurable (Table 3.3). In addition, the sub-Antarctic *Patagonotothen* species and *Dissostichus eleginoides* had no hybridisation signal (Figure 3.2A and B), indicating the complete absence of functional AFGP gene in their genome, and this is consistent with their cool-temperate, ice-free environments. These data suggest that *Patagonotothen* species and *D. eleginoides* have been in these warmer environments longer than *P. magellanica* and *L. squamifrons* such that there was sufficient time for the AFGP gene to degenerate completely or become undetectable.

The presence of AFGP or AFGP-like coding sequence in the genomic DNA does not necessarily mean that it is functional (Cheng *et al*. 2003). The functionality of the AFGP sequence is measured by their expression on the mRNA. The AFGP mRNA expression among the three *Lepidonotothen* species that were different in their AFGP gene dosage (Figure 3.3C) was tested in tissues that are known to be strong expression sites of the AFGP mRNA in notothenioids (Cheng *et al*. 2006). Consistent with the presence of large AFGP gene families, *L. nudifrons* and *L. larseni* had stronger mRNA hybridisation signals indicating that these species express functional mRNA AFGP gene. *Lepidonotothen nudifrons* is distributed from the Antarctic Peninsula to the adjacent islands in the Low Antarctic region (DeWitt *et al.* 1990) where the water is cold with infrequent seasonal sea-ice (Murphy *et al.* 2013), such that AFGP protection is needed during ice seasons. *Lepidonotothen larseni* on the other hand is found on both sides of the Antarctic Polar Front (APF) (DeWitt *et al.* 1990), where it experiences different temperature regimes. *Lepidonotothen larseni* specimens used in this chapter to determine AFGP gene functionality were collected in the cold environment of Dallman Bay that have pack ice during most of the year (Table 3.1). There is a need to determine the AFGP gene functionality of *L. larseni* north of APF where the water temperature is warmer.

Lepidonotothen squamifrons, unlike its two congeners, possessed non-functional AFGP genes across its geographic distribution (Figure 3.5; DeWitt *et al.* 1990) where it experiences different temperature regimes. DeVries and Lin (1977) detected no AFGP in blood serum of *L. kempi* Norman 1937 of the Balleny Islands. The merging of these two species (Miller 1993; Schneppenheim *et al.* 1994) extended the distribution of *L. squamifrons* from the warmer ice-free sub-Antarctic waters to the ice-cold Antarctic waters. The specimens used for this study to determine AFGP gene functionality of this species were collected in the ice-cold waters of Hugo Island where AFGP protection is needed. Since notothenioid species can survive undercooling up to -2.5 °C as long as they do not come in contact with ice (O'Grady and DeVries 1982; DeVries and Cheng 2005), *L. squamifrons* may avoid freezing by inhabiting ice-free water layers [160-900 m (Miller 1993)]. However, other species, *Akarotaxis nudiceps*, *Trematomus loennbergii*, *T. scotti*, *Pleuragramma antarctica* and *Notothenia coriiceps*, which were collected in these freezing waters have functional AFGP gene that can prevent them from freezing (Cheng, unpublished; DeVries and Lin 1977).

Although some notothenioid species possess no AFGP gene in their genome (Figure 3.2; Cheng *et al*. 2003; Cheng and Detrcih III 2007), all taxa in this group, including species of the non-Antarctic families, have a trypsinogen-like protease (TLP) gene in their genome (Cheng *et al.* 2003). The TLP gene is considered to be the ancestral protease gene that gave birth to the AFGP gene in the five Antarctic families (Chen *et al.* 1999). The absence of the AFGP gene in the species of non-Antarctic notothenioid families (Table 3.3) indicates that they may have diverged in their warmer environments before the TLP gene has evolved into an AFGP gene. Studies of the origin of notothenioid species distributed in the sub-Antarctic and cool-temperate belonging to the five Antarctic families suggest that these species diverged from their Antarctic sister taxa after the evolution of AFGP gene (Stankovic *et al*. 2002). In the absence of selection pressure in these warmer environments, the AFGP function became unessential, leading to degeneration and/or complete loss of the gene in some notothenioid species.

3.4.2. Origin of the AFGP-bearing ancestor of the sub-Antarctic notothenioids

Although several sub-Antarctic species have lower levels or lack of AFGPs, the mitochondrial and nuclear DNA phylogenetic relationships (Figure 3.3 and 3.5) in this study indicated that they share a common AFGP-bearing ancestor with Antarctic species. Similar results were observed by Cheng *et al.* (2003), who found sub-Antarctic nototheniid species nested within a clade of AFGP-bearing Antarctic species. The notothenioid Antarctic clade (consisting of the five Antarctic families) diverged in the Oligocene-Miocene transition, a period of global cooling and ice sheet expansion in Antarctica (Near 2004; Matshiner *et al*. 2011; Near *et al.* 2012). The several sub-Antarctic and cool-temperate notothenioid species belonging to these five Antarctic families then diverged from their Antarctic sister taxa in the mid-Miocene to Pliocene (Stankovic *et al*. 2002). The divergence time of these sub-Antarctic species correlates with the estimated time of the evolution of the AFGP gene (Miocene) in notothenioid species (Chen *et al*. 1997). The four Antarctic families (Bathydraconidae, Artedidraconidae, Harpagiferidae and Channichthyidae) originated relatively recently with Artedidraconidae (3.0 mya) being the last to diverge (Near *et al.* 2012). With the exception of *Harpagifer georgianus*, all species belonging to these four families that have been studied so far, including sub-Antarctic species (Figure 3.2), possess substantial amounts of AFGP (Ahlgren and DeVries 1984; Cheng and Detrich III 2007; Bilyk and DeVries 2010a; Ghigliotti *et al.* 2013). In agreement with most previous studies (Rustchmann *et al*. 2011; Dettai *et al.* 2012; Near *et al*. 2012), the results of this study supported the paraphyly of family Nototheniidae and revealed complex phylogenetic relationships with regard to AFGP characteristics within this family. For example, the AFGP-null *Dissostichus eleginoides* was clustered in one clade with AFGP-fortified *D. mawsoni* and *Aethotaxis mitopteryx*, indicating that they share a most recent AFGP-bearing common ancestor. This position of *D. eleginoides* in a phylogenetic tree is inconsistent with the possibility that this species diverged before the evolutionary gain of the AFGP gene. Since this species is found in the water column reaching down to about 3000 m in the warmer environments of the Southern Ocean (DeWitt *et al.* 1990; Miller 1993), a more likely parsimonious explanation is that the AFGP gene mutated or was lost at their current warmer habitats.

The nototheniid clade containing the sub-Antarctic species of the genera *Lepidonotothen* and *Patagonotothen* also shared a most recent common ancestor with all of the Antarctic species

of the genera *Trematomus* and *Pagothenia* which possess functional AFGP genes in their genome (Figure 3.4 and 3.5). Nuclear and mitochondrial DNA phylogenetic analyses of the genus *Lepidonotothen* indicated that *L. squamifrons*, with non-functional AFGP genes, is not closely related to the other *Lepidonotothen* species, all of which have functional AFGP genes (Bargelloni *et al.* 2000; Near and Cheng 2008; Tomasziewicz *et al.* 2011; Dettai *et al.* 2012). The S7 intron 1 nuclear gene analyses of Near and Cheng (2008), and the present study did not resolve relationships within this group (Figure 3.5). On the other hand, the mtDNA analyses of Bargelloni *et al.* (2000) and Near and Cheng (2008) indicated that *L. squamifrons* is sister to the other *Lepidonotothen* and the *Patagonotothen* clade, while Tomasziewicz *et al.* (2011), Dettai *et al.* (2012) and this study placed this species only with the *Patagonotothen* clade (Figure 3.4). It has been estimated that the *L. squamifrons-Patagonotothen* group diverged in the late Miocene (Near *et al.* 2012), after the onset of the AFGP gene. Similarly, *N. angustata* with diminished AFGP (Cheng *et al.* 2003) is also more closely related to the AFGP diminished *Paranotothenia* group than it is to the other *Notothenia* species that possess substantial AFGP (Appendix III). The *Paranotothenia-N. angustata* group is also estimated to have divergence time, north of the APF, correlating with the evolution of AFGP (mid Miocene) (Cheng *et al.* 2003). This group, including other *Notothenia* species, shares a more recent common ancestor with the four AFGP-bearing families (Figure 3.4). This relationship is in agreement with the mitochondrial DNA analyses of Near and Cheng (2008), Rustchmann *et al*. (2011) and Dettai *et al*. (2012). The two groups discussed above suggest that sub-Antarctic species in their various relationships may share a most recent AFGP-bearing common ancestor that has undergone the process of AFGP gene degeneration.

The relationships discussed above suggest that sub-Antarctic and cool-temperate species have more likely originated from an ancestor that was distributed south of the APF. There are several possible hypothetical explanations on how notothenioid species ended up north of the APF, including (1) some cool-temperate species of New Zealand and South American waters, such as the *Patagonotothen* species of the Patagonian region, may have migrated with the northward shifting of the APF in the late Miocene and stayed behind when it retreated (Kennett 1982; Petricorena and Somero 2007); (2) the APF is suspected to be leaky and may allow passive transport of planktonic larvae of notothenioid species, such as the sub-Antarctic *Champsocephalus esox*, to the north (Clarke *et al*. 2005); and (3) since the APF effects extend to only 1000 m depth, it does not serve as a barrier for deep-sea notothenioids, like

Dissostichus eleginoides inhabiting depths down to about 3000 m. Deep-sea notothenioids should swim freely without the AFP influence to the north below the 1000 m depth (Miller 1993; Bargelloni *et al*. 2000). The latter species has also been caught in the sub-Arctic waters where it was suspected to be an occasional migrant that gets carried by deep-sea current that is part of the global conveyor belt (Møller *et al.* 2003). However, a combination of all three explanations could account for the dispersal of notothenioid species north of APF. The observed large AFGP gene dosage in DNA of some sub-Antarctic species is inconsistent with the possibility that these species migrated to the warmer environment as a survival mechanism. This however is consistent with the origin of these species in these less harsh waters after the evolution of AFGP.

Mapping the geographic distribution of the notothenioid species onto phylogenetic trees indicated variable presence of the AFGP trait. However, this study revealed that species that have small AFGP gene dosage or no detectable AFGP sequence in their genome are almost exclusively sub-Antarctic. The exception was *Patagonotothen guntheri*, which, despite having no detectable AFGP sequence in its DNA (Cheng and Detrich III 2007), is also found (depth 120-250 m) at Shag Rocks near South Georgia Island where the seawater gets below freezing point and there is infrequent sea-ice in winter. In the absence of ice, notothenioid fishes do not need AFGP protection and these species can survive undercooling up to -2.5 °C (O'Grady and DeVries 1982; DeVries and Cheng 2005). Therefore, *P. guntheri* do not need AFGP protection to survive in their almost ice-free environments. In contrast, the channichthyid *Champsocephalus esox* that is found in the warmer waters around South America had strong hybridisation signal indicating that it still possesses a large AFGP gene family in its genome (Figure 1A). The detectable AFGP sequence of *C. esox* suggests that this species may have arrived recently in these warmer environments, such that insufficient time has elapsed for degeneration of the AFGP genes. Furthermore, individuals of species that are widely distributed around different latitudes in the Southern Ocean, where they are exposed to different thermal regimes, had similar AFGP gene family sizes (Figure 3.2). For example, *Notothenia coriiceps* specimens from ice-cold waters of Arthur Harbour in the Palmer Archipelago of Antarctica (Murphy *et al.* 2013) and those from ice-free waters of Bouvet Island in the lower Antarctic area (Perissinotto *et al.* 1992) had equal hybridisation signal indicating that they possess equivalent levels of AFGPs. Similar results were observed from specimens of the latitudinally widely distributed *Notothenia rossii* which had substantial

levels of AFGPs among the different thermal environments from which they were collected (Chapter Two; Miya *et al.* 2014).

This study and others that carried out molecular phylogenetic analyses of family Nototheniidae have indicated that this family is genetically diverse and paraphyletic (Runtschmann *et al.* 2011; Dettai *et al.* 2012). There is therefore a need to revise its taxonomy in a manner that reflects phylogenetic relationships. Dettai *et al.* (2012) suggested that this paraphyly can be corrected by either splitting the Nototheniidae into a number of new families, or integrating it with the other four Antarctic families and creating subfamilies to conserve the four Antarctic clades. However, in both suggestions sub-Antarctic species will still be nested in clades with Antarctic species, sharing with the latter a most recent AFGP-bearing common ancestor. This association is found in several phylogenetic studies and therefore strongly indicates that sub-Antarctic notothenioid species originated from an Antarctic ancestor that possessed the AFGP gene, and this is supported by their recent origin in their current habitat (Stankovic *et al.* 2002). AFGP protection is not needed in the sub-Antarctic environments and in the absence of selection pressure, the AFGP gene and function in sub-Antarctic species would have become reduced and eventually completely lost as this study (and other studies) demonstrated. Given that sub-Antarctic species have different AFGP gene dosages, with few species still carrying a large AFGP gene family in their genome, these findings suggest that the maintenance and loss of AFGP function is not only coupled to the thermal environment but also subject to the history and evolutionary time of divergence of a species.

Chapter Four

Re-evaluation of the taxonomic status of three nototheniid fishes that are distributed on both sides of Antarctic Polar Front

Abstract

Notothenioid species that are found south and north of the Antarctic polar front (APF) have previously been split into separate species and/or subspecies in their respective localities. These splits were largely based on morphological characteristics. However, the analyses of more recent morphological studies disagreed with these taxonomic acts. Molecular systematic studies conducted on notothenioid species so far have concentrated largely on the Atlantic Ocean sector. This study aimed to re-evaluate the taxonomic status of the three nototheniids, *Lepidonotothen squamifrons*, *L. larseni* and *Gobionotothen marionensis* at the DNA level, by comparing specimens from different localities in the Southern Ocean. Sequence divergences using two mitochondrial genes (ND2 and COI) and one nuclear gene (S7 intron 1) were estimated among different taxonomic levels of notothenioid species focusing on the genera *Lepidonotothen* and *Gobionotothen*. *Lepidonotothen kempi* was nested within *L. squamifrons* in the phylogeny of *Lepidonotothen* with sequence divergences, for all genes, between these two species ranging from 0% to 0.5%, suggesting that *L. kempi* and *L. squamifrons* are populations of one species. The sequence divergence between *L. squamifrons* and other *Lepidonotothen* species was higher (0.8% to 18.7%) indicating that they are different species. The *L. larseni* specimens also represented one population (0.3% to 0.6%) with low geographic variation between Atlantic and Indian Ocean specimens. This study therefore does not support the splitting of *L. squamifrons* and *L. larseni* into different species. The phylogeny of *Gobionotothen* clearly separated individuals of *G. acuta* from Heard and MacDonald Islands from *G. marionensis* individuals into different clades, with sequence divergence of 1.4% (COI) between these clades suggesting they are different species. Therefore, this study supports the existence of two species in Balushkin's (1991) 'marionensis' group, *G. marionensis* and *G. acuta*, and suggests that the populations which were called G. *angustifrons* belong to *G. marionensis*.

4.1. Introduction

The perciform suborder Notothenioidei has several wide-ranging species distributed north and south of the Antarctic Polar Front (APF). Most of these species are in the family Nototheniidae, which is the most speciose family of the suborder (DeWitt *et al*. 1990; Eastman 2005). Currently, this family consists of 59 species in 12 genera that are found throughout the Antarctic and sub-Antarctic regions, as well as in the coastal waters of New Zealand and South America (Eastman 1993, 2005; Eastman and Eakin 2000; Eschmeyer and Fong 2014). There has been some taxonomic uncertainty with regards to the latitudinally widespread species of the Nototheniidae that resulted, over the years, in splitting either into subspecies or separate species. This started with the work of Nybelin (1947, 1951) who divided *Notothenia rossii* into two subspecies and *N. coriiceps* into two species, separating populations of the Atlantic Ocean sector of the Southern Ocean from those of the Indian Ocean sector. For example, he retained the name *N. coriiceps* Richardson 1844 for the Indian Ocean sector and named the Atlantic Ocean sector population *N. neglecta*. When describing these two species, Nybelin (1951) compared 89 specimens from the Scotia Arc islands in the Atlantic Ocean sector with four specimens from Kerguelen Island in the Indian Ocean sector. However, Gon and Klages (1988) and DeWitt *et al*. (1990), using more representative samples in the comparison of the geographical variants, observed that the supposed differences between split species or subspecies were within the range of ontogenetic and individual variation. They therefore, considered *N. coriiceps* and *N. neglecta* as synonymous, and also disagreed with the splitting of *N. rossii* into subspecies (*N. r. rossii* and *N. r. marmorata*).

Systematic reviews after Nybelin (1947, 1951), involving species in the genera *Lepidonotothen* and *Gobionotothen*, led to more taxonomic splitting. DeWitt (1966) described the 'squamifrons' group within *Lepidonotothen*, which included *L. squamifrons* found around the Indian Ocean islands and the Atlantic Ocean's South Georgia and South Sandwich Islands; *L. kempi* distributed along the Scotia Arc islands and around the Antarctic continent; and *L. macrophthalma* found around the Falkland Islands and Burdwood Bank in the South Atlantic Ocean. *Lepidonotothen squamifrons* was further divided into two subspecies, i.e. *L. s. squamifrons* in the Indian Ocean sector and *L. s. atlantica* in the Atlantic Ocean sector (Figure 4.1) by Permitin and Sazonov (1974). The study of Schneppenheim *et*

al. (1994), using protein electrophoresis, concluded that the 'squamifrons' group was 'probably' comprised of one species, *L. squamifrons.* A more recent population genetic study of Jones *et al.* (2008), using the mitochondrial DNA NADH dehydrogenase sub-unit 2 (ND2) gene, found genetic differentiation among *L. squamifrons* individuals in the Atlantic Ocean sector. There is however still no clarity as evident from the separation between *L. squamifrons* and *L. kempi* in several recent papers (Wohrmann 1996; Voskoboinikova and Kellermann 1997; Balushkin 2000; Langenbuch and Portner 2003; Petrov 2011). Balushkin (1976) coined the 'larseni' group also within *Lepidonotothen* when he divided *L. larseni* into four geographically restricted species. This group included *L. larseni* from the South Georgia and Bouvet Islands; *L. nybelini* in the South Orkney and South Shetland Islands; *L. loesha* found around the Balleny Islands in the Pacific Ocean sector; and *L. tchizh* in the Indian Ocean sector islands (Figure 4.2). Balushkin (2000) retained these four species as valid in his last morphological revision of the Nototheniidae. However, Jones *et al*. (2008) found no significant genetic differentiation among the Atlantic sector specimens of the 'larseni' group, implying that *L. larseni* and *L. nybelini* are one species and indicating that this group needs taxonomic revision.

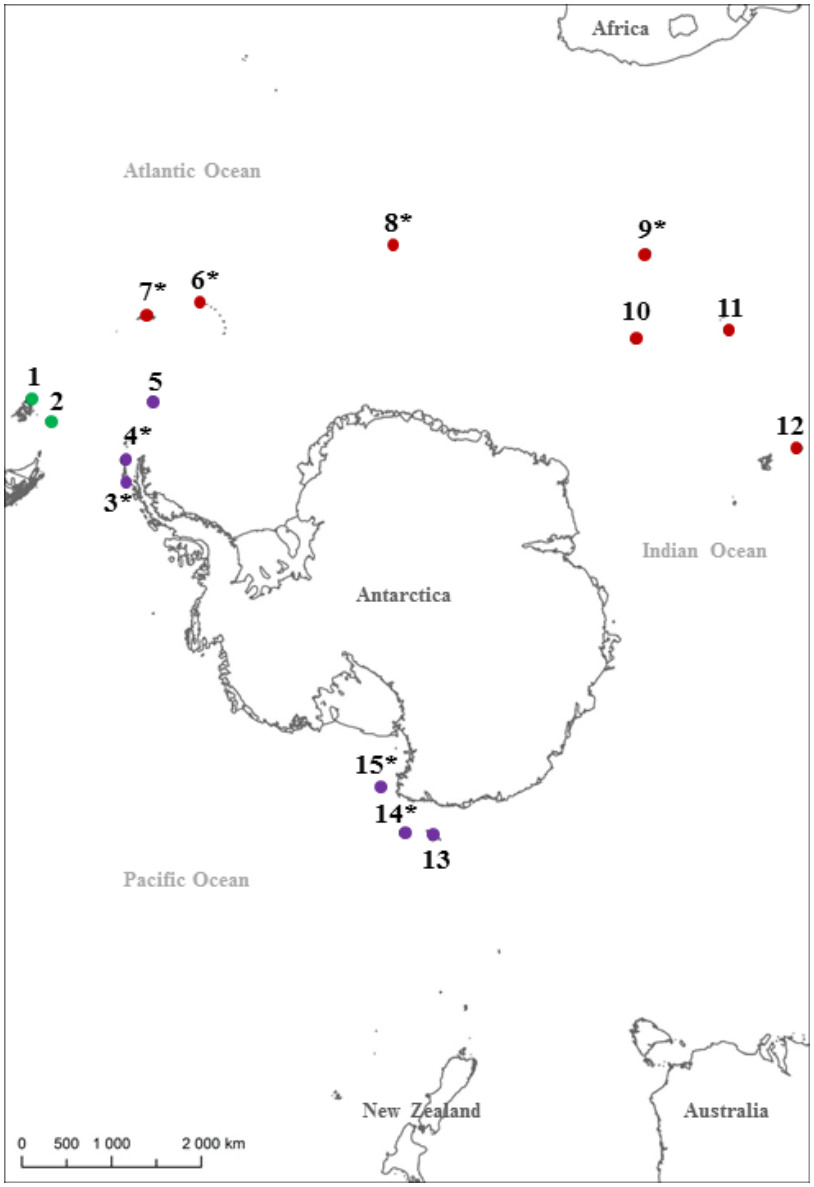


Figure 4.1: Distribution of the three species belonging to DeWitt's (1966) 'squamifrons' group *Lepidonotothen squamifrons* (●), *L. kempi* (●) and *L. macrophthalma* (●).**1**: Falkland Islands, **2**: Burdwood Bank, **3**: Low Island, **4**: Elephant Island, 5: South Orkney Islands, **6**: South Sandwich Islands, 7: South Georgia Island, **8**: Bouvet Island, **9**: Prince Edward Islands (only Marion Island was sampled for the present study), **10**: Lena Seamount, **11**: Crozet Island, **12**: Kerguelen Island, **13**: Balleny Islands, **14**: Scott Island and **15**: Mawson Bank. The sample sites for this study are marked with asterisk.

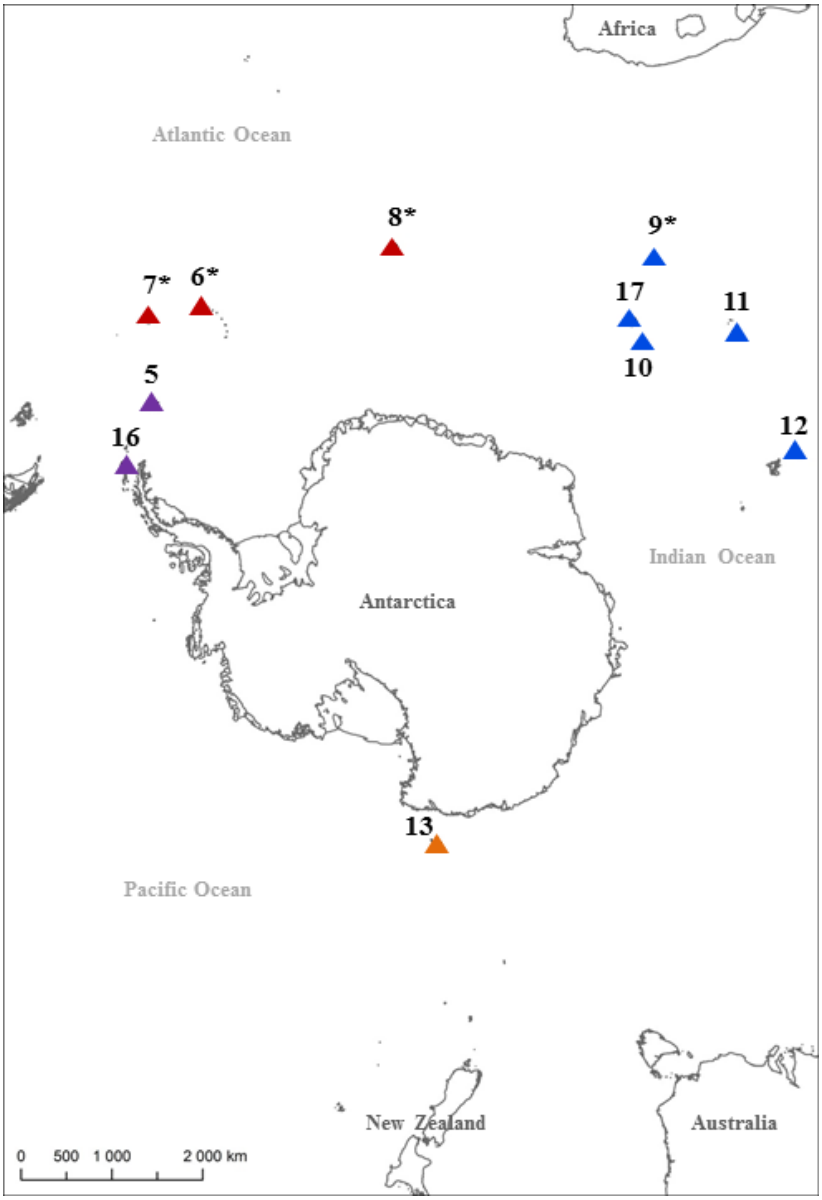


Figure 4.2: Distribution of the four species belonging to Balushkin's (1976) 'larseni' group; including *Lepidonotothen larseni* (▲), *L. nybelini* (▲), *L. loesha* (▲) and *L. tchizh* (▲). Names of localities **5-13** are listed in figure 4.1; **16**: South Shetland Islands and **17**: Ob' Seamount. Asterisk indicates sampling sites for this study.

There has also been taxonomic uncertainty in some species of *Gobionotothen*. *Gobionotothen marionensis* was described as *G. marionensis* (Günther 1880) in the Prince Edward and Crozet Islands of the Indian Ocean sector, and *G. angustifrons* (Fischer 1885) in the South Georgia and South Sandwich Islands of the Atlantic Ocean sector. Later morphological reviews of these species by Boulenger (1902), Lönnberg (1905), Gon and Klages (1988) and DeWitt *et al.* (1990) found insufficient evidence to justify two different taxa, and considered them as synonyms. However, Balushkin (1991) supported the *G. marionensis* splitting and also added *G. acuta*, found in the Kerguelen Islands, to what he called the 'marionensis' group (Figure 4.3). He also described *G. barsukovi* from Heard Island, which Duhamel *et al.* (2005) later recognised as a manifestation of clinal variation in meristics of *G. acuta*. Furthermore, Voskoboynikova and Balushkin (1991) accepted Nybelin's (1947) division of the *G. angustifrons* into subspecies, i.e. *G. angustifrons angustifrons* in the South Georgia Island and *G. angustifrons sandwichensis* of the South Sandwich Islands.

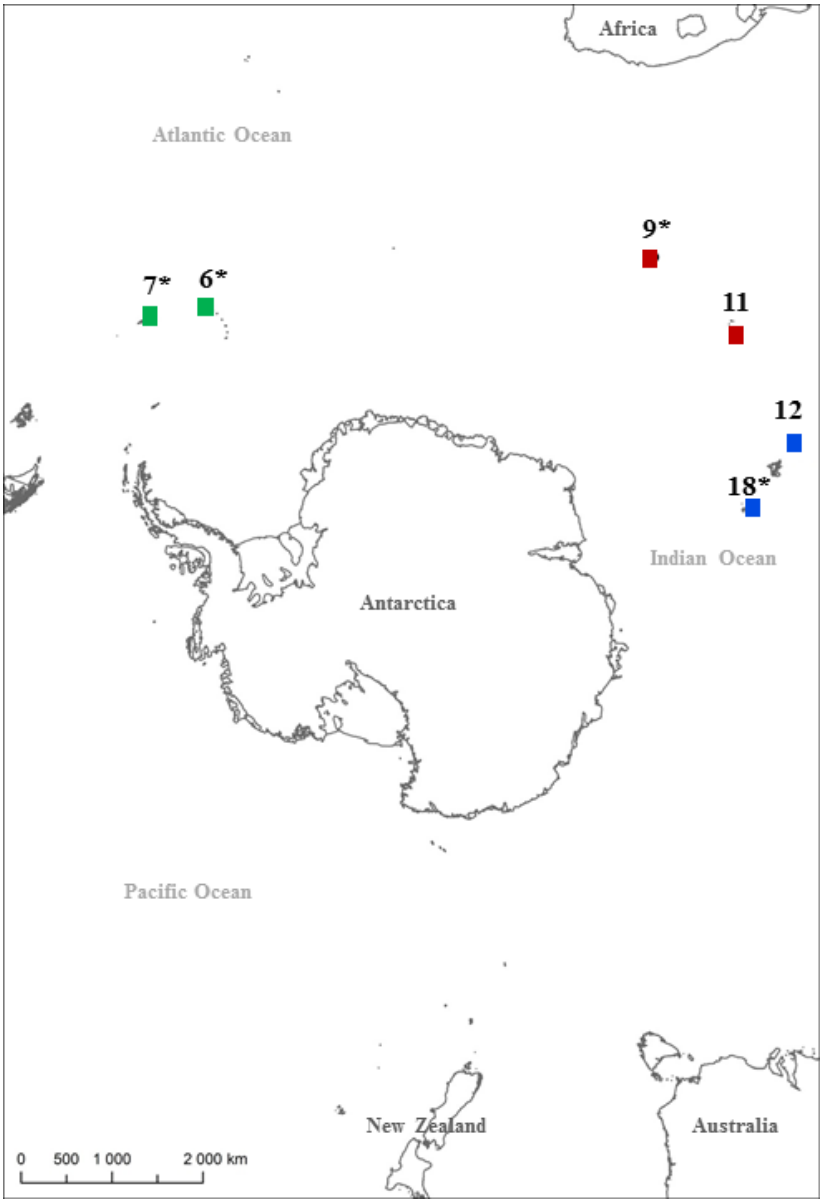


Figure 4.3: Distribution of the three species belonging to Balushkin's (1991) 'marionensis' group *Gobionotothen marionensis* (■), *G. angustifrons* (■) and *G. acuta* (■). **6**: South Sandwich Islands, 7: South Georgia Island, **9**: Prince Edward Islands (only Marion Island was sampled for the present study), **11**: Crozet Island, **12**: Kerguelen Island, **18**: Heard and McDonald Islands. Asterisk indicates sampling sites for this study.

The taxonomic splitting of notothenioid species that are distributed on both side of the APF reviewed above was largely based on morphological characteristics, before the advent of molecular genetics. For example, decisions on the status of *G. marionensis* were based on different anal-fin ray counts and the inter-orbital width (Regan 1913; Norman 1938; Hureau

1985e). However, Gon and Klages (1988) found that the inter-orbital width of this species is subjected to individual variation. Another problem associated with these morphologically based splits was the inability of the keys to identify small fishes as their diagnostic characters were mainly based on the presence or absence of scales on the head (Balushkin 1976; Permitin and Sazonov 1974). Gon and Klages (1988) observed that *L. squamifrons* juveniles at Marion Island lack scales on top of the eye balls, on a large part of the snout and anterior part of the cheeks, and are therefore difficult to identify to subspecies level. Furthermore, the difference in coloration between specimens from different localities was one of the reasons that led to the splitting of species such as *L. squamifrons* and *L. larseni* (Permitin and Sazonov 1974). The problem with colour characters when using museum specimens is that the fixation, preservative and duration in preservative may affect the colour of a fish (Gon and Klages 1988).

It is quite clear from the brief review above that the taxonomy of nototheniids distributed on both side of the APF needs revision. Although a few molecular genetic studies have attempted to review this splitting (Jones *et al.* 2008; Damerau *et al.* 2012), these studies have generally been geographically restricted as they have concentrated on specimens from populations that are distributed around the Atlantic Ocean sector islands. However, it is necessary to compare Atlantic Ocean sector specimens with specimens from the Indian Ocean sector and, when possible, material from type localities to resolve the taxonomic status of these geographically wide-ranging species. The present study reviewed the taxonomic status of three such nototheniid species, namely *Lepidonotothen squamifrons, L. larseni* and *Gobionotothen marionensis* at the DNA level. The advantage of DNA analyses is that this method clusters species based on very similar sequences that allow unambiguous identifications (Lautredou *et al.* 2010), enabling scientists to elucidate relationships that could not otherwise be inferred from the morphological analysis (Bargelloni *et al.* 1994; Klingenberg and Ekau 1996; Ritchie *et al.* 1997; Near *et al.* 2004; Dettai *et al.* 2012). Consequently, this method has become a useful tool in recent studies on the delineation of individuals of the same species and identification of different species (Jones *et al.* 2008; Kuhn and Near 2009; Lautredou *et al.* 2010; Damerau *et al.* 2012). The genetic analysis method will therefore enable the re-assessment of the taxonomic status of the three selected nototheniid species by estimating genetic divergences and the phylogenetic analysis of

geographically distinct individuals between these split species using selected mitochondrial and nuclear markers.

4.2. Materials and methods

4.2.1. Sampling

Tissue samples of *Lepidonotothen* and *Gobionotothen* species were collected in the Atlantic and Indian Ocean sectors of the Southern Ocean during the American ICEFISH2004 cruise aboard the RVIB Nathaniel B. Palmer and the South African relief cruise to Marion Island (2011), respectively. Additional tissue samples were obtained from the Museum of New Zealand, Te Papa Tongarewa (including samples from the Pacific Ocean sector), the Peabody Museum of Natural History, Yale University and the Government of South Georgia and the South Sandwich Islands (GSGSSI). The sampling sites for the three nototheniids groups, i.e. 'squamifrons', 'larseni' and 'marionensis', are indicated in figures 4.1, 4.2 and 4.3, respectively. These tissue samples were collected from 45 specimens (Table 4.1) and were stored in 96% ethanol prior to DNA extraction. Additional DNA sequence data of *Gobionotothen acuta* and *Lepidonotothen mizops* were obtained from the GenBank database (Table 4.1; Appendix V).

Table 4.1: Collection localities and number of tissues harvested from *Lepidonotothen* and *Gobionotothen* species.

Locality	**Numbers of specimens**							
	L. squamifrons	*L. larseni*	*L. kempi**	*L. nudifrons*	*L. mizops*	*G. marionensis*	*G. acuta*	*G. gibberifrons*
Atlantic Ocean localities								
Low Island	1[a]	-	-	-	-	-	-	-
Elephant Island	1[a]	-	-	-	-	-	-	-
South Sandwich Islands	4[b]	2[c]	-	-	-	3[c]	-	-
South Georgia Island	3[c]	3[c]	-	3[c]	-	2[c]	-	3[c]
Bouvet Island	3[c]	2[c]	-	-	-	-	-	-
Indian Ocean localities								
Marion Island	3[d]	4[d]	-	-	-	4[d]	2[d]	-
Head and MacDonald Islands	-	-	-	-	3[f]	-	2[f]	-
Pacific Ocean localities								
Scott Seamount	-	-	2[e]	-	-	-	-	-
Mawson Bank	-	-	1[e]	-	-	-	-	-
Unknown	1[e]	-	-	-	-	-	2[g]	-
Total	**14**	**11**	**3**	**3**	**3**	**9**	**6**	**3**

*The current status of *L. kempi* as junior synonym of *L. squamifrons* is noted (Eschmeyer 2014), the specimens called *L. kempi*, throughout this chapter, were identified as such upon collection in 2008.

Superscript alphabets are specimens from [a]the Peabody Museum of Natural History, [b]the GSGSSI, [c]ICEFISH2004 cruise, [d]Marion Island relief cruise 2011, [e]the Museum of New Zealand, and the GenBank sequences by [f]Smith *et al.* (2012) and [g]Near *et al.* (2012).

4.2.2. DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

DNA was extracted using the DNeasy QIAGEN tissue extraction kit (Qiagen Inc., Valencia, CA) following the manufacturer's specifications. The three gene regions, including NADH dehydrogenase subunit 2 (ND2), cytochrome oxidase subunit 1 (COI) and the first intron of the S7 ribosomal nuclear DNA (S7 intron 1), were amplified by PCR from the extracted DNA. Polymerase chain reactions (PCR), for the genes regions, were performed in 25 µl reactions containing 1 × reaction buffer, 0.8 mM of dNTPs, 1.5-2.5 mM of $MgCl_2$, 0.2 µM of each primer, 1 U Super-Therm Taq DNA polymerase (ABGene, USA) and 2-3 µl DNA template. The complete protein-coding region of the ND2 gene was amplified with the Gln (CTA CCT GAA GAG ATC AAA AC)/Trp (GAG ATT TTC ACT CCC GCT TA) primer pair published by Kocher *et al*. (1995). Thermal cycling conditions for the ND2 gene consisted of an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55-59 °C for 45 sec and extension at 72 °C for 1 min 10 sec, then followed by a final extension at 72 °C for 4 min. The partial COI gene region was amplified with the universal primer pair, dLCO1490 (GGT CAA CAA ATC ATA AAG AYA TYG G)/dHCO2198 (TAA ACT TCA GGG TGA CCA AAR AAY CA), published by Folmer *et al.* (1994). The PCR used the following cycling conditions: an initial denaturation of 2 min at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 54-58 °C for 30 sec and 72 °C for 50 sec, then a final extension of 7 min at 72 °C. The partial first intron of the S7 ribosomal protein gene was amplified with S7RPEX1F/S7RPEX2R primer pair (primer sequences are listed in Table 3.2 of Chapter Three) published by Chow and Hazama (1998). The profile for amplification of this gene included an initial denaturation at 94 °C for 8 min followed by 35 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1min 50 sec, then followed a final extension at 72 °C for 4 min. PCRs were performed in a Mastercycler gradient (Eppendorf, USA).

PCR products of the three gene regions (ND2, COI and S7 intron 1) were sequenced at Macrogen Inc, South Korea. The complete genomic DNA sequence of the ND2 gene region was sequenced with both forward (Gln) and reverse (Trp) primers sequences; while partial genomic DNA of COI and S7 intron 1 gene regions were sequenced with forward primer sequences, dLCO1490 and S7PPEX1F, respectively. Sequences generated in this study were edited manually using ChromasPro version 1.5 (Technelysium). These sequences were

aligned with sequence from GenBank and DNA barcoding, in COI analyses (Appendix V), using Mega 5.05 (Tamura *et al*. 2011) and Clustal X version 2.0 (Larkin *et al.* 2007) software. The S7 intron 1 gene alignment had gaps and these were treated with the simple insertion/deletion (indel) coding method incorporated in DnaSP v5.10.01 software (Librado and Rozas 2009). The indels with the same 5′ and 3′ termini were considered homologous and indels of different length were treated as different events.

4.2.3. Divergence levels among DNA sequences

The estimates of evolutionary distance among sequences were analysed by pairwise genetic distance calculations implemented in Mega 5.05 (Tamura *et al*. 2011) software. This calculates the number of nucleotides substitutions per site between sequences using the Kimura 2-parameter model (Kimura 1980). The rate of variation among sites was modelled with a gamma distribution (shape parameter = 1). The DNA sequence divergence was calculated for different taxonomic levels including family, genus and species, and at the individual level. The genetic divergence levels within notothenioid families and nototheniid genera were analysed using gene sequences obtained in Chapter Three. In species identification analyses, the average genetic distance among individuals within sister species should not exceed average genetic distance between sister species (Hebert *et al.* 2003). Furthermore, since the study species (*Lepidonotothen squamifrons, L. larseni* and *Gobionotothen marionensis*) were separated into different species in their respective geographic range, sequence divergences among the different localities were also calculated.

4.2.4. Phylogenetic analyses

Maximum likelihood (ML) and parsimony analyses were used to reconstruct a phylogenetic tree from the aligned DNA sequences. Maximum likelihood analyses, to determine branch lengths, were performed using the computer program PAUP* 4.0b10 (Swofford 2002). The best model of molecular evolution for the ML phylogeny was determined using Modeltest version 3.7 (Posada and Crandall 1998). The likelihood scores and parameters, as estimated by the Akaike information criterion (AIC), were then used in subsequent ML analyses. Heuristic searches algorithm with tree-bisection-reconnection (TBR) branch swapping method and 10 random addition replicates were used to find the best ML tree. Parsimony

trees were also constructed on PAUP* following the same method as in ML analyses, with 100 random addition replicate datasets. Supports for nodes, in parsimony trees, was assessed with non-parametric bootstrap analysis using 1000 pseudoreplicates. Bootstrap support results were defined as weak (50-69%), moderate (70-89%) and strong support (90-100%); bootstrap support values below 50% were considered to be inconclusive (Felsenstein 1985). The number of variable sites and parsimony-informative characters were also estimated with parsimony criterion. Two *Patagonotothen* species (*P. wiltoni* and *P. tessellata*) were used as outgroup taxa, since this genus is sister to *Lepidonotothen* species [Chapter Three (Figure 3.4)]; Dettai *et al.* (2012); Near *et al.* (2012) and Near and Cheng (2008) found *Patagonotothen* to be sister to genus *Gobionotothen*.

4.3. Results

4.3.1. Estimates of DNA sequence divergences

The aligned sequences were trimmed to equal lengths, which resulted in 1047 base pairs (bp) for ND2, 618 and 603 bp in COI for *Gobionotothen* and *Lepidonotothen* datasets respectively. The trimmed sequences of S7 intron 1 nuclear gene consisted of 699 bp in *Lepidonotothen* and 700 bp in *Gobionotothen* datasets, with gaps. The S7 intron 1 gene had much lower DNA sequence divergence levels [mean ranging from 0.1% (among individuals) to 7.6% (among families)] than of that the two mtDNA genes [mean ranging from 0.3% (among individuals) in COI to 34.1% (among families) in ND2] (Table 4.2). Nonetheless, in all three gene regions the intra-specific variability was smaller than the inter-specific variability such that an increase of genetic variation through increasing taxonomic levels within the suborder Notothenioidei was observed. However, the minimum sequence divergence between sister species was very low ranging from 0% in S7 intron 1 (*Lepidonotothen*) to 0.7% in COI (*Gobionotothen*), indicating that some species within these genera are genetically similar and represent lineages that share a most recent common ancestor. The maximum sequence divergence among individuals of the same species (0.3-1.1%) was much lower than the variation between species of the same genus (1.3-18.7%), and these followed the prerequisite for species identification (Hebert *et al.* 2003). Furthermore, the sequence pairwise comparisons between localities were generally lower than the comparisons among individuals of the same species. The genetic distance between localities of *L. squamifrons* ranged from 0% to 0.9% (Table 4.3), *L. larseni* from 0.2% to

0.6% (Table 4.4) and *G. marionensis* ranged from 0.4% to 0.6% (Table 4.5). These results with zero percent divergence in some cases suggest that *Lepidonotothen squamifrons*, *L. larseni* and *Gobionotothen marionensis* across their sampled localities consist of one population, respectively.

Table 4.2: A summary of sequence divergence of notothenioid species at different taxonomic levels, as well as among individuals of the same group.

Comparisons within	**Genes**	**Taxa**	**Min (%)**	**Max (%)**	**Mean (%)**	**SE* (%)**
Notothenioid families	ND2	8	10.6	51.5	34.1	1.6
	COI	7	9.0	33.5	17.0	1.4
	S7	8	2.4	37.7	7.6	0.6
Nototheniid genera	ND2	9	16.4	48.9	31.5	1.7
	COI	9	5.9	24.7	15.9	1.2
	S7	8	1.4	8.4	4.1	0.4
Lepidonotothen species	ND2	4	0.5	18.7	10.5	0.9
	COI	5	0.2	12.6	6.6	0.9
	S7	4	0.0	1.3	0.8	0.2
Gobionotothen species	ND2	3	0.5	7.2	3.0	0.3
	COI	3	0.7	5.9	2.4	0.4
	S7	3	0.2	1.7	0.7	0.2
'squamifrons' group	ND2	17†	0.0	1.1	0.5	0.1
	COI	18†	0.0	1.0	0.3	0.1
	S7	17†	0.0	0.3	0.1	0.0
'larseni' group	ND2	11†	0.0	0.7	0.3	0.1
	COI	11†	0.0	1.1	0.3	0.1
	S7	11†	0.0	0.9	0.5	0.1
'marionensis' group	ND2	11†	0.1	0.9	0.5	0.1
	COI	11†	0.0	1.1	0.5	0.2
	S7	10†	0.0	0.7	0.4	0.1

*Standard error.

†Number of specimens.

Table 4.3: Pairwise sequence divergence comparison between localities of *Lepidonotothen squamifrons* and *L. kempi*. Highlighted in bold are the highest percentage of sequence divergences.

Localities	**Genes**	**1**[†]	**2**	**3**	**4**	**5**	**6**	**7***	**8***	**9**
2. Bouvet Island	ND2	0.4	-							
	COI	0.5	-							
	S7	0.1	-							
3. South Georgia Island	ND2	0.1	0.4	-						
	COI	0.2	0.3	-						
	S7	0.1	0.0	-						
4. South Sandwich Islands	ND2	0.7	0.6	0.7	-					
	COI	0.3	0.4	0.3	-					
	S7	0.1	0.0	0.0	-					
5. Low Island	ND2	0.8	0.7	0.8	0.3	-				
	COI	0.4	0.7	0.5	0.2	-				
	S7	0.1	0.0	0.0	0.0	-				
6. Elephant Island	ND2	-	-	-	-	-	-			
	COI	0.4	0.3	0.2	0.1	0.3	-			
	S7	0.2	0.1	0.2	0.1	0.1	-			
7. Mawson Bank*	ND2	0.2	0.3	0.3	0.6	0.8	-	-		
	COI	0.2	0.3	0.0	0.3	0.5	0.2	-		
	S7	01.	0.0	0.0	0.0	0.0	0.1	-		
8. Scott Seamount*	ND2	0.3	0.4	0.4	0.7	**0.9**	-	0.2	-	
	COI	0.2	0.3	0.0	0.3	0.5	0.2	0.0	-	
	S7	0.1	0.0	0.0	0.0	0.0	0.1	0.0	-	
9. Unknown	ND2	0.2	0.3	0.3	0.6	0.8	-	0.0	0.2	-
	COI	0.4	0.5	0.3	0.3	0.5	0.2	0.3	0.3	-
	S7	0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.1	-

**L. kempi* localities.

[†]Marion Island

Table 4.4: Pairwise sequence divergence comparison between localities of *Lepidonotothen larseni*. Highlighted in bold are the highest percentage of sequence divergences.

Localities	Genes	1†	2	3	4
2. Bouvet Island	ND2	0.5	-		
	COI	0.5	-		
	S7	**0.6**	-		
3. South Georgia Island	ND2	0.2	0.5	-	
	COI	0.4	0.4	-	
	S7	0.4	**0.6**	-	
4. South Sandwich Islands	ND2	0.3	0.5	0.3	-
	COI	0.4	0.4	0.3	-
	S7	0.4	0.4	0.3	-

†Marion Island

Table 4.5: Pairwise sequence divergence comparison between localities of *Gobionotothen marionensis*. Highlighted in bold are the highest percentage of sequence divergences.

Localities	Genes	1†	2	3
2. South Georgia Island	ND2	**0.6**	-	
	COI	0.4	-	
	S7	0.5	-	
3. South Sandwich Islands	ND2	0.4	**0.6**	-
	COI	0.5	0.4	-
	S7	0.4	0.4	-

†Marion Island

4.3.2. Phylogenetic relationships within *Lepidonotothen* and *Gobionotothen* species

The parsimony-uninformative and informative sites for all three gene regions are indicated in Table 4.6. The ND2 gene has the most parsimony-informative sites which accounted for about 22% and 23% of the *Lepidonotothen* and *Gobionotothen* sequences, respectively. The lower number of parsimony-informative sites (2% and 3%) observed in S7 intron 1 gene may be due to the fact that nuclear genes evolve at a slower rate than mitochondrial genes (Simmons and Ocheterena 2000; Near and Cheng 2008). The ND2 gene phylogenetic relationships were more resolved with strong bootstrap support values while the S7 intron 1 gene displayed low phylogenetic resolution (Figures 4.4 to 4.9). The phylogenetic relationships estimated by the maximum likelihood and parsimony trees were congruent for the *Lepidonotothen* and *Gobionotothen* phylogenies, respectively. Of the five *Lepidonotothen* species analysed, *L. nudifrons*, *L. mizops* (in COI analysis only) and *L. larseni* were clearly genetically separated in different clades from each other, while *L. kempi* was nested within the *L. squamifrons* lineages (Figure 4.4 to 4.6). Similarly, the individuals of *G. acuta* from Marion Island were nested within *G. marionensis*, while *G. acuta* from the unknown locality, and the Heard and MacDonald Islands and *G. gibberifrons* were in distinct monophyletic lineages clearly separated from each other in the ND2 and COI analyses (Figure 4.7 and 4.8). In the S7 intron 1 analysis, the *G. acuta* from the unknown locality was also nested within *G. marionensis* lineage (Figure 4.9).

Table 4.6: The number of parsimony uninformative and information sites for *Lepidonotothen* and *Gobionotothen* analyses. The percentages indicated in the parentheses are calculated from the total data length of the DNA sequences for each gene region.

Genus	Gene	Data length (bp)	Parsimony-uninformative sites	Parsimony-informative sites
Lepidonotothen	ND2	1047	43 (4%)	234 (22%)
	COI	603	36 (6%)	84 (14%)
	S7	699	9 (1%)	20 (2%)
Gobionotothen	ND2	1047	80 (8%)	247 (23%)
	COI	618	37 (6%)	99 (16%)
	S7	700	19 (3%)	37 (5%)

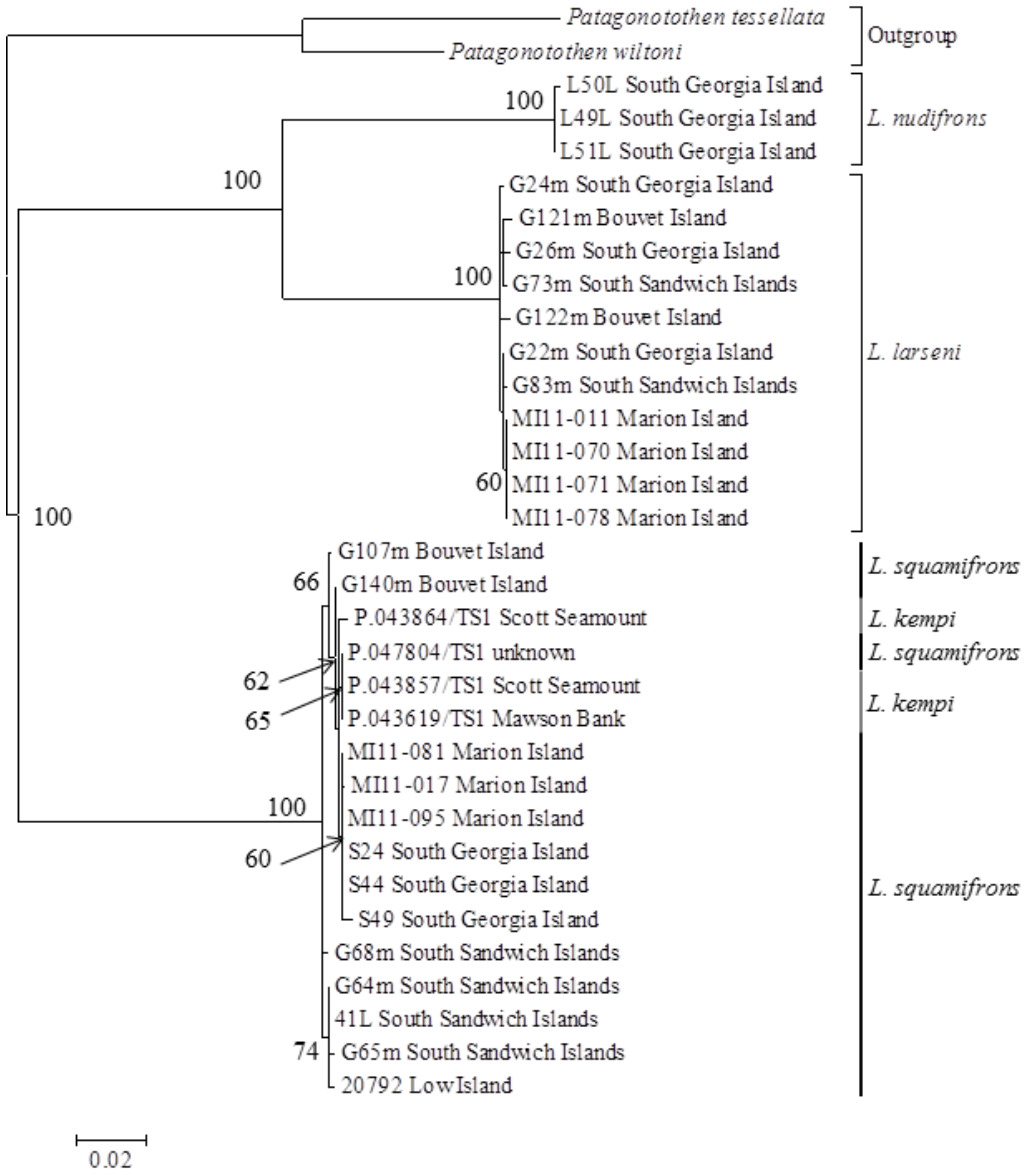


Figure 4.4: Mitochondrial ND2 gene maximum likelihood phylogenetic tree of *Lepidonotothen* species. The bootstrap support values estimated from the parsimony analyses are displayed. The taxon names represent the specimen number followed by the locality name.

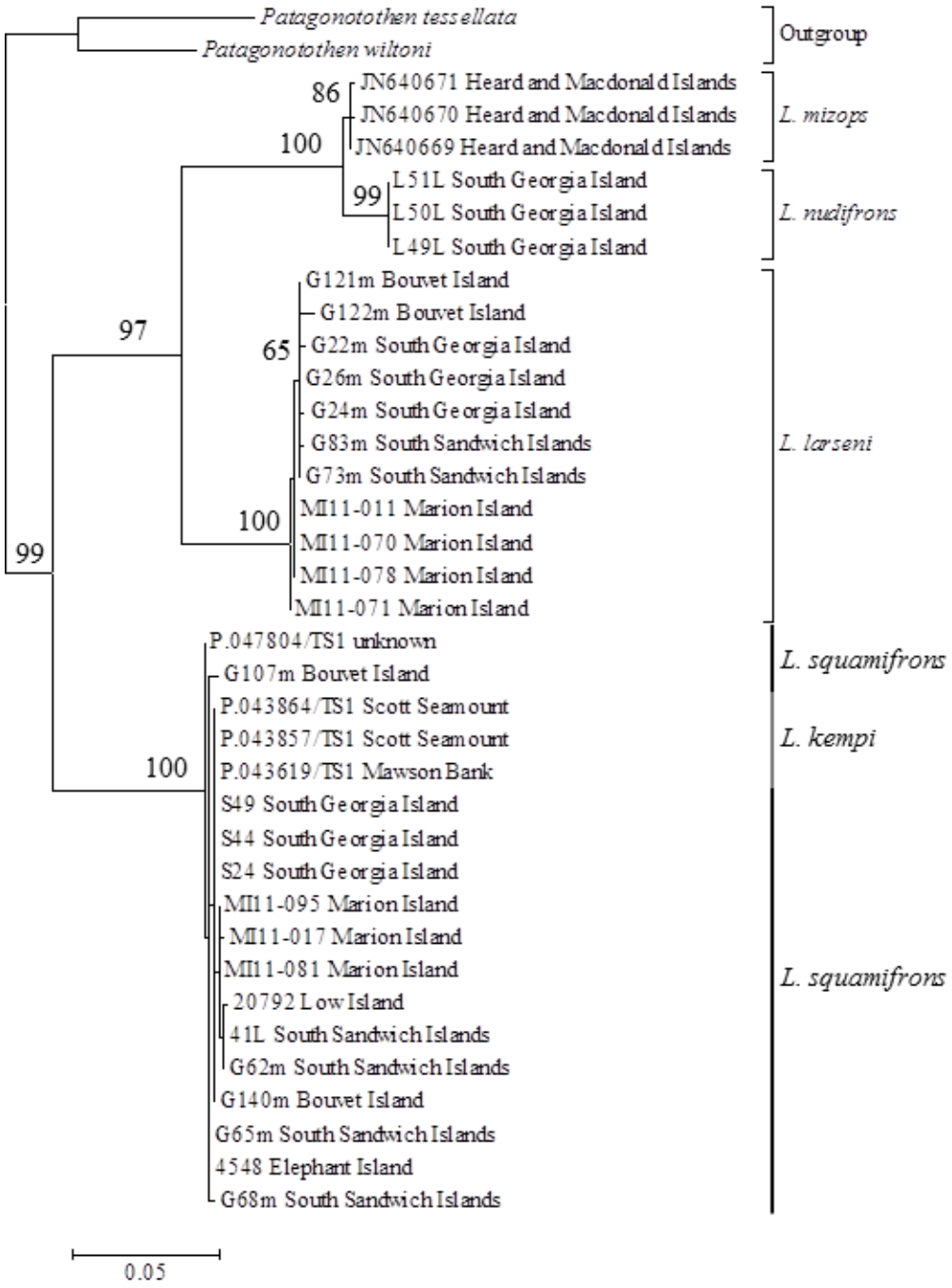


Figure 4.5: Mitochondrial COI gene maximum likelihood phylogenetic tree of *Lepidonotothen* species. The bootstrap support values estimated from the parsimony analyses of this data. The taxon names represent the specimen number followed by the locality name.

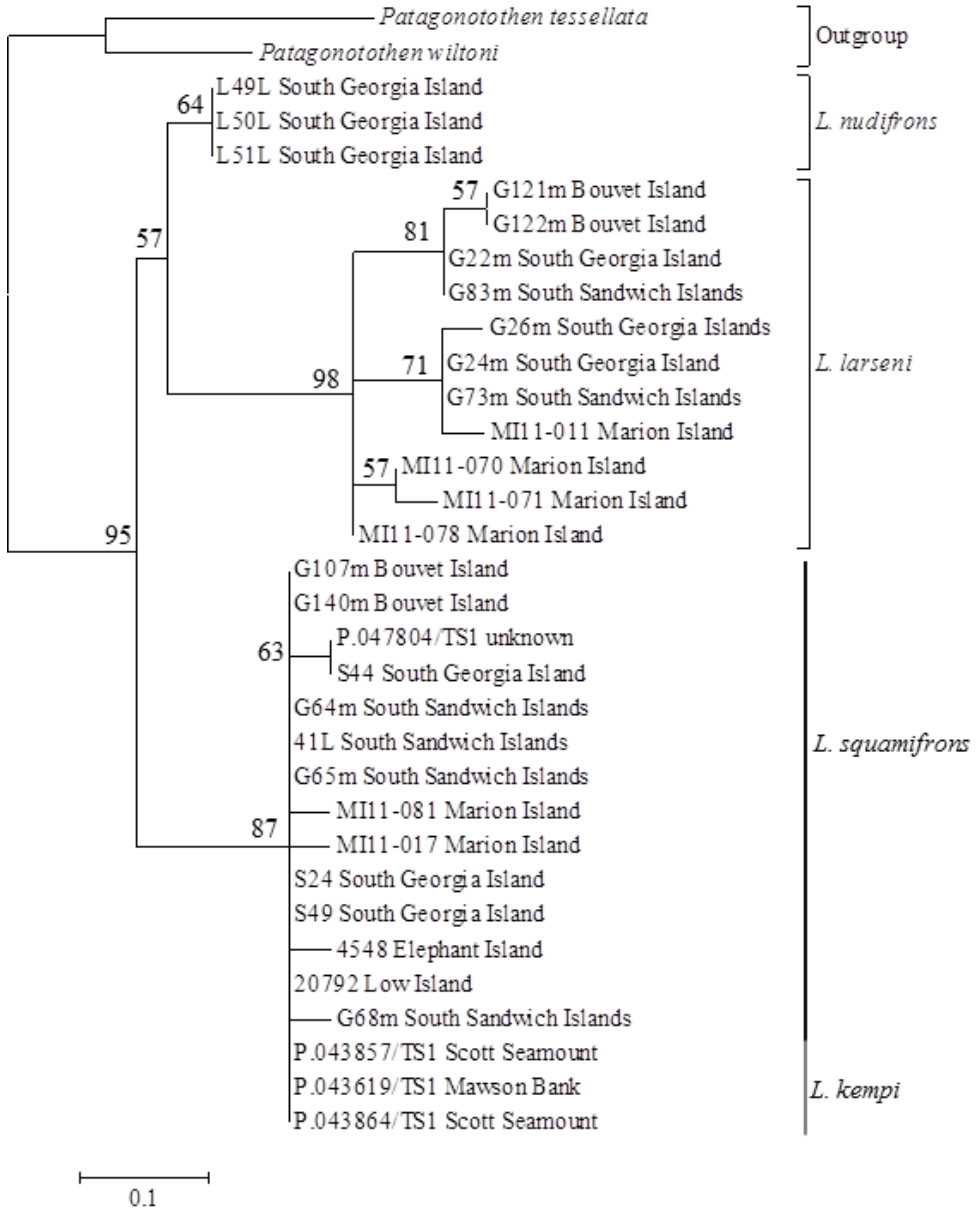


Figure 4.6: The S7 intron 1 gene maximum likelihood phylogenetic tree of *Lepidonotothen* species. The bootstrap support values from the parsimony analyses of this gene are displayed. The taxon names represent the specimen number and the locality name.

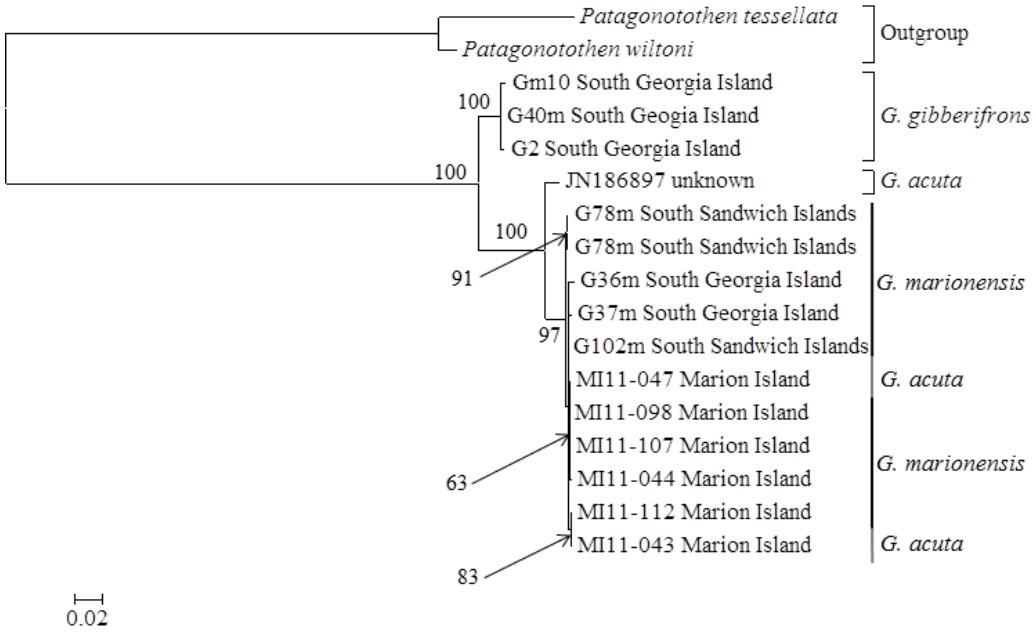


Figure 4.7: Mitochondrial ND2 gene maximum likelihood phylogenetic relationships among *Gobionotothen* species. The bootstrap support values from the parsimony analyses are displayed. The taxon names represent the specimen number followed by the locality name.

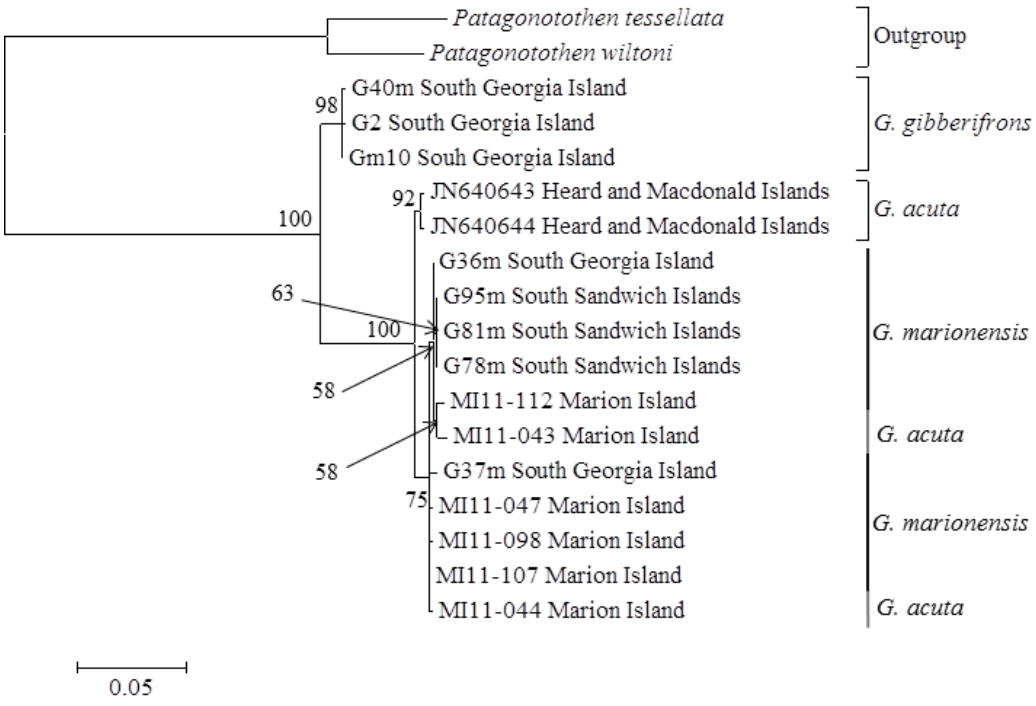


Figure 4.8: Mitochondrial COI gene maximum likelihood phylogenetic tree showing relationships among *Gobionotothen* species. The bootstrap support values estimated from the parsimony analyses are displayed. The taxon names represent the specimen number and the locality name.

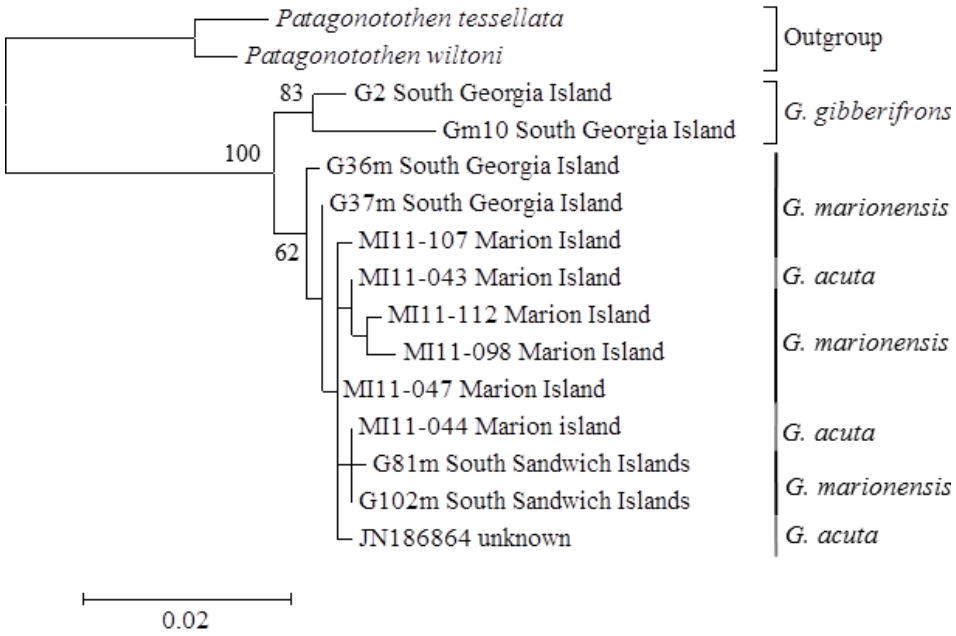


Figure 4.9: The S7 intron 1 gene maximum likelihood phylogenetic tree showing relationships among *Gobionotothen* species. The bootstrap support values from the parsimony analyses are displayed. The taxon names represent the specimen number followed by the locality name.

4.3.2.1. *Lepidonotothen squamifrons*

All phylogenetic analyses suggested that *Lepidonotothen squamifrons* was paraphyletic with *L. kempi* nested within it. This lineage had bootstrap support of 100% in the mtDNA genes (ND2 and COI), and 87% in a nuclear gene (S7 intron 1) analyses (Figure 4.4 to 4.6). The ND2 phylogenetic tree had two main lineages or clades that were moderately supported with bootstrap score of 66% and 74% (Figure 4.4). The first clade, with the bootstrap support of 66%, consisted of specimens from a wide geographical range, including Marion Island in the Indian Ocean, South Georgia and Bouvet Islands in the Atlantic Ocean and *L. kempi* from the Pacific Ocean sector. Although the *L. kempi* specimens grouped together within this clade (bootstrap score of 65%) in the DN2 tree (Figure 4.4), the sequence divergences between localities within this first clade was very low and varied from 0.1% to 0.3% (Table 4.3). Furthermore, the sequence divergence between *L. kempi* and all *L. squamifrons* individuals ranged from 0%, in S7 intron 1, to 0.5%, in ND2 (Table 4.7) suggesting that these species are not genetically different, but constitute populations of the same species. The second ND2

clade, with bootstrap support of 74%, contained specimens from two Atlantic Ocean localities, South Sandwich and Low Islands with sequence divergence of 0.3% (Table 4.3). This relationship between the two Atlantic Ocean localities was also observed with the mtDNA COI phylogenetic analyses (Figure 4.5), though not supported with sequence divergences of 0.2% (Table 4.3). This clade is interesting because it contains specimens which were previously called *L. kempi* (DeWitt 1966), and these localities are also closer to the Antarctic Peninsula, a type locality of the '*L. kempi*' (Norman 1937). However, the sequence divergence between species in the 'squamifrons' group, which include *L. squamifrons* and *L. kempi*, was very low and ranged from 0% to 1.1% in all three gene regions analyses (Table 4.2). These results suggest that the populations within this group share a most recent common ancestor that evolved once before the dispersal of species within this lineage occurred.

Table 4.7: The percentages of sequence divergence among *Lepidonotothen* species. Highlighted in bold are the highest percentage sequence divergence values

Species		**1**[†]	**2**	**3**	**4**
2. *L. larseni*	ND2	12.2	-		
	COI	8.3	-		
	S7	0.9	-		
3. *L. squamifrons*	ND2	**18.7**	18.4	-	
	COI	12.9	10.6	-	
	S7	0.8	1.3	-	
4. *L. kempi*	ND2	**18.7**	18.5	0.5	-
	COI	12.7	10.7	0.2	-
	S7	0.7	1.3	0.0	-

[†]*L. nudifrons*

4.3.2.2. *Lepidonotothen larseni*

The monophyly of the *L. larseni* group was strongly supported in all analyses with high bootstrap scores of ≥98% (Figure 4.4 to 4.6). The S7 intron 1 phylogenetic tree grouped *L. larseni* individuals into different paraphyletic lineages which are weakly to moderately supported (57% to 81%) (Figure 4.6). A lineage consisting exclusively of the Marion Island specimens was congruent in all three gene analyses, with ND2 and S7 intron 1 genes showing weak bootstrap scores of 60% and 57%, respectively. The pairwise sequence divergence between these Marion Island and Atlantic Ocean sector specimens was however low and ranged from 0.2% to 0.6% (Table 4.5 and 4.6). On the other hand, the phylogenetic analysis of the S7 intron 1 gene grouped one Marion Island (MI11-011) fish in a lineage with specimens from the South Georgia and South Sandwich Islands, with bootstrap support of 71% for this clade (Figure 4.6). However, the S7 intron 1 gene provided limited phylogenetic resolution to either support or reject all the clades supported by the mtDNA genes phylogenies, since it evolves slower than the mtDNA genes (Near and Cheng 2008). A lineage with exclusively Atlantic Ocean sector specimens had bootstrap support of 66% in the COI gene phylogenetic analyses. The results of the mtDNA gene analyses suggest that there may be low geographic structuring between *L. larseni* specimens from Indian and Atlantic Ocean sectors.

4.3.2.3. *Gobionotothen marionensis*

The ND2 and COI phylogenetic trees of the 'marionensis' group had two lineages with bootstrap support of strong 100% (Figure 4.7 and 4.8). The first lineage consisted of the *G. acuta* specimens from the unknown locality in the ND2 tree, and the Heard and MacDonald Islands in the COI trees. The sequence divergence between this lineage and *G. marionensis* ranged from 2.8% to 1.4% in ND2 and COI (Table 4.8), respectively, indicating that these species are closely related. The second lineage on the other hand consisted of the Marion Island *G. acuta* individuals and *G. marionensis* specimens. This lineage had strong bootstrap support of 97% in the ND2 and 75% in the COI trees. However, the sequence divergence between the two species within this lineage was very low and ranged from 0.2% to 0.7% (Table 4.7 and 4.8), suggesting that these species represent one population. Similar to the *L. larseni* analyses, the S7 intron 1 gene was not resolved and included the 'marionensis' group species in one clade (Figure 4.9). Although the *G. marionensis* specimens from Marion Island

and Atlantic Ocean localities grouped in a separate lineage, Marion Island specimens also formed two supported clades in the mtDNA analyses. The clade with specimens MI11-112 and MI11-043 was slightly different from other *G. marionensis* specimens from Marion Island with the bootstrap scores of 84% and 64% in ND2 and COI analyses, respectively (Figure 4.7 and 4.8). However, the sequence divergence between these two Marion Island groups range from 0.4% to 1.0% (Table 4.5), suggesting that there is genetic sub-structuring within the *G. marionensis* population of this island. Furthermore, the pairwise sequence divergence between *G. marionensis* specimens from Marion Island and Atlantic Ocean localities islands was very low varying from 0.4% to 0.6% (Table 4.5). Therefore, these data suggest that these specimens may represent one population with geographic variation.

Table 4.8: The percentages of sequence divergence among *Gobionotothen* species. Highlighted in bold are the highest percentage sequence divergence values

Species		**1**[†]	**2**	**3**	**4**
2. *G. marionensis*	ND2	**7.2**	-		
	COI	5.9	-		
	S7	1.6	-		
3. *G. acuta*[a]	ND2	**7.2**	0.5	-	
	COI	5.8	0.7	-	
	S7	1.7	0.2	-	
4. *G. acuta*[b]	ND2	7.0	2.8	2.8	-
	COI	5.5	1.4	1.5	-
	S7	1.7	0.3	0.3	-

[†]*G. gibberifrons*

[a]*G. acuta* from Marion Island; [b]*G. acuta* from the unknown locality, and Heard and MacDonald Islands.

4.4. Discussion

The results of this study followed a genetic variability pattern, as the intra-specific variability was smaller than the inter-specific variability, within all the analysed levels in the suborder Notothenioidei (Table 4.2), indicating a reliability of the datasets (Hebert *et al.* 2003). However, there was overlap in genetic distance between conspecific individuals and congeneric species, which may have resulted from low sister species divergence and/or misidentified species. Furthermore, phylogenetic trees resulting from all three gene analyses (ND2, COI and S7 intron 1) clustered specimens of the 'squamifrons', 'larseni' and 'marionensis' groups together in separate clades (Figure 4.4 to Figure 4.9). Although the S7 intron1 nuclear gene trees did not resolve relationships within these groups (Figure 4.6 and 4.9), the mtDNA analyses and their sequence divergence results, have provided a convincing identification of species within these groups. The results of this study also support the genetic study of Jones *et al.* (2008) that found no significant differentiation in species studied, except for *L. squamifrons*, when comparing populations in the Atlantic Ocean sector. Furthermore, the inclusion of more populations from the Atlantic, Indian and Pacific Ocean sectors of the Southern Ocean in the present study adds more information with regard to relationships among geographically distant individuals of the same species.

Lepidonotothen kempi, a member of DeWitt's (1966) 'squamifrons' group, was nested within the *L. squamifrons* lineage in the phylogenetic analyses of all three gene regions (ND2, COI and S7 intron1) (Figure 4.4 to 4.6). The ND2 phylogenetic tree revealed two main clades within this lineage, with *L. kempi* being grouped in a clade (66% bootstrap support) consisting of the *L. squamifrons* specimens from South Georgia, Bouvet and Marion Islands (Figure 4.4). This is unexpected since DeWitt's (1966) distribution of *L. kempi* did not include any of these islands (Figure 4.1). One would expect this *L. kempi* to group with the specimens from Low Island since this island (an island of the South Shetland Islands) lies at the tip of the Antarctic Peninsula, the type locality of *L. kempi* (Norman 1937). The Low Island specimen was, however, nested within the exclusively *L. squamifrons* clade that had 74% bootstrap support (Figure 4.4). In the notothenioid phylogenetic tree (see Chapter Three), *L. squamifrons* and *L. kempi* were part of a monophyletic lineage that was strongly supported (100% bootstrap support). Correspondingly, the AFGP studies of these two species indicated that they both possess no functional AFGP gene in their different inhabits (see

Chapter Three; DeVries and Yin 1977). Although some taxonomic studies still separate these species (Voskonoinikova and Kellermann 1997; Balushkin 2000), the position of *L. kempi* in the *Lepidonotothen* phylogenetic tree and the low DNA sequence divergence among all genes between these two species [from 0% in S7 intron 1 to 0.5% in ND2 (Table 4.7)] support the conclusion of Schneppenheim *et al.* (1994) and Eastman and Eakin (2000) that they are one species, *L. squamifrons*. Furthermore, similar to Jones *et al.* (2008) results, the two clades of *L. squamifrons* (Figure 4.4) indicate that the populations within this species are genetically heterogeneous. The phylogenetic relationship and sequence divergence among all other *Lepidonotothen* species [0.7-18.7% (Table 4.7)] clearly distinguish *L. nudifrons* and *L. larseni* from the specimens of the *L. squamifrons* group. However, the inclusion of *L. squamifrons* in the *Lepidonotothen* resulted in a paraphyletic genus (see Chapter Three, Figure 3.5), hence sequence divergences between this species and its congeners *L. larseni* and *L. nudifrons* is high (>18.7%).

The mitochondrial (ND2 and COI) phylogenetic trees of *L. larseni* indicated that there may be genetic sub-structuring between populations from the Atlantic and Indian Ocean sectors (Figure 4.4 and 4.5). The genetic homogeneity observed among Atlantic Ocean populations agree with the results of Jones *et al* (2008), who found no genetic differentiation among *L. larseni* populations of South Georgia, Bouvet and South Shetland Islands. Moreover, the results of Chapter Three of this thesis revealed that the Atlantic Ocean *L. larseni* populations possess equivalent levels of AFGPs in their different thermal habitats. These therefore suggest that the Atlantic Ocean populations of the 'larseni' group (*L. larseni* and *L. nybelini*) of Balushkin (1976) represent one species. The sequence divergence between localities in Atlantic and Indian oceans was very low ranging from 0.3% to 0.5% in both COI and ND2 genes (Table 4.4). These analyses indicate that the Atlantic Ocean *L. larseni* specimens and the Indian Ocean *L. tchizh* are one species, *L. larseni*, with intra-specific variation. Unfortunately, the Pacific Ocean species (*L. loesha*) of the 'larseni' group was not available for this study to review its relationship with other members of the group. However, given the observed relationship between of the Atlantic and Indian oceans species, it is conceivable that *L. loesha* represents a *L. larseni* population that exists in the Pacific Ocean sector of the Southern Ocean.

In the ND2 and COI analyses *Gobionotothen acuta* specimens from the unknown locality and Heard and MacDonald Islands belonged to a lineage that was differentiated from the *G. marionensis* specimens (Figure 4.7 and 4.8, respectively). It is important to note that the Heard and MacDonald Islands are part of the Kerguelen Plateau and are close to Kerguelen Islands, the type locality for the *G. acuta* (Günther 1880). The sequence divergence between the *G. marionensis* specimens, and the *G. acuta* specimens from Heard and MacDonald Islands and the unknown locality ranged between 1.4% in COI and 2.8% in ND2 (Table 4.8). The level of these sequence divergences and the position of these two lineages in the phylogenetic trees suggest that this group represents two distinct species. Therefore these data support Balushkin's (1991) conclusion that *G. marionensis* and *G. acuta* are different species. The specimens of *G. acuta* from Marion Island on the other hand were nested in a lineage with all the *G. marionensis* specimens in all three gene analyses (Figure 4.7 to 4.9). Given the position of the *G. acuta* specimens from Marion Island in the *Gobionotothen* phylogenetic analyses and the low sequence divergence [0.2% to 0.7% (Table 4.8)] between these *G. acuta* and *G. marionensis*, these specimens are probably misidentified *G. marionensis*. Since Duhamel *et al.* (1983) collected *G. acuta* at the Prince Edward Islands no other specimens have been reported from there suggesting a possible misidentification. Similar to *L. larseni*, the ND2 phylogenetic tree of *G. marionensis* distinguished between Atlantic and Indian Ocean clades (Figure 4.7), which could support the previous splitting of this species into *G. angustifrons* and *G. marionensis* in these oceans (Regan 1913; Norman 1938; Hureau 1985e; Balushkin 1991), respectively. However, the sequence divergence between localities in these two oceans is too low [0.4-0.6% (Table 4.5)] to conclusively support a taxonomic split and favours the conclusion of Gon and Klages (1988), based on morphological analyses, that *G. marionensis* and *G. angustifrons* are one species.

The results discussed above indicate that the geographically separated populations of *L. squamifrons*, *L. larseni* and *G. marionensis* found in Atlantic and Indian oceans represent one species. Since these three studied nototheniid species have a long pelagic stage, they are good candidates for dispersal to distant localities (DeWitt *et al.* 1990). The dispersal of pelagic eggs, larvae and juveniles of these species is promoted by the Antarctic Circumpolar Current (ACC) (Kellermann and Kock 1984; Loeb *et al*. 1993). This current is driven by the westerly winds and extend uninterrupted around the Antarctic continent (Orsi *et al.* 1995), such that the pelagic eggs, larvae and juvenile of these nototheniids could be transported throughout

the Southern Ocean. This explains the genetic homogeneity observed in the *L. squamifrons* lineage that includes populations from the Atlantic, Indian and Pacific oceans sectors (Figure 4.5).

In conclusion, this study does not support the splitting of the widely distributed *L. squamifrons* and *L. larseni* into different species. It agrees with the previous morphological and genetic studies that disagreed with the splitting of the nototheniid species found on both sides of the APF (Gon and Klages 1988; Schneppenheim *et al*. 1994). In addition, this study confirms that *G. marionensis* and *G. acuta* are different species but disagrees with the splitting of *G. marionensis* into *G. angustifrons* in the Atlantic Ocean and *G. marionensis* in the Indian Ocean. Therefore the 'marionensis' group of Balushkin (1991) consists of only two species, *G. marionensis* and *G. acuta*. The species of the two studied genera were clearly separated, i.e. *Lepidonotothen* consisted of *L. squamifrons*, *L. larseni*, *L. nudifrons* and *L. mizops* (COI analysis); and *Gobionotothen* composed of *G. marionensis*, *G. acuta* and *G. gibberifrons* (taxonomic status of *G. barsukovi* needs to be confirmed). The results of this study highlight a need for population genetic studies that will assess, in more detail, the effect of distance on gene flow among widely distributed, discontinuous populations in the Southern Ocean.

Chapter Five

General discussion and conclusion

5.1. Introduction

The aim of this study was to characterise the antifreeze attributes of sub-Antarctic notothenioid fishes and explore the potential association with their taxonomic status. This evaluation was done firstly by investigating the antifreeze glycoprotein (AFGP) traits of the widely distributed *Notothenia rossii*, a species which was previously split into two subspecies (Nybelin 1947, 1951) in its different thermal environments (Chapter Two). The analyses of *N. rossii* indicated that although this species possessed AFGP proteins in body fluids at the different latitudes around the Southern Ocean, the AFGP levels differed slightly and correlated with environmental temperature severity. The results also indicated that *N. rossii* populations inhabiting the waters north and south of the Antarctic Polar Front (APF) share a most recent AFGP-bearing common ancestor. Subsequently, the AFGP attributes of several sub-Antarctic notothenioid species belonging to the five Antarctic families were characterised and the observed AFGP characteristics of these species were mapped onto mitochondrial and nuclear genes phylogenetic trees (Chapter Three). Although the general hypothesis is that the AFGP gene is a synapomorphic character shared by the five Antarctic families (Chen *et al.* 1997; Cheng 1998b), the analyses in this study indicated that several of the sub-Antarctic species belonging to this clade possessed no AFGP gene in their genome. There were also differences in AFGP levels among species and within groups in the family Nototheniidae (Figure 3.5 and 3.6) that was paraphyletic in all resultant trees supporting previous studies that suggested that this family needs revision (Runtshmann *et al.* 2011; Dettai *et al.* 2012).

The taxonomic status of the three nototheniids, *Lepidonotothen squamifrons*, *L. larseni* and *Gobionotothen marionensis*, which are found on both the north and the south sides of APF, was reviewed by constructing their mitochondrial and nuclear phylogenetic relationships (Chapter Four). The S7 intron 1 nuclear gene phylogenetic tree was less resolved compared to the mtDNA genes (ND2 and COI) and these differences have also been observed by several other molecular studies on notothenioid species (Cheng and Near 2008; Kuhn and Near 2009). This is because nuclear genes evolve at a slower rate than the mtDNA genes and

are therefore more useful in solving deeper phylogenetic questions, such as those at the genus and family levels, and questions concerning the evolution of species (Simmons and Ocheterena 2000; Near *et al.* 2004). Mitochondrial genes on the other hand are more useful for analyses of the intraspecific relationships in a phylogenetic tree (Jones *et al*. 2008; Damerau *et al*. 2012) as also indicated by this study. Therefore, based on the mitochondrial genes analyses results, this study does not support the past taxonomic splitting of *L. squamifrons* and *L. larseni* into species and agrees that *G. marionensis* and *G. acuta* are different species.

5.2. Antifreeze glycoprotein (AFGP) biology and sub-Antarctic notothenioid species

This study demonstrated that in the absence of selection pressure the AFGP gene in notothenioid species degenerates, a process of mutational changes in the gene that leads to reduction and ultimately disappearance of function (Miya *et al.* 2014). Notothenioid species inhabiting warmer and ice-free waters of the Southern Ocean possessed no or lower levels of AFGP in their body fluid, compared to species from ice-cold waters. Other, similar studies have also demonstrated this difference in levels of AFGP among notothenioid species inhabiting different environmental conditions (Ahlgren and DeVries 1984; Jin and DeVries 2006). Furthermore, the present study is the first to investigate the effect of thermal habitat on AFGP levels among latitudinal populations of a widely distributed notothenioid species. The AFGP levels in *Notothenia rossii* specimens were congruent with environmental severity, with the specimen from the warmer ice-free waters of Ob' Seamount possessing the lowest AFGPs levels and specimens from the ice-cold waters of Dallman Bay having the highest levels. Similarly, the warm acclimation study on *Pagothenia borchgrevinki* also recorded a decrease in levels of serum AFGPs with increasing environmental temperature (Jin and DeVries 2006). Although these studies indicate that levels of serum AFGPs can be modulated in response to thermal changes in the environment, they further indicate that the maintenance of high levels of blood circulating AFGPs in a fish relies on the selection pressure exerted by freezing environments.

Analyses also recorded large AFGP gene families in the DNA of some sub-Antarctic notothenioid species even though antifreeze protection is not needed in these warmer

environments (Chapter Three). *Champsocephalus esox* a species occurring in the warmer ice-free waters of Falkland Island was one of the notothenioid species with a large AFGP gene dosage. This species has originated recently at this warm environment with estimated divergence time of 1.7 mya (Stankovic *et al.* 2002) suggesting that not enough time has elapsed for degeneration of the AFGP gene to take place. Unfortunately there was no available AFGP phenotype data for *C. esox* to infer the functional status of this AFGP gene family. However, since this study revealed that species possessing a large AFGP gene family in their genome produce high levels of AFGPs in their body fluids, *C. esox* might also possess high levels of active AFGPs in its blood.

Unlike *C. esox, Harpagifer georgianus* and *Paranotothenia magellanica* of the ice-free waters of Marion Island have an almost undetectable dosage of the AFGP gene in their genome. Since Marion Island is a small, young island [~1 mya (McDougall *et al.* 2001; Chown and Froneman 2008)], it is evident that these species settled recently at this island. These species also have a distribution that is restricted to the warmer ice-free waters of the Southern Ocean (DeWitt *et al.* 1990; Hureau 1990), where antifreeze protection is unessential. In the South American waters *P. magellanica* possessed similar undetectable AFGP gene in genomic DNA (Cheng *et al.* 2003 and Cheng and Detrich III 2007). Consistent with the AFGP gene results, *P. magellanica* have unmeasurable levels of AFGP proteins in its body fluid (Table 3.3), suggesting that this species possesses non-functional AFGP. These results suggest that *P. magellanica* has inhabited these warm ice-free waters for an extended period of time such that the AFGP gene has greatly mutated. This is the first report on the AFGP attributes of *H. georgianus* and there is a need for a follow up study that will determine levels of circulating AFGPs. The difference in AFGP gene dosage that was observed between sub-Antarctic species *C. esox* and *P. magellanica* and *H. georgianus* also indicate that the maintenance of large family of AFGP genes in a fish does not only rely on selection pressure but also on the evolutionary time of divergence of a species.

Although sub-Antarctic notothenioid species diverged after the evolution of the AFGP gene in the Miocene (5-14 mya) (Stankovic 2002), a number of these species have undetectable AFGP gene sequence in their genome (Chapter Three; Cheng *et al.* 2003; Cheng and Detrich III 2007), and non-measurable AFGP proteins in their body fluids. These include species in

the genus *Patagonotothen* that are found in the Patagonian region of Southern America and diverged around 7 mya. Another AFGP null species is *Dissostichus eleginoides* found around the warmer regions of the Southern Ocean, which diverged around 10 mya (Near 2004; Near *et al.* 2012). The possibility that these species diverged before the evolutionary gain of the AFGP gene is unlikely given their position in the estimated notothenioid phylogenies (Figure 3.5 and 3.6), where they share an AFGP-bearing common ancestor with the Antarctic sister species. Alternatively, *Patagonotothen* and *D. eleginoides* had a primordial AFGP gene, which was completely lost due to the absence of selection pressure for its function when they arrived at their new non-freezing environments. On the other hand, *Notothenia angustata* of the ice-free waters of New Zealand, which diverged from its Antarctic sister 11 mya (earlier than the above species; Cheng *et al.* 2003), still possesses some AFGP gene sequences though they are non-functional (Cheng *et al*. 2003). Similarly, *Lepidonotothen squamifrons* from Bouvet and South Sandwich Islands, with the estimated divergence of about 10 mya (Near *et al*. 2012), had reduced AFGP sequences that are non-functional (Figure 3.3). The implication of all of these results of the studies above is that, although the maintenance and loss of AFGP function is coupled with the thermal environment and evolutionary time of divergence of a species, the rate at which this gene becomes completely lost differs among notothenioid species.

5.3. AFGP levels and taxonomy of notothenioid species

In agreement with previous studies (Chen *et al*. 2003; Near and Cheng 2008; Matshiner *et al*. 2011; Near *et al.* 2012), the phylogenetic trees obtained by this study indicated that the suborder Notothenioidei is monophyletic (Chapter Three). However, two families of this suborder, Bathydraconidae and Nototheniidae, were paraphyletic. All of the bathydraconid species that have been studied so far possess substantial AFGP levels in their body fluids (Chapter Four; Ahlgren and DeVries 1984; Wohrmann 1996), while nototheniid species have varying AFGP gene dosages that are represented in the four main monophyletic clades that make this family. Mapping of the geographic distribution of the available notothenioid species and their AFGP characteristics onto phylogenetic trees revealed that each of the four Nototheniidae clades contained High Antarctic, Low Antarctic and sub-Antarctic species with large, small or no AFGP gene families in their genome. This suggests that these clades are not differentiated based on their physiology and/or geographic distribution, and therefore

they share the Nototheniidae common ancestor that evolved before the diversification of these clades. This study however supports the morphologically based phylogenetic study of Balushkin (2000) as well as the molecular studies of Dettai *et al* (2012) and Near *et al.* (2012) that proposed subfamilies or a taxonomic review of the family Nototheniidae.

The phylogenetic relationships within nototheniid clades and genera were complex and also revealed that the taxonomic status of some species needs revision. With the exception of *Dissostichus mawsoni*, species with a similar AFGP gene dosage grouped together in distinct clades and were closely related to each other within each clade (Figure 3.5). This was also observed among species belonging to the genus *Lepidonotothen* where *Lepidonotothen squamifrons* with non-functional AFGP was more closely related to the AFGP-null species of *Patagonotothen* than to its congeners *L. larseni* and *L. nudifrons* that have functional AFGP. Consequently, the genus *Lepidonotothen* is paraphyletic with *L. larseni*, *L. nudifrons* and *L. mizops* (AFGP attribute unknown) (Dettai *et al.* 2012) forming a separate monophyletic clade (Figure 3.5) excluding *L. squamifrons* and indicating that the latter species' placement in this genus should be re-evaluated. The paraphyly of *Lepidonotothen* can be corrected by either moving *L. squamifrons* to *Patagonotothen* or placing it in its own monotypic genus. The sequence divergence between *L. squamifrons* and *Patagonotothen* is about 7% (Appendix IIIB), which is equivalent to sequence divergence of some basal species, like in the genus *Gobionotothen* (Table 4.8). In addition, the sequence divergence between *L. sqamifrons* and its congeners is too high (>18%) to support that they are sister species, therefore, all these results suggest that *L. squamifrons* could be a basal species of the genus *Patagonotothen*. A similar situation was observed in the genus *Notothenia*, which is paraphyletic with *Paranotothenia magellanica* included in it. The ND2 phylogenetic data revealed two lineages within this genus. The first lineage consisted of *N. angustata*, *N. microlepidota* and *Paranotothenia magellanica*, all of which possess a small AFGP gene dosage, while the second lineage had *N. coriiceps* and *N. rossii* which have large AFGP gene families (Figure 3.5). The differences between these two lineages [sequence divergence of about 4% (Appendix IIIB)] suggest that *Notothenia* might consist of two subgenera or genera, which are *Paranotothenia* and *Notothenia*. Since *N. angustata* and *N. microlepidota* share the most recent common ancestor with *Paranotothenia magellanica*, they should be classified in the genus *Paranotothenia*. Therefore the genus *Notothenia* should only consist of the monophyletic AFGP-bearing *N. coriiceps* and *N. rossii*.

Given the difference in magnitude of the AFGP gene dosage within sub-Antarctic notothenioid species belonging to the five Antarctic families, this study demonstrated that they share a most recent AFGP-bearing common ancestor with their Antarctic sisters (Chapter Three). A majority of the species belonging to the notothenioid Antarctic clade have been estimated to have originated less than 10 mya (Near *et al.* 2012), which is after the estimated late Miocene evolution of the AFGP gene. This suggests that sub-Antarctic notothenioid species may have diverged after the onset of the AFGP gene and established themselves in the warmer environments where AFGP protection is not needed. However, it is important to note that AFGP originated as a single gene which later duplicated to more than one copy (Chen *et al*. 1997; Cheng and Chen 1999). It is possible that some sub-Antarctic species might have diverged before the expansion of this gene into a large gene family. The *Patagonotothen* ancestor might be a case in point, as in the absence of selection pressure its AFGP gene did not duplicate, but degenerated and was ultimately completely lost. The sub-Antarctic species that still possess a large AFGP gene family appear to have diverged sometime after the duplication of the AFGP gene. Several of these species have settled or colonised their current warmer environments more recently in the Pliocene period such that there has not been enough time for their AFGP gene to degenerate.

5.4. Species variation

The paraphyletic family Nototheniidae has the highest number of wide-ranging species that are distributed on both sides of the APF and also has species which are exclusively sub-Antarctic (Appendix II, III and IV). With the APF acting as a barrier, populations south and north of it could become reproductively isolated such that over time due to genetic drift they may develop different characteristics that may lead to the splitting of a species. For example, the splitting of widespread nototheniids was based on differences in biological features, such as growth rates, and morphological characteristics including differences in colouration and other meristic characters. Although the environmental temperature may affect the growth rate of a fish, this is also dependant on the availability of food and variations in food-particles size, i.e. larger food particles may lower growth rate (Knox 2007; Azaza *et al.* 2010). For example, the reason for the slower growth rate of *Lepidonotothen squamifrons* in Kerguelen Island compared to that observed in South Georgia Island may be due to food competition,

since this species is abundant and dominates the fish species at Kerguelen Island (DeWitt *et al.* 1990; Knox 2007). Consequently, differences in growth rate can cause slight differences in the meristic characters among populations of a species, resulting in large overlaps in characters that preclude the splitting of species (Gon and Klages and 1988, DeWitt *et al.* 1990).The colour of a fish is influenced by their surrounding habitats and their diet, as well as preservatives and duration in preservative after collecting, (Gon and Klages 1988). Fishes can also change their colour, using chromatophores, as predation survival strategy (Fuji 2000). Unless these effects are not taken into account, these methods alone cannot be reliably used to resolve the taxonomy of the widely distributed notothenioids species.

The present study reviewed the taxonomic status of the three latitudinally widespread nototheniids *Lepidonotothen squamifrons*, *L. larseni* and *Gobionotothen marionensis* at the molecular level (Chapter Four). The results of *L. squamifrons* observed in this study are similar to those of Jones *et al.* (2008) who found significant genetic differentiation between South Shetland and Bouvet Islands populations. Furthermore, the addition of data from other oceans indicated that the Bouvet population is similar to Indian and Pacific Ocean populations. Since sequence divergence between the two *L. squamifrons* populations was too low (0.6% to 0.8%) to consider them as different species, a possibility is that they might represent two or more subspecies (Figure 4.4). A previous study of Permitin and Sazonov (1974) described two subspecies within this species which were *L. squamifrons squamifrons* in the Indian Ocean sector and *L. squamifrons atlantica* from around South Georgia Island. In the present study specimens from these localities were grouped in one clade (Figure 4.5), disagreeing with their splitting. The subspecies implied by this study will have to include populations from South Georgia Island, Bouvet Islands, Indian and Pacific Ocean sectors and populations from South Shetland and South Sandwich Islands. However, additional specimens from other localities, including the Falkland Islands and Indian Ocean islands, are needed to confirm the presence of subspecies in *L. squamifrons*.

The phylogenetic trees of *L. larseni* and *G. marionensis* clustered individuals from Indian Ocean sector and Atlantic Ocean sector in separate clades, respectively (Figure 4.4 and 4.7). These separations were surprising considering the young age of Marion Island [less than million year old (McDougall *et al.* 2001; Chown and Froneman 2008)], a representation of

Indian Ocean islands, suggesting that dispersal of these species into this island happened recently. However, the differences observed between populations from these oceans were too low to support splitting species and therefore populations in these oceans represent consists of one species. The possibility is that since Marion Island is the warmest among the studied localities, temperature might have played a role in genetic sub-structuring between individuals of these oceans. The 'marionensis' group of Balushkin (1991) consist of two species, the *Gobionotothen marionensis* and *G. acuta* (Figure 4.8 and 4.9) with the Atlantic Ocean *G. angustifrons* being included in the *G. marionensis* population. Furthermore, there was a genetic sub-structuring within *G. marionensis* of Marion Island (Figure 4.5 and 4.6), which is a type locality for this species (Günther 1880), and some individuals were misidentified as *G. acuta* (Chapter Four). Therefore, specimens from all geographic localities of *G. marionensis* should be included in genetic analyses to have a better understanding of the population structure of this species in the Southern Ocean.

Although this study does not support the splitting of notothenioid species that are found on both sides of the APF, the analyses indicated that these widespread species exist in several distinct populations in their different geographic habitats (Chapter Four). Since the APF is considered to be a barrier for fish migration from either side, it is now evident that it is 'leaky' and allows transport or dispersal of fish to the north (Clarke *et al.* 2005; di Prisco *et al.* 2007). The westerly wind driven Antarctic Circumpolar Current (ACC) promotes eastwards dispersal of pelagic eggs, larvae and juveniles from the northern Scotia Arc islands to Bouvet Island and beyond. The three nototheniid species, i.e. *L. squamifrons*, *L. larseni* and *G. marionensis*, studied here have a long pelagic early life history stage that improves the chance of dispersal to distant localities, as well as ensuring uninterrupted gene flow (Sponaugle *et al.* 2002). These were also supported by the genetic homogeneity of *L. squamifrons* lineage that included populations from all three ocean sectors of the Southern Ocean (Figure 4.5). This dispersal potential explains the distribution of these species on both sides of the APF, and the genetic distinction observed between populations in the Atlantic and Indian Ocean sectors (Chapter Four). Furthermore, the westerly direction of ACC, the young age of Marion Island and position of this Island west of Kerguelen Island (type locality of *G. acuta*) explain why *G. acuta* is unlikely to be in water near this island.

5.5. Final conclusion and recommendations

This study contributed in closing the research gap between the notothenioid species of the Antarctic and sub-Antarctic regions of the Southern Ocean with regard to their AFGP attributes and systematics. It provided the first report on AFGP gene attributes of seven Low and sub-Antarctic notothenioid species. These include *Gobionotothen gibberifrons, G. marionensis, Lepidonotothen larseni, L. nudifrons, Artedidraco mirus, Parachaenichthys georgianus* and *Harpagifer georgianus*. Although these species are found in the warmer environments of the Southern Ocean, with the exception of *H. georgianus*, they still possess large AFGP gene families in their genomic DNA. Therefore, there is a need for a study that will determine the functional status of their AFGP gene since the freezing protection function of this gene is not needed in the warmer ice-free waters. Furthermore, this is the first study that used AFGP attributes of notothenioid species to assess their taxonomic status. The phylogenetic tree of notothenioid species indicated that species with the same levels of AFGPs share the most recent common ancestor, except *D. eleginoides*. This is evidence that the AFGP characteristics of a species or taxon can be used to resolve the taxonomy of notothenioid species, especially for the species belonging to the paraphyletic family Nototheniidae.

Although this study added more information and understanding on the adaptations and systematics of sub-Antarctic notothenioid species, there is still more to be done in this region of the Southern Ocean. This includes the determination of the functional status of the AFGP gene in more of the latitudinally widespread species that experience different weather regimes, such as *Notothenia rossii.* For example, although the *Lepidonotothen larseni* specimen from the icy-cold waters of Dallman bay and the less severe waters around South Georgia Island both possessed large AFGP gene family, the functional status in the latter area is unknown. Determining the functional status of the AFGP gene in the non-freezing environment where it is not needed will provide a better understanding on the biology and evolution of AFGP attributes among populations of a geographically widely distributed species. There is also lack of information with regard to genetic structuring within populations of a species that is distributed in the Atlantic, Indian and Pacific Ocean sectors of the Southern Ocean, since the previous population studies concentrated on the Atlantic Ocean populations. Therefore, there is a need for such studies to review the morphologically based

splitting of latitudinally widespread species and thereby providing information which can be used when conserving and managing the relevant species.

Appendices:

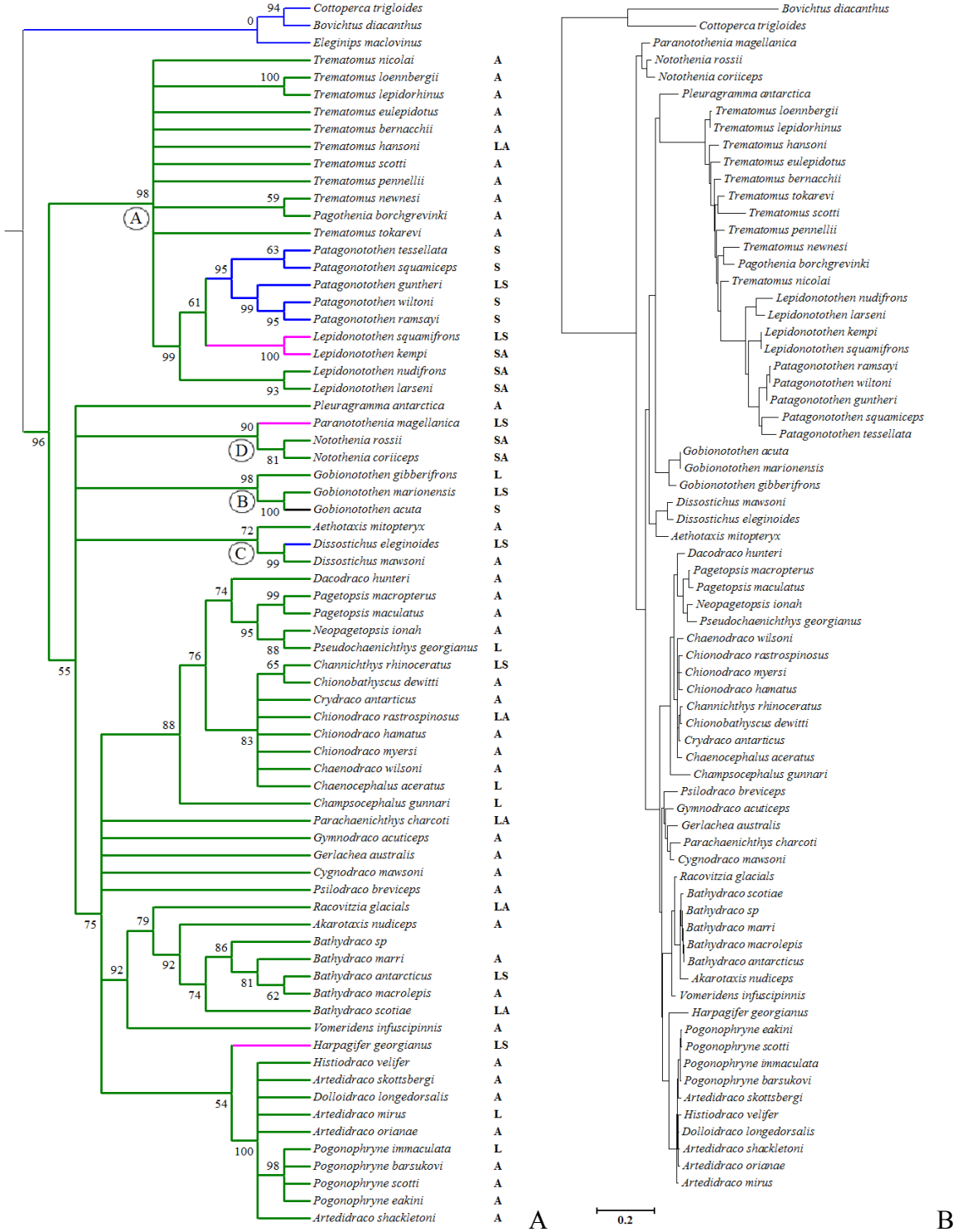
A**ppendix I:** Notothenioid species sequences downloaded from GenBank and their accession numbers.

Species	**GenBank accession numbers**		
	ND2[a]	**COI**	**S7**[a]
Pseudaphritidae			
Pseudaphritis urvillii	JN186885	-	JN186845
Bovichtidae			
Bovichtus variegatus	JN186882	-	JN186842
Artedidraconidae			
Artedidraco orianae	JN186888	HQ712830[b]	JN186848
Artedidraco shackletoni		HQ712857[b]	JN186849
Artedidraco skottsbergi	FJ973333	HQ712874[b]	JN186850
Dolloidraco longedorsalis	FJ647587	HQ712975[b]	FJ647619
Histiodraco velifer	FJ973335	HQ713024[b]	JN186851
Pogonophryne barsukovi	FJ973337	JN641106[c]	JN186852
Pogonophryne immaculata	FJ973344	JN641108[c]	JN186856
Pogonophryne eakini	FJ973342	JN641107[c]	JN186854
Pogonophryne marmorata	FJ973350	-	JN186857
Pogonophryne scotti	JN186994	HQ713182[b]	JN186858
Bathydraconidae			
Akarotaxis nudiceps	HQ170108	HQ712805[b]	HQ170152
Bathydraco antarcticus	HQ170113	HQ712880[b]	JN186859
Bathydraco macrolepis	HQ170110	JN640779[c]	-
Bathydraco marri	HQ170111	HQ712882[b]	HQ170156
Bathydraco scotiae	HQ170115	JN640790[c]	HQ170159
Cygnodraco mawsoni	HQ170116	HQ712951[b]	HQ170160
Gerlachea australis	HQ170118	HQ713004[b]	HQ170162
Gymnodraco acuticeps	HQ170120	HQ713010[b]	HQ170165
Parachaenichthys charcoti	HQ170122	EU326403[d]	HQ170167
Parachaenichthys georgianus	HQ170123	-	HQ170168
Psilodraco breviceps	HQ170128	HQ712804[b]	HQ170173
Racovitzia glacialis	HQ170130	HQ713217[b]	HQ170174
Vomeridens infuscipinnis	JN186893	JN641174[b]	JN186860
Channichthyidae			

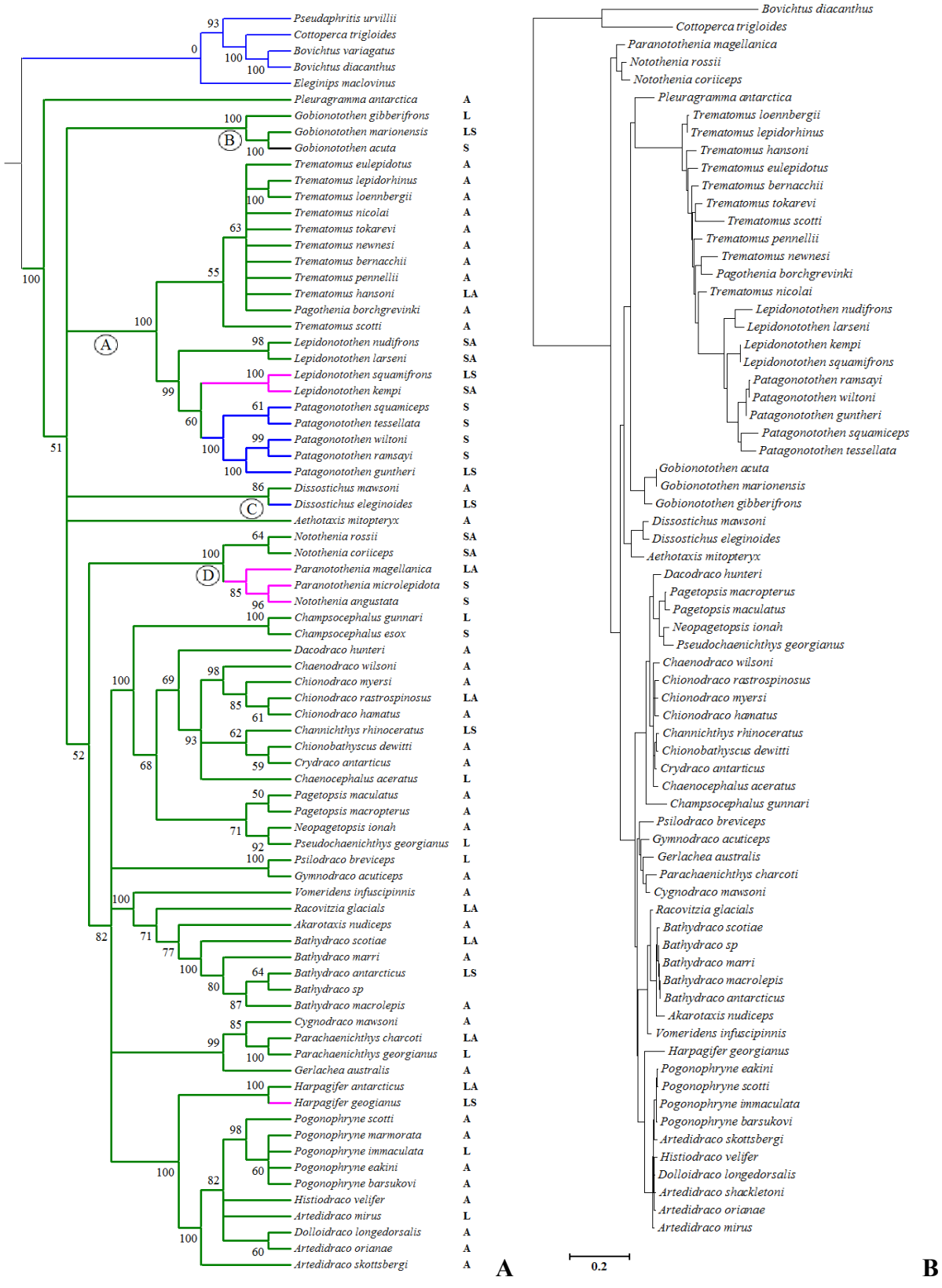
Chaenocephalus aceratus	AY249493	EU326330 [d]	HM166091
Chaenodraco wilsoni	AY249504	HQ172902 [b]	HM165755
Champsocephalus esox	-	-	HQ170135
Channichthys rhinoceratus	AY249503	HQ170139 [b]	-
Chionobathyscus dewitti	HQ170099	HQ172908 [b]	HQ170140
Chionodraco hamatus	HQ170101	HQ172910 [b]	HQ170142
Chionodraco myersi	HQ170103	HQ172925 [b]	HQ170145
Chionodraco rastrospinosus	AY249502	EU326337 [d]	HM165855
Cryodraco antarcticus	AY249494	HQ712935 [b]	HM165959
Dacodraco hunteri	AY249479	HQ712963 [b]	HQ170148
Nepagetopsis ionah	HM165731	HQ713091 [b]	HM165690
Pagetopsis macropterus	AY249511	HQ713119 [b]	HM165520
Pagetopsis maculatus	AY249512	HQ713120 [b]	HQ170150
Harpagiferidae			
Harpagifer antarcticus	JN186896	-	JN186861
Nototheniidae			
Aethotaxis mitopteryx	JN186895	JN640756 [c]	JN186862
Dissostichus mawsoni	AY517753	EU326340 [d]	AY517753
Notothenia angustata	AY256562	-	JN186870
Pagothenia borchgrevinki	FJ647716	HQ713126 [b]	FJ647674
Patagonotothen ramsayi			JN186876
Patagonotothen squamiceps	JN186910	-	-
Pleuragramma antarctica	JN186912	HQ713170 [b]	-
Trematomus bernacchii	AY256569	EU326421 [d]	FJ647676
Trematomus eulepidotus	FJ647718	HQ713243 [b]	FJ647680
Trematomus hansoni	FJ647748	EU326427 [d]	FJ647683
Trematomus lepidorhinus	JN186913	JN641150 [c]	JN186871
Trematomus loennbergii	FJ647725	JN641154 [c]	FJ647689
Trematomus newnesi	FJ647729	EU326429 [d]	FJ647692
Trematomus nicolai	FJ647731	JN641164 [c]	FJ647697
Trematomus pennellii	FJ647742	HQ713272 [b]	-
Trematomus scotti	FJ647733	EU326434 [d]	FJ647709
Trematomus tokarevi	FJ647740	HQ713353 [b]	FJ647710

[a]Near *et al.* (2012); [b]Dettai *et al.* (2011); [c]Smith *et al.* (2012); [d]Rock *et al.* (2008)

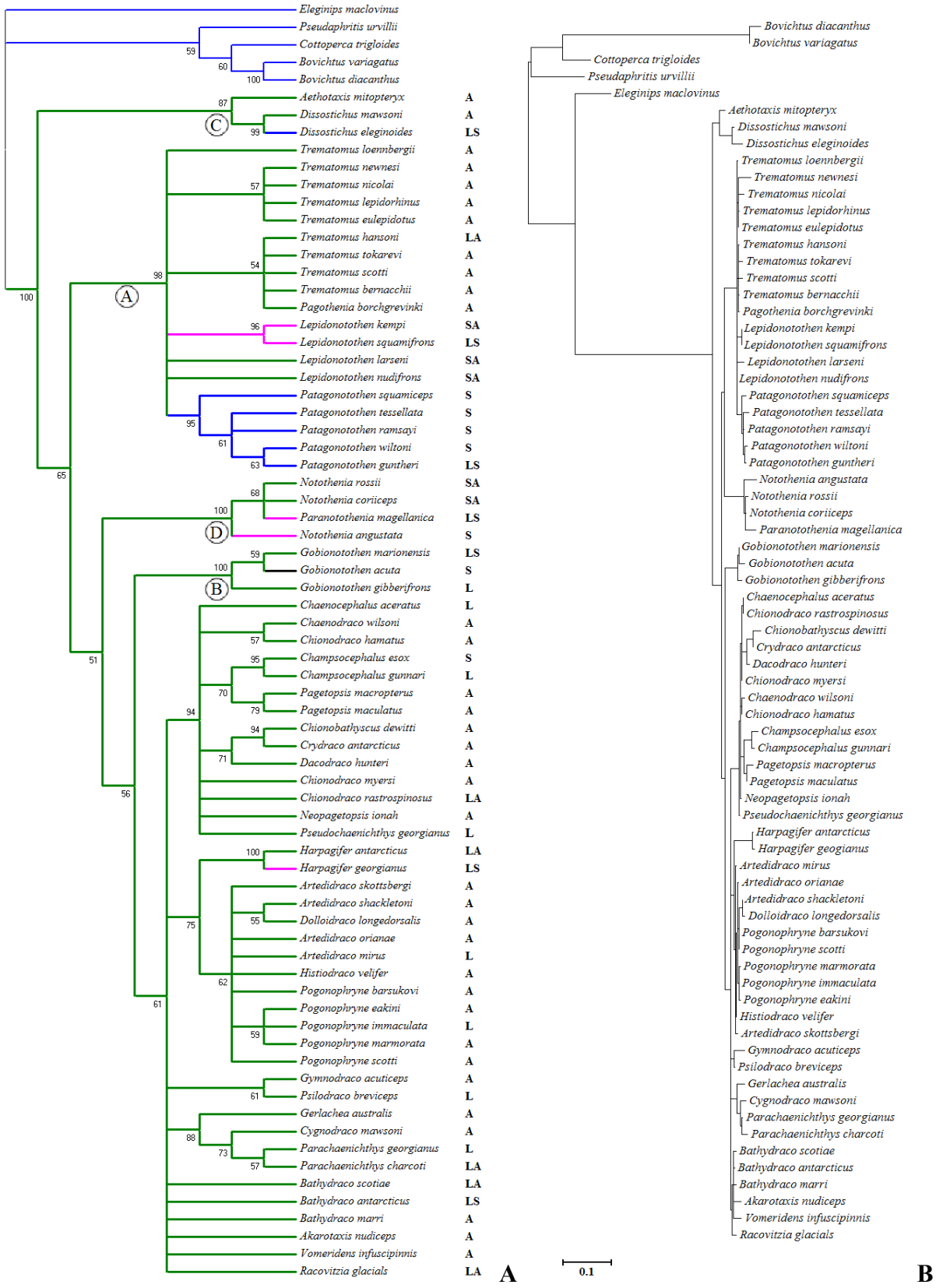
Appendix II: Mitochondrial COI gene parsimony (A) and maximum likelihood (B) phylogenetic trees.



Appendix III: Mitochondrial ND2 gene parsimony (A) and maximum likelihood (B) phylogenetic trees.



Appendix IV: Nuclear S7 intron 1 gene parsimony (A) and maximum likelihood (B) phylogenetic trees.



Appendix V: GenBank accession numbers of all sequences, including sequences from other authors, used in both *Lepidonotothen* and *Gobionotothen* molecular analyses; and the COI barcoding numbers of the sequence upload in barcoding database.

Species name	**Tissue no.**	**GenBank accession no.**			**COI barcoding no.**
		S7	**ND2**	**COI**	
L. squamifrons	20792				-
	4548				-
	G64m				SAANF018
	G65m				-
	G68m				-
	41L		KF412880		-
	S24				-
	S44				-
	S49				-
	G107m				SAANF019
	G140m				SAANF022
	MI11-017				SAAMF021
	MI11-081				SAAMF023
	MI11-095				SAAMF025
	P.047804/TS2				-
L. larseni	G83m				SAANF017
	G73m				-
	G26m				-
	G22m				-
	G24m				-
	G121m				SAANF015
	G122m				-
	MI11-011				SAAMF014
	MI11-070				-
	MI11-071				-
	MI11-078	KF412842	KF412887	KF412863	SAAMF017
L. kempi	P.043857/TS1				-
	P.043864/TS1				-
	P.043619/TS1	KF412844	KF412886	KF412862	-
L. nudifrons	L49L	KF412841	KF412888	KF412864	-
	L50L				-

	L51L				-
L. mizops	BWA4538			JN640669	-
	BWA4533			JN640670	-
	BWA4540			JN640671	-
G. marionensis	G78m				SAANF011
	G81m				SAANF012
	G102m				-
	G36m				SAANF010
	G37m				-
	MI11-047	KF412849	KF412885	KF412860	SAAMF003
	MI11-098				SAAMF009
	MI11-112				SAAMF006
	MI11-107				SAAMF005
G. acuta	MI11-043				SAAMF001
	MI11-044	KF412848	KF412883	KF412861	SAAMF002
	YFTC2339	JN186897	JN186864		-
	BWA4466			JN640643	-
	BWA4495			JN640644	-
G. gibberifrons	G39m				-
	G2				-
	G10m				-

References:

Ahlgren, J.A. and DeVries, A.L. 1984. Comparison of antifreeze glycopeptides from several Antarctic fishes. *Polar Biology*. **3**: 93-97.

Akaike, H. 1973. Information theory as an extension to the maximum likelihood principle. In: Petrov, B.N. and Csaki, F. (eds) *Second international symposium on information theory*. Akademiai Kiado, Budapest. pp. 267-281.

Andriashev, A.P. 1965. A general review of Antarctic fish fauna. In: van Oye, P. and van Mieghen, J. (eds) *Biogeography and ecology in Antarctic. Monographiae Biologicae 15*. Junk, The Hague, pp. 591-550.

Ansorge, I.J., Roman, R., Durgadoo, J.V., Ryan, P.G., Dlamini, L., Gebhardt, Z., Rainier, S., Smith, M., Mtonsti, T. and Lutjeharms, J.R.E. 2008. The first oceanographic survey of the Conrad rise. *South African Journal of Science*. **104**: 333-336.

Azaza, M.S., Dhraief, M.N., Kraiem, M.M. and Baras, E. 2010. Influences of food particle size on growth, size heterogeneity, food intake and gastric evacuation in juvenile Nile tilapia, *Oreochromis niloticus*, L., 1758. *Aquaculture*. **309**: 193-202.

Balushkin, A.V. 1976. A review of the "larseni" group of species of the genus *Notothenia* Rich. *Journal of Ichthyology*. **16**(1): 1-12.

Balushkin, A.V. 1991. Review of green notothenias, *Gobionotothen*, Balushkin (Nototheniidae) of the Antarctic and sub-Antarctic. *Journal of Ichthyology*. **31**(4): 531-542.

Balushkin, A.V. 1992. Classification, phylogenetic relationships, and origins of the families of the suborder Notothenioidea (Perciformes). *Journal of Ichthyology*. **32**(3):3-19.

Balushkin, A.V. 1994. *Proeleginops grandeastmanorum* gen. et sp. nov. (Perciformes, Notothenioidei, Eleginopsidae) from the late Eocene of Seymour Island (Antarctica) is a fossil Notothenioid, not a Gadiform. *Journal of Ichthyology*. **34**(8): 298-307.

Balushkin, A.V. 2000. Morphology, classification and evolution of notothenioid fishes of the Southern Ocean. *Journal of Ichthyology*. **40**:S74-S109.

Balushkin, A.V. and Eakin, R.R. 1998. A new species toad plunderfish *Pogonophryne fusca* sp. nova (Fam. Artedidraconidae: Notothenioiddei) with notes on species composition and species groups in the genus *Pogonophryne* Regan. *Journal of Ichthyology*. **38**: 574-579.

Balushkin, A.V., Petrov, A.F. and Prutko V.G. 2010. *Pogonophryne brevibarbata* sp. nov. (Arterdidraconidae, Notothenioidei, Percifromes)- a new species of toadlike plunderfish from the Ross Sea, Antarctica. *Proceedings of the Zoological Institute of the Russian Academy of Science.* **314**: 381-386.

Balushkin, A.V. and Korolkova, E.D. 2013. New species of plunderfish *Pogonophryne favosa* sp. n. (Artedidraconidae, Notothenioidei, Perciformes) from the Cosmonauts Sea (Antarctica) with description in artedidraconids of unusual anatomical structures-convexitas superaxillaris. *Journal of Ichthyology*. **53**(8): 562-574.

Bargelloni, L., Ritchie, P.A., Patarnello, T., Battaglia, B., Lambert, D.M. and Meyer, A. 1994. Molecular evolution at subzero temperatures: Mitochondrial and Nuclear phylogenies of fishes from Antarctica. *Molecular Biology and Evolution*. **11**(6): 854-863.

Bargelloni, L., Marcato, S., Zane, L. and Patarnello, T. 2000. Mitochondrial phylogeny of notothenioids: A molecular approach to Antarctic fish evolution and biogeography. *Systematic Biology*. **49**(1): 114-129.

Barker, P.F. and Thomas, E. 2004. Origin, signature and palaeoclimatic influence of the Antarctic Circurmpolar Current. *Earth-Science Reviews*. **66**: 143-162.

Barnes, D.K.A., Fuenets, V., Clarke, A., Schloss, I.R. and Wallace, M.I. 2006. Spatial and temporal variation in shallow seawater temperatures around Antarctica. *Deep-sea Research II*. **53**:853-865.

Bilyk, K.T. and DeVries, A.L. 2010(a). Freezing avoidance of the Antarctic icefishes (Channichthyidae) across thermal gradients on the Southern Ocean. *Polar Biology*. **33**: 203-213.

Bilyk, K.T. and DeVries, A.L. 2010(b). Delayed onset of adult antifreeze activity in juveniles of the Antarctic icefish *Chaenocephalus aceratus*. *Polar Biology*. **33**: 1387-1397.

Bilyk, K.T., Evans, C.W. and DeVries, A.L. 2012. Heat hardening in Antarctic notothenioid fishes. *Polar Biology*. **35**(9): 1447–1451.

Black, V.S. 1951. Some aspects of the physiology of fish. II. Osmotic regulation in teleost fishes. *University of Toronto: Biology Series 59 no. 71*. pp. 53-89.

Boulenger, G.A. 1902. Pisces. In: Report on the collection of Natural History made in the Antarctic region during the voyage of the "Southern Cross". *London, British Museum of Natural History*. pp. 174-189.

Buchett, M.S. and Ricketts, C. 1984. The population dynamics of *Notothenia rossii* from South Georgia (Antarctica). *Polar Biology*. **3**:35-38.

Buckley, T.R. and Cunningham, C.W. 2004. The effects of nucleotide substitution model assumptions on estimates of nonparametric bootstrap support. *Molecular Biology and Evolution*. **19**(4): 394-405.

Burnham, K.P. and Anderson, D.R. 2002. *Model selection and multi-model inferences, a practical information-theoretic approach, 2nd edn*. Springer, New York.

Chen, L., DeVries, A.L. and Cheng, C.-H.C. 1997. Evolution of antifreeze glycoprotein gene from trypsinogen gene in the Antarctic notothenioid fish. *Proceedings of the National Academy of Sciences of the United States of America*. **94**: 3811-3816.

Chen, W.-J., Bonillo, C. and Lecointre, G. 1998. Phylogeny of the Channichthyidae (Notothenioidei, Teleostei) based on two mitichondrial genes. In: di Prisco, G., Pisano, E. and Clarke, A. (eds) *Fishes of Antarctica: a biological overview*. Springer, Milan Berlin Heidelberg, pp. 287-298.

Chen, W.-J., Bonillo, C. and Lecointre, G. 2003. Repeatability of clades as a criterion of reliability: a case study for molecular phylogeny of Acanthomorpha (Teleostei) with larger number of taxa. *Molecular phylogenetics and evolution.* **26**(2): 262–88.

Cheng, C.-H. C. 1996. Genomic basis for antifreeze glycopeptide heterogeneity and abundance in Antarctic fishes. In: S. Ennion and G. Goldspink (eds) *Gene expression and manipulation in aquatic organisms*, Cambridge University Press, Cambridge. pp. 1–20.

Cheng, C.-H.C. 1998(a). Evolution of the diverse antifreeze proteins. *Current Opinion in Genetics and Development*. **8**: 715-720.

Cheng, C.-H.C. 1998(b). Origin and mechanism of evolution of antifreeze glycoproteins in polar fish. In: G. di Prisco and E. Pisano (eds) *Evolution of the Antarctic Ichthyofauna*, Springer-Verlag, Italy, pp. 311-328

Cheng, C.-H.C. and DeVries, A.L. 1991. The role of antifreeze glycopeptides and peptides in the freezing avoidance of cold-water fish. In: di Prisco, G. (ed) *Life Under Extreme Conditions.* Springer-Verlag, Berlin-Hetdelberg. pp. 1-l4.

Cheng, C.-H.C. and Chen, L. 1999. Evolution of an antifreeze glycoprotein. *Nature*. **401**: 443-444.

Cheng, C-H.C., Chen, L., Near T.J. and Jin Y. 2003. Functional antifreeze glycoprotein genes in temperate-water New Zealand Notothenioid fish infer an Antarctic origin. *Molecular Biology and Evolution*.**20**:1897-1909.

Cheng, C-H.C., Cziko, P.A and Evans, C.W. 2006. Nonhepatic origin of notothenioid antifreeze reveals pancreatic synthesis as a common mechanism in polar fish freezing avoidance. *Proceedings of the National Academy of Science of the United States of America.* **103**(27):10491-10496.

Cheng, C.-H.C. and Detrich, H.W.III. 2007. Molecular ecophysiology of Antarctic notothenioid fishes. *Philosophical Translation of Royal Society London Series B.* **362**: 2215-2232.

Chow, S. and Hazama, K. 1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. *Molecular Ecology*. **7**:1247-1263.

Chown, S.L. and Froneman, P.W. 2008. The Prince Edward Islands in a global context. In: Chown, S.L. and Froneman, P.W. (eds) *The Prince Edward Islands: Land-sea interactions in a changing ecosystem*. Sun Press, Stellenbosch. pp 1-16.

Clark, M.S., Fraser, K.P.P., Burns, G. and Peck, L.S. 2008. The HSP70 heat shock response in the Antarctic fish *Harpagifer antarcticus*. *Polar Biology*. **31**: 171-180.

Clarke, A., Doherty, N., DeVries, A.L. and Eastman, J.T. 1984. Lipid content and composition of three species of Antarctic fish in relation to buoyancy. *Polar Biology*. **3**:77-83.

Clarke, A., Barne, D.K.A. and Hodgson, D.A. 2005. How isolated is Antarctica? *Trends in Ecology and Evolution*. **20** (1):1-3.

Cziko, P.A. and Cheng, C.-H.C. 2006. A new species of nototheniid (Perciformes: Notothenioidei) fish from McMurdo Sound Antarctica. *Copeia.* **2006**(4): 752-759.

Damerau, M., Matshiner, M., Salzburger, W. and Hanel, R. 2012. Comparative population genetics of seven notothenioid fish species reveals high levels of gene flow along ocean currents in the southern Scotia Arc, Antarctica. *Polar Biology*. **35**: 1073-1086.

De Broyer, C., Danis, B., with 64 SCAR-MarBIN taxonomic editors. 2011. How many species in the Southern Ocean? Towards a dynamic inventory of the Antarctic marine species. *Deep-Sea Research II*. **58**: 5-17.

Deng, G., Andrews, D.W., and Laursen, R.A. 1997. Amino acid sequence of a new type of antifreeze protein from the long horn sculpin *Myoxocephalus octodecimspinosis*. *FEBS Letter*. **402**: 17–20.

Derome, N., Chen, W.-J., Dettai, A., Bonillo, C. and Lecointre, G. 2002. Phylogeny of Antarctic dragonfishes (Bathydraconidae, Notothenioidei, Teleostei) and related families based on their anatomy and two mitochondrial genes. *Molecular phylogenetics and evolution.* **24**(1): 139–52.

Dettai, A., Adamowizc, S.J., Allcock, L., Arango, C.P., Barnes, D.K.A., Barratt, I., Chenuil, A., Couloux, A., Cruaud, C., David, B., Denis, F., Denys, G., Diaz, A., Eleaume, M., Feral, J.-P., Froger, A., Gallut, C., Grant, R., Griffiths, H.J., Held, C., Hemery, L.G., Hosie, G., Kulklinski, P., Lecointre, G., Linse, K., Lozouet, P., Mah, C., Monniot, F., Norman, M.D., O'Hara, T., Ozouf-Costaz, C., Piedallu, C., Pierrat, B. Poulin, E., Puillandre, N., Riddle, M., Samadi, S., Saucede, T., Schubart, C., Smith, P.J., Stevens, D.W., Steinke, D., Strugnell, Tarnowska, K., Wadley, V. and Ameziane, N. 2011. DNA barcoding and molecular systematics of the benthic and demersal organisms of the CEAMARC survey. *Polar Science*. **5**:298-312.

Dettai, A., Berkani, M., Lautredou, A.-C., Couloux, A., Lecointre, G., Ozouf-Costaz, C. and Gallut, C. 2012. Tracking the elusive monophyly of nototheniid fishes (Teleostei) with multiple mitochondrial and nuclear markers. *Marine Genomics*. **8**: 49-58.

DeVries, A.L. 1971. Glycoprotein as biological antifreeze agents in Antarctic fishes. *Science*. **172**: 1152-1155.

DeVries, A.L. 1982. Biological antifreeze agents in coldwater fishes. *Comparative Biochemistry and Physiology*. **73A**: 627-640.

DeVries, A.L. 1988. The role of glycopeptide and peptide antifreeze in the freezing avoidance of Antarctic fishes. *Comparative Biochemistry and Physiology*. **90B**: 611-621.

DeVries, A.L., Komatsu, S.K. and Feeney, R.E. 1970. Chemical and physical properties of freezing point-depressing glycoproteins from the Antarctic fishes. *Journal of Biological Chemistry*. **245**:2901-2908.

DeVries, A. L. and Lin, Y. 1977. The role of glycoprotein antifreezes in the survival of Antarctic fishes. In G. A. Llano (ed) *Adaptation within Antarctic Ecosystems.* The Proceedings of the Third Symposium on Antarctic Biology, Gulf Publishing Co., Houston. pp. 439-458.

DeVries, A.L. and Cheng, C-H.C. 2005. Antifreeze proteins and organismal freezing avoidance in polar fishes. In: Farrell, A.P. and Steffensen, J.F. (eds) *Fish Physiology*, vol 22, Academic Press, San Diego, pp. 155-201.

DeVries, A.L and Steffensen, J.F. 2005. The Arctic and Antarctic polar marine environments. In: Farrell, A.P. and Steffensen, J.F. (eds) *Fish Physiology*, vol 22, Academic Press, San Diego, pp. 1-24.

DeWitt, H.H. 1966. Revision of the Antarctic and southern genus Notothenia (Pisces, Nototheniidae). Unpublished Ph.D. thesis, Stanford University, Stanford. xiii+469 pp.

DeWitt, H.H. 1971. Coastal and deep-water benthic fishes of the Antarctic. In: Bushnell, V.C. (ed) *Antarctic map folio series, Folio 15*. American Geographic Society, New York, pp. 1-10.

DeWitt, H.H., P.C. Heemstra and O. Gon, 1990. Nototheniidae. In: O. Gon and P.C. Heemstra (eds.) *Fishes of the Southern Ocean,* J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa. pp. 279-331.

Di Prisco, G. 2000. Life style and biochemical adaptation in Antarctic fishes. *Journal of Marine Systems.* **27**(1-3): 253–265.

Di Prisco, G., D'Avino, R., Caruso, C., Tamburini, M., Camardella, L, Rutigliano, B., Carratore, V. and Romano, M. 1991(a). Biochemistry of oxygen transport in the red-blooded Antarctic fish. In di Prisco, G., Maresca, B. and Tota, B. (eds) *Biology of Antarctic Fishes*, Springer-Verlag, Berlin. pp. 263-281.

Di Prisco, G., Maresca, B. and Tota, B. (eds) 1991(b). *Biology of Antarctic Fishes*. Springer-Verlag, Berlin.

Di Prisco, G., Pisano, E. and Clarke, A. (eds) 1998. *Fishes of Antarctica: A Biological Overview*. Springer-Verlag, Milano.

Di Prisco, G., Giardina, B. and Weber, R.F. (eds) 2000. *Hemoglobin Function in Vertebrates: Adaptation in Extreme and Temperate Environments*. Springer-Verlag, Milano.

Di Prisco, G., Eastman, J.T., Giordano, D., Parisi, E. and Verde, C. 2007. Biogeography and adaptation of notothenioid fishes: Hemoglobin function and globin-gene evolution. *Gene*. **398**: 143-155.

Duhamel, G., Hureau, J.-C and Ozouf-Costaz, C. 1983. Ecological survey of the notothenioid fishes in the Southern Ocean from Bouvet to Kerguelen Islands. In Nemoto, T. and Matsuda, T. (eds) *Proceedings of the biomass colloquium in 1982*. Memoirs of national institute of polar research, special issue 27, Tokyo. pp. 176-182.

Duhamel, G., Gasco, N. and Davaine, P. 2005. Poissons des îles Kerguelen et Crozet: Guide régional de l'océan Austral. Muséum national d'Histoire naturelle. Paris.

Duman, J.G. 1994. Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade Solanum dulcamara. *Biochimica et Biophysica Acta*. **1206**: 129-135.

Duman, J.G., Li, N., Verleye, D., Goetz, F.W., Wu, D.W., Andorfer, C.A., Benjamin, T. and Parmelee, D.C. 1998. Molecular characterization and sequencing of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. *Journal of Comparative Physiology B*. **168**: 225-232.

Duman, J.G. and DeVries, A.L. 1976. Isolation, characterization, and physical properties of protein antifreezes from the winter flounder, *Pseudopleuronectes americanus*. *Comparative Biochemistry and Physiology B*. **53**: 375–380.

Eakin, R.R. 1990. Artedidraconidae. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 332-356.

Eakin, R.R. 1981. Osteology and relationship of the fishes of the Antarctic family Harpagiferidae (Pisces, Notothenioidei). *Antarctic Research Series*. **31**(3): 81-147.

Eakin, R.R., Eastman, J.T. and Matallanas. 2008. New species of *Pogonophryne* (Pisces, Artedidraconidae) from the Bellingshausen Sea, Antarctica. *Polar Biology*. **31**: 1175-1179.

Eakin, R.R., Eastman, J.T. and Near, T.J. 2009. A new species and a molecular phylogenetic analysis of the Antarctic fish genus *Pogonophryne* (Notothenioidei: Artedidraconidae). *Copeia*. **4**: 705-713.

Eastman, J.T. 1993. *Antarctic Fish Biology.* Academic Press, San Diego. 178 pp.

Eastman, J.T. 2005. The nature of biodiversity of Antarctic fishes. *Polar Biology*. **28**: 94-107.

Eastman, J.T. and DeVries, A.L. 1982. Buoyancy studies of notothenioid fishes in McMurdo Sound, Antarctica. *Copeia*. **2**: 385-393.

Eastman, J.T. and Grande, L. 1991. Late Eocene gadiform (Teleostei) skull from Seymour Island, Antarctic Peninsula. *Antarctic Science*. **3**: 87-95.

Eastman, J.T. and Eakin, R.R. 2000. An updated species list for notothenioid fish (Perciformes; Notothenioidei), with comments on Antarctic species. *Archive of Fishery and Marine Research.* **48**(1): 11-20.

Eastman, J.T. and McCune, A.R. 2000. Fishes on the Antarctic continental shelf: evolution of a marine species flack? *Journal of Fish Biology*. **57**: 84-102.

Eschmeyer, W.N. (ed).: Species and genera in the Catalog of fishes. http://research.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Version update 5 February 2014.

Eschmeyer, W.N. and Fong, J.D. (eds).: Species by family/subfamily in the Catalog of fishes. http://research.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp. Version update 5 February 2014.

Evans, C.W., Hellman, L., Middleditch, .M., Wojnar, J.M., Brimble, M.A. and DeVries, A.L. 2012. Synthesis and recycling of antifreeze glycoproteins in polar fishes. *Antarctic Science*. **24**(3): 259-268.

Felsentein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. **39**(4): 783-791.

Fischer, J.G. 1985. Ichthyologische und herpetologische Bemerkungen. I. Über Fische von Süd-Georgien. *Jahrb Wissench Anastalten zu Hamburg*. **2**: 49-65.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*. **3**(5): 294-299.

Foster, T.D. 1984. The marine environment. In: Laws, R.M. (ed) *Antarctic ecology, vol. 2*. Academic Press, London, pp. 345-371.

Fuji, R. The regulation of motile activity in fish chromatophores. 2000. *Pigment Cell Research*. **13**: 300-319.

Garnham, C.P., Campbell, R.L. and Davies, P.L. 2011. Anchored clathrate waters bind antifreeze proteins to ice. *Proceedings of Natural Academy of Science United States of America*. **108**: 7363–7367.

Gerday, C. 1997. Relationship linking ecology, lifestyle, and adaptive evolution of Antarctic fishes. *Comparative Biochemistry and Physiology*. **118A**: 975-1102.

Ghigliotti, L., Cheng, C.-H.C., Bonillo, C. Coutanceau, J.-P. and Pisano, E. 2013. In situ gene mapping of two gene supports independent evolution of sex chromosomes in cold-adapted Antarctic fish. *BioMed Research International*. 1-8.

Gon, O. 1990. Bathydraconidae. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 364-380.

Gon, O. and Klages, N. 1988. The marine fish fauna of the sub-Antarctic Prince Edward Islands. *South African Journal of Antarctic Research*. **18**(2): 32-54.

Gon, O. and Heemstra, P.C. (eds) 1990. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown.

Gon, O. and Mostert, D. 1992. Aspects of the ecology of two nototheniid fish species in the inshore zone of the sub-Antarctic Marion Island. *South African Journal of Antarctic Research.* **22**: 59-67.

Gon, O., Hendry, D.A. and D. Mostert. 1994. Glycoprotein antifreeze in *Notothenia coriiceps* (Pisces: Nototheniidae) from the sub-Antarctic Marion Island. *South African Journal of Antarctic Research.* **24**(1and2): 53-56.

Grande, L. and Eastman, J.T. 1986. A review of Antarctic ichthyofaunas in the light of new fossil discoveries. *Palaeontology*. **29**(1): 113-137.

Griffith, M. and Yaish, M.W.F. 2004. Antifreeze proteins in overwintering plants: a tale of two activities. *Trends in Plant Science*. **9**(8): 399-405.

Günther, A. 1880. Report on the shore fishes procured during the Voyage of H.M.S. 'Challenger' in the years 1873-1876. *Challenger Report of Zoology*. **22**: xiv+268 pp.

Harding, M.M., Anderberg, P.I. and Haymet, A.D.J. 2003. Antifreeze glycoproteins from polar fish. *European Journal of Biochemistry.* **270**(7): 1381–1392.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and Dewaard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*. **270**: 313-322.

Hew, C.L., Slaughter, D., Joshi, S., Fletcher, G.L. and Ananthanarayanan, V.S. 1984. Antifreeze polypeptides from the Newfoundland ocean pout, *Macrozoarces americanus*: Presence of multiple and compositionally diverse components. *Journal of Comparative Physiology: B.* **155**: 81–88.

Hofmann, G.E., Lund, S.G., Place, S.P. and Whitmer, A.C. 2005. Some like it hot, some like it cold: the heat shock response is found in New Zealand but not Antarctic notothenioid fishes. *Journal of Experimental Marine Biology and Ecology.* **316**(1): 79–89.

Hsiao, K.C., Cheng, C.-H.C., Fernandes, I.E., Detrich, H.W. and DeVries, A.L. 1990. An antifreeze glycopeptide gene from the Antarctic cod *Notothenia corriceps neglecta* encodes a polyprotein of high peptide copy number. *Proceedings of the Natural Academy of Sciences of the United State of America*. **87**: 9265-9269.

Hureau, J.-C. 1985(a). Artedidraconidae. In: Fischer, W. and Hureau, J.C. (eds.) *FAO species identification sheets for fishery purposes. Southern Ocean (Fishing areas 48, 58 and 88), vol. 2*. Food and Agriculture Organisation, Rome, pp. 245-251

Hureau, J.-C. 1985(b). Bathydraconidae. In: Fischer, W. and Hureau, J.C. (eds.) *FAO species identification sheets for fishery purposes. Southern Ocean (Fishing areas 48, 58 and 88), vol. 2*. Food and Agriculture Organisation, Rome, pp. 252-256.

Hureau, J.-C. 1985(c). Channichthyidae. In: Fischer, W. and Hureau, J.C. (eds.) *FAO species identification sheets for fishery purposes. Southern Ocean (Fishing areas 48, 58 and 88), vol. 2*. Food and Agriculture Organisation, Rome, pp. 261-277.

Hureau, J.-C. 1985(d). Harpagiferidae. In: Fischer, W. and Hureau, J.C. (eds.) *FAO species identification sheets for fishery purposes. Southern Ocean (Fishing areas 48, 58 and 88), vol. 2*. Food and Agriculture Organisation, Rome, pp. 282-284.

Hureau, J.-C. 1985(e). Nototheniidae. In: Fischer, W. and Hureau, J.C. (eds.) *FAO species identification sheets for fishery purposes. Southern Ocean (Fishing areas 48, 58 and 88), vol. 2*. Food and Agriculture Organisation, Rome, pp. 323-385.

Hureau, J.-C. 1990. Harpagiferidae. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 357-363.

Hureau, J.-C. (ed.) 1997. Proceedings, 3rd workshop of the European Science Foundation Network on "Antarctic fishes: Ecology, lifestyle, and adaptive evolution. Comparison with Arctic fish." *Cybium*. **21**(4): 334-450

Hureau, J.-C. and Slosarczyk, W. 1990. Exploitation and conservation of Antarctic fishes and recent ichthyological research in the Southern Ocean. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 52-63.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H. and Hebert, P.D.N. 2007. Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*. **7**:544-548.

Iwami, T. 1985. Osteology and relationships of the family Channichthyidae. *Memoirs. National Institute of Polar Research, Series E. Biology and Medical Science.* **36**: 1-69.

Iwami, T. and Kock, K.-H. 1990. Channichthyidae. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 381-399.

Iwami, T., Numanami, H. and Naito, Y. 1996. Behaviour of three species of the family Artedidraconidae (Pisces, Notothenioidei) with reference to feeding. *Proceedings of NIPR Symposium of Polar Biology*. **9**: 225-230.

Jai, Z. and Davies, P.L. 2002. Antifreeze proteins: an unusual receptor-ligand interaction. *Trends in Biochemical Sciences*. **27**(2): 101-106.

Jin, Y. 2003. Freezing avoidance of Antarctic fishes: the role of a novel antifreeze potentiating protein and the antifreeze glycoproteins. Unpublished PhD thesis. University of Illinois, Urbana-Champaign, USA.

Jin, Y. and DeVries, A.L. 2006. Antifreeze glycoprotein levels in Antarctic notothenioid fishes inhabiting different thermal environment and the effects of warm acclimation. *Comparative Biochemistry and Physiology.* **144B**: 290-300.

Johnson, G.D. and Patterson, C. 1993. Percomorph phylogeny: A survey of Acanthomorphs and a news proposal. *Bulletin of Marine Science*. **52**(1): 554-626.

Jones, C.D., Anderson, M.E., Balushkin, A.V., Duhamel, G., Eakin, R.R., Eastman, J.T., Kuhn, K.L., Lecointre, G., Near, T.J., North, A.W., Stein, D.L., Vacchi, M. and Detrich, H.W. III. 2008. Diversity, relative abundance, new locality records and population structure of the Antarctic demersal fishe from the northern Scotia Arc islands and Bouvetøya. *Polar Biology*. **31**: 1481-1497.

Katz, M.E., Cramer, B.S., Toggweiler, J.R., Esmay, G., Liu, C., Miller, K.G., Rosenthal, Y. Wade, B.S. and Wright, J.D. 2011. Impact of Antarctic Circumpolar Current development on late Paleogene ocean structure. *Science*. **332**: 1076-1079.

Kellermann, A. and Kock, K.-H. 1984. Postlarval and juvenile notothenioids (Pisces, Perciformes) in the Southern Scotia Sea and Northern Weddell Sea during FIBEX 1981. *Meeresforschung.* **30**:82–93.

Kennett, J.P. 1982. *Marine geology*. Prentice-Hall. Englewood Cliffs. New Jersey.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*. **16**: 11-120.

Klingenberg, C.P. and Ekau, W. 1996. A combined morphometric and phylogenetic analysis of an ecomorphological trend: Pelagization in Antarctic fishes (Perciformes: Nototheniidae). *Biological Journal of Linnean Society*. **59**: 143–177.Knight, C.A., Driggers, E. and DeVries, A.L. 1993. Adsorption to ice of fish antifreeze glycopeptides 7 and 8. *Biophysical Journal*. **64**: 252-259.

Knight, C.A., Driggers, E. and DeVries, A.L. 1993. Adsorption of fish antifreeze glycopeptides to ice, and effects on ice crystals growth. *Biophysical Journal*. **64**: 252-259.

Knox, G. A. 1970. Antarctic marine ecosystem. In: Holdgate, M.W. (ed) *Antarctic ecology, vol. 1*. Academic Press, London, pp. 69-96.

Knox, G.A. 2007. *Biology of the Southern Ocean, 2nd edn*. New Zealand.

Kocher, T.D., Conroy, J.A., Stauffer, J.R. and Luckwood, S.F. 1995. Evolution of NADH dehydrogenase subunit 2 in East African Cichlid fish. *Molecular Phylogenetics and Evolution.* **4**(4):420-432.

Kock, K.-H. 2005. Antarctic icefishes (Channichthyidae): a unique family of fishes. A review, Part I. *Polar Biology*. **28**(11): 862–895.

Kock, K.-H. and Kellermann, A. 1991. Review: Reproduction in Antarctic notothenioid fish. *Antarctic Science*. **3**(2): 125-150.

Kondo, H., Hanada, Y., Sugimoto, H., Hoshino, T., Garnham, C.P., Davies, P.L. and Tsuda, S. 2012. Ice-binding site of snow mold fungus antifreeze protein deviates from structural regularity and high conservation. *Proceedings of Natural Academy of Science United States of America.* **109**: 9360–9365

Kuhn, K.L. and Near, T.J. 2009. Phylogeny of *Trematomus* (Notothenioidei: Nototheniidae) inferred from mitochondrial and nuclear gene sequences. *Antarctic Science*. **21**: 565–570.

Langenbuch, M. and Pörtner, H.O. 2003. Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as afunction of ambient CO_2: pH-

dependent limitations of cellular protein biosynthesis? *Journal of Experimental Biology*. 206: 3895-3903.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*. **23**: 2947-2948.

Lautredou, A.-C., Bonillo, C., Denys, G., Cruaud, C., Ozouf-Costaz, C., Lecointre, G. and Dettai, A. 2010. Molecular taxonomy and identification within the Antarctic genus Trematomus (Notothenioidei, Teleostei): How valuable is barcoding with COI? *Polar Science*. **4**: 333-352.

Lee, J.K., Park, K.S., Park, S., Park, H., Song, Y.H., Kang, S.H. and Kim, H.J. 2010. An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast. *Cryobiology*. **60**: 222–228.

Librado, P. and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*. **25**(11):1451-1452.

Loeb, V., Kellermann, A. K., Koubbi, P., North, A. W. and White, M.G. 1993. Antarctic larval fish assemblages: a review. *Bulletin of Marine Science*. **53**:416–449.

Lönnberg, E. 1905. The fishes of the Swedish South Polar Expedition. *Südpolar-Expedition*. 5(6): 1-69.

Lutjeharms, J.R.E. 1990. The Oceanography and fish distribution of the Southern Ocean. In: Gon, O. and Heemstra, P.C. *Fishes of the Southern Ocean*. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa, pp. 6-27.

Matschiner, M., Hanel, R. and Salzburger, W. 2011. On the origin and trigger of the notothenioid adaptive radiation. *PLoS One*. **6**(4):e18911.

McDougall, I., Verwoerd, W. and Chevallier, L. 2001. K-AR geochronology of Marion Island, Southern Ocean. *Geological Magazine*. **138**(1): 1-17.

Middleton, A.J., Marshall, C.B., Faucher, F., Bar-Dolev, M., Braslavsky, I., Campbell, R.L., Walker, V.K. and Davies, P.L. 2012. Antifreeze protein from freeze-tolerant grass has a β-roll fold with an irregularly structured ice-binding site. *Journal of Molecular Biology*. **416**: 713–724.

Miller, R.G., 1993. *A history and atlas of the fishes of the Antarctic Ocean*. Foresta Institute, Nevada.

Miya, T., Gon, O., Mwale, M. and Cheng, C-H.C. 2014. The effect of habitat temperature on serum antifreeze glycoprotein (AFGP) activity in *Notothenia rossii* (Pisces: Nototheniidae) in the Southern Ocean. *Polar Biology*. **37**: 367-373.

Møller, P.R., Nielsen, J. and Fossen, I. 2003. Patagonian toothfish found off Greenland. *Nature*. **421**: 599.

Montgomery, J. and Clements, K. 2000. Disaptation and recovery in the evolution of Antarctic fishes. *Trends in ecology and evolution*. **15**(7): 267-271.

Morley, S.A., Griffiths, H.J., Barnes, D.K.A. and Peck, L.S. 2010. South Georgia: a key location for linking physiological capacity to distributional changes in response to climate change. *Antarctic Science*. **22**: 774-7781.

Murphy, E.J., Hfmann, E.E., Watkins, J.L., Johnston, N.M., Pinones, A., Ballerini, T., Hill, S.L., Trathan P.N., Tarling, G.A., Cavanagh, R.R., Young, E.F., Thorpe, S.E. and Fretwell, P. 2013. Comparison of the structure and function of the Southern Ocean regional ecosystems: The Antarctic Peninsula and South Georgia. *Journal of Marine Systems*. **109-110**: 22-42.

Naganobu, M., Katayama, T., Ichii, T., Ishii, H. and Nasu, K. 1993. Characteristics of oceanic structure in the waters around the South Shetland Islands of the Antarctic Ocean between December 1990 and February 1991: Outstanding coastal upwelling? *Proceedings of the NIPR Symposium on Polar Biology*. **6**: 166-170.

Near, J.N. 2004. Estimating divergence times of notothenioid fishes using a fossil-calibrated molecular clock. *Antarctic Science*. **16**(1):37-44.

Near, T.J., Pesavento, J.J. and Cheng, C-H.C. 2004. Phylogenetic investigation of Antarctic notothenioid fishes (Perciformes: Notothenioidei) using complete gene sequences of the mitochondrial encoded 16S rRNA. *Molecular phylogenetics and evolution*. **23**(3):881-891.

Near, T.J. and Cheng, C.-H.C. C. 2008. Phylogenetics of notothenioid fishes (Teleostei: Acanthomorpha): Inference from mitochondrial and nuclear gene sequences. *Molecular Phylogenetic and Evolution.* **47**: 832-840.

Near, T.J., Dornburg, A., Kuhn, K.L., Eastman, J.T., Penniington, J.N. Patarnello, T., Zane, L., Fenandez, D.A. and Jones, C.D. 2012. Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes. *Proceedings of the National Academy of Science of the United States of America.* **109**(9): 3434-3439.

Nelson, J.S. 2006. *Fishes of the world, 4th edition*. John Wiley and Sons, Inc., Hoboken, New York.

Nickell, P.K., Sass, S., Verleye, D., Blumenthal, E.M. and Duman, J.G. 2013. Antifreeze proteins in the primary urine of larvae of the beetle *Dendroides canadensis*. *Journal of Experimental Biology*. **216**: 1695-1703.

Nicodemus-Johnson, J., Silic, S., Ghigliotti, L., Pisano, E. and Cheng, C.-H.C. 2011. Assembly of the antifreeze glycoprotein/trypsinogen-like protease genomic locus in the Antarctic toothfish *Dissostichus mawsoni* (Norman). *Genomics*. **98**: 194-201.

Norman, J.R. 1937. Diagnoses of new nototheniiform fishes collected by the 'Discovery' Expedition. Annals and Magazine of Natural History. 20(10): 475-476.

Norman, J.R. 1938. Coast Fishes. Part III. The Antarctic zone. *Discovery Reports*. **18**: 1-104.

Nybelin, O. 1947. Antarctic fishes. *Scientific Results of the Norwegian Antarctic Expedition*. 1927-1928, **26**: 1-76.

Nybelin, O. 1951. Sub-Antarctic and Antarctic fishes. *Scientific Results of the "Brategg" Expedition*. 1947/48, **2**: 1-32.

O'Brien, K.M. and Mueller, I.A. 2010. The unique mitochondrial form and function of Antarctic channichthyid icefishes. *Integrative and Comparative Biology*. **50**(6): 993-1008.

O'Grady, S.M. and DeVries, A.L. 1982. Osmotic and ionic regulation in polar fishes. *Journal of Experimental Marine Biology and Ecology.* **57**: 219-228.

O'Grady, S.M., Ellory, J.C. and DeVries, A.L. 1982. Protein and glycoprotein antifreezes in the intestinal fluid of polar fishes. *The Journal of Experimental biology.* **98**: 429–38.

Orsi, A. H., Whitworth, TIII and Nowlin, W.D. Jr. 1995. On the meridional extent and fronts of the Antarctic Circumpolar Current. *Deep Sea Research I.* **42**:641–673.

Park, K.S., Do, H., Lee, J.H., Park, S.I., Kim, S.-J., Kang, S.-H. and Kim, H.J. 2012. Characterization of the ice-binding protein from Arctic yeast *Leucosporidium* sp. AY30. *Cryobiology*. 64: 286–296.

Perissinotto, R., Laubscher, R.K. and McQuaid, C.D. 1992. Marine productivity enhancement around Bouvet and the South Sandwich Islands (Southern Ocean). *Marine Ecology Progress Series.* **88**: 41-53.

Permitin, Y.Y. and Sazonov, Y.I. 1974. The systematics of *Notothenia squamifrons* Günther and related species. *Journal of Ichthyology*. **14**(4): 503-514.

Petricorena, Z.L.C and Somero, G.N. 2007. Biochemical adaptations of notothenioid fishes: Comparisons between cold temperate South American and New Zealand species and Antarctic species. *Comparative Biochemistry and Physiology, Part A.* **147**:799-807.

Petrov, A.F. 2011. New data on depths inhabited by striped-eyed rock cod *Lepidonotothen kempi* (Norman) (Nototheniidae) off Bouvet Island. *Journal of Ichthyology*. **51**(8): 683-685.

Pointer, M.A, Cheng, C.-H.C., Bowmaker, J.K., Parry, J.W.L., Soto, N., Jeffery, G., Cowing, J.A and Hunt, D.M. 2005. Adaptations to an extreme environment: retinal organisation and spectral properties of photoreceptors in Antarctic notothenioid fish. *The Journal of experimental biology*. **208**: 2363–76.

Posada, D. 2003. Using Modeltest and PAUP* to select a model of nucleotide substitution. In: Baxevanis, A.D., Davison, D.B., Page, R.D.M, Petsko, G.A., Stein, L.D. and Stormo, G.D. (eds). *Current protocols in bioinformatics*. John Wiley and Sons, New York. pp. 6.5.1-6.5.14.

Posada, D. and Crandall, K.A. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics*. **14**: 817-818.

Prirodina V.P. 2000. On the systematic position of littoral and dee-water species of the genus *Harpagifer* (Harpagiferidae, Notothenioidei) from Macquarie island with a description of two new species. *Journal of Ichthyology*. **40**: 488-494.

Prirodina V.P. 2002. Redescription of littoral and deep-sea species of the genus *Harpagifer* (Harpagiferidae, Notothenioidei) off islands of the Indian Ocean Sector of the Southern Ocean with the description of a new species. *Journal of Ichthyology*. **42**: 701-712.

Prirodina V.P. 2004. *Harpagifer crozetensis* sp. nova (Harpagiferidae, Notothenioidei), a new species from the littoral of the Crozet Islands (Indian Ocean Sector of the Antarctic). Journal of Ichthyology. **44**: 395-399.

Ramer, J.C., MacWilliams, P. and Pual-Murphy, J. 1995. Effects of hemolysis and frozen storage on serum electrolyte and chemistry values in cotton-top tamarins (*Saguinus oedipus*). *Journal of Zoo and Wildlife Medicine*. **26**:61-66.

Raymond, J.A., Lin,Y. and DeVries, A.L. 1975. Glycoprotein and protein antifreezes in two Alaskan fishes. *Journal of Experimental Zoology*. **193**: 125–130.

Raymond, J.A. and DeVries, A. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proceedings of the National Academy of Science of the United States of America. **74**: 2589-2593.

Raymond, J.A., Christner, B.C. and Schuster, S.C. 2008. A bacterial ice-binding protein from the Vostok ice core. *Extremophiles*. **12**: 713–717.

Raymond, J.A. and Janech, M.G. 2009. Ice-binding proteins from enoki and shiitake mushrooms. *Cryobiology*. **58**: 151–156.

Regan, C.T. 1913. The Antarctic fishes of the Scottish National Antarctic Expedition. *Transactions of the Royal Society of Edinburgh*. **49**(15): 229-292.

Richardson, J. 1844-48. Ichthyology of the voyage of H.M.S. 'Erebus' and 'Terro'. In: Richardson, J. and Grey, J.E (eds) *The zoology of the voyage of H.M.S. 'Erebus' and 'Terro' 1839-43,2*. London, pp viii+139pp.

Ritchie, P.A., Lavoue, S. and Lecointre, G. 1997. Molecular phylogenetics and the Evolution of Antarctic notothenioid fishes. *Comparative Biochemistry and Physiology A*. **118**:1009-1025.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F. and Carvalho, G.R. 2008. DNA barcodes of fish of the Scottia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. *Antarctic Science*. **20**:253-262.

Ronquist, F. and Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. **19**(12):1572-1574.

Rosen, D.E. 1973. Interrelationships of higher euteleostean fishes. In Greenwood, P.H., Miles, R.S. and Patterson, C. (eds) *Interrelationships of fishes*. Academic Press, New York. pp. 397-513.

Rutschmann, S., Matschiner, M., Damerau, M., Muschick, M., Lehmann, M.F., Hanel, R. and Salzburger, W. 2011. Parallel ecological diversification in Antarctic notothenioid fishes as evidence for adaptive radiation. *Molecular Ecology*. **20**:4707-4721.

Ruud, J.T. 1954. Vertebrates without erythocytes and blood pigment. *Nature London*. **173**: 848-850.

Sambrook, J. and Russell, D.W. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York.

Schneppenheim, R., Kock, K.-H., Duhamel, G. and Janssen, G. 1994. On the taxonomy of the *Lepidonotothen squamifrons* group (Pisces, Perciformes, Notothenioidei). *Archive of Fishery and Marine Research*. 42: 137-148.

Shandikov, G.A. 2008. *Channichthys mithridatis*, a new speices of icefishes (Perciformes: Notothenioidei: Channichthyidae) from the Kerguelen Islands (East Antarctica), with comments on the taxonomic status of *Channichthys normani*. *Journal of Karazin Kharkiv National University, Series: Biology*. **814**: 123-131.

Shandikov, G.A. 2011. *Channichthys richardsoni* sp. n., a new Antarctic icefish (Perciformes: Notothenioidei: Channichthyidae) from the Kerguelen Islands area, Indian sector of the Southern Ocean. *Journal of Karazin Kharkiv National University, Series: Biology*. **971**: 125-134.

Shandikov, G.A and Eakin, R.R. 2013. *Pogonophryne neyelovi* , a new species of Antarctic short-barbeled plunderfish (Perciformes, Notothenioidei, Artedidraconidae) from the deep Ross Sea. *ZooKeys.* **77**(296): 59–77.

Shandikov, G.A., Eakin, R.R. and Usachev, S. 2013. *Pogonophryne tronio*, a new species of Antarctic short-barbeled plunderfish (Perciformes: Notothenioidei: Artedidraconidae) from the deep Ross Sea with new data on *Pogonophryne brevibarbata*. *Polar Biology*. **36**(2): 273–289.

Shotton, R and Tandstad, M. 2011. Southern Ocean: FAO statistical areas 48, 58 and 88. In: *Review of the state of wold marine fishery resources*. FAO and Fisheries Aquaculture Technical Paper, 569. B16: 217-224.

Simmons, M.P. and Ocheterena, H. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology*. **49**(2): 369-381.

Slaughter, D., Fletcher, G.L., Ananthanarayanan, V.S. and Hew, C.H. 1981. Antifreeze proteins from the sea raven, *Hemitripterus americanus*. *Journal of Biology and Chemistry*. **256**: 2022–2026.

Smith, P.J., Steinke, D., Dettai, A., McMillan, P., Welsford, D., Stewart, A. and Ward, R.D. 2012. DNA barcodes and species identifications in Ross Sea and Southern Ocean fishes. *Polar Biology*. **35**:1297-1310.

Somero, G.N. and DeVries, A.L. 1967. Temperature tolerance of some Antarctic fishes. *Science*. **156**(3772): 257-258.

Sponaugle, S. Cowen, R.K., Shanks, A., Morgan, S.G., Leis, M.J., Pineda, J. Boehlert, G.W., Kingsford, M.J., Lindeman, K.C., Grimes and C. Munro, J.L. 2002. Predicting self-recruitment in marine populations: biophysical correlates and mechanisms. *Bulletin of Marine Science*. **70**: 341-375.

Stankovic, A., Spalik, K., Kamler, E., Borsuk, P. and Welfenski, P. 2002. Recent origin of sub-Antarctic notothenioids. *Polar Biology*. **25**:203-205.

Swofford D.L. 2002. PAUP* v4: Phlyogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution*. **20**(10):2731-2739.

Tankevich, P.B. 1994. Growth and age of Antarctic cod, *Notothenia rossii rossii*, on the Ob bank (Indian Ocean Sector of Antarctica). *Journal of Ichthyology*. **34**(4): 66-71.

Tomasziewicz, M., Hautecoeur, M., Coutanceau, J.-P., Bonillo, C., Dettai, A., Mazzei, F., Ghigliotti, L., Pisano, E., Detrich, H.W., Duhamel, G., Lecointre, G. and Ozouf-Costaz, C. 2011. Comparative cytogenetic studies of the Nototheniidae (Teleostei : Acanthomorpha)

from the Indian (Kerguelen-Heard plateau) and Atlantic sectors (South Georgia, South Sandwich, Falklands/Malvinas, Bouvet Islands) sectors of the Southern Ocean. In: Duhamel, G., Welsford, D. (eds.) *The Kerguelen Plateau, Marine Ecosystems and Fisheries.* pp. 109–121.

Verde, C., Parisi, E. and di Prisco, G. 2006. The evolution of thermal adaptation in polar fish. *Gene*. **385**: 137–45.

Voskoboinikova, O.S. and Kellermann, A. 1997. The osteological development of nine species of Nototheniidae (Perciformes: Notothenioidei). *Cybium*. **21**(3): 231-264.

Voskoboinikova, O.S. and Balushkin, A.V. 1998. Morphological characteristics and distribution of Antarctic Joan's flatenose Bathydraco joanne (Bathydraconidae, Notothenioidei) with descreption of young fish from Kerguelen Island. *Journal of Ichthyology*. **38**: 673-676.

Voskoboynikova, O.S. and Balushkin, A.V. 1991. On the taxonomic status of *Gobionotothen angustifrons sandwichensis* (Nototheniidae). *Journal of Ichthyology*. **31**(9): 71-78.

Wöhrmann, A.P.A. 1996. Antifreeze glycopeptides and peptides in Antarctic fish species from the Weddell Sea and the Lazarev Sea. *Marine Ecology Progress Series*. **130**: 47-59.

Yang, S.-H., Wojnar, J.M., Harris, P.W., DeVries, A.L., Evans, C.W. and Brimble, M.A. 2013. Chemical synthesis of a masked analogue of the fish antifreeze potentiating protein (AFPP). *Organic and Biomolecular Chemistry*. **11**(30): 4901-5048.