Functional Antifreeze Glycoprotein Genes in Temperate-Water New Zealand Nototheniid Fish Infer an Antarctic Evolutionary Origin

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The fish fauna of the Antarctic Ocean is dominated by five endemic families of the Perciform suborder Notothenioidei, thought to have arisen in situ within the Antarctic through adaptive radiation of an ancestral stock that evolved antifreeze glycoproteins (AFGPs) enabling survival as the ocean chilled to subzero temperatures. The endemism results from geographic confinement imposed by a massive oceanographic barrier, the Antarctic Circumpolar Current, which also thermally isolated Antarctica over geologic time, leading to its current frigid condition. Despite this voluminous barrier to fish dispersal, a number of species from the Antarctic family Nototheniidae now inhabit the nonfreezing cool temperate coasts of the southern continents. The origin of these temperate-water notothenioids is not completely understood. Since the AFGP gene apparently evolved only once, before the Antarctic notothenioid radiation, the presence of AFGP genes in extant temperate-water notothenioids can be used to infer an Antarctic evolutionary origin. Genomic Southern analysis, PCR amplification of AFGP genes, and sequencing showed that *Notothenia angustata* and *Notothenia microlepidota* endemic to southern New Zealand have two to three AFGP genes, structurally the same as those of the Antarctic notothenioids. At least one of these genes is still functional, as AFGP cDNAs were obtained and low levels of mature AFGPs were detected in the blood. A phylogenetic tree based on complete ND2 coding sequences showed monophyly of these two New Zealand notothenioids and their inclusion in the monophyletic Nototheniidae consisted of mostly AFGP-bearing taxa. These analyses support an Antarctic ancestry for the New Zealand notothenioids. A divergence time of approximately 11 Myr was estimated for the two New Zealand notothenioids, approximating the upper Miocene northern advance of the Antarctic Convergence over New Zealand, which might have served as the vicariant event that lead to the northward dispersal of their most recent common ancestor. Similar secondary northward dispersal likely applies to the South American notothenioid *Paranotothenia magellanica*, which has four AFGP genes in its DNA, but not to the sympatric notothenioid *Patagonotothen tessellata*, which does not appear to have any AFGP sequences in its genome at all.

Introduction

The perciform suborder Notothenioidei is a monophyletic group of fishes indigenous to the southern ocean and largely endemic to the Antarctic region, with no relatives known in the northern hemisphere (Gon and Heemstra 1990; Eastman 1993; Bargelloni et al. 2000). Currently, eight families encompassing 122 species are recognized (Eastman and Eakin 2000). Three small basal families (Bovichtidae, Pseudaphritidae, and Eleginopidae) with 12 species are non-Antarctic, 11 of which reside in the southern coastal waters of New Zealand, Australia, and South America. The other five families (Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae) make up the rest of the suborder, consisting of 94 species that are endemic to the Antarctic and 15 species that occur along the cool-temperate southern coast of South America and New Zealand (Gon and Heemstra 1990; Eastman and Eakin 2000).

The non-Antarctic and Antarctic notothenioid fishes are separated by a major oceanographic barrier, the Antarctic Circumpolar Current (ACC), which is a massive clockwise and northeastward moving oceanic current 200 to 1,000 km wide and reaching the ocean floor, creating a polar front zone (Foster 1984). The ACC is both a thermal barrier, decoupling the warm subtropical gyres from the cold Antarctic waters, resulting in a sharp temperature drop across the polar front, and a physical barrier against migration of fish in either direction. The unrestricted flow of the ACC was estimated to have been established around 25 to 22 MYA, leading to the thermal isolation and subsequent glaciation of the Antarctic continent and freezing of its coastal water; current frigid conditions were reached around mid-Miocene (10 to 15 MYA) (Kennett 1977, 1982; Anderson 1999).

The basal non-Antarctic notothenioids (Bovichtidae, Pseudaphritidae, and Eleginopidae) presumably diverged and became established in the brackish coastal water of the southern continental blocks before the isolation of Antarctica, whereas the Antarctic species are thought to have arisen in situ within the Antarctic (Eastman 1993; Eastman and McCune 2000). The survival of the endemic Antarctic species in their ice-laden, subzero marine environment depends on the presence of bloodborne antifreeze glycoproteins (AFGPs), which bind to ice crystals that enter their bodies and arrest further ice growth, thus preventing their blood and other body fluids from being frozen (DeVries 1988; Cheng and DeVries 1991). AFGPs in Antarctic notothenioids occur as a family of many size isoforms, comprising four to over 55 repeats of a basic glycotripeptide unit, Ala/Pro-Ala-Thr, with each Thr linked to a disaccharide of galactose-N-acetylgalactosamine (DeVries 1988; Cheng and DeVries 1991). The AFGP gene evolved from a functionally unrelated pancreatic trypsinogen-like serine protease gene through recruitment of the front segment (exon 1 and intron 1) and the tail segment (exon 6 and 3′ flanking sequence) of the ancestral protease gene, de novo iterative duplications of a 9-nt ThrAlaAla coding element that straddled the intron

Key words: AFGP gene, non-Antarctic notothenioids, South American notothenioids, evolution, biogeography.

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1 and exon 2 junction creating the repetitive ice-binding AFGP coding region, and deletion of exons 2 through 5 of the protease ancestor (Chen, DeVries, and Cheng 1997*a*; Cheng 1998; Cheng and Chen 1999). The evolution of AFGP apparently occurred once, before the diversification of the five Antarctic families (Cheng 1998; Eastman and Clarke 1998). This antifreeze protection has been considered to be a key evolutionary innovation, which enabled the ancestral notothenioid to undergo adaptive radiation and diversification into icy water column niches vacated by the extinction of unprotected fish taxa to become the dominant fish group in today's Antarctic fish fauna and the only known marine species flock (Eastman and Clarke 1998; Eastman and McCune 2000). Contemporary Antarctic notothenioids are endowed with a large AFGP gene dosage, by virtue of their large AFGP multigene families, with many member genes each encoding a large AFGP polyprotein precursor that yields multiple mature AFGP molecules (as many as 46) after posttranslational removal of the three-residue spacers that link them in tandem (Hsiao et al. 1990; Chen, DeVries, and Cheng 1997*a*, 1997*b*).

Given the ACC barrier to fish dispersal, how the notothenioid species from the predominantly endemic Antarctic group became distributed in the cool-temperate coasts of the southern land masses has been an ongoing question. All these species belong to the most speciose Antarctic family, Nototheniidae (Eastman and Eakin 2000). The genus *Notothenia* has two non-Antarctic members, *N. angustata* and *N. microlepidota* (commonly known as New Zealand black cods), which occur in the cool temperate shallow water of the southeastern coast of South Island (Otago Harbor, 45.5°S) and the islands of the Campbell Plateau (50°S to 54°S) farther south, respectively. These two species resemble their Antarctic relatives in hemoglobin multiplicity (D'Avino and Di Prisco 1997), absence of swim bladder, and negative buoyancy associated with a typically benthic life style (MacDonald, Montgomery, and Wells 1987) but differ with respect to hematological properties (Fago, D'Avino, and Di Prisco 1992), the presence of kidney glomeruli, and the apparent absence of antifreeze activity in blood, instead of the completely aglomerular kidneys associated with renal conservation of circulatory AFGPs, particularly the small isoforms in Antarctic members (Eastman and DeVries 1986). This mixture of anatomical and physiological similarities and differences cast ambiguity over whether the New Zealand black cods are of an Antarctic origin.

The most recent, comprehensive molecular phylogenetic hypothesis of Notothenioidei, based on partial 12S and 16S rRNA gene sequences from 33 taxa, shows that two other non-Antarctic nototheniids, *Dissostichus eleginoides* and *Patagonotothen tessellata*, which occur along the southern coasts of South America, are sister taxa to the endemic Antarctic species, leading to the general inference that non-Antarctic nototheniids originated from dispersal events of Antarctic ancestors to the north, presumably associated with the northward excursions of the polar front (Bargelloni et al. 2000). This phylogenetic analysis did not include the two New Zealand black cods (Bargelloni et al. 2000), and thus an analysis including these two taxa would be informative in inferring their evolutionary origin and mechanism of dispersal.

Since the evolution of the notothenioid AFGP apparently occurred once, before the five Antarctic families diverged (Cheng 1998; Eastman and Clarke 1998), and the evolution was directly linked to the freezing of the Antarctic water, determining whether AFGP gene sequences are present in the New Zealand nototheniids and other non-Antarctic notothenioids would allow us to infer their geographic evolutionary origin. The apparent lack of antifreeze in the two NZ species may be due to insufficient sensitivity in the prior detection method used, thus, more sensitive assays are needed to determine whether AFGP is indeed present in their blood, which will ascertain whether the AFGP genes if present are still functional. In addition, a phylogenetic hypothesis including these two taxa would clarify their relationship with their Antarctic congeners and other endemic Antarctic species. This study sought to ascertain the evolutionary origin of the New Zealand black cods through a combination of molecular, phylogenetic, and protein biochemical methods.

Materials and Methods

Specimens

The New Zealand nototheniids *Notothenia angustata* and *N. microlepidota* were collected with traps in the shallow water near the entrance of Otago Harbor (45.5°S, 170°E) of South Island in August and September and held till early October at ambient temperatures (14°C to 10°C) at the Portobello Marine Station, Otago University, before being transported to McMurdo Station, Antarctica for cold acclimation. *N. microlepidota* occurs predominantly around the islands of the Campbell Plateau, and Otago Harbor represents the northern limit of its distribution range (Stewart 2002), where an occasional specimen is caught. Two other non-Antarctic nototheniids in this study, *Paranotothenia magellanica* and *Patagonotothen tessellata*, were collected in the Beagle Channel near Ushuaia, Argentina, and generously provided to us by Dr. Jorge Calvo. The basal non-Antarctic notothenioids—*Bovichtus variegatus* (Bovichtidae), *Pseudaphritis urvilli* (Pseudaphritidae), and *Eleginops maclovinus* (Eleginopidae)—were collected from Otago Harbor, New Zealand, Tasmania, Australia, and Punta Arenus, Chile, respectively. For endemic Antarctic notothenioids, nototheniids *N. coriiceps* and *N. rossii* were collected from the Antarctic Peninsula water, and *Dissostichus mawsoni* and *Trematomus bernacchii* were collected from McMurdo Sound. *Pogonophryne scotti* (Artedidraconidae) was from the Ross Sea, *Harpagifer antarcticus* (Harpagiferidae) was from South Georgia, and *Gymnodraco acuticeps* and *Akarotaxis nudiceps* (Bathydraconidae) were from McMurdo Sound and the Ross Sea, respectively. *Chaenocephalus aceratus* and *Pagetopsis macropterus* (Channichthyidae) were from the Antarctic Peninsula and McMurdo Sound, respectively. Tissues and serum collected from these fish were flash frozen in liquid nitrogen and stored at –80°C until use.

Genomic Southern Analysis

High-molecular-weight genomic DNA was isolated from liver or spleen. About 5 μg of genomic DNA from Antarctic notothenioid species, and 20 to 25 μg of genomic DNA from non-Antarctic species were digested with *Eco*RI or *Taq*I and electrophoresed on 1% agarose gel and vacuum-transferred to Hybond-N nylon membrane (Amersham). The membrane was hybridized in QuickHyb solution (Stratagene) to a ^{32}P-labeled probe derived from a 2.1-kb fragment of an AFGP gene (clone *Dm*1a) from *D. mawsoni* (Chen, DeVries, and Cheng 1997a) that contains only the intronless, repetitive AFGP polyprotein coding region. The membrane was hybridized at 55°C overnight, washed to 65°C in 0.5% SDS/0.1 × SSC (15 mM NaCl, 1.5 mM Na citrate), and autoradiographed on X-ray film. The blot was then completely stripped of the AFGP probe by immersing it in boiling 0.1% SDS and allowing the solution to cool slowly to room temperature. The stripped blot was reautoradiograped on X-ray film to ensure that all AFGP probe has been removed, then rehybridized to a ^{32}P-labeled probe derived from a 455-nt fragment of a cDNA of the trypsinogen-like protease (TLP) from *D. mawsoni* (Chen, DeVries, and Cheng 1997*a*) that included only the coding sequence from exon 2 through exon 5. The hybridization and washing conditions were similar to those for the AFGP probe. This AFGP gene probe and TLP cDNA probe cover the nonhomologous regions of these two evolutionarily related genes (Chen, DeVries, and Cheng 1997*a*; Cheng and Chen 1999), and thus each probe will hybridize to its specific target only (i.e. AFGP and TLP sequences, respectively).

Amplification of AFGP Genes

Partial AFGP genes were amplified from genomic DNA by PCR (polymerase chain reaction) with a pair of primers designed from AFGP genes of the Antarctic notothenid *D. mawsoni*. Complete AFGP genes consist of two exons intervened by a single intron of about 2 kb; exon 2 encodes the AFGP polyprotein precursor (Chen, DeVries, and Cheng 1997*a*, 1997*b*). The primers used here amplified complete exon 2, the AFGP polyprotein coding region, by annealing to conserved sequences at the 3′ end of intron 1 and the 5′ end of the 3′ flanking sequence. The reaction mixture consisted of 1 μg of genomic DNA, 0.8 μM of each primer, 0.25 mM of dNTPs, 1.6 mM of $MgCl_2$, 6 units of *Taq* polymerase, 6 units of *Taq* Extender (Stratagene), and 1X *Taq* Extender buffer, in a 60 μl volume. The PCR reaction condition consisted of denaturation at 94°C for 4 min followed by 19 cycles of 94°C for 1 min and 70°C for 4 min, empirically determined to successfully amplify through the highly repetitive AFGP polyprotein coding region. Southern blot analysis of the PCR products was performed similar to that for genomic DNA described above, using the AFGP-specific probe. One AFGP-positive PCR product each from *N. angustata* and *N. microlepidota* was recovered from agarose gel, cloned into pCR2.1 (Invitrogen), and sequenced.

RT-PCR Amplification of AFGP cDNA

Total RNA was isolated from pancreatic tissue of *N. angustata* and *N. microlepidota* (after holding at 2°C to 4°C for 2 months) using the Ultraspec RNA isolation reagent (Biotecx). PolyA+ RNA was isolated from total RNA using Dynabeads (Dynal) and treated with 1 unit of RNase-free DNase I (Life Technologies). The DNA-free polyA+ RNA was reverse transcribed with the Superscript Preamplification System (Life Technologies) to produce the first strand cDNA, and double-stranded, partial AFGP cDNA was generated by PCR amplification using a forward and a reverse primer that anneals to the conserved 5′ UTR (forward) and AFGP spacer (reverse) sequences of AFGP genes. Thirty-five amplification cycles of 1 min at 95°C, 50 s at 55°C, and 2 min at 72°C were performed. The RT-PCR products were cloned as a pool in pCR2.1 (Invitrogen). AFGP-positive clones were isolated by screening with the AFGP-specific probe, and several were selected for sequencing.

Detection of Mature AFGP in New Zealand Nototheniid Serum by Nanoliter Osmometry and ELISA

Native serum from three environmental specimens of *N. angustata* (10°C to 14°C) and from three other specimens after 2 months of 2°C to 4°C acclimation were examined for thermal hysteresis (antifreeze activity), which is the difference between the melting and freezing temperatures of a minute, single-crystal seed ice ($< 5\mu M$) in the serum using the Clifton nanoliter osmometer. The nanoliter osmometer is equipped with a light microscope that allows direct observation of the growth habit of the seed ice crystal under fine temperature control.

Actual concentrations of AFGPs in the blood of *N. angustata* were estimated by enzyme-linked immunosorbent assay (ELISA). Polyclonal antibodies against purified AFGPs from *D. mawsoni* were raised in rabbit and used in the ELISA. Serum samples were collected from 11 environmental (10°C to 14°C) individuals of *N. angustata* in New Zealand and again after acclimation at 2°C to 4°C water for 2 months at McMurdo Station, Antarctica. In each assay, a 96-well ELISA plate was coated with a series of known concentrations of antigen standards (purified AFGPs) and diluted (usually 1:1000) *N. angustata* serum samples. The plate was rinsed with phosphate buffer saline (PBS) three times and then blocked with PBSBT (PBS, 0.1% bovine serum albumin, 0.1% Tween). After washing the plate with PBSBT three times, rabbit anti-AFGP serum (diluted 1:500 in PBSBT) was added. After 30 min of incubation at room temperature and rinsing with PBSBT, the secondary antibody, goat–anti-rabbit IgG-HRP (horse radish peroxidase) diluted 1:4000 in PBSBT, was added to bind the primary antibody. The chromogenic peroxidase substrate tetramethylbenzidine (TMB) was added and incubated at room temperature for 5 min. The reactions were stopped by adding 4M H_2SO_4, and the absorbance at 450 nm of the samples was read with a plate reader (Dynatech MR5000). The concentration of AFGPs in *N. angustata* samples was determined against the standard curve of purified AFGPs, and the physiological

concentration in black cod serum was calculated by taking into account the dilution factors used in the sample preparation.

Phylogenetic Relationship of *Notothenia* Species

Relationships of species in the genus *Notothenia* were estimated using a maximum likelihood (ML) analysis of the complete ND2 gene sequences of 10 of the notothenioid taxa listed above. Based on previous phylogenetic analyses of notothenioid relationships (Balushkin 2000; Bargelloni et al. 2000), one species each from the Bathydraconidae and Channichthyidae were used as outgroups in the phylogenetic analysis. The complete protein-coding region of the ND2 gene was amplified using previously published primers (Kocher et al. 1995) in a 50-μl volume. PCR products were treated with 1 unit of Exonuclease I and shrimp alkaline phosphatase for 15 min at 37°C, followed by heating for 20 min at 80°C to inactivate the enzymes. The treated PCR products were then used as template for Big Dye Terminator (Applied Biosystems) cycle sequencing, and the sequencing reactions were read with an ABI 3100 automated sequencer. Individual sequence files were edited using EditView version 1.0.1 software, and complete sequence overlaps were constructed from edited sequence files using the program Sequencher version 4.0 (Gene Codes Corp.). Complete ND2 sequences were aligned by eye, as there were no observed insertions or deletions in the sequences. Phylogenetic analysis of the complete ND2 sequences used ML optimality criteria and tree searches were carried out using PAUP* version 4.0 (Swofford 2000). Before ML analysis, the optimal model of sequence evolution for ND2 was determined from a total of 56 progressively complex models by using a hierarchical likelihood ratio test (LRT) (Huelsenbeck and Crandall 1997). The computer program Modeltest version 3.0 was used to calculate ML scores for each model and execute the LRTs (Posada and Crandall 1998). The best ML tree was found using PAUP* 4.0 with the model of sequence evolution and parameters selected using the LRTs with a heuristic tree search using 10 random addition sequence replicates and TBR branch swapping. The support of inferred nodes was assessed using a nonparametric bootstrap analysis with 100 pseudoreplicates.

The sequences reported in this study have been deposited at NCBI under accession numbers AF514871 to AF514874 and AY256561 to AY256570.

Results

Differential AFGP Gene Dosage in Antarctic and Non-Antarctic Notothenioids

Figure 1 shows the Southern blot of *EcoR*I digested genomic DNA from 14 notothenioid species representing all eight families, hybridized to the AFGP-specific probe (panel *A*), stripped and rehybridized to the trypsinogen-like protease (TLP)-specific probe (panel *B*). The DNA of the notothenid *Dissostichus mawsoni* (both panels, lane 5) was also digested with the more frequent cutting 4-bp restriction enzyme *Taq*I to produce shorter DNA fragments that would include a single AFGP gene per fragment. Neither *EcoR*I nor *Taq*I cleaves within the AFGP genes we have characterized (Hsiao et al. 1990; Chen, DeVries, and Cheng 1997*a*, 1997*b*); thus, each positive band in panel *A* represents one or more complete AFGP genes. *EcoR*I does cleave once in the TLP cDNA of several species (unpublished results), and thus each positive band in panel *B* represents a partial or complete TLP gene. The multiple weakly hybridizing bands of *Taq*I digested *Dm* DNA (fig. 1*B*, lane 5) are the result of multiple cleavage of the TLP gene sequence by this 4-bp restriction endonuclease, and therefore do not represent gene number.

Figure 1*A* illustrates the following with regard to the AFGP gene dosage in non-Antarctic and Antarctic notothenioid fishes. First, the three basal non-Antarctic notothenioid species, one each from the families Bovichtidae, Pseudaphritidae, and Eleginopidae, do not appear to possess any AFGP gene sequences in their DNA, as indicated by the complete absence of hybridization to the AFGP-specific probe (fig. 1*A*, lanes 1 to 3). This is the first experimental confirmation of the generally accepted belief that these basal taxa diverged before the isolation of Antarctica and the evolution of the AFGP gene. Second, the Antarctic members of the five Antarctic families all show multiple AFGP-positive bands, some with very strong hybridization intensity, indicating presence of multiple colocalized AFGP gene–bearing DNA fragments of similar size (fig. 1*A*, lanes 4 to 6 and 11 to 14). These results verify that notothenioid species across all five families occupying freezing habitats possess large AFGP gene families. For example, the 10 or more *Taq*I-resolved AFGP-positive bands of *D. mawsoni* (*Dm*, fig. 1*A*, lane 5) plus the highly intense bands between 6 kb to 7.5 kb indicate that its AFGP gene family has many more than 10 member genes. Third, the non-Antarctic members of the predominantly Antarctic Nototheniidae have few or no AFGP gene sequences in their genome, as indicated by the few and much weaker hybridizing bands (fig. 1*A*, lanes 7 to 9), or complete absence of hybridization (fig 1*A*, lane 10), although four to five folds of digested genomic DNA over Antarctic species were applied per lane. For the New Zealand black cods, *N. angustata* (*Na*, fig. 1*A*, lane 7) has only two weakly hybridizing bands, and *N. microlepidota* (*Nm*, fig. 1*A*, lane 8) has three, as opposed to their Antarctic congener *N. coriiceps* (*Nc*, fig. 1*A*, lane 6), which has an AFGP gene family size akin to *D. mawsoni*. Of the two South American notothenids included in figure l*A*, *Paranotothenia magellanica* (*Pm*, fig. 1*A*, lane 9) has four very weakly hybridizing bands, whereas the sympatric nototheniid *Patagonotothen tessellata (Pt*, fig. 1*A*, lane 10) does not appear to have any AFGP sequences at all in its DNA.

Regardless of the presence or absence of AFGP gene sequences, the same Southern blot, stripped and rehybridized with the TLP-specific cDNA probe (fig.1*B*) derived from *D. mawsoni* under high stringency, shows that all notothenioid taxa, whether basal, derived, non-Antarctic, or Antarctic, possess TLP genes (from one to at most four) in their genomes. This lends strong support to the common ancestry of the notothenioid group as a whole

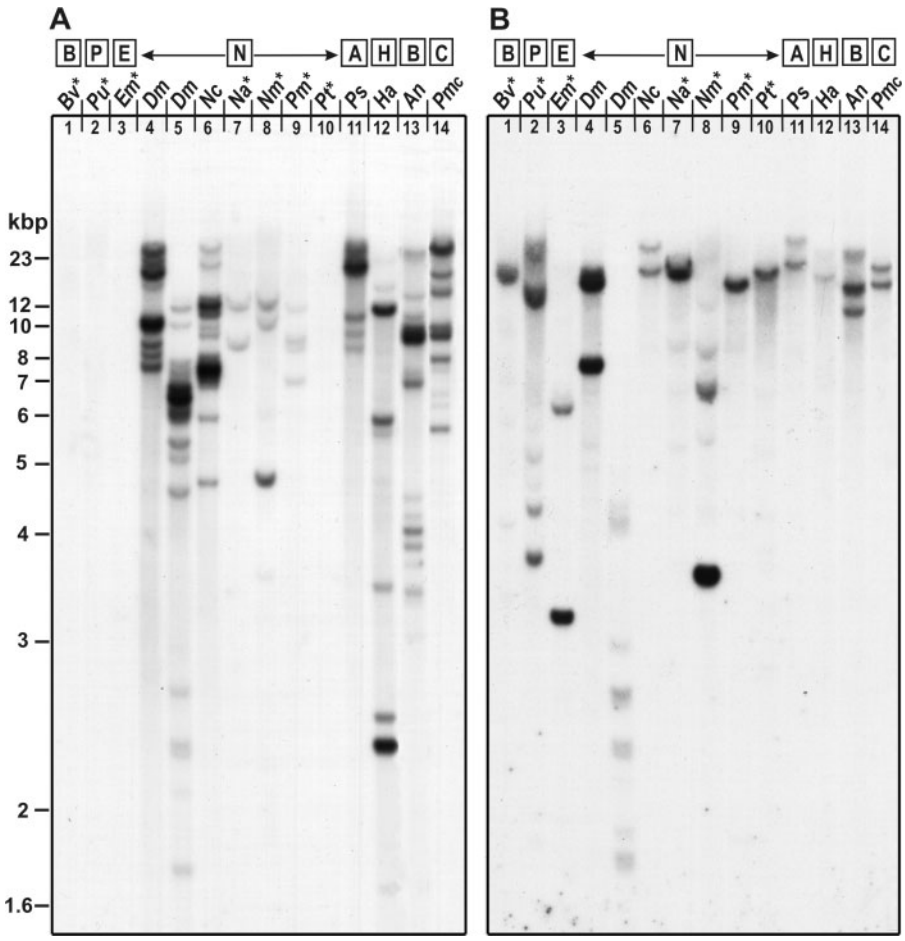

FIG. 1.—Southern blot of digested genomic DNA of Antarctic and non-Antarctic notothenioid fish species from all eight Notothenioid familes, hybridized to (*A*) AFGP-specific gene probe, stripped, and rehybridized to (*B*) trypsinogen-like protease-specific cDNA probe. Five μg and 20 to 25 μg of DNA for Antarctic and non-Antarctic species, respectively, digested with *Eco*RI or *Taq*I (lane 5 only) were used in the blot. Non-Antarctic species are indicated with asterisk (*), and the family each species belongs to is indicated by the boxed single letter. The species are *Bv*, *Bovichtus variegatus* (Bovichtidae); *Pu*, *Psuedaphritis urvilli* (Pseudaphritidae); *Em*, *Eleginops maclovinus* (Eleginopidae); *Dm*, *Dissostichus mawsoni*; *Nc*, *Notothenia coriiceps*; *Na*, *Notothenia angustata*; *Nm*, *Notothenia microlepidota*; *Pm*, *Paranotothenia magellanica*; *Pt*, *Patagonotothen tessellata* (Nototheniidae); *Ps*, *Pogonophryne scotti* (Artdedidraconidae); *Ha*, *Harpagifer antarcticus* (Harpagiferidae); *An*, *Akarotaxis nudiceps* (Bathydraconidae); and, *Pmc*, *Pagetopsis macropterus* (Channichthyidae). Lane numbers were included for ease of description in the text.

and again to the divergence of the basal families before an ancestral TLP gene evolved into an AFGP gene.

PCR amplification of AFGP genes from genomic DNA of *D. mawsoni* and the two New Zealand black cods shows a gene dosage pattern (fig. 2) that correlates with the results of the genomic Southern blot (fig. 1, panel *A*). At least seven AFGP-positive PCR products were obtained from the Antarctic species *Dm*, reflecting its large AFGP gene family, whereas only two and three AFGP-positive products were obtained from *Na* and *Nm*, respectively, consistent with the number of positive genomic fragments observed in the genomic Southern blot (fig. 1*A*). The PCR primers used amplify complete exon 2 containing the entire AFGP polyprotein-coding regions of AFGP genes. This region ranges from approximately 1.6 kb to as long as approximately 3.8 kb in *Dm* (fig. 2), indicating very large AFGP polyproteins are encoded. In contrast, the largest AFGP-positive PCR products in *Na* and *Nm* are about the same size as the smallest *Dm* product (one at approximately 1.8 kb for *Na* and two at approximately 1.6 kb for *Nm*), whereas the smaller products are only about 0.7 to 0.8 kb (fig. 2).

AFGP Genes in New Zealand Black Cods

The sequencing of highly repetitive AFGP polyprotein genes entails labor-intensive generation and sequencing of a large overlapping set of unidirectional deletions because the "primer-walk" sequencing strategy cannot be applied for repetitive sequences, and often only limited number of clear nucleotide reads (200 to 300) can be obtained per sequencing reaction from these difficult templates. Thus, the two smaller PCR-amplified AFGP genes (0.7 to 0.8 kb) from the black cods that would require a smaller set of deletions were chosen for sequencing, assuming their sequences have similar features as the larger PCR-amplified genes. Figure 3 shows the nucleotide sequence and conceptual translation of the PCR-amplified AFGP coding regions from *Na* and *Nm*. Similar to the AFGP genes of Antarctic notothenids *Nc* and *Dm* (Hsiao

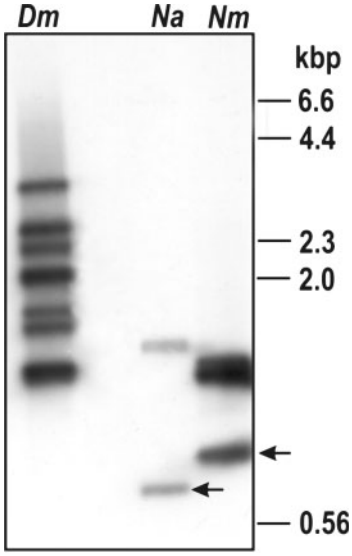

FIG. 2.—Southern blot of PCR amplification of genomic DNA for AFGP genes from Antarctic notothenid *Dm* and New Zealand notothenids *Na* and *Nm*, hybridized to the AFGP-specific gene probe. Arrows indicate the AFGP genes from the New Zealand species that were cloned and sequence.

et al. 1990; Chen, DeVries, and Cheng 1997*a*, 1997*b*), the partial genes from *Na* and *Nm* encode an AFGP polyprotein containing multiple AFGP molecules linked in tandem by conserved three-residue spacers, predominantly Leu-ILe/Asn-Phe. Also, as in Antarctic notothenid AFGP genes, the New Zealand black cod AFGP genes contain the characteristic long run of $(gt)_n$ sequence in the intron immediately ahead of the AFGP polyprotein-coding sequence, as well as the same two amino acids (Arg-Gly) at the carboxyl terminus of the polyprotein (fig. 3), and they share more than 90% identity in the 3′ flanking sequence with *Dm* and *Nc* AFGP genes (Hsiao et al. 1990; Chen, DeVries, and Cheng 1997*a*, 1997*b*). These three sequence elements were derived from the ancestral TLP gene that gave rise to the AFGP gene (Chen, DeVries, and Cheng 1997*a*; Cheng and Chen 1999), indicating that the New Zealand and Antarctic notothenid AFGP genes have the same evolutionary origin. There are 10 and 11 AFGP molecules in the *Na* and *Nm* AFGP polyprotein, respectively (fig. 4), and all are small size isoforms comprising two to six tripeptide (Ala/Pro-Ala-Thr) repeats. Most of the *Na* AFGPs (six out of 10) contain amino acid substitutions that disrupt the Ala/Pro-Ala-Thr pattern. The *Nm* AFGPs have more regular tripeptide repeats with four of 11 molecules containing amino acid substitution.

AFGP cDNA from New Zealand Black Cods

AFGP-positive products were obtained from RT-PCR amplification of pancreatic mRNA, indicating that one or more of the AFGP genes are functional at the transcriptional level in these two New Zealand species. Figure 5 shows the sequence and conceptual translation of one partial AFGP cDNA each from *N. angustata* and *N. microlepidota*. The partial cDNA is derived from the 5′ end of an AFGP mRNA and encodes a signal peptide near identical in sequence to that of *D. mawsoni* AFGP genes (Chen, DeVries, and Cheng 1997*a*, 1997*b*), plus a partial AFGP polyprotein. The signal peptide sequence was an additional component inherited from the ancestral TLP gene (Chen, DeVries, and Cheng 1997*a*; Cheng and Chen 1999), further confirming the common ancestry of the New Zealand and Antarctic notothenid AFGP genes. The encoded partial AFGP polyprotein from *Na* and *Nm* contains three and five AFGP molecules, respectively, ranging in size from three to nine Ala/Pro-Ala-Thr tripeptide repeats, and the molecules are linked by the conserved three-residue spacer, mostly Leu-Ile/Asn-Phe (fig. 5).

Circulatory Levels of AFGPs in New Zealand Black Cods

Thermal hysteresis (antifreeze activity) values of native serum from three environmental specimens of *N. angustata* were 0.056°C, 0.065°C, and 0.065°C (mean = 0.062 ± 0.005°C), and those of three postacclimation serum samples were more variable, 0.046°C, 0.093°C, and 0.158°C (mean = 0.099 ± 0.056°C). Five of these six values are near the margin of error for hysteresis measurements with the nanoliter osmometer and may not be reliable; thus, hysteresis values were not obtained for all individuals. However the presence of antifreeze proteins in the native serum is clearly indicated by the growth of the small single-crystal seed ice into hexagonally faceted ice crystal, which is a manifestation and confirmation of adsorption of antifreeze molecules to the prism faces of ice and retardation of a-axes growth (Knight, Drigger, and DeVries 1993). The actual concentration of AFGPs in the serum of *N. angustata* was quantified by ELISA. AFGP concentrations in serum samples from all 11 environmental specimens range from 0.12 to 0.46 μg/ml (mean ± S.E. = 0.23 ± 0.04 μg/ml), whereas those in serum samples from the same individuals after 2 months of 2°C to 4°C acclimation range from 0.50 to 4.37 μg/ml (mean ± S.E. = 2.60 ± 0.77 μg/ml) (fig. 6). Unpaired Student's *t*-test shows that the difference is significant ($P = 0.012$). Thus, the transcribed AFGP genes of *N. angustata* are translated into small amounts of circulatory protein, and the expression is augmented to slightly higher levels by holding in temperatures equivalent to the lowest winter water temperatures *N. angustata* experiences in the wild.

Monophyly and Divergence Time of New Zealand Black Cods

The aligned ND2 data set contained 1,047 nucleotide sites. The optimal model of sequence evolution determined from the LRTs was GTR+I+G. ML analysis resulted in a single tree, with most nodes supported with very high bootstrap scores (fig. 7). The two New Zealand *Notothenia* species are monophyletic with strong bootstrap support. However, the genus *Notothenia* is not monophyletic, as the two New Zealand *Notothenia* species are more closely related to the South American notothenid *Paranotothenia magellanica* than to the Antarctic species *N. coriiceps* and *N. rossii* (fig. 7). *N. angustata* was placed in the genus

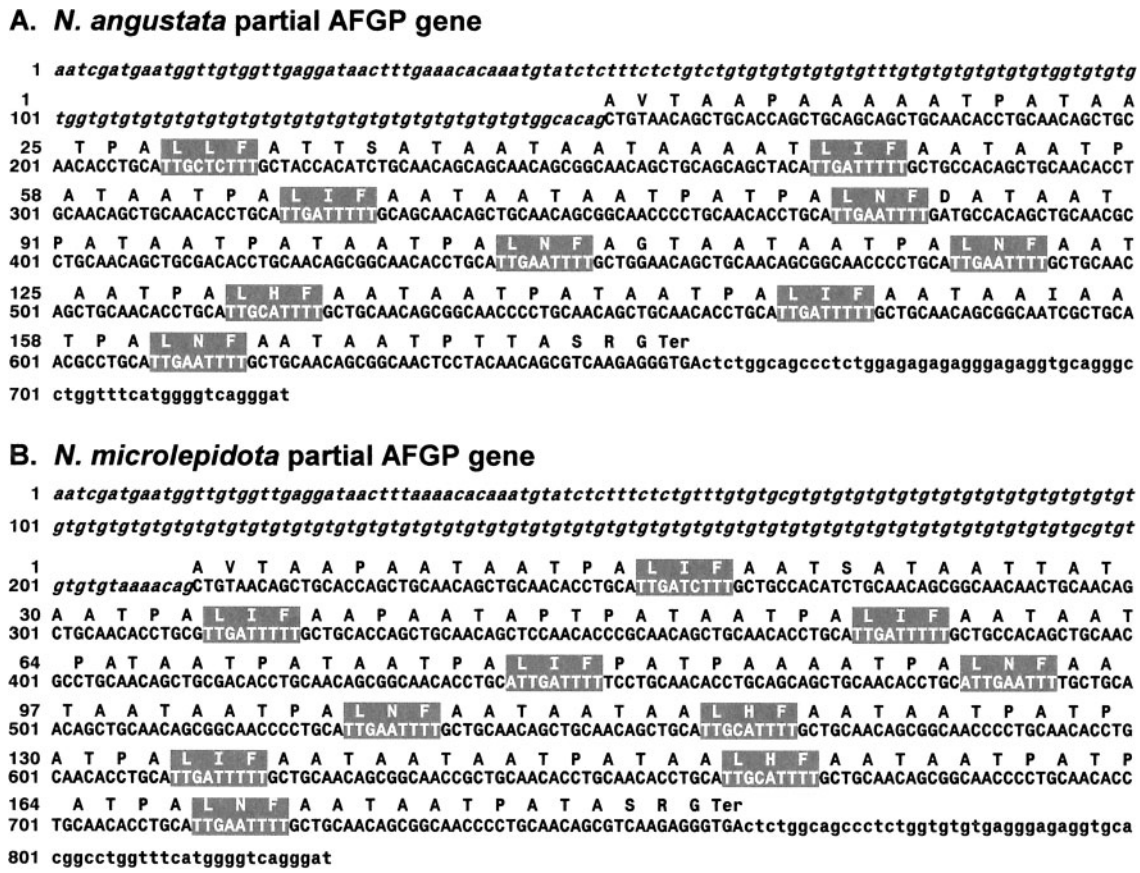

FIG. 3.—DNA sequence and conceptual translation of PCR-amplified partial AFGP genes of New Zealand nototheniids (indicated in figure 2). The partial gene covers 3′ end of intron 1 (in italics) and complete exon 2, which encodes the AFGP polyprotein precursor. Individual AFGP molecules are linked by conserved 3–amino acid spacers boxed in gray.

Paranotothenia in older accounts (Hutton 1875), but more recent taxonomic revision returned it to *Notothenia* (Dewitt 1970; Eastman and Eakin 2000). No prior phylogenetic hypothesis has included *P. magellanica* or the two New Zealand black cods. The nonmonophyly of *Notothenia* as revealed in the ND2 tree in this study indicates that taxonomic definition of this genus has not relied on shared derived characters, and future systematic and phylogenetic investigations should test the validity of *Notothenia* and provide any necessary taxonomic rearrangements. The ML model and parameters selected from the LRTs were used to calculate ML-corrected ND2 genetic distances between *Notothenia* species. The observed genetic distance between *N. angustata* and *N. microlepidota* was 14.4%. Using a "universal" rate of mtDNA substitution (about 1.3% per Myr for complete ND2 and ATPase genes at all sites) that is similar to fossil and geological molecular clock calibrations in other perciform fishes (Bermingham, McCafferty, and Martin 1997), a divergence time of about 11 Myr is estimated for the two New Zealand black cods.

Discussion

Antarctic Origin of New Zealand Nototheniids

The evolution of the teleost suborder Notothenioidei was inextricably linked to the evolution of the geology of the Antarctic region. Antarctica was once part of the supercontinent Gondwanaland, which began to fragment around 160 MYA (Kennett 1977, 1982; Anderson 1999). Paleoceanographic data suggest that the last southern continental block (South America) detached from Antarctica at the Eocene/Oligocene boundary (~38 MYA), commencing the unrestricted flow of the Antarctic Circumpolar Current (ACC) and the cooling of the Antarctic region. By 25 to 22 MYA, the ACC had become fully established, and the resultant thermal isolation lead to a rapid drop in temperatures over land and sea; present day icy, freezing marine conditions presumably were reached at about mid-Miocene (10 to 15 MYA) (Kennett 1977, 1982; Lawver, Gahagan, and Coffin 1992; Anderson 1999).

The three non-Antarctic notothenioid families, Bovichtidae, Pseudaphritidae, and Eleginopidae, presumably diverged and became established in New Zealand, Australia, and South America before Antarctica became isolated (Eastman 1993). Consistent with this is the absence of any detectable AFGP coding sequence in *Bovichtus variegatus*, *Pseudaphritis urvilli*, and *Eleginops maclovinus* from these three families, respectively, based on genomic Southern blot analysis (fig. 1*A*, lanes 1 to 3). However, all three carry the trypsinogen-like protease gene (fig. 1*B*, lanes 1 to 3), consistent with their

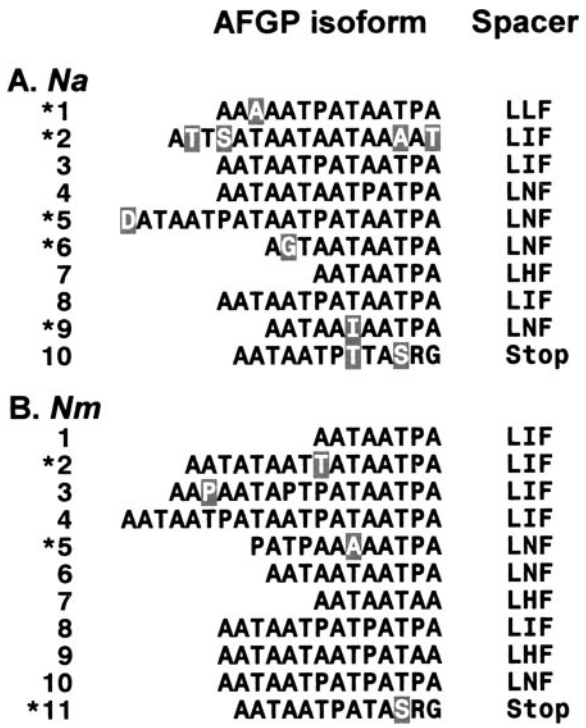

FIG. 4.—AFGP molecules in the polyprotein precursor encoded in the partial AFGP gene from New Zealand notothenioids (*A*) *N. angustata* and (*B*) *N. microlepidota* (sequence also in figure 3). Asterisk (*) indicates molecules with amino acid substitution (boxed in gray) that disrupts the Ala/Pro-Ala-Thr tripeptide repeat, the building block of the repetitive peptide backbone of AFGP isoforms.

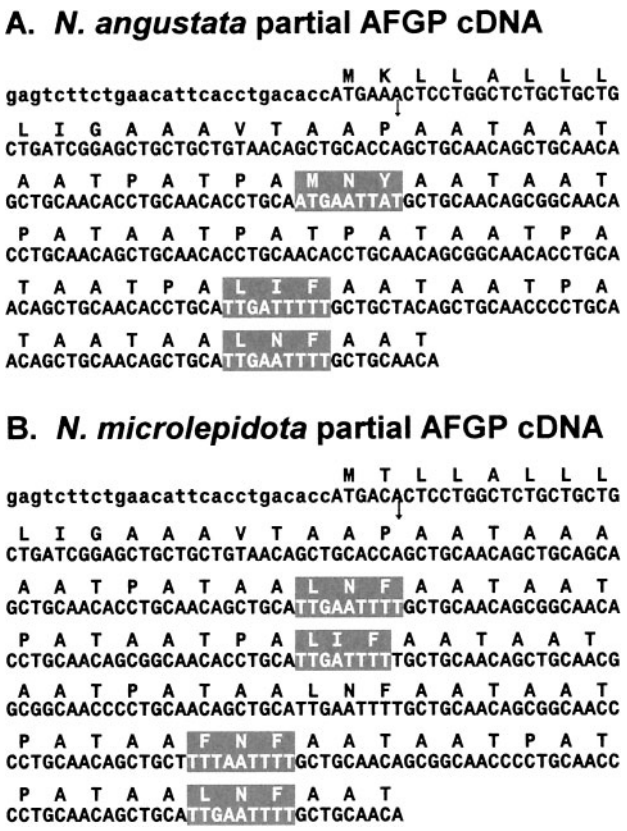

FIG. 5.—Sequence and conceptual translation of partial AFGP cDNA from NZ notothenids (*A*) *N. angustata* and (*B*) *N. microlepidota*. The partial cDNA covers the signal peptide and 5′ of AFGP polyprotein precursor. Arrow indicates the putative cleavage site of signal peptide sequence. The conserved three-residue spacers are boxed in gray.

diversification before the evolution of AFGP gene from an ancestral TLP gene. The other five notothenioid families, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae, are believed to have evolved within the Antarctic water from a shallow benthic stock (Eastman 1993), and extant Antarctic members of these families all synthesize AFGPs and maintain them at high circulatory levels (DeVries 1988; Cheng 1996). Correspondingly, the Antarctic members of these families have large AFGP gene families, as shown in the genomic Southern blot (fig. 1*A*). This is strong evidence that the evolution of the AFGP gene took place before the diversification of the five Antarctic families, and the expansion of their AFGP gene families occurred under freezing selection in the Antarctic region. The family Nototheniidae, which includes the eight species used in this study (*D. mawsoni*, *N. coriiceps*, *N. rossii*, *N. angustata*, *N. microlepidota*, *P. magellanica*, and *P. tessellata*), is the most basal of the five Antarctic families, and *Dm (D. mawsoni*) is a member of the most basal notothenid subfamily, Eleginopinae (Eastman 1993). From our previous analyses of *Dm* AFGP genes, which led to the discovery of the TLP origin of the notothenioid AFGP gene, the time of AFGP evolution was estimated to be about 5 to14 MYA, based on the amount of sequence divergence between the homologous sequence segments of *Dm* AFGP and TLP genes (Chen, DeVries, and Cheng 1997*a*). This time frame is consistent with that of the freezing of the Antarctic Ocean at 10 to 15 MYA (Kennett 1977) and the phyletic diversification of the five Antarctic notothenioid families at 7 to 15 MYA (Bargelloni et al. 1994) into all icy water column niches made possible by their antifreeze protection.

Given that the evolution of the AFGP gene was a fairly recent event and that it apparently occurred only once, before the diversification of the Nototheniidae, one would expect that even non-Antarctic notothenioids, including the New Zealand black cods, *N. angustata* and *N. microlepidota*, would still carry some form of AFGP sequences in their DNA. In keeping with this argument is the phylogenetic positions of the black cods in the ND2 tree (fig. 7), which are nested within the notothenioid clade that includes the Antarctic species, indicating they share a common AFGP-bearing ancestor. Southern analysis of genomic DNA (fig. 1*A*), and PCR amplification of genomic DNA for AFGP-coding sequences (fig. 2) and sequencing of the PCR products (figs. 3 and 4) confirm that both New Zealand notothenioids indeed carry AFGP-coding sequences in their genomes. In addition, at least one of these sequences from each fish is functional and transcribed, as RT-PCR amplification produced AFGP cDNAs encoding functional AFGP molecules (fig. 5). The same gene structures between the AFGP genes of the New Zealand and Antarctic notothenioids, including elements

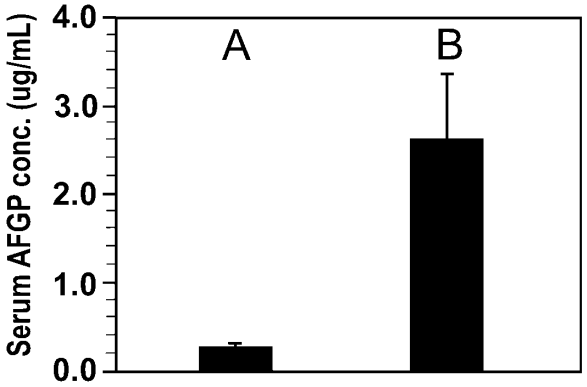

FIG. 6.—Concentration of mature AFGPs in the serum of *N. angustata* from (*A*) ambient temperatures (10°C to 14°C) and (*B*) after 2 months of cold acclimation at 2°C to 4°C, as determined by immunoassays (ELISA). Values are plotted as mean ± S.E. The higher AFGP levels after cold acclimation is statistically significant ($n = 11$, $P = 0.012$).

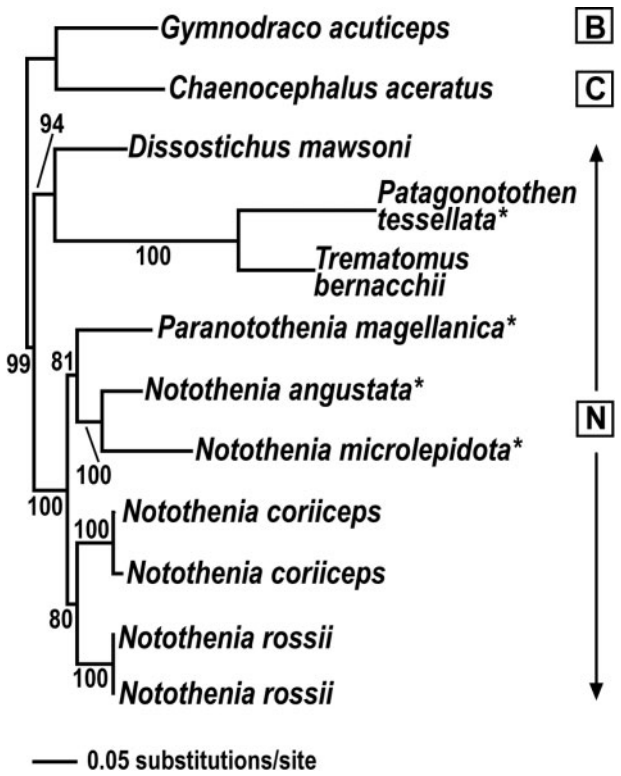

FIG. 7.—Tree resulting from maximum-likelihood analysis of the mtDNA ND2 gene. The boxed single letter indicates the family of the species as in figure 1. The GTR+I+G model of sequence evolution was used. Model rate parameters were R(a) [A-C] = 1.8455, R(b) [A-G] = 12.3845, R(c) [A-T] = 0.7093, R(d) [C-G] = 0.8956, R(e) [C-T] = 6.4088, R(f) [G-T] = 1.0000. Nucleotide frequencies were A = 0.2343, C = 0.3247, G = 0.1693, and T = 0.2630. The gamma shape parameter, alpha = 1.5051, and proportion of invariable sites = 0.3218. Numbers at nodes represent recovery in bootstrap analysis (100 pseudoreplicates), and asterisk (*) indicates non-Antarctic species.

that are signatures of their TLP ancestry, in addition to their highly similar gene sequences, indicate a single evolutionary origin, consistent with the evolution of the AFGP gene before the radiation of the Antarctic notothenioids.

Whether black cod AFGP genes and mRNA are expressed as mature protein was confirmed by the use of nanoliter osmometry and immunoassays. AFGP was previously thought to be absent in the blood of *Na*, inferred from undetectable antifreeze activity (Eastman and DeVries 1986) by the physical method of measuring freezing point depression using a relatively large (~500 μM) polycrystalline seed ice in a microcapillary tube of native serum (DeVries 1986). This method, however, does not have the fine temperature and ice crystallographic resolution for detecting antifreeze activity below 1 mg/ml concentrations. The presence of extremely low antifreeze levels (≤ μg/ml) can be detected by the much more sensitive nanoliter cryoscope osmometer, where the effect of absorbed antifreeze molecules on the growth habit of a minute single-crystal seed ice (< 5 μM) and its melting and freezing points can be observed and monitored at very fine temperature scales. However, thermal hysteresis values at less than 0.1°C observed for five of the six black cod native serum samples are at the threshold of reliable measurement with the nanoliter osmometer. The ELISA using AFGP antibodies, which provides actual quantification of the protein concentration in the nanogram range, is thus the most definitive measurement of the low AFGP concentrations in black cod blood. ELISA assays showed that mature AFGPs are present at the very low levels of less tan 1 μg/ml in *N. angustata* specimens from the ambient environment (10°C to 14°C) and rose to an average of about 2.5 μg/ml by 2 months of cold exposure to 2°C to 4°C, equivalent to the lowest winter water temperatures in their natural habitat (fig. 6). This preacclimation and postacclimation AFGP concentration range probably corresponds to the annual range in the fish in the wild. The concentration increase, however, does not equate an equivalent magnitude of increase in thermal hysteresis, as the absolute amounts of AFGPs in preacclimation and postacclimation sera are both much too low to effect any significant ice growth arrest. The circulatory AFGP levels in the black cods are four to five orders of magnitudes lower than those (30 to 40 mg/ml) present in the Antarctic species to prevent ice growth (DeVries 1988) and, therefore, clearly could not provide a freeze avoidance function, nor would the function be needed in their nonfreezing environment.

Nevertheless, the presence of functional AFGP genes in the New Zealand black cods and the inclusion of these species within the monophyletic nototheniidae encompassing AFGP-bearing Antarctic species (fig. 7) support an Antarctic evolutionary ancestry for the New Zealand black cods. The monophyly of *N. angustata* and *N. microlepidota* (fig. 7) proposes a single origin for the non-Antarctic distribution for these two species and that the diversification of these two species from their most recent common ancestor may have occurred subsequent to the isolation of the lineage north of the Antarctic polar front. The estimated time of divergence for these two species is approximately 11 MYA, which approximates the onset of sea-level glaciation in the Antarctic at approximately 10 to 15 MYA (Kennett 1977) and the upper bound of the estimated 5 to 14 MYA time frame of

evolution of the notothenioid AFGP gene from its TLP ancestor. The northward dispersal of their most recent common ancestor might have occurred before substantial expansion of its AFGP gene family took place, and the few AFGP genes and low level of AFGP expression in the black cods may represent the extant remainder of that AFGP coding capacity.

How the predecessor of the New Zealand black cods escaped the Antarctic is a difficult question to answer definitively. The Antarctic Convergence, which demarcates the oceanographic boundary (present day at approximately 50°S to 55°S) between the Antarctic Ocean and other southern oceans brought about by the Antarctic Circumpolar Current is not static but known to have undergone large-scale north-south movements over geologic time. Water temperatures affect the sinistral (left) versus right coiling directions of the shells of the tiny marine foraminifera planktons (high left/right ratio in cold water and vice versa for warm water), and these ratios are used extensively as thermal tracer in paleoceanography (Kohfeld et al. 1996). Diagnostic of the latitudinal position of the Antarctic Convergence where there is a sharp north-south transition from temperate to cold water is its association with predominantly (≥90%) left-coiling, Antarctic-subAntarctic cold-water forms of the foraminifer *Globigerina pachyderma* (Bé 1969). Stratigraphic records of foraminifera microfossils of the Kapitean Stage in North New Zealand showed the 90% left-coiling *G. pachyderma* isopleth as far north as 39°S during upper Miocene (~12 to 5 MYA), suggesting the Antarctic Convergence advanced as far as north as the midsection of North Island of New Zealand, and the influx of cold Antarctic water masses covered and cooled much of New Zealand seas during that time (Kennett 1968). The foraminifer fossils of the lower Pliocene are primarily right-coiling warmer water forms, suggesting the southward retreat of the convergence and a return to warmer conditions during that time period (Kennett 1968). Within the error of estimation, the geologic dates of this episode of advance and retreat of the Antarctic Convergence over New Zealand correlate with the divergence time of the two New Zealand black cods. It is reasonable to suggest that this paleoceanographic event might have served as the vicariant event that lead to the northward dispersal of the ancestor of the black cods, if it had occurred at the margin of the Antarctic Convergence in the Pacific sector at the time of its northward advance towards New Zealand. This ancestral habitat location is supported by the limited geographic distribution of the New Zealand black cods to southern New Zealand and Islands of the Campbell Plateau (Stewart 2002). At the periphery of the present-day Antarctic Convergence, there are a number of islands populated by a number of notothenioid species in near-shore waters (Gon and Heemstra 1990). In the Pacific sector, the underwater Macquarie Ridge runs from the New Zealand coastal shelf off the west coast of the South Island to the triple plate (Indian/Australian, Pacific, and Antarctic) junction at about 61°S, 162°E. The subduction of the Pacific plate beneath the Indian/Australian plate uplifted the latter at the convergent junction beginning about 12 MYA, forming the Macquarie Ridge complex (Williamson 1988). This ridge could have served as seamount habitats to notothenioid fish living at the Antarctic margin. Today, a series of islands occur in a south-northeastern direction (Macquarie Island, 54.5°S, 159°E; Campbell Island, 52.5°S, 169°E; Auckland Island, 50.5°S, 166°E; Antipodes Islands, 49.7°S, 179°E; and The Snares 48°S, 165°E), some of which are of sufficient geologic age to have been "stepping stones" to the northward migration of the common ancestor of the extant New Zealand nototheniids.

Function of AFGP in New Zealand Black Cods

Southern blot of genomic DNA (fig. 1*A*) illustrates that AFGP gene family size correlates with the severity of the thermal habitat of the fish. The Antarctic notothenioid species from high-latitude freezing environments have large AFGP gene families. Some of the genes are of very large sizes, indicating extensive intragene duplications (*Dm*, fig. 2) of the repetitive AFGP coding sequence, and in addition, the intensely hybridizing bands in the blot (fig. 1*A*) indicate that some of their AFGP genes are arranged in tandem repeats, likely from extensive whole-gene duplications. In contrast, the cool-temperate water New Zealand nototheniids *N. angustata* and *N. microlepidota* have only two to three AFGP-positive bands of weak hybridization (fig. 1*A*), although four to five times the amount of digested genomic DNA over Antarctic taxa was used for these two species. The number of AFGP-positive bands in genomic Southern blot (fig. 1*A*) is the same as the number of AFGP-positive PCR products from amplification of genomic DNA of the New Zealand black cods (fig. 2), indicating these two fish have only two and three AFGP genes, respectively, and the genes are of much smaller sizes as compared with the Antarctic nototheniid *D. mawsoni* (fig. 2). This correlation between AFGP gene family size and cold-habitat temperatures is consistent with environmental selection and maintenance of a crucial freezing protection function. In contrast, the number of TLP genes as indicated by the number of TLP-positive bands in the genomic Southern blot (fig. 1*B*) appear invariant of habitat temperatures, and in fact the temperate-water non-Antarctic taxa may have one or two more TLP genes, perhaps related to greater metabolic activity in warmer water.

The puzzling questions, however, are why the New Zealand blacks cods still retain functional AFGP genes, and what possible function would the residual AFGP serve, in their apparently nonfreezing habitats. The only known function of AFGP is prevention of organismal freezing, by adsorption to ice crystals that enter the fish and preventing them from growing, within the ambient freezing sea water temperature range that the fish lives in. The ice-binding property is dependent on the Ala/Pro-Ala-Thr tripeptide regularity in the protein backbone as it provides the proper molecular spacing for the Thr-linked disaccharides to match the periodicity of water molecules along the a-axis in the ice lattice for hydrogen-bonding to occur between the two, leading to AFGP adsorption on ice (Knight, Driggers, and DeVries 1993). This regularity is highly preserved in the encoded AFGP polyproteins in all

the characterized AFGP genes from Antarctic species. For instance, only one amino acid substitution is found in three *D. mawsoni* AFGP genes encoding a total of 67 AFGP molecules, two of which are very large isoforms (Chen, DeVries, and Cheng 1997*a*, 1997*b*; Cheng and Chen 1999). In contrast, six of the 10 encoded AFGPs in the *N. angustata* gene and four of the 11 in the *N. microlepidota* gene contain amino acid changes (fig. 4). The amino acid changes would lead to reduction or loss in function, since substitution of Thr results in the absence of a disaccharide at that site and substitution of any amino acid in the tripeptide may disrupt the helical structure of the peptide backbone (Berman, Allerhand, and DeVries 1980) and cause spacing irregularity, both of which diminish ice adsorption. Interestingly, there are no mutations in the sequences of the partial AFGP cDNA obtained from the New Zealand nototheniids (fig. 5), indicating at least one other gene in each fish is still intact, at least in the 5′ portion. Overall, the few AFGP genes, amino acid changes in the encoded protein of some genes, and the minuscule protein concentration of the mature protein in the blood collectively mean that although the protein is expressed in the New Zealand black cods, the role of the protein cannot be one of freeze avoidance. Whether the amino acid substitutions in the encoded AFGP reflects coding degeneracy in the absence of freezing selection or an ongoing evolutionary change towards the acquisition of a new function is difficult to ascertain.

South American Nototheniid Fish

This study included two other non-Antarctic nototheniids, *Paranotothenia magellanica* and *Patagonotothen tessellata*, from the southern coast of South America. *P. magellanica* and the New Zealand black cods form a sister group to the Antarctic *Notothenia* species (fig. 7), and its DNA shows four faintly AFGP-positive fragments (fig. 1*A*, lane 9). Whether these AFGP-positive fragments represent functional AFGP genes await testing, but their presence in the genome plus the phylogenetic position of *P. magellanica* within nototheniidae support an Antarctic evolutionary origin for this species, similar to its New Zealand counterparts. Geographically, *P. magellanica* is much more widely distributed than the New Zealand black cods, ranging from the Tierra del Fuego of South America, the Falkland Islands, islands of the southern Indian Ocean, to the Campbell Plateau south of New Zealand (Gon and Heemstra 1990; Stewart 2002), suggesting the mechanism of its northward dispersal was associated with the movements of circum-Antarctic currents. The other South American nototheniid, *Patagonotothen tessellata*, is a member of the largest nototheniid genus comprising 14 species, 13 of which are non-Antarctic (Eastman and Eakin 2000). *P. tessellata* is sister taxon to the very high-latitude, fully-AFGP fortified Antarctic nototheniid *Trematomus bernacchii* in the ND2 tree (fig. 7) and to other high Antarctic taxa (Bargelloni et al. 2000) and thus should be of an Antarctic evolutionary origin. The complete absence of detectable AFGP sequence in its DNA is therefore surprising. More surprisingly, the only Antarctic patagonotothen, *P. guntheri*, appears to have no AFGP sequence in its genome either (unpublished results). How the species of this largest nototheniid genus arrive at their current geographic distribution is puzzling and begs investigation. This biogeographic pattern is counter to that observed for other Antarctic genera where most species are endemic to the Antarctic, and only one or two are non-Antarctic. Thus, secondary escape to the North as a common mechanism of dispersal invoked for Antarctic notothenioids (Bargelloni et al. 2000) may not be applicable to the patagonotothens. A robust phylogenetic analysis of all the *Patagonotothen* taxa combined with the genomic and protein analyses used in this study would be most useful in assessing the biogeographic and evolutionary origins of this group of nototheniids.

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