

Adaptations and lifestyle in polar marine environments: a biological challenge for the study of fish evolution

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The climatic features of Antarctic waters are more extreme and constant than in the Arctic. The Antarctic has been isolated and cold longer than the Arctic. The polar ichthyofaunas differ in age, endemism, taxonomy, zoogeographic distinctiveness and physiological tolerance to environmental parameters. The Arctic is the connection between the Antarctic and the temperate–tropical systems. Paradigmatic comparisons of the pathways of adaptive evolution of fish from both poles address the oxygen-transport system and the antifreezes of northern and southern species. (i) Haemoglobin evolution has included adaptations at the biochemical, physiological and molecular levels. Within the study of the molecular bases of fish cold adaptation, and taking advantage of the information on haemoglobin amino acid sequence, we analysed the evolutionary history of the α and β globins of Antarctic, Arctic and temperate haemoglobins as a basis for reconstructing phylogenetic relationships. In the trees, the constant physico-chemical conditions of the Antarctic waters are matched by clear grouping of globin sequences, whereas the variability typical of the Arctic ecosystem corresponds to high sequence variation, reflected by scattered intermediate positions between the Antarctic and non-Antarctic clades. (ii) Antifreeze (glyco)proteins and peptides allow polar fish to survive at sub-zero temperatures. In Antarctic Notothenioidei the antifreeze gene evolved from a trypsinogen-like serine protease gene. In the Arctic polar cod the genome contains genes which encode nearly identical proteins, but have evolved from a different genomic locus—a case of convergent evolution.

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Although high latitudes and cold climates are common to both the Antarctic and the Arctic, in many respects the two regions are more dissimilar than similar. Due to the Antarctic Polar Front (APF), an oceanographic system producing a barrier responsible for the isolation of marine organisms, the climatic features of the Antarctic waters are more extreme and constant than those of the Arctic. In the Arctic isolation is less pronounced, and the range of temperature variations is wider.

The colonized terrestrial portions are extensive; they are directly linked to temperate areas, greatly facilitating adaptation and redistribution of terrestrial organisms on one hand and, on the other, producing wide and complex terrestrial mechanisms of feedback to the climate, which add to those originating from ocean and atmosphere circulation. In addition, the anthropogenic impact is greater. Tectonic and oceanographic events played a key role in delimiting the two polar eco-

systems and in influencing the evolution of their faunas, whose composition and diversity are strongly linked to geological history. The Antarctic has been isolated and cold longer than the Arctic, with ice sheet development preceding that in the Arctic by at least 10 My. The modern polar faunas differ in age, endemism, zoogeographic distinctiveness, taxonomy and physiological tolerance to various environmental parameters.

The differences in the two polar environments are reflected in global changes. Accordingly, studies of ecosystems are likely to provide answers to different questions, often complementary to one another. In summary, the Arctic is the connection between the more extreme, simpler Antarctic system and the more complex temperate and tropical systems.

“Evolution is the major unifying principle of biology, and evidence of evolutionary processes pervades all levels of biological organisation from molecules to ecosystems” (Eastman 2000: 276). In studying evolution, the comparative approach permits examination of convergent and parallel evolutionary trends at levels ranging from molecules to organisms.

Key aspects of evolution comprise molecular, physiological and behavioural mechanisms of adaptation by which organisms are enabled to survive, grow and reproduce. Current investigations are increasingly exploring links between evolutionary processes, lifestyle and molecular/organ system adaptations.

Two paradigmatic topics have been selected for this article, namely antifreeze compounds and haemoglobin, including loss of expression of globin genes and systematics. Genes coding identical antifreeze compounds in northern cods and southern Notothenioidei have different evolutionary histories; recent studies show that Arctic fish globins diverge from those of notothenioids and display paralogous relationships.

Antifreeze compounds in Antarctic and Arctic fish

The biosynthesis of antifreeze (glyco)proteins and peptides (AFGPs, AFPs), which allows polar fish to survive at sub-zero temperatures, is one of the most intriguing evolutionary adaptations (reviewed in Cheng & DeVries 1991) and meets the criteria for a “key innovation” (Eastman 2000).

Recent studies address the evolution of AFGP genes in Antarctic Notothenioidei. The AFGP gene evolved from a functionally unrelated pancreatic trypsinogen-like serine protease gene, through a molecular mechanism by which the ancestral gene provided the front and tail of the emerging AFGP gene (Chen et al. 1997a). The finding in the notothenioid genome of a chimeric AFGP-protease gene intermediate, a protease gene still bearing the incipient coding element, and independent AFGP genes, reveals a fascinating case of “evolution in action” (Cheng & Chen 1999). It was deduced that the conversion of the ancestral gene to the first AFGP gene occurred 5-14 Mya. This value agrees well with the generally accepted time frame (10-14 Mya) in which the Antarctic water reached the present freezing conditions.

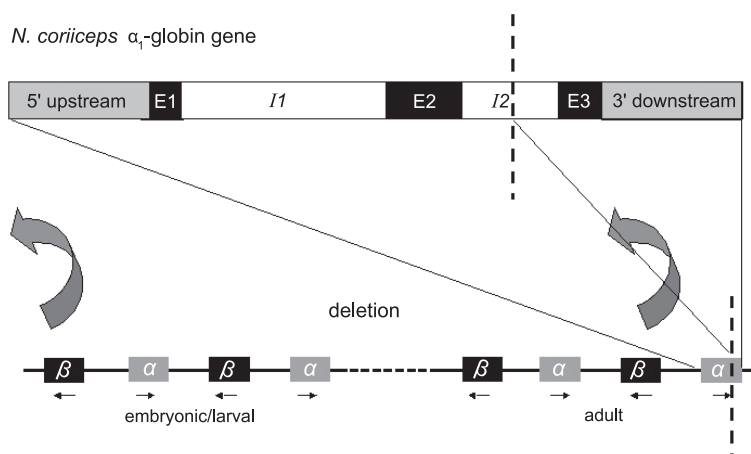
Analysis of AFGP in the polar cod (*Boreogadus saida*, family Gadidae) showed that the genome of this species (phylogenetically unrelated to notothenioids, which belong to different superorder and order) contains genes which encode nearly identical proteins. This would suggest a common ancestry. However the genes of the two fish groups are not homologous, hence have not followed the same evolutionary pathway. Assuming an endogenous, yet unknown genetic origin, the cod AFGP genes have evolved from a different, certainly not trypsinogen-like, genomic locus (Chen et al. 1997b). An example of convergent evolution has thus been discovered.

The haemoglobin system

The Antarctic suborder Notothenioidei comprises eight families. Ninety-six of the 213 (45%) species of the continental shelf, and 122 (including non-Antarctic species) of the 313 (39%) Southern Ocean species described to date are notothenioids (Gon & Heemstra 1990; Eastman 1993, 2000). The dominance of a single taxonomic group of fish is unparalleled in any other oceanic ecosystem.

The coastal Antarctic waters are cold and oxygen-rich. The metabolic demand of fish for oxygen is low, the solubility of oxygen in their plasma is high, and the energetic cost associated with blood circulation is large. With the selective pressure for erythrocytes and haemoglobin (Hb) relaxed and with the cells posing a rheological disadvantage in the temperature-driven increase

Fig. 1. Abrogation of Hb synthesis as a result of a single, large-scale deletional event that removed all globin genes with the exception of the 3' end of the α_1 -globin gene of adult Hb 1. The latter gene of *N. coriiceps* is represented in the upper part.



in blood viscosity, over evolutionary time notothenioids have developed reduced haematocrits (Hct), Hb concentration/multiplicity and oxygen affinity.

In this regard, one of the most unusual phenotypes of vertebrates was reported by Ruud (1954): the colourless blood of the “icefish” species of the family Channichthyidae, the most phyletically derived notothenioids, was devoid of Hb. Icefishes maintain normal metabolic function by delivering oxygen physically dissolved in the blood to tissues. Hct reduction to near zero appears selectively advantageous because it diminishes the energetic cost associated with circulation of a highly viscous, corpuscular blood fluid (Wells et al. 1990; di Prisco et al. 1991; Eastman 1993). The remaining seven notothenioid families are red-blooded.

Channichthyids diverged from other notothenioids approximately 7–15 Mya, but the radiation of species within the icefish clade appears to have been confined to the last one million years (Bargelloni et al. 1994). The development of compensatory adaptations that reduce oxygen demand and enhance oxygen delivery (modest suppression of metabolic rates, large, well perfused gills, scaleless and highly vascularized skin, large capillary diameter, large increase in cardiac output and blood volume) argues that the loss of Hb and erythrocytes, under conditions of physiological stress, was probably maladaptive. Therefore, the most plausible evolutionary scenario is that the phylogenetic trend to reduced Hct and Hb synthesis in notothenioids developed concurrently with enhancements to their respiratory and circulatory systems, leading ultimately to the acorpuscular, Hb-less condition of the icefishes. Alterna-

tively, loss of Hb production may have occurred independently of the trend as a nonadaptive, yet non-lethal mutation in the oxygen-rich Antarctic waters.

Globin genes in notothenioids; evolutionary loss of expression in icefish

The globin-gene organization in red-blooded notothenioids has been characterized, the status of globin genes in channichthyid genomes has been studied, and potential evolutionary mechanisms leading to the Hb-less phenotype have been evaluated (Cocca et al. 1995, 1997, 2000; Zhao et al. 1998; di Prisco et al. 2002). It was found that icefish retain genomic DNA sequences closely related to the adult α -globin gene(s) of its red-blooded notothenioid ancestors and contemporaries, whereas its ancestral β -globin-gene sequences have either been deleted or have diverged beyond the limits of detection. No mRNA transcripts were detected in any tissue; thus, the α -globin-related DNA sequences of icefishes probably are inactive remnants of red-blooded notothenioids.

The differential loss of globin-gene sequence suggests that the most plausible mechanism leading to the Hb-less phenotype is deletion of the single adult β -globin locus of the ancestral channichthyid. To establish a framework for genetic comparison, the adult α -globin gene (corresponding to the major component Hb 1) of red-blooded *Notothenia coriiceps* was characterized (Zhao et al. 1998) and compared with the structures of icefish α -globin-positive genomic clones. The observations are consistent with deletional loss of 5'-upstream α -globin sequences (5'-untranslated region, exons 1 and 2, intron 1 and part of 2). On

the grounds of deletion of the adult β -globin gene and 5' truncation of the major adult α -globin gene, a simple mechanism for icefish globin gene loss was proposed (Fig. 1): expression of adult globins was abrogated by a single deletional event (with chromosomal breakpoints located within I2 of the α -globin gene and downstream of the 3'-untranslated region of the β -globin gene) in the ancestral channichthyid, possibly removing almost the entire notothenioid globin gene complex (Cocca 2000; di Prisco et al. 2002). Thus, failure to synthesize α globin is due to deletional loss of the 5' end of the notothenioid α -globin gene. The transcriptionally inactive remnant, no longer under positive selection pressure for expression, subsequently underwent random mutational drift, without, as yet, complete loss of sequence information.

Hbs in Notothenioidei (Antarctic and temperate) and Arctic fish

Hb, being a direct link between the environment and body oxygen-transport requirements, has been under major evolutionary pressure to adapt and modify its functional features. The capacity of fish to colonize a large variety of habitats appears to be strictly related also to the molecular and functional differences in their Hb systems. A common feature of benthic red-blooded Notothenioidei is the markedly reduced Hb multiplicity. Thirty-four species (di Prisco 1997, 1998) have a single major Hb (Hb 1; 95% of the total) and often a second, functionally similar minor component (Hb 2; 5% of the total, having one of the chains—usually the β —in common with Hb 1). All these Hbs have been characterized structurally and functionally in terms of pH and endogenous-organophosphate regulation of oxygen binding, and thermodynamic analysis. Three species of the family Nototheniidae (active, cryopelagic *Trematomus newnesi* and *Pagothenia borchgrevinki*; *Pleuragramma antarcticum*, a pelagic, sluggish but migratory fish) differ in lifestyles from the 34 sluggish benthic species investigated (di Prisco 1998, 2000). Each species has evolved a unique oxygen-transport system, and each system appears adjusted to the specific mode of life. The more phyletically derived Harpagiferidae, Artedidraconidae and Bathydraconidae mostly have a single Hb (di Prisco et al. 1991). The Hbs of two species of artedidraconids lack oxygen-binding cooperativity (Tamburrini et al. 1998), in a way

similar to that of the ancestral haemoproteins of primitive organisms, raising intriguing questions on the mode of function of multi-subunit molecules and on evolutionary implications.

Comparison between cold-adapted and non-cold-adapted (non-Antarctic) notothenioids may help to elucidate their evolutionary history. The monotypic family Pseudaphritidae is one of the most basal notothenioid families. *Pseudaphritis urvillii* (di Prisco 2000) is a catadromous relict species from coastal waters, estuaries and rivers of southern Australia. Most likely, its ancestors became associated with the Australian component of Gondwana 40-50 Mya, when the water temperature was still mild; the separation of Australia from Antarctica is likely to be the event leading to speciation of euryhaline *P. urvillii* (Bargelloni et al. 2000). Because of its distribution, *P. urvillii* is especially important for the calibration of the molecular clock.

Unlike cold-adapted notothenioids, *P. urvillii* has glomerular kidneys (Eastman 1993) and has neither AFGPs nor the genes encoding AFGPs (Cheng et al 2003); the oxygen affinity of its major Hb is much higher than that of Antarctic notothenioids. On the other hand, although *P. urvillii* is not cold-adapted, some haematological features resemble those of cold-adapted fish: the Hb multiplicity is similar to that of Antarctic bottom dwellers, namely Hb 1 and Hb 2 are in the typical proportions and have one chain (the α) in common. Furthermore, the amino acid sequences of both Hbs reveal high identity with Antarctic Hbs, and contain most of the residues which differentiate Antarctic notothenioids from temperate fish. In Hb 1, the identity with Antarctic notothenioids is higher than with any temperate fish (60-66%), following the general trend of notothenioids (di Prisco et al. 1991). However the identity between Hb 1 of *P. urvillii* and other Antarctic fish (Table 1) is close to, or lower than, the low extreme of the range of values of Antarctic notothenioids (82-99% and 77-93% for the α and β chains, respectively). This argues in favour of a common origin within notothenioids, and also suggests that Hb 1 has undergone modifications only to a limited extent. If sequence mutations in Antarctic fish are indeed related to the development of cold adaptation, they would relate to divergence during the first stages of the cooling process, before the event which gave origin to AFGPs.

The comparison of *P. urvillii* with the more

Table 1. Sequence identity (%) between the globins of Hb 1 in a selection of Notothenioidei (di Prisco 1998).

Species	<i>P. urvillii</i> ^a	<i>N. ang.</i> ^a	<i>P. ant.</i>	<i>G. acut.</i>	<i>C. maw.</i>	<i>A. mit.</i>	<i>T. bern.</i>	<i>T. newn.</i>
<i>N. coriiceps</i>	80	99	95	82	83	83	89	87
<i>T. newnesi</i>	78	94	89	92	90	90	97	
<i>T. bernacchii</i>	80	96	92	91	91	90		
<i>A. mitopteryx</i>	73	91	88	84	84			
<i>C. mawsoni</i>	78	89	87	93				
<i>G. acuticeps</i>	76	89	85					
<i>P. antarcticum</i>	81	94						
<i>N. angustata</i> ^a	78							
<i>N. coriiceps</i>	77	93	86	80	88	82	90	86
<i>T. newnesi</i>	77	85	84	80	84	83	93	
<i>T. bernacchii</i>	82	91	90	83	87	86		
<i>A. mitopteryx</i>	77	84	84	77	80			
<i>C. mawsoni</i>	75	87	82	85				
<i>G. acuticeps</i>	75	81	80					
<i>P. antarcticum</i>	77	88						
<i>N. angustata</i> ^a	81							

^a Temperate notothenioid.

recent *Notothenia angustata*, common near the coast of southern New Zealand, has provided useful insights. Similar to *P. urvillii*, *N. angustata* (family Nototheniidae) is endowed with features typical of both temperate and cold-adapted fish. Some haematological parameters (high Hct, erythrocyte number and Hb content/cellular concentration) favour oxygen transport in a temperate environment (Macdonald & Wells 1991), but Hb multiplicity and structural/functional features closely resemble those of Antarctic notothenioids (Fago et al. 1992). The sequence identity with cold-adapted *N. coriiceps* of the same genus is the highest ever found among notothenioids. Thus *N. angustata* is an ideal link between temperate and Antarctic habitats. The two nototheniids diverged evolutionarily after APF establishment. The genome of *N. angustata* contains antifreeze genes, able to become activated upon cold acclimation, suggesting that—unlike *P. urvillii*—this fish was cold-adapted prior to its recent migration to temperate waters (Cheng et al. 2003). In fact, at the end of the Miocene (5 Mya) and during the Pliocene, APF moved northwards up to 39°S, the latitude of northern New Zealand, favouring fish migration.

The first molecular characterization of the oxygen-transport system of an Arctic species, the benthic spotted wolffish (*Anarhichas minor*), has recently been reported (Verde et al. 2002).

This research showed that the features of the Hb system of three Arctic species (*A. minor*, the polar cod [*B. saida*], the Atlantic cod [*Gadus morhua*]) differ from those of the phyletically unrelated Antarctic benthic species. The Hb multiplicity (three major Hbs) is similar to that of *P. antarcticum*, but there the similarity ends. For instance, only one Hb of *A. minor* is strongly pH-regulated, with organophosphates producing a dramatic affinity decrease for oxygen also at alkaline pH, accompanied by loss of cooperativity.

Table 2 reports the comparison of the sequence identity of the globins of the three *A. minor* Hbs with those of major and minor Antarctic Hbs, as well as of temperate fish. Hb 2 (the Hb present in higher amount) is the only Hb in which both chains have higher similarity with the Antarctic major Hbs. In Hb 1 and Hb 3, only one of the chains is either more similar to major, or to minor Antarctic Hbs. These findings are illustrated in

Table 2. Sequence identity (%) between *A. minor* Hbs and Antarctic and temperate fish Hbs.

<i>A. minor</i> Hbs	Antarctic major Hbs	Antarctic minor Hbs	Temperate Hbs
α_1 (Hb 1)	62-68	75-80	58-68
α_2 (Hb 2, Hb 3)	73-77	65-68	56-64
β_1 (Hb 1, Hb 2)	74-80	62-70	60-66
β_2 (Hb 3)	70-77	78-84	58-65

more detail in the phylogenetic trees (Figs. 2, 3).

Phylogenetic analysis of globin genes

Recent years have witnessed rapid development of molecular phylogenetic analysis based on sequences of proteins (e.g. Hb) and nucleic acids.

The sequences of major and minor Antarctic Hbs cluster in two groups. In each group, the sequence identity is very high: 73-99% and 84-100%, respectively (di Prisco 1998). The identity between major and minor Hbs is lower, ranging between 61% and 73%. The sequences of the α and β chains of Antarctic fish Hbs, together with those of several temperate and Arctic species, have been employed to build phylogenetic trees, using the neighbour-joining (NJ) method (Saitou & Nei 1987).

A number of sequences fail to be grouped in any of the clades; these include globins of temperate *Chelidonichthys kumu* and *Thunnus thynnus*. The globins of the Arctic species *A. minor* are close to the notothenioid clades. In the tree of Fig. 2, the α chain shared by Hb 2 and Hb 3 of *A. minor* is close to the major Antarctic globins, but also to the two temperate globins, while the α of Hb 1 appears more closely related to the minor Antarctic globins. The α chain of Hb 2 of *G. morhua* (Atlantic cod) is close to the α chain of *Oncorhynchus mykiss* (trout) Hb IV. In the tree of Fig. 3, the position of the *A. minor* β chain shared by Hb 1 and Hb 2 is placed into the group of the major Antarctic globins, whereas the β chain of Hb 3 appears well separated from the subclades of major and minor Antarctic globins. The β chain of Hb 1 and Hb 2 of the polar cod *B. saida* outgroups with respect to the other sequences; in *G. morhua*, the β chain (shared by Hb 2 and Hb 3), and an additional β chain (whose sequence was deduced from DNA, and possibly belongs to a larval Hb) constitute a clade characterized by a node supported by a high bootstrap value.

The position in the tree of the β chain of the polar cod *B. saida* is surprising. It is the most cold-adapted of the three Arctic species, therefore one would expect the position to be closer to the Antarctic globins. However it is very difficult to find objective criteria to identify links between a phenotypic trait (e.g. the primary structure) and adaptation, in this case to temperature. The globin trees are indeed the combination of gene histories (duplication) and taxon history (speciation), mixed with convergence of proteins due to

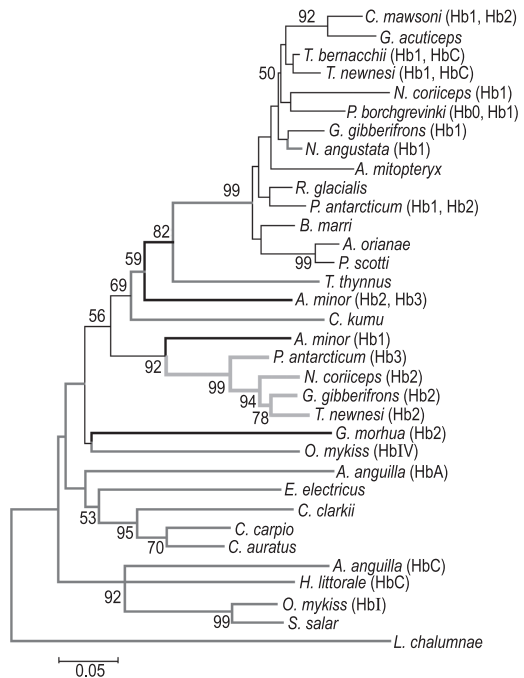


Fig. 2. Condensed phylogenetic trees of α -globin sequences of Arctic, Antarctic and temperate fish. The numbers on branch nodes (bootstrap), obtained by the internal branch test implemented in MEGA 2, correspond to confidence probability that the branch length is significantly higher than zero (after 10 000 replicates). The species used are: *Cygnodraco mawsoni*, *Gymnodraco acuticeps*, *Trematomus newnesi*, *T. bernacchii*, *Notothenia coriiceps*, *Pagothenia borchgrevinki*, *Gobionotothen gibberifrons*, *Aethotaxis mitopteryx*, *Racovitzia glacialis*, *Bathyrdraco marri*, *Artedidraco orianae*, *Pogonophryne scotti*, *Pleuragramma antarcticum* (Antarctic); *N. angustata* (temperate notothenioid); *Anarhichas minor*, *Boreogadus saida*, *Gadus morhua* (Arctic), *Chrysophrys auratus*, *Thunnus thynnus*, *Chelidonichthys kumu*, *Oncorhynchus mykiss*, *Electrophorus electricus*, *Anguilla anguilla*, *Catostomus clarkii*, *Cyprinus carpio*, *Carassius auratus*, *Hoplosternum littorale*, *Salmo salar*, *Latimeria chalumnae* (temperate). Simple lines refer to globins from major Antarctic Hbs; lines in bold, light grey and dark grey refer to globins from Arctic, minor Antarctic and temperate Hbs, respectively.

adaptation.

Molecular phylogenetic analysis thus indicates that the Arctic globins are divergent from those of Antarctic fish, displaying paralogous relationships. Indeed all non-Antarctic globins, including the Arctic ones (other than *A. minor*), occupy scattered positions in the trees.

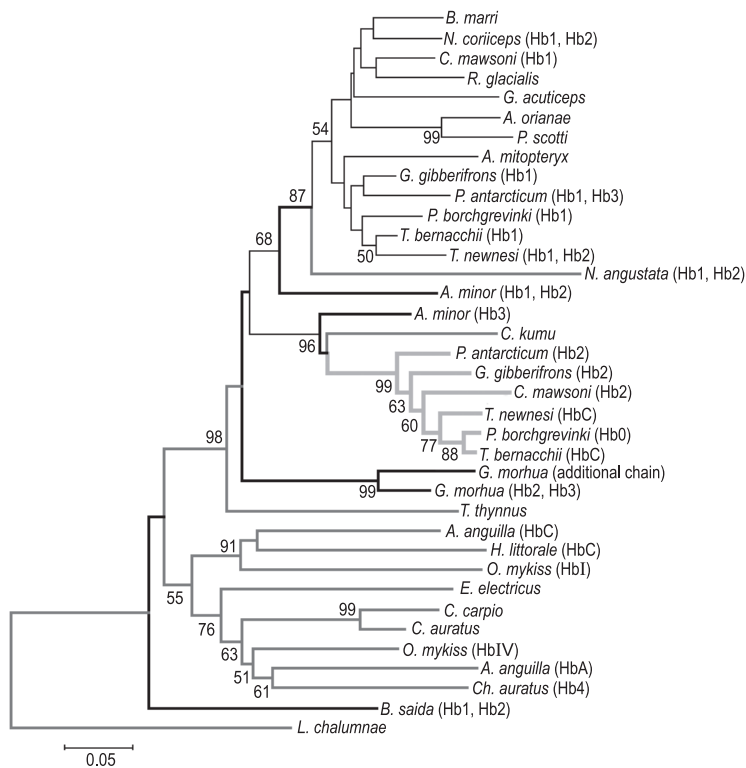


Fig. 3. Condensed phylogenetic trees of β -globin sequences of Arctic, Antarctic and temperate fish. See Fig. 2 for details.

Concluding remarks

In conclusion, the remarkable differences in the oxygen-transport system between Arctic and Antarctic fish indicates that distinct evolutionary pathways in the regulatory mechanisms of the fish oxygen-transport system have been followed in the two polar environments. The different phylogenetic histories of Arctic and Antarctic fish depend on the respective habitats. As a result of the isolation of Antarctica, the genotype of the Notothenioidei diverged with respect to other fish groups. The evolution of the genes of AFGPs is a pertinent example: Arctic and Antarctic AFGPs are the result of a case of convergent evolution, as each type derives from a distinct ancestral gene sequence. Although both are cold, the Arctic and Antarctic habitats differ in many aspects. Indeed, in the Arctic isolation is less pronounced and the range of temperature variations is wider than in the Antarctic. Therefore, it is not surprising that the Arctic ichthyofauna, being a much more complex system than the Antarctic one, which is dominated by a single taxonomic group, is characterized by high diversity, reflected in the phylogeny

of a given trait. The lifestyle of a benthic species such as *A. minor*, unlikely to disperse across wide latitude and temperature gradients, corresponds to higher similarity of Hb evolution with Antarctic notothenioids. In contrast, the two species of Gadidae occupy intermediate positions between the Antarctic and temperate clades, in keeping with their active, pelagic and migratory lifestyle. In short, the constant physico-chemical conditions of the Antarctic ocean are matched by clear grouping of fish globin sequences, whereas the variations typical of the Arctic ocean correspond to high sequence divergence.

The evolution of polar marine organisms is a highly complex scenario, and great difficulties are to be expected when venturing into this topic. In the field of polar biology, however, this is an exciting challenge which will keep us quite busy for many decades.

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