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Article

# The Oxygen-Transport System of Polar Fish: The Evolution of Hemoglobin

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**Abstract :** Organisms living in the Arctic and Antarctic regions are exposed to strong constraints, of which temperature is a driving factor. Evolution has led to special adaptations, some with important implications at the biochemical, physiological, and molecular levels. The northern and southern polar oceans have very different characteristics. Tectonic and oceanographic events have played a key role in delimiting the two polar ecosystems and influencing evolution. Antarctica has been isolated and cold longer than the Arctic; its ice sheet developed at least 10 million years earlier. As an intermediate system, the Arctic is a connection between the more extreme, simpler Antarctic system and the very complex temperate and tropical systems. By studying the molecular bases of cold adaptation in polar fish, and taking advantage of the information available on hemoglobin structure and function, we analysed the evolutionary history of the $\alpha$ and $\beta$ globins of Antarctic and Arctic hemoglobin using the molecular clock hypothesis as a basis for reconstructing the phylogenetic relationships among species.

**Key words :** Antarctic, Arctic, fish, cold adaptation, phylogeny

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## 1. Introduction

Although high latitudes and cold climates are common to both polar habitats, in many respects the two regions are more dissimilar than similar. Antarctica has been isolated and cold longer than the Arctic, its ice sheet developing at least 10 million years earlier. The modern polar ichthyofaunas differ in age, endemism, taxonomy, zoogeographic distinctiveness, and range of physiological tolerance to environmental parameters. The climatic features of the Antarctic waters are more extreme than those of the Arctic; due to the Antarctic Polar Front, its ichthyofauna is almost completely isolated. As an intermediate system, the Arctic has a connection between the more extreme, simpler Antarctic system and the very complex temperate and tropical systems.

The modern Antarctic ichthyofauna is largely endemic and, unlike the populations of the other continental shelves, is dominated by the suborder Notothenioidei. Ninety-six of the 213 species living on the shelf or upper slope of the Antarctic continent are notothenioids (Eastman 2000). Indirect indications suggest that notothenioids appeared in the early Tertiary period, filling the ecological void on the shelf left when most of the other fish fauna became locally extinct during a glacial maximum, and began to diversify in the middle Tertiary. Low competition and increasing isolation favoured speciation. Notothenioids fill a variety of ecological niches normally occupied by taxonomically diverse fish communities in temperate waters.

The ancestral notothenioid stock was probably a sluggish, bottom-dwelling teleost that evolved some 40-60 million years ago in the Antarctic shelf waters, which at that time were not cold. Only recently, have molecular phylogenies begun to provide indications about when the Antarctic radiation occurred.

With cold adaptation, evolutionary trends in the notothenioids have led to unique specialisations, including hematological modifications. Notothenioids differ from temperate and tropical species in having fewer erythrocytes and reduced hemoglobin (Hb) concentration and multiplicity

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*Corresponding author. E-mail : c.verde@ibp.cnr.it in the blood. The vast majority of species of the largely endemic dominant suborder Notothenioidei have a single Hb, sometimes accompanied by a minor component (di Prisco 1997).

Oxygen carriers are one of the most interesting systems for studying the interrelationships between environmental conditions and molecular evolution. Hb, a direct link between the exterior and the requirements of the body, is under strong evolutionary pressure. To ensure an adequate supply of oxygen, Hbs share a common molecular mechanism based on a ligand-linked conformational change in a multi-subunit structure. Within the framework of this common mechanism, the respiratory proteins of polar organisms have adapted to meet special needs.

Taking advantage of the wealth of information available on Hbs of a large number of Antarctic notothenioid species (e.g. amino acid sequences), this suborder is suitable for studying the evolution of the oxygen carrier in fish, using a molecular phylogenetic approach.

The Arctic and Antarctic oceans have very different characteristics. Tectonic and oceanographic events played a key role in delimiting the two polar ecosystems and in influencing evolution.

The ichthyofaunas of both polar environments are currently under investigation. In the Arctic, isolation is less stringent; the range of temperature variations is wider, both in the ocean and on the surrounding land, which is linked directly to temperate areas, facilitating migration and redistribution of the ichthyofauna. Arctic fish are characterised by higher biodiversity and, unlike Antarctic notothenioids, contain several Hbs. For instance, the blood of the spotted wolffish *Anarhichas minor*, a benthic, sedentary fish in the family Anarhichadidae (suborder Zoarcoidei) contains three functionally distinct major Hb components, the amino acid sequences and oxygen-binding properties of which were described recently (Verde *et al.* 2002). High multiplicity and functional differences have also been observed in two species of the family Gadidae: *Boreogadus saida* (polar cod) and *Gadus morhua* (Atlantic cod) (Verde *et al.* 2003).

## Antarctic Notothenioidei: adaptations in the blood and oxygen-transport system

The suborder Notothenioidei includes the families Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae (Balushkin 1992; Pisano *et al.* 1998). Notothenioids are red-blooded, with the exception of Channichthyidae (Ruud 1954), the only known adult vertebrates whose blood is devoid of Hb. Bovichtidae and Nototheniidae, together with monotypic Pseudaphritidae and Eleginopidae, include species that also inhabit waters north of the Antarctic and sub-Antarctic.

Notothenioids are by far the most thoroughly characterised group of fish in the world. The hematological features of many Antarctic Notothenioidei have been investigated extensively in the past few decades. Our studies of the oxygen-transport system of fish (di Prisco 1998) have examined 35 of the 80 known red-blooded Antarctic notothenioids and have correlated sequence, multiplicity, and oxygen binding with ecological constraints to infer phylogenetic information. This sample encompasses all the major families. As illustrated in the next Chapter, two species of non-Antarctic notothenioids have also been investigated for comparative purposes.

As subzero seawater temperatures greatly increase the viscosity of blood, which could have potentially negative physiological effects, the reduction or elimination of erythrocytes and Hb evolved as an adaptation to offset this increased viscosity and reduce the amount of energy needed to circulate the blood. The Hb-less Channichthyidae represent the extreme in this trend. In these fish, no other carrier has replaced Hb and the oxygen-carrying capacity of blood is only 10% that of red-blooded fish. However, the Channichthyidae are not at a disadvantage due to their lack of Hb, because they have developed physiological and anatomical adaptations enabling them to prosper without Hb (low metabolic rate; large, well-perfused gills; large blood volume, heart, stroke volume, and capillary diameter; cutaneous respiration).

Thirty-two red-blooded notothenioid species (all sluggish bottom-dwellers) have a single major Hb (Hb 1) and often a second, functionally similar minor component (Hb 2), which accounts for less than 5% of the total Hb and usually shares the $\beta$ chain in common with Hb 1 (di Prisco and Giardina 2000). Another component (Hb C) forms less than 1% of Hb in all species.

The amino acid sequences appear to follow another general trend. Those of major and minor Hbs cluster in two groups, within which the sequence identity is high (73%-99% and 84%-100%, respectively). However, the identity between the major and minor Hbs is lower, between 61% and 73%. The results of sequence analysis, together with the similar functional features of major and minor Hbs in a given species, led us to conclude that the minor Hbs are vestigial remnants or larval components, devoid of physiological significance in adult fish (di Prisco *et al.* 1991; di Prisco 1998).

Three species of the family Nototheniidae (active and cryopelagic *Trematomus newnesi* and *Pagothenia borchgrevinki*, and *Pleuragramma antarcticum*, a sluggish, pelagic, migratory fish) do not follow the pattern of low multiplicity, but have three to five functionally distinct Hb components. *T. newnesi* is the only species in which Hb C is not present in traces, and it has two major, functionally distinct Hbs (D'Avino *et al.* 1994). This more active fish may require such a Hb system to ensure oxygen binding at the gills and controlled delivery to tissues when its active behavior produces acidosis. With three major Hbs, *P. antarcticum* has the highest multiplicity among notothenioids. These Hbs display almost identical effector-enhanced Bohr and Root effects, but differ thermodynamically in terms of heat levels given off in oxygenation (Tamburrini *et al.* 1996), allowing optimal energy savings in the oxygenation-deoxygenation cycle during migration across water masses where low temperatures are likely to differ significantly and fluctuate. *P. borchgrevinki*, has five Hbs (Riccio *et al.* 2000) with different pH levels and organophosphate regulation, and heat levels occurring during oxygenation. The complexity of this oxygen-transport system (the most specialised among notothenioids) suggests that this species is adapted to a large variety of conditions.

### Arctic fish: the Hb system of *Anarhichas minor*

The hematological features of the Arctic spotted wolffish *Anarhichas minor*, a benthic sedentary species, differ markedly from those of the Notothenioidei. The three Hbs of *A. minor* (Hb 1, Hb 2, Hb 3) differ functionally in pH and organophosphate regulation, subunit cooperativity, and oxygen-binding responses to temperature (Verde *et al.* 2002). The evolution of the oxygen-transport system of *A. minor* appears to have produced adaptations suitable to reconciling respiration with the variety of conditions experienced in the Arctic marine environment.

Hb 1 and Hb 2 display a low, effector-enhanced Bohr effect (Riggs 1988), and no Root effect (Brittain 1987). In contrast, Hb 3 displays pronounced Bohr and Root effects, accompanied by strong organophosphate regulation. By virtue of the Root effect (an exaggerated Bohr effect), the large decrease in oxygen affinity at low pH prevents Hb from becoming fully saturated with oxygen even at very high oxygen tensions, and moreover subunit cooperativity is lost.

The Root effect originates from strong, proton-dependent stabilisation of the low-affinity T (tense) quaternary structure relative to the high-affinity R (relaxed) state (Perutz and Brunori 1982; Perutz *et al.* 1987). Its physiological role is to secrete oxygen into the swimbladder, which is absent in all Antarctic fish, as well as in *A. minor*, and into the choroid rete against high oxygen pressures following local acidification of the blood, in a countercurrent capillary system. Among Antarctic fish, only the few species possessing Hbs without Root effect and the Hb-less Channichthyidae lack the choroid rete, which is probably the most ancient anatomical structure associated with the presence of Root-effect Hbs (Farmer *et al.* 1979). In contrast to mammalian retinas, those of fish are poorly vascularised, yet the eyes of these fish may have oxygen tensions exceeding 800 mmHg (Fairbanks *et al.* 1969). Clearly, a secretory mechanism must be in operation to achieve this.

The important role of Hb in carrying oxygen to vertebrate tissues is probably the origin of its adaptation to most different environmental conditions, but its specialised function imposes severe structural constraints on the molecule. Hence, it is not surprising that only a small fraction of the residues of the polypeptide chains are modified during evolution. Although the structural basis of the Root effect is still elusive, some residues of the $\beta$ chain are thought to be involved in its molecular mechanism. Although a small number of $\alpha$-chain residues may also be involved, it is widely accepted that the $\beta$-chain primary structure plays the most crucial role. According to Perutz and Brunori (1982), the constellation of polar residues needed to produce the Root effect and the accompanying large Bohr effect in teleosts comprises Lys $\beta$82, Ser $\beta$93, Glu $\beta$94, Arg $\beta$143, and His $\beta$146.

Like Antarctic fish Hbs, each *A. minor* Hb shares one of the two chains in common with another Hb. Hb 1 and Hb 2 have identical $\beta$ chains (indicated as $\beta^1$) and different $\alpha$ chains ($\alpha^1$ and $\alpha^2$, respectively), Hb 3 differs from Hb 2 only in the $\beta$ chain ($\beta^2$), and Hb 1 and Hb 3 have no chain in common. The composition of Hb 1, Hb 2, and Hb 3 are $\alpha^1{}_2\beta^1{}_2$, $\alpha^2{}_2\beta^1{}_2$, and $\alpha^2{}_2\beta^2{}_2$, respectively.

The molecular basis for the overstabilisation of the T state in Root-effect Hbs is not yet understood. *T. newnesi* Hb 1 (D'Avino *et al.* 1994) has no Root effect, despite 95% sequence identity with *Trematomus bernacchii* Root-effect Hb 1 and the presence of His $\beta$146. At the molecular level, the presence of Ser $\beta$93 in Root-effect Hbs is undoubtedly of primary importance, although not even this criterion holds in absolute terms, as Cys has replaced Ser in *Cygnodraco mawsoni* Root-effect Hb 2. In many fish Hbs, most or all of these essential residues (including Ser) have been conserved, even when they lack the Root effect. In some instances, this happens because their

**Table 1. Sequence identity (%) between the $\alpha$ and $\beta$ globins of *A. minor* Hbs and those of Antarctic and temperate fish Hbs[a].**

| *A. minor* globins | Antarctic fish major Hbs (Hb 1) | Antarctic fish minor Hbs (Hb 2, Hb C) | Temperate fish Hbs |
|---|---|---|---|
| $\alpha^1$ (Hb 1) | 62-68 | 75-80 | 58-68 |
| $\alpha^2$ (Hb 2/Hb 3) | 73-77 | 65-68 | 56-64 |
| $\beta^1$ (Hb 1/Hb 2) | 74-80 | 62-70 | 60-66 |
| $\beta^2$ (Hb 3) | 70-77 | 78-84 | 58-65 |

[a] All sequences are from Stam *et al.* (1997) and Verde *et al.* (2002).

influence is balanced by a substitution elsewhere.

As expected, a comparison with Antarctic Hbs does not reveal a simple pattern. The $\alpha^1$ and $\beta^2$ globins of *A. minor* share higher identity with the corresponding chains of Antarctic minor Hbs (Hb 2 and Hb C), whereas the $\alpha^2$ and $\beta^1$ chains share higher identities with the chains of major Hbs (Table 1). Hb 2 is the only component of *A. minor* sharing overall higher identity with the major Antarctic fish Hbs. In all cases, the identities are consistently higher than those with Hbs of temperate species (Verde *et al.* 2002). Whether these differences are evolutionarily significant is an important question that remains unanswered.

## Antarctic notothenioids and Arctic fish: phylogenetic analysis

The amino acid sequences of the $\alpha$ and $\beta$ chains of Antarctic fish together with those of several non-Antarctic species, have been analysed using maximum parsimony to build cladograms and phylogenetic trees (Stam *et al.* 1997, 1998). The trees are in agreement with those obtained in morphological analyses and studies of mitochondrial RNA sequences (Ritchie *et al.* 1996) and strongly support the monophyly of Antarctic notothenioids, with non-Antarctic *Pseudaphritis urvillii* as their sister taxon.

Further phylogenetic analysis was performed on multiple alignments constructed with the programme Clustal X. The inferred neighbour-joining (NJ) trees for $\alpha$ and $\beta$ globins are shown in Figs. 1A and 2A, respectively. The sequences marked with asterisks evolved significantly slower or faster than the average rate at the 1% significance level in the branch length test (Takezaki *et al.* 1995). These sequences were removed from the data set, and the linearised trees depicted in Figs. 1B and 2B were constructed with the remaining sequences under the

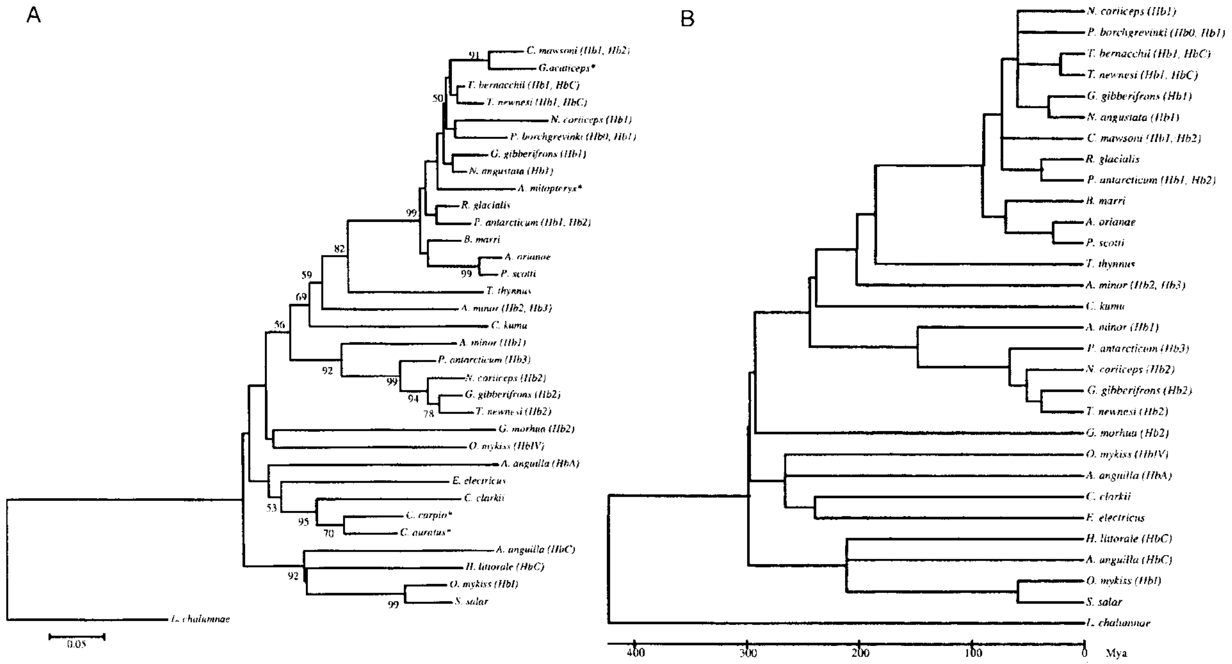

Fig. 1. (A) Phylogenetic tree of amino acid sequences for $\alpha$ globins from Antarctic, Arctic, and temperate fish Hbs. Bootstrap values (percentages of 10,000 replicates) are given at the nodes. An asterisk near a taxon name indicates a sequence evolving significantly slower or faster than the average for all sequences, (B) Linearised tree inferred after removing deviant sequences, showing the time scale.

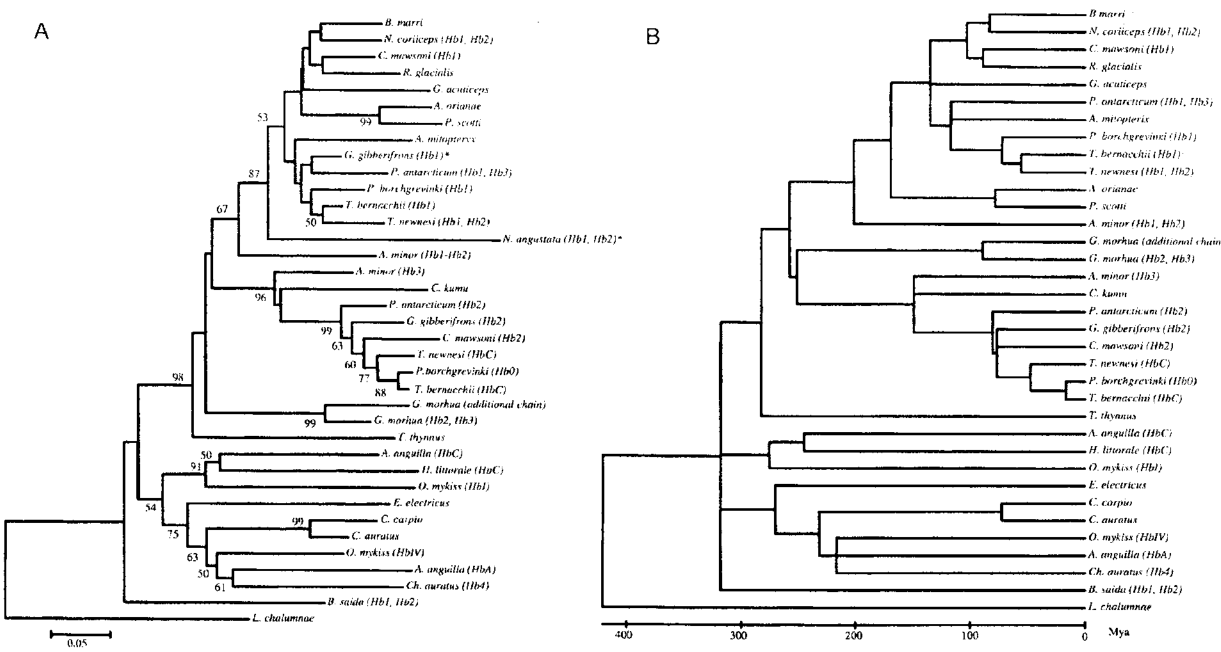

**Fig. 2. (A) Phylogenetic tree of amino acid sequences for $\beta$ globins from Arctic, Antarctic, and temperate fish Hbs. Bootstrap values (percentages of 10,000 replicates) are given at the nodes. An asterisk near a taxon name indicates a sequence evolving significantly slower or faster than the average for all sequences, (B) Linearised tree inferred after removing deviant sequences, showing the time scale.**

molecular-clock assumption. The time scale was calibrated by assuming a divergence time of about 420 million years for *Latimeria chalumnae*. According to this time scale, both globins diverged about 240 million years ago, before cooling. The time of divergence indicates that Hb diversification was not strictly correlated with changes in habitat temperature.

The topologies of the two trees suggest different evolutionary histories for the $\alpha$ and $\beta$ globins (Verde *et al.* 2003). The chains of Antarctic minor Hbs appear to have diverged from the major components before cold adaptation; they are a monophyletic group, which forms an unresolved cluster, well separated from that of the chains of the major Hbs, and from the Hbs of temperate fish. This analysis led to the tentative hypothesis that at least two gene duplications of an adjacent $\alpha$ and $\beta$ pair occurred in ancient teleosts.

In another representation, the trees in Fig. 3 show a synopsis of the phylogenetic analyses. The duplication event that gave rise to the two clades containing the globins of Antarctic major and minor Hbs occurred very early, presumably at the onset of the Mesozoic.

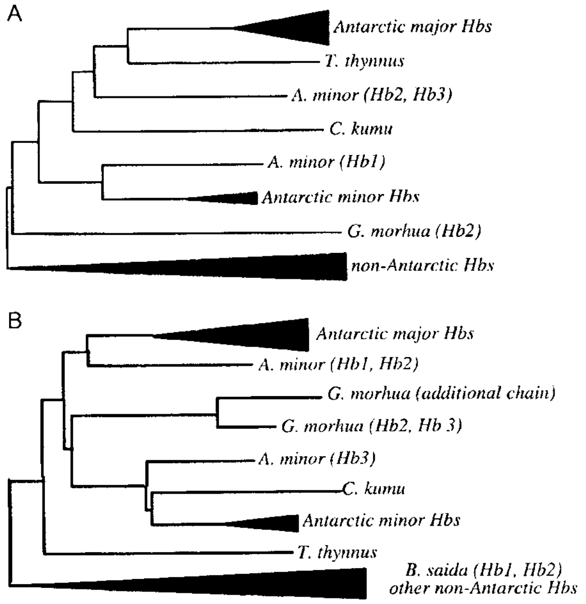

**Fig. 3. Synoptic trees summarising the results of Figs. 1 and 2. (A), $\alpha$ globins. (B), $\beta$ globins.**

A number of sequences fail to group in any of the clades; these include the globins of temperate *Chelidonichthys kumu* and *Thunnus thynnus*. In addition, the globins of the Arctic species that occupy different positions in both trees, with the exception of *A. minor*, which is close to the notothenioid clades, have independent evolutionary histories. For example, the $\beta$ chain of *B. saida* is included in the clade of other non-Antarctic species (Fig. 3B), but its position is very distant from all the other globins (see also Fig. 2A). The $\alpha^2$ chain of Hb 2 of *G. morhua* (Atlantic cod) is close to the $\alpha$ chain of *Oncorhynchus mykiss* (trout) Hb IV (see also Fig. 1A), whereas the two $\beta$ chains appear closely related, probably resulting from a relatively recent gene duplication event.

Apart from the Antarctic globins, which are grouped in two distinct clades, consisting of the major and minor Hbs, respectively, most of the other globins fail to cluster in well-defined clades.

In the tree in Fig. 2A, the $\alpha^2$ chain shared by *A. minor* Hb 2 and Hb 3 is close to the major Antarctic globins, and also to the two temperate globins, while $\alpha^1$ of Hb 1 appears more closely related to the minor Antarctic globins. In the tree in Fig. 2B, the *A. minor* $\beta^1$ chain shared by Hb 1 and Hb 2 is grouped with the major Antarctic globins, whereas the Hb 3 $\beta^2$ chain is well separated from the subclades of major and minor Antarctic globins.

The $\beta^1$ chain of Hb 1 and Hb 2 of the polar cod *B. saida* does not group with the other sequences, whereas $\beta^2$ (Hb 2, Hb 3) and another $\beta$ chain (possibly belonging to a larval sequence which was deduced from DNA) of the Atlantic cod *G. morhua* constitute a clade with high bootstrap support. Interestingly, the divergence of these two sequences was more recent than that of other paralogous globin families, such as those of the major and minor Antarctic Hbs.

## 2. Conclusion

The importance of the Arctic in contributing to the overall ensemble of adaptive processes influencing the evolution of marine organisms calls for investigations of adaptations taking place in the main biological systems of Arctic fish (e.g. the respiratory system). A wealth of knowledge is available on the oxygen-transport system of fish inhabiting Antarctic waters, but very little is known about the structure and function of the Hbs of fish from the other polar marine environment, which has such different physico-chemical features.

The amino acid sequences of the *A. minor* Hbs clearly show low levels of identity with temperate fish species, which may imply some degree of correlation with cold adaptation. These observations are in keeping with the results of the phylogenetic analysis. The study of the structure/function relationship in the Hbs of *A. minor* has revealed several important features, again suggesting that the main characteristic of the Hb system of *A. minor*, which does not resemble any system found in Antarctic fish, is its response to the need to optimally adapt to Arctic waters, where the low temperatures both differ and fluctuate to greater extents than in the Antarctic.

There seems to be no single molecular explanation for the Root effect. Instead, a combination of several factors arising from local structural differences, which may vary from one species to another, is likely to have a determining role. Although in some cases it has been possible to assign functional shifts to single amino acid replacements, the structural basis of the Root effect is far from being fully understood, and even X-ray crystallography has provided no unequivocal explanation (Ito *et al.* 1995; Mazzarella *et al.* 1999). It is important to note, however, that a reduced or absent Root effect should not be regarded as a "loss of function", but rather as a physiological adaptation to living habits.

In conclusion, the marked differences in the oxygen-transport systems of Arctic and Antarctic fish indicate that distinct evolutionary pathways in the regulatory mechanisms of the fish respiratory system have been followed in the two polar environments. The different phylogenetic histories of Arctic and Antarctic fish depend on their respective habitats. As a result of the isolation of Antarctica, the Notothenioidei acquired a completely different genotype with respect to other fish groups. Although both are cold, the Arctic and Antarctic habitats differ in many ways. Indeed, isolation is much less stringent and temperature variations are wider in the Arctic than in the Antarctic. Consequently, the complex Arctic ichthyofauna, unlike that of the Antarctic (dominated by one taxonomically uniform group), is characterised by high diversity, reflected in the phylogeny of a given trait. The 'lifestyle' of a benthic species, such as *A. minor*, which is unlikely to encounter wide temperature gradients, appears to result in the greater similarity it exhibits to the Hb evolutionary traits of Antarctic notothenioids. In contrast, the two Gadidae occupy an intermediate position between the Antarctic and non-Antarctic clades, in keeping with their active, pelagic, migratory 'lifestyle'. In short, the constant physico-chemical conditions of the Antarctic Ocean are matched by a clear grouping of fish globin sequences, whereas the variations typical of the Arctic Ocean matches the wide sequence variation.

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