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Review

The adaptive evolution of polar fishes: Structure, function and molecular phylogeny of hemoglobin

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Abstract: Temperature affects all molecular processes and is the major determinant of habitat suitability. Whilst there is an increasing understanding of evolutionary adaptation to temperature in some processes, several key questions often remain open about the structure-function relationships associated with protein thermal adaptation. Proteins, such as hemoglobin, are highly sensitive to temperature and therefore, their structural and functional properties mirror the thermal conditions encountered by species during their evolutionary histories. The most stable thermal environments are aquatic; research on polar fishes has provided important insights into the details of thermal adaptation. In polar fishes, the evolution of hemoglobin includes adaptations with implications at the biochemical, physiological and structural levels. Although both are cold, the Northern and Southern polar oceans have very different oceanographic features. In comparison with Antarctic fish of the suborder Notothenioidei, Arctic fish are characterised by higher biodiversity and hemoglobin multiplicity. Within the study of the molecular bases of cold adaptation in fish inhabiting the polar habitats, and taking advantage of the information available on hemoglobin structure and function, the evolutionary history of the α and β globins of Arctic and Antarctic fish hemoglobins has been analysed, under the assumption of the molecular-clock hypothesis.

key words: polar fish, hemoglobin, molecular phylogeny

1. The polar marine environments

During the Paleozoic and Mesozoic eras, Antarctica together with South America, Africa, India, Australia and New Zealand, was part of the supercontinent Gondwana. The fragmentation of Gondwana began approximately 135 million years ago (mya). The continental drift carried Antarctica close to the present position at the beginning of the Cenozoic era, about 65 mya (Kennett, 1977). The isolation of Antarctica became complete 22–25 mya with the opening of the Drake passage and the development of the Polar Front, a well-defined, roughly circular, oceanic frontal system, currently running between 50°S and 60°S, where the surface layers of the north-flowing Antarctic waters sink beneath the less cold, less dense waters of more northern latitudes. The cooling of the environment steadily proceeded, reaching the present, extreme climatic conditions.

Throughout the year, the temperature of the Antarctic coastal waters, in which fish from temperate waters would rapidly freeze and could not survive, is −1.87°C, the equilibrium temperature of the ice-salt water mixture. Temperature is the most important physical factor affecting organisms. For the study of temperature adaptations, Antarctica more than any other habitat on earth is indeed a unique natural laboratory.

The biosynthesis of antifreeze (glyco) proteins and peptides (AFGPs, AFPs), is one of the most intriguing evolutionary adaptations (reviewed in Cheng and 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, b). The finding in the notothenioid genome of (i) a chimeric AFGP-protease gene intermediate, (ii) a protease gene still bearing the incipient coding element, and (iii) independent AFGP genes, reveals a fascinating case of "evolution in action". 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.

A classical example of how temperature adaptation has influenced evolution is the marine teleost family Channichthyidae (icefish). This clade has lost the ability to express the genes encoding the globin chains of hemoglobin (Hb) (di Prisco *et al*., 2002). The increased solubility of oxygen in water may allow these fish to transport sufficient oxygen in physical solution rather than via an oxygen-binding carrier.

In the process of cold adaptation, the evolutionary trend of Antarctic fish has led to unique specialisations, including modification of the hematological characteristics *e.g.* the level and multiplicity of Hbs (di Prisco, 2000). The vast majority of species of the dominant suborder Notothenioidei have a single Hb, sometimes accompanied by a minor component accounting for approximately 5% of the total and easily separated by ion-exchange chromatography. Structural and functional characterisation of Antarctic fish Hbs was initiated in the early eighties.

Although high latitudes and cold climates are common to the Arctic and the Antarctic, in many respects the two regions are more dissimilar than similar. For instance, in the Arctic the range of temperature variation is wider, both in the ocean and in the surrounding lands, which are directly linked to temperate areas, facilitating migration and redistribution of the ichthyofauna.

The study of the structure and function of Arctic fish Hbs was initiated only recently, when the first molecular characterisation of the oxygen-transport system of an Arctic species (*Anarhichas minor*) was reported (Verde *et al*., 2002). The evolution of oxygen transport by this Hb system, which has three functionally distinct components, appeared to be the response to the need to optimally adapt to the temperature differences and fluctuations of Arctic waters, much larger than in the Antarctic.

Although Europe began to separate from Greenland in the late Cretaceous, the exchange of Atlantic and Arctic waters through the passage between Greenland and the Svalbard islands was not possible until 27 mya. The Arctic region was in a high-latitude position by the early Tertiary, but the climate remained temperate with water

temperatures of 10–15°C. The Antarctic has been isolated and cold longer than the Arctic, with ice sheet development preceding that in the Arctic by at least 10 mya.

In an attempt to link protein evolution, molecular adaptation and polar environmental conditions, this review aims to survey the current knowledge of molecular phylogeny and adaptations in the oxygen-transport system of Antarctic and Arctic fishes.

2. Hemoglobin

Hb is one of the most interesting systems for studying the relationships between environmental conditions and molecular evolution. Being a direct link between the exterior and body requirements and fulfilling its primary function under extremely variable conditions, it has experienced a major evolutionary pressure to adapt and modify its functional features. Hence Hbs and the globin genes encoding them are an important system for investigating many biochemical and evolutionary issues.

Oxygen-binding molecules are ancient proteins. When multicellular organisms increased in size, the simple diffusion of oxygen across the body wall was inadequate to reach all cells. The evolution of simple oxygen-binding proteins into multisubunit, circulating proteins, in combination with the advent of circulatory systems, made the transport of oxygen possible on a significant scale from the periphery of the organism to cells (Terwilliger, 1998). Thus, Hbs have evolved diversities (both structural and functional); they exhibit functional repertoires especially in fish expressing more than one.

How did Hbs adapt in polar fish in order to provide adequate oxygen supply to the tissues under extreme environmental conditions?

Changes in levels of allosteric effectors modulating the oxygenation properties can modify oxygen delivery. Fish have higher ability than other organisms to control concentrations of modulators (namely organophosphates). Regulation of the function of oxygen-transport proteins by allosteric effectors in response to relatively short-term environmental changes helps the fish to properly deal with environmental changes and metabolic demands (Terwilliger, 1998).

Changing the rate of Hb synthesis obviously has an impact on how much oxygen the blood can transport. However, rather than regulating the rate of expression of a single oxygen carrier, many organisms, including fish, express multiple respiratory proteins with different oxygen affinities. These Hbs are produced and function either simultaneously to optimise oxygen transport, or sequentially, if they have different oxygen-binding properties, in order to be able to meet oxygen demand despite rapidly changing environmental conditions (*see below*, Arctic fishes). The presence of multiple Hbs may allow higher total Hb concentration to be maintained in the erythrocyte than if there were only a single one. In many cases, polymorphism has no visible phenotypic effect and may have no obvious correlation with environmental conditions (*see below*, Arctic fishes).

In summary, changes in synthesis of oxygen-transport protein, whether up- or down-regulation or expression of a particular combination of gene products, are often considered to be a long-term response to developmental or environmental change, while allosteric effectors are thought to be responsible for more immediate, short-term perturbations.

3. Antarctic Notothenioidei

Fishes of the perciform suborder Notothenioidei, mostly confined within Antarctic and sub-Antarctic waters, are the dominant component of the Southern Ocean ichthyofauna. According to a recently revised classification (Balushkin, 1992; Pisano *et al*., 1998), Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae are the families of the suborder. Notothenioids are red-blooded except Channichthyidae (Ruud, 1954).

Unlike temperate and tropical species, notothenioids have evolved reduced Hb concentration and multiplicity. Blood has a reduced erythrocyte number and a lower Hb content (Everson and Ralph, 1968; Hureau *et al*., 1977; Wells *et al*., 1980); the number of Hb components is also reduced. The red-blooded families of the suborder generally have a single Hb (Hb 1) accounting for 95–99% of the total, and often a functionally similar minor component Hb 2 (di Prisco, 1998). It is unknown whether the synthesis of Hb 2 is under ontogenetic or environmental control.

In Antarctic notothenioids, a single Hb in lower amounts is regarded as the consequence of a lesser role for the oxygen carrier, possibly due to their sluggish mode of life, slower metabolism, and to the peculiarity of the environmental conditions (high stability and constancy of physico-chemical features).

Studies on the oxygen transport in all Antarctic species investigated have shown that the oxygen affinity of Hb (a property which controls both the binding of oxygen at the gill exchange surface and its release to the tissues) is quite low (di Prisco *et al*., 1988), as indicated by the high values of p_{50} (the partial pressure of oxygen required to achieve half saturation of Hb). This feature is presumably related to the high concentrations of dissolved oxygen in the cold Antarctic waters.

When the oxygen affinity decreases in the lower physiological range of pH, the Bohr effect is present (Riggs, 1988); if the extent of the decrease is such that a given Hb can no longer become saturated even when exposed to pressures of several atmospheres of pure oxygen, and the cooperativity is totally lost, this Hb displays the Root effect (Brittain, 1987). Such effect is often observed in fish Hbs. The presence of the Root effect implies the presence of the Bohr effect, but not *vice versa*. The presence or absence of these effects dictate how easily Hb may bind sufficient oxygen at the gills or adequately release it to the tissues. Bohr and Root effect are displayed by the Hbs of all Notothenioidei examined, with the exception of two of the three components of *Trematomus newnesi*, and of the single Hb of the bathydraconid *Gymnodraco acuticeps* (Tamburrini *et al*., 1992) and the nototheniid *Aethotaxis mitopteryx* (D'Avino *et al*., 1992).

4. Arctic fishes

The modern polar ichthyofaunas differ in age, endemism, taxonomy, zoogeographic distinctiveness (Fig. 1 and 2), and range of physiological tolerance to environmental parameters (Eastman, 1997).

If the comparison between cold-adapted and non-cold-adapted species is a useful approach to understanding the fish evolutionary history, as well as the molecular

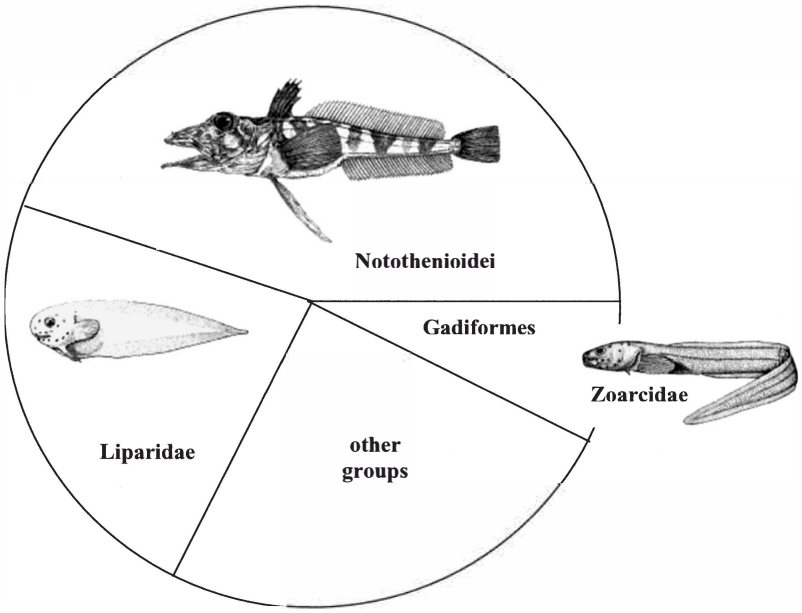

Fig. 1. In the Antarctic, five groups account for approx. 74% of the ichthyofauna (Notothenioidei, Myctophidae, Liparidae, Zoarcidae and Gadiformes).

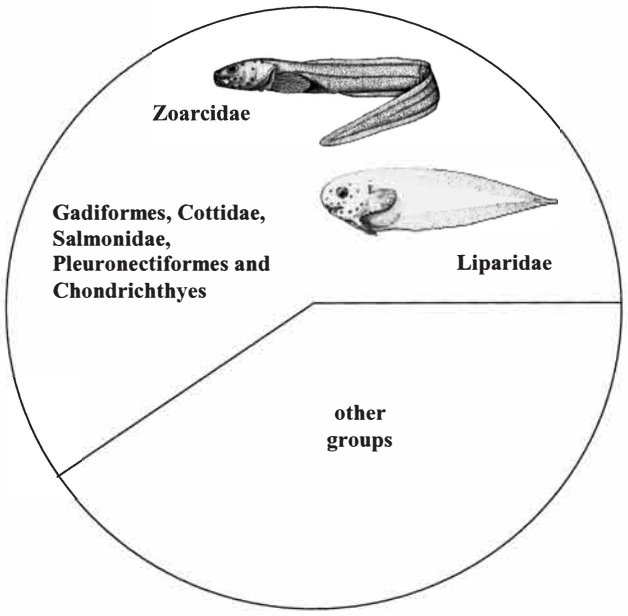

Fig. 2. In the Arctic, six dominant groups account for 58% of the fauna (Liparidae, Zoarcidae, Gadiformes, Cottidae, Salmonidae, Pleuronectiformes and Chondrichthyes).

mechanisms of cold adaptation, a logical extension of this argument also includes the comparison of the fish faunas of both polar northern oceans.

Arctic fishes share many similarities in freezing avoidance strategies with Antarctic notothenioids. However, notothenioids constitutively synthesise AFGPs, whereas Arctic fishes producing AFGPs exhibit seasonal patterns of synthesis. This is an energy-efficient strategy that avoids costly synthesis when freezing is not a danger.

The Arctic fish species investigated so far are characterised by higher biodiversity and, unlike Antarctic notothenioids, have high Hb multiplicity. For instance, the blood of the spotted wolffish *A. minor*, a benthic, sedentary fish of the family Anarhichadidae (order Perciformes, suborder Zoarcoidei) contains three functionally distinct major components, whose amino-acid sequences and oxygen-binding properties have been recently described (Verde *et al*., 2002). High multiplicity and functional differences have also been observed in *Arctogadus glacialis* (Arctic cod), *Boreogadus saida* (polar cod) and *Gadus morhua* (Atlantic cod), all belonging to the family Gadidae (Verde *et al*., unpublished).

5. Phylogenetic analysis of Hbs of Antarctic notothenioids and Arctic fishes

Within the study of the molecular bases of cold adaptation in fish inhabiting the polar habitats, and taking advantage of the information available on Hb structure and function, we analysed the evolutionary history of the α and β globins of Antarctic and Arctic Hbs, under the assumption of the molecular-clock hypothesis, as a basis for reconstructing the phylogenetic relationships among species. Table 1 lists the 29 species examined in this study and the accession numbers of 36 α-globin and 37 β-globin amino-acid sequences used in the phylogenetic analysis. Sequences not available in data banks are indicated by references.

Phylogenetic analysis was performed on the multiple alignments constructed with the programme CLUSTAL X (Verde *et al*., 2004). The inferred Neighbour-Joining (NJ) trees for α and β globins are reported in Fig. 3A and Fig. 4A. The genetic distances were measured according to the *p*-distance model. The sequences marked with an asterisk 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 Fig. 3B and Fig. 4B were constructed with the remaining sequences under the molecular-clock assumption.

Calibration of the time scale was performed by assuming a divergence time of about 420 mya for *Latimeria chalumnae* (Benton, 1997). According to this time scale, the globins of Antarctic fish diverged approximately 250 mya, *i.e.* at the onset of the Mesozoic (Verde *et al*., 2004). According to two interpretations, the appearance of AFGP dates back to 5–14 mya (Chen *et al*., 1997a, b), or to late Eocene, 38 mya (Lecointre, personal communication). In both cases, the appearence of AFGP is related to cooling, whereas Hb diversification does not appear to be correlated to such changes in the environmental conditions.

The time of the gene duplication event that gave origin to the two paralogous groups of major and minor Hbs is also similar, suggesting that they diverged long before the first stock of ancestral notothenioids. This event concomitantly involved also a

Table 1. List of species and globin sequences investigated. Taxa are arranged according to Nelson (1994).

Order and species	Subunit	Accession number/reference
Coelacanthiformes (outgroup)		
Latimeria chalumnae[d]	a, ß	P23740, P23741
Scorpaeniformes		
Chelidonichthys kumu[d]	a, ß	P80270, P80271
Perciformes		
Thunnus thynnus[d]	a, ß	P11748, P11749
Anarhichas minor[c]	a (Hb 1), a (Hb 2, Hb 3)	P83270, P83271
	ß (Hb 1, Hb 2), ß (Hb 3)	P83272, P83273
Chrysophrys auratus[d]	a, ß (Hb 4)	Stam et al. 1997
Notothenia coriiceps[a]	major a (Hb 1), ß (Hb 1, Hb 2)	P10777, P16309
	minor a (Hb 2)	P16308
Notothenia angustata[b]	major a (Hb 1), ß (Hb 1, Hb 2)	P29624, P29628
	minor a (Hb 2)	P16308
Pleuragramma antarcticum[a]	major a (Hb 1, Hb 2), ß (Hb 1, Hb3)	Stam et al. 1997
	minor a (Hb 3), ß (Hb 2)	Stam et al. 1997
Pagothenia borchgrevinki[a]	major a (Hb 1, Hb 0)	P82344
	major ß (Hb 1)	P82346
	minor ß (Hb 0)	P83245
Gobionotothen gibberifrons[a]	major a, ß (Hb 1)	P83611, P83612
	minor a, ß (Hb 2)	P83613, P83614
Aethotaxis mitopteryx[a]	a, ß	Stam et al. 1997
Trematomus newnesi[a]	major a, ß (Hb 1)	P45718, P45720
	minor a (Hb 2), ß (Hb C)	P45719, P45721
Trematomus bernacchii[a]	major a, ß (Hb 1)	P80043, P80044
	minor ß (Hb C)	P45722
Cygnodraco mawsoni[a]	major a (Hb 1, Hb 2), ß (Hb 1)	P23016, P23017
	minor ß (Hb 2)	P23018
Gymnodraco acuticeps[a]	a, ß	P29623, P29625
Racovitzia glacialis[a]	a, ß	Stam et al. 1997
Bathydraco marri[a]	a, ß	Stam et al. 1997
Pogonophryne scotti[a]	a, ß	Stam et al. 1997
Artedidraco orianae[a]	a, ß	Stam et al. 1997
Salmoniformes		
Salmo salar[d]	a	P11251
Oncorhynchus mykiss[d]	a, ß (Hb I)	P02019, P02142
	a, ß (Hb IV)	P14527, P02141
Gadiformes		
Gadus morhua[c]	a (Hb 2)	this study
	ß (Hb 2, Hb 3)	this study
	ß (additional chain)	O13077
Boreogadus saida[c]	ß (Hb 1, Hb 2)	this study
Anguilliformes		
Anguilla anguilla[d]	a, ß (Hb C)	P80726, P80727
	a, ß (Hb A)	P80945, P80946
Gymnotiformes		
Electrophorus electricus[d]	a, ß	P14520, P14521
Siluriformes		
Hoplosternum littorale[d]	a, ß (Hb C)	P82315, P82316
Cypriniformes		
Cyprinus carpio[d]	a, ß	P02016, P02139
Carassius auratus[d]	a, ß	P02018, P02140
Catostomus clarkii[d]	a	P02017

[a]Antarctic Notothenioidei; [b]Non-Antarctic Notothenioidei; [c]Arctic species; [d]Temperate freshwater and marine species

Fig. 3A.

Fig. 3. A. Phylogenetic tree of amino acid sequences of α chains from Antarctic, Arctic and temperate fish Hbs. Bootstrap values (percentage of 10000 replicates) are given at the nodes. The asterisk on taxon names indicates the deviant sequences, evolving significantly slower or faster than the average of all sequences. B. Linearised tree inferred after removal of deviant sequences, showing the time scale. Major Hbs (normal lettering), minor Hbs from Antarctic fish (bold), Arctic fish (italic), temperate fish (underlined). The coelacanth L. chalumnae (outgroup) is in bold and underlined.

number of Arctic and temperate sequences, such as those of *A. minor*, *Chelidonichthys kumu*, *Thunnus thynnus* and *G. morhua* (β chain only), because they fall in the same clade of the Antarctic globins.

Unlike the globins of Antarctic species, the Arctic globins occupy scattered positions in both trees, suggesting independent evolutionary histories, with the exception of *A. minor* which is close to the notothenioid clades. For example, the β^1 chain of the

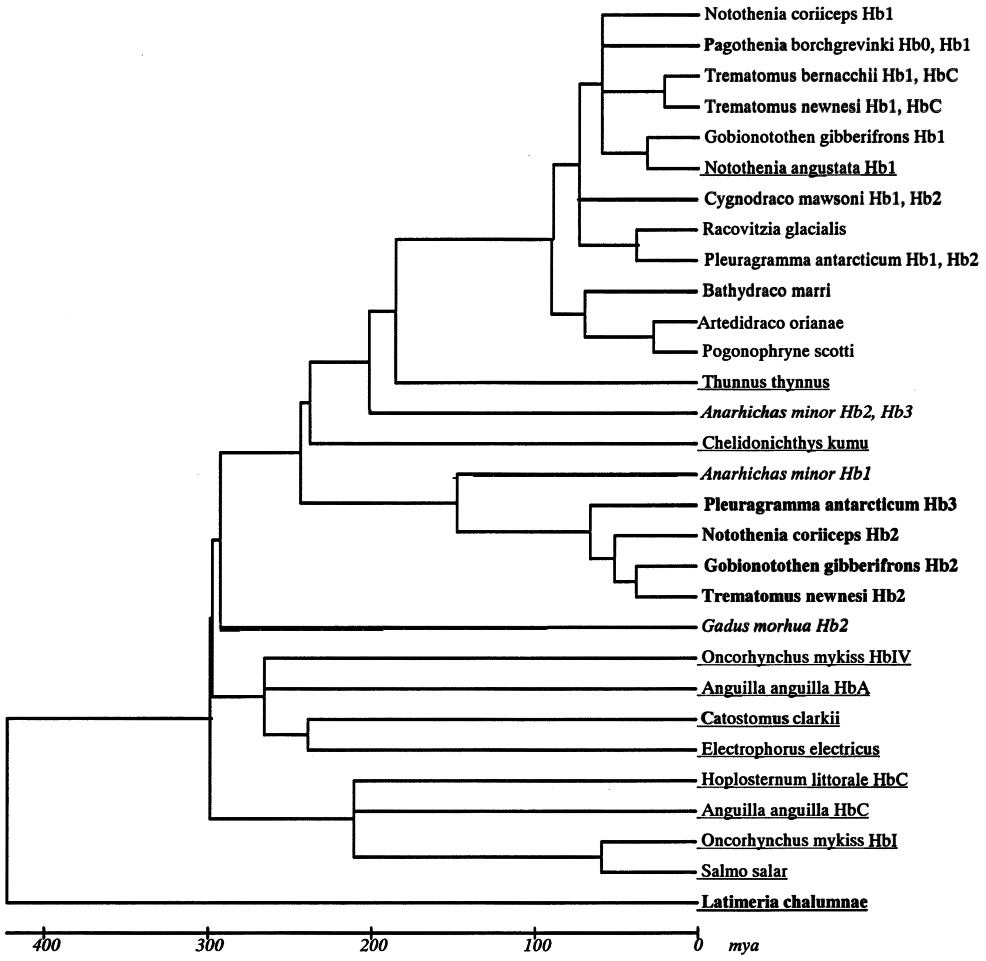

Fig. 3B.

polar cod *B. saida* is included in the clade of the other non-Antarctic species, but its position is remote from all the other globins (Fig. 4A). The α^2 chain of Hb2 of the Atlantic cod *G. morhua* is close to the α chain of trout (*Oncorhynchus mykiss*) HbIV (Fig. 3A), whereas the two β chains appear closely related, probably as a result of a relatively recent gene duplication event.

In the tree of Fig. 3A, the α^2 chain shared by *A. minor* Hb2 and Hb3 is close to the major Antarctic globins, whereas α^1 of Hb 1 appears more closely related to minor Antarctic globins. In the tree of Fig. 4A, the position of the *A. minor* β^1 chain shared by Hb1 and Hb2 falls into the group of the major Antarctic globins, whereas the Hb3 β^2 chain appears well separated from the subclades of major and minor Antarctic globins.

The β^1 chain of Hb1 and Hb2 of cold-adapted *B. saida* outgroups with respect to the other sequences. This is a surprising finding, but it is very difficult to find objective

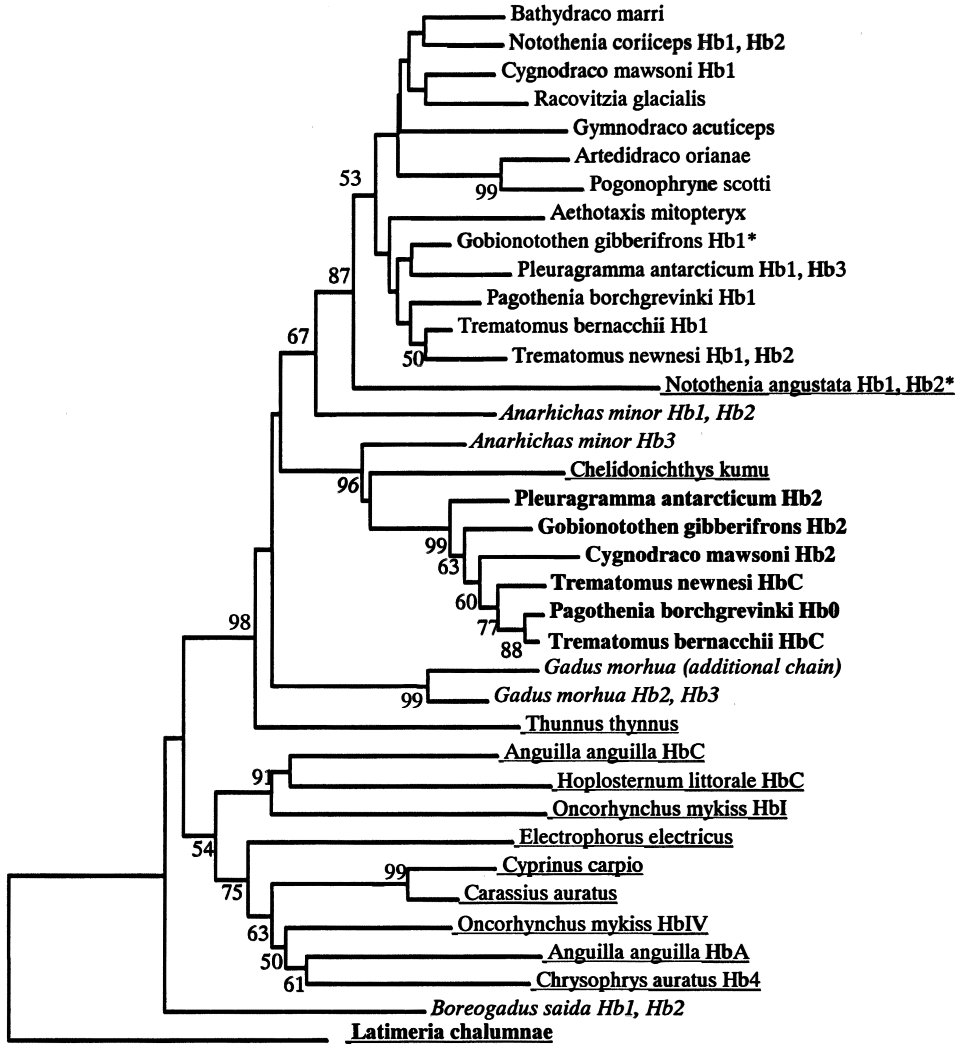

Fig. 4A. Same as Fig. 3A except β chains.

criteria to identify links between a phenotypic trait (*e.g.* the primary structure) and adaptation, in this case to temperature. In *G. morhua*, β^2 (Hb2, Hb3) and another β chain (possibly belonging to a larval Hb, and whose sequence has been deduced from DNA) constitute a clade characterised by a node supported by a high bootstrap value. Interestingly, the divergence time of these two sequences is more recent than other paralogous globin families, such as those of major and minor Antarctic Hbs.

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

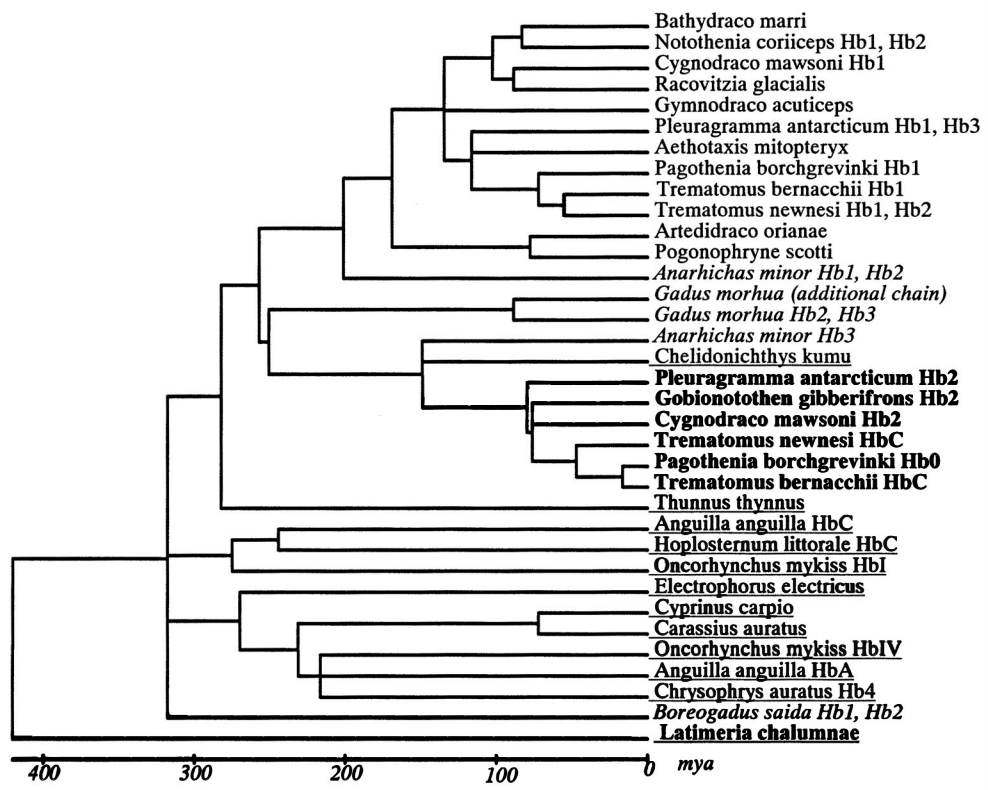

Fig. 4B. Same as Fig. 3B.

6. Concluding remarks

The remarkable differences in the oxygen-transport system between Arctic and Antarctic bony fishes indicate 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 fishes depend on the respective habitats. As a result of the isolation of Antarctica, the genotype of Antarctic Notothenioidei diverged with respect to other fish groups. In this perspective, the evolution of the genes of AFGPs is an intriguing process, and identical Arctic and Antarctic AFGPs are the result of a case of convergent evolution, as each type derives from a distinct ancestral gene sequence (Chen *et al.*, 1997b).

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, distributed across a much more complex oceanographic system than the Antarctic one (dominated by a single taxonomic group), is characterised by high diversity, also reflected in the phylogeny of a given trait. The life style of *A. minor* a benthic Arctic species, unlikely to disperse across wide latitude and temperature gradients, corresponds to higher similarity of Hb evolution with Antarctic notothenioids. In con-

trast, Gadidae, including *A. glacialis* (Verde *et al*., unpublished), occupy intermediate positions between the Antarctic and temperate clades, in keeping with their active, pelagic and migratory life style.

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 environment correspond to high sequence divergence.

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