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Comparison of Arctic and Antarctic teleost haemoglobins: primary structure, function and phylogeny

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Abstract: Organisms living in the Arctic and Antarctic are exposed to strong environmental constraints, especially temperature. Consequently, haemoglobin evolution has included adaptations with implications at the biochemical, physiological and molecular levels. The northern and southern polar oceans have very different oceanographic characteristics. Within the study of the molecular bases of cold adaptation in fish inhabiting polar habitats, and taking advantage of the information available on haemoglobin structure and function, we analysed the evolutionary history of the α and β globins of Antarctic and Arctic haemoglobins, under the assumption of the molecular-clock hypothesis, as a basis for reconstructing the phylogenetic relationships between species. Temperate fish, including two non-Antarctic notothenioids of special evolutionary interest, were also considered. Phylogenetic analysis was performed on the multiple sequence alignments constructed with the programme Clustal X. Tree topologies indicate that the chains of Antarctic major and minor haemoglobins cluster in two well separated groups and diverged prior to cold adaptation, forming a monophyletic group. In Arctic haemoglobins, the structure/function relationship reveals important differences in comparison with Antarctic ones, indicating a distinct evolutionary pathway. The Arctic ichthyofauna (unlike the Antarctic, dominated by one taxonomically uniform group) is characterized by high diversity, reflected in the phylogeny of a given trait. The constant physico-chemical conditions of the Antarctic waters are matched by a clear grouping of fish globin sequences, whereas the variability typical of the Arctic Ocean corresponds to high sequence variation, reflected in the trees by scattered intermediate positions between the Antarctic and non-Antarctic clades. The evolutionary history of the Root effect, an important physiological feature of fish haemoglobin, was investigated. Analysis of the fate of the residues of the β chains suggested to be correlated with the Root effect indicate that they should rather be regarded as ancestral characters, inherited by some species but not by others.

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Introduction

Although high latitudes and cold climates are common to the Arctic and Antarctic, in many respects the two regions are more dissimilar than similar. Antarctica has been isolated and cold longer than the Arctic; the ice sheet developed 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 and the ichthyofauna is virtually isolated by the Antarctic Polar Front. Due to less extreme and less constant climatic conditions, the Arctic ecosystem may fill an intermediate position between the Antarctic and the temperate and tropical ones.

The modern Antarctic fish fauna is largely endemic and, unlike faunas 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, filling the ecological void on the shelf left by most of the other fish fauna (which became locally extinct during maximal glaciation), and began to diversify in the middle Tertiary. Reduced competition and increasing isolation favoured speciation. Notothenioids fill a varied range of ecological niches normally occupied by taxonomically diverse fish communities in temperate waters.

The ancestral notothenioid stock probably arose as sluggish bottom-dwellers that evolved some 40–60 million years ago in the Antarctic shelf waters, which at that time were not cold. Molecular phylogenetics has recently begun to provide indications about the time of radiation in the Antarctic. The initial divergence of some of the basal families took place during the early fragmentation of Gondwana. At this time stocks appear to have become established in isolated continental blocks (e.g. the monotypic family Pseudaphritidae in Australia).

During the process of cold adaptation, the evolutionary trend of notothenioids has led to unique specializations, including modification of haematological features. Notothenioids differ from temperate and tropical species in

having fewer erythrocytes and reduced haemoglobin (Hb) concentration and multiplicity 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. Haemoglobin, a direct link between the exterior and the oxygen requirements of the body, experienced major evolutionary pressure for functional adaptation. In order to ensure an adequate supply of oxygen to the entire organism, Hbs have developed a common molecular mechanism based on ligand-linked conformational change in a multi-subunit structure. Within the framework of this common mechanism, the respiratory proteins of polar organisms have acquired adaptive features to meet special needs.

Taking advantage of the wealth of information (e.g. Hb amino acid sequences) available for a large number of notothenioids, including two non-Antarctic species, this suborder is an excellent case study for the evolution of the oxygen carrier in fish in a molecular phylogenetic context.

The characteristics of the Arctic Ocean are very different from those of the Southern Ocean. Tectonic and oceanographical events played a key role in delimiting the two polar ecosystems and in influencing evolution.

The Hbs of the ichthyofaunas from both polar environments are currently the focus of our work. 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. Arctic fish are characterized by higher biodiversity and, unlike Antarctic notothenioids, have high Hb multiplicity. For instance, the blood of the spotted wolffish *Anarhichas minor*, a benthic, sedentary fish of the family Anarhichadidae (superorder Acanthopterygii, suborder Zoarcoidei) contains three functionally distinct major Hb 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 two gadids, *Boreogadus saida* (polar cod) and *Gadus morhua* (Atlantic cod).

In addition to utilizing Antarctic and Arctic Hb primary structures in a phylogenetic analysis, we also considered the evolutionary history of one of the most important and ancient physiological features of many fish Hb, the Root effect, and traced the fate of the ancestral amino acid residues.

Adaptations in the blood and oxygen-transport system

Antarctic notothenioids

According to the current classification (Balushkin 1992, Pisano *et al*. 1998), the suborder comprises the families Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae. Notothenioids are red-blooded except the Channichthyidae (Ruud 1954), the only known adult vertebrates whose blood is devoid of Hb. The Bovichtidae and Nototheniidae, together with monotypic Pseudaphritidae and Eleginopidae, comprise species which also inhabit waters north of the Antarctic and sub-Antarctic regions.

With respect to Hb, Notothenioids are by far the most thoroughly characterized group of fish in the world. The haematological features of many Antarctic Notothenioidei have been extensively investigated in the past few decades. Our studies on the oxygen-transport system of fish (di Prisco 1998) have so far encompassed 35 out of a total of 80 red-blooded Antarctic notothenioids and are aimed at correlating sequence, multiplicity and oxygen binding with ecological constraints and at obtaining information on evolution. This highly representative number includes all major families. As indicated in the section below, two species of non-Antarctic notothenioids have also been investigated for comparative purposes.

Because the subzero seawater temperature would greatly increase the viscosity of blood, with potentially negative physiological effects, reduction or elimination of erythrocytes and Hb was developed as an adaptation to offset this increase and reduce the amount of energy needed for circulation. The Hb-less Channichthyidae represent the extreme of this trend. In these fish, Hb has not been replaced by another carrier and the oxygen-carrying capacity of blood is only 10% of that of red-blooded fish. However, the Channichthyidae are not at all at a disadvantage from the lack of Hb, because they have developed physiological and anatomical adaptations enabling them to survive 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 Hb (Hb 1) and often a second, functionally similar minor component (Hb 2), accounting for less than 5% of the total and usually having the β chain in common with Hb 1 (di Prisco 1998). Another component (Hb C) is present at less than 1% in all species.

The amino acid sequences appear to follow another general trend. Those of major and minor Hbs cluster in two groups; in each group, identity is high (73–99% and 84%–100%, respectively). However, the identity between major and minor Hbs is lower, ranging between 61% and 73%. The analysis of sequences, together with the similar functional features of major and minor Hbs in a given species, led us to conclude that 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

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cryopelagic *Trematomus newnesi* and *Pagothenia borchgrevinki*, and *Pleuragramma antarcticum*, a pelagic, sluggish but migratory fish) do not follow the pattern of low multiplicity, but have three to five functionally distinct Hb components. *Trematomus 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). Such an Hb system may be required by this more active fish to ensure oxygen binding at the gills and controlled delivery to tissues when the active behaviour produces acidosis. *Pleuragramma antarcticum* has the highest multiplicity of major Hbs (three) among notothenioids; these Hbs display almost identical effector-enhanced Bohr and Root effects, but differ thermodynamically in the values of oxygenation heats (Tamburrini *et al*. 1996). This allows optimal energy savings in the oxygenation-deoxygenation cycle during migration across water masses where the low temperature is likely to show significant differences and fluctuations. *Pagothenia borchgrevinki* has five Hbs (Riccio *et al*. 2000) with different pH and organophosphate regulation, as well as oxygenation heats. The complexity of this oxygen-transport system (the most specialized among notothenioids) suggests that the life style of this species is adapted to a large variety of conditions.

Non-Antarctic notothenioid species

From the evolutionary standpoint, the analysis of the oxygen-transport system of non-Antarctic notothenioids is of great interest. In particular, the comparison of *Pseudaphritis urvillii* (amongst the most primitive species of the suborder) with the more recent *Notothenia angustata* provides useful insights.

Pseudaphritis urvillii, of the monotypic family Pseudaphritidae, is common in estuaries and lower portions of Australian rivers, and is considered a relict species, whose ancestors were associated with the Australian component of Gondwana about 40–50 million years ago. Similar to most Antarctic notothenioids, *P. urvillii* has Hb 1 (95%) and Hb 2 (less than 5%). The oxygen affinity of Hb 1 is strongly regulated by pH and organophosphates (Bohr and Root effects). This species is euryhaline and may migrate upstream as far as 120 km from the sea (Andrews *et al*. 1980). Because of its distribution, it is especially important for the calibration of the molecular clock. This Gondwanian species may have drifted northward reaching Australian freshwaters, subsequently succeeding in becoming established in an area with a depauperate freshwater fauna.

Besides the life style, some features differentiate this species from cold-adapted Antarctic notothenioids, e.g. it has glomerular kidneys and has neither antifreeze glycoproteins (AFGPs) nor the genes encoding AFGPs (Cheng *et al*. 2003). On the other hand, other features are consistent with cold adaptation. In freshwater *P. urvillii* is almost neutrally buoyant (Eastman 1993); the major and minor Hbs are in the typical proportions observed in benthic notothenioids and have one chain (the α) in common. The primary structures reveal high identity with the amino acid sequences of Antarctic globins; however, the values are at the lower end of the identity ranges of 73–99% and 84–100% for major and minor Hbs respectively (Table I). Although *P. urvillii* has never become fully cold adapted, this identity is higher than with any temperate fish (60–63% for the α and 60–66% for the β chains, following the general trend shown by notothenioids). Similar to Antarctic notothenioids, the identity between major and minor Hbs is lower (64–68%). The data argue in favour of a common origin, but also suggests that the major component has undergone modifications only to a limited extent. If sequence mutations in Antarctic fish are indeed related to the development of cold adaptation, this may imply that *P. urvillii* diverged and migrated during the first stages of the cooling process and in any case before the event which gave origin to the biosynthesis of AFGPs. It is worth recalling that, based on the age of 15–5 million years inferred from the divergence of molecular sequences between trypsinogen and antifreeze (Chen *et al*. 1997), the acquisition of antifreeze genes responsible for the synthesis of AFGPs in the nototheniid *Dissostichus mawsoni* is coincident with the cooling and appearance of ice in the Southern Ocean.

The nototheniid *N. angustata* inhabits coastal waters of the southern island of New Zealand. It is a sedentary bottom feeder and experiences minimum temperatures of about 5°C. This species also has haematological features typical of both temperate and cold-adapted fish. High haematocrit, erythrocyte number, Hb content and cellular concentration (MCHC) typically favour oxygen transport in a temperate

Table I. Sequence identity (%) between the α and β chains of *P. urvillii* Hbs and of Antarctic fish Hbs[a].

P. urvillii	Antarctic major Hbs (Hb 1)	Antarctic minor Hbs (Hb 2, Hb C)
α (Hb 1, Hb 2)	70–80	64–65
β^1 (Hb 1)	70–78	65–68
β^2 (Hb 2)	70–77	80–88

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

Table II. Sequence identity (%) between the α and β chains of *Anarhichas minor* Hbs and of Antarctic and temperate fish Hbs[a].

A. minor	Antarctic fish major Hbs (Hb 1)	Antarctic fish minor Hbs (Hb 2, Hb C)	Temperate fish 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

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

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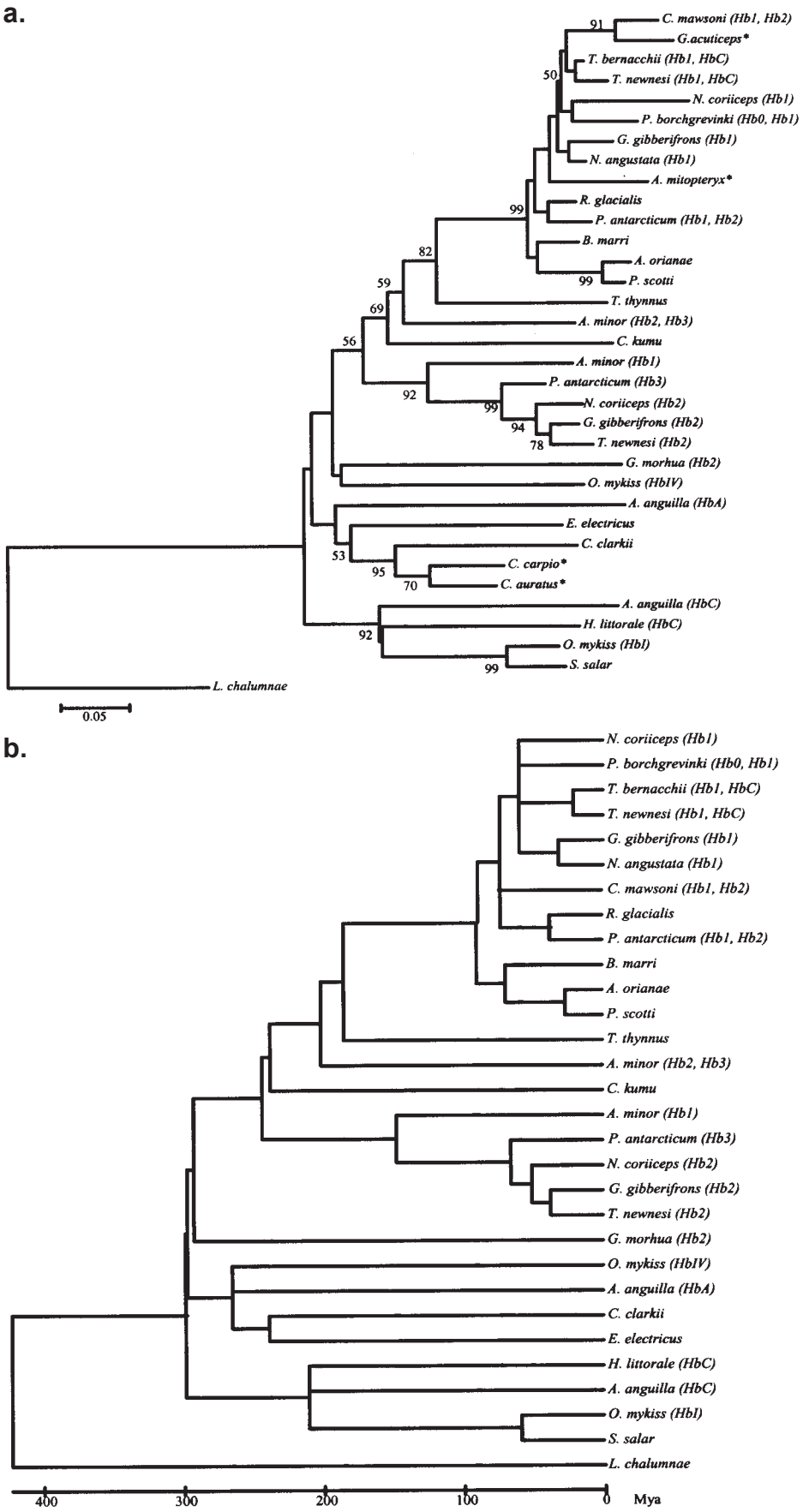

Fig. 1. a. Phylogenetic tree of amino acid sequences of α chains from Antarctic, Arctic and temperate fish Hbs, indicated in parentheses at each branch. Bootstrap values (percentage of 10 000 replicates) are given at the nodes. The asterisk near taxon names indicates the sequences evolving significantly slower or faster than the average of all sequences.
b. Linearized tree inferred after removal of deviant sequences, showing the time scale.

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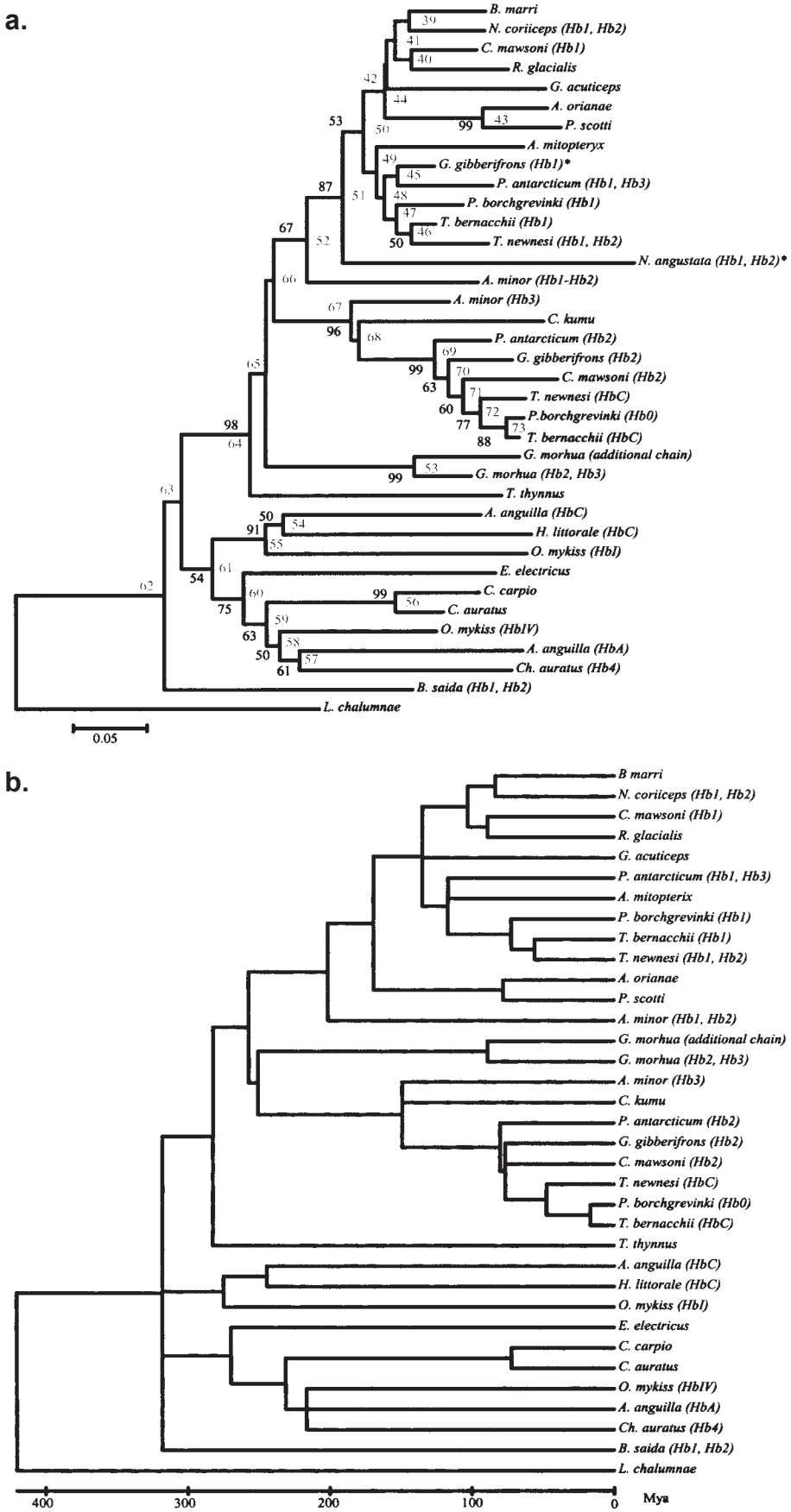

Fig. 2. a. Phylogenetic tree of amino acid sequences of β chains from Arctic, Antarctic and temperate fish Hbs, indicated in parentheses at each branch. Bootstrap values (percentage of 10 000 replicates) are given at the nodes. Numbers in light type indicate nodes where functionally important ancestral residues of Root-effect HBs are conserved - see also Table IV. The asterisk near taxon names indicates the sequences evolving significantly slower or faster than the average of all sequences. **b.** Linearized tree inferred after removal of deviant sequences, showing the time scale.

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Table III. List of species and globin sequences investigated.

Order and species	Family	Subunit	Accession number/reference
Coelacanthiformes (outgroup)			
Latimeria chalumnae[d] Smith	Coelacanthidae	α, β	P23740, P23741
Scorpaeniformes			
Chelidonichthys kumu[d] Linnaeus	Triglidae	α, β	P80270, P80271
Perciformes			
Thunnus thynnus[d] Linnaeus	Scombridae	α, β	P11748, P11749
Anarhichas minor[c] Olafsen	Anarhichadidae	α (Hb 1), α (Hb 2, Hb 3)	P83270, P83271
		β (Hb 1, Hb 2), β (Hb 3)	P83272, P83273
Chrysophrys auratus[d] Forster	Sparidae	α, β (Hb 4)	Stam *et al.* 1997
Notothenia coriiceps[a] Richardson	Nototheniidae	major α (Hb 1), β (Hb 1, Hb 2)	P10777, P16309
		minor α (Hb 2)	P16308
Notothenia angustata[b] Hutton	Nototheniidae	major α (Hb 1), β (Hb 1, Hb 2)	P29624, P29628
		minor α (Hb 2)	P16308
Pleuragramma antarcticum[a] Boulenger	Nototheniidae	major α (Hb 1, Hb 2), β (Hb 1, Hb3)	Stam *et al.* 1997
		minor α (Hb 3), β (Hb 2)	Stam *et al.* 1997
Pagothenia borchgrevinki[a] Boulenger	Nototheniidae	major α (Hb 1, Hb 0)	P82344
		major β (Hb 1)	P82346
		minor β (Hb 0)	P83245
Gobionotothen gibberifrons[a] Lönnberg	Nototheniidae	major α, β (Hb 1)	Marinakis *et al*. 2003
		minor α, β (Hb 2)	Marinakis *et al.* 2003
Aethotaxis mitopteryx[a] DeWitt	Nototheniidae	α, β	Stam *et al*. 1997
Trematomus newnesi[a] Boulenger	Nototheniidae	major α, β (Hb 1)	P45718, P45720
		minor α (Hb 2), β (Hb C)	P45719, P45721
Trematomus bernacchii[a] Boulenger	Nototheniidae	major α, β (Hb 1)	P80043, P80044
		minor β (Hb C)	P45722
Cygnodraco mawsoni[a] Waite	Bathydraconidae	major α (Hb 1, Hb 2), β (Hb 1)	P23016, P23017
		minor β (Hb 2)	P23018
Gymnodraco acuticeps[a] Boulenger	Bathydraconidae	α, β	P29623, P29625
Racovitzia glacialis[a] Dollo	Bathydraconidae	α, β	Tamburrini *et al*. unpublished
Bathydraco marri[a] Norman	Bathydraconidae	α, β	Stam *et al.* 1997
Pogonophryne scotti[a] Regan	Artedidraconidae	α, β	Stam *et al*. 1997
Artedidraco orianae[a] Regan	Artedidraconidae	α, β	Stam *et al*. 1997
Salmoniformes			
Salmo salar[d] Linnaeus	Salmonidae	α	P11251
Oncorhynchus mykiss[d] Walbaum	Salmonidae	α, β (Hb I)	P02019, P02142
		α, β (Hb IV)	P14527, P02141
Gadiformes			
Gadus morhua[c] Linnaeus	Gadidae	α (Hb 2)	Verde *et al*. unpublished
		β (Hb 2, Hb 3)	Verde *et al.* unpublished
		β (additional chain)	O13077
Boreogadus saida[c] Lepechin	Gadidae	β (Hb 1, Hb 2)	Verde *et al*. unpublished
Anguilliformes			
Anguilla anguilla[d] Linnaeus	Anguillidae	α, β (Hb C)	P80726, P80727
		α, β (Hb A)	P80945, P80946
Gymnotiformes			
Electrophorus electricus[d] Linnaeus	Electrophoridae	α, β	P14520, P14521
Siluriformes			
Hoplosternum littorale[d] Hancock	Callichthyidae	α, β (Hb C)	P82315, P82316
Cypriniformes			
Cyprinus carpio[d] Linnaeus	Cyprinidae	α, β	P02016, P02139
Carassius auratus[d] Linnaeus	Cyprinidae	α, β	P02018, P02140
Catostomus clarkii[d] Baird & Girard	Catostomidae	α	P02017

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

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environment (Macdonald & Wells 1991), but Hb multiplicity and structural/functional features closely resemble those of Antarctic notothenioids (Fago *et al*. 1992). For example, the amino acid sequence identity between Hbs of temperate *N. angustata* and cold-adapted *Notothenia coriiceps* of the same family is the highest ever found among notothenioids, suggesting that the sequence similarity in *N. angustata* with Antarctic notothenioids may indeed be correlated with prior cold adaptation.

Unlike Antarctic notothenioids, this fish has pauciglomerular kidneys; it lacks the swimbladder, but is not neutrally buoyant (Eastman 1993). It does not synthesize AFGPs but the genome of *N. angustata* does have the AFGP genes, and these genes may become activated at low temperatures (Cheng *et al*. 2003). The presence of AFGP genes suggests that this fish migrated from Antarctic to temperate waters in more recent geological time than *P. urvillii*, and, unlike the latter fish, was cold adapted prior to radiation.

Arctic fish

The haematological features of the Arctic spotted wolffish *Anarhichas minor*, a benthic sedentary species, differ markedly from those of Notothenioidei. The three Hbs of *A. minor* (Hb 1, Hb 2, Hb 3) are functionally different 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 reconcile 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 the oxygen affinity at low pH impairs Hb from being fully saturated with oxygen even at very high oxygen tensions; moreover, subunit cooperativity is lost.

Similar to Antarctic fish Hbs, each *A. minor* Hb has one of the two chains in common with another one. Hb 1 and Hb 2 have identical β chains (indicated as β^1) and differ by the α chain (α^1 and α^2), Hb 3 differs from Hb 2 only by the β chain (β^2), and Hb 1 and Hb 3 have no chain in common. Thus the chain composition of Hb 1, Hb 2 and Hb 3 is $\alpha^1{}_2\beta^1{}_2$, $\alpha^2{}_2\beta^1{}_2$, and $\alpha^2{}_2\beta^2{}_2$, respectively.

As expected, the comparison with Antarctic Hbs does not reveal a simple pattern. The α^1 and β^2 globins of *A. minor* display higher identity with the corresponding chains of Antarctic minor Hbs (Hb 2 and Hb C), whereas the α^2 and β^1 chains have higher identity with the chains of major Hbs (Table II). Hb 2 is thus the only component of *A. minor* displaying 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 and unanswered question.

A detailed analysis of these structural features follows in the next section.

Phylogenetic analysis of Antarctic notothenioids and Arctic fish

The amino acid sequences of the α and β chains of Antarctic fish and *P. urvillii*, together with those of several non-Antarctic species, have been analysed using maximum parsimony to construct notothenioid cladograms (Stam *et al*. 1997, 1998). The trees are in agreement with those obtained by morphological analysis and sequence studies on mitochondrial RNA (Ritchie *et al*. 1996) and give strong support to the monophyly of Antarctic notothenioids, with non-Antarctic *P. urvillii* as their sister taxon.

Table III lists the 29 species examined in this study and the accession numbers of 36 α-globin and 37 β-globin sequences used in the phylogenetic analysis. The sequences not available in data banks are indicated by references.

Further phylogenetic analysis was performed on the multiple alignments constructed with the programme Clustal X. The inferred Neighbour-Joining (NJ) trees for α and β globins are reported in Figs 1a & 2a. 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 linearized trees depicted in Figs 1b & 2b 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 million years for *Latimeria chalumnae* (Benton 1997). According to this time scale, globins currently found in Antarctic fish diverged approximately 250 m.y.a., i.e. at the onset of Mesozoic; hence, unlike AFGP, whose appearance coincided with cooling of the Antarctic continent, Hb diversification appears less stringently correlated to 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. Such an event involved also a number of non-Antarctic 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 forming two distinct compact groups, the Arctic globins occupy scattered position in both trees, suggesting independent evolutionary histories, with the exception of *A. minor* which is close to the notothenioid clades.

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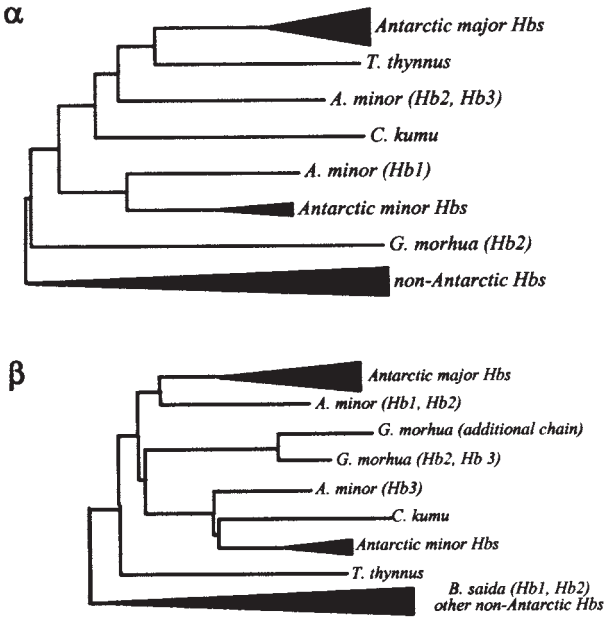

Fig. 3. Synoptic trees summarizing the results shown in detail in Figs 1 & 2. **a.** α chains, **b.** β chains.

For example, the β^1 chain of *B. saida* can be included in the clade of the other non-Antarctic species (Fig. 3b), but its position is remote from all the other globins (see also Fig. 2a). The α^2 chain of Hb 2 of *G. morhua* (Atlantic cod) is close to the α? chain of *Oncorhynchus mykiss* (trout) Hb IV (see also Fig. 1a) whereas the two β chains appear closely related, probably as a result of a relatively recent gene duplication event.

In the tree of Fig. 1a, the α^2 chain shared by *A. minor* Hb 2 and Hb 3 is close to the major Antarctic globins, but also to the two temperate globins, whilst α^1 of Hb 1 appears more closely related to minor Antarctic globins. In the tree of Fig. 2b, the position of the *A. minor* β^1 chain shared by Hb 1 and Hb 2 is placed in the group of the major Antarctic globins, whereas the Hb 3 β^2 chain appears well separated from the subclades of major and minor Antarctic globins.

The β^1 chain of Hb 1 and Hb 2 of the polar cod *B. saida* outgroups with respect to the other sequences, whereas β^2 (Hb 2, Hb 3) and another β chain (possibly belonging to a larval Hb, and whose sequence has been deduced from DNA) of the Atlantic cod *G. morhua* constitute a clade characterized 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 the major and minor Antarctic Hbs.

In another representation, the trees in Fig. 3 show a synopsis of the phylogenetic analyses, graphically summarizing the cladograms in a visually simplified manner.

Table IV. Conservation of functionally important ancestral residues in Root-effect Hbs at the internal nodes of Fig. 2a.

Node	Residues and position in the ß chain
39	Asn2, Trp3, Ser43, Val82, **Ser93**, Glu94, His97, Lys143, **His146**
40	Lys2, Trp3, Ser43, Val82, **Ser93**, Glu94, His97, Lys143, **His146**
41	Asn2, Trp3, Ser43, Val82, **Ser93**, Glu94, His97, Lys143, **His146**
42	Asn2, Trp3, Ser43, Val82, **Ser93**, Glu94, His97, Lys143, **His146**
43	Gln2, Trp3, Ser43, Met82, **Ser93**, Glu94, His97, Lys143, **His146**
44	Glu2, Trp3, Ser43, Val82, **Ser93**, Glu94, His97, Lys143, **His146**
45	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
46	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
47	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
48	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
49	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
50	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
51	Glu2, Trp3, Ser43, Ala82, **Ser93**, Glu94, His97, Lys143, **His146**
52	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Lys143, **His146**
53	Glu2, Trp3, Gly43, Lys82, **Ser93**, Asp94, His97, Arg143, **His146**
54	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, Asn97, Ser143, ***Phe146***
55	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Ser143, ***Phe146***
56	Glu2, Trp3, Ala43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
57	Glu2, Trp3, Ala43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
58	Glu2, Trp3, Ala43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
59	Glu2, Trp3, Ala43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
60	Glu2, Trp3, Ala43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
61	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
62	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
63	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
64	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
65	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
66	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
67	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
68	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
69	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
70	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
71	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
72	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**
73	Glu2, Trp3, Gly43, Lys82, **Ser93**, Glu94, His97, Arg143, **His146**

The two residues reported having an essential role in the Root-effect are in bold. A non-conservative replacement is indicated in italic and underlined.

Ancestral residues and the Root effect

In an attempt to shed light on the molecular bases of the Root effect (one of the most important and ancient physiological features of many fish Hbs), its evolutionary history has also been investigated in parallel, analyzing the fate of the relevant ancestral residues in the β chains (Table IV). The Root effect originates from a strong, proton-dependent stabilization of the low-affinity T (tense) quaternary structure relative to the high-affinity R (relaxed) state (Perutz & Brunori 1982, Perutz *et al*. 1987). The physiological role is to secrete oxygen into the swimbladder (when present) and the ocular choroid rete against high oxygen pressures following local acidification of the blood in a counter current capillary system. Among Antarctic fishes (all lacking the swimbladder), only the few species possessing Hbs without a Root effect, as well as the Hb-less Channichthyidae, lack the choroid rete. The choroid rete is probably the most ancient anatomical structure associated

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with the presence of Root effect Hbs (Farmer *et al*. 1979). In contrast to mammalian retinas, those of fishes are poorly vascularized and yet the eyes of these fishes may show oxygen tensions of over 800 mmHg (Fairbanks *et al*. 1969). It is clear that a secretory mechanism must be in operation in order to achieve this situation.

The important role of Hb in carrying oxygen to vertebrate tissues is probably the origin of its adaptation to different environmental conditions, but its specialized 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 β chain have been suggested to be involved in its molecular mechanism. Although a small number of α-chain residues may also be involved, it is widely accepted that the β-chain primary structure has the most crucial role. According to Perutz & Brunori (1982), the constellation of polar residues needed to produce the Root effect and the accompanying large Bohr effect in teleosts comprises Lys β82, Ser β93, Glu β94, Arg β143 and His β146, which are part of the ensemble of Root effect residues of Table IV.

We have attempted to trace the possible modifications of these residues during the evolutionary history of β chains. Our results indicate that the residues suggested to be correlated with the Root effect should rather be regarded as ancestral characters, inherited by some fish species but not by others. The replacement of some residues is not always accompanied by the disappearance of the Root effect, indicating the existence of a complex molecular mechanism, probably involving yet unidentified factors. It is however relevant that some species appear to have conserved the residues and the Root effect, whilst others have lost both. Thus, the clade grouping the major Antarctic globins as well as those of *A. minor* Hb 1 and Hb 2 contains five chains of Hbs devoid of the Root effect (only two Hbs, *Aethotaxis mitopteryx* Hb and *A. minor* Hb 1 and Hb 2, show replacements in β146) and ten chains of Root-effect Hbs (a few of these sequences display some conservative replacements). The members of the clade containing the minor Antarctic globins, as well as those of the temperate fish *C. kumu* and of *A. minor* Hb 3, have *in toto* retained the Root effect and all the canonical residues, with the exception of the β chain of *Cygnodraco mawsoni* Hb 2 (Caruso *et al.* 1991), in which Ser β93 has been replaced by Cys, and of *T. newnesi* Hb 2, lacking the effect.

The molecular basis for the overstabilization of the T state in Root-effect Hbs is not understood as yet. *Trematomus 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 β146. Undoubtedly at the molecular level the presence of Ser β93 in Root-effect Hbs is of primary importance, although not even this criterion holds in absolute terms; in fact, in *C. mawsoni* Root-effect Hb 2, Cys has replaced Ser. In many fish Hbs, most or even all these essential residues (including Ser) have been conserved even when they lack the Root-effect. In some instances this happens because their influence is balanced by a substitution elsewhere.

Conclusions

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

The amino acid sequences of Hbs of the zoarcoid *A. minor* clearly show low identity levels with temperate fish species, which may imply some degree of correlation with cold adaptation. On the other hand, the suborder Zoarcoidei has been proposed to be closely related to Notothenioidei (Anderson 1990). Consequently, although this is controversial (Chen *et al*. 2003, Miya *et al*. 2003), phylogenetic relatedness cannot be excluded. The study of the structure/function relationship in the Hbs of *A. minor* has revealed several important features, again suggesting (without excluding phylogeny) that the main characteristic of the Hb system of *A. minor* (which does not resemble any system found in Antarctic fish) is the response to the need to optimally adapt to the Arctic waters, where temperatures show larger differences and fluctuations than in the Antarctic.

There seems to be no single molecular explanation of the Root effect. Instead, the combination of several factors arising from local structural differences, that may vary from one species to another, is likely to have the driving 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 life style.

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 respiratory system have been followed in the two polar environments. The different phylogenetic histories of Arctic and Antarctic fish reflect their respective habitats. As a result of the isolation of Antarctica, the Notothenioidei acquired a completely

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different genotype 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 convergent evolution, as each type derives from a different ancestral gene sequence (Chen *et al*. 1997). Although both are cold, the Arctic and Antarctic habitats differ in many aspects. Indeed, in the Arctic isolation is not complete and the range of temperature variation 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 (dominated by one taxonomically uniform group), is also characterized by high diversity, reflected in the phylogeny of a given trait. The life style of a benthic species, such as *A. minor*, unlikely to cross wide latitude and temperature gradients, shares more similarity in Hb evolution with Antarctic notothenioids.

In contrast, the two gadid species occupy an intermediate position between the Antarctic and non-Antarctic clades, in keeping with their active, pelagic and migratory life style. In short, the constant physico-chemical conditions of the Antarctic ocean is matched by clear grouping of fish globin sequences, whereas the variability typical of the Arctic oceans corresponds to high sequence variation.

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