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Polar Science 4 (2010) 295–308

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The hemoglobins of sub-Antarctic fishes of the suborder Notothenioidei

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Received 16 December 2009; revised 19 April 2010; accepted 19 April 2010
Available online 12 May 2010

Abstract

Fishes of the perciform suborder Notothenioidei provide an excellent opportunity for studying the evolution and functional importance of evolutionary adaptations to temperature. To understand the unique biochemical features of high-Antarctic notothenioids, it is important to improve our knowledge of these highly cold-adapted stenotherms with new information on their sub-Antarctic relatives.

This paper focuses on the oxygen-transport system of two non-Antarctic species, *Eleginops maclovinus* and *Bovichtus diacanthus*.

Unlike most Antarctic notothenioids, the blood of *E. maclovinus* and *B. diacanthus* displays high hemoglobin (Hb) multiplicity. *E. maclovinus*, the sister group of Antarctic notothenioids, has one cathodal (Hb C) and two anodal components (Hb 1, Hb 2). *B. diacanthus*, one of the most northern notothenioids, has three major Hbs. The multiple Hbs may have been maintained as a response to temperature differences and fluctuations of temperate waters, much larger than in the Antarctic. Although non-Antarctic notothenioids have never developed cold adaptation, the amino-acid sequence reveals high identity with the globins of Antarctic notothenioids.

Hbs of sub-Antarctic notothenioids are characterised by high oxygen affinity and Root effect. Phylogenetic analyses are consistent with the hypothesis that Bovichtidae and Eleginopidae diverged after they became established in more temperate waters north of the Antarctic Polar Front.
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Keywords: Hemoglobin; Fish; Hemoglobin multiplicity; Root effect

1. Introduction

The formation of the Southern Ocean, which surrounds Antarctica and includes the Weddell and Ross Seas, was marked by the creation of a large mass of water, uniquely cold and thermally stable. The opening of the Drake Passage, occurred approximately 41 million years ago, and the formation of the Tasmanian Gateway few millions years after, are the two key events (Scher and Martin, 2006). The separation of the southern landmasses led to the development of the Antarctic Circumpolar Current (ACC), and this in turn was at least partially responsible for cooling of

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doi:10.1016/j.polar.2010.04.007

Antarctic waters from ~20 °C to the present extreme values near −1.8 °C (Clarke, 1983). Ocean changes played a crucial role in establishing the thermal conditions that have driven evolution of the Antarctic biota (Eastman, 1993). The cold and oxygen-rich waters south of the Antarctic Polar Front (APF; the northern boundary of the ACC, a well-defined roughly circular oceanic system, running between 50°S and 60°S) serve as important effectors of evolution of the Antarctic marine biota. The APF acts as a cold "wall" that hinders mixing of the waters of the Southern Ocean with those of the Indian, Pacific and Atlantic Oceans and limits migration of the fauna of the temperate ocean to the south, and vice versa (Coppes and Somero, 2007). Because of this, the APF acts as a barrier for gene flow, causing evolutionary processes to occur in isolation.

Diversification of the major group of Antarctic fishes of the perciform suborder Notothenioidei, largely confined within Antarctic and sub-Antarctic waters, has occurred in parallel with the climatic changes. This suborder comprises eight families and 122 species. Five families and 96 species are Antarctic, whereas three families and 26 species are non-Antarctic (Eastman, 2005). Among the five notothenioid families, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae, 15 species occur along the cool-temperate southern coast of South America and New Zealand (Eastman and Eakin, 2000). These cold-temperate species encounter water temperatures of approximately 5–15 °C. Three small basal families, 10 of 11 species in the family Bovichtidae, monotypic Pseudaphritidae and Eleginopidae are non-Antarctic and presumably diverged during the Eocene and became established in waters around areas corresponding to New Zealand, Australia and high-latitude South America. They essentially never experienced near-freezing water temperatures, and absence of detectable antifreeze-glycoprotein (AFGP) coding sequence in Bovichtidae, Pseudaphritidae and Eleginopidae supports this scenario for the basal notothenioid lineages (Cheng et al., 2003).

To understand the unique biochemical features of high-Antarctic notothenioids, it is important to enrich our knowledge of these highly cold-adapted stenotherms with new information on their sub-Antarctic relatives.

The survival of polar fish depends on the presence of AFGPs because they avoid freezing by binding water to AFGPs, thus preventing growth of ice crystals in their blood and other body fluids (DeVries, 1988; Cheng and DeVries, 1991). The occurrence of AFGPs in non-Antarctic notothenioids from South America and New Zealand waters has been examined in several studies (Cheng et al., 2003; Cheng and Detrich, 2007). The copy numbers of AFGP genes and the numbers of AFGPs reflect the harshness of the freezing threat that Antarctic notothenioids have to face, in comparison with temperate notothenioids, which reached temperate waters before the production of the high levels of AFGPs found in current Antarctic notothenioids (Cheng et al., 2003).

In *Notothenia angustata* and *Notothenia microlepidota*, living in cool-temperate waters, the AFGP system is reduced, with very low blood AFGP concentration and only two to three genes showing some replacements in the functional repeat Thr–Ala–Ala (Cheng et al., 2003). *Dissostichus eleginoides*, a non-Antarctic notothenioid of the family Nototheniidae, appears to have no functional AFGP sequences, consistent with its non-Antarctic distribution. The hypothesis is that this species had the primordial AFGP genotype, lost or mutated following its migration to non-Antarctic habitats (Cheng et al., 2003).

Another remarkable example of adaptation to extreme cold found in Antarctic notothenioid fishes is the apparent loss of inducible heat-shock response (HSR) at constantly cold temperatures (Hofmann et al., 2000). Interesting results showed that *Bovichtus variegatus* (family Bovichtidae) expresses heat-shock proteins (hsp) in response to heat stress, whereas *N. angustata,* a non-Antarctic notothenioid living in New Zealand waters, does not display the stress-inducible hsp synthesis at the protein-level (Hofmann et al., 2005). These results suggest that HSR, the up-regulation of heat-stress sensitive genes, was lost after evolution in the subzero, stenothermic environment of Antarctic waters during the divergence of Bovichtidae from the other Antarctic notothenioid families.

Red-blooded high-Antarctic notothenioid families have evolved a suite of physiological and molecular adaptations, accompanied by a decrease in Hb oxygen affinity and concentration (Verde et al., 2006b). Hb-less Channichthyidae represent the extreme of this trend (Ruud, 1954).

This paper provides preliminary data on the oxygen-transport system of two non-Antarctic species, *Eleginops maclovinus* and *B. diacanthus*.

E. maclovinus, locally known as mullet or róbalo, is a notothenioid belonging to the family Eleginopidae and is the only species of its genus. It is an important component of the ichthyofauna in the coastal temperate and sub-Antarctic waters of South

America; it is also found in coastal waters around the Falkland Islands (Falkland Islands Government, 2003; Brickle et al., 2005). *E. maclovinus* is the sister group of Antarctic notothenioids that dominate the cold shelf waters of Antarctica and it is of interest to understand notothenioid diversification representing the "starting point" for the notothenioid radiation (Eastman and Lannoo, 2008). The status of *Eleginops* as sister group of Antarctic notothenioids is supported by phylogenetic analyses employing both morphological (Balushkin, 2000) and molecular data, including partial (Bargelloni et al., 2000) and complete 16S rRNA (Near et al., 2004) mtDNA gene sequences.

South Atlantic *B. diacanthus*, belonging to the phyletically basal family Bovichtidae, is the klipfish from Tristan da Cunha (37°S) and lives near the northern limit for notothenioids (Eastman, 1993). Here this species experiences mean monthly temperatures of 13–19 °C, and can tolerate summer tide-pool temperatures up to 27.4 °C (Andrew et al., 1995).

2. Materials and methods

2.1. Collection of specimens

Specimens of *E. maclovinus* and *B. diacanthus* were collected during ICEFISH 2004. Adult *E. maclovinus* was collected near the Falkland Islands and adult *B. diacanthus* in the temperate waters of Tristan da Cunha. Blood was taken by heparinised syringes from the caudal vein. Hemolysates were prepared as described previously (D'Avino and di Prisco, 1988). Saline-washed erythrocytes were frozen at −80 °C until use.

2.2. Purification of Hbs and oxygen binding

Separation of *E. maclovinus* Hbs was achieved by FPLC anion-exchange chromatography on a Mono Q-Tricorn column (1.0 × 10 cm), equilibrated with 20 mM Tris–HCl pH 7.6. Purification of *B. diacanthus* Hbs was achieved by chromatofocusing with Polybuffer Exchangers (PBE) 94 (Amersham Biosciences), equilibrated with 25 mM Tris-acetate pH 8.3. All steps were carried out at 0–5 °C. For oxygen binding, aliquots of CO-Hbs were stored at −80 °C. For each experiment, an aliquot was thawed, converted to the oxy form by exposure to light and oxygen, and immediately used; no oxidation was spectrophotometrically detectable, indicating that final Met-Hb formation was negligible (<2%).

2.3. Amino-acid sequencing

Globins of *E. maclovinus* and *B. diacanthus* were purified by reverse-phase HPLC on C_4 Vydac (4.6 × 250 mm) columns. Before loading, samples were incubated in a denaturating solution of 5% β-mercaptoethanol and 1% TFA at room temperature. Fractionation of tryptic peptides and subsequent amino-acid sequencing were carried out as previously described (Verde et al., 2002). Nucleotide sequences were established by cloning and sequence analysis of globin cDNAs:

a) *B. diacanthus*: total RNA was isolated from the spleen using RNA easy Extraction (Qiagen). First-strand cDNA synthesis was performed according to the manufacturer's instructions (BioLabs New England) using an oligo(dT)-adaptor primer. The β-globin cDNAs were amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Primer sequences are available from the authors upon request. Amplifications were performed with 25 μl Hot StarTaq Master Mix Qiagen (2.5 units Taq Polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP), 5 pmol each of the above primers. The PCR programme consisted of 30 cycles of 1 min at 94 °C, 1 min at temperatures between 42 ÷ 54 °C, 1 min at 72 °C, ending with a single cycle of 10 min at 72 °C. The N-terminal regions of *B. diacanthus* Hbs were obtained by amino-acid sequencing. Amplified cDNA was purified and ligated in the pGEM-T Easy vector (Promega). *Escherichia coli* (strain JM109) was transformed with the ligation mixtures. Standard molecular-biology techniques (Sambrook et al., 1989) were used in the isolation, restriction and sequence analysis of plasmid DNA;
b) *E. maclovinus*: total RNA was isolated from the spleen using TRI Reagent (Sigma). First-strand cDNA synthesis was performed according to the manufacturer's instructions (Promega) using an oligo(dT)-adaptor primer. The β-globin cDNA was amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Primer sequences are available from the authors upon request. Amplifications were performed with 2.5 units EuroTaq (EuroClone Genomics), 1X Reaction Buffer, 3 mM $MgCl_2$, 200 mM of each dNTP, Reverse primer 0.6 μM, Forward primer 1.2 μM. The PCR programme consisted of 40 cycles of

1 min at 94 °C, 1 min at 38 °C, 1 min at 72 °C, ending with a single cycle of 10 min at 72 °C. The N-terminal regions of *E. maclovinus* Hbs were obtained by amino-acid sequencing. Amplified cDNA was purified and ligated in the pDrive Cloning Vector (Qiagen). *E. coli* (strain TOP 10) was transformed with the ligation mixtures. Standard molecular-biology techniques (Sambrook et al., 1989) were used in the isolation, restriction and sequence analysis of plasmid DNA.

2.4. Oxygen affinity and Root effect

Hemolysate stripping was performed as described previously (Tamburrini et al., 1994). Oxygen equilibria were measured in 100 mM Hepes/Mes in the pH range 6.25–8.4 at 5 °C, at a final Hb concentration of 0.5–1.0 mM on a heme basis. Experiments were performed in duplicate, a standard deviation of ±3% for p_{50} values was calculated. In order to achieve stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (Weber et al., 1987). Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP were measured at a final ligand concentration of 3 mM, a large excess over the tetrameric Hb concentration. Oxygen affinity (measured as p_{50}) and cooperativity (n_{Hill}) were calculated from the linearised Hill plot of log $S/(1 - S)$ against log pO_2 at half saturation, where S is the fractional oxygen saturation. The Root effect was determined in 100 mM Hepes/Mes in the pH range 6.25–8.4 at room temperature by calculating the mean absorbance difference at three wavelengths (540, 560 and 575 nm) between the spectra at pH 8.4 (fully oxygenated Hb) and pH 6.25, and the spectra after deoxygenation using sodium dithionite.

2.5. Phylogenetic analysis

Multiple alignments of α and β globin amino-acid sequences were performed with the program CLUSTAL X (Thompson et al., 1997). The sequence of *Latimeria chalumnae* was included in the analysis as out-group. Phylogenetic trees of globin sequences were inferred using the neighbour joining (NJ) method implemented in the program MEGA 3 (Kumar et al., 2004). The genetic distances were measured according to the *p*-distance model. Robustness of the NJ trees was assessed by bootstrap analysis with 10,000 replications.

3. Results and discussion

3.1. Purification of Hbs: the Hb multiplicity

Unlike most Antarctic notothenioids, the blood of *E. maclovinus* and *B. diacanthus* displays high Hb multiplicity.

The electrophoretic pattern of the hemolysate of *E. maclovinus* shows the presence of one cathodal (Hb C) and two anodal components (Hb 1 and Hb 2). Hb 1 and Hb 2 account for approximately 65–70% and 5% of the total, respectively (data not shown); Hb C, always found in Antarctic notothenioid species in trace amounts (i.e. less than 1%), was 20–25% of the total in *E. maclovinus*, similar to the high-Antarctic notothenioid *Trematomus newnesi* (D'Avino et al., 1994). Purification of *E. maclovinus* Hbs was achieved by ion-exchange chromatography, showing three components, Hb C, Hb 1, and Hb 2 (data not shown).

The globins of the hemolysate of *E. maclovinus* were separated by reverse-phase HPLC (data not shown). The elution profile indicates four globins, two α chains (α^1 and α^2) and two β chains (β^1 and β^2) as established by amino-acid sequencing and mass spectrometry. The chains composition of Hb 1 is $(\alpha^1\beta^1)_2$. Hb C and Hb 2 have the α chain and β chain, respectively, in common with Hb 1.

High multiplicity was also observed in *B. diacanthus*, one of the most northern notothenioids. The chromatofocusing of the hemolysate showed three major Hbs. The elution profile of the hemolysate of *B. diacanthus*, by reverse-phase HPLC, indicated four globins (data not shown), two α chains (indicated as α^1 and α^2, respectively) and two β chains (β^1 and β^2).

Fish commonly exhibit pronounced Hb multiplicity with marked differences in the oxygen binding properties and in their sensitivities to allosteric effectors, a feature that may serve to adapt oxygen transport to environmental variations and metabolic requirements (Weber, 1990; di Prisco and Tamburrini, 1992; Feuerlein and Weber, 1994; Fago et al., 2002; Weber et al., 2000). Hb multiplicity is usually interpreted as a sign of phylogenetic diversification and molecular adaptation resulting from gene-related heterogeneity and gene duplication events, probably maintained as a response to temperature differences and fluctuations of temperate waters, much larger than in the Antarctic. This may explain why high-Antarctic notothenioids have a single major Hb, whilst sub-Antarctic notothenioids, such as *E. maclovinus* and *B. diacanthus*, retained Hb multiplicity, presumably to cope with the small or large temperature changes in the respective

habitats north of the Polar Front (di Prisco et al., 2007). These properties may reflect the dynamic life history of fish and the different environmental conditions that species encounter. Similar to the other sub-Antarctic notothenioids, *Cottoperca gobio* (family Bovichtidae) displays Hb multiplicity, with two major Hbs in the hemolysate, probably required to cope with environmental variability (Giordano et al., 2006, 2009). In contrast, *Pseudaphritis urvillii*, similar to most Antarctic notothenioids, has a single major Hb and a minor component. The low amount of minor Hb can be considered a synapomorphy, connecting *P. urvillii* to the other notothenioids (Verde et al., 2004).

3.2. Primary structure and amino-acid-sequence identities

The amino-acid sequences of α^1, β^1 and β^2 of *E. maclovinus* Hb 1 and Hb C were deduced by sequencing peptides produced by trypsin digestion and the fragments generated after cleavage of the single Asp–Pro bond (D'Avino et al., 1994) (data not shown). The N termini of the α chains are not available to Edman degradation due to the presence of an acetyl group, similar to all teleost Hbs. The C terminus of β^2 was deduced from the sequence of cDNA. The amino-acid sequences of the α and β chains of *B. diacanthus* Hb 1, Hb 2 and Hb 3 were established by alignment of tryptic peptides for α^1 and α^2, and on the basis of nucleotide sequences, using primers designed on sequence stretches of β^1 and β^2 (data not shown).

Table 1 is an overview of sequence identities of α and β chains of notothenioids, Arctic and temperate fish Hbs. In notothenioids, the identity among the major components (Hb 1) is very high (right-hand). The minor components Hb 2 and Hb C usually have the β and α chain in common, respectively, with Hb 1. The chains which are not in common have low identity with Hbs of temperate non-notothenioids (as expected), but also with Hb 1 of all notothenioids. On the other hand, the identity with one another is high (left-hand). In many non-Antarctic notothenioids, the globin sequences have high similarity with major Hbs of Antarctic notothenioids. The sequence identity of the α chains of *E. maclovinus* Hb 1 and Hb C and of the α chains of *B. diacanthus* reaches values of 70–80% compared with major Hbs of Antarctic notothenioids.

Although non-Antarctic notothenioids have never developed cold adaptation, the amino-acid sequence reveals high identity with the globins of Antarctic notothenioids. This argues in favor of a common phylogenetic origin within notothenioids and suggests that the primary structure of the Antarctic Hbs have undergone modifications only to a limited extent. Unlike changes in amino-acid sequence that occurs at a much slower overall rate, other modifications in hematological features of Notothenioidei, such as Hb multiplicity, might be considered a short-term response to environmental changes. Short-term responses, such as regulatory processes or enhanced protein synthesis, may well be additional mechanisms of temperature compensation and represent phenotypic-plasticity response to environmental changes. When faced with new selection pressures, organisms can basically respond in several ways; one of these may be to adjust to the changed conditions (i) by means of phenotypic plasticity without altering their genetic constitution, or (ii) by genetic changes through the process of evolution occurring in the long run (Holt, 1990; Davis et al., 2005).

3.3. Oxygen affinity and Root effect

The oxygen affinity of Hbs of many high-Antarctic species is quite low (di Prisco et al., 2007), as indicated by the values of p_{50} (the oxygen partial pressure required to achieve half saturation). In contrast, the affinity in Hbs of the non-Antarctic notothenioids *C. gobio*, *B. diacanthus*, *P. urvillii* and *E. maclovinus* is higher. The relationship between oxygen affinity of notothenioid Hbs and habitat features is still not well understood. Spectroscopic and modelling studies on *P. urvillii* Hb 1 have shown that all the non-conservative replacements in the primary structure of α and β chains leave the conformation and electrostatic field surrounding the heme pocket essentially unmodified (Verde et al., 2004) with respect to Hb 1 of the high-Antarctic *Trematomus bernacchii* (Ito et al., 1995; Mazzarella et al., 2006b). Spectroscopic studies have demonstrated that the heme pocket of *E. maclovinus* Hb 1 is similar to that of *P. urvillii* Hb 1 (G. Smulevich, personal communication).

In many Hbs of teleost fishes, the complete loss of cooperativity (indicated by a Hill coefficient equal to one) and the inability to saturate the ligand sites at low pH, even at high oxygen pressure, is a distinctive property with respect to the Bohr effect. This feature is known as the Root effect (reviewed by Brittain, 1987, 2005).

A general reduction in the Root effect, is noticed during the evolution of the Antarctic notothenioids (di Prisco et al., 2007) corresponding to a variable scenario pertaining to the choroid *rete*. The physiological role of the Root effect is to secrete oxygen

Table 1

Sequence identity (%) of α (A) and β (B) chains of Hbs of notothenioids, Arctic and temperate fish. The abbreviations on top of the columns (*Ca*, *Ee*, etc) from left to right, correspond to the species from bottom to top. Adapted from Verde et al. (2008). Non-Antarctic notothenioids are highlighted in grey. The list of names of species are in Table 2, with the exception of *Gadus morhua* (accession numbers: α^2 O41425; β^2 P84611), *Thunnus thynnus* (accession numbers: α P11748; β P11749) and *Boreogadus saida* (accession number: β^1 P84607).

A. α chains

Identity (%)

Species	*Ccl*	*Ca*	*Ee*	*Hl*	*Ss*	*Aa*	*Aa*	*Cc*	*Tt*	*Om*	*Om*	*Gm*	*Amin*	*Amin*	*Tn*	*Nc*	*Ck*	*Rg*	*Ps*	*Ga*	*Bm*	*Ao*	*Amyt*	*Cm*	*Pu*	*Cg*	*Cg*	*Na*	*Nc*	*Bd*	*Bd*	*Em*	*Tn*
Hemoglobin/Globin				C		A	C			IV	I	2	1	2, 3	2	2								1, 2	1, 2	2	1	1	1	a^2	a^1	1, C	1, C
T. bernacchii Hb 1, C	59	64	59	56	54	54	52	63	76	61	54	59	66	74	62	66	69	92	88	90	90	87	90	91	91	64	83	96	89	80	66	88	97
T. newnesi Hb 1, C	58	62	57	55	63	53	52	61	76	61	53	60	64	72	63	66	58	90	85	91	88	85	90	90	76	61	80	93	87	78	64	86	
E. maclovinus Hb 1, C	61	66	61	59	55	57	54	67	76	60	57	62	68	74	86	69	71	85	84	83	85	84	85	84	82	66	83	88	81	84	70		
B. diacanthus α^1	60	66	62	63	62	57	63	68	67	62	62	65	82	68	82	86	66	68	66	64	68	66	65	66	66	88	66	65	62	69			
B. diacanthus α^2	60	64	64	58	60	53	56	64	76	57	61	61	71	77	65	67	72	77	76	75	79	76	76	76	79	67	87	78	73				
N. coriiceps Hb 1	55	61	54	53	51	50	51	59	71	57	52	56	62	68	61	62	62	86	83	81	83	83	83	83	71	61	74	90					
N. angustata Hb 1	59	66	60	56	57	54	53	64	76	61	56	59	67	75	64	66	69	93	90	88	92	90	91	89	77	64	83						
C. gobio Hb 1	60	64	63	61	61	54	54	66	76	57	61	58	71	81	64	66	73	79	78	78	80	77	79	78	79	66							
C. gobio Hb 2	57	66	64	64	64	57	64	66	67	61	63	64	81	69	85	87	66	66	66	63	67	64	61	64	66								
P. urvillii Hb 1, 2	57	63	62	61	58	54	57	61	76	58	59	57	66	76	63	65	70	76	75	75	76	74	72	77									
C. mawsoni Hb 1, 2	58	63	57	56	54	53	52	63	76	61	55	61	64	72	64	69	69	88	85	92	88	84	84										
A. mitopteryx	58	61	56	53	53	53	50	61	73	58	53	61	64	70	64	63	67	88	84	85	85	84											
A. orianae	61	66	62	58	58	56	53	66	73	59	59	58	66	73	64	66	68	90	96	81	89												
B. marri	59	65	61	57	59	54	55	64	78	63	60	62	68	74	68	71	68	94	90	86													
G. acuticeps	57	61	55	54	54	52	52	61	74	61	54	59	64	71	65	66	66	86	82														
P. scotti	61	66	63	58	58	54	54	66	73	59	59	58	68	73	64	66	69	90															
R. glacialis	59	67	60	57	59	57	54	65	76	61	59	60	67	73	65	67	68																
C. kumu	64	70	63	58	59	57	55	71	69	57	59	61	67	71	63	66																	
N. coriiceps Hb 2	56	65	61	60	61	56	61	65	66	63	61	66	80	68	93																		
T. newnesi Hb 2	55	64	59	61	63	57	61	64	64	61	62	66	57	66																			
A. minor Hb 2, 3	61	69	64	63	62	57	56	67	73	59	61	58	72																				
A. minor Hb 1	59	66	64	67	69	57	64	66	68	61	67	65																					
G. morhua Hb 2	59	64	61	56	58	52	55	64	61	62	57																						
O. mykiss Hb I	64	67	67	71	91	56	68	66	61	60																							
O. mykiss Hb IV	63	68	65	58	63	60	54	66	58																								
T. thynnus	61	65	61	62	59	54	54	64																									
C. carpio	81	90	73	64	66	67	62																										
A. anguilla Hb C	54	64	59	68	69	54																											
A. anguilla Hb A	61	66	61	56	57																												
S. salar	62	67	69	73																													
H. littorale Hb C	58	63	63																														
E. electricus	66	76																															
C. auratus	76																																
C. clarkii																																	

B. β chains

Identity (%)

Species	*Ca*	*Ee*	*Hl*	*Aa*	*Aa*	*Cc*	*Tt*	*Om*	*Om*	*Bs*	*Gm*	*Amin*	*Amin*	*Tb*	*Tn*	*Pu*	*Cm*	*Ck*	*Rg*	*Ps*	*Ga*	*Bm*	*Ao*	*Amyt*	*Pu*	*Cm*	*Cg*	*Na*	*Nc*	*Bd*	*Bd*	*Em*	*Em*	*Tn*
Hemoglobin/Globin			C	A	C			IV	I	1, 2	2, 3	3	1, 2	C	C	2	2								1	1	1, 2	1, 2	1, 2	β^2	β^1	C	1	1, 2
T. bernacchii Hb 1	63	59	56	55	60	60	67	65	56	60	71	76	75	69	69	69	66	67	84	78	82	86	80	85	81	88	71	91	88	73	82	71	81	93
T. newnesi Hb 1, 2	59	56	54	55	57	56	65	60	53	57	70	72	75	67	67	67	64	63	82	77	80	84	78	82	77	84	69	84	84	71	80	69	76	
E. maclovinus Hb 1	65	63	54	56	64	63	68	64	61	63	71	75	81	72	71	71	67	66	77	73	76	77	76	78	82	78	73	82	80	72	83	70		
E. maclovinus Hb C	62	53	53	57	58	59	61	63	54	64	71	80	64	90	89	86	82	78	66	64	65	67	67	67	67	67	84	71	71	84	67			
B. diacanthus β^1	62	60	53	57	64	61	67	63	59	61	70	71	75	70	69	69	67	65	76	75	72	77	76	74	83	77	71	80	79	72				
B. diacanthus β^2	63	57	54	57	63	60	64	67	57	66	71	86	69	86	86	90	83	78	67	69	68	69	71	70	71	69	88	73	72					
N. coriiceps Hb 1, 2	58	56	56	54	60	57	66	63	53	58	69	75	78	70	70	69	65	68	86	82	80	89	82	81	78	87	72	93						
N. angustata Hb 1, 2	60	57	58	54	60	58	65	63	55	60	71	76	78	70	70	70	65	69	87	82	80	89	83	84	80	87	72							
C. gobio Hb 1, 2	62	57	54	59	63	59	65	66	60	65	71	84	71	86	85	89	82	77	68	66	68	70	68	71	69	68								
C. mawsoni Hb 1	58	56	53	54	59	55	66	61	52	58	70	73	75	68	68	67	67	63	89	83	84	87	82	80	76									
P. urvillii Hb 1	64	59	54	56	60	62	69	65	59	64	71	71	77	69	68	68	65	65	76	72	74	76	73	76										
A. mitopteryx	57	55	56	54	59	56	67	59	53	58	68	71	78	66	67	66	62	63	79	75	78	79	76											
A. orianae	58	56	55	53	58	57	65	61	52	60	69	71	74	67	67	67	63	65	80	90	77	81												
B. marri	58	54	55	54	59	56	65	60	53	59	67	73	76	67	67	67	65	67	87	78	84													
G. acuticeps	58	56	53	54	58	56	63	58	54	61	65	70	71	66	67	65	65	63	79	78														
P. scotti	57	57	54	52	57	56	63	60	50	60	67	69	73	65	65	65	62	61	80															
R. glacialis	56	54	54	53	56	54	65	60	50	57	68	71	75	66	65	66	61	63																
C. kumu	59	52	54	55	55	58	59	60	52	60	69	79	66	76	75	80	73																	
C. mawsoni Hb 2	57	54	48	56	60	54	60	59	54	58	67	78	71	89	89	84																		
P. urvillii Hb 2	60	53	51	56	60	57	63	63	52	64	71	84	68	88	86																			
T. newnesi Hb C	60	54	52	56	60	58	60	61	56	61	68	81	66	95																				
T. bernacchii Hb C	61	54	52	56	58	58	62	63	54	62	70	82	75																					
A. minor Hb 1, 2	63	58	57	56	59	60	71	63	58	60	71	73																						
A. minor Hb 3	63	56	54	57	58	61	65	64	58	65	72																							
G. morhua Hb 2, 3	63	56	52	58	58	60	69	63	54	62																								
B. saida Hb 1, 2	64	56	58	59	61	64	58	61	59																									
O. mykiss Hb I	63	50	55	53	66	63	58	58																										
O. mykiss Hb IV	78	70	58	72	60	72	62																											
T. thynnus	61	54	54	54	60	60																												
C. carpio	91	68	60	66	59																													
A. anguilla Hb C	64	63	69	56																														
A. anguilla Hb A	71	59	50																															
H. littorale Hb C	62	59																																
E. electricus	72																																	
C. auratus																																		

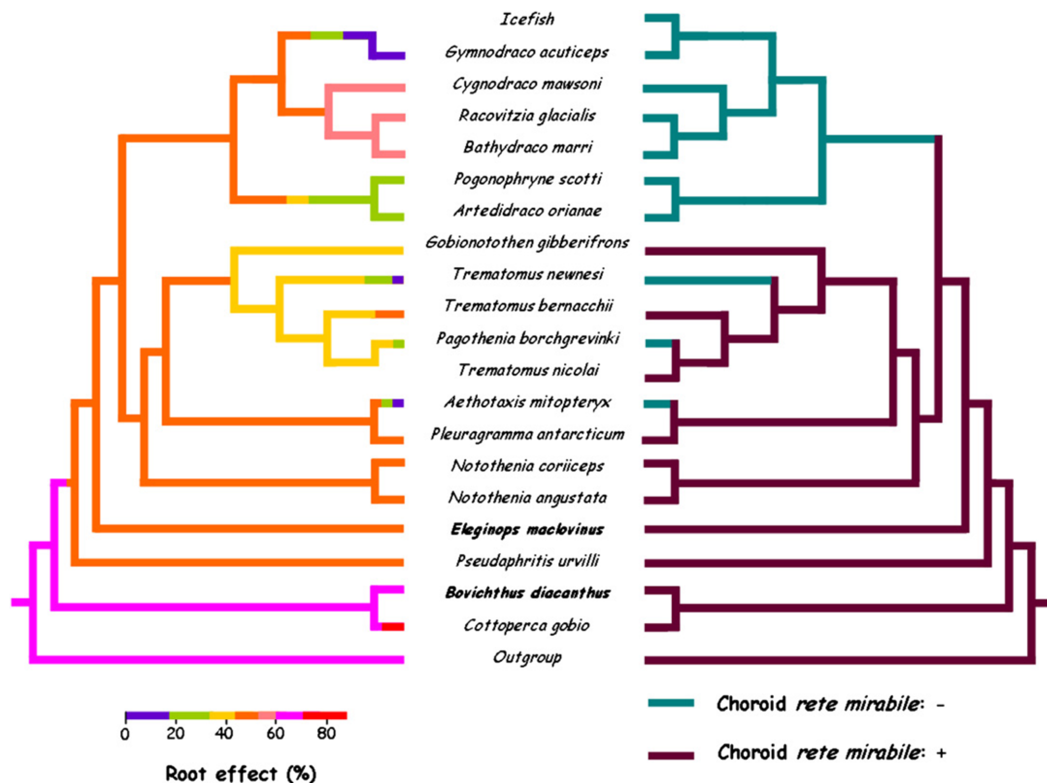

Fig. 1. Reconstruction of the evolutionary history of the Root effect. Adapted from Verde et al. (2008).

against high oxygen pressures into the swimbladder (when present) and the choroid *rete* (Wittenberg and Wittenberg, 1961; Wittenberg et al., 1964). It is likely that the eye choroid *rete* represents the most ancient anatomical structure associated with the presence of Root-effect Hbs (Wittenberg and Haedrich, 1974).

Antarctic fish lack the swimbladder. Among high-Antarctic notothenioids, many species have lost the choroid *rete*, although several retain portions of the *rete* and/or small vestigia of the choriocapillaris (Eastman, 1988, 1993, 2006). Fig. 1 shows the presence of the choroid *rete* and Root effect in Nototenioidei. The comparison of two phylogenetically related groups of non-Antarctic and high-Antarctic species shows that the choroid *rete* is very well developed in sub-Antarctic notothenioids *E. maclovinus*, *P. urvillii*, *C. gobio* and *B. diacanthus*, which are the most basal of the suborder. The Root effect drops to low values in the Artedidraconidae lineage, as well as in one Bathydraconidae (*Gymnodraco acuticeps*), as expected, but it is found at unexpectedly high levels in two species of the latter family (di Prisco et al., 2007). Hbs of sub-Antarctic notothenioids of the most basal notothenioid family are characterised by high Root effect (Fig. 1).

Since the identity among the major components (Hb 1) is very high in notothenioids (Table 1), some general structural explanations of the heterotropic proton regulation of oxygen affinity observed in *B. diachantus* and *E. maclovinus* major Hbs can be proposed. Indeed, the high sequence similarity suggests that some structural properties of these sub-Antarctic Hbs can be predicted according to the crystals structures available for high-Antarctic Hbs. A partial list of the available X-ray crystal structures of Antarctic fish Hbs is reported in a recent review (Verde et al., 2008). These structural studies strongly suggest that the key region responsible for pH modulation of oxygen affinity and cooperativity includes the aspartic triad at the $\alpha_1\beta_2$ interface (Mazzarella et al., 2006a, 2006b) and the CD corner of the α chain (Mazzarella et al., 2006b; Vergara et al., 2009). At the $\alpha_1\beta_2$ interface, two protons per tetramer are released upon oxygenation due to the breakage of an inter-Asp hydrogen bond between Asp95α and Asp101β (Ito et al., 1995; Mazzarella et al., 2006a, 2006b). This same structural evidence is also common to tuna fish (Yokoyama et al., 2004). At the CDα, the typical switch region in tetrameric Hbs (Baldwin and Chothia, 1979), an important order–disorder transition takes place upon pH increase due to deprotonation of His55α.

Table 2
Species and globin sequences investigated. Adapted from Giordano et al. (2006).

Species	Family	Subunit	Accession number/reference
Eleginops maclovinus[b]	Eleginopidae	Major α, β (Hb 1)	Unpublished
		Minor α (Hb C), β (Hb 2)	Unpublished
Bovichtus diacanthus[b]	Bovichtidae	α, β	Unpublished
Latimeria chalumnae[d]	Coelacanthidae	α, β	P23740, P23741
Chelidonichthys kumu[d]	Trigilidae	α, β	P80270, P80271
Anarhichas minor[c]	Anarhichadidae	α (Hb 1), α (Hb 2, Hb 3)	P83270, P83271
		β (Hb 1, Hb 2), β (Hb 3)	P83272, P83273
Chrysophrys auratus[d]	Sparidae	α, β (Hb 4)	Stam et al., 1997
Cottoperca gobio[b]	Bovichtidae	α (Hb 1)	P84653
		β (Hb 1)	P84652
Pseudaphritis urvillii[b]	Pseudaphritidae	α (Hb 1, Hb 2)	P83623
		β (Hb 1)	P83624
		β (Hb 2)	P83625
Notothenia coriiceps[a]	Nototheniidae	major α (Hb 1)	P10777
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P16309
Notothenia angustata[b]	Nototheniidae	major α (Hb 1)	P29624
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P29628
Pleuragramma antarcticum[a]	Nototheniidae	α (Hb 1, Hb 2)	Stam et al., 1997
		β (Hb 1, Hb 3)	Stam et al., 1997
		minor α (Hb 3), β (Hb 2)	Stam et al., 1997
Pagothenia borchgrevinki[a]	Nototheniidae	α (Hb 1, Hb 0)	P82344
		major β (Hb 1)	P82346
		minor β (Hb 0)	P83245
Gobionotothen gibberifrons[a]	Nototheniidae	major α, β (Hb 1)	P83611, P83612
		minor α, β (Hb 2)	P83613, P83614
Aethotaxis mitopteryx[a]	Nototheniidae	α, β	Stam et al., 1997
Trematomus newnesi[a]	Nototheniidae	major α, β (Hb 1)	P45718, P45720
		minor α (Hb 2), β (Hb C)	P45719, P45721
Trematomus bernacchii[a]	Nototheniidae	major α, β (Hb 1)	P80043, P80044
		minor β (Hb C)	P45722
Cygnodraco mawsoni[a]	Bathydraconidae	α (Hb 1, Hb 2)	P23016
		major β (Hb 1)	P23017
		minor β (Hb 2)	P23018
Gymnodraco acuticeps[a]	Bathydraconidae	α, β	P29623, P29625
Racovitzia glacialis[a]	Bathydraconidae	α, β	Tamburrini et al., unpublished
Bathydraco marri[a]	Bathydraconidae	α, β	Stam et al., 1997
Pogonophryne scotti[a]	Artedidraconidae	α, β	Stam et al., 1997
Artedidraco orianae[a]	Artedidraconidae	α, β	Stam et al., 1997
Salmo salar[d]	Salmonidae	α	P11251
Oncorhynchus mykiss[d]	Salmonidae	α, β (Hb I)	P02019, P02142
		α, β (Hb IV)	P14527, P02141
Anguilla anguilla[d]	Anguillidae	α, β (Hb C)	P80726 P80727
		α, β (Hb A)	P80945, P80946
Electrophorus electricus[d]	Electrophoridae	α, β	P14520, P14521
Hoplosternum littorale[d]	Callichthyidae	α, β (Hb C)	P82315, P82316
Cyprinus carpio[d]	Cyprinidae	α, β	P02016, P02139
Carassius auratus[d]	Cyprinidae	α, β	P02018, P02140
Catostomus clarkii[d]	Catostomidae	α	P02017
Oryzias latipes[d]	Adrianichthyidae		Maruyama et al., 2004

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

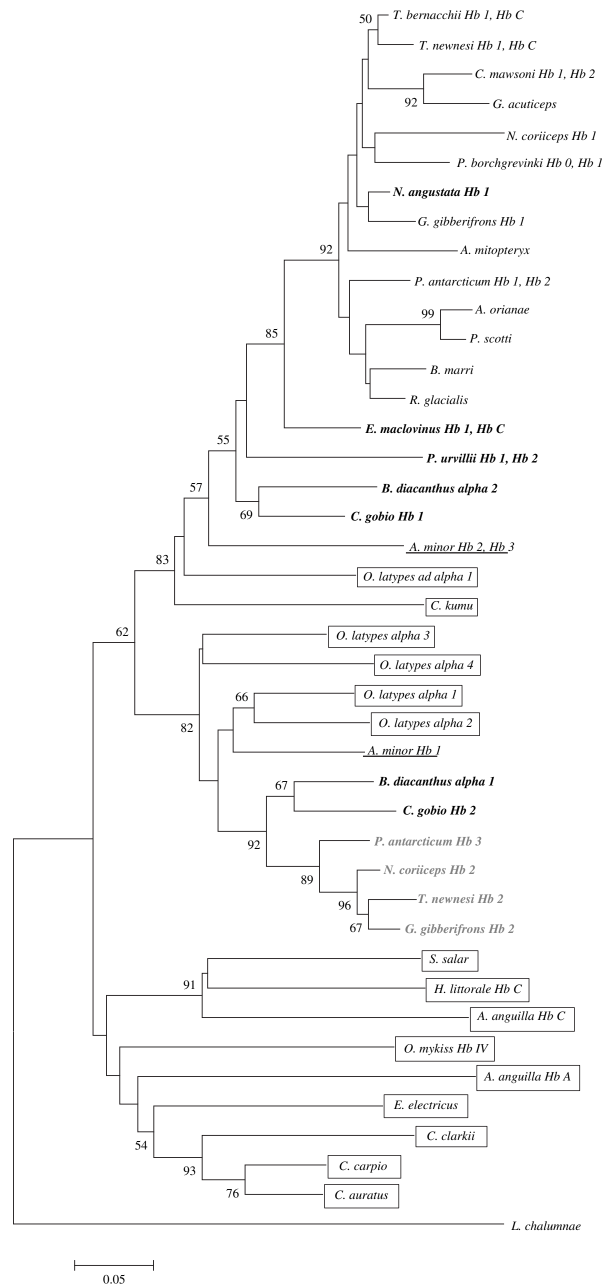

Fig. 2. Phylogenetic tree of amino-acid sequences of α chains of Arctic, Antarctic and temperate fish Hbs. Bootstrap values (percentage of 10,000 replicates) are given at the nodes. Notothenioid major globins are in black, notothenioid minor globins in grey, non-Antarctic notothenioid globins in bold, Arctic globins underlined, and temperate globins in box.

A punctual structural explanation of the higher affinity herein reported in sub-Antarctic Hbs than in high-Antarctic Hbs requires the establishment of the crystal structure of the former in its ligated and un-ligated forms. This study is in progress in *E. maclovicnus* Hb 1.

Interestingly, high-Antarctic Hbs exhibit at least three stable quaternary structures. Indeed, the classical relaxed state (R) (Camardella et al., 1992; Mazzarella et al., 1999), the tense (T) (Mazzarella et al., 2006a, 2006b) and an R/T intermediate quaternary structure [typical at least of the ferric (Riccio et al., 2002; Vergara et al., 2007) and partially oxidised states (Merlino et al., 2009; Vitagliano et al., 2008)] have been observed. Currently, the R/T quaternary structure is considered associated to ferric or partially oxidised states, though it cannot be excluded that the ferrous

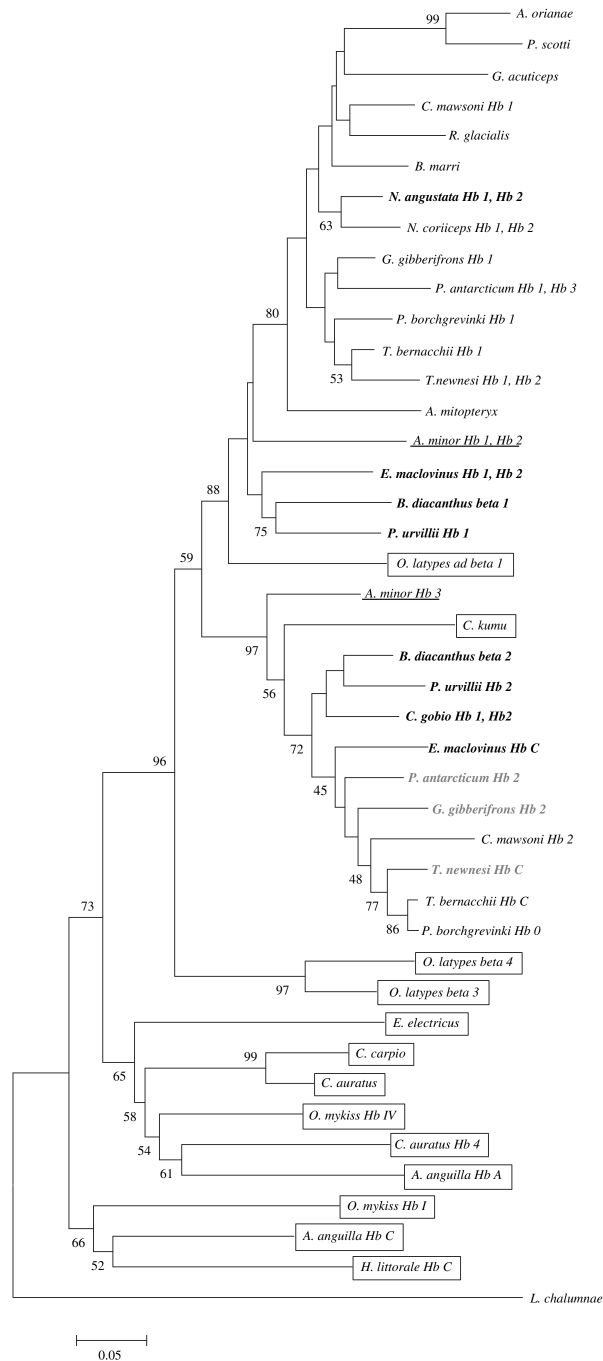

Fig. 3. Phylogenetic tree of amino-acid sequences of β chains of Arctic, Antarctic and temperate fish Hbs. Bootstrap values (percentage of 10,000 replicates) are given at the nodes. Notothenioid major globins are in black, notothenioid minor globins in grey, non-Antarctic notothenioid globins in bold, Arctic globins underlined, and temperate globins in box.

state may adopt this structure. Antarctic fish Hbs have an heterogeneous ferric state with α-heme in the aquomet state, and with β-heme in a bis-histidyl (hemichrome) state (Riccio et al., 2002; Vergara et al., 2007). It is an open question whether the accessibility to these multiple structural states are only a peculiarity of Antarctic fish Hbs or it extends to sub-Antarctic fish Hbs. Sequence similarity between the Hbs of *P. urvillii* and *C. gobio*, where hemichrome forms (Verde et al., 2004; Giordano et al., 2009), suggests that the

formation of the bis-histidyl adduct should be expected also in sub-Antarctic fish Hbs.

3.4. Molecular phylogeny

Table 2 reports the list of the species examined in this study and the accession numbers of α-globin and β-globin sequences used in the phylogenetic analysis. The sequences not available in data banks are indicated by references.

Two phylogenetic trees, one for α globins (Fig. 2) and one for β globins (Fig. 3) were obtained. Phylogenetic analysis was performed on the multiple alignments constructed with the program CLUSTAL X (Thompson et al., 1997; Verde et al., 2006a). The genetic distances were measured according to the *p*-distance model to evaluate to what extent the resulting tree differs from the expected interrelationships among species in a given cluster of orthologs (i.e. gene copies diversified by speciation). In both trees, anodal and cathodal globins of temperate fish Hbs form the first divergence lineage; the globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups. As a result of the isolation of Antarctica, the genotype of Notothenioidei diverged with respect to other fish groups in a way interpreted as typical of a species flock (Eastman and McCune, 2000).

The obtained topology is in general agreement with the maximum-likelihood method (Giordano et al., 2006) and the hypothesis of four globin groups (Maruyama et al., 2004), which include α and β globins, belonging to notothenioid minor Hbs, in the "Embryonic Hb Group", and α and β globins (shared by Hb 1 and Hb 2 in most Antarctic notothenioids) of the major notothenioid Hbs in the "Notothenioid Major Adult Hb Group".

The position of the *C. gobio* globins appears congruent with the phylogenetic evidence from nuclear and mitochondrial genes (Bargelloni et al., 2000), suggesting that *C. gobio* is the sister taxon of *P. urvillii*, *E. maclovinus*, and also of Antarctic notothenioids. The basal position of *E. maclovinus* and *B. diacanthus* Hbs is in agreement with the postulated divergence before the appearance of AFGPs. Their basal position in notothenioids is consistent with the hypothesis that Bovichtidae and Eleginopidae diverged after they became established in more temperate waters north of the APF. The α chain of *E. maclovinus*, shared by Hb 1 and Hb C, branches off the clade of major Antarctic Hbs, and the same applies to the β chain of Hb 1. The β chain of *E. maclovinus* Hb C is in a basal position with respect to the clade of Antarctic minor Hbs.

Anarhichas minor, an Arctic fish, is close to the notothenioid clades, according to the teleostean phylogeny (Verde et al., 2002, 2006a).

Acknowledgements

The ICEFISH cruise was supported by the National Science Foundation grant OPP 0132032 to H. William Detrich (Chief Scientist, Northeastern University). This study is financially supported by the Italian National Programme for Antarctic Research (PNRA). It is in the framework of the SCAR programme Evolution and Biodiversity in the Antarctic (EBA). It is partially supported by the Ministero Italiano dell'Università e della Ricerca Scientifica (PRIN 2007SFZXZ7 "Structure, function and evolution of heme proteins from Arctic and Antarctic marine organisms: cold adaptation mechanisms and acquisition of new functions").

The authors thank Sandra Parker and Prof. H William Detrich for their collaboration during the cloning and sequence analysis of *B. diacanthus* globins.

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