A Genomic Fossil Reveals Key Steps in Hemoglobin Loss by the Antarctic Icefishes

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Antarctic icefishes are the only vertebrates that do not have hemoglobin and erythrocytes in their blood. These startling phenotypes are associated in several icefish species with deletions of juvenile and adult globin loci, which in red-blooded teleosts are typically composed of tightly linked pairs of α- and β-globin genes. It is unknown if the loss of hemoglobin expression in icefishes was the direct result of such deletions or if other mutational events compromised globin chain synthesis prior to globin gene loss. In this study, we show that 15 of the 16 icefish species have lost the adult β-globin gene but retain a truncated α-globin pseudogene. Surprisingly, a phylogenetically derived icefish species, Neopagetopsis ionah, possesses a complete, but nonfunctional, adult αβ-globin complex. This cluster contains 2 distinct β-globin pseudogenes whose phylogenetic origins span the entire Antarctic notothenioid radiation, consistent with an origin via introgression. Maximum likelihood ancestral state reconstruction supports a scenario of icefish globin gene evolution that involves a single loss of the transcriptionally active adult αβ-globin cluster prior to the diversification of the extant species in the clade. Through lineage sorting of ancestral polymorphism, 2 types of alleles became fixed in the clade: 1) the α-globin pseudogene of the majority of species and 2) the inactive αβ-globin complex of N. ionah. We conclude that the globin pseudogene complex of N. ionah is a “genomic fossil” that reveals key intermediate steps on the pathway to loss of hemoglobin expression by all icefish species.

Introduction

Possession of red blood cells containing the oxygen transporter hemoglobin is virtually synonymous with vertebrate life. Imagine the surprise of the scientific community when Ruud (1954) published his observation that the blood of Antarctic icefishes was devoid of oxygen-transporting proteins. In his study, Ruud (1954) characterized the blood of the blackfin icefish, Chaenocephalus aceratus, as nearly transparent, lacking erythrocytes and hemoglobin, containing leucocytes at <1% by volume, and iron poor. Noting that the blood of at least 3 other icefishes, Champsocephalus esox, Champsocephalus gunnari, and Pseudocheaenichthys georgianus, is also “colourless,” he suggested that loss of the vertebrate oxygen transporter is a common character of icefishes (Ruud 1958). Indeed, subsequent work has demonstrated that the absence of hemoglobin is a characteristic of all the extant icefish species that diversified ~8.5 MYA (di Prisco et al. 2002; Near 2004). Ruud (1954) surmised that loss of red cells and hemoglobin is possible only in the very cold (−1.9 to +1°C) and oxygen-saturated waters of the Southern Ocean but did not speculate on underlying genetic mechanisms.

Icefishes (Channichthyidae) are a phylogenetically derived clade of the Notothenioidei, a lineage that as a whole represents an adaptive radiation in the frigid waters of the Southern Ocean surrounding Antarctica (Eastman 1993; Near et al. 2003, 2004; Eastman 2005). With the exception of the 16 icefish species, all other notothenioid species possess red blood and express hemoglobin (di Prisco et al. 1991; D’Avino and di Prisco 1997; di Prisco 1998; Verde et al. 2004). As in most other teleosts, the globin loci of the red-blooded notothenioids are organized as αβ-gene pairs that are linked 5′ to 5′, or head-to-head, with regulatory elements located in the intergenic region (Cocca et al. 1995; Lau et al. 2001; Gillelman et al. 2003). Our previous work has shown that the genomes of 2 icefish species, C. aceratus and Chionodraco rastrospinosus, lack most of the adult αβ-globin complex, retaining only a pseudogene that consists of a 3′ fragment of the α-globin gene (Cocca et al. 1995; Zhao et al. 1998).

Did the absence of hemoglobin expression in icefishes occur via a single mutational event prior to the diversification of the extant icefish species that deleted all but a small remnant of the αβ-globin gene cluster or were multiple mutational steps involved? To address these questions, we investigated the evolutionary history of the αβ-globin genes in icefishes through comparative phylogenetic analyses that included all icefish species as well as other species from several lineages of red-blooded, hemoglobin-expressing Antarctic notothenioids. We report here that a derived icefish species, Neopagetopsis ionah, retains a complete, but nonfunctional, adult αβ-globin complex, which we interpret as an intermediate evolutionary event that abrogated globin expression prior to near total globin gene extinction. This genomic fossil must have arisen through interspecific introgression between lineages that span the entire notothenioid radiation.

Materials and Methods

Specimen Collection, DNA Sequencing, and Analysis of α- and β-Globin Genes

Notothenioid specimens were collected through the course of several Antarctic expeditions. Species sampled, tissue collection numbers, and museum voucher catalogue numbers (if available) are shown in table 1. Many of these specimens are identical to those used in a previous analysis of icefish phylogenetic relationships (Near et al. 2003).

Genomic DNA was prepared from testis, spleen, pro-nephric kidney, or muscle by use of DNAzol (Molecular Research Center, Inc., Cincinnati, OH). Globin genes and pseudogenes were amplified by polymerase chain reaction (PCR); reactions contained 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.4 µM of each primer, 3.0 mM MgCl2, 2.5 units of Qiagen Taq DNA
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**NOTE.**—NA, not available.
polymerase, 1× Q solution, 1× Qiagen PCR buffer, and 0.3 μg of genomic DNA. Primer sequences are given in table 2, and primer pairs used to amplify globin genes for each specimen are listed in table 1. Touchdown PCR was performed for 11 cycles using the following parameters: 1) denaturation for 1 min at 94°C; 2) primer annealing for 1 min, ramping temperature from 60°C to 50°C in one-degree increments per cycle; and 3) extension for 1 min at 72°C. Subsequently, 15 cycles of conventional PCR were performed using the following profile: 1) denaturation for 1 min at 94°C, 2) primer annealing for 1 min at 50°C, and 3) extension for 1 min at 72°C. A final extension was performed for 5 min at 72°C. Amplified products were used as templates for automated DNA sequencing (University of Maine DNA Sequencing Facility), either directly or following subcloning.

ClustalX (Thompson et al. 1997) was used to align the DNA sequences with minor adjustments made by eye. All DNA sequences have been submitted to GenBank (table 1). Modeltest 3.0 (Posada and Crandall 1998) was used to estimate likelihood parameters for the α- and β-globin DNA alignments (F81 substitution model with among-site rate variation following a gamma distribution, shape parameter = 0.1948). PAUP* 4.0 (Swoford 2003) was used to construct Neighbor-Joining and maximum parsimony trees with bootstrap analyses including 2,000 pseudoreplicates. We also obtained trees and posterior probabilities for nodes in the analysis of β-globin gene sequences with the computer program MrBayes 3.0 (Ronquist and Huelsenbeck 2003) using 4 × 10^6 Markov chain Monte Carlo generations. We discarded the first 1 × 10^6 generations as “burn-in” trees.

Phylogenetic Relationships of Icefishes

The conclusions drawn in this study rely upon the validity of the reference icefish phylogeny, specifically the placement of N. ionah as a derived species. In a previous study, Near et al. (2003) investigated the phylogenetic relationships of the 16 icefish species using mtDNA gene sequences (the complete 16S large-subunit ribosomal RNA and the complete coding region of reduced nicotinamide adenine dinucleotide dehydrogenase subunit 2 [ND2]) and 58 discrete morphological characters. The total evidence data set was sufficiently robust to reject 2 of 4 previous hypotheses of icefish relationships. Furthermore, 22 characters from external morphology and osteology supported the derived phylogenetic placement of N. ionah (Near et al. 2003).

In the present study, we used these mtDNA gene alignments and maximum parsimony trees (Near et al. 2003) to assess alternative phylogenetic relationships among icefishes, specifically the hypothesis that N. ionah is a basal lineage and represents the sister species of all other icefishes. The best tree that depicted N. ionah as basal in the clad was determined using maximum parsimony constraint searches in PAUP* 4.0 (Swoford 2003). To test the null hypothesis that the published (Near et al. 2003) and constrained trees are equally good explanations of the data, we applied a maximum likelihood Shimodaira–Hasegawa test as implemented in PAUP*. The optimal molecular evolutionary model and parameter values for the mtDNA data set were determined by using Akaike information criteria as executed in the computer program Modeltest 3.0 (Posada and Crandall 1998, 2001).

Ancestral Character State Reconstruction

To estimate ancestral character states of the globin cluster in icefishes and other closely related Antarctic notothenioid clades, we employed a maximum likelihood strategy (Sclhuler et al. 1997; Pagel 1999). The phylogeny presented in figure 1b was rooted with Harpagifer antarcticus (Harpagiferidae) and Dolloidi Draco longedorsalis (Arctidraconidae) and included 5 dragonfish species (Bathydraconidae) that were used in a previous phylogenetic analysis of icefishes (Near et al. 2003). The maximum likelihood branch lengths of this phylogeny were converted to relative divergence times by “calibrating” the basal node to 100 arbitrary time units and using penalized likelihood as executed in the computer software r8s (Sanderson 2002, 2003). The optimal smoothing parameter value (31.62) was determined with the cross-validation analysis available in r8s. The rate-smoothed phylogeny with branch lengths as relative divergence times was used as the model tree in subsequent ancestral state reconstructions.

The computer program Mesquite v. 1.11 (Maddison WP and Maddison DR 2006) was used to implement ancestral state reconstruction and calculation of maximum likelihood scores. One of two character states was scored for all species in the analysis: 1) a complete or nearly complete αβ-globin cluster present or 2) a truncated α-globin pseudogene present. We used a likelihood ratio test with 1 degree of freedom (df) to discriminate between the one-parameter Markov k-state model (Mk1) that is a generalization of the Jukes–Cantor model (Lewis 2001) and the asymmetrical 2-parameter Markov k-state model that has 2 different rates of change for character losses and gains (Pagel 1997; Mooers and Schluter 1999).

Maximum likelihood scores from the reconstructions were also employed to test the hypothesis that the αβ-globin cluster was transformed to the α-globin pseudogene on 4 different occasions in the course of icefish diversification. Using a modified asymmetric 2-parameter model, we assumed that the rate of regaining the αβ-globin cluster after transformation to the α-globin pseudogene was zero
(Kohlsdorf and Wagner forthcoming) in accordance with Dollo’s law of irreversibility (once a complex character is lost, it cannot be regained [Simpson 1953; Gould 1970; Bull and Charnov 1985]). The rate of character loss was adjusted to require 4 losses of the $\alpha$-globin cluster. This procedure constrained several internal nodes in the icefish phylogeny to be reconstructed as possessing the $\alpha\beta$-globin cluster. We compared the likelihood scores of this 4-loss model with the Mk1 model using a likelihood ratio test with df = 5.

Results and Discussion

Status of Icefish $\alpha$- and $\beta$-Globin Genes

Using PCR on genomic DNA extracts, we determined the nucleotide sequences of the $\alpha$-globin pseudogene for all 16 icefish species (table 1). We also sequenced the adult $\alpha\beta$-globin cluster for the hemoglobin-expressing dragonfish, Parachaeoichthys charcoti (Bathydraconidae), a species in the sister lineage of icefishes (Balushkin 2000; Derome et al. 2002; Near et al. 2004). We found a breakpoint in intron 2 of the $\alpha$-globin pseudogene that was identical in 15 of these species. Furthermore, the genomic DNA conjoined to the pseudogene, via nonhomologous recombination or intrachromosomal rearrangement, was very similar in sequence across all species. However, an isoleucine transfer RNA (tRNA) found upstream of intron 2 in 14 icefish species was absent in the $\alpha$-globin pseudogene of Dacodraco hunteri (fig. 1a).

Unexpectedly, the genome of the phylogenetically derived icefish species, N. ionah, contained 2 $\beta$-globin pseudogenes and a full-length $\alpha$-globin gene (fig. 1a and c). The first $\beta$-globin pseudogene consisted of the 3’ portion of exon 3 and the 3’ untranslated region (UTR). The second $\beta$-globin pseudogene in N. ionah was essentially complete, containing the 3 exons and 2 introns seen in Notothenia coriiceps, Notothenia angustata, and P. charcoti; however, there was a splice-site mutation at the junction of intron 1 and exon 2 (10-bp deletion that includes the CAG/G acceptor site) that undoubtedly renders this $\beta$-globin gene non-functional (fig. 1a). The validity of the N. ionah gene organization was confirmed by recovery of identical globin gene complexes from 2 specimens, the first captured near Enderby Land and the second from nearshore habitats of the South Shetland Islands (Kock and Stransky 2000).

Due to the derived position of N. ionah in the icefish phylogeny (fig. 1b), one might postulate a scenario in which the $\alpha$-globin pseudogene present in all other icefishes is...
explained by 4 independent losses of the αβ-globin cluster (4-loss model) whose breakpoints occurred at precisely the same locations during the course of icefish diversification. A more likely interpretation is that the αβ-globin cluster in *N. ionah* is an ancestral condition that represents an intermediate state between the functional globin cluster seen in most teleosts, including all non-icefish notothenioids, and the 5'-truncated α-globin gene present in all other icefish species (single loss–retention model). We propose that the presence of this intermediate condition in the phylogenetically derived *N. ionah* is explained through retention of ancestral genetic polymorphism (Hudson 1990; Hudson and Coyne 2002), specifically the lack of coalescence among ancestral icefish αβ-globin alleles. This interpretation is supported by the fairly young evolutionary age of the icefish radiation (Near 2004) and by the fact that, among 20 allozyme loci, more than half of the alleles were shared by 3 or more icefish species (Clément et al. 1998).

Alternative Phylogenetic Relationships of Icefishes—Might *N. ionah* Be Ancestral to Other Icefishes?

Our conclusion that the αβ-globin cluster of *N. ionah* is an ancestral intermediate on the path to nearly complete elimination of the locus in other icefish species rests on the validity of the currently accepted phylogeny of the clade. The phylogeny of icefish species presented by Near et al. (2003) (fig. 1b), with the 2 *Champsoscephalus* species forming the most basal icefish lineage and *N. ionah* nested within a derived clade, is supported by multiple phylogenetic studies that analyzed discrete morphological characters (Iwami 1985; Voskoboinikova 2000; Near et al. 2003), mtDNA genes (Chen et al. 1998; Near et al. 2003), or both (Near et al. 2003). Nevertheless, we have reexamined the possibility that *N. ionah* may occupy a more ancestral position in the icefish clade.

The robustness of the icefish phylogenetic tree presented in figure 1b was confirmed when we tested this tree against alternative phylogenetic hypotheses. A constrained maximum parsimony search that depicts *N. ionah* as the most basal icefish species resulted in 2 trees. When compared with the phylogeny depicted in figure 1b (ln L = −9864.65), both of these alternatives were rejected by the Shimodaira–Hasegawa test (ln L = −9.885.39 and −9.891.69, P = 0.047 and 0.028, respectively). Therefore, we consider the derived position of *N. ionah* in the icefish phylogeny (fig. 1b) valid and a basal position for this species to be significantly incompatible with the phylogenetic data.

**Ancestral Character Reconstruction**

The evolutionary model of a single loss and retention of the αβ-globin cluster in *N. ionah* is supported by
ancestral state reconstruction. Figure 2 shows that maximum likelihood ancestral state reconstruction using the Mk1 model (left) resulted in significant optimizations for all internal nodes in the icefish phylogeny as lacking the αβ-globin cluster and possessing the α-globin pseudogene. A likelihood ratio test failed to detect a significant difference between the Mk1 model and the more complex asymmetric 2-parameter model ($\chi^2 = 0.20, df = 1, P = 0.65$). To test the model that the αβ-globin cluster was lost through 4 independent events in icefishes, the asymmetric 2-parameter model was modified in Mesquite to constrain reconstruction of internal nodes as possessing the αβ-globin cluster by using a forward rate of 0.667 and a backward rate equal to 0 (fig. 2, right). The maximum likelihood score for this model was $-384.91$ and was rejected when compared with the Mk1 model in a likelihood ratio test ($\chi^2 = 755.22, df = 1, P < 0.001$).

Phylogenetic Origins of Icefish Globin Genes

In addition to the ancestral state reconstruction, phylogenetic analyses of the α- and β-globin gene sequences revealed the importance of the N. ionah αβ-pseudogene cluster in understanding the evolution of hemoglobin loss in icefishes. We aligned the 5’-truncated α-globin pseudogene (129 bp) present in 15 icefish species with orthologous α-globin sequences sampled from N. ionah and from hemoglobin-expressing Antarctic notothenioid families, including 9 dragonfish species, 2 species of Arctiidae, and 2 species of Nototheniidae. Despite limited sequence variability among the icefishes (6 variable nucleotide sites among the 129 bp), Neighbor-Joining analysis demonstrated the monophyly of all icefish α-globin pseudogenes relative to the α-globin genes sampled from the 13 red-blooded notothenioid species (fig. 3). Thus, the icefish α-globin gene fragments share a common ancestry.

Although the α-pseudogene phylogeny is poorly resolved (due to short sequence length and limited variation) compared with the mtDNA morphology tree (fig. 1b), the sharing of 3 pseudogene alleles among multiple icefish species (fig. 3) further supports our hypothesis that allelic sorting of ancestral globin gene sequences is incomplete when compared with mtDNA genes (Brown et al. 1979), and ancestral polymorphism of allelic variation in nuclear genes takes much longer to sort among lineages relative to mtDNA genes (Moore 1995; Palumbi et al. 2001; Hudson and Coyne 2002; Hudson and Turelli 2003). Of particular note with respect to the relatively young evolutionary age of the icefish clade (Near 2004), the sharing of mtDNA haplotypes has yet to report support in bootstrap analysis (2,000 pseudoreplicates). Duplicate sequences for single species were determined from independent specimens and are reported here as an indication of intraspecific variability.

-nearly complete β-globin pseudogene is phylogenetically related to the β-globin genes sampled from notothenioid species that are distantly related to both icefishes and dragonfishes (fig. 4b). Because the placement of the 2 N. ionah pseudogenes in the phylogeny is supported by significant Bayesian posterior probabilities and high parsimony bootstrap values, we have designated the inner β-globin pseudogene of the N. ionah globin complex as “β CHAN” and the outer pseudogene “β NOTO” to reflect their phylogenetic origins (figs. 4a and 4a). Gene duplication in an ancestral icefish cannot explain the origin of the 2 β-globin pseudogenes because the divergence of β CHAN and β NOTO spans the most recent common ancestor of the entire Antarctic nototheniid radiation that diversified ~24 MYA (fig. 4a and b) (Balushkin 2000; Near 2004; Near et al. 2004). Rather, we propose that gene transfer as the result of introgression via interspecific hybridization between an ancestral icefish lineage and a nototheniid explains the phylogenetic placement of β NOTO in the β-globin gene tree (fig. 4a).

Scenario for Evolutionary Loss of the αβ-Globin Gene Cluster by Icefishes

The evolution of the globin loci in icefishes from an ancestral condition presumably similar to that of...
hemoglobin-expressing notothenioids appears to have involved multiple mutational events. We hypothesize that the ancestral icefish globin complex, prior to loss of functionality, was similar in length and organization to that of the dragonfish *P. charcoti* (figs. 1 and 5). Formation of a chimeric, “‘*N. ionah–like,’” globin locus required at least 2 steps, introgression of *b NOTO* into an ancestral icefish globin locus via nonhomologous recombination and deletion of the 5′ end of *b CHAN* (fig. 5). Subsequent deletions eliminated the *β CHAN* and *β NOTO* genes and juxtaposed the residual 3′ *α*-globin fragment with a tRNA-containing chromosomal segment seen in most icefish species. The *α*-globin pseudogene allele of *D. hunteri* departs from this condition via excision of the tRNA element from the contiguous chromosomal DNA (fig. 5).

The predisposition of the notothenioid *αβ*-globin complex to rearrange can be inferred from the structure of the adult globin locus of the representative nototheniid *N. coriiceps*. The ~4-kb intergenic region between the *α* and *β*-globin genes of this species contains 2 tripartite direct repeats and 2 tripartite indirect repeats (Lau et al. 2001), sequences that facilitate intrachromosomal DNA rearrangements via recombination–repair activities at non–B DNA slipped hairpins and cruciform conformations (Bacolla and Wells 2004), respectively. Thus, it is not surprising that the globin intergenic region of red-blooded notothenioids shows a disparity in length of ~2 kb between the phylogenetically divergent nototheniids and dragonfishes (fig. 1a).

We conclude that genomic evolution of the functional *αβ*-globin gene cluster found in most teleosts to the 5′-truncated *α*-globin pseudogene observed in most icefish species was not the result of a single mutation event. The events leading to the loss of the *αβ*-globin cluster preceded the diversification of the extant icefish species, a conclusion supported by rejection of a multiple loss model using phylogenetic analyses and by ancestral state reconstruction (fig. 2). Due to the stochastic nature of lineage sorting, a “genomic fossil” was preserved in one icefish species that provides insight to the mechanism of hemoglobin loss in this clade. Phylogenetic analysis of the notothenioid *β*-globin genes indicates that interspecific hybridization between divergent Antarctic notothenioid lineages was an important factor in the loss of hemoglobin in icefishes (fig. 4a and b). We note that hemoglobin loss in icefishes was not selectively neutral but rather maladaptive as shown by the development of compensatory adaptations that enhance oxygen delivery, including increases in cardiac output and blood volume, cutaneous uptake of oxygen, and decreases in metabolic oxygen demand (Hemmingsen 1991). Interestingly, the loss of hemoglobin in icefishes is
The ancestral icefish adult \(\alpha\)-globin locus contained the notothenioid \(\alpha\)-globin gene (blue shading as in fig. 1a) and \(\beta\) CHAN, a \(\beta\)-globin gene phylogenetically related to the bathydraconid \(\beta\) gene (green shading as in fig. 1a). We propose that a hybrid icefish globin cluster was formed by introgression of a notothenioid \(\beta\)-globin gene, \(\beta\) NOTO (red), into the icefish globin locus through interspecific hybridization and nonhomologous recombination and by deletion of the 5' portion of \(\beta\) CHAN to yield the pseudogene \(\beta\) CHANA. The temporal order of these events cannot be determined from this data set. The \(\beta\) NOTO gene of \(N.\ ionah\) also acquired a splice-site mutation during evolution of the hybrid locus. Other deletions formed an allele in which the \(\beta\)-globin loci and most of the \(\alpha\)-globin gene were lost such that the remaining 3' \(\alpha\)-globin fragment was juxtaposed with a tRNA-containing chromosomal segment; this allele presumably experienced an additional deletion event to give the Dacodraco hunteri \(\alpha\)-globin remnant. Persistence of the hybrid allele in \(N.\ ionah\) is attributed to retention of an ancestral polymorphism (Hudson 1990; Hudson and Coyne 2002). paralleled by the loss of myoglobin, the intracellular oxygen-binding protein, in 6 icefish species through at least 4 different mutational events (Sidell et al. 1997; Grove et al. 2004; Sidell and O’Brien 2006). Despite the costs associated with loss of hemoglobin and myoglobin in icefishes, the chronically cold and oxygen-saturated waters of the Southern Ocean provided an environment in which vertebrate species could flourish without oxygen-binding proteins.

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