



## SYMPOSIUM

# Antarctic Notothenioid Fishes: Genomic Resources and Strategies for Analyzing an Adaptive Radiation

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**Synopsis** The perciform suborder Notothenioidei provides a compelling opportunity to study the adaptive radiation of a marine species-flock in the cold Southern Ocean that surrounds Antarctica. To facilitate genome-level studies of the diversification of these fishes, we present estimates of the genome sizes of 11 Antarctic species and describe the production of high-quality bacterial artificial chromosome (BAC) libraries for two, the red-blooded notothen *Notothenia coriiceps* and the white-blooded icefish *Chaenocephalus aceratus*. Our results indicate that evolution of phylogenetically derived notothenioid families (e.g., the crown group Channichthyidae [icefishes]), was accompanied by genome expansion. Six species from the basal family Nototheniidae had *C*-values between 0.98 and 1.20 pg, a range that is consistent with the genome sizes of proposed outgroups (e.g., percids) of the notothenioid suborder. In contrast, four icefishes had *C*-values in the range 1.66–1.83 pg. The BAC libraries VMRC-19 (*N. coriiceps*) and VMRC-21 (*C. aceratus*) comprise 12× and 10× coverage of the respective genomes and have average insert sizes of 138 and 168 kb. Paired BAC-end reads representing ~0.1% of each genome showed that the repetitive element landscapes of the two genomes (13.4% of the *N. coriiceps* genome and 14.5% for *C. aceratus*) were similar. The availability of these high-quality and well-characterized BAC libraries sets the stage for targeted genomic analyses of the unusual anatomical and physiological adaptations of the notothenioids, some of which mimic human diseases. Here we consider the evolution of secondary pelagicism by various taxa of the group and illustrate the utility of Antarctic icefishes as an evolutionary-mutant model of human osteopenia (low-mineral density of bones).

## Introduction to the notothenioid radiation

The modern ichthyofauna of the Southern Ocean is severely restricted in species diversity due to dramatic paleoclimatic and paleogeographic changes in Antarctica (Eastman 1993) that occurred during the Cenozoic Era. The onset of widespread glaciation in Antarctica ~34 million years ago (mya) (Zachos et al. 2001), attributable to (1) declining atmospheric CO<sub>2</sub> (DeConto and Pollard 2003); (2) establishment of the Antarctic Circumpolar Current due to opening of the Drake Passage ~40–34 mya (Kennett 1977; Livermore et al. 2005; Scher and Martin 2006); and (3) development of the Antarctic Polar Front (between ~50 and 60°S), decoupled the Southern Ocean

from warmer, subtropical waters to the north (Kennett 1977). As the Southern Ocean cooled to the freezing point of seawater (−1.9°C), the shallow-water, cosmopolitan, and temperate fish fauna of the late Eocene (38 mya) became largely extinct due to destruction of inshore habitat and changes in trophic structure caused by repeated ice-sheet scouring of the continental margin (Eastman 2005). Today, species of a single perciform suborder, the Notothenioidei, constitute 46% of the ~300 fish species of the Southern Ocean and, at the highest latitudes, represent 77% of species diversity and 90% of biomass (Eastman 2005). Given their geographical restriction, high endemism, and rapid speciation (average time for speciation of 0.76–2.1 million years), the Notothenioidei are

considered the best example of a marine species flock (Eastman 2000; Eastman and McCune 2000).

The ancestral notothenioid stock was a negatively buoyant, bottom-dwelling perciform species that arose ~40–60 mya (DeWitt 1971; Eastman 1993; Eastman and Clarke 1998). As competing taxa became locally extinct, the notothenioids diversified to occupy the vacated ecological niches, many of which were present in the water column. Lacking a swim bladder, the Notothenioidei evolved pelagic, or partially pelagic, lifestyles by reduction of skeletal mineralization and enhancement of lipid deposition (Eastman 1993). Tailoring of morphology for life in the water column, termed secondary pelagicism, is the hallmark of the notothenioid radiation, has arisen independently several times in different clades (Eastman 1997, 1999; Near et al. 2007), and reflects the retention of larval characteristics in the adult (paedomorphism) (Eastman 1997).

The long residence of the notothenioids in a freezing marine environment has also driven the evolution of biochemical and physiological adaptations that resist or compensate for the effects of extreme cold. The evolution of the antifreeze glycoprotein genes from a pancreatic, trypsinogen-like gene (Chen et al. 1997; Cheng and Chen 1999) is a remarkable genetic “resistance” innovation that fostered survival of the group (Montgomery and Clements 2000). Major examples of “compensatory” adaptation in notothenioids include efficient microtubule assembly (Williams et al. 1985; Detrich et al. 1989, 1992, 2000; Paluh et al. 2004) and protein translocation at low temperatures (Römisch et al. 2003), homeoviscous adaptation of membrane lipids to preserve membrane fluidity (Logue et al. 2000), and cold-stable lens crystallins that prevent formation of cataracts (Kiss et al. 2004). Expansion of gene families involved in metabolic processes (e.g., biosynthesis, folding, and degradation of proteins; lipid metabolism) that are critical to the physiological fitness of these organisms has also been reported (Chen et al. 2008).

Less well appreciated are the regressive changes or losses of function (“disaptations” per Montgomery and Clements [2000]) that evolved in the notothenioids due to relaxed selection pressure in their stable, cold environment. Striking regressive changes include the loss of erythrocytes (Ruud 1954) and the respiratory transport protein hemoglobin (Cocca et al. 1995, 2000; Zhao et al. 1998; Near et al. 2006) by all species of the icefish family and independent losses of cardiac myoglobin in a subset of those species (Sidell et al. 1997; O’Brien and Sidell 2000; Sidell and O’Brien 2006). Antarctic

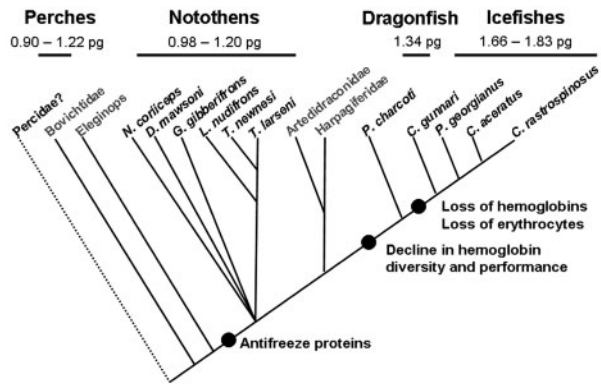
notothenioids have also lost the inducible heat-shock response (Hofmann et al. 2000; Buckley et al. 2004), which appears to have been recruited to a constitutive status to deal with elevated denaturation of proteins caused by cold stress (Place et al. 2004; Place and Hofmann 2005; Todgham et al. 2007).

## Enablement of the genomes of the Notothenioidei

The novel phenotypes evolved by the high-Antarctic notothenioids beg mechanistic explanation, which arguably may best be approached using comparative genomics. In 2003, we collected high-molecular-weight DNA from many species of these hard-to-obtain fishes with the aim of enabling genomic studies in the group. We then submitted a white paper to the Bacterial Artificial Chromosome (BAC) Resource Network at the National Human Genome Research Institute (NHGRI) to request construction of high-quality and high-representation BAC genomic libraries from two notothenioid species, the blackfin (Scotia Arc) icefish *Chaenocephalus aceratus* and the bullhead notothen (yellowbelly rock cod) *Notothenia coriiceps*. Our choice of these two species was driven strategically both by their hematological and skeletal differences and by their relative numerical dominance among large notothenioids that can be collected near Palmer Station. *Chaenocephalus aceratus* represents a highly derived lineage (icefishes) that is devoid of erythrocytes, lacks functional globin genes, and has poorly ossified skeletons, whereas *N. coriiceps* represents a more basal lineage whose members possess erythrocytes, synthesize functional hemoglobins, and are robustly ossified. Thus, we anticipate that targeted genomic comparisons of these two species will illuminate the physiological, anatomical, and gene-regulatory changes associated with diversification within the notothenioid suborder. We illustrate our approach by considering the evolution of reduced mineralization of bone in the secondarily pelagic icefish. We also emphasize that utility of this naturally selected trait as a model for a common, maladaptive human condition, osteopenia (low density of minerals in bone), which predisposes to the disease osteoporosis.

## Genome expansion correlates with phyletic diversification in the suborder Notothenioidei

As a first step in developing genome resources for the Notothenioidei, we measured the sizes of the genomes of 11 Antarctic species by flow cytometry (Detrich et al. 2010). Figure 1 shows the phylogenetic



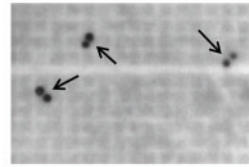
**Fig. 1** Phylogenetic relationships among the 11 notothenioid species examined in this study. Six nototheniids of the basal, red-blooded family Nototheniidae (*Dissostichus mawsoni*, *Gobionotothen gibberifrons*, *Lepidonotothen nudifrons*, *N. coriiceps*, *Trematomus larseni*, and *T. newnesi*) and four icefishes of the white-blooded notothenioid crown group Channichthyidae (*C. aceratus*, *Champscephalus gunnari*, *Chionodraco rastrospinosus*, and *Pseudochaenichthys georgianus*) are shown with the size ranges of their genomes. One species (*Parachaenichthys charcoti*) of the red-blooded dragonfishes (Bathydraconidae), the sister group to the icefishes, is also presented. The perches (Percidae) are the probable sister group to the Notothenioidae (Dettai and Lecointre 2004), and the size range of their genomes is based on *Perca flavescens* (Hinegardner and Rosen 1972; Hardie and Hebert 2004), *P. fluviatilis* (Vialli 1957; Vinograd 1998), and *Sander lucioperca* (Vinograd 1998). The positions of the notothenioid families Bovichtidae, Eleginops, Artedidraconidae, and Harpagiferidae (gray font; not examined herein) are shown for comparison. The closed circles indicate key evolutionary events during the diversification of the Notothenioidae: the acquisition of antifreeze proteins; the decline in hemoglobin multiplicity and performance; and the losses of hemoglobins and of red blood cells by the icefishes. The tree is based on the studies of Lecointre et al. (1997), Balushkin (2000), Near et al. (2004), and Near and Cheng (2008). Reproduced from Detrich et al. (2010). Copyright (2010 Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.); Reprinted with permission of John Wiley & Sons, Inc.

relationships of the species relative to other notothenioid clades and indicates that values of *C* increase substantially as one moves from basal (Nototheniidae) to derived (Bathydraconidae, Channichthyidae) families. The basal Nototheniidae have genomes whose sizes are comparable to those of outgroups Percidae, Serranidae, and Gasterosteidae, which suggests that a *C*-value of  $\sim 1$  pg is ancestral for the notothenioid suborder. Our estimates of genome size for the 11 notothenioids fall in a range that is consistent with the limited number of prior measurements (Morescalchi et al. 1992, 1996; Hardie and Hebert 2003, 2004; Chen et al. 2008). Taken together, these results support the hypotheses: (1) that genome expansion accompanied the evolution of phylogenetically derived notothenioids; and (2) that

### 1. BAC Library Construction



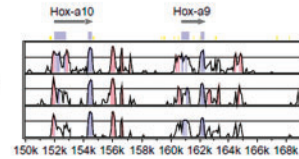
### 2. Isolation of Target BAC



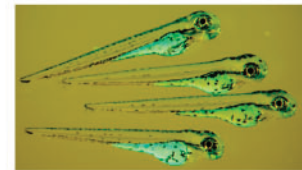
### 3. Obtain Sequences

```
...GGTCTGACAGCGGTAGTCC
CCAGGGGTCTGCCTATACC
CCACCTGGTGGTGTCTGGGG...
```

### 4. Global Alignments & Plots



### 5. Functional Experiments



**Fig. 2** Flowchart for targeted comparisons of genomic sequences. BAC libraries are used to isolate orthologous regions of interest from the genomes of two species (1, 2). Sequences are obtained from the respective BAC clones (3) and aligned for comparison (4). Testable hypotheses derived from the sequence comparisons, such as putative regulation of gene expression by conserved DNA sequence elements, are then analyzed by functional experiments *in vivo* and *in vitro*. See Miyake and Amemiya (2004) and Amemiya and Gomez-Chiarri (2006) for in-depth reviews of the comparative genomics pipeline. Modified from Miyake and Amemiya (2004) with permission. Copyright (2004) Elsevier, Inc.

genome expansion may have been driven in ways that offset reduction (Bathydraconidae) or loss (Channichthyidae) of hemoprotein function.

## BAC libraries for *N. coriiceps* and *C. aceratus*

Although new technologies continue to reduce the costs of DNA sequencing, non-traditional vertebrate models, such as the Antarctic notothenioids, are unlikely to be chosen for complete genomic analysis in the foreseeable future. Nonetheless, one can envision targeted strategies (Fig. 2) whereby BAC libraries are generated from the genomes of two or more organisms with known phylogenetic relationships, BAC clones containing orthologous genomic regions

of interest are isolated and their inserts sequenced; and computational tools are used to compare the regions to identify conserved and nonconserved DNA elements. The results could then be used to derive hypotheses amenable to empirical testing. One large-scale example of the targeted genomic sequencing strategy is the Encyclopedia of DNA Elements (ENCODE) initiative, which evaluated the regulatory logic of the human genome by comparison of selected regions encompassing 1% of the genome to orthologous regions from many other vertebrate species (Birney et al. 2007). Conversely, BAC libraries can be applied, at minimal cost, to quasi-global analyses, such as sequencing the ends of randomly selected BAC inserts to compare the compositions of repetitive elements of the genomes of two or more species (cf Shedlock et al. [2007]). Here we describe BAC libraries for *N. coriiceps* and *C. aceratus* and illustrate their use in both quasi-global and targeted genomic studies.

BAC libraries are based on plasmid vectors that contain an *F*-factor origin of replication (Shizuya et al. 1992). The *F*-factor replicon ensures that the plasmid replicates as a single-copy entity in *Escherichia coli*, a prerequisite for stable propagation of cloned inserts >100 kb. Large inserts are desirable for many genomic applications, including physical mapping of genomes, positional cloning of mutated genes, targeted genomic sequencing, and generation of transgenic animals (Yang et al. 1997; Amemiya et al. 1999; Miyake and Amemiya 2004).

Construction of the notothenioid BAC libraries was performed under the auspices of the NHGRI and followed rigid standards of quality control that mandated: (1) 10× genomic coverage; (2) average insert size of ~150 kb; (3) assurance that the library contained a minimum of 95% insert-containing clones; and (4) little or no detectable contamination by bacterial DNA. The most critical challenge was to prepare high-molecular-weight DNA from blood cells of the two species, erythrocytes in the case of *N. coriiceps* and leukocytes for *C. aceratus*, using the laboratory facilities at Palmer Station, Antarctica. Because the laboratory lacks a pulsed field-gel apparatus, we operated “blind” and could not assess the quality of the DNAs until the samples were returned to our laboratories. Analyses of these DNAs through high-resolution, pulsed field-gel electrophoresis verified that the DNAs were suitable for BAC library construction, which subsequently followed slight modifications of published methods (Osoegawa et al. 1998; Danke et al. 2004); greater detail may be found in Detrich et al. (2010).

**Table 1** Summary statistics for the notothenioid BAC libraries

	<i>Chaenocephalus aceratus</i> (VMRC-21)	<i>Nototothenia coriiceps</i> (VMRC-19)
Vector used	pCC1BAC	pCC1BAC
Restriction enzyme used	EcoRI	EcoRI
DNA source	Leucocytes	Erythrocytes
Number of 384-well plates	336	288
<i>Escherichia coli</i> strain used	DH10B T1 resistant	DH10B T1 resistant
Empty wells	0	0
Estimated non-recombinant clones and vector artifact clones, %	6451 (5)	8847 (8)
Estimated insert-containing clones, %	122 573 (95)	101 745 (92)
Average insert size	168 kb	138 kb
Median insert size	168 kb	155 kb
Estimated genome coverage	10X	12X
Restriction enzyme used	EcoRI	EcoRI

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Table 1 provides summary statistics for the two libraries, VMRC-19 (*N. coriiceps*) and VMRC-21 (*C. aceratus*). The libraries comprise 12× and 10× coverage of the respective genomes based on the total number of clones in the library, the average insert sizes (138 kb for *N. coriiceps*, 168 kb for *C. aceratus*), and the genome sizes determined for the taxa. A survey of randomly selected BAC clones showed that non-recombinant and vector-artifact clones are rare (8% for VMRC-19, 5% for VMRC-21), and inserts of bacterial origin are not detectable. Thus, the two libraries meet nearly all of the criteria mandated by the NHGRI and are sufficiently robust to be used in targeted or global genomic studies. For inquiries regarding access to the BAC libraries, please contact Pieter de Jong (pdejong@chori.org).

## Repetitive-element landscapes of the *N. coriiceps* and *C. aceratus* genomes

Large-scale BAC-end sequencing of *C. aceratus* and of *N. coriiceps* (samples represent ~0.1% of each genome) indicated that the GC contents of the two species are identical (42%) and agree with prior estimates of notothenioid genomes (41–43%) obtained by analytical ultracentrifugation (Bucciarelli et al. 2002). The proportion of repetitive elements in the two samples (14.5% for *C. aceratus* and 13.4% for *N. coriiceps*) is similar, as are the percentages of most individual elements (Table 2). However,



**Table 2** Repetitive elements in a sample of BAC end sequences<sup>a</sup>

Repetitive element class	n (%)	
	<i>Chaenocephalus aceratus</i>	<i>Notothenia coriiceps</i>
Transposable elements	1390	618
DNA transposons	566 (40.9)	253 (41.1)
Mariner/Tc1	27 (1.9)	32 (5.2)
Kolobok	32 (2.3)	17 (2.8)
Harbinger	30 (2.2)	7 (1.1)
hAT	211 (15.2)	91 (15.0)
Other	266 (19.1)	106 (17.0)
LTR retrotransposons	258 (18.3)	80 (13.0)
Gypsy	156 (11.0)	49 (7.9)
Copia	25 (1.8)	1 (0.2)
DIRS	46 (3.3)	17 (2.8)
Other	31 (2.2)	13 (2.1)
Non-LTR retrotransposons	468 (33.4)	241 (38.6)
CR1	337 (24.0)	157 (25.0)
L1	46 (3.3)	31 (5.0)
SINE	52 (3.7)	37 (6.0)
Other	33 (2.4)	13 (2.6)
Endogenous Retrovirus	98 (7.0)	44 (7.0)
SSRs and satellites	474	246

<sup>a</sup>Repetitive elements in paired-end reads of the BAC inserts were evaluated by use of RepeatMasker v3.0 (Smit et al. 2004), CENSOR (Kohany et al. 2006), and the Repbase Update (Jurka et al. 2005). Reproduced from Detrich et al. (2010). Copyright (2010 Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.); Reprinted with permission of John Wiley & Sons, Inc.

CR1, chicken repeat 1; DIRS1, *Dictyostelium* intermediate sequence repeat 1; ERV, endogenous retroviral sequence; hAT, *hobo*, *Ac* and *Tam3* family; L1, long-interspersed element 1; LTR, long-terminal repeat; SINE, short-interspersed element; SSR, simple sequence repeat; TE, transposable element.

genomic representation of the Mariner/Tc1 transposon, of the Gypsy and Copia LTR retrotransposons, and of SINE elements appears to differ between *C. aceratus* and *N. coriiceps*. Whether these differences in retrotransposon composition contributed to the differential expansion of the icefish genomes and of individual protein-coding gene families, as postulated for LINEs by Chen et al. (2008), remains to be tested by large-scale, whole-genome sequencing and by functional approaches.

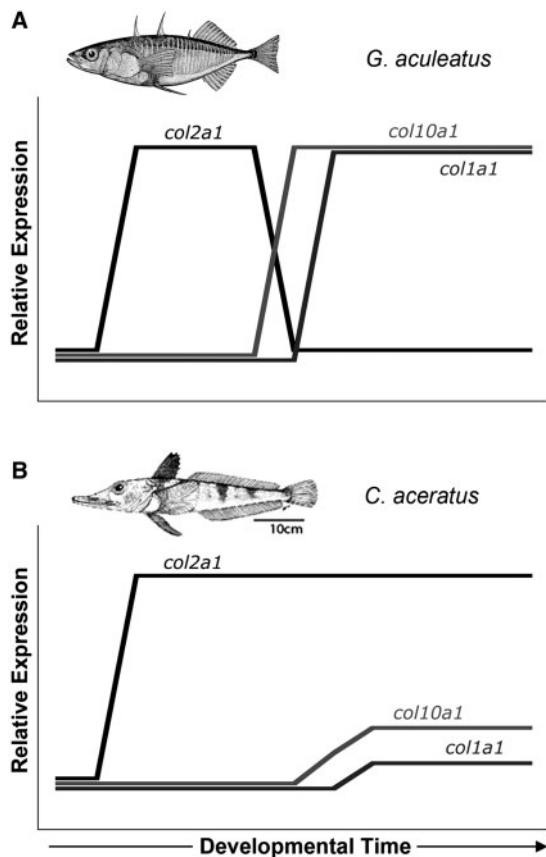
### An evolutionary-mutant model for human osteopenia: paedomorphism in notothenioid skeletal development

Albertson et al. (2009) have proposed that the analysis of non-traditional “evolutionary-mutant models” that mimic human disorders provides a

complementary approach to standard forward genetics (induction of mutations in traditional laboratory animals [e.g., mice, zebrafish, flies, and worms]) for discovering genes and mechanisms that contribute to human disease. The severity, early onset, and coding-sequence bias of induced mutations often mask developmental pleiotropy and preclude phenotypic examination at the later life stages relevant to many human diseases. In contrast, many naturally occurring mutations in humans that either cause or predispose to disease result from alterations to the *cis*-regulatory regions of genes, whose combinatorial complexity and tissue specificity can yield normal gene activity early in development but abrogate gene regulation in adults. Given that most genetic pathways are functionally conserved in animals, one may reasonably anticipate that a molecular understanding of atypical phenotypes favored by natural selection in wild populations will contribute to identifying novel genetic factors and environmental interactions that affect human health. Here we consider the evolution of secondary pelagicism by Antarctic notothenioids and its relationship to maladaptive osteopenia in humans.

Notothenioids evolved secondary pelagicism by paedomorphism, the retention of ancestrally juvenile traits by adults of a descendant taxon (Eastman 1997). Paedomorphism results from heterochronic processes that change the schedule of developmental events (Gould 1992). When compared to benthic notothenioids, pelagic and benthopelagic species of the suborder show several paedomorphic skeletal characters: (1) delayed and reduced skeletal ossification; (2) partial or complete retention of the notochord; (3) reduction of the pterygoid process of the palatoquadrate; and (4) reduced numbers of teeth and of tooth rows.

To address the molecular mechanisms that cause skeletal reduction and morphological change in notothenioids, Albertson et al. (2010) examined craniofacial development in a true pelagic species, *Pleuragramma antarcticum*, a benthopelagic species, *C. aceratus*, and a benthic species, *N. coriiceps*. The closely related percomorph, *Gasterosteus aculeatus* (three-spine stickleback), and the distantly related cyprinid, *Danio rerio* (zebrafish), were selected as outgroups. Using collagen genes as markers of cartilage development (*col2a1*) and bone formation (*col1a1* and *col10a1*), they found that pelagic and benthopelagic notothenioid larvae exhibit delays in osteogenic development. Figure 3 shows schematically that expression of these genes in the stickleback and zebrafish follows the typical vertebrate pattern; *col2a1* is expressed early in development (in



**Fig. 3** Schematic illustration of heterochronic alteration of collagen gene expression in the notothenioid larval skeleton relative to “conventional” teleost outgroups. (A) Expression in the three-spine stickleback *G. aculeatus* and in the zebrafish *D. rerio* follows the typical vertebrate pattern, with *col2a1* expressed first in differentiating chondrocytes, followed by down-regulation of *col2a1* and subsequent up-regulation of the bone markers, *col10a1*, and *col1a1*. (B) The notothenioid pattern is quite different, with sustained high levels of *col2a1* expression throughout later periods of larval development, expression of *col10a1* limited to a small subset of skeletal elements, and weak *col1a1* expression throughout the pharyngeal skeleton. The x-axis denotes developmental time starting just before chondrocyte differentiation, and the y-axis represents relative (not quantitative) levels of collagen gene expression. Adapted from Albertson et al. (2010) with permission of the authors.

differentiating chondrocytes) and then is down-regulated as expression switches to the bone marker genes *col1a1* and *col10a1*, which are up-regulated and maintained at high levels (panel A). The pattern for pelagic/benthopelagic notothenioids differs dramatically, with early and sustained expression of *col2a1* throughout larval development (panel B). Low levels of *col10a1* were expressed in a subset of craniofacial elements, and *col1a1* was expressed weakly in the pharyngeal skeleton (morphological features not shown). Thus, the evolution of bone loss in Antarctic notothenioids can be

explained in part by prolongation of the early chondrogenic developmental program through extended periods of larval development. These data provide an initial molecular view of the signaling cascades that modulate bone density and suggest that changes in the regulation of suites of genes are linked to the adaptive radiation of Antarctic notothenioids into pelagic habitats. [The strong conservation of collagen protein sequences between the osteopenic *C. aceratus* and the strongly mineralized *N. coriiceps* reinforces the deduction that the critical evolutionary changes occurred in gene regulatory regions (Albertson et al. 2010)]. They are also likely to provide insights into the development of human osteopenia, which in turn may suggest new therapeutic approaches to prevent or treat this condition.

We are now ready to use the targeted genomic strategy to test the hypothesis that alteration of the regulation of collagen gene expression is involved in adaptive modulation of bone density. Using our BAC libraries for the benthic notothenioid *N. coriiceps* and the benthopelagic *C. aceratus*, we will isolate orthologous BAC clones whose inserts contain the *col1a1*, *col2a1*, and *col10a1* genes. Sequence analysis of these clones and bioinformatic comparison of non-coding regions should reveal whether or not the regulatory motifs governing the expression of orthologous collagen genes in the two species have diverged. Simultaneous comparison of the notothenioid regulatory elements to those of collagen genes from stickleback and zebrafish is necessary to ensure that sound, phylogenetically controlled, inferences are drawn. Functional testing by generation of transgenic stickleback or zebrafish whose collagen gene regulatory motifs have been swapped for those of the two notothenioids would complete the formal chain of logic.

Recently, Chan et al. (2010) showed that the pelvic reduction in freshwater populations of the three-spine stickleback has evolved repeatedly through deletion of a tissue-specific enhancer for the *Pituitary homeobox transcription factor 1* (*Pitx1*) gene. Restoration of *Pitx1* expression by transgenic insertion of the enhancer into a pelvic-reduced laboratory line rescued pelvic girdle and spine formation. We are tempted to speculate that a similar mechanism for deletion may underlie the evolutionary transition between the switching of collagen gene expression observed in robustly mineralized notothenioids and the loss of switching observed in osteopenic species.

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