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Cytochrome b gene sequences from two eelpouts (perciformes, zoarcidae) from McMurdo Sound (Antarctica): Implications on the antifreeze gene structure

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The fish family Zoarcidae (eelpouts) comprises approximately 200 species, living mainly in the Northern Hemisphere (Nelson 1984). Nine genera with 22 species occur in the southern oceans (Anderson 1990, pp. 256–276). It is the second largest group of antarctic ocean fishes (Nothoteniidae are represented by at least 32 species). All zoarcid species are benthic except *Lycodapus* and *Melanostigma*, which are bentho- or mesopelagic.

To avoid freezing, several eelpout species that live at subzero temperatures synthesize antifreeze peptides (AFPs). Eelpout AFPs were characterized from *Macrozoarces americanus, Pachycara brachycephalum, Lycodichthys dearborni,* and *Lycodes polaris* (Li et al., 1985; Schrag et al, 1987; Cheng and DeVries, 1989). Thus far, the AFPs that have been characterized have been found to be encoded within large gene families and although the gene products are heterogeneous with respect to composition, they are approximately the same size (7 kilodalton) with the exception of an additional 14 KDa variant found in *L. dearborni* (Wang et al., in press).

The sequences of the 7 KDa AFPs characterized from different eelpout species can be both similar and quite different depending upon the species. In the case of *L. dearborni*, the 14 KDa variant is a unique major component, which is species specific and appears to be made up by joining of two 7 KDa AFPs with a nine amino acid residue linker. The carboxyl 7 KDa portion is nearly identical to the minor AFP found in another eelpout, *P. brachycephalum*, and quite unlike the 7 KDa AFPs found within its own family. Because orthologous proteins cannot be found in other species, and because the sequence identities between species is often extremely variable, it is difficult to infer phylogenies based on antifreeze genes. For these reasons, antifreeze gene trees and organism trees are not always concordant. Nevertheless, some phylogenetic inferences can be drawn based on other traits such as the genomic organization of the antifreeze gene family. We postulate that the extreme compositional and size heterogeneity present in a species like *L. dearborni* has resulted from gene amplification and modification of the amplified genes and represents a relatively old antifreeze gene family. The gene family of *P. brachycephalum*, on the other hand, would appear to be young because it is largely made up of copies of a single 7 KDa protein and only a few of a very similar minor one. This major AFP accounts for approximately 95 percent of the circulationg AFP (Cheng and DeVries 1989). This gene family organization suggests a recent origin because there has been insufficient time for recognizable modification of the many copies present in the family. Thus, in an attempt to further infer phylogenetic relationships between these two species and the possible relationship of the age of the AFP gene families, we accessed an independent locus, the mitochondrially encoded cytochrome b (Cyt b) gene of both McMurdo Sound species.

P. brachycephalum and *L. dearborni* were collected using fish traps in 600 meters of water in McMurdo Sound, Antarctica. DNA was extracted from liver tissue according to a method already described (Bernardi and Bernardi 1990). The polymerase chain reaction (Saiki et al. 1988) was used to amplify a 836-base-pair (bp) region of the Cyt b gene. Primers and protocols were from Kocher et al. (1989) and Palumbi et al. (1991). Approximately 100 nanograms of DNA was used as template for 100-milliliter PCR reactions that contained 10 millimolar (mM) Tris, hydrogen chloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01 percent (weight-to-volume) gelatin, 200 mM of each dNTP, 2.5 units of *Taq* DNA polymerase (Perkin-Elmer, Cetus), and 1 mM of each amplification primer. PCR products were sequenced directly using the femtomole sequencing kit (Promega) and dATP33 (NEN) as the radiolabeled nucleotide. Sequencing primers used were CB3-H, CB2-H, CB1-L, and GLUDG-L (Palumbi et al. 1991). Cyt b sequences were aligned

using the Geneworks (IntelliGenetics) program. Because of a high degree of sequence conservation, refinements were unnecessary.

Cyt b sequences, starting at aminoacid position 1, are given in the figure. Of 382 aligned positions, 10 were variable (sequence divergence is 2.6 percent). When using the standard rate of mutation for fish cytochrome b of 2.5 percent per million years (Meyer et al. 1990), the divergence between the two species was estimated at about 1 million years. Considering the extreme environment in which antarctic fish live, especially the very low water temperature, it is possible that the standard rate of mutation is not applicable in our case. Transitions were more frequent than transversions (eight transitions, two transversions). An implication is that the sequences compared are not deeply within the multiple-hit zone (Brown et al. 1982; Meyer and Wilson 1990). Three amino acid positions were different between the two sequences. One position, a Glycine in position 65 is unique to eelpouts (Degli Esposti et al. 1993). Further studies will tell us if this amino acid is common to eelpouts or if it is unique to antarctic species.

Lycodichthys has a long history of residence in antarctic waters, possibly dating from the Miocene 25–5 million years ago (Anderson 1990). This is consistent with our hypothesis that the heterogeneous AFP gene family of *L. dearborni* is relatively old. Based on the Cyt b gene analysis, *P. brachycephalus*, which has a homogeneous AFP gene family, is estimated to have diverged from *L. dearborni* about 1 million years ago. This dating suggests that the heterogeneity found in *L. dearborni* was acquired recently.

A greater understanding of the genomic organization of the AFP gene family, their origin (ancestral molecule), the processes of amplification, and rate of change is needed before one can infer any phylogenetic relationships from AFP gene sequence comparisons.

L. dearborni and *P. brachycephalum* DNAs were extracted, PCR amplified, and sequenced at the Crary Laboratory, as part of the McMurdo Biology Course, supported by National Science Foundation grant OPP 93-17696 to the University of Southern California.

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	M T S L R K T H P L L K I A N N A L V D	
P. brachycephalum	ATG ACA AGC CTA CGA AAA ACC CAC CCG TTA CTA AAA ATC GCA AAT AAC GCA CTA GTT GAC	60
L. dearborni	— — — — — — -.. ... ... C..	
	L P A P S N I S V W W N F G S L L G L C	
P. brachycephalum	CTG CCC GCC CCC TCC AAC ATC TCC GTG TGA TGA AAC TTT GGC TCC CTG CTA GGC CTC TGC	120
L. dearborni	... ...T ...	
	L I I Q I L T G L F L A M H Y T P/A D I T	
P. brachycephalum	TTA ATT ATT CAA ATC CTT ACA GGG CTC TTC CTC GCC ATA CAT TAT ACC CCC GAC ATC ACA	180
L. dearborni	... ...C ... ... G..	
	T A F S S I/V G H I C R D V N Y G W L I R	
P. brachycephalum	ACT GCC TTC TCC TCT ATT GGT CAC ATT TGT CGA GAC GTC AAC TAC GGC TGG CTC ATC CGC	240
L. dearborni	... ... G..	
	N L H A N G A S F F F I C I Y M/L H I G R	
P. brachycephalum	AAC CTC CAC GCC AAC GGC GCC TCC TTC TTC TTC ATC TGC ATT TAC ATG CAC ATC GGC CGC	300
L. dearborni	... ...T ... ...C ... C..	
	G L Y Y G S Y L Y K E T W N I G V I L L	
P. brachycephalum	GGA CTG TAT TAC GGC TCT TAC CTC TAT AAA GAA ACA TGA AAC ATC GGT GTT ATT TTA CTA	360
L. dearborni	... ...C	
	L L V M N T A F V G Y V L P	
P. brachycephalum	TTA CTC GTA ATA ATA ACA GCC TTC GTG GGC TAC GTT TTA CC	401
L. dearborni	... ...A	

Cyt b sequence alignment for two zoarcid species. The putative amino acid sequence is shown above the alignment. When different between the two species, both amino acids are shown, with the left amino acid corresponding to the *P. brachycephalum* sequence. Dots (.) correspond to identical nucleotides. Dashes (-) correspond to nondetermined nucleotides.

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Dynamic instability of microtubules from antarctic fishes

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Microtubules are dynamic cellular polymers that undergo alternating periods of growth and shortening, both *in vivo* and *in vitro*, through the end-dependent addition and loss of tubulin alpha-beta dimers. Although this "dynamic instability" has been analyzed extensively using microtubules from mammalian brain tissues (Mitchison and Kirschner 1984; Walker et al. 1988; Gildersleeve et al. 1992), the dynamic properties of microtubules from cold-living poikilotherms are only partially understood (Himes and Detrich 1989; Simon, Parsons, and Salmon 1992). In this context, microtubules from the ectothermic fishes of antarctic coastal waters are particularly interesting. As the southern ocean began to cool approximately 40 million years ago, the antarctic fishes diverged from temperate fishes (DeWitt 1971) and evolved molecular, cellular, and physiological adaptations that preserve metabolic efficiency and macromolecular function at their now chronically low body temperatures (–1.86°C to + 2°C). Recent work from my laboratory has shown that the molecular adaptations that enable the microtubules of antarctic fishes to assemble and to remain stable in this extreme thermal regime reside in their tubulin subunits (Detrich, Johnson, and Marchese-Ragona 1989; Detrich et al. 1990, 1992; Detrich and Parker 1993). Here we describe the dynamic instability of brain microtubules from two antarctic rockcods, *Gobionotothen gibberifrons* and *Notothenia coriiceps*. Our results suggest that functional adaptation of tubulins to low temperatures yields microtubules characterized, at both physiological and nonphysiological temperatures, by unusually slow subunit exchange. For a more detailed analysis, see Billger et al. (1994).

Tubulin free of microtubule-associated proteins (MAPs) was purified from brain tissues of the two rockcods by methods previously described (Detrich and Overton 1986; Detrich et al. 1989). To assess the dynamic instability of microtubules assembled from these tubulins, we measured *in vitro* the rates of growth and shortening of individual antarctic fish microtubules (nucleated from sea urchin flagellar axonemes), and the frequencies of interconversion between these states, by video-enhanced differential interference contrast microscopy (cf. Walker et al. 1988; Gildersleeve et al. 1992) at temperatures between 5°C and 25°C. The table shows that microtubules from *G. gibberifrons* (*Gg*) or from *N. coriiceps* (*Nc*) display dynamic instability at the near-physiological temperature of 5°C. Their rates of growth and shortening, however, as well as their frequencies of catastrophe (transition to shortening) and rescue (resumption of growth), are an order of magnitude (or more) smaller than those observed for cow (*Bos taurus; Bt*) brain microtubules at 37°C. Thus, the fish microtubules exhibit dynamic instability but at slower rates and with smaller transition frequencies than their mammalian counterparts. Attempts to assemble microtubules from bovine tubulin at 5°C were unsuccessful. Therefore, no direct, isothermal comparison of piscine and bovine microtubule dynamics was possible.

Antarctic fish brain microtubules also grow slowly at 25°C, and two rate classes, presumably corresponding to the distinct plus (+) and minus (–) ends of the polar microtubule polymer, are observed (*see* table). Disassembly rates also

Kinetic parameters for microtubule dynamic instability

Species	Temperature (°C)	Assembly rate (μm/min)	Disassembly rate (μm/min)	Catastrophe frequency (per minute)	Rescue frequency (per minute)
Gg/Nc[a]	5–8	0.21±0.16	–1.9±0.6	0.008	<0.0004
Gg/Nc[a]	25	(+) 0.53±0.08 (–) 0.12±0.07	–0.24±0.11	ND	ND
Bt (cow)[b]	37	2.5±0.58	–37±28	0.06–0.3	1.2–3.0

[a]Measurements were made as described in Billger et al. (1994).
[b]Assembly and disassembly rates for bovine tubulin are from Gildersleeve et al. (1992), and catastrophe and rescue frequencies (+ ends) are from Walker et al. (1988).
ND, not determined.