Molecular Evolution at Subzero Temperatures: Mitochondrial and Nuclear Phylogenies of Fishes from Antarctica (Suborder Notothenioidei), and the Evolution of Antifreeze Glycopeptides

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Most fishes of the perciform suborder Notothenioidei are endemic to the subzero marine waters of Antarctica. A number of remarkable physiological attributes allow them to inhabit this extreme environment; for example, the blood of almost all notothenioid species contains antifreeze glycopeptides. To establish a molecular phylogenetic hypothesis for these fishes, DNA sequences from two mitochondrial genes, portions of the 12S and 16S ribosomal genes (928 base pairs [bp]), were determined for 18 species. These belong to 15 genera in five families of the suborder. The DNA data suggest that two of these families are unnatural groups and consequently that the classification and phylogeny of this suborder is in need of revision. In terms of DNA variation, the Bovichtidae are a distantly related sister group to the other families of the suborder that includes the icefishes, the only vertebrates without hemoglobin. The fishes of the suborder (except the Bovichtidae) seem to have speciated rapidly, forming an adaptive radiation in the Antarctic waters. A phylogenetic analysis of published hemoglobin amino acid sequences for other notothenioid fishes supports these results from mtDNA. On the basis of molecular phylogeny, the evolution of antifreeze glycopeptides was studied. The age of the radiation of notothenioid fishes had been estimated to be at least 38 Mya. However, the level of mtDNA variation detected in notothenioid fishes appears to be too low to agree with this date of origin and might instead suggest a younger age (10–15 Mya). Alternatively, the low level of detected mtDNA variation would agree with the traditional old-age estimate if an extremely slow rate of mtDNA evolution is postulated for this group. This slow-rate hypothesis, if true, could be explained by decreased metabolic rates slowing down the tempo of molecular evolution.

Introduction

The waters around Antarctica are inhabited by more than 260 species of fishes (Gon and Heemstra 1990; Miller 1993). Although this is only about 1% of all known fishes it still seems a surprisingly high number for such an extreme marine environment, with constant subzero temperatures (Eastman 1991, 1993, p. 55). The fish fauna on the continental shelf of Antarctica is dominated in terms of numbers (more than 50% of all species) and biomass by species of the perciform suborder Notothenioidei. This suborder comprises the families Bovichtidae, Nototheniidae, Artedidraconidae, Harpagiferidae, Bathydraconidae, and Channichthyidae. Compared with all other groups of fishes in Antarctica, only notothenioids successfully formed a "species flock"; they have the highest degree of endemism and occupy the largest number of habitats by far (Kock 1992; Eastman 1993, p. 67). These fishes show a large degree of ecological diversification, including feeding in the most extreme environment, the cryopelagic, the undersurface of the ice cover that is composed of ice platelets (Eastman and DeVries 1986).

The cold Antarctic marine environment, with temperatures as low as −2°C, slows down physiological and biochemical processes. Other characteristics of the Antarctic ocean, the small degree of fluctuation in temperature, the high viscosity, and the increased oxygen solubility, differentiate it from the waters of other oceans. Notothenioids, like other marine teleost fishes, are hyposmotic to seawater. They possess remarkable physiological characteristics, most notably antifreeze glycopeptides (AFGPs) that cause the depression of the freezing point of their body fluids and allow them to cope with these environmental challenges (e.g., see DeVries 1988). None of the 11 basal bovichtid species have AFGPs, and only one of these lives within the Antarctic Convergence, a circum-Antarctic current (47°–63°S). The blood of icefishes (family Channichthyidae) has a reduced viscosity due to the complete absence of

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hemoglobin, a unique condition in vertebrates (Ruud 1954; Hamoir 1988). Even blood in other notothenioid species that possess hemoglobin has a decreased viscosity, compared with that of temperate fishes through lowering of the hematocrit (Wells et al. 1980).

The unique Antarctic marine ecosystem is believed to have originated in isolation from Gondwana during the progressive cooling of circumpolar waters about 40–60 Mya (see Eastman 1991). Antarctica was completely separated from South America in the late Cenozoic (30–23 Mya) (Elliot 1985) through the Antarctic Convergence. This current reduced the exchange of the surface water in a north-south direction and thus caused the isolation of the southern ocean. About 10–15 Mya the water temperatures are believed to have reached 0°C (Kennett 1977; Clarke 1990). It has been argued that this might have created a void through the extinction of the previous fish fauna. Furthermore, this cold temperature might have acted as effective barrier for potential immigrants into this environment (reviewed in Eastman 1993, pp. 120–144).

Traditionally, the suborder Notothenioidei has been divided in five or six families (fig. 1 and table 1), yet

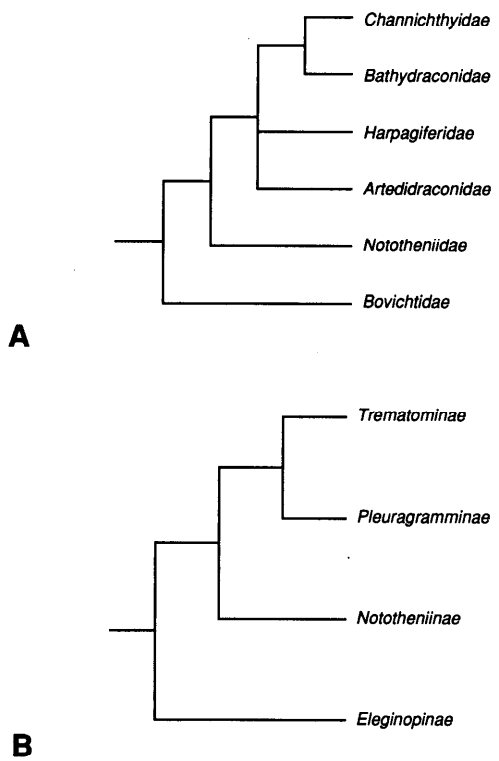

FIG. 1.—*A*, Traditional phylogenetic hypothesis, from Eastman (1993), of fishes of the suborder Notothenioidei, based on a cladistic analysis of morphological characters. *B*, Traditional family-level phylogenetic hypothesis showing relationships among the subfamilies in the family Nototheniidae, based on morphological characters which were not analyzed cladistically (Andersen 1984; Balushkin 1984).

Table 1
Species Included in This Study from Which mtDNA Sequences Were Determined, and Their Current Systematic Position (based on De Witt et al. 1990; Eastman 1993)

Order Perciformes
- Suborder Notothenioidei
  - Family Bovichtidae
    - *Bovichtus variegatus*
  - Family Nototheniidae
    - Subfamily Trematominae
      - *Trematomus eulepidotus*
      - *T. hansoni*
      - *T. nicolai*
      - *T. pennellii*
      - *Pagothenia borchgrevinki*
    - Subfamily Nototheniinae
      - *Notothenia coriiceps neglecta*
      - *Gobionotothen gibberifrons*
    - Subfamily Eleginopinae
      - *Dissostichus mawsoni*
  - Family Artedidraconidae
    - *Histiodraco velifer*
    - *Pogonophryne scotti*
  - Family Bathydraconidae
    - *Gymnodraco acuticeps*
    - *Cygnodraco mawsoni*
    - *Parachaenichthys charcoti*
  - Family Channichthyidae
    - *Chaenocephalus aceratus*
    - *Chionodraco hamatus*
    - *Cryodraco antarcticus*
    - *Pagetopsis macropterus*
- Suborder Zoarcoidei
  - Family Zoarcidae
    - *Lycodichthys dearborni*
    - *Pachycara brachycephalum*

their monophyly has only recently been demonstrated by cladistic methodology (Iwami 1985). Notothenioid fishes are believed to have evolved from an ancestor, endemic to the Antarctic, that gradually adapted to the cooling conditions and finally radiated and successfully filled many ecological niches in Antarctic waters (Eastman 1993, p. 28). Unfortunately, no fossil record exists to aid in the understanding of the evolution of the present fish fauna (reviewed in Eastman 1993, pp. 23–38). Phylogenetic hypotheses based on molecular characters might allow one to estimate tempo and mode of evolution and the times of origin for particular clades, which is not possible for phylogenies based on morphology. This study provides a molecular phylogenetic hypothesis for fishes of this suborder and attempts to establish a temporal perspective for the evolutionary history of notothenioids.

Material and Methods

MtDNA sequences were determined for 18 notothenioid species (*Pagothenia borchgrevinki* and *Trema-*

Table 2
Matrix of Sequence Differences for the Partial 12S and 16S Mitochondrial Ribosomal Genes

	1	2	3	4	5	6	7	8
1. *Bovichtus varigatus*		15.4	15.9	15.6	16.1	15.3	14.9	15.
2. *Trematomus eulipidotus*	134/41		0.9	0.5	1.1	0.9	3.4	3.
3. *T. hansoni*	138/42	8/1		1.1	1.4	1.4	3.8	4.
4. *T. nicolai*	135/41	4/0	10/1		1.1	0.9	3.4	3.
5. *T. pennellii*	140/45	10/4	12/3	10/4		1.4	3.8	4.
6. *Pagotheniz borchgrevinki*	133/41	8/2	12/3	8/2	12/6		3.4	3.
7. *Notothenia coriiceps*	129/42	30/9	33/10	30/9	33/13	30/11		2.
8. *Gobionotothen gibberiforns*	133/44	34/7	35/8	32/7	38/11	32/9	17/6	
9. *Dissostichus mawsoni*	137/44	40/9	39/10	38/9	42/13	36/11	24/6	19,
10. *Histiodraco velifer*	127/40	36/7	40/8	36/7	41/11	36/9	20/6	22,
11. *Pogonophryne scotti*	127/41	34/6	38/7	34/6	39/10	34/8	18/5	20,
12. *Gymnodraco acuticeps*	127/44	36/9	38/10	34/9	39/13	34/11	18/6	19,
13. *Cygnodraco mawsoni*	137/45	49/12	50/13	49/12	48/14	47/14	33/11	38,
14. *Parachaenichthys charcoti*	136/44	46/11	48/12	46/11	49/15	45/13	32/10	37,
15. *Chaenocephalus aceratus*	128/42	37/13	39/14	38/13	42/17	39/15	25/12	26,
16. *Chlonodraco hamatus*	126/42	38/11	40/12	39/11	43/15	36/13	24/10	25,
17. *Cryodraco antarcticus*	127/42	36/11	38/12	37/11	41/15	38/13	24/10	25,
18. *Pagetopsis macropterus*	128/43	41/12	43/13	40/12	46/16	41/14	26/11	28,
19. *Lycodichthys dearborni*	141/55	112/41	111/42	113/41	116/45	114/43	102/38	102
20. *Pachycarz brachycephalum*	140/53	107/39	106/40	108/39	112/43	109/41	97/36	97

NOTE.—Values above the diagonal are uncorrected percentage differences including substitutions; below are the total number of substitutions and transversions.

tomus hansoni two individuals each were sequenced) (table 1). Specimens were preserved in 70% ethanol and total genomic DNA was extracted from white muscle, liver, or spleen tissue by proteinase K/SDS dissolution and purified by phenol:chloroform extraction and ethanol precipitation (Kocher et al. 1989; Sambrook et al. 1989). The polymerase chain reaction (PCR) (Saiki et al. 1988) was used to amplify two segments, one of the large (16S) and one from the small (12S) mitochondrial ribosomal genes. Double-stranded amplifications were performed in 25-μl vol containing 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 1 mM of each dNTP, 1 μM of each primer, 10–1,000 ng genomic DNA, and 0.5 units of Taq polymerase (Cetus Corp.). DNA sequences of the PCR primers used have been published elsewhere (Kocher et al. 1989; Palumbi et al. 1991). Gel purification (2.5% NuSieve-Agarose, in TAE buffer) of the double-stranded PCR products was followed by generation of single-stranded DNA of both strands for direct sequencing, using asymmetric PCR (Gyllensten and Erlich 1988). Single-stranded DNA was concentrated and desalted in spin columns (Millipore: Ultrafree-MC 30,000), and both strands were sequenced by the dideoxy method using a commercial kit (Sequenase, United States Biochemical). Three hundred seventy-five base pairs of the 12S gene and 553 bp of the 16S gene were determined (for a total of 928 bp for each of the 20 taxa) (GenBank accession numbers Z32702-Z32739 and Z32747-Z32748). The orthologous DNA sequences were aligned with a multiple sequence editor (ESEE; see Cabot and Beckenbach 1989) and by CLUSTAL, using default settings (Higgins and Sharp 1988). The alignment was unambiguous, with the exception of a 45-bp region in the 16S sequence (positions 223–267 in the data set); this region was judged not to be well aligned and hence, excluded from the phylogenetic analyses.

Phylogenetic analyses were performed using maximum parsimony (MP) implemented in PAUP (Swofford 1993). Because of the large number of taxa, heuristic search procedures were necessary to search for the most parsimonious tree(s). The reliability of the heuristic searches was improved by using the option "random addition of taxa" with 100 replications in PAUP. Several different character-weighting schemes were used. Analyses were done either with transitions and transversions equally or with transversions only. A priori weighting schemes included weighting transversions more than transitions (2:1 and 3:1 according to the observed ratio between outgroup and ingroup taxa and within-ingroup taxa, respectively). A successive-approximation approach to weighting of characters was also performed according to their "cladistic reliability" (Farris 1969; Williams and Fitch 1989). Reweighting was based on both the mean rescaled and the consistency index of all equally parsimonious trees found in parsimony analysis and a base weight of 1,000. In addition, we estimated the frequency of the six classes of nondirectional mu-

9	10	11	12	13	14	15	16	17	18	19	20
14.6	14.6	14.6	15.8	14.7	14.7	14.5	14.6	14.7	16.2	16.1	
4.1	3.9	4.1	5.6	5.3	4.2	4.4	4.1	4.7	12.9	12.3	
4.5	4.6	4.4	4.4	5.7	5.5	4.5	4.6	4.4	4.9	12.8	12.2
4.4	4.1	3.9	3.9	5.6	5.3	4.4	4.5	4.3	4.6	13.0	12.4
4.8	4.7	4.5	4.5	5.5	5.6	4.8	4.9	4.7	5.3	13.3	12.9
4.1	4.1	3.9	3.9	5.4	5.2	4.5	4.1	4.4	4.7	13.1	12.5
2.8	2.3	2.1	2.1	3.8	3.7	2.9	2.8	2.8	3.0	11.7	11.2
2.2	2.5	2.3	2.2	4.4	4.2	3.0	2.9	2.9	3.2	11.7	11.2
	3.1	2.9	2.8	4.2	4.6	3.4	3.2	3.4	3.8	11.6	11.3
27/6		0.5	1.6	3.2	3.3	2.0	2.1	2.1	2.4	12.3	11.7
25/5	4/1		1.4	3.9	3.2	2.0	1.8	1.8	2.2	11.9	11.3
24/6	14/6	12/5		2.9	3.1	1.5	1.4	1.4	1.7	12.0	11.4
37/9	28/9	25/8	25/7		3.2	3.4	2.9	3.3	3.7	12.4	12.0
40/8	29/8	287	27/6	28/7		3.6	3.0	3.3	3.7	12.9	12.3
30/10	17/8	17/9	13/8	30/11	31/8		0.8	0.3	1.1	12.4	11.6
28/8	18/8	16/7	12/6	25/9	26/6	7/2		0.5	0.8	12.3	11.5
30/8	18/8	16/7	12/6	29/9	29/6	3/2	4/0		0.8	12.3	11.5
33/9	21/9	19/8	15/7	32/10	32/7	10/3	7/1	7/1		12.2	11.5
101/40	107/42	103/41	104/38	108/45	112/44	108/44	107/42	107/42	106/43		0.9
98/38	102/40	98/39	99/36	104/43	107/42	101/42	100/40	100/40	100/41		

tations (A-G, T-C, A-C, A-T, G-C, G-T) with two different methods. From our data set 1,000 random-joining trees were constructed with MacClade (Maddison and Maddison 1992) and unambiguous character changes were counted in all trees; the mean change frequency for each class was corrected for base composition and the reciprocal of this value was applied as weight. An alternative approach, a modified version of the best-fit rate test (Knight et al. 1993), was also used. Here, the total number of mutations in all pairwise comparisons was scored; under the null hypothesis of equal probability the expected number of mutations (E) for each class represented 1/6 of the total number with the only correction for the base composition of each class. The number of observed mutations per class (O) was calculated in all pairwise comparisons, and E/O ratio of each class was used as weight in the MP analyses.

Neighbor-joining (NJ) analyses (Saitou and Nei 1987) were performed with MEGA (Kumar et al., 1993). Different methods were used to estimate evolutionary distances that accounted for multiple substitutions: Jukes-Cantor (Jukes and Cantor 1969), Kimura's two-parameter model (Kimura 1980), and Tamura-Nei's method (Tamura 1992). We also performed NJ analyses considering both transitions and transversions or transversions only.

Also, previously published hemoglobin sequences (di Prisco et al. 1991; Fago et al. 1992) were phylogenetically analyzed. An MP analysis was performed using the PROTPARS option in PAUP (Swofford 1993). The shortest trees were found using the branch and bound search. Statistical confidence of MP and NJ evolutionary trees was assessed using bootstrapping (Felsenstein 1985) with 400 replications for each method.

Results and Discussion

Phylogenetic Results

Table 2 shows the pairwise percentage sequence divergence and number of nucleotide substitutions that were observed in this study; of the 883 nucleotides analyzed, 217 sites were variable and 136 sites were phylogenetically informative. The MP analysis yielded a phylogenetic topology that, at the level of relationships among families, is in good agreement with the traditional noncladistic (Eakin 1981) and cladistic analyses (Eastman 1993, p. 108; Iwami 1985) of morphological and physiological characters (figs. 1*A*, 2). In the MP analysis a priori and a posteriori (successive weighting; see Farris 1969) weighting yielded essentially the same results. The only difference that resulted from different weights for transitions and transversions in the MP analyses is the position of the genus *Notothenia* (fig. 2). When transversions are weighted a priori more strongly than transitions, *Notothenia* is identified to be the sister to the clade *Dissostichus* + *Gobionotothen,* the same grouping that was determined in the NJ tree (fig. 3). Generally, MP results were closely matched by the NJ analysis (fig. 3); in addition, all methods to calculate evolutionary distances in several NJ analyses resulted in identical topologies.

In agreement with the traditional phylogeny, the molecular data strongly suggest that the family Bovichtidae is the most basal clade of the suborder. It is, in terms of DNA divergence, almost as distantly related to the other notothenioid families as the outgroups used in this study, the family Zoarcidae (eelpouts) (fig. 2 and table 1). The monophyly of the suborder is also strongly supported by our molecular data. Members of the family Zoarcidae are represented in Antarctica with 22 species, but most species in this group live outside Antarctica. Other fishes, for example, blennioids and gadiforms, are also about equally distant to the notothenioids as the eelpouts (data not shown).

The monophyly of the families Artedidraconidae and Channichthyidae seems well supported by the molecular data (figs. 2, 3). The major difference between the molecular phylogeny and the traditional one is the systematic position of the family Nototheniidae and the relationship among nototheniid species. The monophyly of this family is not supported by MP or NJ analyses of mtDNA sequences (figs. 2, 3). The molecular phylogeny suggests that the family status of the Nototheniidae and even that of the whole suborder Notothenioidei might be in need of revision since here the subfamily Notothenioinae was identified as the sister group to all notothenioids (Artedidraconidae + Bathydraconidae + Channichthyidae). Although the bootstrap values were not high (fig. 4), the MP analysis of hemoglobin protein sequences also suggests that the family Nototheniidae is a paraphyletic assemblage (fig. 4). Some genera that have been traditionally assigned to the family Nototheniidae (*Notothenia, Dissostichus,* and *Gobionotothen*) (table 1) appear to be more closely related to the Artedidraconidae + Bathydraconidae + Channichthyidae clade than to species of the genera *Trematomus* and *Pagothenia.*

The classical taxonomy of the notothenioids has always been controversial (Andersen 1984). The DNA data further suggest that the classification and the phylogenetic relationships among the four subfamilies in the family Nototheniidae might need to be revised (figs. 1*B*, 2, 3 and table 1). All molecular data suggest that the genera *Dissostichus* and *Gobionotothen,* traditionally assigned to distinct subfamilies, the Eleginopinae and the Nototheniinae (table 1 and fig. 1*B*), are more closely related to each other than *Notothenia* and *Gobionotothen* are to each other (table 1). This is surprising, since *Dissostichus,* a large pelagic piscivorous predator, is the sister group to *Gobionotothen,* a small benthic species. This grouping would imply that major shifts in ecology can occur within small evolutionary time spans. *Pagothenia* is the sister group of *Trematomus* (figs. 2, 3) in agreement with earlier data (Andersen 1984; McDonald et al. 1992; Ritchie 1993). *Pagothenia* is a specialized cryopelagic species; its ecology is closely linked to the ice cover of Antarctica (Kennett 1977).

The phylogenetic position of *Gymnodraco* as a member of the family Bathydraconidae is unresolved in the MP analysis; the NJ analysis suggests that it is the sister group to the Channichthyidae (figs. 2, 3). This finding would indicate that the family Bathydraconidae might also not be a natural group, that is, it is a paraphyletic assemblage. Unfortunately, representatives of the family Harpagiferidae were not represented in this study; this family had, by various authors, been considered to be a subfamily of the family Artedidraconidae, and its relationship to the other notothenioid families remained unclear on the basis of morphological data (Gon and Heemstra 1990; Eastman 1993, p. 108).

The Loss of Hemoglobin

Vertebrates typically have several forms of hemoglobin, which differ in their oxygen-binding properties allowing for survival under differing environmental and physiological conditions (Wells et al. 1980). In notothenioid fishes a general trend toward a reduction in the number of different hemoglobin forms and low amounts of variation in the amino acid sequences of the remaining hemoglobin has been observed. The more derived notothenioids, the harpagiferids, artedidraconids, and bathydraconids, have only one type of hemoglobin (di Prisco et al. 1991). On the basis of the traditional assumption of an old age for these fishes (more than 38 Mya), the constancy of environmental conditions has been proposed to be a stringent selective force acting to keep the amount of variation in hemoglobins of notothenioids low (Fago et al. 1992). Alternatively, the observed low hemoglobin diversity among notothenioids might be explained by a recent origin of these fishes, which would not have allowed enough time for hemoglobin sequences to diverge (Fago et al. 1992) (see below).

As a presumed adaptation to the low-temperature environment in Antarctica, notothenioids have a low hematocrit that reduces the viscosity of their blood (Wells et al. 1980). An extreme reduction of the viscosity is achieved in one of the most derived groups of notothenioids, the icefishes (family Channichthyidae), which have no hemoglobins at all. In addition to decreasing the viscosity, these white-blooded fishes are colorless, decreasing their visibility to predators (Ruud 1965). Whether or not the loss of hemoglobin should be interpreted as an adaptive or nonadaptive feature is unclear (Wells 1990). The loss of hemoglobin as an oxygen carrier was counteracted through compensatory modifications of the cardiovascular system (e.g., the cardiac output rate is extremely large, the pumped blood

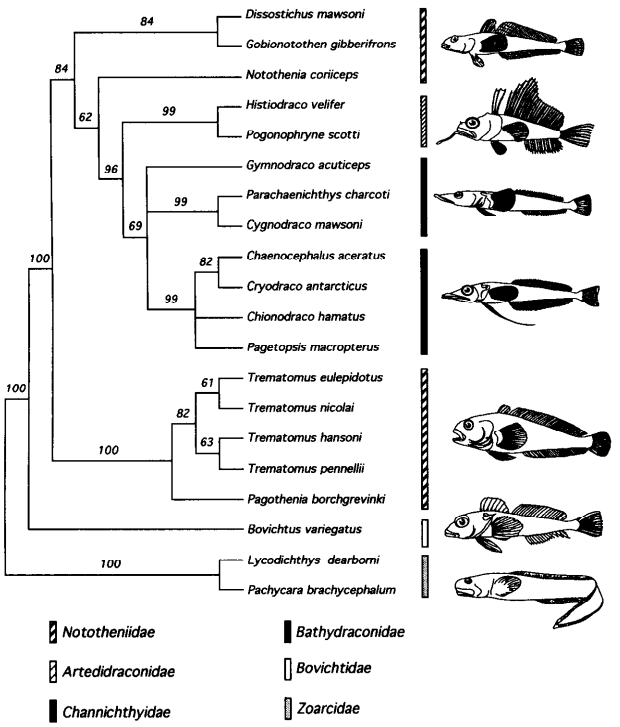

FIG. 2.—50%-majority-rule consensus MP bootstrap tree based on a posteriori weighting method (Farris 1969; Williams and Fitch 1989). An MP analysis with all characters unweighted yielded 15 shortest trees of a length of 344 steps (CI = 0.791; rescaled CI = 0.635). Numbers indicate bootstrap values (Felsenstein 1985). A 50%-majority-rule consensus tree of the most parsimonious trees yielded a topology in agreement (with one more polytomous node) with the tree shown. The bars in this figure and in figs. 3–5 indicate the current classification (according to Gon and Heemstra 1990) of the fishes into families. The outlines of the fish (redrawn from Eastman 1993), from top to bottom, are *Gobionotothen gibberifrons, Histiodraco velifer, Gymnodraco acuticeps, Cryodraco antarcticus, Trematomus hansoni, Bovichtus variegatus,* and *Lycodichthys dearborni.*

volume is two to four times larger than that of other teleosts), ensuring the transport of oxygen to tissues (Eastman 1993, p. 237).

The Bohr effect is the lowered oxygen affinity in hemoglobin induced through a lowered pH. It is more pronounced in active than in sedentary species. *Gymnodraco acuticeps,* a sluggish sit-and-wait predator, is the only known fish without a Bohr effect, and it has only one type of hemoglobin (di Prisco et al. 1990, 1991). The NJ analysis of the DNA sequences (fig. 3) identifies *Gymnodraco* as the sister group to the Channichthyidae. Therefore, the molecular data suggest that the family Bathydraconidae may not be a natural group, that is, *Gymnodraco* may actually be more closely related to the Channichthyidae than to other members of the family Bathydraconidae. This species could hence represent a phylogenetic and physiological intermediate condition between the possession of hemoglobin in basal notothenioids and the loss of hemoglobin in the derived icefishes, the Channichthyidae (figs. 2, 3) (Eastman 1993, p. 236). The molecular phylogeny might provide a framework on the basis of which the evolution and the biological significance of amino acid substitutions in hemoglobin can be studied.

The Evolution of the AFGPs

At least four different types of AF peptides have been identified in fishes belonging to many distinct taxonomic groups (Scott et al. 1986; Davies and Hew 1990) (fig. 5). Although acting in a similar way through a noncolligative ice growth reduction effect, there is clear evidence that this character evolved repeatedly in separate taxa. Notothenioid AFs have been investigated in great detail by DeVries and co-workers (Cheng and DeVries 1991 and references therein). Antifreezes consist of eight distinct AFGPs, different in molecular weight and composed of variable number of repeats of a basic triglycopeptide unit. Molecular (Hsiao et al. 1990) and biochemical evidence suggests that AFGPs are encoded by a gene family. The presence of AFGPs has been documented in all notothenioids tested so far, with the only exceptions being the family Bovichtidae and three species

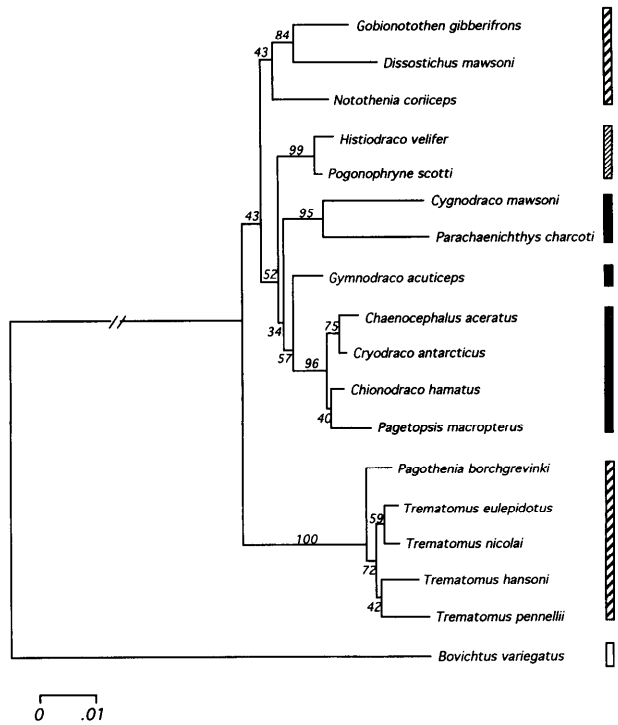

FIG. 3.—Neighbor-joining phylogenetic hypothesis based on a distance matrix that was corrected for multiple substitutions based on the Kimura two-parameter model. The bar indicates 1% corrected sequence distance, and the branches are drawn according to the number of inferred substitutions. Numbers indicate bootstrap values (Felsenstein 1985).

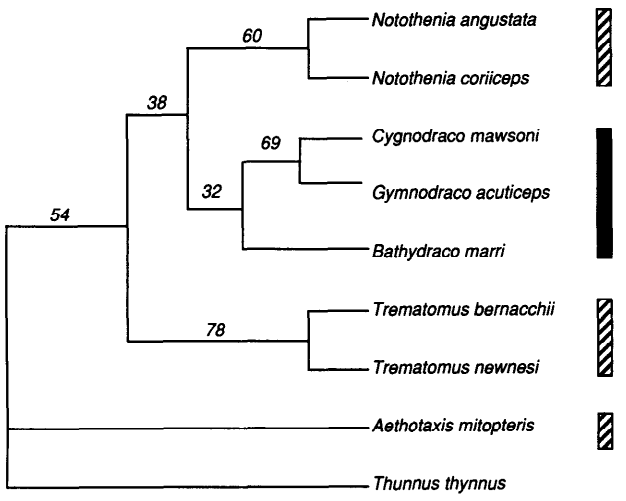

FIG. 4.—Majority-rule consensus MP (Swofford 1993) bootstrap tree of notothenioid fishes, based on published hemoglobin sequences (DiPrisco et al. 1991; Fago et al. 1992). The bluefin tuna (*Thunnus thynnus*) was used as outgroup in this analysis.

from three genera, *Dissostichus eleginoides, Lepidonotothen kempi,* and *Notothenia angustata* (Eastman 1993). Interestingly, these taxa without AFGPs are found outside the subzero Antarctic waters.

The evolution of AFGPs was investigated, on the basis of our molecular phylogeny, with MacClade (Maddison and Maddison 1992) (fig. 5). The resulting pattern suggests that AFGPs originated at a single point after the separation of the family Bovichtidae but prior to the diversification of the remaining groups and were subsequently lost independently at least twice in genera that are variable with respect to the presence of AFGPs (fig. 5). The hypothesis of a single origin of AFGPs is also in agreement with the molecular hypothesis of a recent radiation of notothenioids. Since the water temperature only dropped to 0°C in the middle and late Miocene (about 10–15 Mya) (Kennett 1977; Clarke 1990), the evolution of AFGPs might be a recent event enabling the notothenioids to survive the freezing temperature. The AFGPs might be one of the factors contributing, possibly as a "key innovation," to their evolutionary success.

Tempo of Molecular Evolution and the Age and Biogeographic History of the Notothenioid Radiation

Knowledge of the date of origin of notothenioids would aid in the identification of the evolutionary forces that might have permitted these fishes to speciate and to occupy many ecological niches in Antarctica. Age estimates are important in the determination of the likely paleoenvironmental conditions that might have prevailed during the notothenioid radiation. Fossils of notothenioid fishes are lacking and hence cannot assist in the reconstruction of the phylogeny of these fishes and the calibration of the rates of molecular evolution.

Biogeographic evidence, the occurrence of a few nototheniids, and harpagiferids outside the Antarctic Convergence were used to argue for the origin of the suborder prior to the isolation of Antarctica around the boundary of the Eocene and Oligocene (about 38 Mya) (see Eastman 1993, p. 126). Since the bovichtids are only represented by a single species in Antarctica and because they are distant from other notothenioids in molecular terms (between 14.6%–16.1% uncorrected sequence divergence; see table 2), we do not consider them to be a part of that radiation. The only species of the Bovichtidae found in Antarctica is likely to have immigrated to this region recently. Within the family Nototheniidae the subfamily Trematominae are exclusively found in Antarctica. The molecular phylogenies (figs. 2, 3) suggest that they are the sister group to all other notothenioid species. This suggestion might imply that the species currently classified as members of the family Nototheniidae that are found outside Antarctica are likely to have originated in Antarctica and emigrated

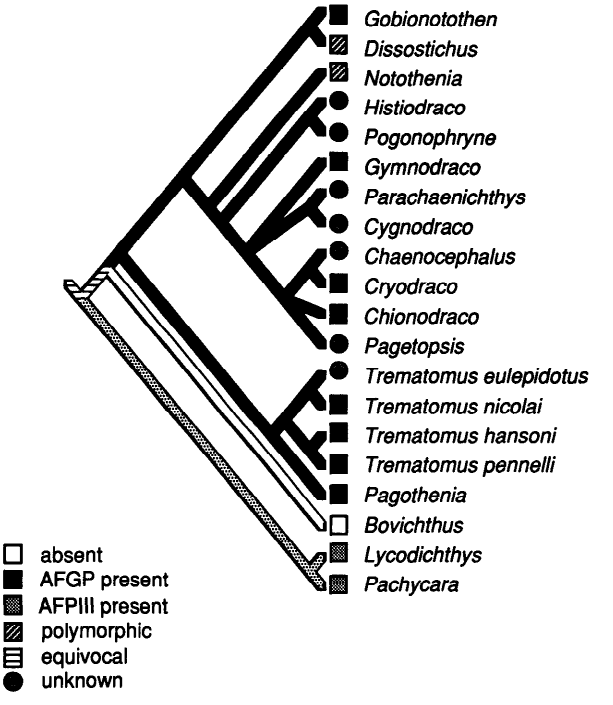

FIG. 5.—Tracing of the evolution of different antifreeze glycopeptides with MacClade (Maddison and Maddison 1992). Phylogenetic topology is as in fig. 2. Circles indicate genera for which the presence of antifreeze glycopeptides has not been tested. The character reconstruction based on the phylogeny indicates that AFGPs are likely to have evolved only once in the common ancestor of all notothenioids (with the exception of the family Bovichtidae). AFGPs might have been lost at least twice independently in those members of the genera *Dissostichus* and *Notothenia* that live outside Antarctica.

from it, instead of having originated outside the Antarctic Convergence and then immigrated to it.

The DNA sequence variation detected in these two mitochondrial genes among the major groups of notothenioid fishes (all members of the suborder except the Bovichtidae) does not exceed 6% (uncorrected for multiple substitutions) total DNA divergence and 1.7% transversion differences. These values are surprisingly small, as are the internode distances (fig. 3), suggesting that the notothenioid diversification could have occurred more rapidly and recently than previously thought.

Typically, overall mtDNA evolution is believed to occur at a rate of 1%–2% million years (Myr) (DeSalle et al. 1987 and references therein). Transversion mutations accumulate more slowly and linearly over time (DeSalle et al. 1987; Irwin et al. 1991) in ungulates at a divergence rate of about 0.14%/Myr (Allard et al. 1992). If an ungulate transversion rate is assumed for notothenioid fishes, the origin of their radiation would be estimated to be less than 12 Mya. That date would be an underestimate if the rate of molecular evolution in notothenioids is slower than that of ungulates. However, the notothenioid rate would have to be extremely slow for it to be brought into agreement with the traditional estimate of origin of these fishes. If an origin of 38 Mya is assumed, the transversion rate would be about three times slower than the ungulate rate (less than 0.05% Myr) and the overall rate would be only between 0.15–0.2%/Myr, which would be 10–20 times slower than the typically assumed rate of mtDNA evolution.

Recently, the rate of mtDNA evolution has been tied to metabolic rate, and it was suggested that ectotherms might have a slower rate of DNA and amino acid evolution than endotherms (Kocher et al. 1989; Martin et al. 1992; Martin and Palumbi 1993; Rand 1993, 1994). A typical ectotherm divergence rate of mtDNA evolution ranges between 0.3–0.7%/Myr (Martin et al. 1992). The metabolic rate of Antarctic fishes in absolute terms is lower than that of temperate fishes (reviewed in Eastman 1993, pp. 147–155) and certainly lower than typical endotherm metabolic rates. If there were a causal link between metabolic rate and the rate of molecular evolution (e.g., through oxidative damage by radicals; Richter et al. 1988), it might not be surprising that rates of molecular evolution might be slow in notothenioid fishes even compared with other ectotherms (Martin et al. 1992; Martin and Palumbi 1993; Rand 1993, 1994).

Recent allozyme work on nototheniid fishes (McDonald et al. 1992) found little variation within and among species of the genus *Trematomus,* suggesting that they are only about 2.4–1.3 Myr old. Likewise, the mtDNA sequence divergence in the *Pagothenia* + *Trematomus* clade was only about 0.5%–1%, suggesting an age of 1–3 Myr (assuming a typical DNA clock rate) for this genus (fig. 3). The topologies of the allozyme and mtDNA phylogenies also agree for the species included in both studies (figs. 2, 3). The allozyme data (McDonald et al. 1992) estimated that the split between *Dissostichus* and *Trematomus* is much older (28–20 Mya) than the age of the genus *Trematomus* itself. Divergence time estimates may be risky since "allozyme clocks" are not reliable timekeepers (Avise and Aquadro 1982). *Trematomus* differs from *Dissostichus* by only 4.5%–5% mtDNA sequence divergence.

If, prima facie, the rate of mtDNA evolution in notothenioids is assumed to be comparable to that of other ectotherms, the age estimate of that split would be only about 7–15 My old. Since this is the deepest cladogenic event in the notothenioid phylogeny (figs. 2, 3) it would seem to suggest that the notothenioid radiation started not earlier than the middle to late Miocene, a much younger age for the whole radiation than had been traditionally assumed (about 38 Mya). At this time the Antarctic ice sheet developed and the water temperatures dropped significantly to the 0°C range (Kennett 1977; Clarke 1990). If the hypothesis for the recent origin of notothenioid fishes is corroborated, the evolution of these species was possibly associated with a different set of ecological and paleoenvironmental factors than thought before. The adaptive diversification of the notothenioid fishes might have proceeded at a much more rapid pace then previously thought.

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