Metallothioneins in Antarctic Fish: Evidence for Independent Duplication and Gene Conversion

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In the present paper, we examine eight species of Antarctic fish belonging to the suborder Notothenioidei, using reverse-transcriptase polymerase chain reaction, to investigate the presence of mRNAs encoding metallothionein (MT) isoforms. A total of 168 bp from the coding region and the complete (133–165 bp) 3′ untranslated region (UTR) was obtained for all species (for three of them, we also sequenced the full-length cDNA, including the 5′ UTR). Phylogenetic analyses carried out on the MT-coding region suggest monophyly for Antarctic fish MTs with respect to other teleost MT genes. Analyses also revealed that notothenioid MTs can be divided into at least two groups of paralogy, MT-1 and MT-2. These results indicate that notothenioid MT isoforms arose from at least one gene duplication event occurring in the ancestral lineage of the Notothenioidei. This duplication occurred independent of the one which gave origin to two metallothionein isoforms in the rainbow trout. In addition, an instance of gene conversion was observed between MT-1 and MT-2 genes in *Notothenia coriiceps.* Analyses of the 5′ UTR, combined with quantitative assay of differential expression of MT-1 and MT-2, indicate that only the 3′ UTR underwent a gene conversion event in the mentioned species. These findings, together with the observation of a differential pattern of expression for the two MT isoforms, disclose an unexpected complexity in the evolution and function of notothenioid MTs; as in most teleost species examined (apart from the rainbow trout), a single MT form is present.

Introduction

It has been proposed that genes of extant species have evolved from ancestral sequences by duplications, mutations, and internal rearrangements of the genomic material (Ohno 1978). Paradigmatic is the case of metallothioneins (MTs), cysteine-rich metal-binding proteins which are believed to play an essential role in regulating the intracellular concentration of the ionic forms of zinc and copper and in protecting cells from the deleterious effects of harmful heavy metals such as cadmium and mercury (Kägi et al. 1981; Kägi and Schaffer 1988; Kägi 1993). In mammals, four MT isoforms have been found: MT-1 and MT-2 are the major ones, being ubiquitous, although differently accumulated in the various tissues (Kägi 1991, 1993); MT-3 is mostly expressed in the nervous system (Masters et al. 1994), whereas MT-4 is abundant in the epithelium (Quaife et al. 1994). For humans and other mammals, a large number of MT isoforms have been isolated, apparently originating from events of gene duplication which may have occurred many times independently (Griffith et al. 1983; Hunziker et al. 1995). An example of the high multiplicity of MT isoforms is offered by the observation that in humans, at least 10 distinct MT-1-type genes have been identified as part of a single gene cluster on chromosome 6, adjacent to MT-2, MT-3, and MT-4 genes (Quaife et al. 1994). However, although MT isoforms

might differ in amino acid sequence, in general they do not vary in cysteine content and metal-binding capacity (Winge et al. 1984; Ebadi and Babin 1989).

Compared with the multiplicity of mammalian MTs, a different situation is observed in lower vertebrates, in which only one or two distinct MTs have been isolated in each species examined (Fernando, Wei, and Andrews 1989; Roesigadi 1993), thus suggesting that the evolution of MT genes may have followed different pathways in different groups of taxa. In the teleost fish, for example, apparently only one MT gene is expressed (Hamer 1986, Kägi 1993; Chan 1994), with the single exception of rainbow trout, which shows two MT isoforms (Bonham and Gedamu 1984; Zafarullah, Bonham, and Gedamu 1988). This observation might be explained, at least in part, by the scarcity of data on teleost MTs, but it also may indicate a strikingly different evolutionary history of MTs in the teleost fish. In either case, a comparative study of MTs focusing on several closely related fish species might shed some light on evolution of MTs in teleosts. In particular, sequence analysis of piscine MTs might contribute to answering the question of whether multiple MT genes are present in teleosts, and, if this is the case, how multiplicity arose.

As mentioned above, in mammalian species, MT isoforms are expressed differentially in different tissue, and the expression of MT proteins is regulated at both the transcriptional and the translational levels. A further question is therefore whether this also holds true for lower vertebrates and what the relationship is between sequence and expression of the different isoforms. Moreover, a study on MT evolution in the teleost fish might be extremely valuable, because teleosts diverged early during the evolution of vertebrates, as part of the actynopterigian lineage.

In the present paper, we attempt to address the above questions by investigating the pattern of MT evolution in a group of closely related teleost fish, the Notothenioidei. They form a perciform suborder, comprising more than 120 species, mostly endemic to the waters surrounding Antarctica (Eastman 1993). Notothenioids show remarkable modifications of their anatomy and physiology in relation to the peculiar conditions of the Antarctic environment, namely the extreme water temperature ($-2°C$) and the presence of sea ice. Among other characteristics, most notable are the expression in all body fluids of antifreeze glycopeptides (which depress the freezing point, allowing notothenioids to cope with the cold temperature; DeVries 1988), and the reduction of hematocrit and hemoglobin content (to contrast the increased blood viscosity; MacDonald and Wells 1991). The tendency toward reduction of oxygen carriers reaches its extreme with the notothenioid family Channichthyidae (icefishes), which are the only vertebrates completely lacking hemoglobin (Ruud 1954).

The ecological importance of notothenioids in the Antarctic marine ecosystem and their remarkable adaptations to this extreme environment have meant that great efforts have been devoted to studying notothenioid physiology, producing a great wealth of data at the anatomical, functional, and biochemical levels. With regard to MTs, recent studies showed a peculiar transcriptional and posttranscriptional control in the expression of MT genes in both white-blooded and red-blooded notothenioids. In fact, differential expression of MT isoforms has been reported, together with the observation in some tissues of absence of MT at the protein level in the presence of MT mRNA expression (Scudiero et al. 1997; Carginale et al. 1998). Moreover, similar to what has been observed for hemoglobin, another striking feature has been described for the icefish: the lack of endogenous MTs in liver (Scudiero et al. 1997), contrary to the general pattern in vertebrates. In addition, phylogenetic relationships among notothenioid taxa have been reconstructed on the basis of morphological (see Eastman 1993) and molecular data (Bargelloni et al. 1994; Chen, Bonillo, and Lecointre 1998). From all of the above, it is apparent that notothenioids, which have also diversified quite recently (10–15 MYA; Bargelloni et al. 1994), likely from a single ancestor, represent the ideal subject for evolutionary studies at the comparative level.

In the present paper, we examine eight species of notothenioids using reverse-transcriptase polymerase chain reaction (RT-PCR) to detect the presence of transcripts encoding distinct metallothionein isoforms. The results obtained show the existence of two MT isoforms in each of the fish species examined, and phylogenetic analyses on notothenioid MTs, together with the MT nucleotide sequences available for other teleost fish, indicate that at least two paralogous MT genes are present in notothenioids. The present data suggest that this multiplicity originated after divergence of the notothenioid ancestor from the other fish lineages examined, and that this event is distinct from the duplication which gave origin to MT isoforms in salmonids.

Materials and Methods
Samples

Adult fish were collected in the proximity of Terra Nova Bay Station (Italy) and in Palmer Station (U.S.A.) in Antarctica and kept in aquaria supplied with aerated seawater at approximately $-1°C$. Specimens were sacrificed, and samples of liver and brain tissue were quickly removed, frozen in liquid nitrogen, and stored at $-70°C$ until the analysis. Total RNA was isolated from frozen livers by the method of Chomczynski and Sacchi (1987). RNA concentration and purity were analyzed spectrophotometrically.

Reverse Transcription of RNA and PCR Amplification of MT cDNA

First-strand cDNA was synthesized from 5 μg total RNA. Briefly, RNA was denatured at 70°C for 3 min, mixed with 10 pmol of dNTPs, 20 U of RNasin (Promega), 50 pmol Oligo(dT)-adapter primer (5′-CGGA-GATCTCCAATGTGATGGGAATTC(T)$_{17}$-3′) (synthesized by Pharmacia Biotech.), and 200 U of M-MLV reverse transcriptase (Promega), and incubated for 2 h at 42°C. The reaction was stopped by heating at 65°C for 5 min. The reverse transcription mixture was amplified by PCR using as primers the 20mer N-terminal primer PK70 (5′-AAATGGATCCCTGCGAITGY-3′, where I = inosine and Y = C + T), derived from the N-terminal amino acid sequence of piscine MT, and the adapter primer described above. Amplification of reverse-transcriptase mixture, containing single-stranded cDNA derived from 0.5 μg of total RNA, was performed with 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), 50 pmol of each of the above primers, and 0.2 mM dNTPs (final concentration) in 10 mM Tris/HCl (pH 8.3) containing 50 mM KCl and 1.5 mM MgCl$_2$. Following a 3-min denaturation at 95°C, the PCR steps consisted of 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, followed by 15 min at 72°C and a 4°C hold.

Cloning and Sequencing of PCR-Amplified cDNA

The PCR fragments were purified using the Qiaquick gel extraction kit (Qiagen). After purification, the PCR-amplified cDNAs were ligated in the pGEM-T vector (Promega) with T4 DNA ligase at 16°C overnight. *Escherichia coli* (strain TG2) cells were transformed with the ligation mixture. The recombinant clones were used for nucleotide sequencing according to the method of Sanger, Nicklen, and Coulson (1977) using T7 DNA polymerase. All sequences were determined on both strands.

Rapid Amplification of 5′ cDNA Ends (5′-RACE)

A 5′-RACE protocol was carried out using a Marathon cDNA amplification kit (Clontech Laboratories) in order to obtain the 5′ untranslated region (UTR) of *Notothenia coriiceps, Chaenocephalus aceratus,* and *Trematomus bernacchii* MT mRNAs. Single-stranded cDNA was obtained as described above, and second-strand synthesis was performed according to manufacturer's instructions. The resulting double-stranded DNA was ligated

with the Maratahon adapter overnight at 16° by T4 DNA ligase. A portion of the ligated DNA (1/20) was then used as template for a PCR reaction using as primer IN2 (5'-GCAGGAGGCAGTTTGTGCAAGTGCA-3'), which is complementary to bases 52–75 of the fish MT-coding sequence. PCR conditions were set according to Marathon kit protocol. The amplified fragments containing the 5' UTRs were sequenced as described above.

Relative Quantification of MT mRNA Isoforms

In order to quantify the relative amounts of the two MT mRNA isoforms in *N. coriiceps* and *C. aceratus,* RT-PCR reactions were performed on total RNA from brain tissue as described above, except for the presence of 5 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) in the mix reaction and the use, as forward primer, of Ncor-1 (5'-TGCAACTGCGGGAG-GATCCTGC-3'), which exactly matches from base +34 to base +54 of both MT isoforms of *N. coriiceps* and *C. aceratus.* Due to the presence of a discriminating *Pvu*II restriction site, it was possible to estimate the relative amounts of the two isoforms in both species. PCR products were digested with 10 U of *Pvu*II restriction enzyme (New England Biolabs) at 37°C for 1 h. The digested products were size-fractionated in a 2% agarose gel. The expected bands for *N. coriiceps* are of 269 bp (uncut fragment), and 222 + 47 bp (digested fragments), whereas in *C. aceratus,* they are of 304 bp (uncut) and 227 + 47 bp. These differences between the two species in MT mRNA size are due to the different lengths of their 3' UTRs. The gel was dried up and exposed on a Kodak phosphor screen. The visualized bands were quantified using the Molecular Imager FX System apparatus (Bio-Rad) and MultiAnalist software (Bio-Rad).

Sequence Analysis and Phylogenetic Reconstruction

Multiple alignments of the sequences obtained were performed using two different programs: MALIGN (Wheeler and Gladstein 1994) and CLUSTAL W 1.6 (Thompson, Higgins, and Gibson 1994). In CLUSTAL W, several schemes of gap weighting were used, with alternatively high and low costs for opening and extending a gap between two or more sequences. In MALIGN, different gap penalties were used, and optimization of multiple alignments was conducted either on 1,000 random trees or through a heuristic search for the most parsimonious cladograms.

We inferred the relationships among the investigated taxa by two different methods of phylogenetic analysis: the neighbor-joining (NJ) analysis was performed as implemented in the program MEGA (Kumar, Tamura, and Nei 1993), while maximum-parsimony (MP) reconstructions were obtained using PAUP*, version 4d64 (D. L. Swofford, personal communication). In the NJ analysis, genetic distances were measured correcting for multiple hits according to Jukes-Cantor model and Kimura model. In MP, two alternative strategies were used to find the most parsimonious trees. When analyzing data set α (see below), given the number of sequences examined (25), a heuristic search was performed with random addition of taxa (100 replicates).

When data set β (see below) was considered, an exhaustive search was implemented, all character changes weighted equally, with a gap considered a fifth state. Robustness of both MP and NJ trees was assessed by bootstrap analysis (Felsenstein 1985), with 2,000 replications. Nucleotide bias within and among sequences was assessed with a χ^2 approach as implemented in PAUP*.

The potential existence of transition (TS)–transversion (TV) bias was evaluated following two approaches. In the first one, 10,000 trees were generated, resolving at random a star phylogeny where only the position of the outgroup(s) was resolved. For each random tree, the total number of unambiguous TSs and TVs was calculated using an MP approach as implemented in MacClade 3.0 (Maddison and Maddison 1992). The TS/TV ratio was estimated as the ratio between the number of TSs (averaged over the 10,000 values) and the average number of TVs. A second method used a maximum-likelihood (ML) approach applied a posteriori on the most parsimonious tree(s). That is, once tree(s) had been reconstructed using MP, the obtained topology was evaluated through ML, and the value of the TS/TV ratio was estimated as the one which gave the highest ML score.

A similar approach using 10,000 random trees was used to evaluate the distribution of changes (all changes, transversions and transitions separately) among the three codon positions in the coding region. Such analysis was performed separately for data set α and data set β. For data set α, the ratio of average inferred changes for each codon position was 1:1.3:5.3 (first, second, and third codon positions) for all changes. Bias in the TV/TS ratio was observed only at the third codon position (TV : TS = 1:2.3). Similar results were obtained for data set β: 1:1.4:5 (first, second, and third codon positions, all changes), and a biased TV/TS ratio was present only at the third codon position (TV : TS = 1:4). Different weighting schemes in MP reconstructions were adopted according to the above results: in both data set α and data set β, the first and second codon positions were given a weight five times higher than that of the third position and noncoding region. Under this position-weighting scheme, limited to third codon positions, TVs were considered equal to TSs or, alternatively, were given a higher weight (TV : TS = 2:1 or TV : TS = 4:1).

Rate Homogeneity

Rate constancy was assessed according to the method of Takezaki, Rzhetsky, and Nei (1995), which was implemented in a computer program made available by its authors. The test can be performed following production of a tree topology by a tree- building method (without the assumption of the rate constancy) after rooting the tree using one or more outgroup(s). In our analyses, we used the program option "branch length" test, which estimates the sum of branch lengths from the root to each sequence (root-to-tip distance). Deviations (*d*) from the average root-to-tip distance, calculated over all ingroup sequences, are then estimated for each sequence. Under the rate constancy hypothesis, the differ-

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                                                    5'UTR
                  ┌──────────────────────────────────────────────────────────────────┐
                  1              30                   60                 90
C.hamatus      MT1 GACAA-----AACGCTCTCATCTCAGACCAAAGACACTGCACCTACTCACGAGGACAAGAGAGACATCACCTGAGAACATGGACCCTTGCGA
T.bernacchii   MT1 ........G................................................................C.
N.coriiceps    MT1 ...............--------...T.....................G....................T.....T..
N.coriiceps    MT2 ...G......G.T.............................G....T..............T..C.....
C.hamatus      MT2 .....CACAAC..........G......G.T...........................G..................C.....

                  91             120                  150                180
C.hamatus        MT1 CTGCTCCAAGAGTGGGACCTGTAACTGCGGAGGATCCTGCACTTGCACAAACTGCTCCTGCAAAAGCTGCAAGAAG---AGCTGCTGCCC
C.rastrospinosus MT1 ...........................................................................
C.aceratus       MT1 ..............A...C........................................
P.charcoti       MT1 ..............A...C........................................
G.acuticeps      MT1 ............A...C..........................................
P.borchegrevinki MT1 ............A...C..........................................
T.bernacchii     MT1 G...............C..........................................
N.coriiceps      MT1 A.........A.....C..........................................
N.coriiceps      MT2 G..T.....A.....C........................................CC...
C.hamatus        MT2 ........A.....C........................................CC...
C.rastrospinosus MT2 ........A.....C........................................CC...
C.aceratus       MT2 ...A.....A.....C.......................................CC...
P.charcoti       MT2 ........A.....C........................................CC...
G.acuticeps      MT2 ........A.....C........................................CC...
P.borchegrevinki MT2 ........A.....C........................................CC...
T.bernacchii     MT2 ........A.....C.....................T....CC..T...............
P.fluviatilis    MT  ........G....A.....C..................G.........CC.C...............

                                                                    3'UTR
                                                                  ┌════════
                  181            210                  240                270
C.hamatus        MT1 ATGCTGCCCATCCGGCTGCACCAAATGCGCCTCTGGCTGCGTGTGCAAAGGGAAGACTTGTGACACAAGCTGCTGTCAGTGAAAGGCCTG
C.rastrospinosus MT1 .....................................................................
C.aceratus       MT1 ..........................................T..............................
P.charcoti       MT1 .....................................................................
G.acuticeps      MT1 .....................................................................
P.borchegrevinki MT1 .........................................................-.....
T.bernacchii     MT1 ...............................T........................G...
N.coriiceps      MT1 ...............................T..................T...........GAA...C
N.coriiceps      MT2 ..................................................T...........GAA...C
C.hamatus        MT2 ..................................................G.A...C
C.rastrospinosus MT2 ..................................................GA-...C
C.aceratus       MT2 ..................................................GAA-..C
P.charcoti       MT2 ..................................................G.A...C
G.acuticeps      MT2 ..................................................GA-...C
P.borchegrevinki MT2 ..................................................G.A...C
T.bernacchii     MT2 ...........................T...........T.............GA.....
P.fluviatilis    MT  ..................C....G.........A..T..C.........A.........G.CGC......C......GGAT...C

                                                    3'UTR
                  ┌══════════════════════════════════════════════════════════════════
                  271            300                  330                360
C.hamatus        MT1 ACC-TCTGCCCGCTTCTGCCTTT-GGGATGGAGCCCTTATGAA---TACCT-GACTAAAT--TTGCTGTTGTGA-ATGTC-TTCAGAGTA
C.rastrospinosus MT1 ................-....................-..........................---
C.aceratus       MT1 .....GCT.........-.......-.................T...................-..
P.charcoti       MT1 ......-....-........................................TTA.---
G.acuticeps      MT1 ..................................T.......................-------
P.borchegrevinki MT1 ....................-......................T.....................-------
T.bernacchii     MT1 .........................T...........C......T......................-..
N.coriiceps      MT1 .G..CT-----........TC.....A.........T..G....C.....T-.---------.------...CA.........A.....A-
N.coriiceps      MT2 .G..CT-----........TC.....A.........T..G....C.....T-.---------.------...CA.........A.....A-
C.hamatus        MT2 .G..C.-----........TC.....A.........T..G....C.....T.T.....C.....CCTA....CA.........A.....A-
C.rastrospinosus MT2 .G..C------........TC.....A.........T..G....C.....T.T.....C.....CCT-...CA.........A.....A-
C.aceratus       MT2 .G..C------........TC.....A.........T..G....C.....T.T..A..C.....CCT-...CGA.........A.....A-
P.charcoti       MT2 .G..C------........TC.....A.........T..G....C.....T.T.....C.....CCT-...CA.........A.....A-
G.acuticeps      MT2 .G..C------........TC.....A.........T..G....C.....T.T.....C.....CCT-...CA.........A.....A-
P.borchegrevinki MT2 .G..C------........TC.....A.........T..G....C.....T.T.....C.....CCT-...CA.........A.....A-
T.bernacchii     MT2 ......CAGT.........TC.....A.........T..G....C.....T.TA....C.....CCT-...CC.........A.....A-
P.fluviatilis    MT  TGGA..A....CG....A..C.CAT.-..T.....GG.G..C.C..C....T..A.T.T.AA....A.C..CA...C.....A....AG.
```
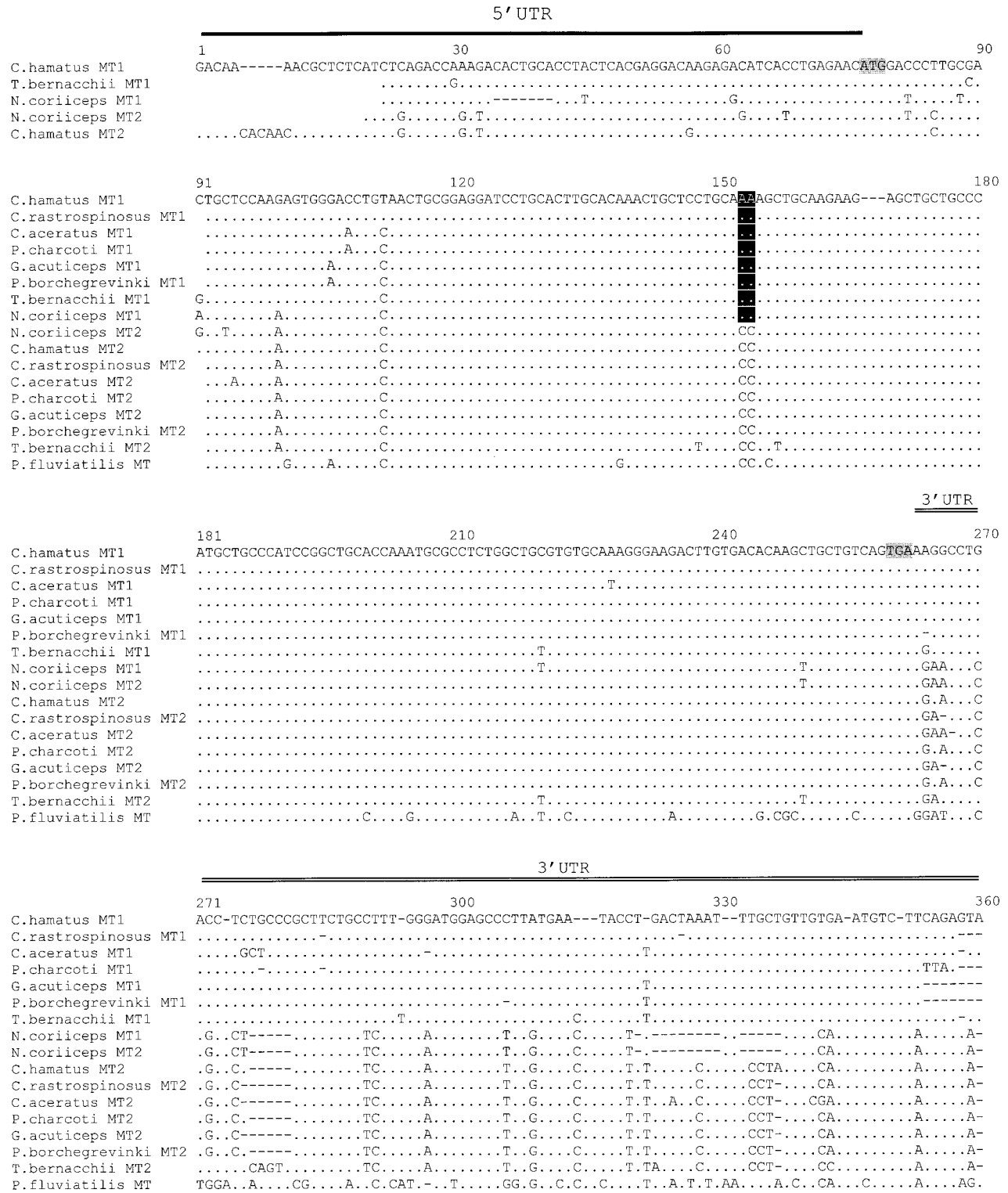
FIG. 1.—Alignment of 16 notothenioid MT sequences plus *Perca fluviatilis* MT. Dots represent identity with reference sequence, dashes refer to insertion-deletion events. Light shading indicates start and termination codons. Dark shading shows sites 153–154, which are involved in the threonin-leucine transition (see text). A single solid line encompasses the 5′ UTR, and a double line encompasses the 3′ UTR.

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                                          3'UTR

                                361               390                   420       429
            C.hamatus MT1       ATAATGTTTTGGTTTTTGTTGGTACTTGTTTTCAA-TGTCGAAATAAATGAACTTAGCCTACTT-----
            C.rastrospinosus MT1 -.........TT..G.................................T................
            C.aceratus MT1      ......A...TT....................................T................
            P.charcoti MT1      ......-..G--..-..T..........C.........G.........T..-----...G....
            G.acuticeps MT1     ----------------.T.............................T..-----...G....
            P.borchegrevinki MT1 ----------------.T.............................T..-----...G....
            T.bernacchii MT1    .....AA..-------..T..T.........................T..T..-----...G....
            N.coriiceps MT1     ---...G.GAA-----.T..-.........A.G.....T..........C.G..-----C...G....
            N.coriiceps MT2     ---...G.GAA-----.T..-.........A.G.....T..........C.G..-----C...G....
            C.hamatus MT2       ---...G.GAA------T.GT.........A.G.....T..........C...-----C...G....
            C.rastrospinosus MT2 ---..TG.-------------.........A.G.....T..........C.G.......-C...G....
            C.aceratus MT2      ---..TG.-------------.........A.T.....T..........C.G..-----C...G....
            P.charcoti MT2      ---...G.GAA-----.T..-.........A.G.....T..........C.G..-----C...G....
            G.acuticeps MT2     ---...G.GAA-----.T..-.........A.G.....T..........C.G..-----C...G....
            P.borchegrevinki MT2 ---...G.GAA-----T.GT.........A.G.....T..........C...-----C...G....
            T.bernacchii MT2    ---...G.GAA-----.T..T.........ATG.A...T..........AC.G..-----C...G....
            P.fluviatilis MT    ...---..CA-----.T..T....G-..C....G....T..........TGA..-----C..AATGTG
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FIG. 1. (*Continued*)

ence between average and observed distances is expected to be zero. For the branch length test, a nonparametric approach was used to estimate the variance of d by analyzing 1,000 bootstrap replicates of the original data set. For further details see Takezaki, Rzhetsky, and Nei (1995).

Two different topologies were examined: (1) the NJ tree reconstructed with no constraints, and (2) the tree reflecting the expected phylogenetic relationships among notothenioid taxa.

Partition Matrix

To reveal possible events of reticulate evolution (i.e., gene conversion), a partition matrix (Jakobsen, Wilson, and Easteal 1997) was constructed for the sequence alignment including the 5′ UTRs of five notothenioid MTs as well as for the alignment of the coding region and 3′ UTRs of 16 notothenioid MTs. Partition matrices were obtained using the program Partmatrix, made available by its authors. In brief, a partition matrix is a condensed graphical summary of the phylogenetic information along the sequence alignment, and it might be helpful in detecting regions of sequence with distinct evolutionary histories and the location of boundaries between them. Among all possible topologically distinct trees that could be reconstructed to represent the phylogeny of sequences, some will contain a branch that defines a certain binary partition of sequences, i.e., a partition dividing the sequences into two mutually exclusive groups. Trees like these are defined as indicative of the bipartition. For any variable site of the sequence alignment, we can identify the possible trees in which the minimum number of nucleotide changes, c, is one less than the number of different nucleotides observed, n. A site is defined as identical to a given partition when all $c = n - 1$ trees are indicative of the partition. If at least one of the trees is indicative of the partition, the site is said to be consistent with the partition, while if none of the trees is indicative of the partition, the site is defined as inconsistent with the partition.

The program used identifies all parsimoniously informative sites, then determines all the partitions supported by the data. Finally, each site is examined to assess whether it is identical, consistent, or inconsistent with regard to each of the partitions. It should be noted that when a site has more than two nucleotides (three or more bases, gaps, or missing information), the program implements the following approach: when more than two nucleotides are found, only TVs are taken into account, and purines (A and G) and pyrimidins (T and C) are considered equivalent. Sites at which information is missing or there is a gap in only one sequence are converted to two temporary sites by assigning the missing sequence in turn to each of the groups formed by the remaining sequences, and a score of 0.5 is added to the support for the bipartitions to which these temporary sites are identical. For sites at which several sequences are missing information, support for any partition is not assigned, but these sites are displayed on the matrix and their consistency with each partition (determined by the other sites) is examined. For further details, the reader should refer to the original paper by Jakobsen, Wilson, and Easteal (1997).

Wilcoxon Signed-Ranks Test

To examine the extent of conflict between the tree topology obtained for notothenioid MTs versus the phylogenetic hypothesis based on mitochondrial sequence data, a heuristic search was performed under MP as described above (exhaustive search, all character changes equal, gap considered as a fifth state), but enforcing topological constraints based on the expected notothenioid phylogeny. The resulting most-parsimonious trees were compared with the unconstrained tree, and the statistical significance of the extra steps required beyond the unconstrained tree was evaluated using a Wilcoxon signed-ranks test (Templeton 1983; Larson 1994) as implemented in PAUP*.

Results and Discussion
Phylogenetic Reconstruction and MT Duplication

In each of the eight notothenioid species examined, two distinct cDNA forms were isolated. Most of the coding region, as well as the entire 3′ UTR were sequenced (fig. 1). As a first step, 25 "homologous" sequences, representing the coding regions and the 3′ UTRs of MT cDNAs from 17 teleost species, were con-

Table 1
List of Species for Which MT Sequences Were Examined

Species	Accession Number(s)
Ostariophysi	
Cypriniformes	
Danie rerio	X97278
Carassius auratus	X97271
Protacanthopterygii	
Esociformes	
Esox lucius.	X59392
Salmoniformes	
Oncorhynchus mykiss	
	X59394, X59395
Paracanthopterygii	
Gadiformes	
Gadus morhua.	X97269
Acanthopterygii	
Pleuronectiformes	
Pleuronectes platessa	X56743
Perciformes	
Perca fluviatilis	X97272
Zooarces viviparus	X97270
(Notothenioidei)	
Notothenia coriiceps	AJ006484, AJ006485
Trematomus bernacchii.	AJ011585, Z72485
Pagothenia borchgrevinki.	AJ007562, AJ007563
Parachaenichthys charcoti	AJ007950, AJ007951
Gymnodraco acuticeps	AJ007560, AJ00756
Chionodraco hamatus.	Y12580, Y12581
Chionodraco rastrospinosus.	AJ011584, Z72484
Chaenocephalus aceratus.	AJ011583, Z72483

FIG. 2.—Consensus tree summarizing results from different phylogenetic methods applied to data set α. Only nodes supported under all methods are indicated. Unresolved phylogenetic placements of sequences are shown as polytomies. Numbers on branch nodes indicate bootstrap values (after 2,000 replicates) depending on the different methods used: NJ on nucleotide data after Jukes-Cantor correction (upper), NJ based on uncorrected pairwise distance between inferred protein (middle), MP using weighting scheme for codon positions (lower). Specific clades (A, B, C) are indicated (see *Results and Discussion* for details). MT sequences from two cyprinid species (*Danio rerio* and *Carassius auratus*) were defined as outgroups. This choice arises from consideration of the known phylogenetic placement of the fish taxa examined (Nelson 1994), as well as from results of UPGMA analysis (not shown) on both DNA and inferred protein data, which place MT sequences from the two cyprinid species as sister group to all the MT forms considered in this study.

sidered (table 1). However, given the large phylogenetic distance among several taxa, with neither method could a reliable alignment be obtained for the 3′ noncoding region. Sequence similarity was extremely low, and ''positional homology'' (Mindell 1991) could not be safely inferred. Consequently, only the coding region was used at this stage (data set α). MP and NJ analyses on data set α, at both the nucleotide and the protein levels and following different weighting schemes (see *Materials and Methods*), yielded similar phylogenetic hypotheses, with the same nodes receiving strong bootstrap support (>90% of replicates) under all methods. A consensus tree is reported in figure 2, summarizing results from different methods. Three highly supported nodes characterize three clades (A, B, and C in fig. 2). Clade A includes two MT sequences of *Oncorhynchus mykiss* (rainbow trout) and one from *Esox lucius* (pike). This is in agreement with the known phylogenetic position of these species, which belong to the superorder Protacanthopterygii. Within clade A, the two MT forms (1 and 2) from rainbow trout appear to have originated before the separation between Esociformes and Salmoniformes, because rainbow trout MT-1 is more closely related to pike MT than is rainbow trout MT-2. However, this hypothesis is supported by relatively low bootstrap values.

The second clade (clade B), which consistently emerges from all of the analyses, contains the MT sequences of all the acanthopterygian species examined, again in agreement with the expected taxonomic placement of these taxa. Included in clade B are all of the

MTs obtained from the eight Antarctic fish species considered in the present study. These latter sequences appear to be monophyletic, forming a third, well-supported, group (clade C). This suggests the existence of a single ancestral sequence from which all of the notothenioid MTs examined might have diverged, but only after the separation of the notothenioid ancestor from the ancestral lineages of the other taxa considered in this work.

Based on this evidence, a subsequent analysis was conducted focusing on the 16 notothenioid sequences and their closest outgroup, including the 3′ UTR, up to a total of 335 bp (data set β). A single sequence (*Perca fluviatilis* MT) was chosen as a reference taxon to the ingroup taxa, judging from the position in the phylogenetic trees based on data set α and considering the similarity to notothenioid sequences at the 3′ UTR. This allowed us to obtain a reliable alignment (fig. 1) for the entire 3′ noncoding region.

As can be observed in figure 2, when the phylogenetic analysis is limited to the coding region, relationships within notothenioid MT sequences cannot be
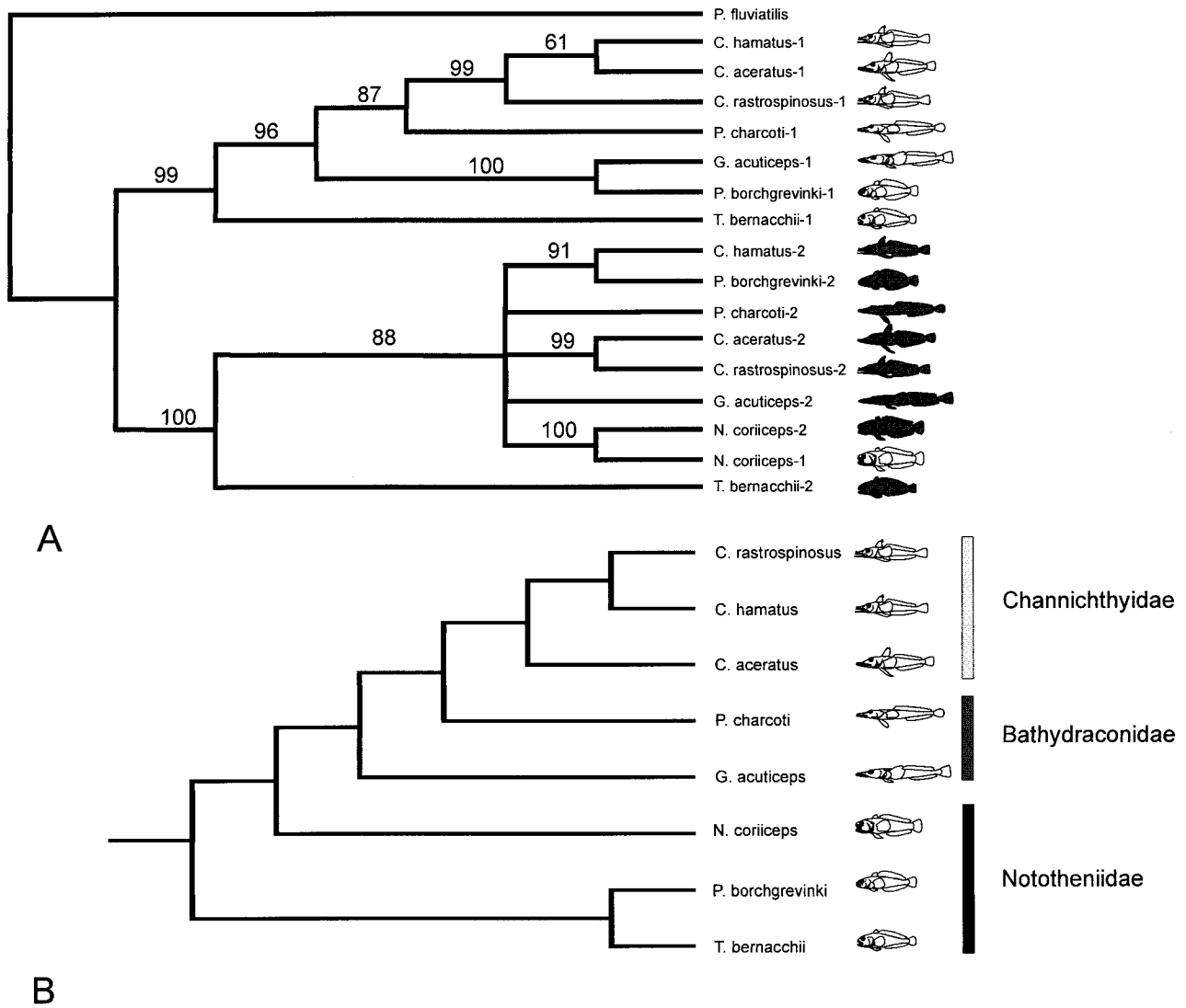
FIG. 3.—*A,* Strict consensus of 7 most-parsimonious trees (87 parsimony-informative sites; for each tree, length = 231, consensus index = 0.827, retention index = 0.888). Data set β was used. All changes were considered equal. Giving higher weight to changes at first and second positions yielded similar results. Numbers on branch nodes indicate bootstrap values (after 2,000 replicates); only values greater than 50% are shown. *Perca fluviatilis* MT was used as the outgroup. *B,* Phylogenetic relationships among the notothenioid species examined in the present work as obtained by merging results from two different molecular studies (Bargelloni et al. 1994; Chen, Bonillo, and Lecointre 1998). Traditional subdivision of the examined species into notothenioid families is represented with shaded bars.

clearly resolved, although a single clade groups all MT-1 sequences (one for each notothenioid species). All notothenioid MT-1 genes show a lysine at position 20, while MT-2s display a threonine at that position, as do all the rest of the nonnotothenioid species examined. The presence of a lysine at site 20 in the place of a threonine indicates a nonconservative amino acid change (polar to charged residue) and appears to be an evolutionary novelty common only to all notothenioid MT-1s. The functional significance of such a change, however, is difficult to determine, and it should be recalled that, in higher vertebrates, differences in the amino acid sequence do not always translate into differences in function (Ebadi and Babin 1989).

The low phylogenetic resolution in the coding region is likely due to the extremely low variability detected among notothenioid MTs at both the nucleotide

and the protein levels (100% amino acid identity is observed in several comparisons). In contrast, the 3′ noncoding region is highly variable, thus carrying more phylogenetic information. However, a large part of the sequence variation detected consists of insertion-deletion events (fig. 1). This poses a problem in using methods based on models of nucleotide substitutions, as distance methods are unable to incorporate gaps, while MP allows one to consider a gap a fifth character state, besides the four bases. Consequently, most of the subsequent analyses were performed using MP. Nevertheless, NJ tree reconstructions (and rate tests based on pairwise distances; see below) were also conducted, excluding from the data set all sites containing gaps.

Figure 3*A* shows results from MP. This method separates notothenioid MTs into two groups, MT-1 and MT-2, with high bootstrap support. Each clade contains only

one of the two MT forms expressed in each notothenioid species, therefore suggesting that at least two paralogous groups of genes, MT-1 and MT-2, exist in these Antarctic fishes. The observed pattern can be explained assuming the occurrence of an ancient MT duplication which took place after the separation of the notothenioid lineage from the rest of the teleosts but before the diversification of the notothenioid species examined. However, when the complete sequence information (coding and 3′ UTR) is considered, MTs from *N. coriiceps* do not follow the separation in two distinct clades, contrary to what is observed for all of the remaining notothenioid MTs. In fact, at variance with evidence from the NJ tree based on protein data (fig. 2), both *N. coriiceps* MT sequences are included in the MT-2 clade (fig. 3*A*). This result is likely due to the complete sequence identity of the 3′ UTR of *N. coriiceps* MT-1 with that of MT-2 in the same species (fig. 1). Evidence from MT gene genealogy, with the placement of *N. coriiceps* MT-1 and MT-2 as sister taxa (with 100% bootstrap support) in the MP analysis (fig. 3*A*) could be explained as the result of a recent duplication event occurring after the divergence of the *N. coriiceps* lineage from the other notothenioid taxa. This hypothesis, however, fails to account for the contrasting evidence that emerges from the phylogenetic analysis of the coding region only (fig. 2). To solve this incongruousness, reticulate evolution, i.e., gene conversion, is proposed to have involved part of the *N. coriiceps* MT-1 gene, as discussed later in the text.

Although the most evident case is that of *N. coriiceps* MT-1, this might not be the only MT sequence which does not fit into the "simple" scheme of two clades, MT-1 and MT-2, each comprising strictly orthologous genes, i.e., genes whose divergence traces back to species divergence. To further investigate this possibility, the tree topology obtained for notothenioid MTs was compared with the phylogeny of the species (fig. 3*B*) based on mitochondrial data (Bargelloni et al. 1994; Chen, Bonillo, and Lecointre 1998). From such a comparison, several discrepancies are observed between MT gene genealogy and species phylogeny. MT-1 and MT-2 from *Pagothenia borchgrevinki* show the most striking exception to the notothenioid species tree. Both sequences from this species emerge as sister group not to the other trematomid taxon (namely *Trematomus bernacchii,* a member of the same subfamily), but, alternatively, to *Gymodraco acuticeps* MT-1 or *Chionodraco hamatus* MT-2, two species belonging to different families (Bathydraconidae and Channichthyidae, respectively).

In addition, since some nodes are not resolved in the MP tree (fig. 3*A*), the obtained tree topology was evaluated through comparison with alternative phylogenetic hypotheses by means of a Wilcoxon signed-ranks test (see *Materials and Methods*). Results of this test show that the phylogenetic position of the investigated taxa within each clade (MT-1 and MT-2) is significantly in contrast with the one expected from the species phylogeny ($P < 0.001$). This suggests that each clade might contain paralogous genes, thus implying

that MT genes in notothenioids might have experienced more than a single duplication event. Further analyses of the 5′-flanking region, as well as introns, might shed light on this issue. In any event, complex relationships within families of duplicated genes are not unusual. Multiple duplication events and successive loss of some duplicated copies might lead to the expression of paralogous genes in different species (as in the case of Hox genes in vertebrates; see Holland and Garcia-Fernandez 1996). Alternatively, if genes within MT-1 and MT-2 clades are indeed orthologous (therefore supporting the hypothesis of a single MT duplication event in the ancestral lineage of these fish), the discrepancy between MT gene genealogy (fig. 3*A*) and species phylogeny (fig. 3*B*) could be an artifact due to extreme differences in substitution rates among sequences, possibly linked to differential selective pressure (see, e.g., Patarnello et al. 1997). In this regard, it worth noting that in both white-blooded and red-blooded notothenioids, there exists a posttranscriptional, tissue-specific control in the expression of these MT isoforms that might be subject to selective pressure (Carginale et al. 1998; Scudiero et al. 1997; unpublished data). In mammalian species, both 5′-UTR- and 3′-UTR-specific sequence stretches have been hypothesized to play a role in posttranscriptional regulation of MT expression (Nishimura et al. 1996). However, none of these putative regulative cassettes is found in the 3′ UTRs of all notothenioid species examined. This does not exclude that different regulative sequences in this region might be important in teleost MT mRNA.

Gene Conversion in *N. coriiceps* MT-1

To further examine the hypothesis of gene conversion, variable phylogenetic signals along the nucleotide alignment of data set β were explored by constructing a partition matrix following the approach of Jakobsen, Wilson, and Easteal (1997). The partition matrix of 16 notothenioid MTs is shown in figure 4*A*. The most support is given to the binary partition with all MT-2s plus *N. coriiceps* MT-1 on one side and all the rest of the MT-1s on the other. Several sites of the 3′ UTR are indicative of or identical to this partition, and the same holds true for a single site in the coding sequence (a synonymous change at position 100; see fig. 1). In the latter region, however, two adjacent sites (two transversional changes at positions 153–154, leading to a substitution at the amino acid level) are identical to a (different) partition, dividing all MT-1s and MT-2s, respectively, into two distinct groups (fig. 4*A*). At this amino acid position, as mentioned above, all MT-1s (including *N. coriiceps* MT-1) share a lysine residue, which appears to be a shared synapomorphy of notothenioids MTs, whereas a threonine is observed in all MT-2s, similar to the remaining teleost MTs examined.

Examination of the partition matrix in figure 4*A* suggests that MT-1 of *N. coriiceps* might be a case of reticulate evolution. This appears to have involved at least the entire 3′ UTR, which is identical to the 3′ UTR of MT-2 of the same species. With regard to the coding region, we are left with two alternative hypotheses: (1)
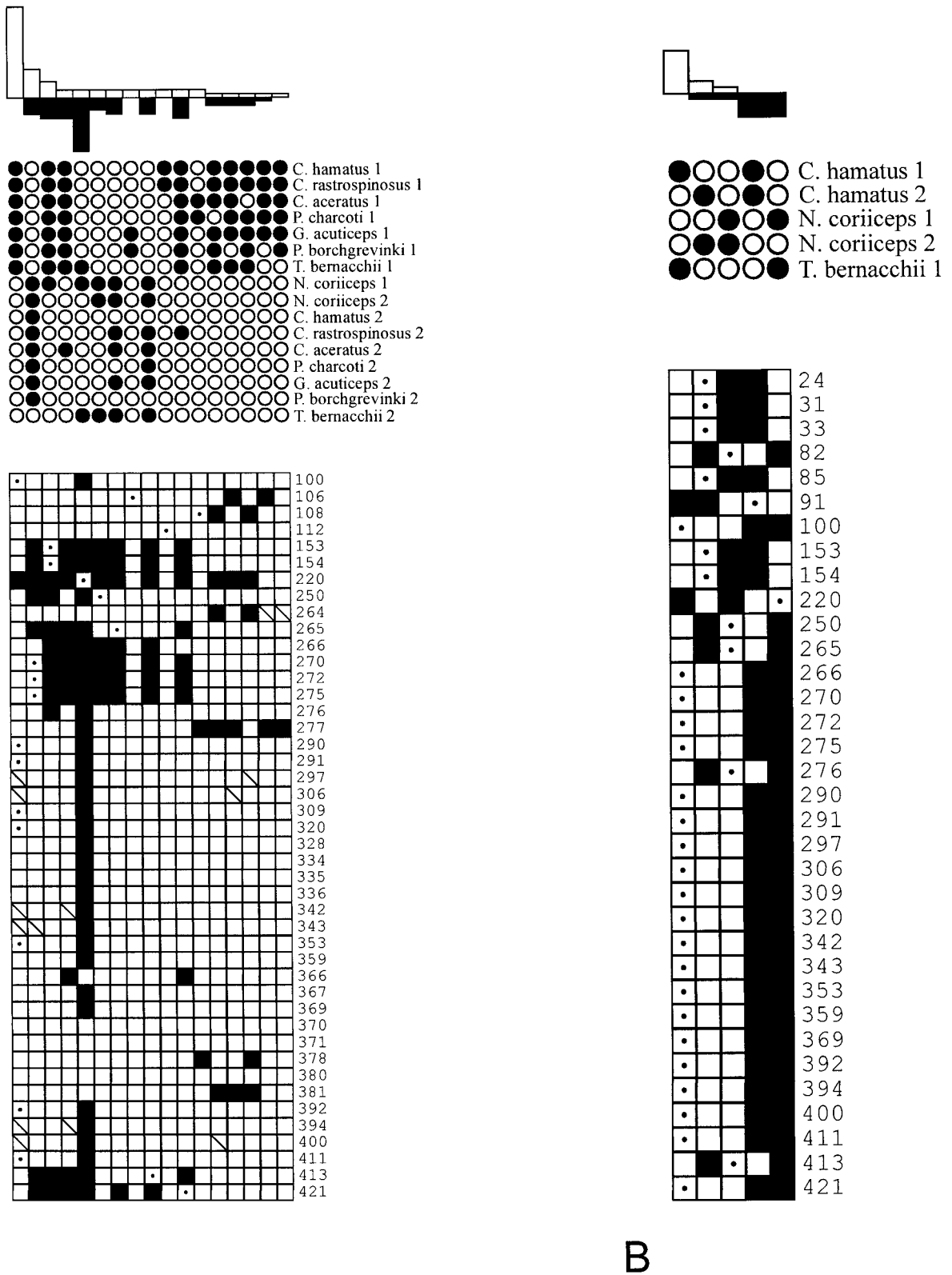
A

B

Fig. 4.—A, Partition matrix of the alignment including part of the coding region and the 3' UTR of 16 notothenioid MTs. Each column represents a partition observed in the data, while each row represents a parsimoniously informative site. A site consistent with a partition is represented by a white square, whereas an inconsistent site is represented by a black square. White squares with a small dot indicate those sites that are identical to a given partition, and squares with a diagonal line represent sites that provide half support for two partitions (see *Materials and Methods* and the detailed description in Jakobsen, Wilson, and Easteal 1997). *B,* Partition matrix of the alignment including the 5' UTRs, the coding regions, and the 3' UTRs of five notothenioid MTs.
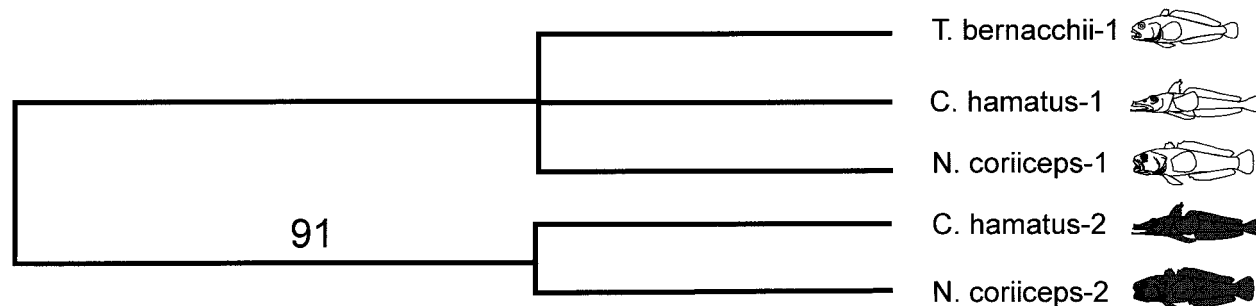
Fɪɢ. 5.—Strict consensus of two most-parsimonious trees based on 256 bp (5′ UTR and complete coding region) of five notothenioid MTs. There were 11 parsimony-informative sites, and for each tree, length = 39, consensus index = 0.897, retention index = 0.636. The tree was arbitrarily rooted to show MT-1s and MT-2s divided into separate clades.

The entire coding region might have met the same fate as the 3′ UTR, undergoing gene conversion as suggested by evidence at site 100, or, alternatively, the entire MT-1 gene might have originated by duplication of MT-2. This hypothesis (duplication or complete gene conversion) requires, however, that two base substitutions occurred at positions 153 and 154 of *N. coriiceps* MT-1, leading to the parallel acquisition of the same amino acid residue (lysine), as independently occurred in the other MT-1s. (2) Alternatively, gene conversion might have been limited to the 3′ UTR, and the synonymous substitution observed at site 100 might be due to parallel evolution.

To elucidate the fate of the 5′ portion of the *N. coriiceps* MT-1 gene, the complete cDNA sequence was sequenced for MT-1 and MT-2 of *N. coriiceps* and *C. hamatus* and for MT-1 of *T. bernacchii.* In the case of recent duplication of MT genes or gene conversion of the entire MT-1 locus in *N. coriiceps,* we would expect very few differences (or even complete sequence identity) between the two *N. coriiceps* MTs and also in the 5′ region, as observed for the 3′ noncoding one. On the contrary, alignment of the 5′ sequences shows several nucleotide differences between MT-1 and MT-2 of *N. coriiceps,* and a phylogenetic analysis of the 5′ UTR and the coding region shows that MT-1 and MT-2 sequences are partitioned into two distinct clades (fig. 5). Moreover, a partition matrix constructed on the five complete MT sequences shows that several sites (24, 31, and 33 in fig. 4*B*) in the 5′ UTR are not compatible with the hypothesis of gene conversion at the 5′ UTR or complete gene duplication and significantly indicates an independent evolution of the 5′ ends of the two MT genes of *N. coriiceps.*

Further support for the latter hypothesis is provided by expression experiments. If transcriptional regulation is dissimilar between these two isogenes, we can assume that the regulative sequences, presumably located upstream of the coding region, are different. The results obtained clearly show that (1) in both species, MT-2 mRNA is expressed at a higher level than is MT-1 (fig. 6*A*), and (2) the ratios between MT-1 and MT-2 mRNAs are very similar in each of the two species (fig. 6*B*). From point 1 we can infer that in *N. coriiceps,* indeed, two distinct genes are present, characterized by different regulative sequences. In addition, evidence from point 2

suggests a similar regulation of the differentially expressed MT-1 and MT-2 (comparable ratio MT-1 : MT-2 mRNAs). The expression pattern, therefore, appears to mirror the phylogenetic placement of MT-1s and MT-2s into two different clusters, as inferred on the basis of 5′-UTR and coding-sequence data. In contrast, evidence of sequence identity between the 3′ UTRs of MT-1 and MT-2 seems to suggest that this region has no role in regulating the differential expression of the two forms.

Rate Homogeneity Test

As a further step to investigate notothenioid MT evolution, evolutionary rates were compared among sequences using the approach of Takezaki, Rzhetsky, and Nei (1995). The branch length test (see *Materials and Methods*) was conducted at the nucleotide level, using *P. fluviatilis* MT as an outgroup. These analyses were carried out on the whole β data set, as well as separately for the coding region and the 3′ UTR.

The results of the test revealed a substantial number of exceptions to the hypothesis of rate homogeneity (table 2). In fact, when the entire β data set (coding and noncoding regions) is examined, eight sequences display a significantly greater or smaller root-to-tip distance than the average one. Sequences with a faster rate all belong to the MT-1 cluster, while those with a slower rate are included in the MT-2 group. This result is insensitive to the inclusion-exclusion of the "odd" MT-1 sequence of *N. coriiceps* and is not dependent on the topology used to perform the test (see *Materials and Methods*). To further investigate this evidence, the data set was partitioned into two subsets, one comprising just the entire coding region and another one comprising only the 3′ UTR. In the first case, the branch length test did not reveal any significant exceptions. In contrast, analysis of the 3′ UTR showed that most of the sequences (excluding two) have root-to-tip distances significantly different from the average one. Again, this result remains insensitive to tree topology and exclusion of *N. coriiceps* MT-1. As in the case of the complete data set, sequences with a greater than average distance belong to the MT-1 cluster, whereas the "slow" ones are all MT-2 sequences. Several hypotheses can be envisaged to explain the evidence of an overall higher substitution rate for the noncoding regions of MT-1 sequences. For example, the two genes MT-1 and MT-2 might be located in dif-
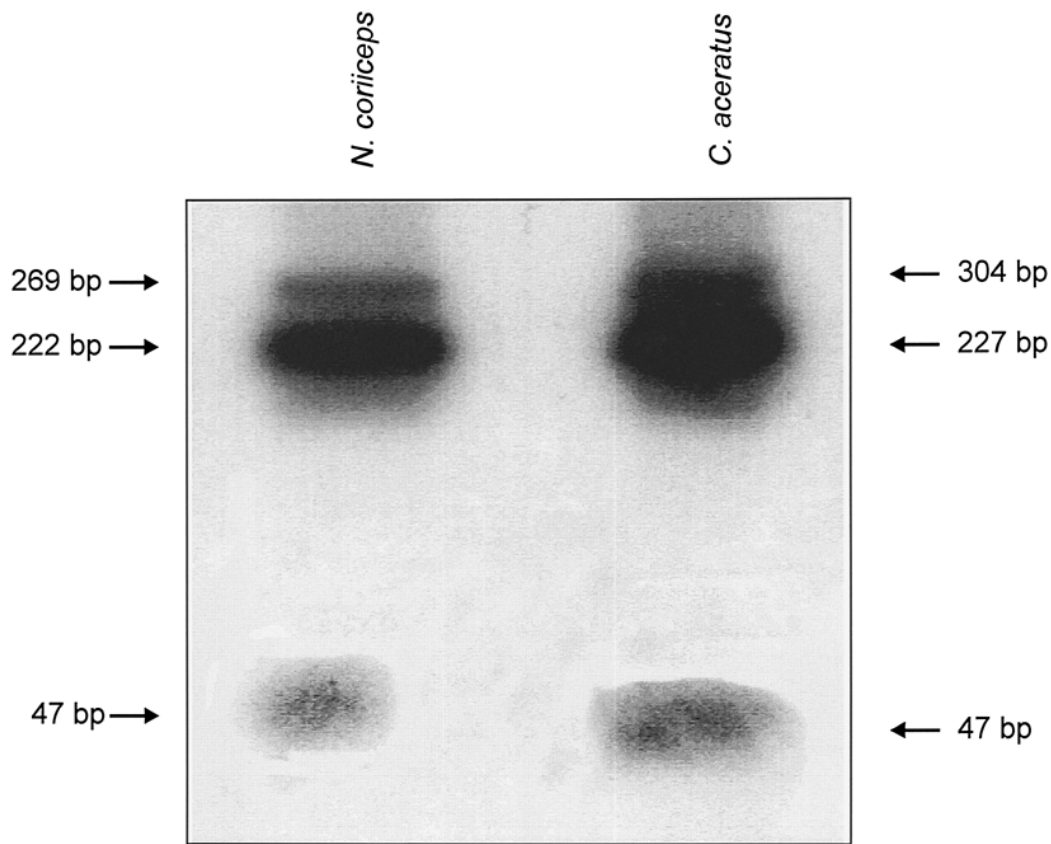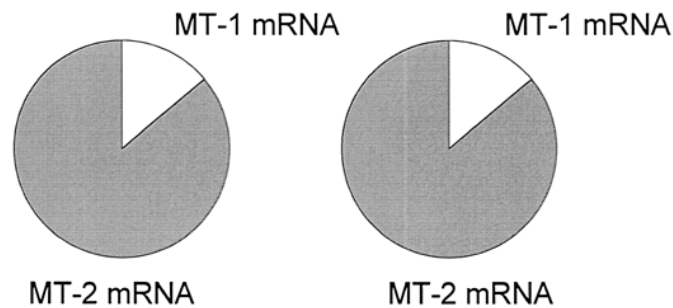
FIG. 6.—*A*, RT-PCR quantification of differentially expressed MT-1 and MT-2 mRNA in *Notothenia coriiceps* (left) and *Chionodraco hamatus* (right). Digestion with *Pvu*II of RT-PCR products generates two fragments (222 + 47 bp or 227 + 47 bp) from MT-1, while it leaves MT-2 uncut (269- or 304-bp fragment). *B*, Relative amounts of expressed MT-1 and MT-2 in the two species as determined when visualized bands were quantified using a Molecular Imager FX System apparatus.

ferent parts of the genome, with different mutation rates, and the lack of rate heterogeneity in the coding region could be due to its extremely low variability. Alternatively, selection might act differentially on the noncoding region, either in terms of different selective constraints or as positive selection. As mentioned above, in higher vertebrates, the 3′ UTR has been suspected to play a regulative role in MT expression, and differential expression of MT-1 and MT-2 has been observed in notothenioids (Carginale et al. 1997; Scudiero et al. 1997; this study). Therefore, functional differences in the 3′ noncoding region might be a likely explanation for the observed rate heterogeneity. This hypothesis, however, is in contrast, at least partially, with evidence of gene conversion in *N. coriiceps.* In this species, in fact, both MT-1 and MT-2 share identical 3′ UTRs.

Table 2
Results of Branch Length Test

	COMPLETE DATA SET		CODING REGION ONLY		3′ UTR ONLY	
SEQUENCE	Root–Tip Distance[a]	*P* Value[b]	Root–Tip Distance[a]	*P* Value[b]	Root–Tip Distance[a]	*P* Value[b]
Trematomus bernacchii MT-1	>	<0.05*	<	<0.94	>	<0.001***
Pagothenia borcvhgrevinki MT-1	>	<0.12	<	<0.89	>	<0.01**
Parachaenichthys charcoti MT-1	>	<0.03*	<	<0.78	>	<0.001***
Gymnodraco acuticeps MT-1	>	<0.12	<	<0.89	>	<0.01**
Chionodraco hamatus MT-1	>	<0.13	<	<0.77	>	<0.02*
Chionodraco rastrospinosus MT-1	>	<0.16	<	<0.77	>	<0.01**
Chaenocephalus aceratus MT-1	>	<0.05*	>	<0.31	>	<0.01**
T. bernacchii MT-2	>	<0.82	>	<0.13	<	<0.10
P. borchgrevinki MT-2	<	<0.01**	<	<0.18	<	<0.001***
P. charcoti MT-2	<	<0.001***	<	<0.18	<	<0.001***
G. acuticeps MT-2	<	<0.01**	<	<0.18	<	<0.001***
C. hamatus MT-2	<	<0.01**	<	<0.18	<	<0.001***
C. rastrospinosus MT-2	<	<0.001***	<	<0.18	<	<0.001***
C. aceratus MT-2	<	<0.75	<	<0.90	<	<0.52
Notothenia coriiceps MT-2	<	<0.41	>	<0.55	<	<0.02*

[a] "˃" indicates that the root-to-tip distance of a particular lineage is greater than the average root-to-tip distance; "˂" indicates a lower-than-average distance.
[b] *P* indicates the probability of rejecting by chance the null hypothesis of no difference between specific and average root-to-tip distance.
* Significant at the 5% level; ** significant at the 1% level, *** significant at the 0.1% level.

Conclusions

In summary, our results indicate that at least two MT genes are present in notothenioids and that this multiplicity originated after their divergence from the other fish lineages examined. This event occurred independent of the duplication which gave origin to metallothionein isoforms in salmonids. MT multiplicity does not appear to be correlated with constitutive expression of MTs, as the two icefish species examined in this study show the same number of MT genes despite a lack of expressed MTs at the protein level.

Furthermore, incongruence between the MT gene tree and the species tree suggests that both MT-1 and MT-2 clusters might include paralogous genes, i.e., genes whose divergence traces back to a duplication event subsequent to the one which gave origin to the two clusters. These findings, together with the observation of gene conversion on *N. coriiceps* MT-1, the differential pattern of expression of the two MT isoforms, and the evidence of rate heterogeneity in the 3′ UTR, disclose an unexpected complexity in the evolution and function of notothenioid MTs.

Acknowledgments

LITERATURE CITED

BARGELLONI, L., P. A. RITCHIE, T. PATARNELLO, B. BATTAGLIA, D. M. LAMBERT, and A. MEYER. 1994. Molecular evolution at subzero temperature: mitochondrial and nuclear phylogenies of fishes from Antarctica (suborder Notothenioidei), and the evolution of antifreeze glycopeptides. Mol. Biol. Evol. **11**:854–863.

BONHAM, K., and L. GEDAMU. 1984. Induction of metallothionein and metallothionein mRNA in rainbow-trout liver following cadmium treatment. Biosci. Rep. **4**:633–642.

CARGINALE, V., R. SCUDIERO, C. CAPASSO, A. CAPASSO, P. KILLE, G. DI PRISCO, and E. PARISI. 1997. Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish which exhibits no basal protein but high endogenous mRNA levels. Biochem. J. **332**:475–481.

CHAN, K. M. 1994. PCR-cloning of goldfish and tilapia metallothionein complementary DNAs. Biochem. Biophys. Res. Commun. **205**:368–374.

CHEN, W.-J., C. BONILLO, and G. LECOINTRE. 1998. Phylogeny of the Channichthyidae (Notothenioidei, Teleostei) based on two mitochondrial genes. Pp. 287–298 in G. DI PRISCO, E. PISANO, and A. CLARKE, eds. Fishes from Antarctica. Springer-Verlag, Milan, Italy.

CHOMCZYNSKI, P., and N. SACCHI. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159

DEVRIES, A. L. 1988. The role of antifreeze glycopeptides and peptides in the freezing avoidance of Antarctic fishes. Comp. Biochem. Physiol. B Comp. Biochem **90**:611–621.

EASTMAN, J. T. 1993. Antarctic fish biology: evolution in a unique environment. Academic Press, San Diego.

EBADI, M., and D. BABIN. 1989. The amino acid composition of the zinc-induced metallothionein isoforms in rat brain. Neurochem. Res. **14**:69–73.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.

FERNANDO, L. P., D. WEI, and G. K. ANDREWS. 1989. Structure and expression of chicken metallothionein. J. Nutr. **119**:309–318.

GRIFFITH, B. B., R. A. WALTERS, M. D. ENGER, C. E. HIL-DEBRAND, and J. K. GRIFFITH. 1983. cDNA cloning and nucleotide sequence comparison of Chinese hamster metal-lothionein I and II mRNAs. Nucleic Acids Res. **11**:901–911.

HAMER, D. H. 1986. Metallothionein. Annu. Rev. Biochem. **55**:913–951.

HOLLAND, P. W. H., and J. GARCIA-FERNANDEZ. 1996. Hox genes and chordate evolution. Dev. Biol. **173**:382–395.

HUNZIKER, P. E., P. KAUR, M. WAN, and A. KANZIG. 1995. Primary structures of seven metallothioneins from rabbit tissue. Biochem. J. **306**:265–270.

JAKOBSEN, I. B., S. R. WILSON, and S. EASTEAL. 1997. The partition matrix: exploring variable phylogenetic signals along nucleotide sequence alignments. Mol. Biol. Evol. **14**:474–484.

KÄGI, J. H. R. 1991. Overview of metallothionein. Methods Enzymol. **205**:613–626.

———. 1993. Evolution, structure and chemical activity of class I metallothioneins: an overview. Pp. 29–55 in K. T. SUZUKI, N. IMURA, and M. KIMURA, eds. Metallothionein III. Birkhauser Verlag, Basel, Switzerland.

KÄGI, J., T. L. COOMBS, J. OVERNELL, and T. M. WEBB. 1981. Synthesis and function of metallothioneins. Nature **292**:495–496.

KÄGI, J. H. R., and A. SCHAFFER. 1988. Biochemistry of metallothionein. Biochemistry **27**:8509–8515.

KUMAR, S., K. TAMURA, and M. NEI. 1993. MEGA: molecular evolutionary genetics analysis. Version 1.02. Pennsylvania State University, University Park.

LARSON, A. 1994. The comparison of morphological and molecular data in phylogenetic systematics. Pp. 371–390 in B. SHIERWATER, B. STREIT, G. P. WAGNER, and R. DESALLE, eds. Molecular ecology and evolution: approaches and applications. Birkhäuser Verlag, Basel, Switzerland.

MACDONALD, J. A., and R. M. G. WELLS. 1991. Viscosity of body fluids from Antarctic notothenioid fish. Pp. 163–178 in G. DI PRISCO, B. MARESCA, and B. TOTA, eds. Biology of Antarctic fish. Springer-Verlag, Berlin.

MADDISON, W. P., and D. R. MADDISON. 1992. MacClade. Version 3.0. Sinauer, Sunderland, Mass.

MASTERS, B. A., C. J. QUAIFE, J. C. ERICKSON, E. J. KELLY, G. J. FROELICK, B. P. ZAMBROWICZ, R. L. BRINSTER, and R. D. PALMITER. 1994. Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. J. Neurosci. **14**:5844–5857.

MINDELL, D. P. 1991. Aligning DNA sequences: homology and phylogenetic weighting. Pp. 73–89 in M. M. MIYA-MOTO and J. CRACRAFT, eds. Phylogenetic analysis of DNA sequences. Oxford University Press, New York.

NELSON, J. S. 1994. Fishes of the world. Wiley, New York.

NISHIMURA, N., G. R. CAM, H. NISHIMURA, C. TOHYAMA, Y. SAITOU, and D. L. ADELSON. 1996. Evidence for developmentally regulated transcriptional, translational, and post-translational control of metallothionein gene expression in hair follicles. Reprod. Fertil. Dev. **8**:1089–1096.

OHNO, S. 1978. Evolution from primordial oligomeric repeats to modern coding sequences. J. Mol. Evol. **25**:325–329.

PATARNELLO T., L. BARGELLONI, E. BONCINELLI, F. SPADA, M. PANNESE, and V. BROCCOLI. 1997. Evolution of Emx genes and brain development in vertebrates. Proc. R. Soc. Lond. B Biol. Sci. **264**:1763–1766.

QUAIFE, C. J., S. D. FINDLEY, J. C. ERICKSON, G. J. FROELICK, E. J. KELLY, B. P. ZAMBROWICZ, and R. D. PALMITER. 1994. Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous ephitelia. Biochemistry **33**:7250–7259.

ROESIGADI, G. 1993. Response of invertebrate metallothioneins and MT genes to metals and implications for environmental toxicology. Pp. 141–158 in K. T. SUZUKI, N. IMURA, and M. KIMURA, eds. Metallothionein III. Birkhauser Verlag, Basel, Switzerland.

RUUD, J. T. 1954. Vertebrates without erythrocytes and blood pigment. Nature **173**:848–850.

SANGER F., S. NICKLEN, and A. R. COULSON. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

SCUDIERO, R., V. CARGINALE, M. RIGGIO, C. CAPASSO, A. CAPASSO, P. KILLE, G. DI PRISCO, and E. PARISI. 1997. Difference in hepatic metallothionein content in red-blooded and haemoglobinless Antarctic fishes. Undetectable metallothionein levels in haemoglobinless fishes is accompanied by accumulation of untranslated metallothionein mRNA. Biochem. J. **322**:207–211,

TAKEZAKI, N., A. RZHETSKY, and M. NEI. 1995. Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. **12**:832–833.

TEMPLETON, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. Evolution **37**:221–244.

THOMPSON, J. D., D. G. HIGGINS, and T. J. GIBSON. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.

WHEELER, W. C., and D. GLADSTEIN. 1994. MALIGN: a multiple sequence alignment program. J. Hered. **85**:417.

WINGE, D. R., K. B. NIELSON, K. D. ZEIKUS, and W. R. GRAY. 1984. Structural characterization of the isoforms of neonatal and adult rat liver metallothionein. J. Biol. Chem. **259**:11419–11425.

ZAFARULLAH, M., K. BONHAM, and L. GEDAMU. 1988. Structure of the rainbow trout metallothionein B gene and characterization of its metal-responsive region. Mol. Cell. Biol. **8**:4469–4476.