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# Biochemical Systematics of Notothenioid Fishes from Antarctica

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**Key Word Index**—Notothenioids; systematics; Antarctica; allozyme variation; cryptic species; genetic distance.

**Abstract**—Genetic variation at 30 protein-coding loci was examined in seven forms of notothenioid fishes from Antarctica. Multilocus heterozygosity varied from 0.018 to 0.078 across taxa. An analysis of the allozyme data revealed the probable existence of an unrecognized cryptic species within *Trematomus bernacchii*. *Pagothenia borchgrevinki* is as closely related to some species of *Trematomus* as are some species of *Trematomus* to each other. Speciation among the species of *Trematomus* and *Pagothenia* appears to have taken place primarily after the separation of Antarctica from Australia.

## Introduction

Notothenioid fishes dominate the coastal zone waters of Antarctica and are circumcontinental in their distribution [1, 2]. These fishes comprise a moderate radiation of teleosts into unoccupied niches and are adapted to the extreme, but thermally stable, environment of subzero waters [1]. There are few deep oceanic islands around Antarctica and no other obvious geographical barriers to gene flow which might promote speciation in this group. As a result, these coastal zone fishes offer the opportunity for studying speciation in a relatively simple community.

Notothenioids are characterized by a suite of morphological characters, primarily involving soft-body parts [2]. Consequently, their evolution is poorly recorded in the fossil record [2]. These fishes have evolved a glycoprotein as an antifreeze mechanism that binds ice and prevents crystal expansion within them at subzero temperatures [1, 3]. Biochemical characterization of antifreeze proteins in notothenioids has been used to assess their general systematic relationships [4, 5], although the systematic value of these glycoproteins is limited because they are probably encoded by a few closely related loci. In contrast, protein electrophoresis samples a larger set of genetic loci. Protein electrophoresis can identify closely related vertebrate species [6, 7] and provide estimates of genetic distance among taxa [8, 9]. A previous survey of 21–26 protein-coding loci was conducted to assess levels of genetic variability for three species of notothenioids [10], but the resulting data were not used for a systematic evaluation.

Our primary objective was to assess the level of genetic variation and divergence among six recognized species of notothenioid fishes from Antarctica. Analysis of the genetic information yielded a summary of overall similarity and the phylogenetic relationships among the species which was compared to the currently accepted taxonomy of the notothenioids.

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## Materials and Methods

Fishes ($n = 148$) were collected near McMurdo Station, Antarctica, in the Ross Sea. There were approximately 20 locations separated by up to 20 km at which holes were drilled in the ice and fish were caught either by trapping or by hook and line. Fish were identified by their external morphology as described by Norman [11]. They were returned to the laboratory and maintained in running sea water aquaria. Fish were killed from one day to several weeks later, and liver, muscle and kidney tissues were removed and placed on dry ice for a few hours prior to their placement in an ultracold freezer (−70°C) for up to six weeks. They were transported back to the United States on dry ice and then placed in another ultracold freezer.

Tissues were prepared for horizontal starch gel electrophoresis according to the methods of Selander *et al.* [12] and Place and Powers [13]. Thirty protein-coding loci were analyzed using more than one buffer in most cases (Table 1). Staining recipes are in Selander *et al.* (12), Nichols *et al.* [14] or Harris and Hopkinson [15]. Substrates for esterase and peptidase are beta-napthyl propionate and leucyl-alanine, respectively. Samples of *Dissostichus mawsoni* were included for comparison on each gel. The common allele in this species was designated as 100 for each locus. Other alleles in this species and other species were designated on the basis of relative mobility of their products compared to that of the product of the 100 allele. Reference photographs of the gels were archived with the data at the Savannah River Ecology Laboratory. In the few cases where pictures were not available, mobilities were estimated based on the named position of an allele relative to other alleles scored for the same gel. Side by side comparisons were made for all species pairs and, where appropriate, for particular rare alleles. Loci were numbered according to the mobility of the products with the most anodal as 1 when one or more isozymes appeared on the same gel or followed traditional nomenclature for teleosts. A locus was defined as polymorphic when the frequency of the common allele was 0.95 or less.

Calculations of allele frequencies, genetic distances and deviations from Castle–Hardy–Weinberg expectations were calculated using BIOSYS-1 [16]. Divergence times between taxa were estimated from Nei's genetic distance [17] using the method of Sarich [18] which takes into account the differentiation caused by fast and slow evolving loci. Stability of the relationships among groups was assessed by jack-knifing the data from variable loci [19]. Jack-knifing involves removing data for one locus at a time, with replacement, and recalculating genetic distances for the remaining 29 loci. Sixteen loci were chosen for perturbation because they differed in their allele frequencies among forms 1 and 2 of *T. bernacchii* and *T. hansoni*. The jack-knife technique was the basis of the computation of standard errors for the genetic distances. A Distance Wagner tree and UPGMA dendrogram were calculated to summarize the genetic relationships between the forms. Statistical significance was indicated by $P \leq 0.05$ and a highly significant difference occurred when $P \leq 0.01$.

## Results

Allelic variation was examined for 30 protein-coding loci. Although tissues were maintained on ice and handled rapidly during preparations of homogenates, certain labile enzymes tended to denature as evidenced by their absence rather than a general streaking on the gel. The products of the following loci (abbreviations explained in Table 1) were particularly susceptible to denaturation: *ADA-1**, *CAT-1**, *EST-2**, *GP-4**, *GUS-1**, *HBDH-1**, *ICDP-2**, *PEP-1** and *IDDH-1**. Thus, sample size within a taxon varied across loci (Table 1). The banding patterns of heterozygotes were consistent with those expected from the known quaternary structure of these enzymes in vertebrates [15, 20]. Multiple bands for PGM activity were suggestive of more than three loci in several of the taxa, but these could not be scored in all species, especially *Dissostichus mawsoni*, the outgroup taxon.

While designating alleles for the 30 loci of the six recognized species of fish, a fixed allelic difference was noted for *MDH-1** within *Trematomus bernacchii*. The two groups recognized on the basis of this difference also had highly significant allele frequency differences for *GPI-B**, *GUS-1** and *MEP-1**. The two forms came from the same area but from collections made at different times. Because of the allele frequency differences, the two groups were treated as individual taxa (forms 1 and 2) for all subsequent analyses.

Of the 30 loci analyzed, only *CK-2** and *EST-2** had the same allele for all seven taxa. Direct count heterozygosity and percentage polymorphic loci tended to be higher for the larger samples. Heterozygosity estimates ranged from 0.044 to 0.078 for samples of the same three recognized species (*Pagothenia borchgrevinki*, *T. hansoni*, and both forms of *T. bernacchii*) sampled by Somero and Soule [10]. They reported percentage polymorphic loci (*P*) less than 15% (0.05 criterion) and direct count heterozygosity measures of 0.005–0.033 for 21–26 loci; these values are within the

TABLE 1. ALLELE FREQUENCIES AND DESIGNATIONS, AND SAMPLE SIZES (in parentheses) FOR 30 PROTEIN-CODING LOCI FOR SEVEN NOTOTHENIOIDS FROM ANTARCTICA

| | Sample† | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Locus/allele‡ | TL | TP | DM | TB1 | TB2 | TH | PB | Buffer/tissue§ |
| *mAAT** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *226 | 0.800 | 0.0 | 0.0 | 0.013 | 0.0 | 0.0 | 0.056 | AC/L |
| *164 | 0.200 | 1.000 | 0.0 | 0.987 | 0.963 | 0.962 | 0.944 | LiOH/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.037 | 0.038 | 0.0 | THCl/M |
| *ADA-1** | (2) | (8) | (2) | (35) | (19) | (23) | (8) | |
| *107 | 0.0 | 1.000 | 0.0 | 0.271 | 0.211 | 0.152 | 0.0 | JRP/L |
| *104 | 0.0 | 0.0 | 0.0 | 0.400 | 0.342 | 0.087 | 0.0 | LiOH/L |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | THCl/L |
| *97 | 0.0 | 0.0 | 0.0 | 0.014 | 0.079 | 0.239 | 0.063 | |
| *94 | 0.0 | 0.0 | 0.0 | 0.314 | 0.368 | 0.196 | 0.0 | |
| *89 | 0.750 | 0.0 | 0.0 | 0.0 | 0.0 | 0.239 | 0.500 | |
| *86 | 0.250 | 0.0 | 0.0 | 0.0 | 0.0 | 0.087 | 0.063 | |
| *83 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.375 | |
| *CAT-1** | (5) | (12) | (2) | (23) | (27) | (25) | (21) | |
| *100 | 1.000 | 1.000 | 1.000 | 1.000 | 0.0963 | 1.000 | 0.976 | THCl/M |
| *85 | 0.0 | 0.0 | 0.0 | 0.0 | 0.037 | 0.0 | 0.024 | |
| *CK-1** | (5) | (12) | (3) | (32) | (27) | (26) | (26) | |
| *100 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.981 | JRP/M |
| *94 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.109 | TC8/M |
| *CK-2** | (4) | (12) | (3) | (39) | (22) | (21) | (24) | JRP/M |
| *100 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | TC8/M |
| *EST-2** | (5) | (12) | (3) | (4) | (27) | (25) | (25) | |
| *100 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | THCl/M |
| *FH-1** | (4) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *107 | 1.000 | 0.0 | 0.0 | 1.000 | 1.000 | 1.000 | 1.000 | JRP/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *87 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *GAPDH-1** | (5) | (12) | (3) | (32) | (27) | (26) | (26) | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | AC/M |
| *86 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.019 | |
| *81 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 0.981 | 0.981 | |
| *GAPDH-2** | (5) | (12) | (3) | (39) | (27) | (26) | (25) | |
| *119 | 0.0 | 0.0 | 0.0 | 0.026 | 0.0 | 0.0 | 0.040 | AC/M |
| *100 | 1.000 | 1.000 | 0.500 | 0.974 | 1.000 | 1.000 | 0.960 | |
| *41 | 0.0 | 0.0 | 0.500 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *G3PDH-1** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *100 | 1.000 | 1.000 | 1.000 | 0.936 | 0.981 | 1.000 | 1.000 | TC6.5/L |
| *88 | 0.0 | 0.0 | 0.0 | 0.064 | 0.019 | 0.0 | 0.0 | JRP/L |
| *GPI-B** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *100 | 0.0 | 0.0 | 0.833 | 0.0 | 0.0 | 0.0 | 0.904 | THCl/M |
| *75 | 1.000 | 1.000 | 0.0 | 0.0 | 0.981 | 0.981 | 0.0 | TC8/M |
| *66 | 0.0 | 0.0 | 0.0 | 1.000 | 0.0 | 0.019 | 0.096 | LiOH/M |
| *48 | 0.0 | 0.0 | 0.167 | 0.0 | 0.019 | 0.0 | 0.0 | |
| *GP-4** | (1) | (4) | (3) | (11) | (9) | (4) | (3) | |
| *270 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 1.000 | LiOH/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | THCl/M |
| *GUS-1** | (5) | (12) | (3) | (34) | (21) | (23) | (26) | |
| *100 | 1.000 | 1.000 | 1.000 | 0.0 | 0.143 | 0.0 | 0.981 | THCl/L |
| *74 | 0.0 | 0.0 | 0.0 | 0.647 | 0.857 | 0.109 | 0.019 | |
| *60 | 0.0 | 0.0 | 0.0 | 0.353 | 0.0 | 0.891 | 0.0 | |
| *HBDH-1** | (3) | (8) | (2) | (30) | (18) | (21) | (23) | |
| *125‖ | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.022 | JRP/L |
| *119 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 0.978 | TC8/L |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |

TABLE 1—*CONTINUED*

| | Sample† | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Locus/allele‡ | TL | TP | DM | TB1 | TB2 | TH | PB | Buffer/tissue§ |
| *ICDP-1** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *120 | 0.0 | 1.000 | 0.0 | 0.949 | 0.944 | 1.000 | 0.981 | TM/M |
| *107 | 1.000 | 0.0 | 0.0 | 0.051 | 0.056 | 0.0 | 0.019 | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *ICDP-2** | (4) | (12) | (3) | (28) | (27) | (26) | (26) | |
| *152 | 0.0 | 0.042 | 0.0 | 0.0 | 0.019 | 0.019 | 0.038 | TM/M |
| *132 | 1.000 | 0.958 | 0.0 | 1.000 | 0.944 | 0.981 | 0.962 | |
| *106 | 0.0 | 0.0 | 0.0 | 0.0 | 0.037 | 0.0 | 0.0 | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *IDDH-1** | (5) | (12) | (3) | (37) | (27) | (18) | (25) | |
| *1800 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | TC8/L |
| *1300 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | LiOH/L |
| *1000 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.000 | |
| *600 | 0.0 | 0.0 | 0.0 | 1.000 | 1.000 | 1.000 | 0.0 | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *LDH-A** | (5) | (10) | (3) | (39) | (27) | (26) | (26) | |
| *118 | 0.0 | 0.050 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | TM/L |
| *100 | 1.000 | 0.850 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | |
| *82 | 0.0 | 0.100 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *MDH-1** | (5) | (12) | (3) | (39) | (27) | (26) | (21) | |
| *109 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.024 | JRP/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.038 | 0.0 | TM/M |
| *83 | 0.0 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | |
| *60 | 1.000 | 1.000 | 0.0 | 0.0 | 1.000 | 0.962 | 0.976 | |
| *MDH-2** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *1000 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | JRP/M |
| *92 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.000 | TM/M |
| *80 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 0.0 | |
| *MDH-3** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | JRP/M |
| *57 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 1.000 | TM/M |
| *MEP-1** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *100 | 0.0 | 0.0 | 1.000 | 0.987 | 0.0 | 0.962 | 0.981 | TC8/M |
| *95 | 1.000 | 0.917 | 0.0 | 0.013 | 0.685 | 0.019 | 0.019 | |
| *90 | 0.0 | 0.083 | 0.0 | 0.0 | 0.315 | 0.019 | 0.0 | |
| *MPI-1** | (5) | (12) | (3) | (36) | (27) | (26) | (26) | |
| *113 | 0.0 | 0.0 | 0.0 | 0.014 | 0.0 | 0.0 | 0.0 | TC8/M |
| *109 | 0.0 | 1.000 | 0.0 | 0.986 | 0.963 | 1.000 | 0.981 | JRP/M |
| *105 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.0 | 0.0 | |
| *100 | 1.000 | 0.0 | 1.000 | 0.0 | 0.019 | 0.0 | 0.019 | |
| *MPI-2** | (3) | (12) | (3) | (36) | (27) | (26) | (26) | |
| *110 | 1.000 | 1.000 | 0.0 | 1.00 | 0.963 | 1.000 | 1.000 | TC8/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.037 | 0.0 | 0.0 | JRP/M |
| *PEP-1** | (5) | (12) | (3) | (16) | (22) | (21) | (19) | |
| *123 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 1.000 | LiOH/L |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *PGD-1** | (5) | (12) | (3) | (27) | (26) | (26) | (26) | |
| *100 | 0.0 | 0.0 | 1.000 | 0.017 | 0.019 | 0.0 | 0.0 | AC/M |
| *92 | 0.0 | 0.0 | 0.0 | 0.017 | 0.019 | 0.077 | 0.058 | |
| *83 | 1.000 | 1.000 | 0.0 | 0.966 | 0.944 | 0.923 | 0.942 | |
| *70 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.0 | 0.0 | |
| *PGM-1** | (5) | (12) | (3) | (39) | (26) | (26) | (26) | |
| *108 | 0.0 | 0.0 | 0.0 | 0.013 | 0.0 | 0.019 | 0.0 | TM/M |
| *100 | 1.000 | 1.000 | 1.000 | 0.974 | 1.000 | 0.981 | 1.000 | |
| *90 | 0.0 | 0.0 | 0.0 | 0.013 | 0.0 | 0.0 | 0.0 | |

TABLE 1—*CONTINUED*

| | Sample† | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Locus/allele‡ | TL | TP | DM | TB1 | TB2 | TH | PB | Buffer/tissue§ |
| *PGM-2** | (5) | (12) | (3) | (32) | (27) | (26) | (26) | |
| *104 | 1.000 | 1.000 | 0.0 | 1.000 | 1.000 | 1.000 | 1.000 | AC/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | JRP/M |
| *PGM-3** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *110 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.962 | JRP/M |
| *100 | 0.0 | 0.0 | 1.000 | 0.013 | 0.0 | 0.038 | 0.038 | |
| *94 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| *81 | 1.000 | 0.0 | 0.0 | 0.987 | 0.981 | 0.942 | 0.0 | |
| *55 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.0 | 0.0 | |
| *SOD-1** | (5) | (12) | (3) | (39) | (27) | (26) | (26) | |
| *231 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.038 | 0.0 | JRP/M |
| *206 | 0.0 | 0.0 | 0.0 | 0.0 | 0.019 | 0.077 | 0.0 | |
| *189 | 1.000 | 1.000 | 0.0 | 1.000 | 0.981 | 0.885 | 0.0 | |
| *100 | 0.0 | 0.0 | 1.000 | 0.0 | 0.0 | 0.0 | 1.000 | |
| H¶ | 0.030 | 0.018 | 0.022 | 0.057 | 0.078 | 0.050 | 0.044 | |
| S.E. | 0.021 | 0.011 | 0.015 | 0.029 | 0.031 | 0.022 | 0.014 | |
| %*P*(0.05) | 6.7 | 10.0 | 6.7 | 36.7 | 46.7 | 40.0 | 53.3 | |
| *A* | 1.1 | 1.1 | 1.1 | 1.5 | 1.7 | 1.6 | 1.6 | |

†TL = *Trematomus loennbergii*, TP = *T. pennelli*, DM = *Dissostichus mawsoni*, TB1 = *T. bernacchii* form 1, TB2 = *T. bernacchii* form 2, TH = *T. hansoni*, PB = *Pagothenia borchgrevinki*.

‡Locus designations and abbreviations are as recommended [31] and are given with Enzyme Commission numbers as follows: *mAAT* (2.6.1.1) = aspartate aminotransferase, *ADA* (3.5.4.4) = adenosine deaminase, *CAT* (1.11.1.6) = catalase, *CK* (2.7.3.2) = creatine kinase, *EST* (3.1.1.1) = esterase, *FH* (4.2.1.2) = fumarate hydratase, *GAPDH* (1.2.1.12) = glyceraldehyde-3-phosphate dehydrogenase, *G3PDH* (1.1.1.8) = glycerol-3-phosphate dehydrogenase, *Gpi* (5.3.1.9) = glucose-6-phosphate isomerase, *GP* = general protein, *GUS* (3.2.1.31) = beta-glucuronidase, *HBDH* = hydroxybuturate dehydrogenase, *ICDP* (1.1.1.42) = isocitrate dehydrogenase, *LDH* (1.1.1.27) = lactate dehydrogenase, *MDH* (1.1.1.37) = NAD-dependent malate dehydrogenase, *MEP-1* (1.1.1.40) = NADP-dependent malic enzyme, *MPI* (5.3.1.8) = mannose-6-phosphate isomerase, *PEP* (3.4.11 or 3.4.13) = peptidase, *PGM* (2.7.5.1) = phosphoglucomutase, *PGD* (1.1.1.44) = phosphogluconate dehydrogenase, *IDDH* (1.1.1.14) = L-iditol dehydrogenase, *SOD* (1.15.1.1) = superoxide dismutase.

§Buffer, tissue and pH conditions: AC = amine citrate 6.2 [29]; TC6.5 = Tris–citrate 6.5, TM = Tris–maleate 7.4, TC = Tris–citrate 8.0, LiOH = lithium hydroxide 8.2, THCl = Tris–HCl 8.5 [12], JRP = Tris–citrate 7.1 [30], L = liver, M = muscle.

‖Estimated mobility (no picture available).

¶*H* = direct count heterozygosity; S.E. = standard error, %*P*(0.05) = percentage polymorphic loci with common allele frequency 0.95 or less, and *A* = average number of alleles per locus.

confidence intervals calculated from our data. Our analyses indicated 40% or more polymorphic loci for these species (Table 1). There were no deviations from Castle–Hardy–Weinberg expectations that could not be attributed to a small sample size of rare genotypic categories.

Overall similarity and genealogical relationships among the taxa are summarized using phenetic (UPGMA) and phylogenetic (Distance Wagner) methods, respectively (Fig. 1). Both methods were employed to assess the phylogenetic relationships among these fishes, even though we are aware that UPGMA is traditionally considered a phenetic clustering method only. Rohlf and Wooten [21] have demonstrated that UPGMA methods may reflect phylogenetic histories more closely than Distance Wagner trees if fewer than 50 variable loci are assayed in a study. Genetic relationships were consistent with what would be expected from currently accepted relationships based on previous taxonomic designations, except for that between the two forms of *T. bernacchii.* Although the overall phenologies were similar, the relationships of *T. bernacchii* forms 1 and 2 with *T. hansoni*, and the connection of *T. pennellii* with the *T. bernacchii–hansoni* group differed between the phenogram and cladogram. When data from the various loci were jack-knifed [19], 13/16 and 12/16 perturbations resulted

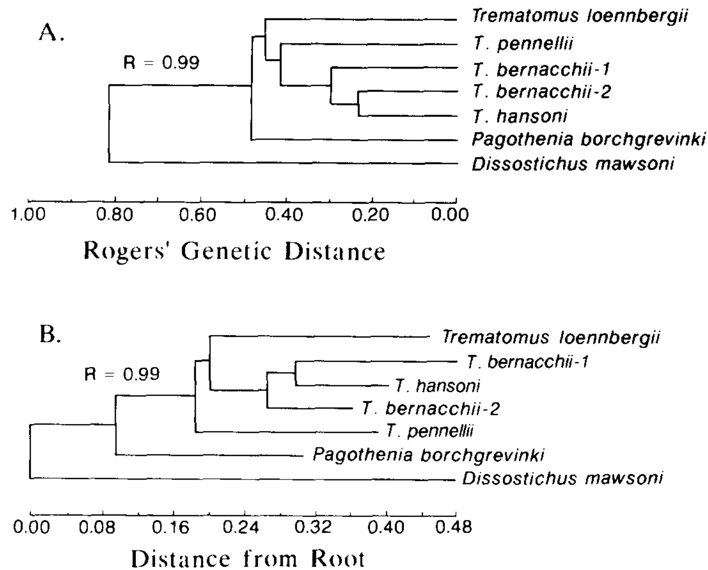


FIG. 1. RELATIONSHIPS OF SEVEN FORMS OF NOTOTHENIOID FISHES FROM ANTARCTICA AS MEASURED BY ROGERS' GENETIC DISTANCE [9] AND SUMMARIZED IN A PHENOGRAM (A) CALCULATED USING THE UNWEIGHTED PAIR-GROUP METHOD OF AVERAGING OR IN A ROOTED DISTANCE WAGNER TREE (B). *R* is the cophenetic correlation coefficient.

in the same phenologies for Distance Wagner tree and the UPGMA phenogram, respectively, as that observed for all 30 loci (Fig. 1). The closest sister group to the *T. bernacchii–hansoni* group was either *T. pennellii* or *T. loennbergii*, followed by *Pagothenia borchgrevinki*, depending on the clustering method used. *Pagothenia* was more closely related to the *Trematomus* group than was *Dissostichus*.

The *T. hansoni–bernacchii* group differed in its phenology from our expectations based on morphological similarities. On both the UPGMA and Distance Wagner dendrograms, *T. hansoni* clustered with one of the two forms of *T. bernacchii*, but the two forms did not cluster together in either case. Because the genetic distances were low, this relationship might have been expected to be unstable when perturbed, but this was not the case. The mean genetic distance and standard error were 0.303±0.006 between the two forms of *T. bernacchii*, 0.276±0.006 between *T. bernacchii* form 1 and *T. hansoni*, and 0.222±0.005 between *T. bernacchii* form 2 and *T. hansoni*. The latter relationship supports the UPGMA phenology over that shown by the Distance Wagner tree.

Intra- and intergeneric genetic distances and estimated divergence times are given in Table 2. For the species of *Trematomus*, the average genetic distance was 0.390; for the comparisons of *Trematomus–Pagothenia*, the mean genetic distance was 0.483, which fell within the range of values reported for comparisons within *Trematomus*. Mean genetic distance was large for comparison of *Dissostichus* to either species of either genus.

## Discussion

Our estimates of heterozygosity for three of the notothenioids tended to be higher than those reported by Somero and Soule [10]. However, confidence limits for these estimates overlap broadly. These higher estimates are probably due to inclusion of different enzymes and buffer conditions in our study. Sampling different loci can affect heterozygosity estimates [22]. Within the confidence intervals for our data, the level of

TABLE 2. MATRIX OF GENETIC DISTANCE MEASURES FOR SEVEN FORMS OF NOTOTHENIOIDS

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| *Trematomus loennbergii* | ***5*** | 0.246<br>(2.0) | 1.120<br>(19.6) | 0.309<br>(2.4) | 0.178<br>(1.4) | 0.217<br>(1.8) | 0.342<br>(2.7) |
| *T. pennelli* | 0.465 | ***12*** | 1.254<br>(25.2) | 0.280<br>(2.2) | 0.158<br>(1.3) | 0.197<br>(1.5) | 0.298<br>(2.3) |
| *Dissostichus mawsoni* | 0.812 | 0.837 | ***3*** | 1.227<br>(24.3) | 1.307<br>(27.5) | 1.206<br>(23.2) | 0.941<br>(12.8) |
| *T. bernacchii* form 1 | 0.508 | 0.486 | 0.824 | ***39*** | 0.105<br>(0.9) | 0.086<br>(0.7) | 0.275<br>(2.2) |
| *T. bernacchii* form 2 | 0.398 | 0.376 | 0.834 | 0.308 | ***27*** | 0.055<br>(0.4) | 0.267<br>(2.1) |
| *T. hansoni* | 0.435 | 0.416 | 0.819 | 0.280 | 0.226 | ***26*** | 0.224<br>(1.9) |
| *Pagothenia borchgrevinki* | 0.531 | 0.500 | 0.766 | 0.478 | 0.470 | 0.437 | ***26*** |

Above the diagonal is Nei's [17] unbiased genetic distance and estimated divergence times [18] (in parentheses) and below the diagonal is the modified Rogers' distances [9]. Sample sizes are italicized for each taxon on the diagonal.

genetic variation in the notothenioids ($P=28.6\%$ and $H=0.043\pm0.008$) is consistent with that for fish in general ($P=20.9\%$ and $H=0.051$; 25).

Notothenioids occur within a narrow range of environmental temperatures (−1.4 to −2.15°C) in subzero waters [2]. Low levels of genetic variation might be expected because of reduced environmental variance under these thermal conditions. Somero and Soule [10] rejected the reduced genetic variation prediction of the niche-variation hypothesis based on comparisons of the notothenioids with temperate and tropical species. Our results are consistent with theirs, but we question the underlying assumption of their prediction. The low variability of water temperature was assumed to be the critical environmental factor for these fishes in the previous study. The more extreme variation in food availability due to lack of sunlight during the austral winter [2] may be much more important than variation in water temperature. Because the niche is not dependent on one variable, an appropriate test of the niche-variation hypothesis requires a better understanding of the environments of a species. Observed differences in species-specific heterozygosities between notothenioids (Table 1) are probably related to the ecological differences among the species examined.

Divergence among these species is reflected not only by differences in heterozygosities but also by the genetic distances between them. The pattern of genetic relationships among the notothenioids is consistent with their current taxonomy [1, 11]. *Dissostichus mawsoni* is clearly the most distantly related form and represents a good outgroup (Table 2, Fig. 1). Although our data suggest that *Pagothenia* is a distant relative of species within *Trematomus*, average intergeneric distances (Rogers' $D=0.483$) fall within the range of intrageneric distances for species of *Trematomus* ($D=0.266$–$0.508$). The species which account for the higher intrageneric distances between species of *Trematomus* were *T. pennellii* and *T. loennbergii*. Although recognition of taxa should be based on a series of characteristics and not just genetic data [23], the taxonomic status of *Pagothenia* may warrant reconsideration. Past taxonomic treatment included *Pagothenia borchgrevinki* within the genus *Trematomus* [11]. We conclude that there are other similarities between *Pagothenia* and species of *Trematomus*, although *Pagothenia* is not a benthic but a benthopelagic feeder [2]. Moreover, *Pagothenia* remains as a sister group to *Trematomus* (Fig. 1). We do not ascribe to using only genetic distances for the purpose of defining species or genera [23]. To evaluate the systematic relationship between *Pagothenia* and *Trematomus*, we suggest using several characters, such as those used for foraging ecology, morphology and chemical physiology, in addition to the genetic data, to determine

whether a taxonomic reconsideration is appropriate for these species of fishes. Our data suggest that the generic status of *Pagothenia* needs further consideration or *T. pennellii* and *T. loennbergii* be considered for separate generic status. Therefore, the higher level taxonomy of the notothenioids needs further study.

Another taxonomic consideration is the relationship of the two forms of *T. bernacchii.* The genetic differences, particularly the fixed allelic differences in *MDH-1**, argue for the existence of two cryptic species. Although strong frequency shifts have been observed between populations of fishes, these normally occur over much larger distances than was the case for the two forms of *T. bernacchii* [e.g. 24–26]. The large allele frequency differences (*GUS-1**, *GPI-B**, *MEP-1**) and the fixed allelic differences (*MDH-1**) suggest that gene flow is limited or non-existent between the two forms. Furthermore, *Trematomus bernacchii* is characterized by two morphotypes with and without a white spot on the top of the head (M. W. S. Smith, personal observation). This polymorphism may be concordant with the genetic differences between the two forms, but additional sampling will be needed to test this hypothesis and to determine the degree of sympatry of the two forms.

Both forms of *T. bernacchii* and *T. hansoni* are a closely related group, and one of the forms (form 2) may be more closely related to *T. hansoni* than to form 1 of *T. bernacchii* based on average genetic distance (Fig. 1A). The exclusion of different amounts of genetic data (i.e. jack-knifing) allows the calculation of confidence intervals for that data matrix and does not alter the relationship substantially. Confidence limits around the average genetic distances are small and the distance between the three forms are significantly different. Our genetic distance data are in support of the UPGMA phenology over the Distance Wagner tree, where *Trematomus bernachii* form 2 is more closely allied to *T. hansoni* than to *T. bernachii* form 1. This is in agreement with the computer simulation studies of Rohlf and Wooten [21] demonstrating that UPGMA methods reflect known phylogenetic histories more accurately than do Distance Wagner trees when fewer than 50 variable loci are assayed. Until a taxonomic re-evaluation of the forms of *T. bernacchii* and *T. hansoni* is completed, care needs to be exercised in studies involving *T. bernacchii.* Evolutionary change in a number of other characteristics may have taken place since the divergence of the two forms.

Important geological events that may have aided or accelerated speciation include: the formation of the circumpolar current *ca.* 23.5 MYBP [2]; ice-cap melting *ca.* 30 MYBP [27] and again around 13 MYBP; and the coastal water temperatures dropping to near freezing about 10 MYBP [2]. Barriers to gene flow might include waters of higher temperatures or lower salinities, glaciers that extend well beyond the coastline, and distance between offshore islands and the mainland. Multiple warming and cooling of the continent created riverine and estuarine habitats [2], which could have played an important role in the speciation of these forms. Rapid radiation into available niches might have occurred but precise information about isolating events is lacking. For example, in the more recently diverged forms, changes in sea level during the Pliocene and Pleistocene [28] and their effects on the continuity of suitable habitat around the continental shelf may have resulted in the isolation necessary for speciation in the absence of obvious geographical barriers to gene flow. Divergence times need to be estimated for other groups in Antarctica. If there is concordance among the divergence events associated with the geological history of Antarctica for major taxonomic groups, these patterns might be used to focus on major vicariant events that enhanced speciation in the Antarctic fauna.

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