

Phylogenetic investigations of Antarctic notothenioid fishes (Perciformes: Notothenioidei) using complete gene sequences of the mitochondrial encoded 16S rRNA[☆]

Thomas J. Near,^{a,*} James J. Pesavento,^b and Chi-Hing C. Cheng^b

^a Department of Ecology and Evolutionary Biology, 569 Dabney Hall, University of Tennessee, Knoxville, TN 37996-1610, USA

^b Department of Animal Biology, 515 Morrill Hall, University of Illinois, Urbana, IL 61801, USA

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Abstract

The Notothenioidei dominates the fish fauna of the Antarctic in both biomass and diversity. This clade exhibits adaptations related to metabolic function and freezing avoidance in the subzero Antarctic waters, and is characterized by a high degree of morphological and ecological diversity. Investigating the macroevolutionary processes that may have contributed to the radiation of notothenioid fishes requires a well-resolved phylogenetic hypothesis. To date published molecular and morphological hypotheses of notothenioids are largely congruent, however, there are some areas of significant disagreement regarding higher-level relationships. Also, there are critical areas of the notothenioid phylogeny that are unresolved in both molecular and morphological phylogenetic analyses. Previous molecular phylogenetic analyses of notothenioids using partial mtDNA 12S and 16S rRNA sequence data have resulted in limited phylogenetic resolution and relatively low node support. One particularly controversial result from these analyses is the paraphyly of the Nototheniidae, the most diverse family in the Notothenioidei. It is unclear if the phylogenetic results from the 12S and 16S partial gene sequence dataset are due to limited character sampling, or if they reflect patterns of evolutionary diversification in notothenioids. We sequenced the complete mtDNA 16S rRNA gene for 43 notothenioid species, the largest sampling to-date from all eight taxonomically recognized families. Phylogenetic analyses using both maximum parsimony and maximum likelihood resulted in well-resolved trees with most nodes supported with high bootstrap pseudoreplicate scores and significant Bayesian posterior probabilities. In all analyses the Nototheniidae was monophyletic. Shimodaira–Hasegawa tests were able to reject two hypotheses that resulted from prior morphological analyses. However, despite substantial resolution and node support in the 16S rRNA trees, several phylogenetic hypotheses among closely related species and clades were not rejected. The inability to reject particular hypotheses among species in apical clades is likely due to the lower rate of nucleotide substitution in mtDNA rRNA genes relative to protein coding regions. Nevertheless, with the most extensive notothenioid taxon sampling to date, and the much greater phylogenetic resolution offered by the complete 16S rRNA sequences over the commonly used partial 12S and 16S gene dataset, it would be advantageous for future molecular investigations of notothenioid phylogenetics to utilize at the minimum the complete gene 16S rRNA dataset.

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1. Introduction

Antarctic notothenioid fishes exhibit remarkable adaptations to polar conditions including antifreeze glycoproteins (AFGP) and enhanced physiological performance at cold temperatures, and are thought to

represent the only known marine species flock (Cheng, 1998; Clarke and Johnston, 1996; Eastman, 1993; Eastman and McCune, 2000). Notothenioids provide a unique model system to investigate mechanisms of adaptive diversification and speciation in the isolated geographic setting of the frigid waters surrounding Antarctica. Well-resolved and robust phylogenetic hypotheses are fundamental to any investigations of notothenioid speciation and adaptive radiation; however, previous molecular phylogenetic studies have sampled a limited number of species and have extensively relied on

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* Corresponding author. Fax: 1-865-974-3067.

E-mail address: tnear@utk.edu (T.J. Near).

partial mtDNA gene sequences (Bargelloni et al., 2000). These efforts have resulted in phylogenetic trees with limited resolution and little power to discriminate among alternative phylogenetic hypotheses.

Despite problems involving the character and taxon sampling in previous molecular investigations of notothenioid relationships, there exists a rich database of comparative morphological characters (Balushkin, 1992, 2000; Eakin, 1981; Iwami, 1985) and DNA sequence data (Bargelloni et al., 1994, 2000; Derome et al., 2002; Lecointre et al., 1997). In general, results between phylogenetic analyses of these datasets are congruent. For example, all published analyses agree that the families Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae form a monophyletic group. Most of the species in these four lineages are restricted to high latitude areas of the Antarctic, and they are recognized here as the High Antarctic clade.

However, there remain key differences between morphological and molecular analyses, and some critical areas of the notothenioid tree remain unresolved regardless of the type of data used. First, there is disagreement between morphological and molecular phylogenies pertaining to higher-level relationships within the Notothenioidei, namely the relationships of basal non-Antarctic lineages in the clade (Balushkin, 2000; Bargelloni et al., 2000; Lecointre et al., 1997). Second, analyses of mtDNA sequence data have suggested that two major notothenioid lineages, Nototheniidae (notothens) and Bathydraconidae (dragonfishes) are paraphyletic (Bargelloni et al., 2000; Derome et al., 2002).

Most notothenioids are endemic to the Southern Ocean with the greatest number of species distributed along the Antarctic Continental Shelf (Eastman, 1993). A number of species are endemic to temperate non-Antarctic areas north of the Southern Ocean such as southern Australia, Tasmania, southern New Zealand, southern South America, and the Falkland Islands. The composition of the non-Antarctic notothenioid fauna includes both phylogenetically basal lineages, and species from clades that are hypothesized to have Antarctic origins but have dispersed to non-Antarctic regions of the Southern Ocean subsequent to the continental separation of Antarctica from Australia, New Zealand, and South America (Eastman, 1993; Stankovic et al., 2002).

Among the primary non-Antarctic notothenioid lineages, the genera *Bovichtus*, *Cottoperca*, and *Pseudaphritis* were traditionally classified in the Bovichtidae, and *Eleginops* was considered a member of the Nototheniidae (Norman, 1938). Phylogenetic analysis of morphological characters revealed that the Bovichtidae was not a monophyletic group (Balushkin, 1992). *Bovichtus* and *Cottoperca* were sister taxa and retained in the Bovichtidae. *Eleginops maclovinus* was proposed as the sister taxon of a clade containing the Nototheniidae and the High Antarctic clade (Harpagiferidae, Artedi-

draconidae, Bathydraconidae, and Channichthyidae), and was removed from the Nototheniidae into the monotypic Eleginopidae (Fig. 1A; Balushkin, 1992). In addition, the morphology inferred phylogeny placed *Pseudaphritis urvillii* as the sister taxon of all other notothenioids (Fig. 1A; Balushkin, 1992, 2000).

Phylogenetic analyses of nuclear (rhodopsin, 28S rRNA) and mtDNA (12S and 16S rRNA) gene sequences have resulted in hypotheses that are similar to morphological analyses across many portions of the notothenioid tree, including the sister taxon relationship between *Eleginops* and the Antarctic notothenioid clades, and the monophyly of the High Antarctic clade (Bargelloni et al., 2000; Lecointre et al., 1997). However, these analyses are incongruent with morphological phylogenetic hypotheses with regard to the phylogenetic relationship of *Pseudaphritis* (Fig. 1B; Balushkin, 2000).

Much of the morphological and ecological diversity in the Notothenioidei that has led investigators to consider the clade an adaptive radiation is present in the Nototheniidae. The morphological and ecological types in the Nototheniidae include small and large benthic species, large pelagic piscivores, and small to medium sized pelagic zooplanktivores (Eastman, 1993). Exploitation of midwater habitats is thought to have been a key component in the diversification of nototheniids. All notothenioids lack a swimbladder; however, two species in the Nototheniidae have evolved neutral buoyancy via significant reduction of skeletal ossification and accumulation of lipid deposits (Eastman and DeVries, 1982; Near et al., 2003b). Other species also have reduced bone ossification, but are not neutrally buoyant. However, some species also utilize pelagic and semipelagic habitats,

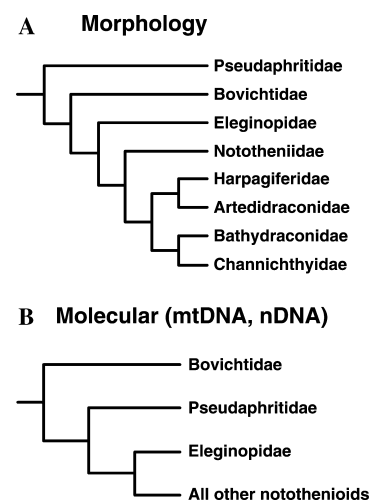


Fig. 1. Alternative hypotheses of higher level notothenioid relationships. (A) Relationships of notothenioid families resulting from analyses of morphological characters Balushkin (1992, 2000). (B) Consensus of relationships among basal notothenioid lineages resulting from analyses of mtDNA and nuclear gene sequences (Bargelloni et al., 2000; Cheng et al., 2003).

presumably aided by modified body and fin shapes that provide lift in swimming and facilitate exploitation of non-benthic habitats (Eastman, 1993; Klingenberg and Ekau, 1996). In addition to the substantial morphological and ecological diversity, the Nototheniidae contains more species than any other notothenioid family comprising 39% (48 of 122) of all recognized species in the Notothenioidei (Eastman and Eakin, 2000).

There is little phylogenetic evidence for the monophyly of the Nototheniidae, whether from morphological or molecular characters (Balushkin, 2000; Bargelloni et al., 2000). In a comprehensive cladistic analysis of discretely coded morphological characters from all notothenioid genera, no character was identified that supported the monophyly of the Nototheniidae (Balushkin, 2000). Also, all published phylogenetic analyses using mtDNA sequence data either resulted in a paraphyletic Nototheniidae (Bargelloni et al., 1997), or lacked adequate phylogenetic resolution (Ritchie et al., 1997). The ability of the partial 12S and 16S mtDNA rRNA dataset used in most of these studies to discriminate among alternative tree topologies may have been overstated, as transversions are either weighted higher than transitions in maximum parsimony (MP) analyses (Bargelloni et al., 2000), or character weighting is a posteriori and based on the consistency index (Bargelloni et al., 1994, 1997). The only unweighted MP analysis of the partial 12S–16S dataset results in trees in which the Nototheniidae appeared not as a paraphyletic group, but as a clade that is poorly resolved (Ritchie et al., 1997). Several lineages of the Nototheniidae along with the High Antarctic clade were collapsed in the strict consensus tree among a collection of 52 most parsimonious trees resulting in an unresolved polytomy (Ritchie et al., 1997).

There is substantial evidence from both morphological (Balushkin, 2000; Iwami, 1985) and mtDNA sequence data (Bargelloni et al., 2000; Derome et al., 2002; Near et al., 2003a) for the monophyly of the 32 species classified in the Bathydraconidae (dragonfishes) and Channichthyidae (icefishes). Phylogenetic relationships of the channichthyids have been investigated with both morphological characters and mtDNA sequence data (Chen et al., 1998; Iwami, 1985; Near et al., 2003a). Two morphological synapomorphies have been identified as support for the hypothesis of monophyly for the bathydraconids, the absence of a spinous dorsal fin and a unique pattern of ossification in the ethmoid (Balushkin, 2000). However, despite evidence of monophyly from these two morphological characters, molecular analyses have resulted in a paraphyletic Bathydraconidae relative to the monophyletic channichthyid icefishes (Bargelloni et al., 2000; Derome et al., 2002). The most robust taxon sampling addressing the molecular phylogenetic relationships of bathydraconids utilized a mtDNA dataset containing just over one kilo base pair

(kbp) of partial gene sequences from both the control region and cytochrome *b*. Parsimony analysis resulted in four well-supported monophyletic groups of bathydraconids; however, relationships among these clades and the relationship of bathydraconids relative to the channichthyids remained unresolved in a basal polytomy (Derome et al., 2002).

Given the lingering ambiguities of notothenioid phylogenetic relationships, we initiated this study to investigate the phylogenetic utility of complete mt rRNA genes in assessing the relationships of the Notothenioidei, specifically examining the phylogeny of primary non-Antarctic lineages, and the monophyly of both the Nototheniidae and Bathydraconidae. Previous analyses of the partial 12S–16S mt rRNA have prompted suggestions of revising the taxonomy of the notothenioids, but such suggestions seemed premature as they were based on partial gene datasets that lack phylogenetic resolution and were unable to discriminate among several key alternative phylogenetic hypotheses (Bargelloni et al., 2000). These previous analyses may also lead to an unwarranted conclusion that mt rRNA genes offer little phylogenetic utility in resolving relationships among notothenioid fishes. Our study critically examines these issues by collecting the largest 16S mtDNA dataset (1.7 kbp) with the most complete taxon sampling to-date for a molecular phylogenetic analysis of the Notothenioidei.

2. Materials and methods

2.1. Data collection

Notothenioid species were collected using a variety of methods in Southern Ocean habitats (Table 1). Tissues were dissected and stored at either -80°C , or preserved in 95% ethanol. Voucher specimens (if available) were deposited at either the Illinois Natural History Survey Fish Collection, or at the University of Tennessee Research Collection of Fishes. Outgroup species were selected from available perciform and scorpaeniform families (Table 1). There is little consensus regarding the sister taxon of the Notothenioidei and we were not attempting to determine which perciform lineages are closely related to notothenioids. Outgroups were used to test the monophyly of the Notothenioidei and provide rooting of the phylogenetic hypotheses generated in this investigation. Nucleic acids were isolated from tissues using standard phenol–chloroform extraction and ethanol precipitation methods. The entire mitochondrial encoded large ribosomal subunit (16S) was amplified using the primers Val-New and Leu-New (Near et al., 2003a). PCR conditions are given in Near et al. (2000). PCR products were prepared for sequencing by digesting with 1.0 unit of Exonuclease I and shrimp alkaline

Table 1
 Notothenioid species sampled and collection localities

Species	Family	Locality (Latitude, Longitude)
<i>Bovichtus variegatus</i>	Bovichtidae	Otago Harbor, New Zealand
<i>Cottoperca gobio</i>	Bovichtidae	Beagle Channel Ushuaia, Argentina
<i>Pseudaphritis urvillii</i> A	Pseudaphritidae	Pedler Creek South Australia, Australia (35°13'S, 138°31'E)
<i>Pseudaphritis urvillii</i> B	Pseudaphritidae	Onkaparinga River South Australia, Australia (35°05'S, 138°44'E)
<i>Eleginops maclovinus</i>	Eleginopidae	Straits of Magellan Punta Arenas, Chile
<i>Aethotaxis mitopteryx</i>	Nototheniidae	Weddell Sea (69°59'S, 5°8'E)
<i>Dissostichus eleginoides</i>	Nototheniidae	Falkland Islands (51°25'.0S, 57°35.0'W)
<i>Dissostichus mawsoni</i> A	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Dissostichus mawsoni</i> B	Nototheniidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Gobionotothen gibberifrons</i>	Nototheniidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Lepidonotothen larseni</i>	Nototheniidae	Livingston Island (52°54'S, 61°8'W)
<i>Lepidonotothen nudifrons</i>	Nototheniidae	Elephant Island (61°11.7'S, 56°29.0'W)
<i>Lepidonotothen squamifrons</i> A	Nototheniidae	Elephant Island (61°10.1'S, 54°33.5'W)
<i>Lepidonotothen squamifrons</i> B	Nototheniidae	Elephant Island (61°16.3'S, 56°29.0'W)
<i>Notothenia angustata</i>	Nototheniidae	Otago Harbor New Zealand (45°30'S, 170°E)
<i>Notothenia coriiceps</i>	Nototheniidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Notothenia rossii</i> A	Nototheniidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Notothenia rossii</i> C	Nototheniidae	Elephant Island (61°25.940'S, 56°07.715'W)
<i>Pagothenia borchgrevinki</i>	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Paranotothenia magellanica</i>	Nototheniidae	Beagle Channel Ushuaia, Argentina
<i>Patagonotothen guntheri</i>	Nototheniidae	Shag Rocks (53°33.0' S, 42°2.0'W)
<i>Patagonotothen ramsayi</i>	Nototheniidae	Falkland Islands (51°25'.0S, 57°35.0'W)
<i>Patagonotothen tessellata</i>	Nototheniidae	Beagle Channel Ushuaia, Argentina
<i>Pleuragramma antarcticum</i> A	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Pleuragramma antarcticum</i> B	Nototheniidae	King George Island (61°39.4'S, 57°5.1'W)
<i>Trematomus bernacchii</i>	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Trematomus loennbergii</i>	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Trematomus newnesi</i>	Nototheniidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Trematomus scotti</i>	Nototheniidae	Deception Island (62°59.3'S, 60°57.8'W)
<i>Harpagifer antarcticus</i>	Harpagiferidae	Signy Island (60°45'S, 46°40'W)
<i>Dolloidraco longedorsalis</i>	Artedidraconidae	Ross Sea (76°30.0'S, 175°56.0'E)
<i>Histiodraco velifer</i>	Artedidraconidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Pogonophryne marmorata</i>	Artedidraconidae	Deception Island (62°59.3'S, 60°57.8'W)
<i>Pogonophryne scotti</i>	Artedidraconidae	Ross Sea (75°30.0'S, 174°56.0'E)
<i>Akarotaxis nudiceps</i>	Bathydraconidae	Ross Sea (75°02.0'S, 166°16.0'E)
<i>Bathydraco marri</i>	Bathydraconidae	Weddell Sea (75°16.0'S, 26°39.0'W)
<i>Cygnodraco mawsoni</i>	Bathydraconidae	Terra Nova Bay, Ross Sea
<i>Gerlachea australis</i>	Bathydraconidae	Weddell Sea (75°00.0'S, 28°00.0'W)
<i>Gymnodraco acuticeps</i> a	Bathydraconidae	McMurdo Sound, Ross Sea (77°51.0'S, 166°40.0'E)
<i>Gymnodraco acuticeps</i> b	Bathydraconidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Parachaenichthys charcoti</i>	Bathydraconidae	Elephant Island (61°15.0'S, 55°36.9'W)
<i>Racovitzia glacialis</i>	Bathydraconidae	Ross Sea (75°30.0'S, 174°56.0'E)
<i>Chaenocephalus aceratus</i>	Channichthyidae	Elephant Island (61°04.0'S, 54°33.6'W)
<i>Champocephalus esox</i>	Channichthyidae	Falkland Islands (51°25'.0S, 57°35.0'W)
<i>Champocephalus gunnari</i>	Channichthyidae	Palmer Archipelago (64°51.0'S, 63°34.0'W)
<i>Chionodraco rastrospinosus</i>	Channichthyidae	Elephant Island (61°10.1'S, 54°33.5'W)
<i>Cryodraco antarcticus</i>	Channichthyidae	Elephant Island (60°58.1'S, 55°04.8'W)
<i>Pagetopsis macropterus</i>	Channichthyidae	South Shetland Islands (62°10.4'S, 60°28.4'W)
Outgroup species		
<i>Hemilepidotus spinosus</i>	Cottidae	Half Moon Bay San Mateo Co. California, USA
<i>Opiodon elongates</i>	Hexagrammidae	Half Moon Bay San Mateo Co. California, USA
<i>Cebidichthys violaceus</i>	Stichaeidae	Half Moon Bay San Mateo Co. California, USA
<i>Applodinotus grunniens</i>	Sciaenidae	Mississippi River, Jo Daviess Co. Illinois, USA
<i>Micropterus treculi</i>	Centrarchidae	Guadalupe River, Kerr Co. Texas, USA
<i>Percina maculata</i>	Percidae	Dismal Creek, Fayette Co. Illinois, USA
<i>Perca flavescens</i>	Percidae	Lake Andrusia, Beltrami Co. Minnesota, USA
<i>Stizostedion vitreum</i>	Percidae	Mississippi River, Rock Island Co. Illinois, USA

phosphatase, for 15 min at 37 °C, followed by 20 min at 80 °C to inactivate the enzymes. Treated PCR products were used as templates for Big Dye (Applied Biosys-

tems) terminator cycle sequencing reactions. Six primers were used to sequence both strands of the 16S gene (primer sequences available upon request). Sequences

were read with an ABI 377 automated sequencer at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois Urbana-Champaign and the Division of Biological Sciences Automated DNA Sequencing Facility at the University of California, Davis. Complete gene sequences were assembled from individual sequencing reactions using the program Sequencher version 3.1 (Gene Codes, Ann Arbor, MI).

2.2. Data analysis

Complete gene sequences 16S rRNA sequences were aligned based on secondary structural elements and conserved motifs, by comparing to existing models of secondary structure for large subunit rRNA (De Rijk et al., 2000; Gutell and Fox, 1988; Gutell et al., 1993). Nucleotide positions were designated as paired and unpaired. Unpaired bases included bulges, loops, and unpaired positions. The pooling of these classes of characters as unpaired positions was justified in that no difference in rate of substitution or nucleotide composition is found among the three classes. These regions of the notothenioid 16S rRNA were initially identified by aligning the *Dissostichus mawsoni* 16S gene with the 16S rRNA secondary structure model for the cyprinid fish *Cyprinus carpio* (De Rijk et al., 2000; Gutell and Fox, 1988; Gutell et al., 1993). The presence of multiple substitutions, or saturation in the 16S sequences was investigated by plotting numbers of observed transitions versus transversions. These plots were constructed for substitutions in both the paired and unpaired regions of the 16S rRNA. Variance of nucleotide composition in each of the designated secondary structural elements among species was estimated using a χ^2 heterogeneity test, both for all sites and variable sites.

Phylogenetic trees were estimated with both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria using the computer program PAUP* 4.0b10 (Swofford, 2000). MP analyses used a heuristic tree search, with TBR branch swapping, and 100 addition sequence replicates. Qualitative support for recovered nodes was assessed using a non-parametric bootstrap analysis with 2000 pseudoreplicates and a heuristic tree search with 10 addition sequence replicates. The computer program Modeltest 3.06 (Posada and Crandall, 1998) was used to determine the optimal model of nucleotide evolution for the ML analysis. Modeltest uses a set of hierarchical likelihood ratio tests (LRTs) to discriminate among 56 progressively complex models of nucleotide evolution. Likelihood scores were calculated and model parameters were estimated on one of the trees recovered from MP analysis. A heuristic tree search with 10 addition sequence replicates and TBR branch swapping was used in PAUP* 4.0b10 to find the best ML tree. Bayesian posterior probability values were determined

for the ML tree using the computer program Mr. Bayes 3.0, with the optimal model of sequence evolution determined from the LRTs. Mr. Bayes 3.0 was run with 1×10^6 generations to ensure the algorithm was run for an appropriate number of iterations, providing convergence in the estimations of the tree topology with the best ML posterior probability, branch lengths, the parameter values of the DNA substitution models, and posterior probability estimates of node support. Four chains were run simultaneously in each analysis and the analysis was repeated four separate times. The effective sample size of the Markov chain was estimated using the computer program Tracer 1.0.1 (A. Rambaut, unpublished, <http://evolve.zoo.ox.ac.uk/software.html?id=tracer>). The burn-in period of the analysis was determined by graphically tracking the ML scores at each generation to determine the point where generations and the ML values reach a plateau. Trees and parameter values resulting from generations prior to the burn-in were discarded. The frequency that a particular clade occurs within the collection of trees after the burn-in was interpreted as a measure of node support.

A Shimodaira–Hasegawa (SH) test was used to determine the significance between the best ML tree and several alternative hypotheses (Goldman et al., 2000; Shimodaira and Hasegawa, 1999). Alternative phylogenetic hypotheses examined with the 16S rRNA dataset include the best tree topology that represents relationships inferred from partial 12S and 16S rRNA sequences, which presents the Nototheniidae as paraphyletic (Bargelloni et al., 2000). Since the taxon sampling used in this study differs from the Bargelloni et al. (2000) analysis, we used backbone constraints to find the best trees that represented the hypothesis presented in that study. We also tested the hypotheses that the family Bathypodaconidae (dragonfishes) is monophyletic, the subfamily classification and relationships of nototheniid genera presented in Balushkin (2000), and the monophyly of *Lepidonotothen*, *Notothenia*, and *Trematomus* as recognized in DeWitt et al. (1990). Maximum likelihood heuristic tree searches with topological constraints were used to find the best trees consistent with these alternative hypotheses of notothenioid relationships.

3. Results

The complete mtDNA 16S rRNA gene ranged in size from 1686 to 1708 bp among the 43 sampled notothenioid species. The aligned DNA sequences consisted of 1764 nucleotide sites (1096 unpaired sites, and 668 paired sites). A total of 72 sites were excluded from all analyses because of problematic alignment. Observed pairwise nucleotide substitutions were more frequent at unpaired sites, occurring as much as three times the frequency observed at paired sites. Uncorrected pairwise

sequence divergence at all sites between notothenioid species ranged from 0.1% (*Pogonophryne scotti* and *P. marmorata*) to 18.0% (*Bovichtus variegatus* and *Trematomus bernacchii*). Nucleotide composition exhibited biases typical of animal mtDNA, but compositional biases did not significantly differ between species at both unpaired and paired sites, or between variable and conserved sites. Compositional biases were very similar to previous observations in channichthyids and characiform fishes (Near et al., 2003a; Orti et al., 1996). Unpaired and paired sites differed in their content of A and G, but not C and T. Unpaired sites exhibited a bias in favor of A and against G. Paired sites were biased in favor of G and C. There was a slight deviation from linearity when unpaired transitions were plotted against unpaired transversions, an indication of multiple substitutions. The only pairwise contrasts of transitions versus transversions that exhibited a signature of multiple substitutions were those at unpaired sites between

Bovichtus or *Cottoperca* and the remaining notothenioid species. There was no indication of multiple substitutions among all other notothenioid contrasts at unpaired sites, nor among any notothenioid contrasts at paired sites (plots not shown).

Phylogenetic analyses of the aligned 16S rRNA dataset using both MP and ML methods resulted in trees that were highly congruent (Figs. 2 and 3). Also, the trees resulting from MP and ML analyses are well resolved and most nodes are supported with high bootstrap pseudoreplicate scores and significant ML Bayesian posterior probabilities (Figs. 2 and 3). The effective sample size of the Markov chain in the Bayesian analysis was 505.3. Congruent with most previous assessments, the Notothenioidei is monophyletic relative to the perciform and scorpaeniform outgroup species sampled, and supported with 100% MP bootstrap scores and significant ML Bayesian posterior probability values (Figs. 2 and 3). Similar to previous molecular

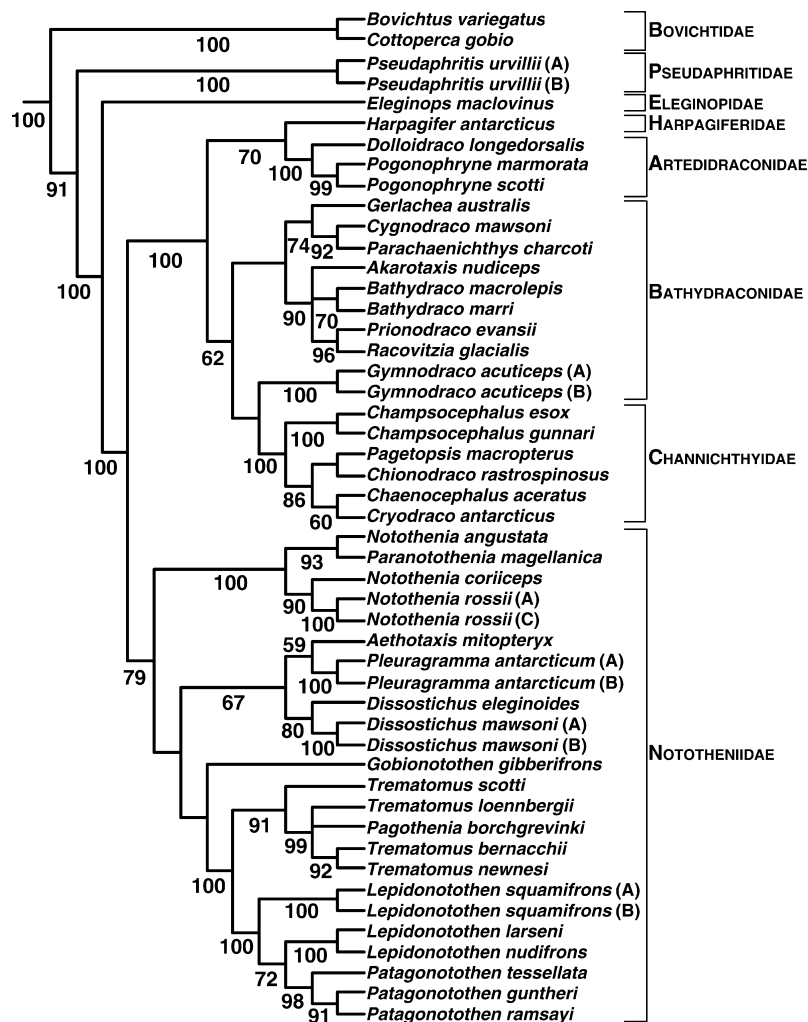


Fig. 2. Strict consensus of four trees resulting from maximum parsimony analysis of the complete gene 16S rRNA dataset. The tree length is 2628 steps and the consistency index (excluding uninformative characters) is 0.399. Numbers at nodes represent percent recovery in bootstrap analysis (2000 pseudoreplicates). Recognized taxonomic families are indicated on the right side of the figure.

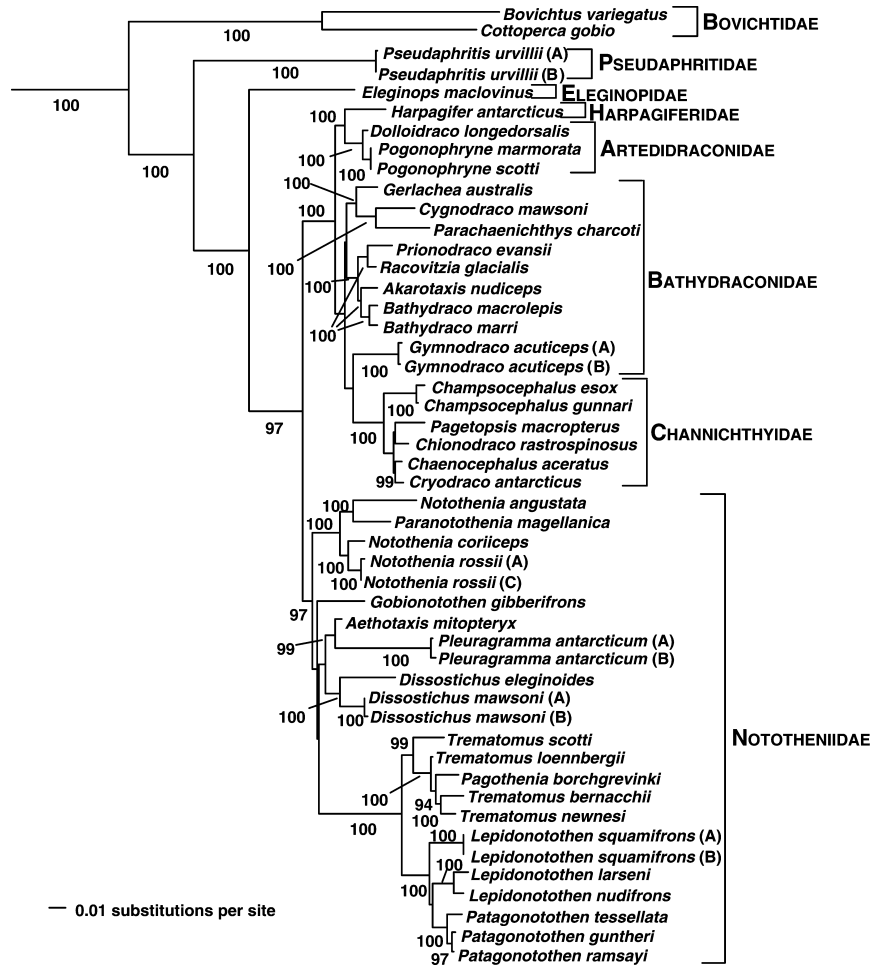


Fig. 3. Tree resulting from maximum likelihood analysis of the complete gene 16S rRNA dataset. Maximum-likelihood score = -14970.49. Parameters for general time-reversible model were R (a) [A-C] = 1.8508, R (b) [A-G] = 6.9370, R (c) [A-T] = 1.8009, R (d) [C-G] = 0.7668, R (e) [C-T] = 8.3020, R (f) [G-T] = 1.0000 with frequency of nucleotides A = 0.3554, C = 0.2584, G = 0.1735, and T = 0.2127, proportion of invariant sites = 0.4168, and the α shape parameter = 0.7483. Branches are scaled to the numbers of substitutions per site. Numbers at nodes represent Bayesian posterior probabilities.

analyses (Lecointre et al., 1997), but incongruent with morphological phylogenetic hypotheses (Fig. 1A; Balushkin, 1992, 2000), the Bovichtidae is the most basal notothenioid lineage (Figs. 2 and 3). However, in contrast to all previously published molecular phylogenetic analyses, the Nototheniidae is monophyletic with substantial nonparametric bootstrap support in MP analysis (Fig. 2) and a significant Bayesian posterior probability in ML analysis (Fig. 3).

The High Antarctic clade containing Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae is monophyletic. The Harpagiferidae and Artedidraconidae are sister taxa, and both the Artedidraconidae and the Channichthyidae are monophyletic (Figs. 2 and 3). The Bathydraconidae is paraphyletic in both MP and ML analyses with *Gymnodraco acuticeps* as the sister taxon of the Channichthyidae (Figs. 2 and 3). The remaining bathydraconids do not group into a single clade in the MP analysis, but are monophyletic in

the ML tree. Regardless, none of the nodes involved with the paraphyly of the bathydraconids are well supported in the bootstrap analysis, and these nodes are not characterized with significant Bayesian posterior probabilities (Figs. 2 and 3).

For each alternative phylogenetic hypothesis, ML constraint tree searches each yielded a single tree. The SH test identified only two of the eight alternative hypothesis examined as significantly different from the ML tree (Table 2). The hypothesis that Pseudaphritidae, and not Bovichtidae, is the sister taxon of the remaining notothenioids was rejected (Balushkin, 1992, 2000). Also, the hypothesis that the nototheniid subfamily, Nototheniinae is monophyletic (Balushkin, 2000) was rejected. The hypotheses that the Bathydraconidae, *Lepidonotothen*, and *Notothenia* each constitute a monophyletic group were not rejected (Table 2). Also, Balushkin's (2000) hypothesis of relationships among the Trematominae, the best representation of the

Table 2
Shimodaira–Hasegawa test of alternative phylogenetic hypotheses of notothenioid fishes

Hypothesis	ln L	Difference in ln L	P
Maximum likelihood tree (Fig. 3)	-14,970.49	Best	—
Bathypagrus monophyletic	-14,974.37	3.88	0.887
<i>Lepidonotothen</i> monophyletic	-14,972.39	1.90	0.916
<i>Notothenia</i> monophyletic	-14,977.59	7.10	0.858
<i>Trematomus</i> monophyletic	-15,000.73	30.24	0.326
Balushkin (1992, 2000)	-15,064.16	93.67	0.013*
Bargelloni et al. (2000)	-15,001.56	31.07	0.316
Trematominae (Balushkin, 2000)	-15,009.51	39.03	0.213
Nototheniinae monophyletic (Balushkin, 2000)	-15,368.72	398.23	<0.001*

Significant results are highlighted with an asterisk.

generally unresolved hypothesis in Bargelloni et al. (2000), and the hypothesis that *Trematomus* is monophyletic were not rejected in the SH test (Table 2).

4. Discussion

In addressing the future of evolutionary research in Antarctic fishes, one of the major proposals was the development of resolved and well-supported phylogenetic hypotheses at both higher-level and species relationships for notothenioid fishes (Eastman, 1995). Our study has included the most extensive taxonomic sampling encompassing species from all of the recognized major lineages of the Notothenioidei, as well as the largest mtDNA dataset for any published molecular phylogenetic analysis of this group. The result is a well-resolved and supported set of hypotheses that reveal strong congruence between traditional taxonomic classifications (Eastman and Eakin, 2000; Norman, 1938), and phylogenetic analyses based on both discretely coded morphological characters (Balushkin, 1992, 2000; Eakin, 1981; Iwami, 1985), and recent molecular analyses using mtDNA and nDNA sequence data (Bargelloni et al., 2000; Derome et al., 2002). The most important result regarding the phylogenetic relationships of notothenioids to emerge from our current analyses is the demonstration of monophyly for both the Notothenioidei and the Nototheniidae.

Despite the general congruence between the results presented in this study and previous investigations, three critical differences remain. First, morphological analyses that result in *P. urvillii* as the sister taxon of all other notothenioids (Fig. 1A; Balushkin, 2000) are incongruent with molecular analyses (Bargelloni et al., 2000; Lecointre et al., 1997). Second, the degree of phylogenetic resolution from our analyses of complete 16S rRNA sequences is substantially greater than previous efforts using partial 12S and 16S rRNA gene sequences. Third, there are significant differences in the relationships proposed among genera in the Nototheniidae between morphology inferred phylogenetic hypotheses and those resulting from MP and ML

analyses of 16S rRNA in this study (Table 2; Figs. 2 and 3).

Results from previous DNA-inferred phylogenetic hypotheses (Bargelloni et al., 2000; Lecointre et al., 1997), and our current results from MP and ML analyses of the complete 16S rRNA dataset (Figs. 2 and 3), consistently resulted in the Bovichtidae as the sister lineage of the remaining notothenioids. Three morphological characters have been proposed as supporting the hypothesis that *P. urvillii* is the sister taxon of the remaining notothenioids (Balushkin, 1992, 2000). However, each one of these proposed morphological characters appears problematic with regard to character polarity, since there is little information regarding character states among outgroup lineages, an issue which apparently has not been fully investigated (Lecointre et al., 1997). On the other hand, Voskoboinikova (1993) identified five morphological characters that could potentially resolve the Bovichtidae as the sister taxon of the remaining notothenioids, and Lecointre et al. (1997) presented two additional morphological apomorphies supporting this relationship. Also, evidence from electrophoretic analysis of hemoglobin subunits demonstrate that bovichtids have two hemoglobin components in equal amounts, as is found in other perciform fishes, unlike *P. urvillii* and the remaining notothenioid fishes which exhibit a derived condition with only one component that accounts for 95% of the total hemoglobin content (D'Avino and Di Prisco, 1997). Finally, the DNA sequence data strongly support the relationship of Bovichtidae as the sister lineage of all other notothenioids (Figs. 2 and 3), as evidenced by the ability of the 16S rRNA dataset to reject the alternative hypothesis proposed from morphological analyses (Table 2; Fig. 1A).

Phylogenetic analysis of the complete 16S rRNA dataset results in trees with much greater resolution than previous analyses using partial 12S and 16S rRNA gene sequences (Bargelloni et al., 1994, 1997, 2000; Ritchie et al., 1997). One apparent consequence from the lack of resolution in these partial gene analyses is the paraphyly of the Nototheniidae (Bargelloni et al., 2000), or a

collection of multiple most parsimonious trees that in the consensus tree several nototheniid lineages are collapsed in a basal polytomy with other notothenioid lineages (Ritchie et al., 1997). Analyses of the complete 16S rRNA dataset in this study not only result in a monophyletic Nototheniidae, but the clade is well supported with both high MP bootstrap pseudoreplicate score and significant ML Bayesian posterior probability (Figs. 2 and 3). The analysis of partial 12S and 16S rRNA gene sequences resolved approximately 79% of all possible interspecific nodes in the phylogeny, with only 45% of all possible interspecific nodes supported with a MP bootstrap score of 70% or greater (Bargelloni et al., 2000). In contrast, MP analysis of the complete 16S rRNA resolved 95% of all possible interspecific nodes and 81% of these nodes were supported with a bootstrap score greater than 70% (Fig. 2).

The fact that adding substantially more data to the question of notothenioid relationships results in a monophyletic Nototheniidae indicates that prior proposals to alter traditional classifications based on the analysis of the partial 12S and 16S rRNA dataset (Bargelloni et al., 2000) may be premature. The complete 16S rRNA dataset, despite the appreciable support for monophyly, cannot reject the hypothesis of a paraphyletic Nototheniidae proposed from the analysis of the partial 12S and 16S gene sequences (Table 2). Greater resolution and increased node support for a monophyletic Nototheniidae may be realized with the addition of mtDNA protein coding gene data, which are known to have a higher rate of substitution than mtDNA rRNA genes (Pesole et al., 1999).

Within the Nototheniidae both MP and ML analyses of the complete 16S rRNA dataset result in four major lineages: First, the species that exhibit neutral buoyancy in the genera *Dissostichus* and *Pleuragramma*, along with *Aethotaxis*; second, the genera *Notothenia* and *Paranotothenia*, with *Notothenia angustata* as the sister taxon of *Paranotothenia magellanica* relative to the other two sampled *Notothenia* species; third, *Trematomus*, *Pagothenia*, and *Lepidonotothen*; and fourth, *Gobionotothen gibberifrons*. *Trematomus* and *Lepidonotothen* are paraphyletic relative to *Pagothenia* and *Patagonotothen* respectively (Figs. 2 and 3). *Gobionotothen gibberifrons* was not placed as the sister species of any of the three other clades with either strong bootstrap support or significant ML Bayesian posterior probabilities (Figs. 2 and 3).

Relationships among the four major clades in the Nototheniidae are not well supported, and while some portions of the MP and ML trees are congruent with previous morphological analyses, other portions are highly incongruent. For example, Balushkin (2000) identified the pelagic neutrally buoyant nototheniid genera as a monophyletic group, named the Pleuragrammatinae. The same result is obtained in both MP

and ML analyses, with *Dissostichus*, *Aethotaxis*, and *Pleuragramma* forming a monophyletic group (Figs. 2 and 3). Although the node was not strongly supported in MP bootstrap analysis or a significant Bayesian posterior probability in ML analysis, the congruence with Balushkin's (2000) pelagic Pleuragrammatinae suggests that adaptations involved with exploiting pelagic habitats via neutral buoyancy (Eastman and DeVries, 1982; Near et al., 2003b) may have a single evolutionary origin. It should be noted that *Aethotaxis* is thought to be neutrally buoyant, but only formalin-fixed specimens have been measured (Eastman and DeVries, 1982; Eastman, 1993).

In the Trematominae, Balushkin (2000) proposed that *Trematomus newnesi* was the sister taxon of *Pagothenia* and *Cryothenia*, and the remaining *Trematomus* species were placed in *Pseudotrematomus*. Although *Cryothenia* was not included in this analysis, the MP and ML analyses did not result in *T. newnesi* as the sister taxon of *P. borchgrevinki* (Figs. 2 and 3). *Trematomus* was paraphyletic in both MP and ML analyses with high MP bootstrap and Bayesian posterior probability support at most nodes (Figs. 2 and 3). However, the SH test using the 16S rRNA dataset did not reject either the relationships among genera of the Trematominae as proposed by Balushkin (2000), or the hypothesis that *Trematomus* is monophyletic, exclusive of *Pagothenia* (Table 2).

The most substantial incongruence between hypotheses based on morphological data and the 16S rRNA dataset within the Nototheniidae pertains to Balushkin's (2000) hypothesis regarding relationships of the Nototheniinae. The monophyly of this clade was strongly rejected in the SH test (Table 2). The morphology based hypothesis of the Nototheniinae nests *Gobionotothen* within *Lepidonotothen*, a result that is incongruent with both the MP and ML analyses of 16S rRNA (Figs. 2 and 3).

In both MP and ML analyses of the 16S rRNA dataset the genera *Notothenia* and *Lepidonotothen* were each paraphyletic. *Notothenia* includes two species with distributions south (*N. coriiceps* and *N. rossii*), and two distributed north (*N. angustata* and *N. microlepidota*) of the Antarctic Polar Front. *N. angustata* does not group with *N. coriiceps* and *N. rossii*, but is the sister taxon of *P. magellanica*, a species distributed on both sides of the Antarctic Polar Front (DeWitt et al., 1990). Despite strong support for this node in MP bootstrap analysis and a significant Bayesian posterior probability in ML analysis, a monophyletic *Notothenia* was not rejected in the SH test (Table 2). *Lepidonotothen* was paraphyletic in both MP and ML analyses of the 16S rRNA dataset, with *L. larseni* and *L. nudifrons* as the sister clade of the three sampled *Patagonotothen* species (Figs. 2 and 3). Similar to *Notothenia*, a monophyletic *Lepidonotothen* was not rejected in the SH test using the 16S rRNA dataset (Table 2).

Despite incongruence between morphology and 16S rRNA inferred phylogenetic hypotheses within the Nototheniidae, much of the higher-level notothenioid relationships are congruent among molecular and morphological studies. For example, in agreement with all published morphological and molecular phylogenetic analyses, MP and ML analyses of the 16S rRNA results in the monophyly of the High Antarctic clade. Within this clade the Harpagiferidae and Artedidraconidae are sister lineages, and the Bathydraconidae and Channichthyidae are in a monophyletic group. However, as has been found in other molecular studies the Bathydraconidae is paraphyletic (Bargelloni et al., 2000; Derome et al., 2002). The monophyly of the Bathydraconidae was not rejected in the SH test (Table 2) and the nodes involved with the paraphyly of bathydraconids are not strongly supported in MP bootstrap analysis and do not have significant Bayesian posterior probabilities in the ML analysis (Figs. 2 and 3). Phylogenetic analyses of the 16S rRNA dataset resulted in three clades of bathydraconids, *Gymnodraco acuticeps*, a clade containing *Gerlachea*, *Cygnodraco*, and *Parachaenichthys*, and a clade with *Prionodraco*, *Racovitzia*, *Akarotaxis*, and *Bathydraco*. These monophyletic groups of bathydraconids are also present in previous analyses of morphology and mtDNA sequence data (Balushkin, 2000; Derome et al., 2002).

With regard to patterns of evolutionary diversification in notothenioids, the complete 16S rRNA inferred phylogenies confirm a single evolutionary origin of antifreeze glycoproteins (AFGPs) in the monophyletic group containing the High Antarctic Clade and the Nototheniidae (Figs. 2 and 3; Bargelloni et al., 1994). The AFGPs prevent blood and other body fluids from freezing in the ice-laden subzero Antarctic marine environment (DeVries, 1988), and are hypothesized to be a key innovation that facilitated the diversification of notothenioid fishes (Cheng et al., 2003). Additionally, the phylogeny reveals a biogeographic pattern where several species from the Channichthyidae and Nototheniidae are secondarily non-Antarctic, distributed in southern South America and New Zealand (Cheng et al., 2003; Stankovic et al., 2002). The timing of the notothenioid radiation has been investigated using molecular clock analyses of the partial gene 12S and 16S rRNA dataset, with appreciable differences resulting from analyses using nucleotide substitution rates of mtDNA rRNA genes in salamanders versus rates inferred from a notothenioid fossil calibration (Bargelloni et al., 2000; Near, in press). Currently we are investigating the tempo of diversification in the Notothenioidei using both internal and external fossil calibrations of molecular clocks at several mtDNA and nuclear genes.

Phylogenetic analysis of the complete mitochondrial encoded 16S rRNA gene sampled across notothenioid diversity produced hypotheses that are much more re-

solved than all previous analyses of the partial 12S and 16S rRNA genes (Bargelloni et al., 1994, 1997, 2000). The increased resolution of the complete 16S rRNA dataset is able to reject certain alternative hypotheses regarding higher-level notothenioid relationships and particular hypotheses of nototheniid relationships (Table 2). Perhaps the most interesting result from these analyses is the well-supported monophyly of the Nototheniidae in trees using both MP and ML optimality criteria. Despite the lack of a clear morphological apomorphy supporting the recognition of the Nototheniidae, intuitive pre-cladistic approaches that initially recognized the Nototheniidae as a “natural” group seems to have accurately identified this clade. Also, the analyses of the complete 16S rRNA demonstrate substantial phylogenetic utility of mtDNA rRNA genes. Previous efforts provided much less evidence for the phylogenetic utility of mtDNA rRNA genes due to an inadequate sampling of characters. The partial 12S and 16S rRNA dataset has been used as the standard dataset in notothenioids to expand taxonomic sampling (Stankovic et al., 2002), or to investigate patterns of diversification in comparative evolutionary studies (Johns and Avise, 1998). The analyses in this investigation reveal clear weaknesses of the partial 12S and 16S rRNA dataset. Since appropriate PCR and sequencing primers that facilitate the collection of the 16S gene sequence from any notothenioid are now available, along with a large number of notothenioid species already sequenced (Table 1), a favorable outcome would be the use of this dataset by investigators who wish to expand the taxon sampling within the Notothenioidei for future molecular phylogenetic studies.

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