

# Phylogenetics of notothenioid fishes (Teleostei: Acanthomorpha): Inferences from mitochondrial and nuclear gene sequences

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Received 11 September 2007; revised 24 November 2007; accepted 28 November 2007

Available online 4 February 2008

## Abstract

Notothenioids represent an adaptive radiation of teleost fishes in the frigid and ice-laden waters of the Southern Ocean surrounding Antarctica. Phylogenetic hypotheses for this clade have resulted primarily from analyses of mtDNA gene sequences, and studies utilizing nuclear gene DNA sequence data have focused on particular sub-clades of notothenioid fishes. In this study, we provide the first phylogenetic analysis of notothenioids using both mtDNA and nuclear gene sequences for a comprehensive sampling of all major lineages in the clade. Maximum parsimony and Bayesian analyses of aligned mtDNA genes, an aligned nuclear gene (S7 ribosomal protein intron 1), and combined dataset containing the mtDNA and nuclear genes resulted in phylogenies that contained the previously identified Antarctic and High Antarctic Clades. There were areas of agreement and disagreement between different datasets and methods of phylogenetic analysis, and the phylogenies resulting from the nuclear encoded S7 ribosomal protein intron 1 sequences were considerably less resolved than those inferred from mtDNA gene sequences. However, we anticipate increased resolution of the notothenioid phylogeny from future analyses that sample DNA sequences from several nuclear genes.

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**Keywords:** Notothenioidae; Ribosomal RNA; ND2; Nuclear gene phylogeny; Nototheniidae; Bathydraconidae; Harpagiferidae; Artedidraconidae; Channichthyidae

## 1. Introduction

Notothenioids are a clade of acanthomorph teleost fishes that contains approximately 129 species (Eastman, 2005). Previous phylogenetic analyses of notothenioids based on both morphological and molecular data have identified an Antarctic Clade that contains Nototheniidae, Artedidraconidae, Harpagiferidae, Bathydraconidae, and Channichthyidae (Balushkin, 2000; Lecointre et al., 1997; Near et al., 2004b). This clade contains 101 of the approximately 129 notothenioid species (Eastman, 2005), and is thought to represent an adaptive radiation in the frigid waters surrounding Antarctica (Cheng et al., 2003; Clarke

and Johnston, 1996; Eastman, 1993, 2005; Eastman and Eakin, 2000). The Antarctic Clade is characterized by the possession of antifreeze glycoproteins that are regarded as a key innovation that facilitates exploitation of the freezing Antarctic marine habitats generally unavailable to temperate-adapted teleost lineages (Cheng, 1998; Cheng et al., 2003; Clarke and Johnston, 1996; Eastman, 1993).

The majority of previous phylogenetic analyses of notothenioids using DNA sequence data have relied exclusively on mtDNA gene sequences (Bargelloni et al., 1994, 2000; Cheng et al., 2003; Czikó and Cheng, 2006; Derome et al., 2002; Near, 2004; Near et al., 2003, 2004b; Ritchie et al., 1996, 1997). A few exceptions include the use of DNA sequences from genes encoded in the nuclear genome to investigate the phylogenetic position of notothenioids relative to other acanthomorph lineages (Chen et al., 2003; Dettai and Lecointre, 2004; Smith and Craig,

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2007), the relationships of major notothenioid clades (Lecointre et al., 1997), and species-level phylogenetic relationships of the subclade Nototheniidae (Sanchez et al., 2007). When interpreting molecular phylogenies, one must bear in mind that conflicts between gene trees and species trees can arise from processes such as differential sorting of ancestral genetic polymorphisms and introgression via interspecific hybridization (Maddison, 1997). One way to assess if gene phylogenies reflect species phylogenies is to determine if similar phylogenies are inferred from genetically unlinked loci (Moore, 1995; Pamilo and Nei, 1988).

Studies of notothenioid phylogenetic relationships that include both mtDNA and nuclear gene sequences have not assessed if analysis of these datasets would result in different or corroborating phylogenetic trees. In previous analyses, data from mtDNA and nuclear genes have been concatenated and treated as a single dataset in phylogenetic analyses (Chen et al., 2003; Dettai and Lecointre, 2004), or trees resulting from separate analyses of datasets were analyzed using supertree methods (Sanchez et al., 2007). In this study we examine the phylogenetic relationships of notothenioids with analyses of a dataset that includes complete gene sequences from protein coding (ND2), transfer RNA, and ribosomal RNA (16S) mitochondrial genes, and a nuclear encoded intron (intron 1 of the S7 ribosomal protein). Our analyses build on the dense taxon sampling presented in Near et al. (2004b) and includes the first assessment of phylogenetic relationships of all major notothenioid lineages using both mitochondrial and nuclear gene sequences.

## 2. Materials and methods

The taxon sampling for this study included 43 species, and is very similar to Near et al. (2004b) with the exception that *Histiadraco velifer* was not sampled in this study, and only a single specimen was sampled for *Pseudaphritis urvillii* (A), *Dissostichus mawsoni* (A), *Lepidonotothen squamifrons* (A), *Notothenia rossii* (C), and *Pleuragramma antarcticum* (A). Two species included in this study were not examined in Near et al. (2004b), *Trematomus nicolai* collected in McMurdo Sound, Ross Sea, Antarctica, and *T. lepidorhinus* collected in the Ross Sea, Antarctica. Collection data on all other specimens used in this study is presented in Near et al. (2004b). Based on the consistent result from previous phylogenetic analyses that Bovichtidae is the sister lineage of all other notothenioid clades, we used the two sampled bovichtid species *Bovichtus variegatus* and *Cottoperca gobio* as outgroups taxa in all analyses.

Nucleic acids were isolated from frozen or ethanol preserved tissues using standard phenol-chloroform and ethanol precipitation methods. The entire mitochondrial encoded large subunit ribosomal subunit (16S) was amplified with primers Val-New and Leu-New (Near et al., 2003). The mitochondrial protein coding gene ND2 was amplified using primers reported in Kocher et al. (1995), and these primers also amplified the complete tRNAs

MET, TRP, and ALA flanking the ND2 gene. The primers used to amplify the nuclear encoded first intron of the S7 ribosomal protein were taken from Chow and Hazama (1998). Conditions for PCR were identical to those reported in Near et al. (2004a). Prior to DNA sequencing PCR products were treated with 1.0 unit of Exonuclease I and shrimp alkaline phosphatase for 15 min at 37 °C to degrade single stranded DNA and primers, and followed by incubation for 20 min at 80 °C to inactivate the enzymes. Treated PCR products were used as templates for Big Dye (Applied Biosystems) 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).

Alignment of the ND2 DNA sequences was performed by eye and guided by the inferred amino acids from conceptual translation. The 16S rRNA gene sequences were aligned based on the secondary structure elements as described in Near et al. (2004b). The computer program MUSCLE (Edgar, 2004) was used to align DNA sequences from the three mitochondrial tRNA genes and the S7 intron.

Maximum parsimony and Bayesian analyses were used to infer phylogenetic trees from the aligned DNA sequences. The computer program PAUP\* 4.0b10 (Swofford, 2003) was used to perform maximum parsimony analyses via heuristic tree searches with TBR branch swapping and 200 addition sequence replicates. Support for nodes in the maximum parsimony phylogenies was assessed with a non-parametric bootstrap analysis using 2000 pseudoreplicates and a heuristic tree search with 10 addition sequence replicates.

The optimal model of DNA sequence evolution was determined for each of seven data partitions identified from the sampled mitochondrial and nuclear genes using the computer program Modeltest 3.0 (Posada and Crandall, 1998). The seven data partitions included one from each of the three codon positions of the ND2 gene, paired and unpaired sites in the 16S rRNA, a single partition for the combined tRNA genes, and a single partition from the S7 intron. MrBayes 3.1 was used to perform a partitioned mixed-model Bayesian phylogenetic analysis, where the optimal molecular evolutionary model was applied to each of the seven data partitions and model parameter values were “unlinked” among partitions assigned the same molecular evolutionary model (Ronquist and Huelsenbeck, 2003). MrBayes 3.1 was run for  $9.0 \times 10^6$  generations. The burn in period of the analysis was determined by plotting the likelihood score against the number of generations.

Nodes in the estimated phylogenies were considered significantly supported if they were recovered with Bayesian posterior probabilities  $\geq 0.95$ .

### 3. Results and discussion

The protein coding ND2 gene was 1047 bp in all individuals sampled and the alignment contained no indels. The pooled three tRNA genes ranged in size from 211 to 212 bp among sampled specimens. The alignment of the tRNA genes included four non-contiguous indels and was 212 bp. The 16S rRNA gene ranged in size from 1686 to 1708 bp and the length of the aligned dataset was 1750 bp. Using the methodology outlined in Near et al. (2004b), 1083 sites in the 16S rRNA were identified as unpaired and 667 bp were identified as paired. The S7 ribosomal protein intron ranged in size from 555 bp in *Patagonotothen guntheri* to 698 bp in *Bovichtus variegatus*. The aligned S7 ribosomal protein intron contained 737 nucleotide sites. The optimal molecular evolutionary models for each data partition are given in Table 1.

Parsimony and Bayesian analyses of the different gene and combined datasets resulted in phylogenies that consistently contained particular clades. However, differences among phylogenies were detected among the mitochondrial and nuclear datasets as well as differences between parsimony and Bayesian analyses of the same datasets. Summary statistics for parsimony analyses are given in Table 2. Similar to previous analyses of notothenioid phylogenetics using molecular and morphological data, all datasets and analyses resulted in phylogenies that contained an Antarctic Clade exclusive of the non-Antarctic *B. variegatus*, *C. gobio*, *P. urvilli*, and *E. maclovinus*, the High Antarctic Clade containing Harpagiferidae, Artedidraconidae,

Bathydraconidae, and Channichthyidae (Near et al., 2004b), a clade containing species sampled from *Notothenia* and *Paranotothenia*, a clade containing *Lepidonotothen*, *Patagonotothen*, and *Trematomus*, a clade containing the species sampled from Channichthyidae, and the unresolved phylogenetic placement of *Gobionotothen gibberifrons* (Figs. 1–4). These nodes were strongly supported in all analyses with either high bootstrap pseudoreplicate scores or significant Bayesian posterior probabilities. Particular differences observed among phylogenies inferred from different analyses and datasets include the monophyly of Nototheniidae and the presence of a clade containing *Aethotaxis mitopteryx*, *Pleuragramma antarcticum*, *Dissostichus eleginoides*, and *D. mawsoni*, all of which are nototheniid species that exhibit neutrally buoyancy (Balushkin, 2000; Near et al., 2007; Near et al., 2004b).

Phylogenies inferred from parsimony and Bayesian analyses of the concatenated mitochondrial genes (Figs. 1 and 2) differed in several ways. The consensus of the six most parsimonious trees contained a monophyletic Nototheniidae and a clade containing all four of the sampled neutrally buoyant species. However, these two nodes in the mtDNA parsimony phylogeny were not strongly supported with high bootstrap pseudoreplicate scores (Fig. 1). In the mtDNA parsimony phylogeny the Bathydraconidae, sampled in this study with *Akarotaxis nudiceps*, *Bathydraco marri*, *Racovitzia glacialis*, *Parachaenichthys charcoti*, *Cygnodraco mawsoni*, *Gerlachia australis*, and *Gymnodraco acuticeps*, was monophyletic with low bootstrap support (Fig. 1). This result is contrary to most other molecular phylogenetic analyses that have resulted in Bathydraconidae as a paraphyletic group (Bargelloni et al., 2000; Derome et al., 2002; Near et al., 2004b). The phylogeny resulting from Bayesian analysis of the mtDNA genes did not contain a monophyletic Nototheniidae, a neutrally buoyant nototheniid clade, or a monophyletic Bathydraconidae (Fig. 2). Nototheniidae was paraphyletic due to the nesting of the High Antarctic Clade, and two nodes lacking significant Bayesian posterior support (0.94 and 0.91) separated the four neutrally buoyant species (Fig. 2).

Bayesian and parsimony analyses of the aligned nuclear encoded DNA ribosomal protein S7 intron 1 sequences resulted in phylogenies that were much less resolved (Fig. 3A and B) than those resulting from analyses of the mitochondrial genes (Figs. 1 and 2). This lack of resolution is reflected by the recovery of several thousand most parsimonious trees in the analysis of the S7 intron sequence data (Table 2). Despite the lack of resolution, the phylogenies resulting from parsimony and Bayesian analyses were broadly congruent with regard to both tree topology and nodes supported with high bootstrap pseudoreplicate scores and significant Bayesian posterior probabilities (Fig. 3A and B). Like all of the other analyses, the parsimony S7 tree contained a monophyletic High Antarctic Clade; however, this node was supported with a low bootstrap pseudoreplicate score in the parsimony analysis

Table 1  
Optimal molecular evolutionary models for data partitions used in Bayesian mixed-model analysis

Partition	Number of substitution types	Invariant sites?	Among site rate variation?
16S Paired	6	Yes	Yes
16S Unpaired	6	Yes	Yes
tRNA genes	2	Yes	Yes
ND2 1st codon position	6	Yes	Yes
ND2 2nd codon position	6	Yes	Yes
ND2 3rd codon position	6	Yes	Yes
S7 Intron 1	6	No	Yes

Table 2  
Summary of maximum parsimony results from mitochondrial, nuclear gene, and combined datasets

Dataset	Number of trees	Tree length	Consistency index
Mitochondrial genes	6	5770	0.364
S7 Intron	8690	703	0.661
Combined data	3	6496	0.387

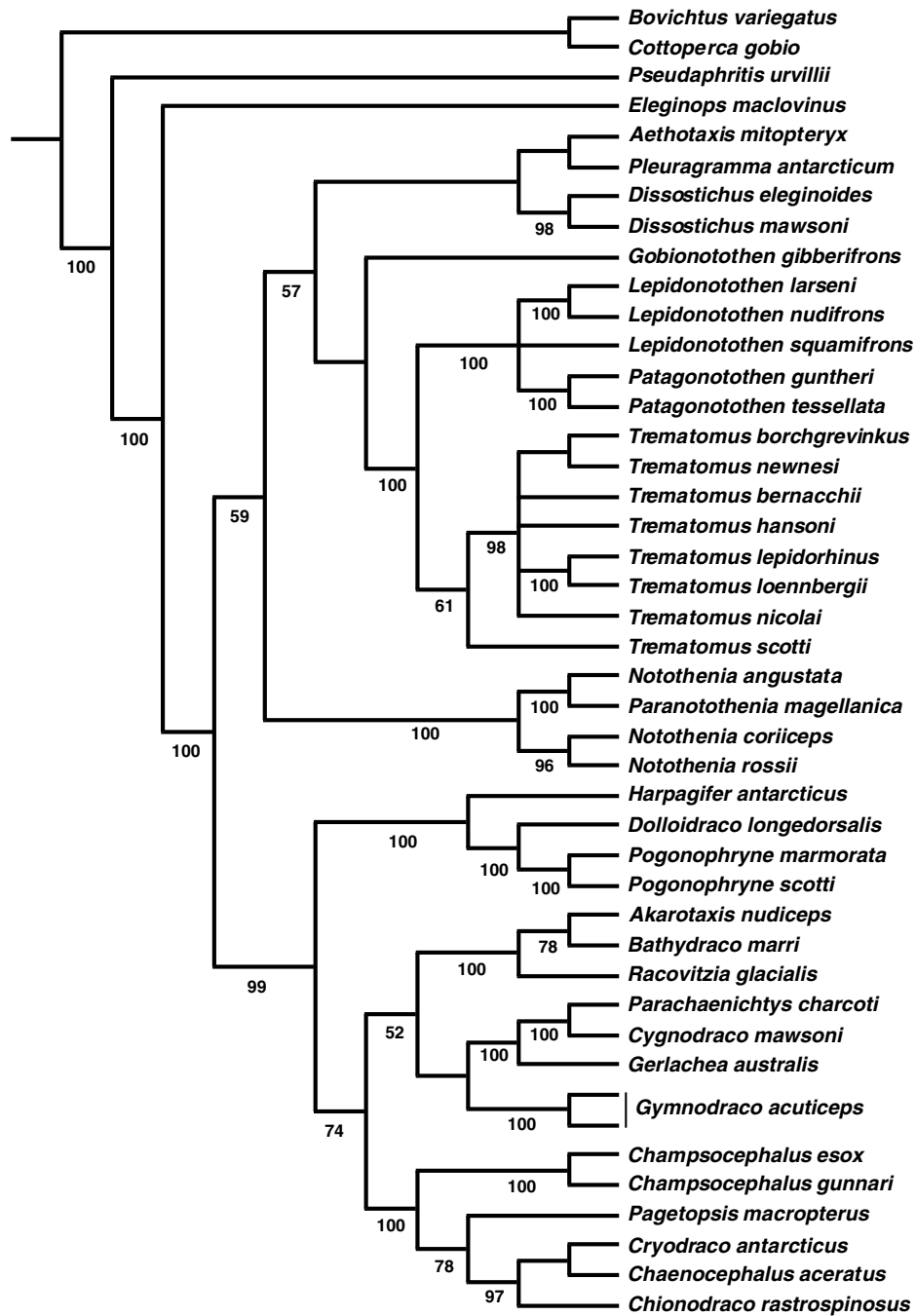


Fig. 1. Notothenioid phylogeny resulting from maximum parsimony analysis of the concatenated mtDNA dataset. Summary statistics are given in Table 2. Numbers at nodes represent bootstrap support values.

(Fig. 3A). The clade of neutrally buoyant species was not present in the S7 phylogenies, but *A. mitopteryx*, *D. eleginoides*, and *D. mawsoni* were present in a clade with high bootstrap and significant Bayesian posterior support. The remaining neutrally buoyant species, *P. antarcticum*, was sister to a clade containing *Notothenia* and *Paranotothenia* in the S7 gene phylogenies (Fig. 3A and B).

The parsimony and Bayesian phylogenies inferred from the combined mitochondrial and nuclear gene dataset were more resolved than the phylogenies resulting from analysis of the nuclear S7 intron, but less resolved than the mtDNA

gene trees (Table 2; Fig. 4). In addition, phylogenies resulting from parsimony and Bayesian analyses of the combined dataset were identical in terms of tree topology and nodes supported with high bootstrap pseudoreplicate scores and significant Bayesian posterior probabilities. Similar to most of the other analyses, the Nototheniidae and the clade containing the neutrally buoyant species were both paraphyletic. The phylogenetic position of *P. antarcticum* was unresolved in a polytomy. Clades present in the combined data analyses included the Antarctic Clade, the High Antarctic Clade, a clade containing *Notothenia* and *Paranotothenia*, and a

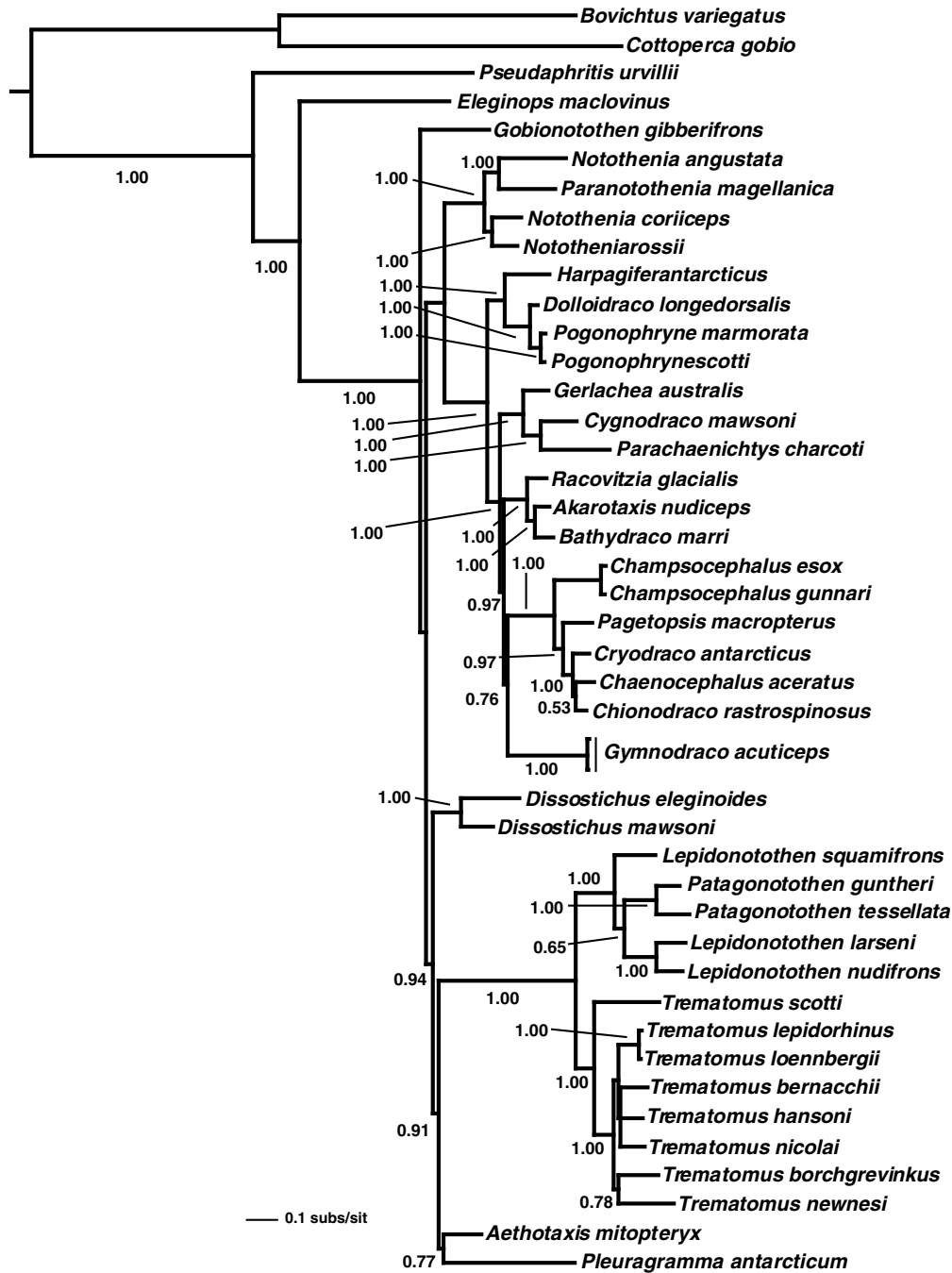


Fig. 2. Notothenioid phylogeny resulting from Bayesian analysis of the concatenated mtDNA dataset. Numbers at nodes represent Bayesian posterior probabilities.

*Lepidonotothen–Patagonotothen–Trematomus* clade. All of these nodes were supported with a bootstrap score of 100 and a Bayesian posterior probability of 1.00 (Fig. 4).

Considering that most investigations of notothenioid phylogeny using molecular data were based on mtDNA sequences, the most important contribution from the new analyses in this study is the support of key phylogenetic hypotheses resulting from analyses of nuclear gene DNA sequences. Specifically, the S7 intron phylogenies strongly supported the monophyly of the Antarctic Clade, the High Antarctic Clade, Channichthyidae, a *Notothenia–Paranoto-*

*thenia* clade, and the *Lepidonotothen–Patagonotothen–Trematomus* clade (Fig. 3A and B). Although some of these relationships have been investigated using two other nuclear genes, the resulting trees from those phylogenetic analysis were not reported, but summarized along with a mtDNA phylogeny in a supertree analysis (Sanchez et al., 2007).

Despite the presence of particular notothenioid clades common in both mitochondrial and nuclear gene phylogenies, substantial differences persist in the phylogenies inferred from each of these two datasets with regard to the

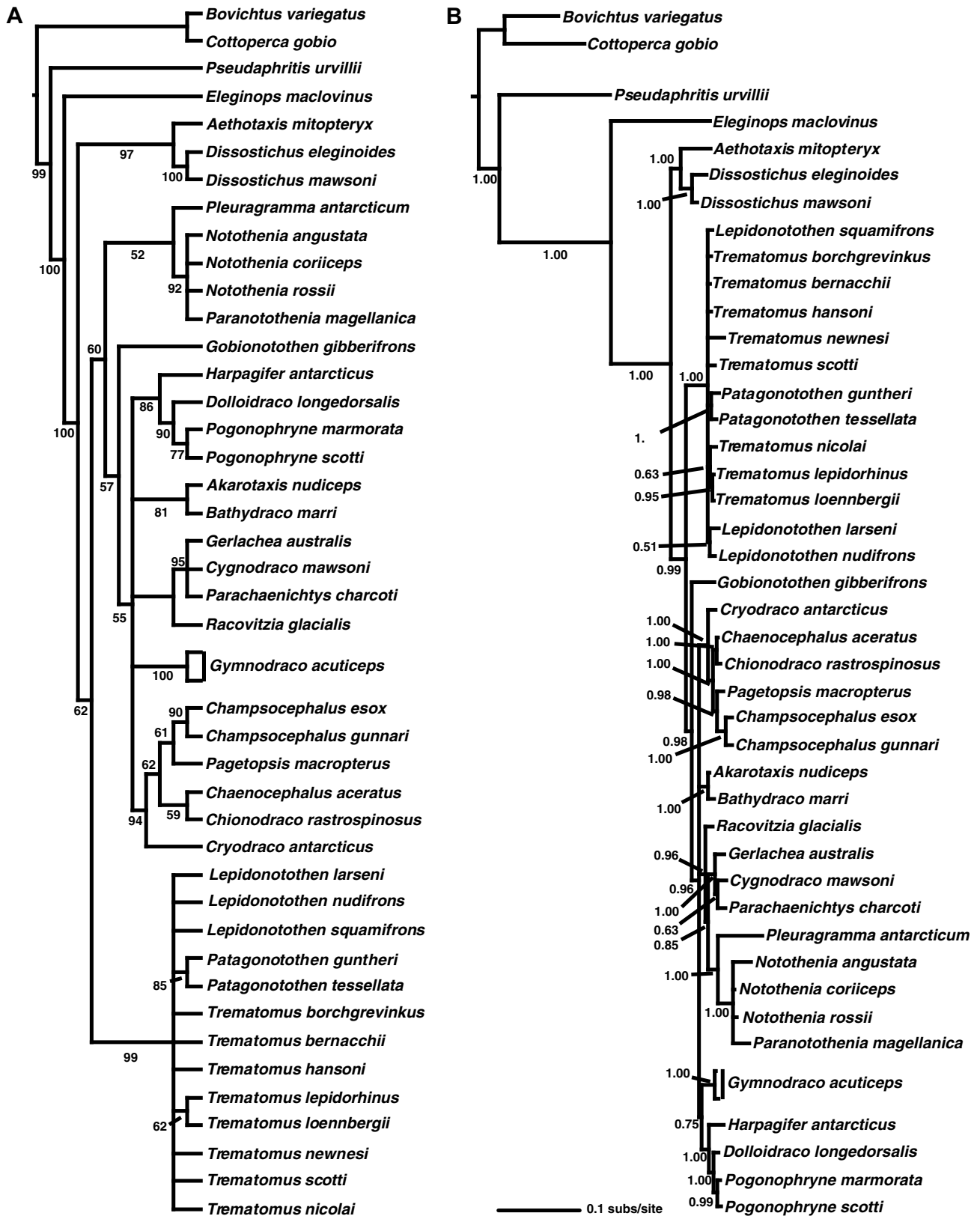


Fig. 3. (A) Notothenioid phylogeny resulting from maximum parsimony analysis of DNA sequences from the nuclear encoded S7 ribosomal protein intron 1. The tree is a strict consensus of 8690 most parsimonious trees. Other summary statistics are given in Table 2. Numbers at nodes represent bootstrap support values. (B) Notothenioid phylogeny resulting from Bayesian analysis of DNA sequences from the nuclear encoded S7 ribosomal protein intron 1. Numbers at nodes represent Bayesian posterior probabilities.

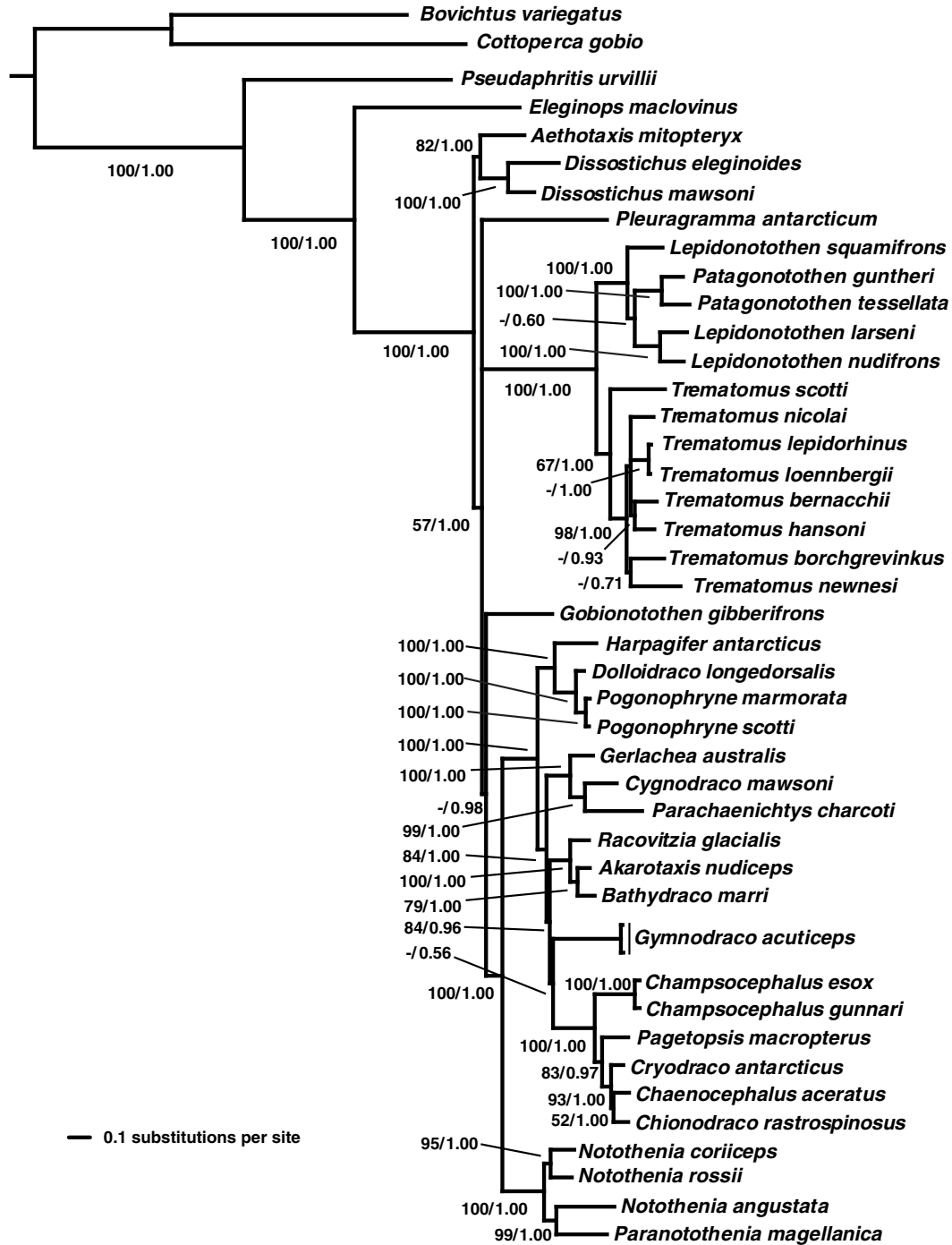


Fig. 4. Notothenioid phylogenies resulting from maximum parsimony and Bayesian analyses of the concatenated mtDNA and nuclear gene datasets. The branch lengths are scaled to the inferred substitution rate from the posterior Bayesian phylogram. Numbers at nodes represent parsimony bootstrap support values followed by Bayesian posterior probabilities. Dashes indicate bootstrap support values less than 50.

presence of particular clades and overall phylogenetic resolution. Specifically, it appears that the absence of a monophyletic Nototheniidae and neutrally buoyant clade in the nuclear gene tree is the result of a lack of phylogenetic resolution and not strong support for the paraphyly of these groups. This explanation is supported by the recovery of over 8000 most parsimonious trees when analyzing the S7 DNA sequences, the presence of several polytomies in the S7 gene trees, and very short internal branch lengths in the

S7 Bayesian phylogeny (Table 2; Fig. 3A and B). Therefore, it is not clear that the mitochondrial and nuclear gene trees presented in this study disagree, or if the S7 nuclear gene provides limited phylogenetic resolution to either support or reject the clades present in the phylogenies resulting from analysis of mtDNA gene sequences.

The higher resolution observed in the notothenioid mitochondrial gene trees is expected, because ancestral polymorphism will sort faster for mitochondrial haplotypes

than nuclear gene alleles (Hudson and Coyne, 2002). The relatively rapid sorting of ancestral haplotypes is a result of the mitochondrial genome having an effective population size that is one quarter that of any set of linked autosomal loci (Avise et al., 1988; Birky et al., 1983; Moore, 1995). Given an origin of the Antarctic Clade approximately 25 million years ago with a possible rapid radiation of the major lineages in the Antarctic Clade (Bargelloni et al., 2000; Near, 2004), internodes in the notothenioid phylogeny may be short when scaled by the ancestral effective population size for specific clades. This indicates that any short nodes that may be difficult to resolve with mtDNA gene sequences will be even more difficult to resolve with DNA sequences sampled from nuclear genes. Despite this seemingly unpromising outlook, the utility of using nuclear genes to resolve notothenioid relationships deserves further exploration since few studies have been published to provide a concrete assessment. As more DNA sequence data are collected from several nuclear genes that exhibit a high relative rate of nucleotide substitution, phylogenetic resolution and node support for the generated notothenioid phylogenies may increase.

### Acknowledgments

We thank C.D. Jones and the United States Antarctic Marine Living Resources Program (AMLR) for providing support for collection of specimens in the South Shetland Islands and Bransfield Strait. Capt. Peter Njardvik and the officers and crew of the R.V. Yuzhmoregeologiya provided logistical field support and laboratory space. B.J. Kendrick provided field and laboratory assistance. Tissue samples for DNA sequencing were kindly provided by J. Calvo, H.W. Detrich, III, A.L. DeVries, K.-H. Kock, G. Lecointre, A.W. North, C. Ozouf-Costaz, and B.D. Sidell. The United States National Science Foundation (DEB-0716155 to T.J. Near and OPP 00-02654 to C.-H.C. Cheng) and the Yale University Peabody Museum of Natural History funded this research.

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