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Estimating divergence times of notothenioid fishes using a fossil-calibrated molecular clock

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Abstract: Hypotheses concerning the diversification of notothenioid fishes have relied extensively on estimates of divergence times using molecular clock methods. The timing of diversification of the cold adapted antifreeze glycoprotein (AFGP)-bearing Antarctic notothenioid clade in the middle to late Miocene has been correlated with the onset of polar climatic conditions along the Antarctic Continental Shelf. Critical examination of the previous molecular clock analyses of notothenioids reveals several problems associated with heterogeneity of nucleotide substitution rates among lineages, the application of potentially inappropriate nucleotide substitution rates, and the lack of confidence intervals for divergence time estimates. In this study, the notothenioid partial gene mtDNA 12S-16S rRNA (PG-rRNA) molecular clock was reanalysed using a tree-based maximum likelihood strategy that attempts to account for rate heterogeneity of nucleotide substitution rates among lineages using the penalized likelihood method, and bootstrap resampling to estimate confidence intervals of divergence time estimates. The molecular clock was calibrated using the notothenioid fossil *Proeleginops grandeastmanorum*. Divergence time estimates for all nodes in the PG-rRNA maximum likelihood tree were substantially older than previous estimates. In particular, the estimated age of the AFGP-bearing Antarctic notothenioid clade predates the onset of extensive sea ice and development of polar conditions by at least 10 million years. Despite caveats involving the fossil calibration and limitations of the PG-rRNA dataset, these divergence time estimates provide initial observations for the development of a novel model of the diversification of cold adapted Antarctic notothenioid fishes.

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Introduction

Phylogenetic and comparative biochemical studies indicate that the diversification of Antarctic notothenioid fishes has been shaped by the dynamic geological and climatic history of the Southern Ocean (Eastman 1993, Chen *et al.* 1997, Bargelloni *et al*. 2000). Precise age estimates for geomorphic changes associated with the isolation of Antarctica through the fragmentation of Gondwana, as well as the timing of Antarctic climate change from temperate to polar conditions have been proposed (Kennett 1982, Eastman 1993, Anderson 1999). Evolutionary biologists have used information regarding the chronological geologic and climatic history of Antarctica to correlate these changes with cladogenic events and the origin of antifreeze proteins in notothenioid fishes (Eastman 1993, Bargelloni *et al.* 1994, 2000, Chen *et al.* 1997, Balushkin 2000). The tree topologies of most morphological and molecular inferred phylogenies of notothenioid fishes are concordant with the hypothesized sequence of geologic events involved with the breakup of Gondwana that ultimately led to the isolation of the Antarctic continent. For example, non-Antarctic notothenioid species classified in the Bovichtidae, Pseudaphritidae, and Eleginopidae are distributed in coastal areas of southern South America, southern Australia, and Tasmania. These lineages are phylogenetically basal to a clade almost exclusively distributed in the Antarctic, providing evidence that vicariance associated with the breakup of Gondwana has been an important factor in the diversification of notothenioid fishes (Eastman 1993, Balushkin 2000, Bargelloni *et al*. 2000).

The majority of notothenioid species are distributed on the Antarctic Continental Shelf. In addition, notothenioids are the dominant component of the Antarctic teleost fish fauna both in terms of species diversity and biomass (Eastman 1993). The Antarctic notothenioids are characterized by the presence of antifreeze glycoproteins (AFGP), which prevent the freezing of body fluids in the subzero Antarctic waters (DeVries & Lin 1977). This character is considered a key innovation that permitted notothenioids to diversify in the ice-laden freezing waters of the Antarctic (Eastman 1993, Clarke & Johnston 1996, Chen *et al*. 1997). The AFGP-bearing Antarctic notothenioids are classified in the Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae. There is substantial evidence from both morphological and molecular phylogenetic analyses for the

monophyly of the AFGP-bearing Antarctic notothenioids (Iwami 1985, Bargelloni *et al*. 1994, 2000, Balushkin 2000).

Molecular clock methods have been used to estimate the timing of diversification of the AFGP-bearing Antarctic notothenioid clade. These efforts have resulted in a variety of age estimates for the most recent common ancestor (MRCA) of the AFGP-bearing notothenioid clade. These range between 11 to 15 millions of years ago (Ma) (Bargelloni *et al*. 1994) or 21 Ma (Bargelloni *et al*. 2000) from analyses of pairwise genetic distances at mtDNA rRNA genes, and between 5 to 14 Ma based on observed sequence polymorphism at a homologous intron between AFGP genes and a trypsinogen-like gene hypothesized to be ancestral to the AFGP genes (Chen *et al*. 1997). The timing of diversification of the AFGP-bearing Antarctic notothenioid clade has been correlated with the onset of major ice sheet growth, drop in ocean surface temperatures, and the formation of sea ice along the Antarctic Continental Shelf at approximately 14 Ma (Kennett 1982, Eastman & McCune 2000). The similarity of the timing between the initial diversification of AFGP-bearing Antarctic notothenioids and the development of freezing oceanic temperatures has provided support for a model of Antarctic notothenioid diversification. In this initial cladogenesis and the origin of an adaptive trait to withstand freezing water temperatures, are directly associated with the onset of polar environmental conditions in the Antarctic during the middle to late Miocene (Chen *et al.* 1997).

The key element in this model of Antarctic notothenioid diversification is the age estimate of the AFGP-bearing Antarctic notothenioid clade. Critical examination of the molecular clock methods used in these age estimates reveals two important issues that require attention before confidence in these divergence time estimates can be fully assessed; the phylogenetic resolution of the mtDNA partial gene 12S-16S rRNA (PG-rRNA) dataset, and the validity of the particular molecular clock methods used in estimating notothenioid divergence times.

Based on evaluation of all published analyses, the phylogenetic resolution of the often used PG-rRNA sequence dataset is limited. Previous analyses have only presented trees resulting from parsimony methods with transversion nucleotide substitutions weighted relative to transitions, with little or no justification (Bargelloni *et al*. 1994, 2000). The result is an apparent inflation of the true phylogenetic resolution offered by the PG-rRNA dataset. Despite character weighting, bootstrap pseudoreplicate support values are weak for most internal nodes in published phylogenies, and several key alternative tree topologies cannot be rejected (Bargelloni *et al*. 2000). Furthermore, the full array of phylogenetic optimality criteria available has not been employed in the analysis of this dataset (e.g. maximum likelihood).

With regard to the molecular clock strategies used to estimate divergence times among notothenioids, there exist four methodological problems. First, the molecular clock has been calibrated at phylogenetic nodes hypothesized to have resulted from vicariant events with an estimated geologic age (Bargelloni *et al*. 2000), or "universal" rates of sequence evolution at mtDNA genes estimated in other taxonomic groups were applied to notothenioids (Bargelloni *et al*. 1994, Ritchie *et al*. 1996, Chen *et al*. 1997, Eastman & McCune 2000, Stankovic *et al*. 2002). Second, most molecular clock methods require the assumption that the rate of nucleotide substitution is uniform among all lineages. Most investigations estimating divergence times in notothenioids have ignored rate heterogeneity (Bargelloni *et al*. 1994, Ritchie *et al*. 1996, 1997, Stankovic *et al*. 2002), which has the potential to significantly effect the estimation of divergence times (Sanderson 1998). When rate heterogeneity has been incorporated into divergence time estimates, relative rate tests have been used to exclude species exhibiting rate heterogeneity from the majority of species contrasts in the analysis (Bargelloni *et al*. 2000). Relative rate tests are known to be problematic in determining rate constancy among lineages, as they measure the substitution rate in only a small portion of the phylogeny. Also, the statistical significance of relative rate tests must be corrected when multiple relative rate tests are used (Sanderson 1998). Third, many attempts using the molecular clock to estimate divergence times in notothenioids have used genetic distances calculated only from transversion nucleotide substitutions (Bargelloni *et al*. 1994, 1997, 2000, Ritchie *et al*. 1996). Since transitions typically occur at a higher rate than transversions in mtDNA genes, the expected variance of divergence time estimates will be lower when using both transitions and transversions (Sanderson & Doyle 2001). Fourth, divergence time estimates in notothenioid fishes have been presented without confidence intervals.

In this paper I provide a critical evaluation of the phylogenetic resolution of the mtDNA partial 12S-16S rRNA gene sequences used in several attempts to estimate divergence times in notothenioids. I also outline a methodology to provide estimates of divergence times using strategies that address the methodological problems outlined above. The molecular clock in notothenioids is calibrated using information from the fossil record. Rate heterogeneity among notothenioid lineages is evaluated using a tree-based maximum likelihood approach. The rate of nucleotide substitution, divergence times of notothenioid lineages, and confidence intervals of lineage ages are estimated using penalized likelihood (PL), a method that facilitates estimation of divergence times despite the presence of significant heterogeneity of nucleotide substitution rates among lineages (Sanderson 2002).

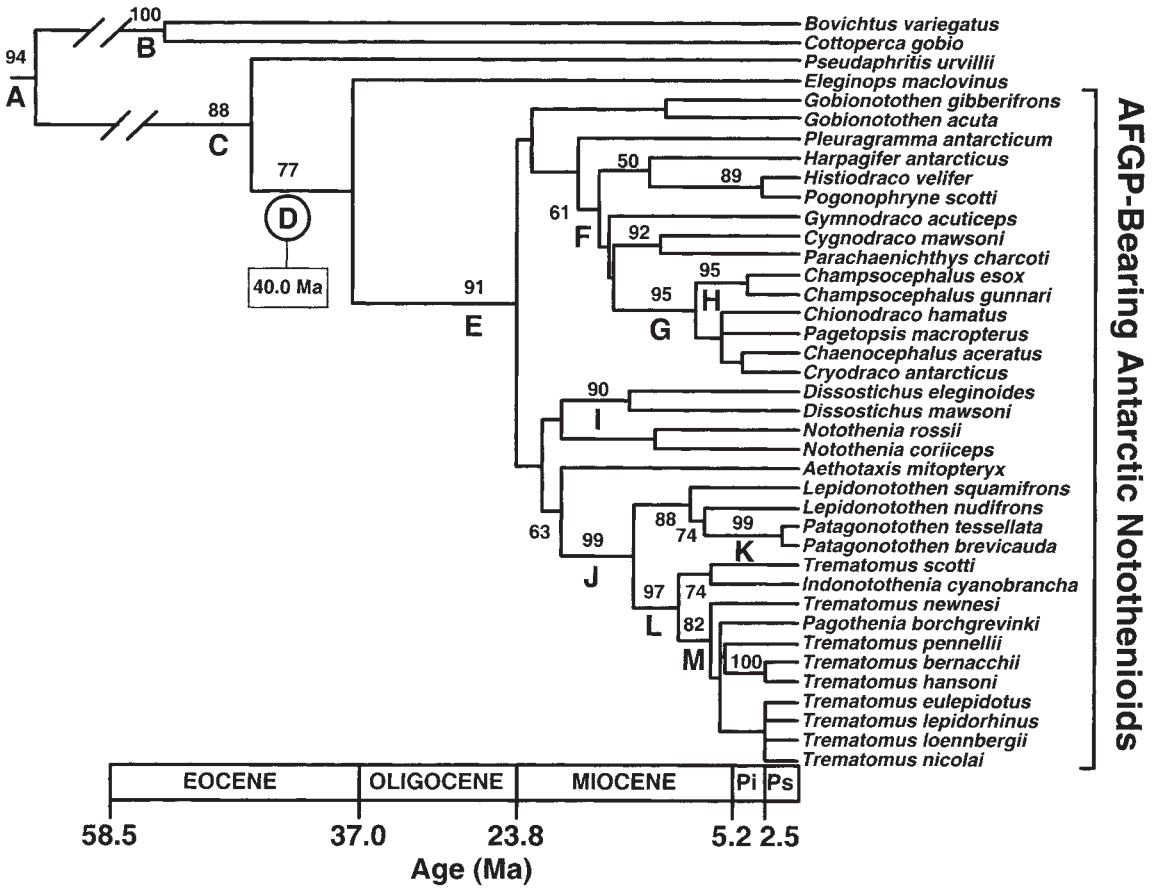

Fig. 1. Chronogram resulting from maximum likelihood analysis of the partial gene 12S-16S rRNA dataset and estimation of divergence times using penalized likelihood. Numbers at nodes represent percent recovery in bootstrap pseudoreplicate analysis. Divergence time estimates with confidence intervals are given in Table I for nodes labeled with capital letters. The letter labeling the node used for the calibration point at 40.0 millions of year ago (Ma) is circled. Branches are drawn to reflect evolutionary ages and are drawn on the geological time scale (Berggren *et al*. 1995). Pi = Pliocene, Ps = Pleistocene.

Materials and methods

Available DNA sequences of the PG-rRNAs were downloaded from Genbank (Appendix). The entire 16S rRNA gene for *Cottoperca gobio*, *Pseudaphritis urvillii*, and *Eleginops maclovinus* was sequenced from PCR products using the same primers and sequencing methods as Near *et al*. (2003). Sequences were aligned using Clustal X (Thompson *et al*. 1997), and adjusted to reflect secondary structure designations of paired and unpaired regions. Phylogenetic relationships were estimated using maximum likelihood (ML) as implemented in the computer program PAUP* 4.0 (Swofford 2000). Heuristic tree searches with TBR branch swapping and ten random sequence addition replicates were used to find the best ML tree. The relative support for a given node was determined with nonparametric bootstrapping (200 pseudoreplicates). Likelihood ratio (LR) tests were used to choose the nucleotide substitution model used in ML analyses (Huelsenbeck & Crandall 1997, Huelsenbeck & Rannala 1997). Likelihood scores and model parameter values used in LR tests were calculated on one of the trees resulting from MP analysis.

Prior to estimating rates of nucleotide substitution with a fossil-based calibration, heterogeneity of substitution rates among lineages was investigated using a tree-wide LR test. The ML scores from rate-variable and rate-constant models of sequence evolution were compared. The LR test statistic was compared to a chi-square distribution with s - 2 degrees of freedom, where s equals the number of sequences in the analysis (Huelsenbeck & Crandall 1997).

After discovering significant heterogeneity of substitution rates among lineages, ages were estimated using the PL method (Sanderson 2002) as implemented in the program r8s version 1.50 (Sanderson 1997). Penalized likelihood is a semi-parametric method that combines a parametric model of having a different rate of substitution on every branch in the phylogeny with a nonparametric roughness penalty that imposes a higher cost on the model if the substitution rate changes too quickly from branch to branch (Sanderson 2002). The optimal smoothing parameter used in PL

analyses was determined from several possible values using cross-validation to determine the smoothing value that minimized the chi-square error (Sanderson 2002). The tree used for these analyses was a ML inferred topology using a Kimura two-parameter model with among-site rate variation and an estimate for the number of invariable sites (K2P+INV+GAMMA). Maximum likelihood branch lengths on the tree used in PL analyses were estimated without enforcing a molecular clock.

The notothenioid fossil record is scarce (Grande & Eastman 1986, Eastman & Grande 1989). However, a fossil assigned to the Eleginopidae provides a potential calibration of the notothenioid molecular clock. The fossil eleginopid *Proeleginops grandeastmanorum* is from the La Meseta Formation on Seymour Island, Antarctic Peninsula (Eastman & Grande 1991, Balushkin 1994). The age of the fossil mammal locality is estimated at approximately 40 Ma through biostratigraphical correlations with megafossils, microfossil invertebrates, and pollen (Woodburne & Zinsmeister 1984, Case 1988, Case *et al*. 1988). Assignment of the fossil inferred age to the appropriate node in the notothenioid phylogeny followed strategies and guidelines discussed in Doyle & Donoghue (1993) and Magallon & Sanderson (2001). The fossil age of *P. grandeastmanorum* (40.0 Ma) was used as the minimal age estimate for clade that includes *Eleginops maclovinus* and its sister lineage, the AFGP-bearing Antarctic notothenioid clade. This fossil inferred age was used to estimate the minimal age of the stem group, and not the crown group of the Antarctic notothenioid diversification (see Magallon & Sanderson 2001).

Confidence intervals on divergence time estimates were estimated by using a nonparametric bootstrap procedure, as outlined in Baldwin & Sanderson (1998). One hundred bootstrap replicate datasets were generated using the CodonBootstrap version 2.1 computer program (Bollback, unpublished) with the normal bootstrap option. This program which facilitates the creation of nexus format data files that include the tree topology and PAUP* commands. Bootstrap replicates were imported into PAUP*, and branch lengths were calculated on the ML tree using the optimal model of sequence evolution, and the distribution of divergence times at each node was assessed using the PL method and the "profile" command in r8s. The central 95% of the distribution of bootstrap age estimates provided the confidence interval for each node (Baldwin & Sanderson 1998, Sanderson 1998).

Results and discussion

A single ML tree resulted from analysis of the 899 aligned nucleotide dataset, using the K2P+INV+GAMMA model of sequence evolution. The ML tree topology is similar to those found in previous MP analyses of this data (Bargelloni *et al.* 2000), as well as phylogenetic analyses of nuclear gene sequence data (Chen *et al*. 2003) and morphological characters (Balushkin 2000). The degree of phylogenetic resolution provided by the partial 12S and 16S rRNA dataset was limited, but several key nodes in the phylogeny were well supported in bootstrap pseudoreplicate analysis (Fig. 1). These nodes, as labelled in Fig. 1, include (A) the MRCA of all notothenioids, (B) the MRCA of the Bovichtidae, (C) the MRCA of *Pseudaphritis urvillii* and the remaining notothenioids, (D) the MRCA of *Eleginops maclovinus* and the Antarctic AFGP-bearing notothenioids, (E) the MRCA of the Antarctic AFGP-bearing

Table I. Estimated ages of Most Recent Common Ancestors (MRCA) and previous estimates of notothenioid divergence times using molecular clock methods given as millions of years ago (Ma). Nodes correspond to those labelled in Fig. 1.

Node	Node description	Age estimate and bootstrap estimate of confidence interval (Ma)	Previous molecular clock divergence time estimates (Ma)
A	MRCA of Notothenioidei	124.8 ± 3.6	45 to 57 [3]
B	MRCA of Bovichtidae	54.1 ± 1.7	No previous estimate
C	MRCA of *Pseudaphritis* and remaining notothenioids	47.0 ± 1.4	38 (calibration point) [5]
D	MRCA of *Eleginops* and Antarctic notothenioids	40.0 (fossil calibration)	27 [5]
E	MRCA of AFGP-bearing Antarctic notothenioids	24.1 ± 0.5	7 to 15 [1] 11 to 15 [3] 5 to 14 [4] 21 [5]
F	MRCA of High Antarctic notothenioid clade	17.0 ± 0.4	No previous estimate
G	MRCA of Channichthyidae	8.5 ± 0.3	2.0 to 2.5 [3] 2.0 to 5.5 [5]
H	MRCA of *Champsocephalus gunnari* and *C. esox*	4.2 ± 0.3	1.7 [6]
I	MRCA of *Dissostichus mawsoni* and *D. eleginoides*	14.5 ± 0.5	No previous estimate
J	MRCA of trematiomines and lepidonotothens	14.0 ± 0.4	No previous estimate
K	MRCA of *Patagonotothen tessellata* & *P. brevicauda*	1.0 ± 0.1	No previous estimate
L	MRCA of *Trematomus scotti* & remaining trematomines	9.8 ± 0.4	2 to 8 [5]
M	MRCA of *Trematomus* sans *T. scotti*	7.4 ± 0.3	3.4 [2] 2.5 to 4.5 [3]

[1] Bargelloni *et al.* (1994), [2] Ritchie *et al.* (1996), [3] Bargelloni *et al*. (1997), [4] Chen *et al.* (1997), [5] Bargelloni *et al.* (2000), [6] Stankovic *et al.* (2002)

notothenioids, (F) the MRCA of the High Antarctic notothenioid clade which includes the Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae, (G) the MRCA of the Channichthyidae (icefishes), (H) the MRCA of the sister species pair *Champsocephalus gunnari* and *C. esox*, (I) the MRCA of the sister species pair *Dissostichus mawsoni* and *D. eleginoides*, and several nodes (J, K, L, and M) that relate the nototheniid genera *Lepidonotothen*, *Patagonotothen*, and *Trematomus* (Fig. 1).

A molecular clock model, where the rate of nucleotide substitution is uniform across the phylogeny, was rejected using the LRT ($\chi^2 = 190.1$, df = 39, $P < 0.001$). Setting the calibration node at 40.0 Ma, and performing PL analysis resulted in divergence time estimates for nodes in the phylogeny that were well supported in ML bootstrap analysis (Fig. 1) and are presented in Table I. The rate of nucleotide substitution for the 12S-16S rRNA partial gene sequences that resulted from the PL analysis was 1.59×10^{-3} substitutions per site per million years.

The 95% confidence intervals for nodes with estimated ages between 124.5 Ma to 14.0 Ma ranged between approximately 2.0 and 3.0% of the age estimate. Confidence intervals around age estimates for nodes younger than 14.0 Ma ranged from approximately 3.5 to 4.5% of the age estimate (Table I). When compared to several molecular clock studies using similar tree-based methods in other organisms, including centrarchid perciform fishes (Near *et al*. 2003), plants (Sanderson 1997), birds (van Tuinen & Hedges 2001), and placental mammals (Springer *et al*. 2003), the confidence intervals for the notothenioid age estimates are much lower. The reason for this difference may involve the much smaller size and relative homogeneity of character types in the nucleotide dataset used in the molecular clock analysis of the notothenioids. The datasets used in the molecular clock analyses listed above are much larger than the notothenioid PG-rRNA, and many of these datasets include nucleotides sampled from several different types of genes (protein coding, introns, ribosomal RNA, etc.). In bootstrap resampling of these larger datasets with nucleotides sampled from different types of genes, the likelihood of generating a dataset with substantial rate variation is greater than when resampling from a smaller dataset that consists of one particular type of gene. If the data sampling strategy is the cause of the small confidence intervals in the notothenioid divergence time estimates, than larger confidence intervals for divergence time estimates would be expected when phylogenetic datasets comprising greater numbers of nucleotide sites sampled across several types of mtDNA and nuclear genes in notothenioids become available.

Two potential problems with the notothenioid divergence time estimates presented in this study involve the calibration using the fossil record. First, the fossil *Proeleginops grandeastmanorum* was identified as a gadiform by Eastman & Grande (1991); however, Balushkin (1994) argued with detailed comparative anatomical evidence and a plausible biogeographical scenario that *P. grandeastmanorum* is a notothenioid assignable to the Eleginopidae. Despite a convincing argument by Balushkin (1994) and lack of any published response to this hypothesis, the initial disagreement between two studies examining this fossil raises suspicion as to the identification of the fossil as a notothenioid. The second problem involving fossil calibration of the notothenioid molecular clock is the presence of only a single calibration point. Ideally, multiple calibration points are used in molecular clock studies to assess the consistency of fossil versus molecular age estimates, as assessed through cross-validation analysis (Near *et al*. 2003). However, given the extremely limited notothenioid fossil record the only strategy that may be available is to rely on externally calibrated molecular clocks from other clades of acanthomorph fishes. If the fossil *P. grandeastmanorum* has been correctly identified as an eleginopid notothenioid, and if the age of the fossil is accurate, then the age this node estimated from any future external calibrations should converge on 40 Ma.

Despite the caveats involving the size and nature of available molecular phylogenetic datasets, and the limitations of a single fossil calibration point, the divergence time estimates presented in this study represent the only published attempt to use tree-based methods that account for nucleotide substitution rate heterogeneity among notothenioid lineages. Even if the conclusions from these sets of divergence time estimates are considered preliminary, they minimally represent the best this particular PG-rRNA dataset has to offer. As larger molecular phylogenetic datasets are gathered for notothenioid fishes, and experiments using external calibrations to estimate divergence times are attempted, the true limitations to notothenioid molecular clock studies will be apparent without the spectre of inadequately sampled datasets and uncomfortable reliance on single fossil calibration points.

Taken at face value the divergence time estimates of notothenioid fishes using the *P. grandeastmanorum* calibration and the PL method offers some very interesting results, which may have substantial bearing on current models of notothenioid diversification (Fig. 1, Table I). Overall, it appears that all previous age estimates for any particular node in the notothenioid phylogeny are younger than estimates from the PL analysis (Table I). In particular, the MRCA of the entire notothenioid radiation, which is the node that separates the Bovichtidae and the remaining notothenioids, is surprisingly ancient at nearly 125 Ma. This is nearly twice the age of the previous estimate between 45 and 57 Ma (Bargelloni *et al*. 1997). If this date is accurate it would indicate that the initial diversification of the notothenioids predates the appearance of acanthomorph

fishes in the fossil record by approximately 30 million years (m.y.) (Patterson 1993). Interestingly, the divergence time estimate between the two sampled bovichtid species (*Bovichtus variegatus* and *Cottoperca gobio*) is also very ancient, dating to the early stage of the Eocene (Fig. 1, Table I). This result indicates that the present distribution of bovichtids in southern South America and New Zealand may represent a pattern of ancient vicariance that predates the diversification of Australian, South American, and Antarctic notothenioids (Fig. 1, Table I).

The age of the MRCA of *Pseudaphritis urvillii* and a clade containing *Eleginops maclovinus* and the AFGP-bearing Antarctic notothenioid clade (Fig. 1, node C) is substantially older than the 38 Ma assigned to this node and used as a calibration point by Bargelloni *et al*. (2000). *Pseudaphritis urvillii* is endemic to freshwater rivers and coastal areas of south-eastern Australia and Tasmania (Eastman 1993). The age estimate of this node at 38 Ma was justified as the geologic age of final separation between Australia and Antarctica (Bargelloni *et al.* 2000). However, the initial separation of Australia and Antarctica is hypothesized to have occurred 95 Ma (Anderson 1999). Therefore, a realistic age estimate for the MRCA of *P. urvillii* and the remaining notothenioids would be between 95 Ma and 38 Ma, since the geographic isolation of organisms occurring across the pre-fragmented Australia–Antarctic component of Gondwana could have occurred at any point during the approximately 55 m.y. between initial and final separation of Australia and Antarctica. The age of this node resulting from the fossil-calibrated PL analysis fits this model (Table I), indicating a 38 Ma estimate for this node may represent a significant underestimate of its true age.

Perhaps the most interesting and potentially controversial result from the fossil-calibrated PL molecular clock analysis is the estimated age of the MRCA of the AFGP-bearing Antarctic notothenioid clade. Most previous estimates have placed the initial diversification of this clade between 7 and 15 Ma, in the middle to late Miocene, and at the time when polar climates and ice sheets formed across the Antarctic (Kennett 1982, Eastman & McCune 2000, Poulin *et al*. 2002). The age resulting from the fossil-calibrated PL analysis was 24.1 ± 0.5 Ma, at the Oligocene–Miocene boundary and substantially older than these other estimates (Table I). This would put the initial diversification of this clade at the time of the development of the unrestricted Antarctic Circumpolar Current and Antarctic Polar Front, which resulted with the separation of South America and Antarctica through sea floor spreading and deepening of the Drake Passage (Kennett 1982, Eastman 1993). These events were the final geomorphic stages that led to the complete geographic and thermal isolation of Antarctica, and the subsequent development of widespread freezing water temperatures along the Antarctic Continental Shelf (Eastman 1993).

Previous age estimates for the AFGP-bearing Antarctic notothenioid clade have correlated closely with the timing of changing environmental conditions towards freezing of Antarctic Continental Shelf habitats (Bargelloni *et al*. 1994, Chen *et al*. 1997). The result from the fossil-calibrated PL molecular clock analysis indicates that the evolution of AFGP in notothenioids may have occurred prior and not simultaneous with the development of the widespread polar Antarctic Continental Shelf climate in the middle Miocene (10 to 14 Ma).

Despite the attention directed towards geomorphic events and climatic changes between 25 and 10 Ma, there is evidence for widespread continental glaciation and sharp drops in oceanic surface temperatures between 38 and 35 Ma (Eastman 1993, Eastman & Clarke 1998, Poulin *et al*. 2002). The correlation of the initial diversification of the AFGP-bearing Antarctic notothenioid clade with the final geological isolation of Antarctica and the potential for freezing water Antarctic Continental Shelf conditions at the Eocene-Oligocene boundary (37 Ma) indicates that a combination of geographic isolation (vicariance) and directional selection driven by exposure to freezing conditions prior to 24.1 ± 0.5 Ma have been important factors in the evolution of Antarctic notothenioids.

If the estimated divergence times presented in this study are accurate, why have all other studies resulted in divergence time estimates for any given node that are much younger? The reason probably involves the fact that several different calibrations have been used in previous studies, and the use of each of these calibrations is potentially problematic. For example, Bargelloni *et al*. (2000) used 38 Ma as the age of the MRCA of *P. urvillii* and the remaining notothenioids based on the separation of Australia and Antarctica. As discussed above, this may be a significant underestimate for the age of this clade, which would have the effect of overestimating the rate of nucleotide substitution, and consequently underestimating divergence times. Another group of potentially problematic calibrations are the ones derived from studies of ungulate mammals and European newts (Allard *et al*. 1992, Caccone *et al*. 1997) that were used in the majority of studies that have estimated divergence times in notothenioids (Bargelloni *et al.* 1994, 1997, Ritchie *et al.* 1996, Stankovic *et al.* 2002). There exists appreciable lineage-specific mtDNA nucleotide substitution rate heterogeneity across major vertebrate groups (Avise *et al*. 1992, Martin *et al*. 1992, Stanley & Harrison 1999), and underestimation of divergence times in notothenioids would result if the rate of nucleotide substitution derived from tetrapods is substantially higher than the actual notothenioid rate. Chen *et al*. (1997) estimated the age of the AFGP-bearing Antarctic notothenioid clade from nuclear encoded introns of the AFGP and a trypsinogen-like gene using a rate derived from RFLP analysis of teleost mtDNA genomes (Martin *et al*. 1992). The fact that the rate of nucleotide substitution in

mtDNA genes can be as much as ten times faster than nuclear genes (Brown *et al*. 1979) would explain the difference in age estimates using the fossil-calibrated PL analysis and those presented in Chen *et al*. (1997).

Ideally future studies of notothenioid phylogenetics and molecular clock analyses of divergence times will use datasets that are much larger in size with regard to both taxon and nucleotide character sampling than the PG-rRNA data analysed here. However, this study has attempted to present molecular clock methods to the community of Antarctic evolutionary biologists that takes into account both the phylogenetic relationships of the organisms in the analysis, as well as the persistent problem of rate heterogeneity. All previous efforts in estimating divergence times in notothenioid fishes have not addressed these problems, which cast a degree of uncertainty on the previous results.

Enthusiasm for the results of the molecular clock analyses presented in this study should be tempered by the caveats regarding the use of a single fossil calibration point and the nature of the PG-rRNA dataset. However, in my opinion this fossil-calibrated PL molecular clock analysis represents a substantially less *ad hoc* and more systematic approach to use this dataset in estimating divergence times of notothenioid fishes than those previously published.

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Appendix

Species data used in this study, Genbank numbers (12S, 16S). Notothenioid families sampled in bold. **Bovichtidae**: *Bovichtus variegatus* (Z32702, Z32721), *Cottoperca gobio* (Z32703, XXXXXX). **Pseudaphritidae**: *Pseudaphritis urvillii* (Z32704, XXXXXX). **Eleginopidae**: *Eleginops maclovinus* (AF145426, XXXXXX). **Nototheniidae**: *Aethotaxis mitopteryx* (AF145423, AF145408), *Dissostichus eleginoides* (AF145425, AF145410), *D. mawsoni* (Z32707, Z32726), *Gobionotothen acuta* (AF145428, AF145413), *G. gibberifrons* (Z32707, Z32727), *Indonotothenia cyanobrancha* (AF145431, AF145416), *Lepidonotothen nudifrons* (AF145429, AF145414), *L. squamifrons* (AF145430, AF145415) *Notothenia coriiceps* (Z32712, Z32731), *N. rossii* (AF145432, AF145417), *Pagothenia borchgrevinki* (Z32715, Z32734), *Patagonotothen brevicauda* (AJ307044, AJ307047), *P. tessalata* (AF145433, AF145418), *Pleuragramma antarcticum* (AF145435, AF145420), *Trematomus bernacchii* (U27520, U27521), *T. eulepidotus* (Z32717, Z32736), *T. hansoni* (Z32718, Z32737), *T. lepidorhinus* (U27523, U27522), *T. loennbergii* (U27525, U27524), *T. newnesi* (U27527, U27526), *T. nicolai* (Z32719, Z32738), *T. pennellii* (Z32720, Z32739), *T. scotti* (U27529, U27528). **Harpagiferidae**: *Harpagifer antarcticus* (U37137, U37136). **Artedidraconidae**: *Histiodraco velifer* (Z32710, Z32729), *Pogonophryne scotti* (Z32716, Z32735). **Bathydraconidae**: *Cygnodraco mawsoni* (Z32706, Z32725), *Gymnodraco acuticeps* (Z32709, Z32728), *Parachaenichthys charcoti* (Z32714, Z32733). **Channichthyidae**: *Chaenocephalus aceratus* (Z32703, Z32722), *Champsocephalus esox* (AJ307046, AJ307045), *C. gunnari* (AF145424, AF145409), *Chionodraco hamatus* (Z32704, Z32723), *Cryodraco antarcticus* (Z32705, Z32724), *Pagetopsis macropterus* (Z32748, Z32747). Genbank accession numbers of percid outgroup species. *Gymnocephalus cernuus* (12S, 16S), *Perca fluviatilis* (12S, 16S).