

# Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes

Thomas J. Near<sup>a,b,1</sup>, Alex Dornburg<sup>b</sup>, Kristen L. Kuhn<sup>b</sup>, Joseph T. Eastman<sup>c</sup>, Jillian N. Pennington<sup>b,d</sup>, Tomaso Patarnello<sup>e</sup>, Lorenzo Zane<sup>f</sup>, Daniel A. Fernández<sup>g</sup>, and Christopher D. Jones<sup>h</sup>

<sup>a</sup>Peabody Museum of Natural History and <sup>b</sup>Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06520 <sup>c</sup>Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, Athens, OH 45701; <sup>d</sup>Ezra Stiles College, Yale University, New Haven, CT 06520 <sup>e</sup>Department of Public Health, Comparative Pathology and Veterinary Hygiene, Università di Padova, 35020 Legnaro, Italy; <sup>f</sup>Department of Biology, Università di Padova, 35131 Padova, Italy; <sup>g</sup>Centro Austral de Investigaciones Científicas, 9410 Ushuaia, Argentina; and <sup>h</sup>Antarctic Ecosystem Research Division, Southwest Fisheries Science Center, National Oceanic and Atmospheric Administration National Marine Fisheries Service, La Jolla, CA 92037

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**The Southern Ocean around Antarctica is among the most rapidly warming regions on Earth, but has experienced episodic climate change during the past 40 million years. It remains unclear how ancient periods of climate change have shaped Antarctic biodiversity. The origin of antifreeze glycoproteins (AFGPs) in Antarctic notothenioid fishes has become a classic example of how the evolution of a key innovation in response to climate change can drive adaptive radiation. By using a time-calibrated molecular phylogeny of notothenioids and reconstructed paleoclimate, we demonstrate that the origin of AFGP occurred between 42 and 22 Ma, which includes a period of global cooling approximately 35 Ma. However, the most species-rich lineages diversified and evolved significant ecological differences at least 10 million years after the origin of AFGPs, during a second cooling event in the Late Miocene (11.6–5.3 Ma). This pattern indicates that AFGP was not the sole trigger of the notothenioid adaptive radiation. Instead, the bulk of the species richness and ecological diversity originated during the Late Miocene and into the Early Pliocene, a time coincident with the origin of polar conditions and increased ice activity in the Southern Ocean. Our results challenge the current understanding of the evolution of Antarctic notothenioids suggesting that the ecological opportunity that underlies this adaptive radiation is not linked to a single trait, but rather to a combination of freeze avoidance offered by AFGPs and subsequent exploitation of new habitats and open niches created by increased glacial and ice sheet activity.**

Notothenioidei | Icefish | Percomorpha | molecular clock | buoyancy

The recent onset of global climate change is causing an increase in temperatures for most regions of the Southern Ocean (1, 2), and is predicted to affect Antarctic marine species through increased physiological stress (3, 4), reduced ice scouring in near-shore habitats (5), and declining phytoplankton and krill populations that comprise the basis of Southern Ocean food webs (3, 6, 7). However, the effect of increasing ocean temperatures on Antarctic fishes is unknown. Teleost fishes are of fundamental importance to Southern Ocean ecology, providing the primary link between high-level vertebrate predators (e.g., toothed whales, penguins, and seals) and lower trophic levels (8). As the teleost fish diversity, abundance, and biomass of the Southern Ocean consists primarily of a single lineage of closely related species, the notothenioids (9), understanding how ancient climatic transitions between periods of global warming and cooling have influenced the patterns of diversification in these fishes is important to the development of forecasts on the impact of present-day climate change on Antarctic biodiversity.

Several characteristics suggest that the living diversity of Antarctic notothenioids was the result of adaptive radiation (10): they exhibit common ancestry (9) and a strong correlation between phenotype and habitat utilization (11, 12), they possess antifreeze glycoproteins (AFGPs) that allow them to occupy freezing habitats unavailable to most other teleosts (13), and

they are more species-rich than their non-Antarctic sister lineage (approximately 100 vs. one species) (9). Molecular divergence time analyses have attempted to correlate the origin of the AFGP-bearing Antarctic notothenioids with a period of global cooling and widespread glaciation of Antarctica that began at the onset of the Eocene–Oligocene boundary (14, 15), approximately 35 Ma (16, 17), leading to the conclusion that the evolutionary innovation of AFGP was the trigger of the notothenioid adaptive radiation (15).

One critical aspect of the Antarctic notothenioid adaptive radiation that requires investigation is the relationship between paleoclimatic change and the tempo of lineage diversification. The AFGP innovation hypothesis predicts that the changing climatic conditions during the Oligocene cooling event, coupled with the evolution of AFGP, triggered increased rates of lineage and phenotypic diversification (15). This suggests that most of the ecological and phenotypic disparity observed among lineages of Antarctic notothenioids evolved early in their history. Subsequent climatic changes in the Southern Ocean, such as warming and glacial retreat during the Middle Miocene climatic optimum, dated between 17 and 14 Ma, or the Middle Miocene climatic transition (MMCT) that initiated the formation of subzero polar conditions between 14.2 and 13.8 Ma (18), will have had a secondary and less pronounced influence on the diversification of Antarctic notothenioids.

In addition to the origin of AFGP, the diversification of structural buoyancy adaptations in notothenioids is another critical component of this Antarctic adaptive radiation that reflects diversification along ecological axes (13) and a correlation between phenotype and habitat utilization (11). Although sharing a benthic common ancestor, Antarctic notothenioid species inhabit all parts of the water column in the Southern Ocean, spanning benthic to epibenthic, semipelagic, cryopelagic, and pelagic habitats (13). As all notothenioid species lack a swim bladder, buoyancy modification has been accomplished through the reduction of skeletal ossification, through alteration of the expression of collagen genes (19), and the evolution of extensive, and in some cases, unique lipid deposits (13, 20). Evolutionary analyses of teleost fish lineages often find a strong phylogenetic signal in ecomorphological traits (21, 22). Therefore, under the AFGP innovation model, disparity in buoyancy and lineage

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<sup>1</sup>To whom correspondence should be addressed. E-mail: thomas.near@yale.edu.

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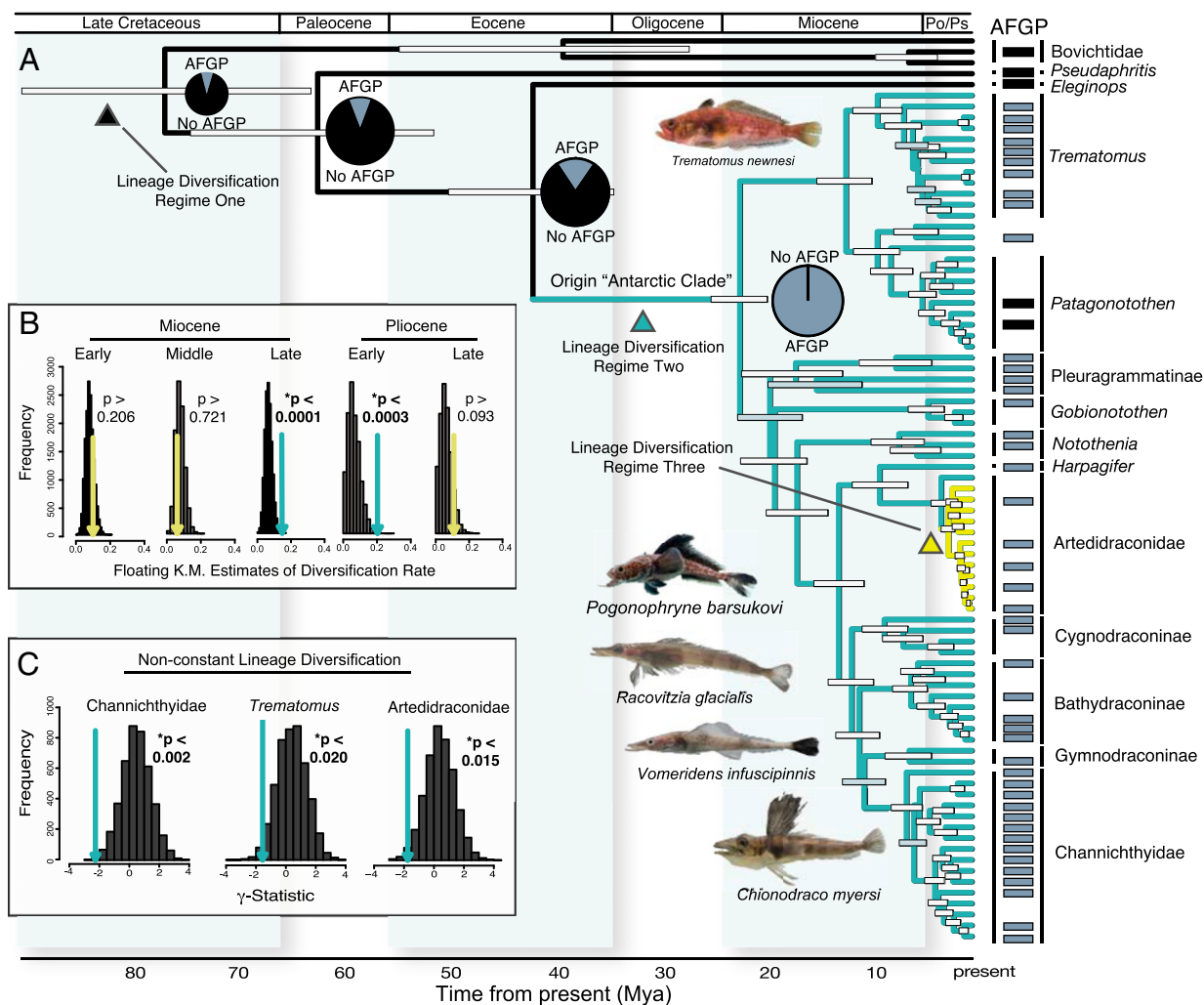
diversification should be intimately linked. This linkage is expected in a niche-filling model whereby lineage and character diversification occur early in a clade's history, and closely related species tend to be more similar in their traits and ecology (10, 23).

We investigated the tempo and mode of Antarctic notothenioid diversification through a set of comparative analyses that used a time-calibrated molecular phylogeny inferred from 83 notothenioid species (Table S1), and information on the paleoclimate of the Southern Ocean, the presence or absence of AFGP in notothenioid species, and field-collected measurements of buoyancy for more than 50 notothenioid species. The results of our analyses show that patterns of lineage and character diversification in notothenioids is more complex than the pattern expected from the conventional Oligocene-aged adaptive radiation scenario. We provide evidence that multiple constituent evolutionary radiations, coincident with more recent climate change events, more accurately characterize the evolutionary history of Antarctic notothenioids. Our results indicate that the evolution of AFGP predates the evolution of the exceptional morphological and ecological diversity in Antarctic notothenioids

by at least 10 million years, and that the origin of this radiation is more closely correlated with climate change since the Late Miocene.

## Results and Discussion

The use of Bayesian and maximum-likelihood methods resulted in strongly supported notothenioid molecular phylogenies (Fig. 1 and Fig. S1), and there was little difference between the phylogenies inferred from nuclear and mtDNA genes (Figs. S2 and S3). By using a Bayesian framework that relaxes the molecular clock (24), we obtained a mean posterior age estimate for the Antarctic clade at 22.4 Ma (Table S2), which corroborates estimates from previous studies (14, 15) and correlates with the Mi1 event (25), a time period of global cooling and ice sheet expansion in Antarctica. However, the mean posterior molecular age estimates for the most species-rich Antarctic notothenioid clades (e.g., *Trematomus*, 9.1 Ma; Channichthyidae, 6.3 Ma; and Artedidraconidae, 3.0 Ma) indicate most of the living diversity of notothenioids originated more than 10 million years after the origin of the Antarctic clade, coincident with the onset of



**Fig. 1.** Notothenioid chronogram with ancestral state reconstructions of AFGP and patterns of lineage diversification. (A) Bayesian inferred time tree of 83 notothenioid species. Bars at nodes represent 95% highest posterior density intervals of age estimates, with white bars showing posterior support 0.95 or greater. Branch colors and triangles correspond to the three best-fit lineage diversification regimes. Taxonomic classifications and presence (gray bar) or absence (black bar) of AFGP for notothenioid species are marked at the tips of the phylogeny. Posterior probabilities of Bayesian ancestral state reconstructions of AFGP origins are indicated by pie charts at key nodes in the notothenioid phylogeny. (B) Kendall–Moran (K.M.) estimates of lineage diversification contrasted with simulated distributions for specified geological time intervals. (C) The frequency distribution of simulated  $\gamma$ -values, with arrows marking the calculated  $\gamma$ -statistic for Channichthyidae, *Trematomus*, and Artedidraconidae.

intensified cooling in the Southern Ocean following the MMCT (Fig. 2). We examined the time-calibrated phylogeny for shifts in lineage diversification with a method that accounts for unsampled species and incrementally fits increasingly complex models of lineage diversification by using a stepwise information theoretic approach (26). Relative to the background diversification rate, our analyses identified three lineage diversification regimes that involve two shifts in lineage diversification rates, corresponding to the branches leading to the common ancestor of the Antarctic clade and to the common ancestor of Artedidraconidae exclusive of *Artedidraco skottsbergi* (Fig. 1 and Fig. S1).

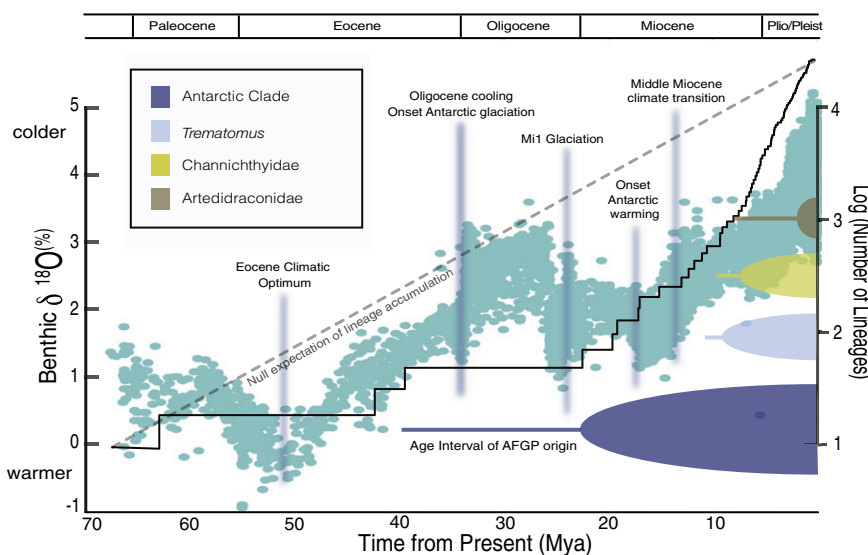
The shift in rate of lineage diversification leading to the Antarctic clade is consistent with the expectations of the AFGP innovation diversification hypothesis; however, this model-fitting approach may mask exceptional pulses of cladogenesis in younger lineages nested in the Antarctic clade. We tested for pulses of diversification that correspond to particular intervals of geologic time from the origin of the Antarctic clade at the Oligocene–Early Miocene boundary through the Pliocene and found that, contrary to the expectations from an AFGP innovation model of notothenioid adaptive radiation, there is an elevated pulse of lineage diversification that corresponds to an interval spanning the Late Miocene through Early Pliocene, and no pulses of diversification from the Early Miocene through the Middle Miocene (Fig. 1B). This temporal pulse of diversification is corroborated by the observation that patterns of lineage diversification within the Antarctic clade are more accurately characterized by pulses of lineage diversification within several nested and younger subclades that originated in the Late Miocene or Early Pliocene, subsequent to the warming period of the Middle Miocene climatic optimum (Fig. 1C and Table S3).

Although AFGP is essential for survival of these fishes, the results of the ancestral reconstruction analyses (Fig. 1A and Table S4) imply that the pulses of lineage diversification observed within derived clades of Antarctic notothenioids (e.g., *Trematomus* and Channichthyidae) are decoupled from the evolutionary origin of AFGP, which minimally dates to the most recent common ancestor (MRCA) of the Antarctic clade (Fig.

1). These combined results do not support the hypothesis that the key innovation of AFGP was the sole driver of the diversification of Antarctic notothenioids. Instead, the origin of the exceptional diversity in this clade is correlated with more recent periods of global climate change, some 10 million years after the origin of Antarctic clade (Fig. 2). Given that notothenioids are poorly represented in the fossil record (9), it is plausible that unobserved extinction has eroded the signature of an early rapid radiation as inferred from the paucity of branching events between the Oligocene cooling event and the MMCT (Fig. 2). Regardless, the results of our analyses provide strong evidence that the bulk of the phylogenetic and ecological diversity of living Antarctic notothenioids originated subsequent to the MMCT.

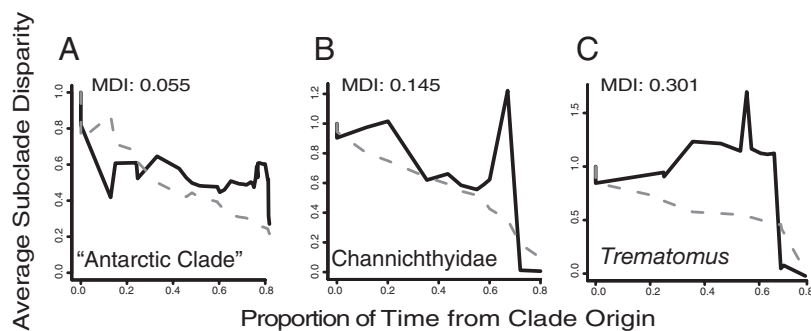
To test predictions of the AFGP innovation adaptive radiation model on ecomorphological diversification, we used field-collected buoyancy measurements from 54 species comprising more than 1,300 specimens (Table S5) in a series of disparity through time analyses (23). The global patterns of subclade disparity in the Antarctic clade, Channichthyidae, and *Trematomus* all resulted in positive mean disparity indices that are indicative of repeated colonization of benthic, epibenthic, semipelagic, and pelagic habitats among closely related lineages (Fig. 3A–C). The rapid lineage diversification within more derived Antarctic notothenioid subclades and the variation in buoyancy among closely related species are marked departures from adaptive radiation theory, which is typically characterized by a pronounced increase in rate of morphological and/or ecological disparity that decreases through time as niches are filled by diversifying lineages (23).

The unexpected pattern of adaptive radiation observed in Antarctic notothenioids may be explained by the appearance of the unique environmental and physical conditions of near-shore marine habitats in the Southern Ocean following the onset of widespread sea ice in the Late Miocene. Approximately 14 Ma ago, the MMCT created the polar conditions characteristic of contemporary Antarctica, intensifying the physical and thermal barriers that isolate the marine fauna of the Southern Ocean (18), initiating the repeated scouring of large sections of the



**Fig. 2.** Patterns of notothenioid lineage diversification and paleoclimate changes. Log-transformed accumulations of lineages through time are shown with a solid line. Expected pattern of lineage accumulations from a Yule pure-birth process is shown with a dashed line. Colored cones and lines match the crown and stem age estimates for the Antarctic clade, *Trematomus*, Channichthyidae, and Artedidraconidae. The interval reconstructed as the most probable evolutionary origin of AFGP is highlighted on the line depicting the stem phylogenetic lineage of the Antarctic clade. Blue filled circles represent oxygen isotope data for specific time periods taken with increased  $\delta^{18}\text{O}$  values corresponding to cooler climatic conditions (7). Major global paleoclimatic events since the Paleocene are highlighted with vertical blue-gray bars.





**Fig. 3.** Patterns of buoyancy disparity through time. Disparity in buoyancy through time is contrasted with simulations for (A) the entire Antarctic clade, (B) Channichthyidae, and (C) *Trematomus*. Mean disparity index (MDI) values were calculated omitting the most recent 20% of the chronogram. Solid lines are the observed patterns of buoyancy disparity, and dotted lines are the median value of the Brownian motion simulations.

continental shelf by glaciers, icebergs (27), and sometimes ice sheets advancing as far as the shelf break (28). This ice activity resulted in periodic extinction of the near-shore benthic fauna (29), allowing for subsequent colonization of previously occupied niche space, as well as creating opportunities for geographic isolation and speciation. The ecological opportunities resulting from repeated creation of open niches through extinction of potential competitors, over substantial expanses of geologic time, could result in the evolution of the unusual pattern of substantial morphological and ecological disparity within Antarctic notothenioid subclades.

Our results highlight the dependence of the primary component of the contemporary Antarctic fish fauna on ecological opportunity associated with historical global climate change and the persistence of subzero polar conditions. We find that the origin of AFGP, despite its being essential for survival in subzero marine habitats, did not alone drive the exceptional diversity of extant Antarctic notothenioids. In contrast, this major component of the Southern Ocean's marine biota has been shaped through evolutionary time by a combination of freeze avoidance offered by AFGP, and abiotic factors initiated by paleoclimatic changes that resulted in continuous ecological divergence into recurrently opening niches. In a tragic twist of fate, the development of polar climatic conditions that shaped the radiation of Antarctic notothenioids is now reversing, and the increasing temperature of the Southern Ocean (1), with the associated potential for the arrival of invasive species and disruption of food webs (3, 6, 30), is the greatest threat to the survival of this unparalleled radiation of teleosts.

## Materials and Methods

**Phylogenetic Data and Analyses.** Standard phenol-chloroform extraction protocol or Qiagen DNeasy Blood and Tissue kits were used to isolate DNA from tissue biopsies sampled from 83 notothenioid species (Table S1). Previously published PCR primers were used to amplify and sequence two mtDNA genes (*nd2* and *16S* rRNA) and five nuclear gene regions (*RPS71*, *myh6*, *sh3px3*, *tbr1*, and *zic1*) that consisted of four unlinked exons and a single intron (31–34). Protein coding gene regions (*nd2*, *myh6*, *sh3px3*, *tbr1*, and *zic1*) were aligned by using the computer program MUSCLE (35) and refined by eye using the inferred amino acid sequences. No frame mutations or DNA substitutions that resulted in stop codons were observed in the aligned sequences. The noncoding genes (*RPS71* and *16S* rRNA) were aligned by using the computer program MUSCLE. The combined seven-gene dataset contained 6,431 bp, and 98.9% of data matrix (taxa and genes) was complete.

Nine data partitions were designated that corresponded to the three separate codon positions for the mtDNA protein coding gene, a single partition for the mtDNA *16S* rRNA, and a single partition for each of the five nuclear genes. Potential partitioning strategies included a single partition for each protein coding gene vs. three codon positions in each of these genes and were assessed by using Bayes factor comparisons of the posterior harmonic mean of the maximum likelihood score from Bayesian phylogenetic analyses.

A GTR+G substitution model was used in a partitioned maximum likelihood analysis by using the computer program RAxML 7.2.6 (36) run with the  $-D$  option. Support for nodes in the RAxML inferred tree was assessed by using a thorough bootstrap analysis (option  $-f$  i) with 2,000 replicates.

**Relaxed Molecular Clock Analyses.** Divergence times of notothenioids were estimated by using an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity implemented in the computer program BEAST version 1.6.1 (24, 37). The seven-gene dataset was partitioned as in the maximum likelihood phylogenetic analysis described earlier, unlinking the UCLN clock and nucleotide substitution models across partitions. Based on the results of a previous UCLN analysis (15), age priors with a normal distribution were applied to four nodes in the notothenioid phylogeny, which included the MRCA of all notothenioids (mean, 71.4; SD, 11.0), the MRCA of *Pseudaphritis urvillii* and all other notothenioids (mean, 63.0; SD, 10.4), the MRCA of *Eleginops maclovinus* and the Antarctic clade (mean, 42.9; SD, 8.0), and the MRCA of the Antarctic clade (mean, 23.8; SD, 1.5). A birth–death speciation prior was used for branching rates in the phylogeny. The BEAST analyses were run five times with each run consisting of  $3.0 \times 10^7$  generations, sampling at every 1,000 generations. The resulting trees and log files from each of the five runs were combined by using the computer program LogCombiner version 1.6.1 (<http://beast.bio.ed.ac.uk/LogCombiner>). Convergence of model parameter values and estimated node heights to their optimal posterior distributions was assessed by plotting the marginal posterior probabilities versus the generation state in the computer program Tracer version 1.5 (<http://beast.bio.ed.ac.uk/Tracer>). Effective sample size values were calculated for each parameter to ensure adequate mixing of the Markov Chain Monte Carlo (MCMC; effective sample size > 200). The posterior probability density of the combined tree and log files was summarized as a maximum clade credibility tree using TreeAnnotator version 1.6.1 (<http://beast.bio.ed.ac.uk/TreeAnnotator>).

**Assessing Patterns of Lineage Diversification.** Diversification rate analyses of notothenioids were performed by using the APE (38), GEIGER (39), and LASER (40, 41) software packages in R. Patterns of lineage accumulation through time were visualized by using a lineage-through-time plot (42). To determine if any notothenioid subclades exhibit departures from a global background rate of lineage diversification, we used MEDUSA, a step-wise Akaike Information Criterion (AIC) approach that incrementally fits increasingly complex models of lineage diversification to a time-calibrated phylogeny (26). This method initially calculates the AIC fit of a two-parameter birth–death model of cladogenesis to the time-calibrated phylogeny and compares this score with the AIC score of a more complex five-parameter model in which two birth rates, two death rates, and an optimal shift point on the phylogeny is estimated. By using an AIC threshold of four to denote a substantial improvement in model fit by the more complex model (43), the step-wise function repeats this model selection process by retaining the more complex parameter rich model and comparing its fit to a model that includes an additional birth, death, and shift-point parameter. This iterative model-building process continues until the addition of new parameters no longer offers an improvement in AIC score. To account for the influence of incomplete taxon sampling on the birth, death, and shift-point parameter estimates, MEDUSA employs a “diversity tree” as its framework. Briefly, a diversity tree is constructed by collapsing lineages with missing taxa and assigning a species richness value to these resulting stem lineages. For clades with complete taxon sampling, tip taxa are not pruned, and

instead assigned a diversity of one (44). Because we sampled more than 75% of the notothenioid species diversity, we assigned most taxa a species richness value of one. Following a list of notothenioid species (45), we collapsed *Harpagifer* to include six species, *Pleuragramma* plus *Aethotaxis* to include three species to account for not sampling *Gvozdarus svetovidovi*, *Notothenia* to include five species as *Notothenia microlepidota* and *Paranotothenia magellanica* were not sampled, *Patagonotothen* to include 14 species, *Psilodraco* to include two species to reflect the lack of *Acanthodraco dewitti* from our analysis, *Bovichtus* to include nine species, and *Trematomus newnesi* to include two species to account for not sampling *Cryothernia*.

To determine if lineage diversification dynamics within notothenioids are best modeled by a single shift point in rates or if inferred shifts in diversification rates were driven by pulses of cladogenesis corresponding to specific time intervals, we calculated floating-point Kendall–Moran estimates of diversification rate for each subdivision of the Miocene and Pliocene, and the Late Miocene and Early Pliocene combined (46). Time interval-specific diversification rate estimates were compared with null distributions generated by simulating 10,000 birth–death trees by using maximum-likelihood values of speciation and extinction. Simulated phylogenies were generated to capture the extant taxonomic diversity of notothenioids, and then resampled to reflect the taxon sampling in the molecular time-calibrated phylogenies.

We investigated if lineage diversification in the major species-rich Antarctic notothenioid subclades was constant over time by assessing the significance of the  $\gamma$ -statistic by using the Monte Carlo constant rates test (MCCR) (47). We compared observed values of  $\gamma$  to a null distribution of 10,000 pure-birth trees simulated under the global estimates of speciation ( $\lambda_G$ ) and extinction ( $\mu_G$ ) that were calculated using a method of moments estimator (48). As the MCCR test is prone to high type I error rates if the initial taxon sampling strategy was nonrandom (49), we repeated all MCCR analyses by using the proportionately older splits simulation protocol that assumes that nonsampled lineages are more likely to be younger (50). All MCCR tests were repeated to incorporate the credible range of maximum likelihood estimates of  $\lambda_G$  and  $\mu_G$ .

**Estimation of AFGP Ancestral States.** We compiled a dataset from the literature on the presence (scored as 1) and absence (scored as 0) of AFGP for 47 notothenioid species (Fig. 1 and Table S4) based on genomic Southern blots (51, 52), freezing-point depression of body fluids (53–55), or isolation of the AFGP (56). Species for which there is no information on the presence of AFGP were pruned from the posterior set of Bayesian-inferred time-calibrated phylogenies (Fig. 1). Ancestral character states for AFGP were estimated by using the modified posterior distribution of trees in the computer program BayesTraits that incorporates a reversible-jump MCMC (RJ-MCMC) with an exponential hyperprior on the rate coefficients to integrate over the uncertainty present in the fit of models of character evolution to the data (57, 58). We ran five replicate sets of BayesTraits analyses with the RJ-MCMC

sampling for 200 million generations, sampling every 2,000 and discarding the first 25% of the runs as burn-in. Log files were processed by using custom-written scripts in R to visualize the posterior distribution of the RJ-MCMC-inferred ancestral reconstructions of AFGP presence or absence.

**Patterns of Disparity in Buoyancy.** Field captured specimens were heavily anesthetized with tricaine methanesulfonate and weighed in seawater at ambient temperatures and weighed in air (11). Buoyancy (%B) was expressed as the percentage of the weight in air ( $W_{Air}$ ) supported in water ( $W_{Water}$ ) as follows (Table S5):

$$\%B = W_{Water}/W_{Air} \times 10^2 \quad [1]$$

Most buoyancy measurements were made during Southern Ocean expeditions in 2001, 2003, 2004, 2006, and 2009; however, some were presented in earlier publications (11, 59, 60). We tested the hypothesis that notothenioid lineages have partitioned more disparity in buoyancy between, rather than within, subclades by calculating the average relative subclade disparity through time of the buoyancy data for the notothenioid Antarctic clade and the major Antarctic subclades (23). In each disparity-through-time analysis, we assessed whether the observed morphological disparity index (MDI) differed from a null model of Brownian evolution by calculating the empirical variance and simulating the evolution of buoyancy on the time-calibrated notothenioid phylogeny 1,000 times by using the Geiger package of computer programs for R (39). Negative MDI values indicate that more morphological disparity is distributed between subclades whereas positive values indicate that subclades have converged on a pattern whereby overall morphological disparity is partitioned among members within each subclade. As incomplete sampling can influence the calculation of the MDI, we restricted our analysis of MDI values to the first 80% of the time-calibrated phylogeny.

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