Historical contingency shapes adaptive radiation in Antarctic fishes

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Adaptive radiation illustrates links between ecological opportunity, natural selection and the generation of biodiversity. Central to adaptive radiation is the association between a diversifying lineage and the evolution of phenotypic variation that facilitates the use of new environments or resources. However, it is not clear whether adaptive evolution or historical contingency is more important for the origin of key phenotypic traits in adaptive radiation. Here we use targeted sequencing of >250,000 loci across 46 species to examine hypotheses concerning the origin and diversification of key traits in the adaptive radiation of Antarctic notothenioid fishes. Contrary to expectations of adaptive evolution, we show that notothenioids experienced a punctuated burst of genomic diversification and evolved key skeletal modifications before the onset of polar conditions in the Southern Ocean. We show that diversifying selection in pathways associated with human skeletal dysplasias facilitates ecologically important variation in buoyancy among Antarctic notothenioid species, and demonstrate the sufficiency of altered trip11, col1a2 and col1a1a function in zebrafish (Danio rerio) to phenocopy skeletal reduction in Antarctic notothenioids. Rather than adaptation being driven by the cooling of the Antarctic, our results highlight the role of historical contingency in shaping the adaptive radiation of notothenioids. Understanding the historical and environmental context for the origin of key traits in adaptive radiations extends beyond reconstructing events that result in evolutionary innovation, as it also provides a context in forecasting the effects of climate change on the stability and evolvability of natural populations.

During adaptive radiation, ecological opportunity and key phenotypic traits interact to facilitate the expansion of populations into new niches. Deciphering the genotype–phenotype relationships of these key traits provides important insight into the historical circumstances that result in phenotypic diversification. Advances in sequencing capabilities now allow for the efficient collection of genomic sequence data for scores of species. This potential for increased taxonomic, genome-wide sampling provides opportunities to investigate the macro-evolutionary mechanisms driving evolution across clades. For example, a major question in the study of adaptive radiation is the relative role of trait novelty versus the modification of existing phenotypes in facilitating the diversification of species. Ecological opportunities, such as those brought about by environmental changes, can promote the origin of novel phenotypes that accelerate lineage diversification. However, trait novelty may arise at any time and adaptive change can result from a shift in selection regimes that enable lineages to opportunistically explore new ecospace using a trait that had previously evolved under different circumstances. These two hypotheses have long been central to the debate concerning the role of punctuated versus gradualistic evolution in the generation of biodiversity, and posit opposing views on how the genomic substrate of subsequent phenotypic diversification evolved. Here, we provide a genomic perspective of ecologically important trait variation and phylogenetic origin in a species-rich adaptive radiation. Through consideration of closely related lineages that are not a part of the adaptive radiation, we can specifically investigate the relative importance of contingency in adaptive radiation and test whether the genomic basis of ecological trait variation coincides with or precedes the onset of radiation.

Results and discussion
To test hypotheses concerning the origin of traits that facilitate adaptive radiation, we focused on Antarctic notothenioids (Cryonotothenioidea), an iconic example of adaptive radiation in marine vertebrates. The diversification of cryonotothenioids followed the progressive cooling of Antarctica that initiated ~33 million years ago, and was coincident with the extinction of a phylogenetically diverse and cosmopolitan fish fauna. This combination of climate change and vacated niches presented notothenioids with the opportunity to diversify into a large range of benthic and water column habitats. All notothenioids lack a swim bladder, the primary buoyancy organ in most teleost fishes; however, there are substantial differences in buoyancy among notothenioid species that are correlated with habitat and resource use. These differences in buoyancy are achieved through the reduction of skeletal density coupled with the accumulation of corporeal lipids that provide static lift. In the context of early-diverging non-Antarctic notothenioid lineages, these ecologically important traits are hypothesized to have arisen during the onset of polar conditions; however, the evolutionary origin of reduced skeletal density in the context of early-diverging non-Antarctic notothenioid lineages challenges this scenario.

To develop a comprehensive phylogenomic perspective on the Antarctic notothenioid adaptive radiation, we employed a
cross-species targeted sequence enrichment approach to specifically sequence conserved and functionally annotated genetic loci. We combined sequence information from the genome of *Notothenia coriiceps* and other percomorph species to design a comparative DNA probe set for systematic targeted enrichment of ~250,000 coding and conserved non-coding elements (CNEs) comprising over 40 Mb of genomic sequence (Supplementary Fig. 1). CNEs were defined as microRNA (miRNA) hairpins, orthologues to human ultraconservative elements and constrained genetic regions identified in the Ensembl compara 11-way teleost genome alignment that did not overlap with protein-coding regions. Using this probe set, we captured coding and non-coding sequences from...
phylogenetically rich sampling of 46 notothenioid species that includes all three early-diverging non-Antarctic lineages and two species from the closely related Percidae (Supplementary Fig. 1). Importantly, we used pooled-population samples of individual species to permit identification of fixed and variable single-nucleotide polymorphisms (SNPs) for each species.

We achieved 88–95% coverage of targeted regions for each sampled species (Supplementary Fig. 1 and Supplementary Tables 1 and 2). This permitted identification of an average of 95,000 fixed species-specific SNPs and 60,000 heterozygous SNPs for each lineage (Supplementary Table 3). As proof of the sensitivity of the dataset, we confirmed known cases of adult haemoglobin gene loss within the icefishes (Supplementary Fig. 2) 16. The power of this dataset was further illustrated by identification of a previously uncharacterized deletion of two putative embryonic haemoglobin genes (Supplementary Fig. 2). Thus, this approach provides a robust and efficient method by which to characterize variation across taxonomically rich clades, even when separated by major evolutionary distances.

Given expectations from the adaptive radiation of East African cichlids 17, we tested whether shifts in the rate of nucleotide evolution would correspond to the onset of rapid lineage diversification in the cryonotothenioid radiation. Using a time-calibrated phylogeny of most living notothenioids and a Bayesian framework to assess shifts in speciation rates 11, 18, we confirmed a shift in lineage diversification at the origin of the antifreeze-bearing Antarctic cryonotothenioids and an additional acceleration in diversification within the Plunderfishes (Artedidraconidae) 10 (Fig. 1a). Intriguingly, these shifts do not correspond to accelerated rates of nucleotide evolution. Instead, we found that the majority of extant cryonotothenioid sequence diversity is derived from a period of very high rates of genomic evolution that occurred in the ancestral lineage that includes the most recent common ancestor of the non-Antarctic distributed Eleginops maclovinus and the Antarctic cryonotothenioids (Eleginopsioidea; Fig. 1b and Supplementary Table 4). This demonstrates that a major change in molecular rate variation preceded the onset of global cooling and adaptive radiation of cryonotothenioids by well over ten million years (Fig. 1c). An assessment of the accumulation of genomic divergence through relaxed molecular clock models (Fig. 1d), along with an analysis of synonymous (dS) and non-synonymous substitution (dN) rates (Fig. 1e), further substantiates a high rate of nucleotide evolution before the origin and diversification of cryonotothenioids. This acceleration of overall genomic change may have been critical for accumulation of the genetic diversity that provided the substrate for phenotypic diversification during the early radiation of cryonotothenioids.

As buoyancy adaptations are key traits that facilitated notothenioid diversification into the water column during the adaptive radiation, we assessed patterns of skeletal density throughout the phylogeny using computerized tomography (Fig. 2a and

**Fig. 2 | Skeletal reduction occurs before the cryonotothenioid radiation.** a, Computed tomography of notothenioid skulls showing relative skeletal density across the phylogeny and decrease in skeletal density before cryonotothenioid radiation. CT, computed tomography. b, Comparison of significance of enrichment for polygenic selection on representative bone density-associated HPO terms between Eleginopsioidea and well-ossified sister group P. urvilli. Dashed line indicates false discovery rate $q < 0.05$ for SUMSTAT gene set enrichment test.
Supplementary Fig. 3). We find that, in parallel with the observed increase in nucleotide substitution rate, a broad reduction in bone density occurred before the recent common ancestor of *E. maclouvinus* and the Antarctic cryonotothenioids (Eleginopsioidea, Fig. 2a) and was retained throughout the clade. Consistent with these decreases in bone density, we found biased diversifying selection in genes associated with human skeletal dysplasias and mineralization defects in Eleginopsioidea relative to the well-ossified sister lineage, *Pseudaphritis urvillii* (Fig. 2b,c). This evolutionary reconstruction is contrary to the expectation of the emergence of novel traits temporally coinciding with the onset of adaptive radiation and changing ecological opportunities. Instead, our results indicate that, before the shift to polar conditions, notothenioid lineages possessed key traits and may have begun to use new habitats in the water column through a modification of buoyancy enabled through a derived reduction in bone density.

To further explore the genetic basis of the evolved reduction in bone density, we assessed patterns of diversifying selection within the phylogeny. Among *E. maclouvinus* and all cryonotothenioids, we identified shared selective signatures in clinically relevant skeletal genes, such as *collagen1a1a* and *collagen1a2* (Fig. 3a, Supplementary Figs. 4–6 and Supplementary Table 5). The function of these genes is conserved among disparate lineages of vertebrates, and non-synonymous mutations in these collagens can lead to severe osteogenesis imperfecta in humans. Notably, previous studies show that *collagen1* expression is reduced in the developing skeleton of notothenioid embryos, providing further evidence that broad changes at *collagen loci* are associated with skeletal variation. In addition to selection on collagens, we identified diversifying selection in an unlikely gene candidate for skeletal constraint in the types of mutations in evolution that can drive changes in the cranium caused by mutations in *colla1admh14* and Supplementary Table 5). This gene is conserved across eukaryotes and functions in vesicle tethering in the cis-Golgi membrane. In humans, mutations in *TRIP11* lead to severe skeletal deficiencies that then result in perinatal lethality. Intriguingly, both *collagen1a1a* and *trip11* are also under diversifying selection and/or accelerated sequence evolution within the further diversification of the Channichthyidae, which have evolved an additional and major reduction in bone density (Figs. 2a and 3a, Supplementary Figs. 4–6 and Supplementary Tables 5–8). This parallelism suggests constraint in the types of mutations in evolution that can drive changes in skeletal density while maintaining viability.

To experimentally test the potential for alterations in *trip11*, *colla1a* and *colla2* function to impart non-lethal skeletal phenotypes that are consistent with those observed in notothenioids, we analysed mutants stemming from genetic screens and
CRISPR-Cas9 gene editing in *D. rerio*. Contrary to expectations from humans, zebrafish homozygous for loss-of-function alleles of *trip11* were viable with no obvious external morphological abnormalities (Fig. 3b,c). However, whereas heterozygous siblings had density patterns comparable to wild-type (WT) zebrafish, similarly sized and age-matched homozygous *trip11* mutants had significantly reduced skeletal density (Fig. 3b,c). In affected individuals, all bones investigated, including those of the skull roof, vertebrae and operculum, were reduced in density (Fig. 3b,c) and generally phenocopied the pattern observed in adult *E. maclovinus* and crynotothenioids (Fig. 2a). Notably, expressivity of the *trip11* skeletal phenotype was variable, which suggests the presence of background genetic modifiers. Similarly, we show that mutant models of *colla1α* (G1144E) and *colla2* (G882N) in zebrafish also cause a reduction in bone density (Fig. 3c)19. These experiments confirm that changes to the loci under selection yield phenotypes consistent with those observed in *E. maclovinus* and species of the crynotothenioid radiation.

Our results reveal that historical contingency was a major factor in shaping the adaptive radiation of notothenioids. Because the onset of polar conditions ~33 Ma (million years ago) decimated the teleost fauna of the Southern Ocean, notothenioids were the only surviving lineage poised to occupy newly open niche space in a range of benthic and water column habitats24. Rather than being driven by directional selection to evolve extreme phenotypes in response to the onset of polar conditions23–25, the genomic substrate for reduced ossifications and buoyancy modifications had long been established, and was selected upon to facilitate the ecological diversification that characterizes the notothenioid adaptive radiation. These results provide an alternative view on the impact of climate change in driving extreme adaptations in the Southern Ocean23–28. As we march further into the Anthropocene, our results caution that as both ecosystems and climate continue to change worldwide, the expectation that rapid adaptation will be the dominant factor in predicting the response of biodiversity requires careful consideration29.

**Methods**

Custom targeted sequence enrichment design. We based the design of the DNA enrichment baits primarily on the *N. coriiceps* genome, the species most closely related to those targeted for sequencing with a published reference assembly30. To account for regions that are unannotated, under drift, or not easily identified within the *N. coriiceps* assembly, we included targeted regions from several outgroup genomes as detailed below. By having the same element potentially represented by more than one genome, this strategy allowed us to mitigate against genome assembly and annotation artefacts while facilitating hybridization of diverse species to the capture probes. Elements were identified in the *N. coriiceps* genome using BLASTN (ncbi-blast-2.2.30+; parameters ‘-max_target_seqs 1 -outfmt 6’). If the BLASTN hit had an E-value <0.001 and covered >80% of the query sequence, we included this region from the *N. coriiceps* genome. If the region was not identified, or had <85% identity in *N. coriiceps*, we retained the version from the genome of origin.

Coding exons were identified from annotations of the *N. coriiceps* stickleback (*Gasterosteus aculeatus*; BROADS1) and European sea bass (*Dicentrarchus labrax*) genomes31. CNEs were defined from the constrained elements identified in the stickleback and tilapia (*Oreochromis niloticus*; Oreinil1.0) genomes from the Ensembl compara 11-way teleost whole-genome alignment32. We also included predicted miRNA hairpins from miRbase33 and ultracconservative non-coding (UCNE) elements from UCNEbase34. miRNA hairpins were padded to be >100 bp. CNEs, miRNAs and UCNEs that overlapped coding exons were removed using Bedtools (v.2.26.0) intersectBed35. CNES ≤100 bp were additionally excluded to facilitate space in the sequence capture design. Where the constrained regions that defined the CNEs overlapped with annotations pertaining to specific miRNAs and UCNEs, the latter annotations were prioritized.

Targeted elements were submitted to Nimblegen for final probe design, and the manufacturing of a Nimblegen SeqCap EZ Developer Library (Roche, No. 06716840101) had 63,838,670 bp of capture space targeting 318,929 elements from four reference genomes (88.9% *N. coriiceps*, 7.7% *G. aculeatus*, 3.0% *D. labrax*, 0.4% *O. nicoleatus*). Accounting for redundancy of orthologous target regions between the genomes, the final design targeted 258,176 unique elements of which 206,303 were predicted-protein-coding exons and 51,673 constrained non-coding regions, with 85.0% coverage of targets not found in the *N. coriiceps* reference genome (47,097 elements; Supplementary Table 2).

Sample preparation and sequencing. Frozen tissue samples were acquired from the HWD and TJN laboratories, and from the Yale Peabody Museum frozen tissue collection (Supplementary Table 9). DNA from each species was isolated using Qiagen DNeasy Blood and Tissue kits, sequencing multiple individuals per species to account for population variation. For each species, equal amounts of DNA from each individual were pooled before sequencing and library preparation. The pooled-population DNA was then sheared to an average size of 200 bp using the Covaris E220 ultrasonicator in 130 µl Covaris microTUBEis (duty cycle, 10%; intensity, 5; cycles/burst, 200; time, 300 s; temperature, 4°C). Shearing was performed in shearing buffer: 10 mM Tris, 0.1 mM EDTA, pH 8.3.

Targeted sequence enrichment and next generation sequencing. Sequencing libraries were constructed from 1µg of DNA using a KAPA Library Prep kit (Roche, No. 07137929001), following standard protocol with barcoding and dual-SPRID (solid phase reversible immobilisation) size selection to generate libraries of 200–450 bp. Sequencing libraries were hybridized, recovered and amplified following the standard protocol of the SeqCap EZ Library SR User Guide v.4.3 with the following changes: hybridization was performed at 45 rather than 47 °C, to allow for more mismatches between sequencing libraries and probes, and we used SeqCap Developer Reagent (Roche, No. 06684335001) rather than Human Cot DNA to block non-specific hybridization as recommended by the protocol. For each species, the majority of the capture probes were designed based on the sequence of *N. coriiceps*, we hybridized species in groups to limit potential competition between sequencing libraries of varied relatedness to the capture baits. Captured libraries were pooled for 100-bp, single-end sequencing using Illumina HiSeq 2500. We targeted multiplexing of eight to nine species per HiSeq 2500 flow cell, totalling six flow cells.

Reference contig assembly. The contig assembly approach is modified from the previously defined Phylomapping pipeline for cross-species targeted sequence enrichment datasets31. Briefly, sequencing reads are grouped into bins by homology to a targeted element (for example, exon, CNE) and then assembled into contigs de novo (Supplementary Fig. 7).

Processing of sequencing reads. Before contig assembly, low-quality bases within sequencing reads were masked using the FASTX-Toolkit36. Identical sequences were then trimmed using Trimmomatic v.0.36 (ref. 36). Identical sequences were then trimmed using Trimmomatic v.0.36 (ref. 36). FASTQ files were collapsed using the FASTX-Toolkit v.0.0.13 (fastx_collapser; parameters ‘-Q 33’).

Read binning into orthology groups by BLAST. Reads were grouped by homology before contig assembly, using both blastn and dc-megablast (v.2.6.0+; parameters ‘-max_target_seqs 2 -outfmt 6’). This dual-BLAST approach accounts for variation between sequencing reads and the reference genome from which the sequencing baits were defined31. As short target exons and CNEs can produce disproportionately small blastn E values, we used an adaptive E value cut-off based on the size of the target region. For target regions >25 bp, the E value cut-off was ≤1 x 10–5; for targets that were ≤25 bp the E value cut-off was ≤1 x 10–10. Reads were further excluded if a substantial portion of the read (>10 bp) overlapped the target interval without being included in a blastn hit. The best resulting E value from either blastn or dc-megablast was selected, with the former selected in the event of a tie. As dc-megablast uses a mismatch-tolerant seed template, inclusion of dc-megablast resulted in the additional recovery of 30,000–50,000 sequencing reads per species (out of an average of 20,000,000 with total blastn hits) and the assembly of 50–150 more target regions than would be assembled by blastn alone.

*De novo* contig assembly. CAP3 was used to assemble contigs de novo from the bins of reads that have high homology to specific target regions (that is, the same exon, CNE and so on) that were identified by BLAST30. Reads were reverse-complementated if necessary to put everything into the same complement strand as the target region from the reference genome. To accelerate CAP3 assembly, overlapping reads were first merged into smaller contigs using Usearch and then mixed with original reads as input for CAP3 (parameters ‘-o 0.97 -fastq_madhit 3 -fastq_minovlen 5’). For CAP3 assembly, we required a minimum read overlap of 16bp and 96% identity between reads during contig assembly (parameters ‘-o 16 -p 96’). This cut-off has an effect of separating the reads stemming from duplication events into separate contigs, providing there is ≥4–6% variance between the parent sequences.

We simulated the ability of this pipeline to distinguish copy number variants (Supplementary Fig. 8a), generating a 300-bp random DNA sequence in silico and making a second copy of this sequence with specific levels of variation from the original. Sequencing reads (100bp) were then generated in silico at a depth of one read every five base pairs. Reads were run through the assembly pipeline to assess whether the original DNA sequences were reconstructed from the read data.
or whether the reads formed a chimeric sequence. This simulation was repeated 250 times. The current assembly approach was able to reassemble the individual paralogues where there was >6% divergence between original paralogous sequences, with inconsistent results at <5% divergence (Supplementary Fig. 8b).

Contig merging and filtering. Sequencing reads were aligned to the assembled contigs using NextGenMap (v.0.5.5; parameters ’-r 40’). This alignment step allows for the recruitment of new reads to the contig that may not have previously been identified by blast due to either high degrees of variance relative to the reference blast database, large indels or low amounts of overlapping sequence with the target region. This allows elongation of the contig to include more of the flanking regions surrounding each target element. Reads were removed from the alignment to the contig if there were more than three mismatches with the exception of indels. To refine and extend the boundaries of the original contig, a second de novo assembly by CAP3 (parameters ’-o 20 -p 85’) was performed using the aligned reads.

Multiple contigs were present in around 65% of target regions after CAP3 assembly. To remove misidentified contigs, we used blastn to compare each contig to the reference genome, removing those whose top hit did not match the original bin from which the reads were assembled. To correct for potential assembly artefacts, the multiple contigs that represent each target were compared to each other and the reference sequence in a multiple sequence alignment using Mafft v.7.313 (ref. [44]) (parameters ’–maxiterate 1000 –localpair’), adding contigs as fragments (parameters ’–addfragments’). Using the read support at each base in the alignment, we generated a consensus contig for each target region. A mismatch between contigs was considered if the read support for the most common base was <80% of all bases present. Contigs were merged only if there were less than 3 mismatches, if the sequence identity between the contigs was >95% or if the contigs did not overlap within the target region. After this refinement step, <2% of target regions were represented by multiple contigs.

Comparison of assembled contigs to reference genome. In comparing our reference exome sequence assembly to the published N. coriceps reference genome, we found 99.8% average percentage identity of exome sequence to the reference target (Supplementary Fig. 9), which included 98.5% of exons in the N. coriceps reference genome (Supplementary Fig. 9). The small differences in sequence identity and putative copy-number variants (CNVs) between this exome and the reference genome (Supplementary Fig. 9). The small differences in sequence identity and putative copy-number variants (CNVs) between this exome and the reference genome (Supplementary Fig. 9). The small differences in sequence identity and putative copy-number variants (CNVs) between this exome and the reference genome (Supplementary Fig. 9).

Estimation of read coverage and depth of targeted regions. Coverage was estimated using REDtools (v.2.23.0) [45]. Reads were first aligned to the assembled contigs with NextGenMap. The coordinates of the read alignments were then lifted to the corresponding position on the reference genome using information from a pairwise sequence alignment between the contig and the orthologous region on the reference genome. Pairwise alignments were performed using Biopython v.1.70 (parameters ’pairwise2; match = 5, mismatch = –4, gap_open = –15, gap_extend = –1’). Alignments were converted to BAM files, sorted and indexed using SAMTools v.1.9 (ref. [46]). Reads alignments were manually inspected in the Integrative Genome Viewer to verify accurate read alignment. Coverage is defined as the percentage of targeted bases in the primary reference genome having at least one read. Depth was estimated using coverageBed (parameters ’-d’).

Distribution of read coverage across the dataset. Most target regions had either 0 or 100% coverage (Supplementary Fig. 10). Although the average depth is similar in the notothenioids compared to the outgroups, there is a wider distribution of depths in outgroup species (Supplementary Fig. 10). Despite global coverage being ≥85% in all species, gene classes associated with the immune system, cell adhesion proteins and extracellular matrix were enriched among regions with relatively poor coverage (<25% coverage in >75% of exons; Supplementary Table 10). This suggests that these fast-evolving gene classes are less likely to be highly represented in these datasets, and is similar to previous findings with cross-species-targeted DNA enrichment [47].

Recovery of population variation. To determine the approach for recovering population variation, we looked for heterozygous SNPs within the targeted regions of the dataset. Sequencing reads were aligned to the reference contig for each species using NextGenMap [48]. SAM files were converted to BAM files using SAMTools v.1.9 (ref. [49]). VarScan were called using SAMTools mpileup and BCTools v.1.9 (parameters ’call –mv’). We considered sites heterozygous in our small population samples if there were at least two reads showing the variant in at least 25% allele frequency within the sequencing reads.

Treatment of exons with a predicted history of duplication. We assembled a single exon/CNE copy for the majority of targets that were directly compared between species. However, less than 3% of targeted regions on average had assembled more than one contig per target region after assembly. For duplicated regions, both exon versions were ignored for that particular species in downstream analyses involving multiple groups, unless those analyses ask specific questions involving copy number.

Identification of orthologous sequences. For orthologue pairing, the contigs generated from the same reference target region were aligned using Mafft v.7.313 (parameters ’–op 10–op 10’), with a maximum likelihood tree topology estimated IQTree [44]. Gene trees were reconciled with the species tree using Notung 2.9 (ref. [50]) (parameters ’–reconcile–tarearrange–silent–threshold 90%–treeoutput nix’) to infer patterns of duplication and loss. The total number of duplication and loss events inferred by Notung was then summed and compared to a null scenario where all copies are local duplicates. If Notung inferred fewer gain/loss events, the duplicate exons were paired based on the reconciled gene tree.

Simulation of orthologue identification approach. To estimate the ability of this approach to parse copy number variation into orthologous groups, we performed a series of simulations (Supplementary Fig. 11a) using a random DNA sequence generated in silico. This ancestral DNA sequence was duplicated, and mutations were added at defined levels to distinguish each parologue. Both paralogues were then evolved according to a specified phylogeny, with variation added to each parologue in increments at each branch point. We varied copy number, length of contig sequence and simulated local losses of an individual parologue within downstream lineages. Each simulation was repeated 250 times. These results suggest that, providing there is 2–4% variation between paralogous sequences, the approach can properly pair orthologous sequences (Supplementary Fig. 11b–d). The approach also compares well with the thresholds at which our pipeline can distinguish copy number variants during contig assembly (see above), meaning that there will not be parologue sequences with <4% divergence in the dataset.

Multiple sequence alignment. All paired orthologous sequences were aligned using Mafft v.7.313 (parameters ’–maxiterate 1000 –localpair’). For coding regions that had out-of-frame or frameshift-causing indels, these alignments were then refined into codon alignments using the frameshift-aware multiple sequence aligner MACv2.03 (parameters ’–prog alignSequences -seq -seq_lr –fs_lr 10 –stop_lr 15’). The multiple sequence alignment was pruned using GUIDANCE v.2.02 to mask residues with score ≤0.6 (parameters ’–bootstrap 25 –mafﬁx-maxiterate 100 –localpair –op 10–op 10’).

Reconstruction of gene sequences from exon data. Single-copy coding exons with orthology to G. aculeatus were concatenated into gene sequences using the alignments of the G. aculeatus genome. The exons for each gene were spliced together in the same order in which they appear in the genome on the strand containing the gene. Transcript isoforms were merged into a non-redundant gene sequence containing all possible exons. A total of 18,600 gene sequences with orthology to G. aculeatus were reconstructed for each species.

Notothenioid phylogeny. We used two approaches to infer a phylogeny for notothenioids. For both analyses, only single-copy genes with >85% coverage in all species were included, resulting in a dataset of 11,627 genes. First, we individually partitioned each gene by codon position and used ModelFinder as implemented in IQTree v.1.6.3 (ref. [44]) to estimate the optimal partitioning scheme and molecular evolution model. The gene set was concatenated into a single sequence, and a maximum likelihood tree was inferred using IQTree v.1.6.3 (ref. [44]). To assess support for the phylogenetic relationships, we performed 1,000 ultra-fast bootstrap replicates [45].

To account for the effects of incomplete lineage sorting and known issues with concatenation for phylogenetic inference [46,47], we also inferred a species tree. Full species tree inference is not computationally feasible with large genomic datasets, so we relied on the summary species tree approach in ASTRA v.5.6.2 (ref. [48]). We first used IQTree v.1.6.3 to infer the maximum likelihood tree for each gene, applying partitioning schemes and molecular evolution models as described above. We then used ASTRA to summarize the distribution of gene trees and estimate the species tree. To assess support for the species tree topology, we estimated local posterior probabilities for each quadpartition in the tree [49].

Quantification of lineage differentiation dynamics. To test for changes in lineage diversification rates across the temporal history notothenioids, we used a Bayesian analysis of macro-evolutionary mixtures implemented in BAMM v.2.5 with a previously published time-tree that sampled all major lineages and 87 of ~120 species of notothenioids [52]. Priors were defined using the function setBAMMprriors contained in the R package BAMMTools v.2.1.0 (ref. [50]). We first used IQTree v.1.6.3 to infer the maximum likelihood tree for each gene, applying partitioning schemes and molecular evolution models as described above. We then used ASTRA to summarize the distribution of gene trees and estimate the species tree. To assess support for the species tree topology, we estimated local posterior probabilities for each quadpartition in the tree [52].
dS and dN estimates of substitution rate. The criteria dN, dS and dN/dS were calculated pairwise between each species and the outgroup Percina caprodes. This was performed for each reconstructed gene of at least 2,000 bp in both species using cat, dn, ds in the BioPython v.1.70 codense module (parameters ‘method = “NG86”’). The values of dN, dS and dN/dS were then averaged across all genes for each species.

Molecular clock models of substitution rate. Substitution rates were estimated from the reconstructed gene sequences using the random local clock model as implemented in BEAST v.2.4.8 (codon partitioned, bModelTest, parameters ‘chain length = 30M’)[55]. To simplify comparisons between gene trees, and because support for the relationships between the included species is high, we fixed the starting tree topology for each gene tree to match our ASTRAL-infered species tree (Supplementary Fig. 13). Only gene trees with an effective sample size (ESS) ≥ 200 were selected for use in downstream analysis (total 1,062). Most recent common ancestor (MRCA) age priors were calibrated based on previous age estimates[56–60]. P. psedaphritis + Eleginopsidae 63.0 Ma (52.6–73.4), Bovichtidae + all notothenioids 85.7 Ma (69.5–102.6), Harpagifer-Pogonomerytide 10.2 Ma (7.7–13.0), Bathyrhochrome-Choeneophalus 11.1 Ma (9.4–13.3), Nototthenia 17.7 Ma (15.2–20.5), Ctenophoridae 21.6 Ma (18.6–23.9), Eleginopsidae 45.9 Ma (37.2–53.2). The maximum clade credibility was constructed for each gene tree using TreeAnnotator.

Detection of diversifying selection. Positive selection was calculated using the adaptive-branch-site random effects likelihood (aBSREL) implemented in Program v.1.51[90]. Single-copy exon alignments were concatenated into genes as input based on the gene order in the stickleback genome. The species tree was used for all comparisons (Supplementary Fig. 13). Accelerated sequence evolution was assessed using phyloP[91] as implemented in PHAST v.1.4 (ref. [92]) (parameters ‘-method LRT-no-prune-features–mode ACC’). The tree model for phyloP was derived separately for the data sets using coding gene phylogeny and the species tree (Supplementary Fig. 13). Tree models for protein-coding regions were based on 3,381 exons ≥1,000 bp with ≥85% coverage in all species. CNE tree models were based on 2,912 elements ≥250 bp with ≥85% coverage in all species.

Ontology enrichment. As there are no gene ontology terms relating to N. coriceps, we generated a custom gene ontology database based on the combined gene ontology data from multiple species. Gene ontology data were mined from chicken, mouse, rat, human, stickleback, medaka and zebrafish in Ensembl BioMart (downloaded December 2016)[63]. All Ensembl gene IDs were converted from human to stickleback orthologues using Ensembl BioMart. Ontology enrichment was performed using Fisher's exact test (Sculp v.18.1; fisher_exact). We also assessed patterns of cumulative polygenic enrichment within ontologies using the SUMSTAT approach as implemented in ref. 64. This approach normalizes the distribution of log-likelihood ratio test values (ΔmL) output from phyloP and Hyphy by taking the fourth root (ΔmL/4). The ΔmL/4 score is then summed for all genes within an ontology, and an enrichment Pvalue is estimated from the empirical sum(ΔmL/4) score through bootstrap resampling (1,500 replicates). For all enrichment analyses, Pvalues were corrected using false discovery rate (python module statsmodels v.0.6.1; fdrcorrection).

Zebrafish husbandry and genetic lines. Zebrafish WT and mutant lines used were housed and maintained as previously described and in accordance with Boston Children's Hospital IACUC regulations[66]. The col1a1a (dmh14) and col1a2 (dmh15) mutants were derived from a forward genetic screen[11].

Zebrafish genome editing. The guide RNA (gRNA) site GGTCAAGAGTTGGCTGAGCGTGG in exon 1 of the zebrafish trip11 gene was targeted. This site is 33 bp downstream of the ATG start codon of trip11. gRNA sequences were cloned in the BsaI site of the DR274 plasmid and in vitro transcribed with the T7 RNAmase kit (ThermoFisher). Cas9 messenger RNA was obtained from SystemBio (CASS001A-1). Injections of fertilized zebrafish embryos were done with 50 ng/μl gRNA and 150 ng/μl Cas9 mRNA. Genotyping was performed using 5′-CTGGTGACAAGTAGTTAG-3′ and forward primer 5′-CA CTCCTACCATTCTCGGTGCTTCAAGATATTTGGTGGAAATTAG AG-3′ as reverse primer. PCR using this primer pair yielded a 178-bp WT band. Fish were identified with a 47-bp deletion spanning the gRNA target site and yielding a 131-bp genotyping band. The deleted sequence is 5′-CTGGTGACAAGTAGTTAG-3′ and generates a frameshift at the 12th amino acid residue of trip11.

Analysis of skeletal density through computed tomography. Adult notothenioid specimens were loaned from the Yale Peabody Museum and Harvard Museum of Comparative Zoology (Supplementary Table 1) and scanned using the Siemens Biograph at Boston Children’s Hospital Department of Nuclear Medicine and Molecular Imaging. Scan data were processed in Siemens PETsyngo VG60A software and analysed in Amira (v.6.0.0, FEI Inc.). Zebrafish were euthanized in 22% MS-222, fixed in 3.7% formaldehyde overnight and rinsed in phosphate buffered saline. The fishes were embedded in 1% agarose to reduce movement during imaging. The fish skulls were scanned as in ref. 11 using a Skyscan 1173 (Bruker), 240-degree scan with 0.2 rotational step; X-ray source voltage set to 70 kV and current set to 80 μA; exposure time was 1,500 s. The resolution of scans was 7.14 microns per pixel. Volume renderings were reconstructed as maximum intensity projections in Amira software. Skeletal density was estimated based on the average pixel intensity measured from the maximum intensity projection of the skull, vertebrate and operculum in ImageJ v1.51s (https://imagej.nih.gov/ij/). A total of n = 6 trip11−/− and n = 10 trip11+− fish were scanned and quantified. The bones of the skull roof (parietal/frontal), operculum and vertebrate were measured from each individual.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The sequencing data have been deposited in the NCBI database as Bioproject PRJNA531677. Assembled contig data have been deposited in the Zenodo repository (10.5281/zenodo.2628936).

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Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- Skeletal computed tomography (CT) scans of notothenioids were processed in Siemens PETsyngo VG60A. No other software was used in the collection of the data

Data analysis

- The following programs were used in the processing and analysis of the data:
  - BAMMTools (v2.1.0) -- lineage diversification rate analysis
  - BEAST (v2.4.8) -- substitution rate analysis
  - HYPHY (v2.3.9) -- positive selection through aBSREL program
  - PHAST (v1.4) -- accelerated sequence evolution through phyloP program
  - Python Scipy (v0.18.1) -- Fisher’s exact test for GO-enrichment (fisher_exact)
  - Python statsmodels (v0.6.1) -- multiple hypothesis test correction (fdrcorrection0)
  - IQTree (v1.6.3) -- gene trees
  - ASTRAL (v5.6.2) -- species tree from gene trees
  - Sequencing read processing and contig assembly programs:
    - Trimmomatic (v0.3)
    - FASTX-Toolkit (v0.0.13)
    - blastn (ncbi-blast-2.2.30+)
    - dc-megablast (v2.6.0+)
    - Biopython (v1.70) -- codonseq and pairwise2 modules
    - BEDtools (v2.23.0)
    - CAP3 (02/10/15)
    - Notung (v2.9)
  - Multiple sequence alignment and alignment pruning:
    - MACSE (v2.03)
Mafft (v7.313)  
GUIDANCE (v2.02)  
Read alignment:  
NextGenMap (v0.5.5)  
SAMtools (v1.9)  
BCFtools (v1.9)  
Processing and analysis of CT scans:  
ImageJ (v1.51s)  
Amira (v6.0.0)  
Siemens PETsyngo VG60A

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The sequencing reads have been deposited on the NCBI SRA (Bioproject PRJNA531677). Assembled contigs and annotations for all species in the dataset have been uploaded to the Zenodo repository (doi: 10.5281/zenodo.2628936).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We started with a goal of n>5 and >10 for control and experimental groups respectively for analysis of trip11 zebrafish mutants, with these numbers selected to capture variation in the phenotype while minimizing the usage of laboratory animals. |
| Data exclusions | No data were excluded from the analysis |
| Replication | Zebrafish trip11 mutant individuals were scanned from two independent genetic crosses. |
| Randomization | As the zebrafish work focused on defined genotypes, we did not randomize into experimental groups for zebrafish experiments. Further, no randomization was necessary in the genomic analysis, as this analysis is predicated on questions at specific places within the phylogeny |
| Blinding | Investigators were not blinded to the genotypic and genomic data analysis. As the research questions focused on specific lineages within the phylogeny, blinding would not be possible. |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
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| ❌  | Eukaryotic cell lines |
| ❌  | Palaeontology         |
| ❌  | Animals and other organisms |
| ❌  | Human research participants |
| ❌  | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ❌  | ChIP-seq              |
| ❌  | Flow cytometry        |
| ❌  | MRI-based neuroimaging |

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | This study involves the use of zebrafish (Danio rerio). Both genders between the ages of 3-6 months were used. Mutant strains include col1a1a (dmh14) and col1a2 (dmh15). CRISPR mutants in trip11 were generated from wild-type strains. |
|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals       | This study did not involve wild animals                                                                                                                                                         |
| Field-collected samples | No tissue samples were directly collected from the field for the purposes of this study. Tissue samples were obtained from the Yale Peabody frozen tissue collection and from the laboratory collection of H.W. Detrich from previously published Antarctic expeditions. Adult notothenioid specimens were also loaned from the Yale Peabody Museum for CT scanning. Full sample and specimen identifiers are available in the Supplement. |
| Ethics oversight   | Boston Children's Hospital Institutional Animal Care and Use Committee (IACUC).                                                                                                                      |

Note that full information on the approval of the study protocol must also be provided in the manuscript.