Comparative Population Transcriptomics Provide New Insight into the Evolutionary History and Adaptive Potential of World Ocean Krill

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Abstract

Genetic variation is instrumental for adaptation to changing environments but it is unclear how it is structured and contributes to adaptation in pelagic species lacking clear barriers to gene flow. Here, we applied comparative genomics to extensive transcriptome datasets from 20 krill species collected across the Atlantic, Indian, Pacific, and Southern Oceans. We compared genetic variation both within and between species to elucidate their evolutionary history and genomic bases of adaptation. We resolved phylogenetic interrelationships and uncovered genomic evidence to elevate the cryptic *Euphausia similis* var. *armata* into species. Levels of genetic variation and rates of adaptive protein evolution vary widely. Species endemic to the cold Southern Ocean, such as the Antarctic krill *Euphausia superba*, showed less genetic variation and lower evolutionary rates than other species. This could suggest a low adaptive potential to rapid climate change. We uncovered hundreds of candidate genes with signatures of adaptive evolution among Antarctic *Euphausia* but did not observe strong evidence of adaptive convergence with the predominantly Arctic *Thysanoessa*. We instead identified candidates for cold-adaptation that have also been detected in Antarctic fish, including genes that govern thermal reception such as *TrpA1*. Our results suggest parallel genetic responses to similar selection pressures across Antarctic taxa and provide new insights into the adaptive potential of important zooplankton already affected by climate change.

Key words: **krill, climate change, population genomics, comparative genomics, genetic adaptation.**

Introduction

The world's oceans have warmed by about 1 °C over the last century as a consequence of anthropogenic greenhouse gas emissions (IPCC 2014). This has strongly impacted pelagic species, causing poleward shifts and accelerated phenologies in fish and zooplankton, such as jellyfish, salps, copepods, and krill (Richardson 2008; Poloczanska et al. 2013, 2016; Ratnarajah et al. 2023), and threatens to destabilize important food webs and ecosystem services (Doney et al. 2012; Baxter and Laffoley 2016). Genetic adaptation could be crucial to sustain populations under climate change, promoting resilience by targeting genes encoding traits such as growth, reproductive timing, and thermal tolerance (Hoffmann and Sgrò 2011; Dam 2013), but is poorly understood in zooplankton. A major obstacle resides in the widespread occurrence of cryptic species in marine zooplankton, which has led to underestimation of diversity (Knowlton 1993; Lee 2000; Bailey et al. 2016; Bucklin et al. 2016; Choquet et al. 2018). Furthermore, while polar oceans are particularly severely impacted by climate change (Huguenin et al. 2022; Rantanen et al. 2022), accessing zooplankton species adapted to these environments can be a logistical challenge and discourage attempts to conduct advanced studies and experiments of adaptation (Bucklin et al. 2018).

Adaptation to cold environments could commonly involve fundamental genetic and physiological alterations and tradeoffs that are maladaptive under rapidly warming temperatures (Pörtner et al. 2007). It is therefore concerning that both Antarctic and Arctic krill are declining or shifting to higher latitudes due to climate

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change (Atkinson et al. 2019; Edwards et al. 2021). Krill (Euphausiacea; "euphausiids"; 86 spp.) are crustacean macrozooplankton and grazers of phytoplankton primary production or smaller zooplankton (Mauchline and Fisher 1969). As important food for fish, mammals, and birds, they play critical roles in transferring nutrients to higher trophic levels in marine ecosystems (Tarling 2010; McBride et al. 2014; Siegel 2016; Johnston et al. 2022). Different species occur throughout tropical, temperate, and polar ecosystems, and their biogeography depends on physiological thermal tolerance, oceanographic conditions, and nutrient availability (Mauchline and Fisher 1969; Cimino et al. 2020). Euphausiids have evolved divergent life cycle strategies across these ecosystems: Low-latitude species associated with nutrient-rich upwellings or near-shore habitats tend to develop and mature quickly, be short-lived (<1 yr or 1 to 2 yr), breed multiple times or continuously throughout the year, and have high productivity rates (Siegel 2000). High-latitude species instead have strategies to cope with long periods of dark, cold, and nutritionally adverse conditions. They are characterized by slow rates of development, extended longevity (e.g. 2+ yr) and larger bodies with long-lasting lipid stores, and have short annual reproductive seasons and low productivity (Falk-Petersen et al. 2000; Siegel 2000, 2016). The molecular mechanisms that govern ecophysiological traits have been studied in some krill, including the photoreceptor and circadian clock gene repertoires (Biscontin et al. 2016; Christie et al. 2017; Palecanda et al. 2022; Urso et al. 2022), genes that regulate the molting cycle (Seear et al. 2010), the gene expression dynamics associated with seasonal growth and reproduction (Seear et al. 2012; Höring et al. 2021; Urso et al. 2022), or heat shock response (Huenerlage et al. 2016; Papot et al. 2016; Toullec et al. 2020). Yet, insights into what genes and variants may contribute to adaptation under environmental change are still highly limited in krill and most other zooplankton (Dam 2013; Bucklin et al. 2018).

Comparative analyses among animals suggest that adaptation may be limited by the supply of beneficial mutations in species with low diversity or "K-selected" life history traits such as long lifespans, large body sizes, low fecundity, and large investments in the quality of offspring, which may indicate a small long-term effective population size ("N_e") (Romiguier et al. 2014; Galtier 2016; Rousselle et al. 2020). Population genetic theory suggests that zooplankton should be on the opposite end of the scale and have high adaptive potential (Peijnenburg and Goetze 2013): (i) They have large populations with many reproductive individuals (i.e. a high "N_e") that can maintain or generate much variation to select from; (ii) due to large N_e, they are expected to be comparably unaffected by genetic drift that may interfere with selection; and (iii) many species have short generation times and high reproductive rates, amenable for adapting to rapid changes. In controlled selection experiments in marine copepods, adaptive genetic responses to increased temperatures have been shown to emerge within 20 generations (Brennan et al. 2022a, 2022b). However, empirical support for these predictions is still largely missing from natural zooplankton populations and conditions, where for example extensive gene flow may constrain natural selection (Lenormand 2002). The large Antarctic krill *Euphausia superba* is among the most abundant animals on Earth (Bar-On et al. 2018) but does not appear to be hypervariable (Bortolotto et al. 2011; Shao et al. 2023). Krill have highly diverse life histories and may not fit the theoretical zooplankton model, which further warrants the need to broadly survey genetic variation and the efficacy of natural selection across many species.

Multigenerational selection experiments are not feasible for highly pelagic species such as krill. Knowledge about the genetic mechanisms and loci that underlie adaptation can alternatively be gained through genome-wide comparisons of natural populations or species native to contrasting conditions (Savolainen et al. 2013; Meek et al. 2023). Due to their remarkable biogeographic history, Antarctic species provide unique opportunities to study environmental adaptation. The formation of the Antarctic Circumpolar Current (ACC) and the Southern Ocean about 30 MYA (Scher et al. 2015) had profound effects on marine biota in the region, creating strong isolation that enabled endemic speciation, novel adaptations to extreme environments, and the development of specialized marine ecosystems (Rogers 2007). Some of the best characterized thermal adaptations have been uncovered by studying Antarctic fauna such as notothenioid fish, including the evolution of antifreeze glycoproteins, amino acid substitutions conferring increased protein flexibility or oxygen tolerance, loss of globins to reduce blood viscosity, and structural changes to muscle tissue (Pucciarelli et al. 2006; Rogers 2007; Berthelot et al. 2019). The ancestor of *E. superba* and the Ice krill *Euphausia crystallorophias* likely split from other species 25 to 27 MYA (Zane and Patarnello 2000), and have since adapted to extreme Antarctic environments. Similar to other Antarctic fauna, they have narrow thermal ranges: *E. superba* inhabits cold waters ranging from −2.0 °C to +4.0 °C (Siegel 2016), while the neritic *E. crystallorophias* is even more restricted (−1.8 °C to 0 °C) (Cuzin-Roudy et al. 2014). Stress tests (e.g. CT50 assays) show that they are sensitive to high temperatures and have low capacity to upregulate inducible and protective heat shock proteins in response to increased temperature (Cascella et al. 2015; Huenerlage et al. 2016; Toullec et al. 2020), which is common among Antarctic ectotherms (Peck et al. 2014; Peck 2016; Chen et al. 2018). In contrast, widespread North Atlantic krill such as *Meganyctiphanes norvegica* and many *Thysanoessa* have thermal ranges spanning 2 to 15 °C or more and occur from the Arctic Ocean to the warm Gulf of Maine (Mauchline and Fisher 1969; Tarling 2010; Ollier et al. 2018), indicating greater thermal tolerance.

So far, only the genome of *E. superba* has been assembled and population-scale scans in this species identified few potentially adaptive variants (Shao et al. 2023), possibly due to extensive panmixia (Bortolotto et al. 2011; Deagle et al. 2015; Shao et al. 2023). Here, we took a different approach. We hypothesized that natural selection has favored genetic variants that influence thermal physiology in Southern

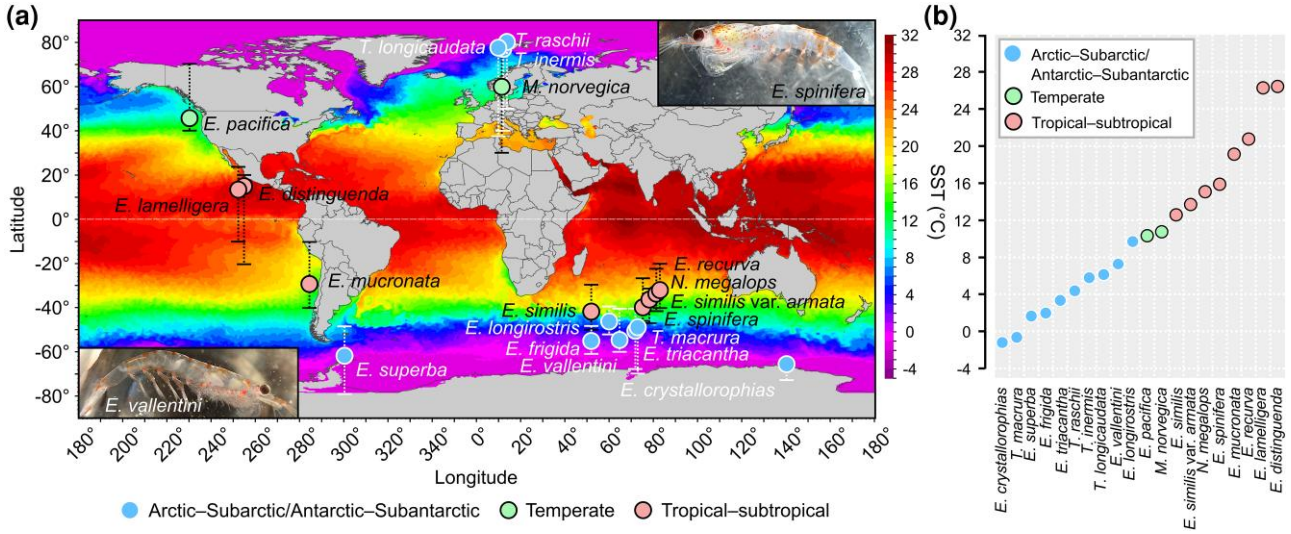

Fig. 1. The geographic distribution of 20 surveyed krill species. a) Circles indicate sampling locations of 1 or more collected specimens (colors represent the thermal conditions associated with the range of each species). The basin-specific latitudinal range of each species is indicated with bars. Ranges are from Mauchline and Fisher (1969). Global Sea Surface Temperature (SST) readouts are a daily snapshot from OISST V2 (2022-06-01) from ERDDAP: https://www.ncei.noaa.gov/erddap/griddap/ (Reynolds et al. 2007; Banzon et al. 2016; Huang et al. 2021). Photos of Indian Ocean krill collected in 2019. b) The approximate mean SST of each species range. See Fig. S1 for full geographic and thermal ranges.

Ocean krill species and that these could be detected using comparative genomics. To this end, we assembled and analyzed the first genome-wide cross-species dataset for krill, including 20 species from the Atlantic, Indian, Pacific, and Southern Oceans, spanning polar to tropical conditions (Fig. 1). Krill have extremely large genomes (11 to 48 Gb), making them very challenging to analyze (Jeffery 2012; Shao et al. 2023). We therefore used RNA-seq data to analyze genetic variation in expressed genes (Romiguier et al. 2014; De Wit et al. 2015; Lenz et al. 2021). To identify candidates, we scanned for signatures of selection in Southern Ocean *Euphausia* species and tested for convergence with *Thysanoessa*. We also performed CT50 experiments to better understand how thermal tolerance varies between species. The hypothesis of high adaptive potential in zooplankton predicts that much of the divergence observed between species is likely to have been shaped by adaptation. This has yet to be tested. In particular, large and potentially "K-selected" Southern Ocean krill like *E. superba* may have constrained effective population sizes and low adaptive potential. To test these hypotheses, we compiled life history information and produced new baseline estimates of genetic diversity and rates of adaptive protein evolution in multiple species. To the best of our knowledge, our study is the first to use comparative genomics methods to uncover the genomic basis of ecological adaptation in zooplankton.

Results

Thermal Tolerance in Krill Is Associated with Habitat Temperature

We used CT50 mobility assays to characterize and compare thermal tolerance in 6 krill species sampled from the Southern, Atlantic, and Indian Oceans. The 2 Antarctic species—*E. crystallorophias* and *E. superba*—had significantly lower CT50 thresholds compared to the 2 subantarctic *Euphausia vallentini* and *Euphausia triacantha* (Fig. 2a). The average CT50 for *E. vallentini* was 17.9 ± 0.42 °C, but could be an underestimate as specimens were fragile and appeared impacted by fishing (reflected in the rapidly subsiding high sigmoid plateau and the flatter slope). CT50 for *E. triacantha*, which appeared healthier, was 18.37 ± 0.39 °C. Fished in 4 °C waters of the high Arctic Spitsbergen (same locality as *Thysanoessa inermis*), *M. norvegica* showed a record CT50 (~23 °C). Overall, CT50s appeared strongly correlated with ambient temperatures (Fig. 2b; Supplementary Fig. S1).

Genome-Wide Datasets to Study Variation and Adaptation

Our dataset spanned 124 transcriptomes from 20 species (102 transcriptomes from 17 species are new for this study). Morphological and molecular species identifications agreed for all samples with barcodes in the MetaZooGene Atlas and Database (MZGdb; Table S1). Our reference transcriptomes contained 20 to 53 K nonredundant coding transcripts per species (Table S2), with high BUSCO completeness levels (median = 92.5%) and low duplication levels (median = 3.3%), amenable for tracing evolutionary patterns using base-level substitutions and polymorphisms. We estimated genetic variation in 9 species, mapping up to 20 specimens per species and detected 260 to 2,200 K high-quality SNPs over 12 to 22 K genes (Table S3).

Genetic Divergence Between Indian Ocean *Euphausia similis* and *Euphausia similis* var. *armata* Suggests that They Are Different Species

Our Indian/Southern Ocean sampling spanned multiple waterfronts, and we found genetic population structure in 2 out of 3 temperate–subtropical species. We detected

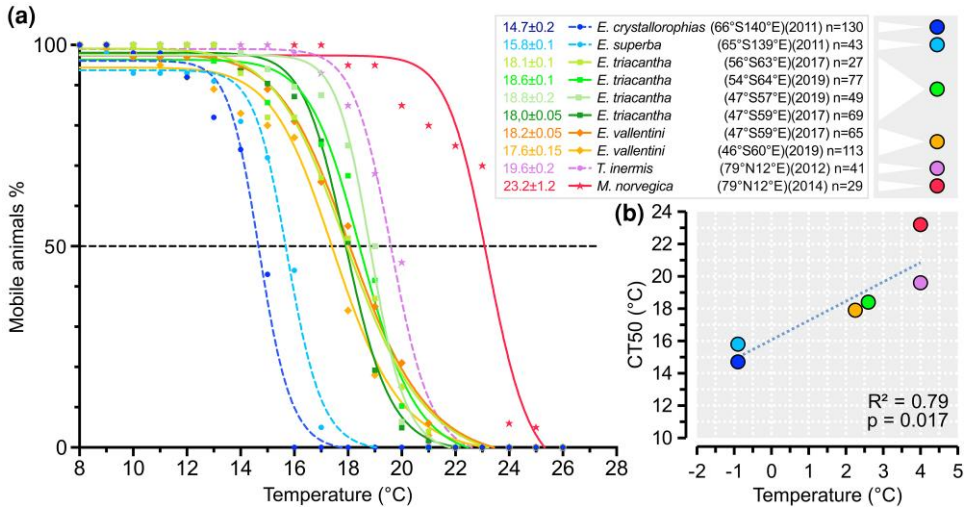

Fig. 2. Thermal tolerance in Southern Ocean and Atlantic Ocean krill. a) The figure shows all the results acquired over several years during campaigns in Antarctica, Arctic, and Indian Oceans. Three species (dashed curves) have been published before (Cascella et al. 2015; Huenerlage et al. 2016), while *E. vallentini* (orange), *E. triacantha* (green), and *M. norvegica* (red) are new to this study. The white legend indicates species/experiment-specific calculated CT50 values with standard errors, fishing coordinates, years, and sample sizes. b) Linear regression correlation between CT50 and the measured ambient sea temperature at the experimental locations. The mean CT50 of all experiments was used for *E. triacantha* ($n = 4$) and *E. vallentini* ($n = 2$).

strong structuring between *Euphausia similis* and its variety *armata* using ancestry analysis and Principal Component Analyses (PCA) (Fig. 3a to c), supported by a high nuclear F_{ST} value (0.68) and mitochondrial divergence (~6.2%; Fig. 3d and e; Supplementary Fig. S3). The specific status of *E. similis* var. *armata* has been unclear since it was first described (Hansen 1911). Using a subset of 2,754 genes sequenced in 4 lineages, we found that F_{ST} between *E. similis* and *armata* was similar to F_{ST} between *Euphausia longirostris* and *Euphausia spinifera* (0.65 vs. 0.7), which are recognized as separate species. The net synonymous divergence *Da* was 1.9% between *E. similis* and *armata* (from 3,939 genes), and 1.89% between *E. longirostris* and *E. spinifera* (from 3,111 genes). A morphological re-assessment of sequenced specimens confirmed that 9 individuals collected mostly to the east matched the *E. similis* var. *armata* variety ("*armata*"), having the diagnostic accessory abdominal spine of variable size (Fig. 3b; Supplementary Fig. S2A) (Hansen 1911; Baker et al. 1990). Western samples matched *E. similis*, without the spines (Fig. 3a and b; Supplementary Fig. S2B; "*similis*"). Because of the consistent molecular and phenotypic differences between *similis* and *armata*, we assembled a separate reference transcriptome for *armata* and subsequently treated the 2 as separate species. We detected weak north–south structuring in our limited *Nematoscelis megalops* sample ($F_{ST} = 0.06$), but none in *E. spinifera*, nor in the subantarctic *E. vallentini* and *E. triacantha* collected from the Antarctic Polar Front (Supplementary Fig. S4).

Two-Fold Difference in Genetic Diversity among 9 Species of Krill

We estimated transcriptome-wide levels of genetic variation and studied how it varied among gene regions and species (Supplementary Figs. S5 and S6). Overall, we detected about 1.6× more variation in untranslated regions (UTRs) compared to coding regions (average $\theta_w = 1.4\%$ vs. 0.9%), and 3.1 to 5.6× more variation at synonymous sites compared to non-synonymous sites (Fig. 4; Supplementary Fig. S5). These patterns likely reflect both direct and linked purifying selection, reducing variation around functionally important sites (Cvijović et al. 2018). We used synonymous variants as representative of neutral variation for estimating N_e and demographic history. All species had variation surpassing 1%/bp, the most diverse being *E. vallentini* ($\pi_S = 2.5\%$; $\theta_w = 3.83\%$), while *E. triacantha* was the least diverse ($\pi_S = 1.1\%$) (Fig. 4; Supplementary Fig. S7). Assuming the same mutation rates among species, estimates of N_e indicate *E. vallentini* (3.6 M) and *E. similis* var. *armata* (3.2 M) have the largest effective population sizes (Table 1). Our *armata* sample had 1.7× as much variation as *similis*, further suggesting that these are separate lineages. Tajima's D (D_T) was negative across all species (−1.2 to −1.9; Table 1), indicating excess of low-frequency variants compared to expectations under neutrality, which could result from recent population expansions. Neither life history traits (e.g. body size) or environmental parameters (e.g. latitude) predicted π_S or D_T (i.e. no correlation $P < 0.1$; Table S10).

Comparative Analyses Establish Comprehensive Gene Orthologies and a Robust Species Tree

To enable direct comparative analyses of molecular evolution, we inferred gene orthology among the 20 krill species and 7 outgroups (Supplementary Material online) and inferred a species tree. We detected 13,255 orthogroups (OGs) spanning 10 or more krill species. We used a subset of 2,280 OGs with ≥18 krill species and >1 M amino acid

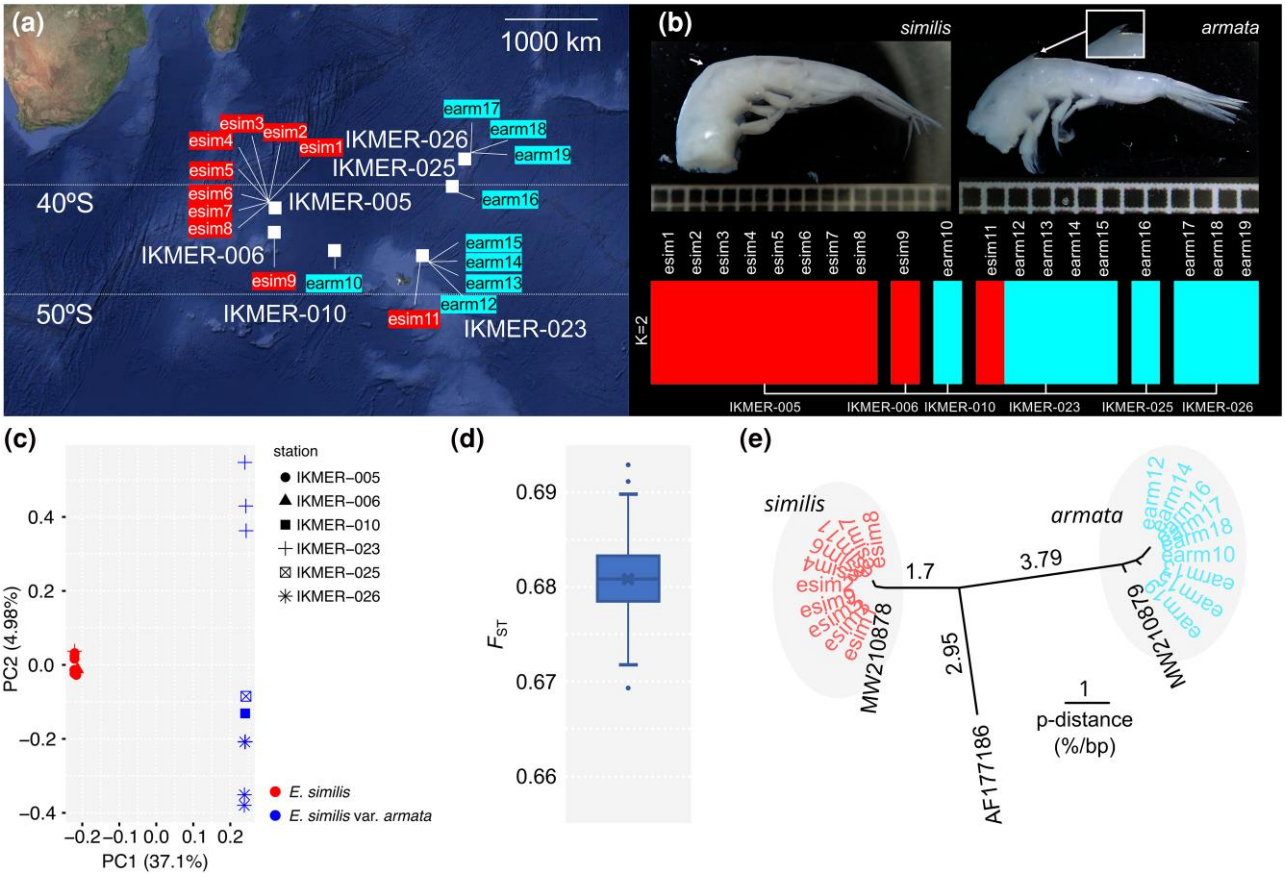

Fig. 3. Genetic structure between *Euphausia similis* samples ("esim"; $n = 10$) and *Euphausia similis* var. *armata* ("earm"; $n = 9$) from the Indian Ocean. a) Sampling stations ($n = 6$). b) Top: representative tails of *E. similis* (without a dorsal spine) and *E. similis* var. *armata* (with a spine on the third abdominal segment). Arrows indicate the spine (if present). Bottom: genetic ancestry and admixture of samples (K = 2 ancestral groups). c) The genetic structure among 19 samples using PCA ($n = 84{,}436$ unlinked nuclear SNPs). d) Weighted F_{ST} estimated from 1,000 random resamples drawing 1 SNP per gene ($n = 6{,}074$ genes). e) A mitochondrial COI neighbor-joining gene tree inferred from uncorrected pairwise genetic distances among 500 to 800 bp *mtCOI* fragments. Distances are shown for the major internal branches. Sequences from MetaZooGene are: AF177186 (north-east of Japan; Kenji Taki; personal communication); MW210878 and MW210879 (from the southern Atlantic Ocean).

positions to reconstruct a fully supported phylogeny (Table S4; Fig. 5a). The topology was mostly consistent with that of Vereshchaka et al. (2019) based on morphology and 4 molecular markers: *Meganyctiphanes* + *Nematoscelis* + *Thysanoessa* form the monophyletic clade *Nematoscelinae*, the sister taxon of *Euphausiinae*. Groupings within *Euphausia* confirm a monophyletic Southern Ocean "*Euphausia superba* group" including *frigida*, *vallentini*, *crystallorophias*, and *superba*. Our clade with *spinifera*, *longirostris*, *similis*, *armata*, and *triacantha* also includes *recurva* and *mucronata*, mixing other species groups.

Scans for Positive Selection Reveal Candidate Genes for Environmental Adaptation in Krill

We compared 5 Southern Ocean *Euphausia* species against the rest of the species tree to study cold-adaptation (Supplementary Fig. S9A). We identified 483 functionally diverse gene candidates containing 1,307 putatively selected codons (likelihood ratio test [LRT] $q < 0.05$; 374 genes matching 335 unique *Drosophila* homologs; Table S6). These genes were marginally enriched for roles in ion homeostasis, muscle development, or temperature detection ($q = 0.087$; Fig. 5b; Table S7). We detected 2 putatively selected sites in the gene encoding the calcium channel TrpA1 (Supplementary Fig. S11A), 3 in the anoctamin ion channel *subdued* (Supplementary Fig. S11B) and 1 in *straightjacket*, all of which are also associated with thermal nociception in animals (Dhaka et al. 2006; Jang et al. 2015; Khuong et al. 2019; Himmel and Cox 2020; Zhang et al. 2022). Crustacean TRP evolution is characterized by widespread gene duplication, and the krill homolog of *Transient receptor potential cation channel A1* is likely orthologous to decapod *TrpA1-like* (Supplementary Fig. S10) (Kozma et al. 2020). The candidates also spanned genes encoding the heat shock protein Hsp110/Hsc70Cb and 2 chaperones (CCT3 and CCT7), which function in folding or protecting proteins from noxious temperatures (Chen et al. 2018). We extended the foreground to include the *Thysanoessa* clade (Supplementary Fig. S9B), aiming to detect evidence of convergent evolution between the 2 groups. We uncovered only 185 candidates (Tables S6 and S7). Among the 483 original candidates, we observed weakened evidence of selection (i.e. reduced LRT scores) in

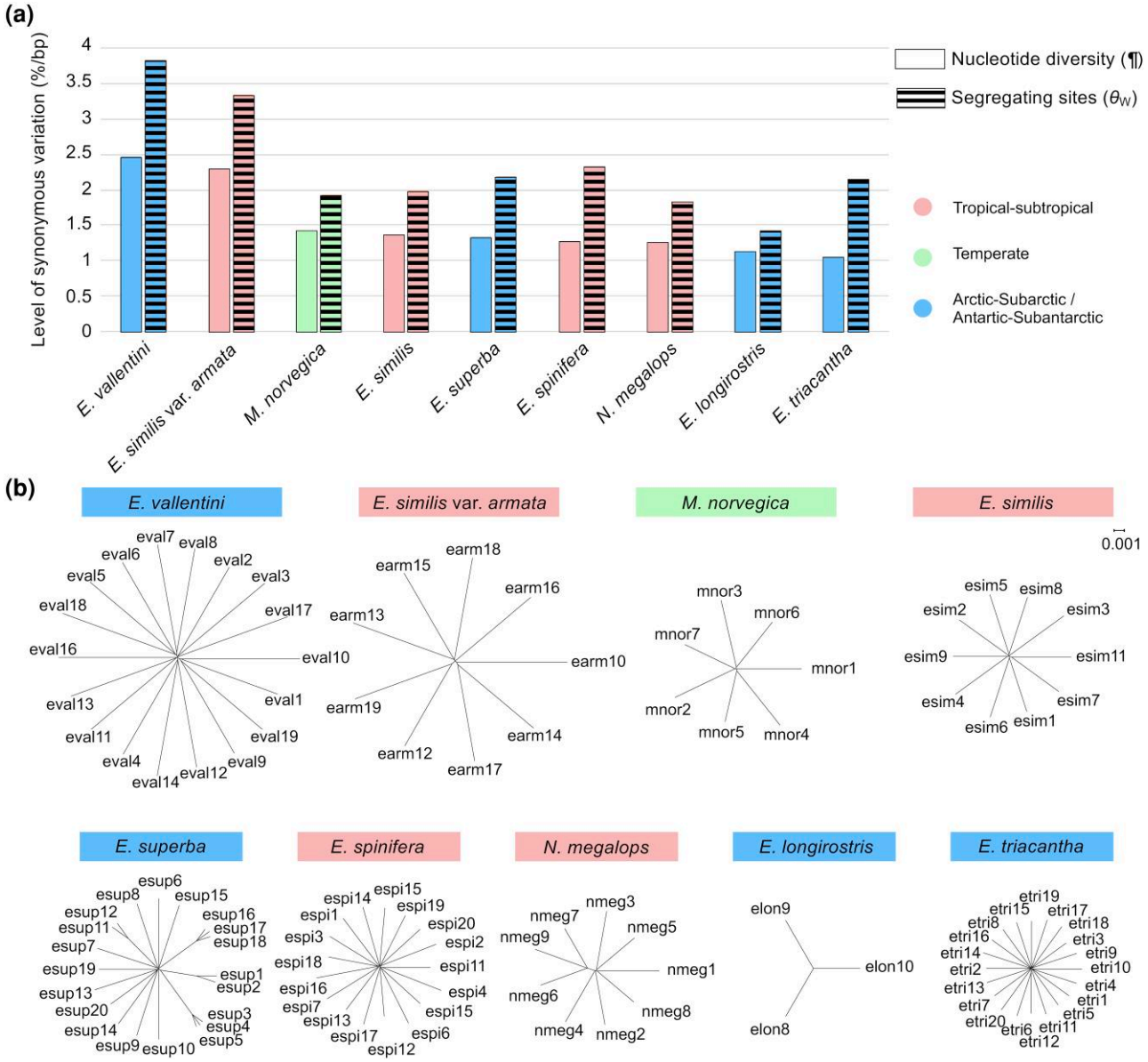

Fig. 4. Genetic variation at synonymous sites in 9 krill species. a) Nucleotide diversity (π_S) and population mutation rate (θ_{WS}) estimated from 74 k to 709 k synonymous SNPs per species, after correction for accessible sites. b) Neighbor-joining trees showing variation and structure across species. Branch lengths are scaled as the average genetic distance per base between samples (π_S), estimated at synonymous loci and corrected for accessible sites and drawn at the same scale.

Table 1 Synonymous variation and demographics in 9 krill species

Ocean	Species	*n*	Genes[a]	SNPs	Total length[b]	Tajima's D (D_T)	N_e[c]
Atlantic	***M. norvegica***	**7**	**15,445**	**236,943**	**3,883,522**	**−1,153**	**1,816,807**
Indian	***E. longirostris***	**3**	**9,535**	**74,381**	**2,278,357**	**−1,402**	**1,353,964**
...	***E. similis***	**10**	**12,731**	**216,303**	**3,072,702**	**−1,289**	**1,878,999**
...	***E. similis* var. *armata***	**9**	**16,165**	**442,254**	**3,849,450**	**−1,335**	**3,163,058**
...	***E. spinifera***	**17**	**11,804**	**254,630**	**2,678,672**	**−1,741**	**2,201,560**
...	***E. triacantha***	**20**	**13,707**	**301,721**	**3,294,790**	**−1,911**	**2,038,746**
...	***E. vallentini***	**18**	**19,509**	**709,573**	**4,470,717**	**−1,362**	**3,624,473**
...	***N. megalops***	**9**	**12,156**	**170,844**	**2,697,988**	**−1,367**	**1,743,387**
Southern	***E. superba***	**20**	**10,777**	**205,319**	**2,203,203**	**−1,488**	**2,074,722**

[a]Expressed genes with at least 1 synonymous SNP.
[b]Counting only accessible sites covered by at least 5× depth of coverage.
[c]N_e = effective population size.

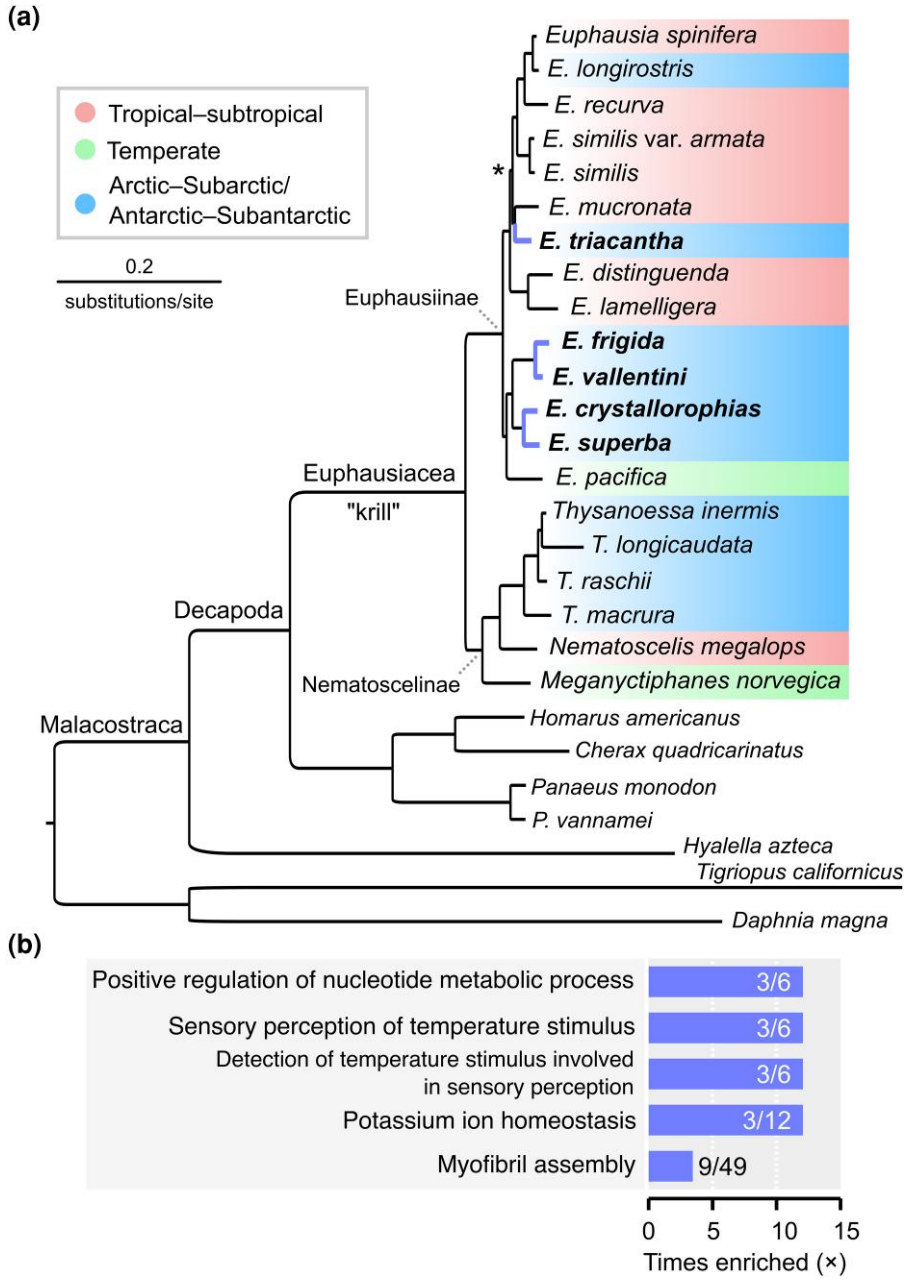

Fig. 5. Phylogenomic inference of interrelationships and molecular evolution in krill. a) A species tree inferred from 2,280 orthologous protein alignments and constructed from 1,000 ultrafast bootstrap replicates (JTTDCMut+F+I+G4 model; lnL = −11,173,074) (Hoang et al. 2018). All nodes in the majority-rule consensus tree but 1 have 100% bootstrap support (* = 99%). Five foreground branches with Southern Ocean species used in the main branch-site test to detect candidate genes for cold-adaptation are highlighted (blue/bold). b) Statistically enriched gene ontologies among the candidate genes ($q < 0.05$; $n = 335$ genes with ≥1 selected sites). Bars indicate enrichment of an ontology in target candidate genes compared to its frequency in background genes. Numbers indicate genes in the target set versus genes in the background test set. Redundant GO terms were removed with *Revigo* (Supek et al. 2011). *Euphausia longirostris* was used as a background species due to its intermediate thermal envelope (Fig. 1; Supplementary Fig. S1).

395 genes and increased LRTs in favor of selection in only 33 genes (Table S6). We analyzed the *Euphausia* and *Thysanoessa* sets independently (Supplementary Fig. S9C and D) to test if more genes than expected by chance had evidence of positive selection in both groups. Among 8,043 common OGs, 582 were significant (LRT $q < 0.05$) in *Euphausia* and 573 in *Thysanoessa*. Only 46 of these were in both sets, marginally more than expected ($n = 41$) and not significant in a hypergeometric probability test ($P = 0.246$).

Slow Rates of Adaptive Protein Evolution in Some Southern Ocean Species

As an indicator of adaptive potential, we estimated α, the proportion of amino acid substitutions that may have

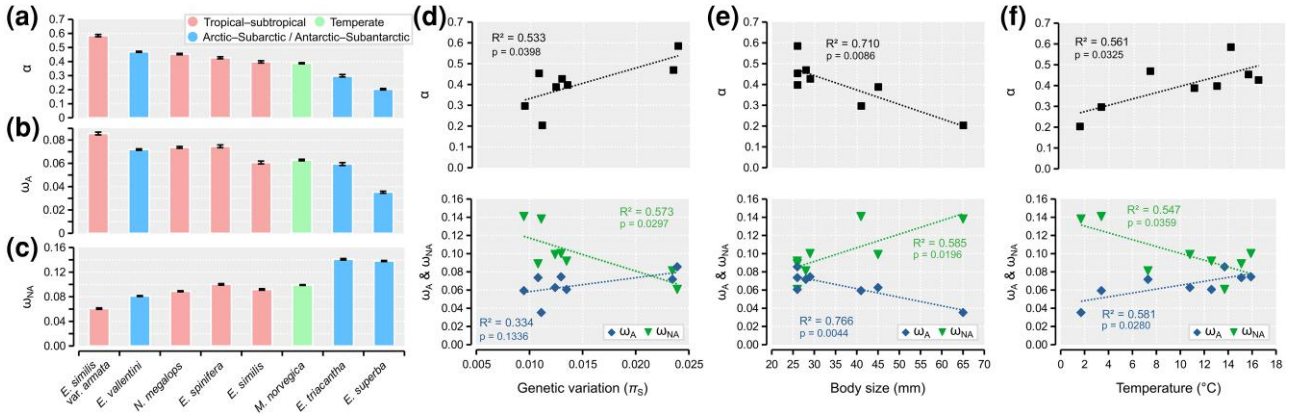

Fig. 6. Estimates of adaptive protein evolution in 8 krill species associated with different environments (GammaZero model). a to c) The proportions and rates of adaptive and nonadaptive amino acid substitutions (α, ω_A, and ω_{NA}), respectively. Whiskers indicate maximum likelihood confidence intervals. d to f) Correlations between genetic variation (π_S), maximum body size and habitat sea-surface temperature, and α (top) and ω_A or ω_{NA} (bottom), respectively, among the 8 species. Lines indicate Pearson linear regressions, and values are r^2 coefficients and significance-values.

evolved through positive selection, in 8 krill species. We inferred *dN* and *dS* between focal species and ancestral nodes (Table S8) and estimated intraspecific polymorphism (*pN*/*pS*) using unfolded site frequency spectra (Supplementary Fig. S12). We used both nonparametric and model-based methods to estimate α and the rates of adaptive and nonadaptive amino acid substitutions (ω_A and ω_{NA}; Table S9; Supplementary Fig. S13). We detected the highest α and ω_A in the subtropical *E. similis* var. *armata* ($\alpha = 0.59$; $\omega_A = 0.086$) (Fig. 6a to c; Table S9), whereas adaptive evolution appeared more limited in the Antarctic krill *E. superba* ($\alpha = 0.20$; $\omega_A = 0.035$) and subantarctic *E. triacantha* ($\alpha = 0.30$; $\omega_A = 0.059$). We then analyzed how genetic diversity (π_S) and N_e-associated life history traits and habitat conditions correlated with adaptive rates. We found that π_S predicted α ($r^2 = 0.53$; $P = 0.04$; Fig. 6d). Our results were more strongly associated with ω_{NA} than ω_A, suggesting that genetic divergence between krill species may be more strongly driven by increased fixation of nonadaptive variants in small populations than by accelerated positive selection in large ones, matching previous observations in animals (Galtier 2016). We also found that small body and propagule (i.e. larval) sizes, traits associated with "r-strategies" and high long-term N_e (Ellegren and Galtier 2016), as well as warmer habitats, were strongly associated with high α (Fig. 6e and f; Table S10). Tajima's D, an indicator of recent demographic events, did not predict α, ω_A, or ω_{NA} (Table S10).

Discussion

Assessments of phylogenetic interrelationships and genetic adaptation among krill species have previously only been based on a few markers (e.g. Jarman et al. 2000; Bucklin et al. 2007; Papot et al. 2016; Vereshchaka et al. 2019). Here, we used comparative population transcriptomics to characterize the interrelationships, genetic variation and evolution of euphausiids. We produced the first phylogenomic species tree for the taxon. It implies that krill have frequently diversified into different climates and supports the idea that *E. triacantha* and *E. longirostris* have expanded into the Southern Ocean independently from other *Euphausia* (Jarman et al. 2000).

Elusive Determinants of Genetic Variation in Krill

Mitochondrial markers have uncovered considerable variability in intraspecific variation among krill species but rarely exceeding 2%/bp (Bucklin et al. 2007; Bortolotto et al. 2011). We estimated diversity across thousands of nuclear genes and found that it varied more than 2-fold among 9 species ($\pi_S = 1.1\%$ to 2.5%). Large and superabundant species like the Antarctic krill *E. superba* and the "Northern krill" *M. norvegica*, that each have enormous biomass estimated to 100 s of Mts (Atkinson et al. 2009; Tarling 2010), were not highly diverse. In fact, the most diverse species was *E. vallentini* ($\pi_S = 2.5\%$), a small (13 to 28 mm) subantarctic, omnivorous, and possibly panmictic species with a 2-yr lifespan (Mauchline and Fisher 1969; Ridoux 1988; Mayzaud et al. 2003). It has a circumpolar distribution mostly distributed north of the Polar Front and can be the dominant species in shelf areas (Palma and Silva 2004; Koubbi et al. 2011; Harkins et al. 2013; Cuzin-Roudy et al. 2014; González et al. 2016). The high level of genetic diversity recorded in this species indicates that *E. vallentini* has the largest population size of all krill analyzed here. In contrast, we found that *E. triacantha* was the least diverse species ($\pi_S = 1.1\%$). This large (24 to 41 mm) and carnivorous krill has a 3-yr lifespan and 2-yr generation time and a circumpolar distribution spanning the Polar Front, across which it can dominate the mesopelagic euphausiid biomass (Mauchline and Fisher 1969; Siegel 1987; Phleger et al. 2002; Cuzin-Roudy et al. 2014). These 2 extremes could be examples of "r-strategist" and "K-strategist" species, whose life history traits (e.g. short vs. long lifespan, small vs. large bodies) have previously been linked to high versus low genetic diversity

(Romiguier et al. 2014; Ellegren and Galtier 2016). However, associated traits like fecundity are not known in either species, and we found no significant correlations between life history traits and levels of variation across all 9 species.

Compared to the neutral levels of genetic diversity of other arthropods, we found that krill have intermediate levels of variation (π_S: mean ± SD): 1.5 ± 0.5% in krill versus 1.4 ± 0.9% in 43 species (Leffler et al. 2012) or 1.4 ± 1.2% in 26 species (Romiguier et al. 2014). Thus, krill are in this sense neither hypo- or hypervariable. Across the tree of life, genetic diversity varies much less than census population sizes (N_c), a largely unexplained phenomenon coined as "Lewontin's paradox" (Lewontin 1974; Buffalo 2021; Charlesworth and Jensen 2022), and many marine species appear to have much lower N_e than expected from abundance (Hedgecock 1994). We inferred long-term N_e for *E. superba* and *M. norvegica* to be only 2 and 1.8 M, respectively, far below expected N_c of hundreds of trillions of individuals. Such discrepancies have been reported for other zooplankton using mitochondrial data (Bucklin and Wiebe 1998), suggesting that it may be common.

Low estimates of N_e could be due to selection for low mutation rates afforded in species with large populations (Sung et al. 2012). For example, mutation accumulation lines in the ubiquitous phytoplankton *Emiliania huxleyi* recovered a low mutation rate ($\mu = 5.6 \times 10^{-10}$), although not low enough to explain its modest levels of variation ($\pi_S = 0.6\%$) (Krasovec et al. 2020). Likewise, Shao et al. (2023) recently estimated low nucleotide diversity in the Antarctic krill ($\pi_{genome} \asymp 0.25\%$; $\pi_{CDS} \asymp 0.17\%$) using genomic data, and also inferred a low μ (6.2×10^{-10}) as a means to explain this. These estimates are extremely low and difficult to reconcile with ours. We measured 5.3× as much variation at synonymous sites than the genome-wide estimate in *E. superba* (1.3% vs. 0.25%), or in coding regions overall (0.88% vs. 0.17%). Insight into genomic variation in euphausiids is still limited, as is baseline data from sources other than next-generation sequencing. Papot et al. (2016) studied variation in 3 PCR-amplified paralogs (A–C) of the nuclear *hsp70* in *E. superba* and *E. crystallorophias*, and our re-analysis of their *E. superba* data gave π_S ranging between 1.4% and 5.0% ($\pi_{S(A)} = 1.4\%$; $\pi_{S(B)} = 5.0\%$; $\pi_{S(C)} = 1.8\%$), closer to our average estimate across 10,777 genes (1.3%). Moreover, Shao et al. (2023) derived low μ assuming a molecular clock based on their estimate of 19.5% divergence from the crab *Eriocheir sinensis*. We estimated distances at synonymous sites (*dS*) between *E. superba* and 13 other *Euphausia* species to be 27 ± 7% (Table S8). This greatly exceeds the distance to the crab, suggesting that their estimate may be downward constrained by unusually conserved alignments. While we do not exclude the possibility of low or variable mutation rates among genomic regions and species, comparable statistics for less constrained sites (e.g. π_S and *dS*) are not available, which calls for additional studies of mutation rate in krill.

Other factors may underlie low N_e/N_c ratios, such as highly skewed reproductive success among individuals, population size changes, linked selection that reduces nearby variation or constantly shifting adaptive peaks (Hedgecock and Pudovkin 2011; Charlesworth and Jensen 2022; Árnason et al. 2023), all of which could shift allele frequencies out of Hardy-Weinberg equilibrium. A recent study in the Atlantic cod found compelling genomic evidence of skewed reproductive success driven by pervasive and recurring selection (Árnason et al. 2023), evidenced for instance by genome-wide negative Tajima's D (D_T). We found strongly negative D_T in all krill and that π is depleted at UTRs and non-synonymous sites, consistent with purifying and linked selection across functional genomic regions. *E. triacantha*, the least diverse species ($\pi_S = 1.1\%$), has the lowest D_T (−1.9), indicating recent population expansion. Likewise, our patterns of variation in *E. superba* (D_T of −1.5) and others (Shao et al. 2023) suggest that it too has likely expanded from a recent bottleneck.

Genome-Wide Assays Provide New Insights into Euphausiid Biodiversity

For Euphausiacea, there are currently 86 described and recognized species, and no new species has been described since 1987 (Baker et al. 1990). However, cryptic variation was reported within *Stylocheiron affine*, with a divergence level of 14% (*mtCOI*) differentiating Red Sea individuals from their Atlantic congeners (Wiebe et al. 2016; Bucklin et al. 2021a), a gap on-par with interspecific levels of divergence in the genus (Bucklin et al. 2007). Another example lies between *E. similis* and its variety *armata* (Hansen 1911), whose specific taxonomic status remains unclear. We detected high mitochondrial divergence between them that co-segregated with the main diagnostic trait. The *armata* variety was first described by Hansen (1911) as differing from *E. similis* by: "[…] *a protruding, acute process on the third abdominal segment*". John (1936) added that "[…] *the process may be variable in size and shape; it varies from being low, rounded and inconspicuous to being a large compressed spine pointing backwards over the fourth segment*", described slight differences in the antennules and male copulatory organs and proposed that reproductively mature *armata* can be smaller than *E. similis*. Mauchline and Fisher (1969) distinguished *armata* as living nearer the surface than *E. similis*. Our morphological assessment focused only on the process on the third abdominal segment and we reported a distinctive process in *armata* with a variable degree of development, in line with John's findings (1936).

We estimated 6.2% of mitochondrial divergence (*mtCOI*) between *E. similis* and *armata*, above the within-species level of variation reported for the genus (average 2.5%) (Bucklin et al. 2007), but below the between-species level (average 16.5%), making it inconclusive. By assessing variation across the nuclear genomes, we found 36,930 SNPs fixed between *E. similis* and *armata* (with $F_{ST} = 1$) across 4,011 genes. These are enriched for terms relating to roles in oocyte development and chromosome

segregation (Table S11), which could indicate barriers to gene flow (Hamaguchi and Sakaizumi 1992; Montecinos et al. 2017; Boynton et al. 2018). Our average estimate of genome-wide F_{ST} between *E. similis* and *armata* (F_{ST} = 0.65) was nearly as high as the one between the species pair *E. longirostris* and *E. spinifera* (F_{ST} = 0.7). This is well above the genome-wide F_{ST} value of 0.41 reported between the most differentiated lineages of the pteropod *Limacina bulimoides* identified by Choo et al. (2023) and proposed as distinct species. Moreover, we found 1.9% of net synonymous divergence (*Da*) between *E. similis* and *armata*, nearly reaching the 2% upper limit of the "grey zone" of speciation, beyond which 2 species are expected to have reached reproductive isolation (Roux et al. 2016; De Jode et al. 2023). In comparison, *Da* was slightly lower with 1.89% between the 2 recognized species *E. longirostris* and *E. spinifera*. Taken together, the evidence motivates us to elevate the *armata* variety to species. *Euphausia similis* and *Euphausia armata* manifest different levels of genetic variation and rates of adaptation. Failure to recognize them as distinct species would underestimate krill biodiversity, inflate intraspecific structure, and skew our understanding of adaptive processes in these species.

The Genetic Basis of Ecological Adaptation in Krill

The environmental changes impacting polar habitats and krill stocks necessitate better insight into how thermal tolerance varies among species and what genetic mechanisms may contribute to adaptation. By analyzing CT50 tolerance data in 6 species, including new observations from subantarctic and temperate species, we found that CT50 thresholds are correlated with the ambient temperatures of both sampling sites and species habitats. However, when accounting for the different ambient starting temperatures, *E. crystallorophias*, *E. superba*, *E. vallentini*, *E. triacantha*, and *T. inermis* show similar absolute CT50s, indicating comparable thermal tolerances beyond the temperatures experienced in their natural habitats. Only *M. norvegica* stands out with a much higher absolute CT50, which can be expected from its ubiquitous distribution (Tarling 2010). Our experiments were performed on animals collected at the colder end of their natural range, which may influence these patterns. A more comprehensive evaluation of plastic thermal tolerance could be achieved by sampling across the entire ranges of each species, including from warmer habitats. Nonetheless, our observations suggest that these krill species may not be, at least at the adult stage, as thermally sensitive and stenothermic as other polar invertebrates (Peck et al. 2014). This is consistent with the findings of experiments conducted on *E. superba* (Toullec et al. 2020), but it may not apply during other stages of their life cycle, such as larval development, where growth rates and survival were shown to start deteriorating already around +3.0 °C (Atkinson et al. 2006; Perry et al. 2020). The observed patterns might reflect either adaptation or acclimation, or possibly both, of krill species to their respective thermal environments. Common-garden experiments would be required to distinguish between acclimatory and adaptive differences among species (e.g. Ljungfeldt et al. 2014; Posavi et al. 2020; Sasaki and Dam 2020). Regrettably, keeping pelagic krill alive and healthy over the course of several generations in an experimental setting to study genetic adaptation is logistically prohibitive. Hence, we used comparative transcriptomics to search for adaptive divergences among krill species.

We compared molecular evolution in Southern Ocean krill inhabiting cold waters and the Polar Front against tropical–temperate species with the aim to detect candidate genes that may have key roles in thermal adaptation. While candidates related to diverse physiological functions, they appeared to be enriched for ion transmembrane transport and homeostasis (Fig. 5; Table S7), functions that were also selectively responsive in copepods (Brennan et al. 2022a). Cold and energy constrained environments affect both enzyme activities and the viscosities of lipid membranes, putting pressure on membrane pumps to uphold ion gradients and essential physiology (Cossins et al. 1995; Pörtner et al. 1998). We detected signatures of selection in several membrane transporters, including *Atpα* that encodes an integral membrane cation antiporter protein (Na^+/K^+ ATPase) and *Calx* (NCX) that encodes a Na^+/Ca^{2+} pump, which have previously been implied in roles in cold-adaptation in Antarctic ectotherms or cold-response across eukaryotes (Galarza-Muñoz et al. 2011; Kon et al. 2021). We also identified multiple candidates involved in thermosensation, including a *TrpA1* homolog. TRP genes encode Transient receptor potential (TRP) Ca^{2+} ion channels, which are considered a "molecular toolkit for thermosensory adaptations" in animals (Hoffstaetter et al. 2018). TrpA1 is associated with harmful heat signaling and heat avoidance behaviors in both invertebrate and vertebrate ectotherms (Akashi 2021; Xiao and Xu 2021), as well as cold sensation in a wide range of animals (Akashi 2021; Zhang et al. 2022). In Southern Ocean notothenioid fish, *TrpA1* has been hypothesized to evolve through duplication and positive selection and be one of the main thermosensors underlying adaptation to cold Antarctic waters (York and Zakon 2022). In addition, we detected potentially adaptive substitutions across genes encoding chaperonin-containing TCP1 (CCTs), which are eukaryotic "cold-shock" proteins and are overexpressed during cold stress (Somer et al. 2002). In notothenioid fish, CCTs may have undergone adaptive evolution to accomplish protein folding in cold and energy-depleted environments but are also upregulated from heat stress (Pucciarelli et al. 2006; Cuellar et al. 2014). Evolutionary change in ion channels, thermosensory genes, and protective proteins could be essential for adaptation to cold. However, the specific functions of these candidate genes are not known in krill, and some identified substitutions may have evolved through nonadaptive processes. The candidates detected here are promising but functional validation is necessary to confirm their roles in adaptation.

Our results suggest that homologous genes may independently have been targeted by natural selection across Antarctic marine taxa, including krill, fish, and octopi, consistent with some degree of convergence in this extreme environment. The independent evolution of similar antifreeze glycoproteins in Antarctic notothenioid fish and Arctic cod (Chen et al. 1997) points to the possibility for adaptive convergence also between taxa of opposite polar regions. However, when analyzing Southern Ocean *Euphausia* and the mostly Arctic-boreal *Thysanoessa* together, we found little support for widespread signatures of adaptive convergence. Genetic adaptation to cold environments may instead have entailed different genes and pathways in the different groups.

On Adaptive Potential in Antarctic Krill

The potential for genetic adaptation ultimately depends on access to genetic variation and favorable demographics, which may vary significantly among species. Empirical studies of protein evolution have indicated that few substitutions among apes have been fixed through positive selection ($\alpha = 0$ to 0.3) (Hvilsom et al. 2012; Galtier 2016), while high α in organisms like *Drosophila* ($\alpha \asymp 0.5$) and sea squirts ($\alpha \asymp 0.8$) indicate strong influence of positive selection (Smith and Eyre-Walker 2002; Tsagkogeorga et al. 2012). Analyses have generally uncovered higher α in invertebrates than in vertebrates and found that adaptive evolution is limited by the supply of variation in low-N_e species (Galtier 2016; Rousselle et al. 2020). Among the 8 species of krill examined here, we found that the proportion of adaptive protein evolution (α) is only 0.40 ± 0.11 (mean $\pm$ SD), suggesting that a majority of amino acid substitutions between species have been fixed through processes other than adaptation, such as genetic drift. Compared to other arthropods, most krill have low α. For example, Galtier (2016) previously estimated α to 0.62 ± 0.13 among 11 arthropod species.

Genetic diversity and life history traits (indicators of N_e), as well as habitat characteristics, predict rates of adaptive protein evolution in krill. Our results indicate that α scales with π_S, but may be more strongly influenced by the high rate of fixation of nonadaptive variants in small populations than the fixation rate of adaptive variants, as also seen in other taxa (Galtier 2016; Moutinho et al. 2020). Nonadaptive processes may contribute more to protein evolution in the Southern Ocean species *E. triacantha* ($\alpha = 0.30$) and *E. superba* ($\alpha = 0.20$). In these 2 species the ratios between *dN/dS* (ω) and *pN/pS* are only 1.10× and 1.01×, respectively, whereas in other krill they range from 1.29× to 1.83×. In addition to comparably low π_S, these 2 species share "K-selected" traits such as large bodies and larvae and longevities of several years, suggestive of life histories associated with low long-term N_e. This suggests that at least in some krill, rates of adaptation may be constrained by effective population size. We find no clear association between Tajima's D and α, ω_A, or ω_{NA}, suggesting that adaptive rates may be unrelated to recent demographic events. However, this does not exclude the possibility that long-term fluctuations in N_e may have influenced these estimates (Rousselle et al. 2018), possibly inflating α or ω_A in short-lived species. An alternative possibility could be that slow rates of adaptation in the Antarctic krill are due to flat environmental gradients and slow rates of environmental change. The Antarctic Circumpolar Current has been a barrier to warm waters and long maintained cold and stable Antarctic conditions (Clarke et al. 1992). Simulations suggest that the rate of environmental change strongly affects adaptive rates (Lourenço et al. 2013). By increasing sample sizes and specifically also analyzing small-bodied Antarctic species such as *E. crystallorophias* and *Euphausia frigida*, it could be possible to disentangle N_e-associated variation and life histories from habitat conditions and identify the main drivers of adaptive rates in Southern Ocean krill.

Conclusions

Thorough understanding of species taxonomy is essential to accurately trace molecular evolution. The significant divergence and distinct patterns of variation uncovered here by genome-wide analyses of *E. similis* and *E. similis* var. *armata* from the Indian Ocean suggest that they are unlikely to exchange genetic material and warrant distinction as separate species.

Our investigation of krill transcriptomes revealed signatures of adaptation to the cold Southern Ocean in multiple genes, including those encoding ion channel proteins with roles in thermosensation or ion homeostasis, some of which have been implied in thermal adaptation in other Antarctic animals before. Adaptive substitutions that extend the thermal range in such locally adapted proteins could be important for fitness and resilience under future conditions (Somero 2010). However, we find that thermal adaptation in Southern Ocean species may not necessarily be representative of adaptation in the Arctic Ocean. This underscores the need to characterize adaptation in krill from many climates and locations. Comparative analyses that include many krill from very warm waters, e.g. the tropical Indian Ocean or Red Sea (Wiebe et al. 2016), have the potential to uncover mechanisms for warm-adaptation. Mapping the distribution of such functional variation among species and populations could provide important insight into how much genetic change is required to adapt to future climates, i.e. the genetic offset (Fitzpatrick and Keller 2015) and inform conservation of the Southern Ocean and beyond (Razgour et al. 2019; Capblancq et al. 2020; Gutt et al. 2021; Teixeira and Huber 2021).

Rates of adaptive protein evolution do not appear to be uniformly high among all krill, suggesting that not all zooplankton may have high adaptive potential. In particular, protein evolution in the Antarctic krill *E. superba* may have been shaped by nonadaptive processes. A combination of comparably small N_e, long generation time (2 to 3 yr) (Siegel 2000) and extensive panmixia (Shao et al. 2023) may limit its rate of adaptation, which could

indicate lower capacity to adapt to rapidly changing environments compared to other species (Peck 2011). Analyzing additional zooplankton taxa could reveal whether these ecologically important species typically exhibit high or low rates of adaptive evolution, which could help forecast how they and marine ecosystems may respond to continued climate change.

Materials and Methods

Sampling and Species Identification

Specimens of 16 krill species were collected from the North Atlantic, Indian, and Pacific Oceans using Isaacs-Kidd midwater trawls during different cruises (details in Table S1). Specimens were preserved in RNA*later* (Invitrogen) or liquid nitrogen, either at the time of collection or after being kept in aquaria at ambient sea temperatures for up to 48 h. We used dissection microscopes and keys for morphological species identification, including Baker et al. (1990). We georeferenced species ranges from Brinton et al. (2000) on a OISST v2 Sea Surface Temperature (SST) map with *QGIS* http://www.qgis.org and computed SSTs for each range from color histograms in *GIMP* https://www.gimp.org/. We compiled life history trait information from John (1936), Mauchline and Fisher (1969), Baker et al. (1990) and Brinton et al. (2000).

CT50 Experiments

Experiments were performed using live *E. triacantha* and *E. vallentini* samples from the Indian Ocean and *M. norvegica* from the Arctic Ocean following Cascella et al. (2015). After acclimation for 24 h at ambient sea temperatures, actively swimming animals were transferred to an experimental tank. Temperature was increased by 1 °C every 10 min. Animals were maintained in the tank until they no longer responded to tactile stimuli of a probing rod, at which point we considered specimens to have reached their critical temperatures. The CT50 was determined through the nonlinear curve fitting option in Prism9 (GraphPad Software, LLC). The mobility/survival curve used was: Survival = c/(1 + (T/CT50), where c is the plateau value before the sharp decrease and CT50 is the threshold temperature at which only 50% of animals are mobile. The program explores the different parameter values and calculates 95% confidence intervals.

Extraction and Sequencing of RNA

For *M. norvegica* and samples collected from the Indian Ocean in 2019, we extracted RNA from abdominal muscle, using the Qiagen RNeasy Plant Mini Kit and following the manufacturer's protocol. The gut was removed to avoid contaminants. Thermo Scientific NanoDrop and Agilent Technologies 2200 TapeStation instruments were used to measure yield, purity and RNA integrity number (RIN)-values. Samples with RIN > 8 were provided (without DNase treatment) to Science for Life Laboratory (Sweden) for the preparation of 94 RNA-seq libraries using Illumina TruSeq Stranded mRNA Library Prep kit with polyA selection. Paired-end libraries (2 × 150 bp) were sequenced on an Illumina NovaSeq 6000 S4 lane. Other samples were prepared as in Huenerlage et al. (2016). In addition, we downloaded and re-used published RNA-seq libraries for the Atlantic *T. inermis* (Huenerlage et al. 2016), the Southern Ocean Ice krill *E. crystallorophias* (Toullec et al. 2013) and for the Antarctic krill *E. superba* from Höring et al. (2021) (Table S1).

Molecular Species Validation

For each library, we queried 1 M forward reads against all krill *Cytochrome c oxidase I* (*COI*) reference barcodes in the MetaZooGene database (MZGDB v3) ($n = 3{,}003$ sequences from 64 fully identified species using *BLASTN*; Tan et al. 2006; Camacho et al. 2009; Bucklin et al. 2021b) and recorded best hits. For barcodes with ≥20 hits, we computed the mean identity scores. We used the barcode with the highest mean score as indication of species.

RNA Trimming, Assembly, and Annotation

We used *Trim Galore!* v0.6.1 https://github.com/FelixKrueger/TrimGalore/and *Cutadapt* v2.3 (Martin 2011) to trim low quality bases (phred < 20) and reads shorter than 50 bp. *Trinity* v2.11.0 (Grabherr et al. 2011) was used with default settings to assemble 1 "reference" transcriptome per species ($n = 19$). We pooled reads from up to 5 specimens to maximize gene completeness, as this produced on average 1.1 to 1.3× as many complete BUSCO genes compared to a single large (44 to 133 M read pairs) or small library (~22 M read pairs). The *Thysanoessa raschii* transcriptome was assembled as in *T. inermis* in Huenerlage et al. (2016). We used a Trinity script to reduce redundancy and keep only the longest splice isoform per gene, *TransDecoder* v5.5.0 https://github.com/TransDecoder/TransDecoder to identify protein-coding transcripts and *TransDecoder.LongOrfs* to detect open reading frames (ORFs) > 300 bp. ORFs were queried for domain homology against Swissprot with BLASTP v2.9.0+ (Camacho et al. 2009) (e-value cutoff $1e^{-5}$) and Pfam (release 34.0) with HMMER3 *hmmscan* v3.3 http://hmmer.org/. We used *TransDecoder.Predict* to identify coordinates for UTRs and coding sequence and *KaKs_Calculator* (Zhang et al. 2006) to enumerate synonymous and non-synonymous sites. Transcriptome completeness was assessed with *BUSCO* v3.0.2b (Simão et al. 2015) and the odb9 arthropod lineage set. We annotated the transcripts using queries against the *Drosophila* FlyBase database (dmel_r6.38) (Larkin et al. 2021) with *DIAMOND* v9.9.0 (Buchfink et al. 2015).

Calling Single Nucleotide Polymorphisms (SNPs)

We measured genetic variation for 9 species/lineages with multiple RNA-seq libraries (Table 1) from SNPs (3 to 20 libraries per species; $n = 113$ in total). We first mapped the trimmed RNA libraries to their respective reference transcriptomes using BWA-MEM v0.7.17-r1188 (Li, unpublished data). Alignments were cleaned with *samtools* v1.14 (Danecek

et al. 2021) to retain reads mapping once and with concordant pairing. Duplicates were removed with *Picard* v2.23.4 http://broadinstitute.github.io/picard/. Variants were called per individual with *GATK* HaplotypeCaller v4.2.0.0 and combined into 1 multi-sample gVCF per species with CombineGVCFs, before joint genotyping with GenotypeGVCFs. We used *vcftools* v0.1.16 (Danecek et al. 2011) to retain only biallelic SNPs genotyped by ≥5 reads at sites with a mean read depth of ≥5× across all individuals.

Inferences of Population Structure with PCA and Admixture

We produced subsets of unlinked high-quality SNPs to assess genetic population structure within each species from the Indian Ocean. Using *vcftools*, we kept SNPs present in at least 80% of the genotypes, with phred > 30 and a minor allele count ≥3 (Linck and Battey 2019). We pruned SNPs based on linkage-disequilibrium using *Plink* v.1.90 www.cog-genomics.org/plink/1.9/(Chang et al. 2015), proceeding in windows of 50 SNPs, sliding by 10 SNPs at a time and with a r^2 threshold of 0.8. We then performed PCA with *Plink* and ancestry analyses with *ADMIXTURE* v.1.3.0 (Alexander et al. 2009), using 2 – N ancestral clusters (K) (N = number of sampling sites per species).

Divergence between *E. similis* ("*similis*") and *E. similis* var. *armata* ("*armata*")

We uncovered extreme structure in a joint SNP call for *similis* and *armata* and investigated this further. First, we estimated the weighted F_{ST} (Weir and Cockerham 1984) between the 2 from 1,000 unique combinations of SNPs comprising 1 SNP randomly drawn per gene, using a custom Perl script from Choquet et al. (2019). To compare with F_{ST} among other sister species, we called SNPs jointly among *similis*, *armata*, *E. longirostris* and *E. spinifera* and estimated pairwise F_{ST} using the same method. We also calculated the net synonymous divergence *Da* (Roux et al. 2016) between *similis* and *armata*, and between *E. longirostris* and *E. spinifera* (Supplementary Material online). Secondly, we assembled mitochondrial *COI* sequences of *similis* and *armata* to assess divergence. RNA-seq reads with best hits against *E. similis* accessions (AF177186, MW210878, or MW210879) were extracted and assembled de novo into *COI* fragments using *SPAdes* v3.15.5 (Prjibelski et al. 2020). The fragments and the barcodes were aligned using *MAFFT* v7.453 (Katoh and Standley 2013). A pairwise distance matrix and neighbor-joining tree was inferred using *SplitsTree* v4.18.2 (Huson and Bryant 2006). Thirdly, we re-assessed the morphology of the samples genetically matching *similis* or *armata*. Using a Leica MZ8 stereomicroscope and Olympus TG-5 camera, we observed and photographed the tails of our best-preserved specimens, including 8 *E. similis* specimens and 7 from *E. similis* var. *armata*. We focused on characterizing the absence or presence of a dorsal accessory spine on the third abdominal segment, described as specific to *E. similis* var. *armata* (Hansen 1911; Baker et al. 1990).

Levels of Intraspecific Genetic Variation

Biallelic SNP datasets were annotated with *SNPeff* v4.3T (Cingolani et al. 2012), i.e. to be synonymous (S), non-synonymous (N), or UTR variants. We estimated variation using the population mutation rate Watterson's theta (θ_w) (Watterson 1975) separately at UTRs, S or N sites, or jointly across full genes, while accounting for accessible sites. We used *SplitsTree* to calculate transcriptome-wide pairwise genetic distance matrices among individuals and deduce π (nucleotide diversity) and BioPerl to calculate Tajima's D as an indicator of demographic history (Stajich et al. 2002). The effective population size (N_e) was calculated as $N_e = \theta_w/4\mu$, where μ is the mutation rate per bp and generation. Because mutation rates are unknown in krill, we used a μ of $2.64e^{-9}$ substitutions/site/year from snapping shrimp (Silliman et al. 2021).

Orthology and Phylogenetic Analyses

We inferred gene orthology among 20 krill species (including *E. similis* var. *armata* as distinct from *E. similis*) and 7 outgroups using *ProteinOrtho* v6.0.14 (Lechner et al. 2011) with *DIAMOND* to detect similarities. For each set of orthologs (an "orthogroup" or "OG") with ≥10 krill, we produced protein-level alignments with MAFFT, using the G-INSI-I method and the "–allowshift –unalignlevel 0.8 –leavegappyregion" variable scoring matrix settings to reduce the risk of over-aligning nonhomologous regions (Katoh and Standley 2016). Single-copy OGs with at least 18 krill species and 2 outgroups were used to make a species tree. First, we trimmed unreliably aligned positions using *Gblocks* (Castresana 2000) (settings: "-t=p -b1=N -b2=N -b4=5 -b5=h -b6=y", where N was 50% + 1 of the number of sequences) and concatenated the OGs. We performed phylogenetic inference under maximum likelihood (ML) across the concatenated data using *IQ-TREE* v2.1.0 (Minh et al. 2020) and the JTTDCMut+F +I+G4 model (chosen using BIC) (Kalyaanamoorthy et al. 2017), and for individual OGs using the JTT+I+G4 model.

Detecting Positive Selection and Candidate Genes for Cold-Adaptation

To identify genes with evidence of selection among cold-adapted krill, we used branch-site models (Yang and Nielsen 2002) in *PAML* v4.9j (Yang 2007) via *ETE Toolkit*. We compared the non-synonymous substitution rate (*dN*—a proxy for selection) against the synonymous substitution rate (*dS*—a proxy for unconstrained or neutral evolution), i.e. the *dN*/*dS* (ω) ratio, between foreground (focal) and background species. Genes with statistically significant evidence of locally elevated ω in the focal species were taken as candidates for episodic positive selection and cold-adaptation. To prepare data, we controlled for spuriously aligned or clustered sequences that may produce false-positive signals.

We removed outgroups and re-aligned all OGs with ≥10 krill species. For OGs with duplicate sequences (e.g. species-specific paralogs), we produced gene trees with *FastTree* (Price et al. 2010) and then used *OrthoSNAP* (Steenwyk et al. 2022) to split alignments into single-copy subsets, only keeping those with ≥10 species. Nucleotide sequences were fitted to the protein alignments using *PAL2NAL* (Suyama et al. 2006). We used *Gblocks* to trim unreliably aligned codons (settings: "-t=c -b5=h -b6=y"). In addition, we masked alignment fragments < 15 bp and sequences around internal indels, replacing 4 codons with gaps around each indel and removing positions where >2 species had missing data. For each OG, we made a pruned unrooted species tree using *Phyutility* (Smith and Dunn 2008). Lastly, as inference of orthology may inadvertently cluster paralogs, we compared the fit between each gene tree (IQ-TREE + HKY model) and the species tree using the normalized Robinson-Foulds distance (nRF) (Robinson and Foulds 1981; Altenhoff et al. 2020). We used the *ETE Toolkit* v3 (Huerta-Cepas et al. 2016) to estimate nRF and observed inflated ω in OGs with high nRF scores (Supplementary Fig. S8), possibly due to spurious clustering of paralogs. We therefore removed OGs with nRF > 0.4 and analyzed 10 to 11 K OGs (Table S5).

We first compared Southern Ocean *Euphausia* against all other krill species (Supplementary Fig. S9A). We then extended the set of focal species to also include the Arctic/Antarctic *Thysanoessa*, and analyzed *Euphausia* and *Thysanoessa* separately, aiming to test for convergent signatures of cold-adaptation between the 2 groups (Supplementary Fig. S9B to D). We analyzed each OG using the bsA1 null model (which models neutral or purifying evolution) and the alternative bsA model, which incorporates positive selection (Zhang et al. 2005). We then performed a LRT, taking the log-likelihood difference between models ($-2\Delta\ln L$) against a χ^2 distribution (1 df) in order to test if the null model could be rejected in favor of the model allowing positive selection ($P < 0.05$; χ^2 critical value = 3.841). As our tests involved scanning thousands of OGs, we used the Adaptive Benjamini and Hochberg method (ABH) to adjust *P*-values for multiple testing, using the R package *multtest* (Benjamini and Hochberg 2000; Pollard et al. 2005). For genes to finally be considered candidates in our main contrast, we required both a significant LRT in favor of the selective model (q-value <0.05) and detection of at least 1 positively selected site using Bayes Empirical Bayes posterior probability > 95% (Yang et al. 2005). To test for shared biological properties among candidate genes, we performed Gene Ontology (GO) enrichment tests of *Drosophila* homologs using *ShinyGO* (Ge et al. 2020), taking the candidates as the target set and all analyzed genes as the background and correcting for multiple testing.

Estimating Overall Rates of Adaptive Evolution

McDonald and Kreitman (1991) proposed that elevated *dN/dS* (ω) between species compared to polymorphisms within species (*pN/pS*) may indicate positive selection in genes. For 8 species with SNP data (*E. longirostris* was excluded due to small sample size), we compared ω to *pN/pS* across thousands of genes to estimate the overall proportion of adaptive amino acid substitutions (α), in which $\alpha = 1 - (pN/pS/\omega)$, i.e. the fraction of the observed divergence that may have been shaped by positive selection as opposed to nonadaptive processes like drift (Smith and Eyre-Walker 2002). For these tests, we estimated lineage-specific ω between the focal species and ancestral sequences (e.g. Rousselle et al. 2020). To infer those, we pruned each OG and the species trees to include only the focal species and 3 other species (Supplementary Table S9). We re-estimated branch lengths using the YN98+F1X4 model in *Bio++ bppML* v2.3.1 (Nielsen and Yang 1998; Guéguen et al. 2013), selected an ancestral node and used *Bio++ bppancestor* v2.3.1 to infer the ancestral gene sequence. The node was selected from pairwise genetic distances between species, avoiding very short distances as shared ancestral polymorphism may distort substitution estimates (Mugal et al. 2020). We estimated the numbers of synonymous and non-synonymous substitutions and sites between the focal and ancestral sequences using *KaKs_Calculator* and the "YN" method to account for unequal base frequencies, transition/transversion rate biases, and multiple substitutions (Yang and Nielsen 2000). Across the same OGs, we then made transcriptome-wide unfolded non-synonymous and synonymous site frequency spectra (SFS) from SNPs. For this, we used the ancestral sequence to polarize the SNPs: The allele shared with this sequence was considered ancestral, while the other was considered derived. This was performed with *basefinder* (a novel tool implemented here; commit adb820c) that cross-references SNP and alignment positions on either plus or minus strands. SFSs were produced from derived allele frequencies. To facilitate unbiased comparisons between species, we downsampled the population datasets to 6 or 8 individuals to have similar SFS resolutions. For each gene, individuals were selected to maximize the overall coverage and genotyped SNPs. We also recomputed π with the same data.

Slightly deleterious non-synonymous variation that is not efficiently removed by purifying selection may lead to overestimation of *pN/pS* and underestimation of α (Charlesworth and Eyre-Walker 2008; Moutinho et al. 2020). Likewise, population structure, bottlenecks, and interference between linked sites may affect fixation rates (Eyre-Walker and Keightley 2009; Messer and Petrov 2013; Galtier 2016; Al-Saffar and Hahn, unpublished data). We therefore used unfolded site frequency spectra of synonymous variants to model demographic history and the distribution of fitness effects among non-synonymous variants to derive more accurate estimates of α as well as the respective rates of adaptive or nonadaptive protein evolution (ω_A and ω_{NA}) (Galtier 2016; Moutinho et al. 2020; Al-Saffar and Hahn, unpublished data). We used the model-based program *grapes* (Galtier 2016) and both nonparametric methods or models originally from *DoFE*

(Eyre-Walker et al. 2006; Eyre-Walker and Keightley 2009) to estimate the SFS and divergence data. The "Basic" method derives the statistics by comparing *dN*/*dS* to *pN*/*pS* without correction, while "FWW" removes non-synonymous SNPs segregating at low frequencies (<15%) (Fay et al. 2001). In addition, we explored 6 model-based methods that estimate population demographics and fitness effects under maximum likelihood (see Galtier 2016 and Al-Saffar and Hahn, unpublished data for details). For each species and method, we ran *grapes* 10 times with random initial values ("-nb_rand_start 10"). For our comparisons of α, ω_A, and ω_{NA} among species, we used the Gamma-Zero model, which fitted a gamma distribution for neutral and deleterious mutations to the data and produced estimates similar to Displaced-Gamma. Both models behaved consistently and were considered accurate in tests (Al-Saffar and Hahn, unpublished data).

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Author Contributions

A.W. and J.-Y.T. conceived and designed the study. A.W., J.-Y.T., A.C., G.T., M.C., and E.C. performed data collection in the field, lab work, or data curation. F.L. contributed software. A.W., M.C., and J.-Y.T. analyzed the data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflict of interest statement. None declared.

Data Availability

The new sequence data underlying this article are available in the ENA at https://www.ebi.ac.uk/ena/browser/home (accession PRJEB61645). SNP and orthology datasets are available in the SciLifeLab Data Repository at https://doi.org/10.17044/scilifelab.c.6620560.

Code Availability

Code for basefinder is available on Github: https://github.com/fellen31/basefinder.

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