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**Original Article**



# Limited interspecific gene flow in the evolutionary history of the icefish genus *Chionodraco*

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## Abstract

Hybridization and introgression are recognized as mechanisms promoting genetic variability during evolutionary radiations. We examined the impact of introgression in the process of speciation, focusing on the Antarctic icefish genus *Chionodraco*. Our analyses confirmed that the three *Chionodraco* species (*Chionodraco hamatus*, *Chionodraco myersi*, and *Chionodraco rastrospinosus*) were genetically distinctive, despite signals of past interspecific gene flow between *C. hamatus* and *C. myersi* that likely occurred during interglacial periods. However, in this study, no recent hybrids were identified. The lack of contemporary hybridization may be due to life-history traits and the type of marker used in the analysis. Our study emphasizes the importance of genomic approaches to detect subtle patterns of past hybridization accurately and highlights the significance of historical climate events in the demographic and evolutionary history of Antarctic notothenioids. Polar regions, and especially the Antarctic Peninsula, are now experiencing the fastest climate changes due to global warming. Understanding the impact of past climate events is fundamental to trace current modifications in species' genetic variability and distributions and predict future evolutionary trajectories. This knowledge is also vital for conservation efforts, including the implementation of marine protected areas.

*Keywords:* hybridization; incomplete lineage sorting; introgression; notothenioidei; RADseq; Southern Ocean

## Introduction

Adaptive radiations represent ideal systems to study the processes that drive speciation. In these situations, new ecological opportunities allow a single ancestral lineage to diversify rapidly (Schluter 2000). Such ecological opportunities can arise for a variety of reasons, including availability of new habitats (e.g. after geological events like tectonic movements or volcanic eruptions), when pre-existing species and competitors go extinct, or when new phenotypes evolve (Schluter 2000, Yoder et al. 2010). The new favourable conditions allow demographic expansion and the relaxation of natural selection, which, in turn, permits an increase in genetic variability, fuelling diversification, and speciation (Naciri and Linder 2020). Increasing evidence demonstrates that hybridization and introgression (the genetic exchange over time between species through hybridization) have played a significant role in adaptive radiations, acting as a mechanism to promote genetic variability during the evolutionary history of many taxa (Abbott et al. 2013, Taylor and Larson 2019).

Cryonotothenioidea, a clade of the suborder Notothenioidei that lives primarily in Antarctic waters, provides a model to investigate the role of hybridization and introgression in promoting adaptation and speciation (Dornburg et al. 2017, Near et al. 2018). Cryonotothenioids are one of the few known examples of adaptive radiation in the marine realm, characterized by exceptionally high rates of species formation compared to most temperate and tropical ray-finned fish taxa (cryonotothenioids include >120 species; Rabosky et al. 2018, Eastman and Eakin 2021). During their rapid radiation over the past 10 million years (Myr; Bista et al. 2023), cryonotothenioids have developed an astonishingly large variety of physiological, ecological, behavioural, morphological, and life-history characteristics. Perhaps the most important among these for their survival are antifreeze glycoproteins (AFGP),

protecting blood and tissues from freezing in <0°C sea water (DeVries and Cheng 2005).

Hybridization has been suggested as a mechanism that has influenced the evolution of the most derived cryonotothenioid family, the Channichthyidae (icefish; Near et al. 2006). While most of the species in this family lack haemoglobin genes, Near et al. (2006) found that *Neopagetopsis ionah* possesses a complete, but non-functional, adult $\alpha\beta$-globin complex. This observation led the authors to hypothesize that interspecific hybridization between divergent Antarctic notothenioid lineages occurred prior to the loss of haemoglobin in icefish and caused the two types of present genomic configuration (an $\alpha$-globin pseudogene in most icefish species and the inactive $\alpha\beta$-globin complex of *N. ionah*; Near et al. 2006). The potential for interspecific breeding, and a permeability of post-zygotic barriers in icefish, was demonstrated by Desvignes et al. (2019) who fertilized *in vitro* eggs from *Chaenocephalus aceratus* with sperm from *Chionodraco rastrospinosus*, obtaining viable larvae up to about four and a half months of development.

Marino et al. (2013) and Schiavon et al. (2021) suggested the occurrence of past and contemporary hybridization and introgression between icefish species of the genus *Chionodraco* (Channichthyidae). Using a panel of 18 microsatellite loci, both studies identified some individuals as putative hybrids. The genus includes three species: *C. hamatus*, *C. myersi*, and *C. rastrospinosus* (Eastman and Eakin 2021). Their current geographic distributions indicate that secondary contacts after allopatric isolation may have represented opportunities for interspecific hybridization (Schiavon et al. 2021). *Chionodraco hamatus* and *C. myersi* have a circumpolar distribution, with the exclusion of the Antarctic Peninsula, and have largely overlapping depth ranges (4–972 m and 99–926 m, respectively; Gon and Heemstra 1990, Eastman 2017). *Chionodraco rastrospinosus*, traditionally described as distributed only off the Antarctic Peninsula and around the islands of the Scotia Arc (South Shetlands, Elephant Island, and South Orkneys, at depths between 200 and 1000 m; Gon and Heemstra 1990, Eastman 2017), was recently reported also from the Weddell Sea (Schiavon et al. 2021). The three species are phylogenetically very close as the genus began to diverge ~2 million years ago (Mya) (Near et al. 2012). Forming such a young group, *Chionodraco* species may be particularly informative in speciation research because interspecific differences could be attributed with some confidence to evolutionary processes operating during recent speciation (Coyne and Orr 2004).

Marino et al. (2013) and Schiavon et al. (2021) studied the occurrence of hybridization among the *Chionodraco* icefish species and posed the following questions: What are the historical patterns of gene flow among the three species? Were they impacted by environmental conditions? And can these patterns be useful to define future scenarios of interspecific gene flow in icefish in a climate change scenario?

The power to resolve these questions was limited by two factors. First, Schiavon et al. (2021) could not unambiguously distinguish between pure and admixed specimens, especially between F1 putative hybrids and backcrosses. Second, Schiavon et al. (2021) reanalysed all individuals considered in Marino et al. (2013) and found a discrepancy in the assignment of individuals as pure or introgressed. None of the individuals identified as hybrids by Marino et al. (2013) appeared as hybrids or introgressed in the larger dataset of microsatellite genotypes by Schiavon et al. (2021). Both Marino et al. (2013) and Schiavon et al. (2021) suggested that past hybridization and introgression may have left little signal in the genomes of *Chionodraco* spp., but the use of microsatellites may have limited the power to detect these signals. Given their large mutation rate, microsatellites show considerable allelic variability, and have been successfully used to study recent interspecific hybridization and introgression (Allendorf et al. 2001, Vähä and Primmer 2006). However, the small number of markers that are usually genotyped limits their applicability. This also combines with general problems related to the detection of hybridization and reconstruction of introgression events between species, like the presence of incomplete lineage sorting (ILS) or delayed coalescent (Tiley et al. 2023). These limitations suggest the need for many markers, such as single nucleotide polymorphisms (SNPs), to increase the resolution of hybridization dynamics, their intertwining with the evolutionary history of the species and the location of regions of the genome involved in the process (e.g. in eels, Barth et al. 2020, in rainbowfish, Brauer et al. 2023). SNPs can reveal ancestral patterns of differentiation (Zhang and Hewitt 2003, Andrews et al. 2016) as well as subtle signals of interspecific admixture.

Therefore, we have adopted a genomic approach based on restriction enzyme-associated DNA-sequencing (RADseq, routinely used to isolate thousands of SNPs; Baird et al. 2008) to confirm the occurrence of past hybridization and the possible identification of hybrid individuals within the genus *Chionodraco*. We also investigated how hybridization may have influenced the relationships among the three *Chionodraco* species in relation to the paleoclimatic variability during species divergence, by testing alternative evolutionary scenarios with various intensities and timings of introgression.

## Materials and methods

### Samples

The samples used in this work were chosen from a previous study by Schiavon et al. (2021) (Table 1). We chose both putatively admixed and pure (unadmixed) individuals based on the microsatellite and D-loop datasets of Schiavon et al. (2021). Individuals were considered pure if their Q-value (membership coefficient: probability that an individual belongs to a certain group–species in our case) was >0.95 and as admixed individuals otherwise (Marino et al. 2013).

Samples of *C. hamatus* and *C. myersi* were collected from the Weddell Sea (Fig. 1; samples from Schiavon et al. 2021). Samples of *C. rastrospinosus* analysed in this study belong to four main geographic areas: South Shetland and Elephant Islands (on the western side and north of the Antarctic Peninsula; both grouped here as 'Antarctic Peninsula' for brevity), Antarctic Sound (on the eastern side of the Antarctic Peninsula), Weddell Sea, and South Orkney Islands (Fig. 1; samples from Schiavon et al. 2021). *Chaenodraco wilsoni*, a geographically widespread sister species of the genus *Chionodraco* (Near et al. 2012), was used as outgroup and specimens were collected from the Weddell Sea and the Antarctic Peninsula (Fig. 1).

The dataset analysed in this study included 65 specimens of the 3 *Chionodraco* species (Table 1): 14 *C. hamatus* (12 pure + 2 putatively admixed with major contribution to their genetic variability from *C. hamatus*); 13 *C. myersi* (10 pure + 2 putatively hybrids as defined in Schiavon et al. 2021,

**Table 1.** List of samples used in this study.

| Species | Cruises | Location | Sample size |
|---|---|---|---|
| *C. hamatus* | PS82 (2014) and PS96 (2016) | Weddell Sea | 14 |
| *C. myersi* | PS82 (2014) | Weddell Sea | 13 |
| *C. rastrospinosus* | ANT XIX/3 (2002) | Antarctic Peninsula# | 11 |
| | PS112 (2018) | | |
| | PS112 (2018) | Antarctic Sound | 7 |
| | ANT XXVII/3 (2011) | South Orkneys | 11 |
| | PS82 (2014) | Weddell Sea | 9 |
| | Total | | 38 |
| *C. wilsoni* | ANT XXVIII/4 (2012) | Antarctic Peninsula# | 10 |
| | PS82 (2014) | Weddell Sea | 10 |
| | Total | | 20 |
| | **Total dataset** | | 85 |

The table reports species names, sampling cruises, general area of sampling (location), and sample size. All samples were collected in collaboration with the Alfred Wegner Institute Helmholtz Centre for Polar and Marine Research (AWI, Bremerhaven, Germany) during R/V Polarstern cruises. #Antarctic Peninsula indicates: South Shetlands and Elephant Island.

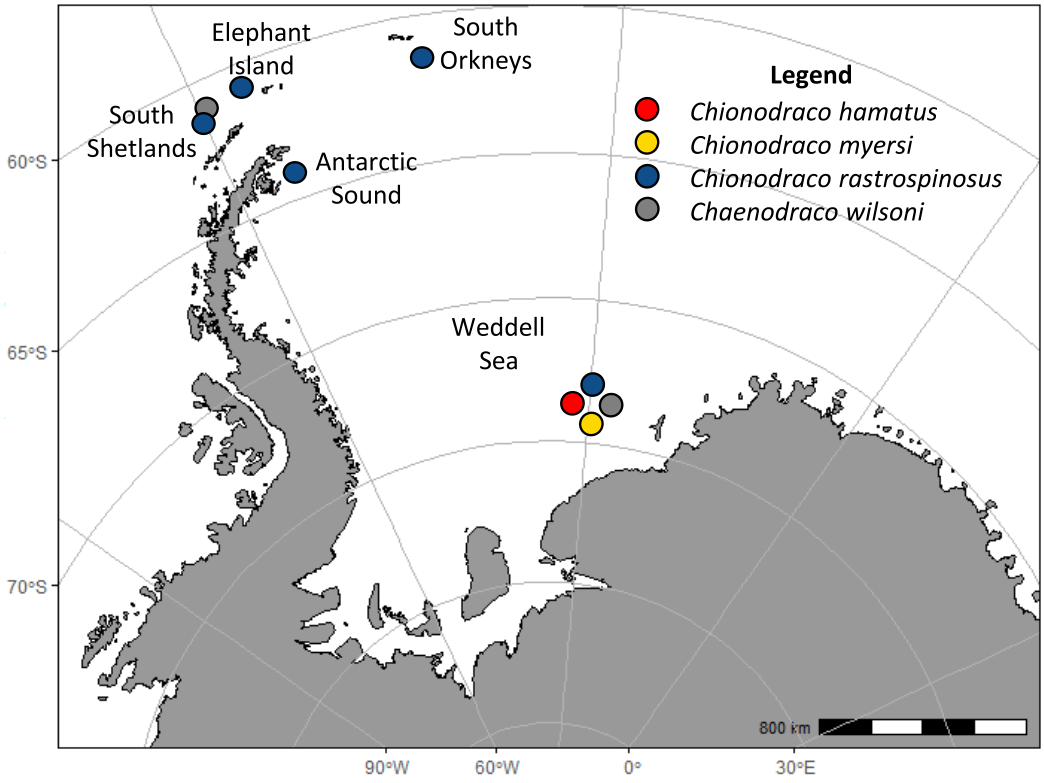


**Figure 1.** Map of sampling sites around the Antarctic Peninsula and in the Weddell Sea. Dots indicate the average locations of sampling for the four species analysed in this study. Map drawn with the R package ggOceanMaps 1.1 (Vihtakari 2024) and modified manually.

and 1 putatively admixed with major genetic contribution from *C. myersi*); and 38 *C. rastrospinosus* (32 pure + 6 putatively admixed with major contribution to their genetic variability from *C. rastrospinosus*). The dataset also included 20 *C. wilsoni*. The following sections report a summary of the methods; see Supplementary Materials for details (section 'Extended Materials and Methods').

## RADseq and filtering

DNA extraction, integrity check, and concentration measurement followed Pardo et al. (2005) and Schiavon et al. (2021). Preparation of the RADseq library using the single restriction enzyme *sbfI* followed Etter et al. (2011) with modifications as described in Ceballos et al. (2019). Paired-end sequencing with a read length of 150 bp was conducted on the Illumina HiSeq4000 platform at the Genomics and Cell Characterization Core Facility (GC3F, University of Oregon, USA). Raw reads were demultiplexed with Stacks 2.53 (Rochette et al. 2019), aligned to the genome of *C. myersi* (Bargelloni et al. 2019), using BWA 0.7.17 (Li and Durbin 2009), and SNPs were called with Stacks 2.53.

Given that *C. rastrospinosus* specimens were collected in different locations, we performed a preliminary assessment of population structure for this species. We repeated the SNP calling as reported in Supplementary Material, but only

with *C. rastrospinosus* to maximize the number of called SNPs.

We prepared datasets for all downstream analysis with VCFtools 0.1.17 (Danecek et al. 2011). Datasets were generated according to the guidelines and properties of the different software as described in the next sections, in Supplementary Table S1 and Supplementary Fig. S1, and in the Supplementary Material.

### Genomic variation, hybrid detection, and introgression

We first estimated the degree of differentiation at the intra- and inter-specific scales to investigate patterns of admixture among species (Supplementary Table S1). We considered samples collected from different geographic sites for a more accurate assessment of interspecific gene flow, whereas patterns of population differentiation will be investigated in a separate study (not discussed here). The relative positions of species (and population samples for *C. rastrospinosus*, given that this species was sampled in more than one geographic location) were visualized in multidimensional genetic space by principal coordinate analysis (PCoA) as implemented in 'adegenet' 2.1.3 (Jombart 2008). Shared coancestry between species was analysed with fineRADstructure (Malinsky et al. 2018) (Supplementary Table S1).

We first applied fineRADstructure and the PCoA to all *Chionodraco* species to identify individuals with putative admixed ancestry. Hence, an unsupervised hierarchical clustering was performed using Admixture 1.3.0 (Alexander et al. 2009). Admixture was run to estimate individual ancestry proportions ($Q$) with cross-validation assuming a value of $K = 2$–11 for the estimation of the optimal number of clusters.

Since PCoA, Admixture, and fineRADstructure do not explicitly fit a historical model and do not allow the inference of number of historical admixture events (Malinsky et al. 2020), we used TreeMix 1.13 (Pickrell and Pritchard 2012) (Supplementary Table S1) to examine introgression between branches of the *Chionodraco* phylogeny, using *C. wilsoni* as outgroup. The method implemented in TreeMix first infers a maximum likelihood (ML) tree to model the relationship among population/species. Then, it adds migration edges between branches that poorly fit to the tree (Brauer et al. 2023).

To assess gene flow between *Chionodraco* species and estimate the proportion of the genome affected by introgression, Patterson's $D$ (ABBA-BABA test) and the $f_4$-ratio statistics were calculated as formal tests for past introgresson in Dsuite (Malinsky et al. 2020) (Supplementary Table S1) and the function *Dtrios*.

Additional analyses with TreeMix (testing 1–10 migration edges) and Dsuite (Supplementary Table S1) were applied to test if certain population samples of *C. hamatus* and *C. rastrospinosus* were more involved in introgression events than others.

To understand whether all genomic regions were equally affected by introgression, we ordered and oriented *C. myersi* scaffolds (not assembled at chromosome level, Bargelloni et al. 2019) into pseudo-chromosomes according to chromosome subdivision in *Dissostichus mawsoni* (Lee et al. 2021, same number of chromosomes as *Chionodraco* spp., Ozouf-Costaz 1987) using RagTag (Alonge et al. 2022) and repeated SNP calling and Dsuite analysis (Supplementary Table S1).

### Phylogenetic reconstruction and demography

To further investigate the relationships among *Chionodraco* species, to reconstruct the species demographics, and to account for the presence of ILS as detected with Dsuite (see Results), we applied Bayesian phylogenetic inference by using the multispecies coalescent model implemented in SNAPP 1.3 (Bryant et al. 2012) for BEAST2 2.6.3 (Bouckaert et al. 2014) (Supplementary Table S1).

Based on the results from Dsuite and SNAPP, we investigated scenarios of gene flow that may have shaped the evolutionary history of the genus *Chionodraco* using fastsimcoal 2.6 (Excoffier et al. 2013) (Supplementary Table S1). The principle behind fastsimcoal is that different patterns of gene flow and demographic events shape the distribution of allele frequencies at polymorphic loci in one or several populations in specific ways (e.g. two populations will have more similar allele frequencies if they are strongly connected or recently split; Bourgeois and Warren 2021). Three population genetics models were tested: (i) complete isolation with no gene flow; (ii) continuous gene flow between *C. hamatus* and *C. myersi*; and (iii) intermittent gene flow between *C. hamatus* and *C. myersi* during interglacial periods (based on Lisiecki and Raymo 2005; from 14 kya to the present, 130–115 kya, 337–300 kya, and 424–374 kya).

Gene flow estimates obtained by fastsimcoal are expressed as the probability for any lineage to move between two populations per generation. To make the implications of these results easier to understand, gene flow estimates are here reported as number of diploid individuals in the sink population that derived from the source population per generation and were calculated as $Nm_{ij} = m_{ij} \times N_{ej}$ (raw migration rate $\times$ effective population size of the sink population).

## Results

### Genomic variation, hybrid detection, and introgression

The first two principal components (PC) of the PCoA recovered four well-separated clusters, corresponding to the four species (Fig. 2). The fourth PC distinguished two groups inside *C. hamatus* (Supplementary Fig. S2).

The PcoA for *C. rastrospinosus* showed intraspecific geographic differences, separating three groups of samples according to geography: (i) Antarctic Peninsula + Antarctic Sound, (ii) South Orkney Islands, and (iii) Weddell Sea (Supplementary Fig. S3).

Estimation of individual ancestries made by Admixture indicated $K = 4$ as the optimal number of genetic clusters (Supplementary Fig. S4). In contrast to Schiavon et al. (2021), all individuals analysed in this study were of pure ancestry, with none showing admixture (Fig. 2, inset).

In agreement with results of Admixture, the co-ancestry matrix inferred by fineRADstructure recovered four clusters corresponding to the number of species expected in the dataset (Fig. 3). Additionally, fineRADstructure supported the existence of two groups within *C. hamatus* (hereafter *C. hamatus*-a, $N = 10$ and *C. hamatus*-b, $N = 4$, Fig. 3). Homogeneous haplotype sharing was inferred among *Chionodraco* species, indicating that none of the individuals could be considered admixed.

The maximum likelihood phylogenetic tree, generated by TreeMix, agreed with the relationships of the four taxa in

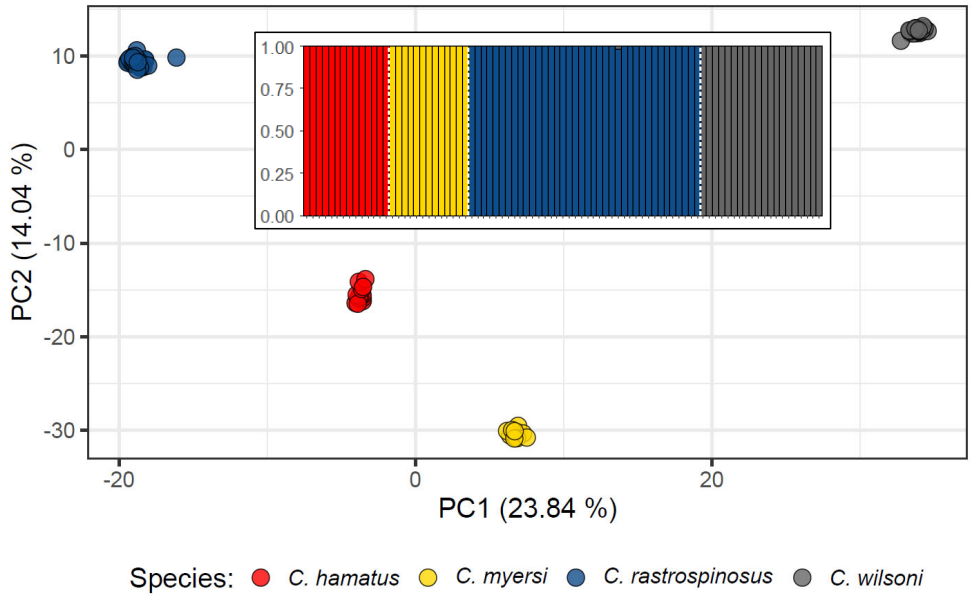


**Figure 2.** Scatter plot of PCoA and admixture plot inset based on 36 582 SNPs for the three *Chionodraco* species and *C. wilsoni*. Labels on the axes indicate the contributions of the displayed principal components to the variance. Admixture plot represents individual membership probabilities to the three *Chionodraco* species and *C. wilsoni* ($K = 4$) given by ADMIXTURE. Each individual is represented by a vertical lane, the proportion of different colours in each lane is proportional to the probability of assignment to each species.

the tree by Near et al. (2018), using RADseq loci. With migration edges added to the tree, the program indicated one gene-flow event from *C. hamatus* to *C. myersi* (Fig. 4 and Supplementary Fig. S5). Plotting the residuals showed that the addition of the migration edge increased the fit of the model to the data (Supplementary Fig. S6). When differentiation within *C. hamatus* and *C. rastrospinosus* was considered based on PCoA and FINERADSTRUCTURE results (Figs 2 and 3, Supplementary Figs S2 and S3), introgression events were inferred for all population pairs, limited only by the number of migration edges allowed (Supplementary Figs S7 and S8).

Analysis with DSUITE showed that only 40.9% of the test-informative sites were concordant with the phylogeny (BBAA sites, which placed *C. hamatus* and *C. rastrospinosus* as sister species), indicating that all species had a large number of shared alleles (Table 2). The ABBA pattern (indicating allele sharing between *C. hamatus* and *C. myersi*) characterized 34.4% of the sites, whereas 24.7% of the sites had the BABA pattern (allele sharing between *C. myersi* and *C. rastrospinosus*). The difference between the numbers of ABBA and BABA patterns is statistically significant ($D = 0.163$, $P < 0.001$), rejecting ILS as the only cause of allele sharing between non-sister species, and thus positing introgression between *C. hamatus* and *C. myersi*. The admixture proportion estimated by the $f_4$-ratio was 13.6% (Table 2).

When considering differentiated populations (for *C. rastrospinosus*) and groups (for *C. hamatus*) (Supplementary Table S2), DSUITE supported introgression between *C. hamatus* and *C. myersi*, but primarily for *C. hamatus*-b. Introgression between *C. rastrospinosus* and *C. hamatus* was detected by including differentiated populations. However, *D* values between *C. hamatus* and *C. rastrospinosus* were ~10 times smaller than those between *C. hamatus* and *C. myersi* (Supplementary Table S2). Of the *C. rastrospinosus* populations, the Weddell Sea population appeared most involved in introgression events (*D*-statistics ranging from 0.013 to 0.027, $f_4$-ratios ranging from 0.007 to 0.022; Supplementary Table S2).

Analysis at chromosome level indicated that 15 of 24 pseudo-chromosomes (and the extra pseudo-chromosome combining all the unmapped contigs) supported introgression between *C. hamatus* and *C. myersi* (Supplementary Table S3).

## Phylogenetic reconstruction and demography

Phylogenetic inference with SNAPP and the molecular clock assumption returned a single tree with full support at each node (topology in agreement with Near et al. 2018) (Fig. 5). The divergence events of the four species were estimated to have occurred 1.5 Mya for *C. wilsoni* (0.8–2.3 Mya 95% height posterior density—HPD interval), 0.9 Mya for *C. myersi* (0.5–1.4 Mya 95% HPD interval), and 0.5 Mya for the split between *C. hamatus* and *C. rastrospinosus* (0.3–0.8 Mya 95% HPD interval). The analysis allowing different $N_e$ per branch indicated an increase of one order of magnitude in the effective population size from basal to terminal branches (Fig. 5).

In the analysis with FASTSIMCOAL, the models with gene flow (models 2 and 3) received the greatest support, whereas the model with no gene flow (model 1) was less probable (Fig. 6). The model with continuous gene flow (model 2) and the model with gene flow during interglacial times (model 3) had overlapping likelihood distributions, indicating that both models fit the observed data similarly well, although the model with gene flow only during interglacial (model 3) had slightly larger likelihood values. The estimated numbers of migrants per generation were small in both cases (model 2: *C. hamatus* → *C. myersi* = 0.25; *C. myersi* → *C. hamatus* = 0.04; model 3: *C. hamatus* → *C. myersi* = 0.76; and *C. myersi* → *C. hamatus* = 0.92).

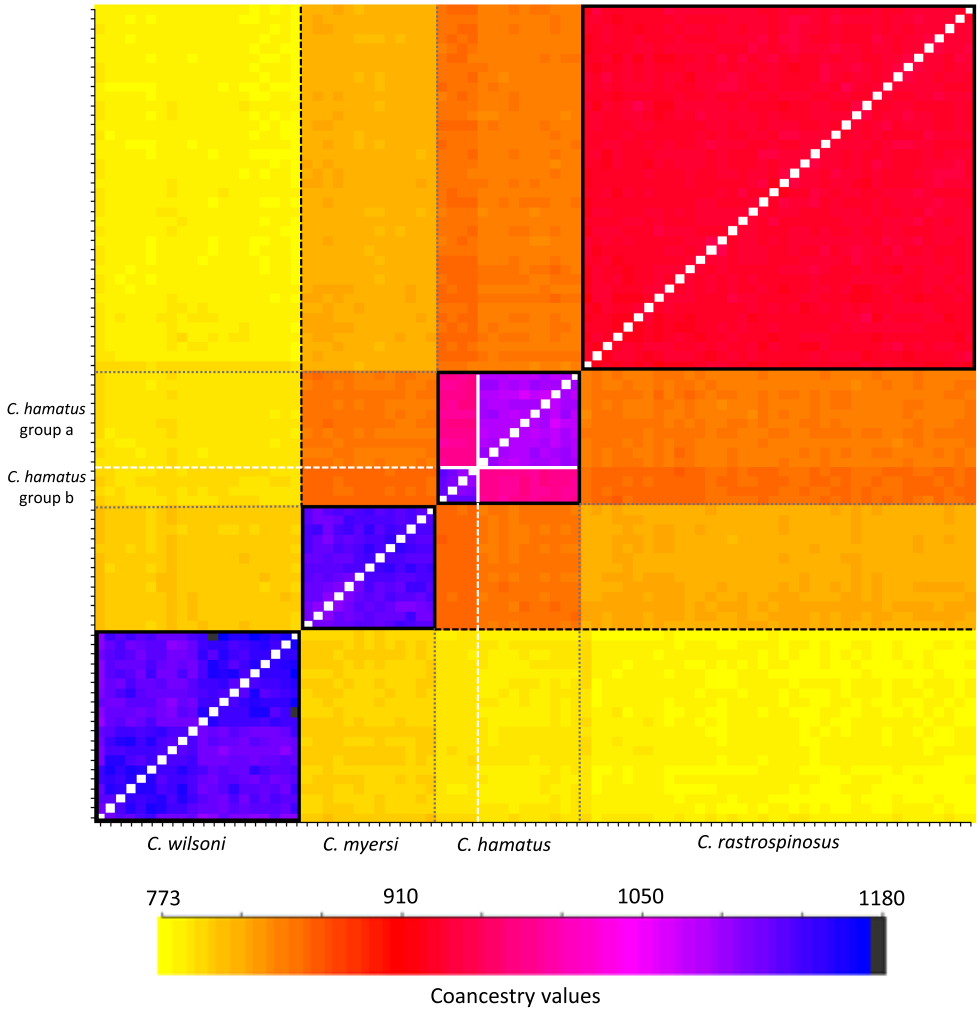


**Figure 3.** Clustered FINERADSTRUCTURE coancestry matrix inferred for the three *Chionodraco* species and *C. wilsoni*. Each row and each column, include the same individuals compared to one another. Coancestry ranges from weak (yellow) to strong (blue) as indicated by the colour scale, with darker blue and purple colours in the matrix indicating larger proportions of loci with shared coancestry. Black solid contour lines indicate the species clusters. Black dashed lines delimit the three species of genus *Chionodraco*. White solid lines indicate two subgroups of *C. hamatus*: *C. hamatus*-a, $N = 10$ and *C. hamatus*-b, $N = 4$.

The site-frequency spectra, SFS, were similar among the three models, and in all cases, there was a good similarity between the observed and the expected SFS (Supplementary Figs S9 and S11). The SFS of *C. hamatus* and *C. rastrospinosus* had the weakest correspondence between observed and expected results for all the models; expected SFS showed less fixed sites than observed for both species.

## Discussion

Distinguishing between alternative scenarios of introgression is important for understanding the speciation process and the evolution of the cryonotothenioid lineage since ancient hybridization may have facilitated the evolution of species diversity in icefish (Near et al. 2006), as in other adaptive fish radiations (e.g. cichlid fish; Meier et al. 2017). With the present work, we aimed to shed light on unresolved questions related to the occurrence of hybridization and introgression in the genus *Chionodraco*. We first confirmed the findings by Marino et al. (2013) and Schiavon et al. (2021) of three genetically distinct *Chionodraco* species. Second, we provide new genomic evidence that past hybridization and introgression played a role in the evolution of this genus of icefish.

The evidence of past introgression brought up by demographic modelling and phylogenetic reconstruction is in agreement with previous studies suggesting that interspecific gene flow characterized the genus *Chionodraco* after species diversification, likely during interglacial periods (Marino et al. 2013). However, we could not confirm the signals of ongoing hybridization observed previously with microsatellite data (Marino et al. 2013, Schiavon et al. 2021). Moreover, this study, for the first time, found evidence that ancient hybridization and introgression occurred mostly between the two sympatric species *C. hamatus* and *C. myersi*. Even though our results do not provide an indication of contemporary interspecific gene flow between *Chionodraco* species, we cannot exclude this possibility. Individuals from areas not sampled by this study might reveal evidence of it. We also caution on using a few markers to draw conclusions about time scale and

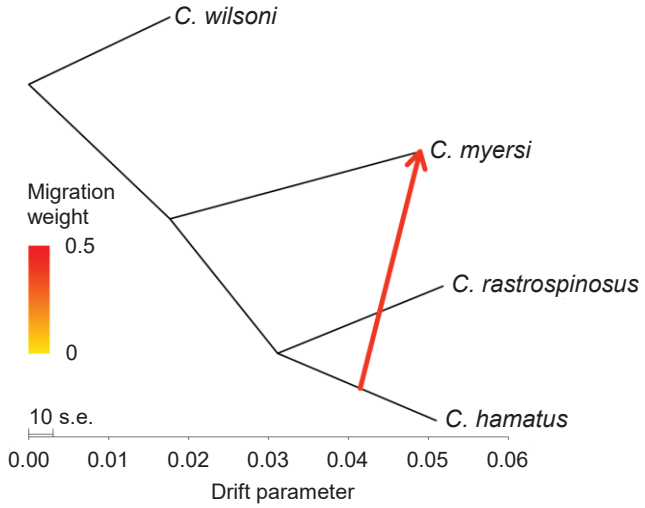


**Figure 4.** Maximum likelihood tree of *Chionodraco* spp. and *C. wilsoni* estimated by TreeMix. The red arrow indicates inferred unidirectional interspecific gene flow from *C. hamatus* to *C. myersi*. Migration weights range from low (yellow) to high (red), as indicated by the colour scale and correspond to the proportion of alleles that the receiving species gained from the donor species.

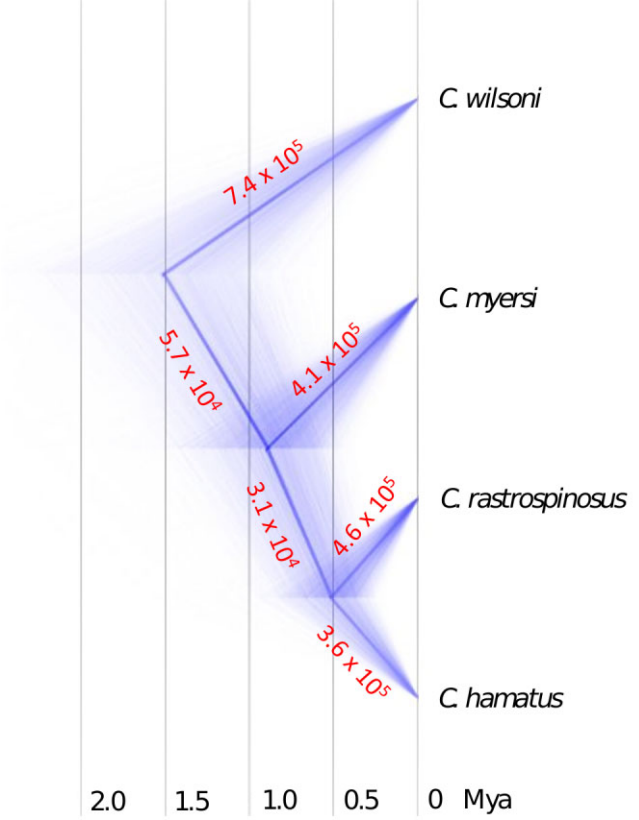


**Figure 5.** Time-calibrated phylogeny of *Chionodraco* spp. and *C. wilsoni* inferred by SNAPP and rendered as a cloud tree. Time is shown on the *x*-axis in Mya. Numbers along the branches indicate the estimates of $N_e$ when SNAPP was set up to allow a different $N_e$ on each branch.

extent of interspecific gene flow. Finally, we emphasize the importance of these types of studies to aid prediction of future interaction and compatibility among species and underscore the conservation value of introgressed populations (Hansen 2023).

RADseq data identified individual genetic clusters for each of the three *Chionodraco* species and *C. wilsoni*, confirming the three species are well separated despite their partially overlapping geographic distributions. In contrast with earlier work with microsatellites (Marino et al. 2013, Schiavon et al. 2021), PCoA showed no individuals with an intermediate genotype between species (putative contemporary hybrids), and the analyses to infer individual ancestry (Admixture and fineRADstructure) did not detect hybrid individuals.

Although the three *Chionodraco* species are clearly separated in the PCoA and Admixture analyses, testing for hybridization using the *D*-statistics showed that fewer than half (~41%) of the sites were concordant with the phylogeny. This result suggests that the three *Chionodraco* species share a great amount of genetic polymorphisms, which, at least partially, is likely due to ILS. *D*-statistics strongly supported interspecific gene flow between *C. hamatus* and *C. myersi* (with an admixture proportion, $f_4$-ratio, 13.6%) and showed that *C. hamatus* and *C. myersi* share more alleles than *C. rastrospinosus* and *C. myersi*. Also, the analysis at chromosome level indicated that introgression is widespread along the genome, as *D* values were statistically different from 0 for 15 of 24 chromosomes. Also considering results from fineRADstructure, which showed a similar amount of coancestry for all pairs of individuals, we posit that interspecific gene flow likely occurred in the past and involved the common ancestors of the individuals we analysed.

Gene flow estimates by TreeMix confirmed evidence of a past introgression event and showed that this has likely occurred from *C. hamatus* to *C. myersi*. TreeMix reconstructed a pattern of interspecific gene flow i.e. asymmetric and different from that originally proposed by Marino et al. (2013) based on microsatellites and approximate Bayesian computation (ABC) simulations. Marino et al. (2013) estimated smaller rates of migrant exchange (about two individuals per generation) between *C. myersi* and *C. hamatus* than between the other two *Chionodraco* species pairs (*C. hamatus*/*C. rastrospinosus* and *C. myersi*/*C. rastrospinosus*).

We tested different scenarios of gene flow between *C. hamatus* and *C. myersi* via demographic modelling (fastsimcoal analysis) to further investigate past introgression under the hypotheses that the species may either have shared geographic ranges continuously (which would have allowed for continuous interspecific gene flow) or been spatially separated tem-

**Table 2.** Results of Dsuite for the detection of introgression among the *Chionodraco* species.

| P1 | P2 | P3 | *D*-statistic | Z-score | *P*-value | $f_4$-ratio | BBAA | ABBA | BABA |
|---|---|---|---|---|---|---|---|---|---|
| *C. rastrospinosus* | *C. hamatus* | *C. myersi* | 0.164 | 9.671 | <0.001 | 0.136 | 1516.190 | 1276.610 | 917.731 |

P1, P2, and P3 indicate the position of the species in the phylogenetic tree. If the value of the *D*-statistic is statistically different from zero, introgression is inferred. The $f_4$-ratio indicates the admixture proportion. "BBAA" indicates the number of sites at which P1 and P2 share the same allele to the exclusion of P3 and P4, "ABBA" the number of sites with alleles shared between P2 and P3, and "BABA" the number of sites with alleles shared between P1 and P3.

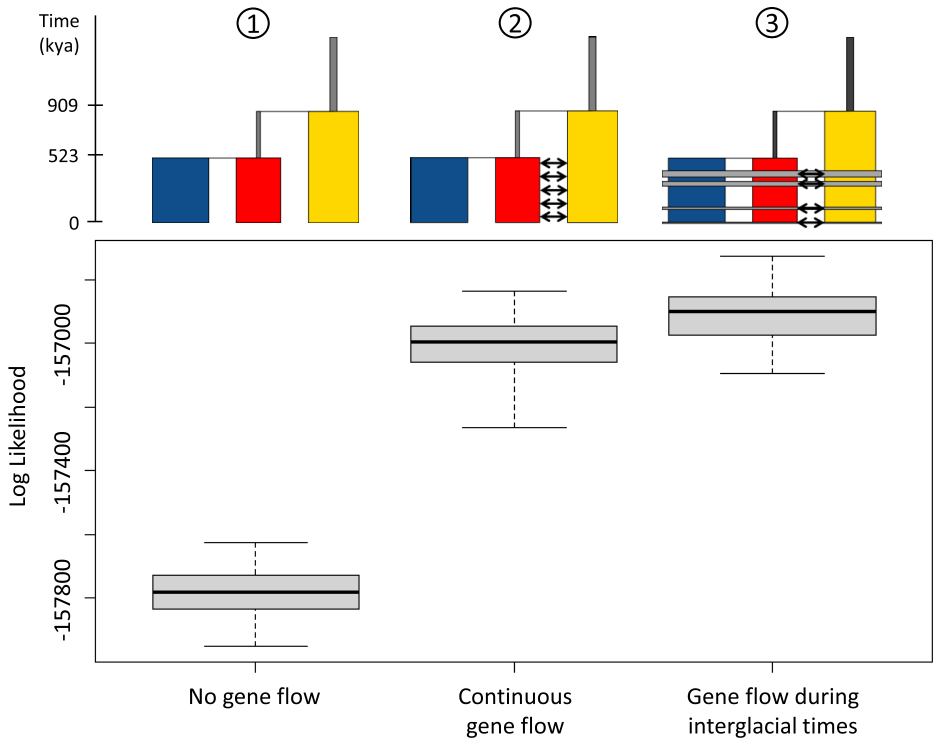


**Figure 6.** Different gene flow models simulated with FASTSIMCOAL and corresponding likelihood support. *Chionodraco myersi* is shown in yellow, *C. hamatus* in red, and *C. rastrospinosus* in blue. The first model portrays the evolutionary tree of the species as inferred by SNAPP, with no gene flow allowed. In the second model, continuous gene flow between *C. hamatus* and *C. myersi* is maintained after the species divergence. In the third model, gene flow between *C. hamatus* and *C. myersi* is allowed only during interglacial times. The width of the rectangles is proportional to the effective population size estimated with SNAPP. The time axis reports the splitting times estimated with SNAPP. In the third model, interglacial periods are marked in orange. The boxplots represent the likelihood distributions of the three simulated models. The black solid line indicates the median value; whiskers extend to minimum and maximum values.

porarily (which would have allowed for interspecific gene flow during secondary contacts). We evaluated three models: no gene flow, continuous gene flow, and gene flow only during interglacial times. The first scenario received the least support, confirming the presence of introgression detected by TreeMix and *D*-statistics. The second and third scenarios received similar support, with slightly larger likelihoods for the scenario with gene flow only during interglacial times. This agrees with results of ABC simulations by Marino et al. (2013), who found similar support for the models with inter-specific gene flow although the greatest probability was observed for the more complex model, in which migration was allowed only during interglacial periods. This lack of power to discriminate among models makes it difficult to understand whether *C. hamatus* and *C. myersi* had the possibility to mate throughout their evolutionary history or whether they were temporarily separated by glaciation events. However, in both cases, interspecific gene flow estimates were very small (less than one migrant per generation). In combination with the absence of contemporary putative hybrid individuals in our dataset, this points toward the occurrence of rare introgression events in the evolutionary history of the genus.

Interspecific gene flow between *C. hamatus* and *C. myersi* may be explained by their phylogenetic proximity. After the divergence of the two lineages, interspecific hybridization may have continued occasionally until reproductive isolation was complete. Nonetheless, although no current hybrids were identified in the RADseq analysis, the possibility that the two species are still interfertile cannot be ruled out. Moreover, we cannot exclude the possibility that limited interspecific gene flow may have occurred for other species combinations. The hypothesis that hybridization and introgression may have characterized other *Chionodraco* species pairs is indirectly supported by experimental evidence showing that even a cross between two genera of icefish (*C. aceratus* and *C. rastrospinosus*) can still produce viable offspring (Desvignes et al. 2019). In addition, even though we cannot exclude that the few cases of mitochondrial-nuclear discordance reported by Schiavon et al. (2021) and Marino et al. (2013) were due to ILS, shared mitochondrial D-loop haplotypes suggest introgression between *C. hamatus* and *C. rastrospinosus*.

Besides phylogenetic proximity, past introgression might have been favoured by the geographic context of speciation in *Chionodraco*. Situations previously ascribed to sympatric speciation have been reinterpreted as allopatric speciation followed by secondary contact (Lucek et al. 2018, Dean et al. 2019). If this were applicable to the genus *Chionodraco*, we would expect that the ancestor of *C. hamatus* and *C. rastrospinosus* initially diverged from *C. myersi* due to geographic isolation (e.g. in glacial refugia, allopatric speciation). Subsequently, a secondary contact during interglacial periods may have allowed introgression between *C. hamatus* and *C. myersi*. However, some introgression may have occurred during the early phases of the divergence between *C. rastrospinosus* and *C. hamatus* (as suggested by *D*-statistics). Subsequently, *C. rastrospinosus* may have remained in geographic isolation

in the Antarctic Peninsula, or may have had small population size and patchy distribution in sympatry, e.g. in the Weddell Sea (Schiavon et al. 2021), thus limiting the potential of introgression with the other *Chionodraco* species. This scenario may explain both the introgression between *C. hamatus* and *C. myersi* found by TreeMix, FineRADstructure, and *D*-statistics and the signal of introgression between *C. rastrospinosus* and *C. hamatus* found only by *D*-statistics.

Differences in life-history traits may explain the low levels of introgression between *C. hamatus* and *C. myersi*. Although living in sympatry, the two species may be reproductively separated by different mating habits. The reproductive period of *C. hamatus* spans from January to March, but for *C. myersi*, it lasts from July to September (Ekau 1991, Vacchi et al. 1996, La Mesa et al. 2013). Additionally, the nesting behaviour that characterizes these fish (Ferrando et al. 2014) is usually species-specific, which may also prevent hybridization (Desvignes et al. 2019). However, reproductive behavioural data for *Chionodraco* spp. come from few, sporadic observations and provide only an incomplete picture of the species' ecology. Moreover, it was observed that the reproductive periods of some notothenioids (e.g. *Gobionotothen gibberifrons*, *Nototheniops larseni*, and the icefish *C. acera-tus*) change with latitude, possibly following a temperature gradient (Papetti et al. 2007). Thus, past climatic oscillations may have promoted occasional overlaps in the times of reproduction in *C. hamatus* and *C. myersi*, in agreement with the notion that notothenioid demographic and evolutionary history is closely linked to climate history. This connection raises concerns about the potential impact of the current warming of the Southern Ocean on the survival of Antarctic marine fauna and the maintenance of genetic diversity. Periodic surveys are essential to monitor the genetic variability of these species, especially in areas of potential secondary contact. Such monitoring is crucial for management and to identify early changes in the distributions of potentially hybridizing species. This is particularly relevant in the implementation of marine protected areas.

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## Supplementary data

Supplementary data is available at *ICES Journal of Marine Science* online.

*Conflict of interest*: The authors have no conflict of interest to declare.

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## Author contributions

LS and CP designed the research. CP, LZ, ER, ML, and FCM conducted the sampling. LS, CP, and SGC conducted molecular lab work. RF and EB provided help for the molecular lab work. LS analysed the data. MM, AW, SGC and ET provided help with data analysis. LS and CP wrote the original draft. All co-authors reviewed the manuscript.

## Ethical statement

Fish were sampled and processed according to and within laws, guidelines, and policies of the German and European Animal Welfare legislation. Sample collection during the cruises with the R/V Polarstern was approved by the competent German authority for Antarctic research, the UBA (Umweltbundesamt).

## Data availability

All the codes, datasets, and accessory files needed to replicate the analyses are available at https://github.com/Papetti-Lab/Chionodraco_gene_flow.git. Raw sequence reads are available under the NCBI BioProject Accession number: PRJNA1041981 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1041981/).

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