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Species distribution, hybridization and connectivity in the genus *Chionodraco*: Unveiling unknown icefish diversity in antarctica

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Abstract

Aim: The species of the genus *Chionodraco* (Notothenioidei) are the most abundant icefish on the continental shelf of the Weddell Sea. While previous studies indicated that only *Chionodraco hamatus* and *Chionodraco myersi* inhabit the Weddell Sea, the third *Chionodraco* species, *Chionodraco rastrospinosus*, was recently sampled in the area. As *C. rastrospinosus* is supposed to be found only at the Antarctic Peninsula and Scotia Arc, this study aimed at confirming the species classification of *C. rastrospinosus* by molecular methods and identifying its putative source population. Given the documented evidence of introgression among the three species, we tested whether the newly found *C. rastrospinosus* shared any genetic variability with the other *Chionodraco* species. To explain the pattern of distribution of the *Chionodraco* species, we aimed at estimating the hydrodynamic connectivity between the Antarctic Peninsula and the Weddell Sea.

Location: Antarctic Peninsula, southern Scotia Arc and the south-eastern Weddell Sea.

Methods: We genotyped 19 microsatellites and sequenced the mitochondrial D-loop for 560 *Chionodraco* individuals. We simulated the dispersal of more than 3 million drifters (Lagrangian model).

Results: The molecular analyses support the presence of *C. rastrospinosus* in the Weddell Sea and its homogeneity with *C. rastrospinosus* from the Antarctic Peninsula. Bayesian clustering identifies three putative hybrids among *C. rastrospinosus* and the other congenerics. Lagrangian simulations do not support connectivity driven by the oceanographic features of the Antarctic Peninsula and Weddell Sea via passive larval dispersal only.

Main conclusions: This study documents, for the first time, the presence of *C. rastrospinosus* in the Weddell Sea unveiling more biodiversity than previously known in this region. The sympatry of the three *Chionodraco* species explains the occurrence of occasional, ongoing events of hybridization in the genus. Alternative possible

hypotheses need to be tested in future studies about the mechanisms maintaining the interspecific connectivity in *Chionodraco* spp.

KEYWORDS
Antarctic continental shelf, clustering analysis, connectivity, gene flow, hybridization, Lagrangian modelling, microsatellite, species distribution, Weddell Sea

1 | INTRODUCTION

The continental shelf of the Weddell Sea (Antarctica) is still largely unprotected. This region is one of the most pristine and relatively unexplored hotspots of marine biodiversity in Antarctica, and it is less affected by sea surface warming compared with other, more exposed, areas as the Antarctic Peninsula (Teschke et al., 2016). Under a climate change scenario, Griffiths et al. (2017) hypothesized that the Weddell Sea continental shelf will be likely a sink area for species' range shifts and a feasible refugium for highly cold-adapted benthic organisms. The Weddell Sea hosts different marine habitats that support a rich fish community dominated by the suborder Notothenioidei. Notothenioids comprise 142 species, 80% of them endemic to Antarctica (calculated from the list of notothenioid species curated by J. T. Eastman and R. R. Eakin, version 15 December 2016, personal communication). They represent ~ 50% of all vertebrate species of the Antarctic continental shelf and constitute the 90%–95% of fish biomass, thus dominating the ichthyofauna of the Southern Ocean (Matschiner et al., 2015; Peck, 2018).

1.1 | The diversity of *Chionodraco* species in the Weddell Sea

Two notothenioid species of the icefish genus *Chionodraco*, *C. hamatus* and *C. myersi* (family Channichthyidae) are among the most abundant icefish species on the Weddell Sea continental shelf (Eastman & Eakin, 2000; Fischer & Hureau, 1985). *Chionodraco hamatus* and *C. myersi* are found on the continental shelf all around Antarctica with the exception of the Antarctic Peninsula and Scotia Sea islands. These two species prefer slightly different but overlapping depth ranges (4–972 m for *C. hamatus* and 99–926 m for *C. myersi*; Eastman, 2017).

The genus *Chionodraco* includes a third species, *C. rastrospinosus*, only found off the Antarctic Peninsula and around the South Shetland and South Orkney islands at up to 1,000 m depth (Eastman, 2017; Gon & Heemstra, 1990; Kock, 1992). While previous studies indicated that *C. hamatus* and *C. myersi* are the only *Chionodraco* species inhabiting the Weddell Sea, a recent expedition on the continental shelf of the south-eastern Weddell Sea has recorded, for the first time, the presence of several individuals identified as *C. rastrospinosus* in sympatry with the other two congeneric species (Knust & Schröder, 2014).

The three species are morphologically very similar, and their identification is based on subtle differences, such as the presence/absence of a rostral spine and number and morphology of gill rakers (Fischer & Hureau, 1985; Gon & Heemstra, 1990). The three *Chionodraco* species are phylogenetically very close, and their divergence was estimated to have occurred ~ 2 mya (Near et al., 2012). *Chionodraco myersi* probably diverged earlier, around 1.8 mya, from the clade that includes the two sister species *C. hamatus* and *C. rastrospinosus* (Near et al., 2012). Given these premises, the first aim of this study was to formally confirm by molecular methods the taxonomic identification of *C. rastrospinosus* specimens collected on the continental shelf of the south-eastern Weddell Sea and to identify the putative source population of this pool of individuals.

1.2 | Incomplete reproductive isolation among *Chionodraco* species in the Weddell Sea

The palaeoclimatic events at which notothenioids have been exposed after the acquisition of the ability to synthesize antifreeze glycoproteins (AFGPs) are generally invoked to explain the mechanisms that could have driven species diversification in these fish (Near et al., 2012). On one hand, repeated cycles of glaciation have periodically isolated populations in remaining ice-free refugia providing a mechanism for allopatric speciation (Barnes et al., 2006; Hewitt, 1997; Rogers, 2007). On the other hand, interglacial times may have provided context for population expansions subsequent to glacial retreat (Janko et al., 2007; Matschiner et al., 2009; 2015; Zane et al., 2006) and for secondary contacts between recently diverged species (Marino et al., 2013). Marino et al. (2013) suggested that secondary contacts between *Chionodraco* species have led to natural events of hybridization that left signatures of introgression in their genomes. The authors found mixed ancestry and similar allelic frequencies at 18 microsatellites, providing molecular evidence of past and present hybridization among the three species. Moreover, approximate Bayesian computation (ABC) simulations suggested that the shared alleles could bear the signature of introgression occurred most likely during the last two major interglacial times (Marino et al., 2013). Despite evidence of interspecific gene flow, no specific hypothesis has been brought up so far for where a geographic contact zone for the three species could be located. If genetically confirmed, the presence of *C. rastrospinosus* in the Weddell Sea would suggest the hypothesis that this area may be a possible contact zone for the three *Chionodraco* species. Therefore, the second aim of this study was to investigate whether the newly found *C. rastrospinosus* specimens may carry any trace of introgression from other *Chionodraco* species and to describe

patterns of contemporary genetic differentiation and hybridization at interspecific scale.

1.3 | Hydrodynamic connectivity

The current system in the area of the Antarctic Peninsula and Weddell Sea is dominated by the Antarctic Circumpolar Current (ACC, Figure 1). The ACC flows eastward over the slope off the western Antarctic Peninsula, moves seaward offshore of the South Shetland Islands (Figure 1) (Ryan et al., 2016) and continues east to form the northern border of the Weddell Gyre (WG, Figure 1), a mainly wind-driven, cyclonic ocean gyre (Ryan et al., 2016). In contrast to the ACC, the Antarctic Slope Current (ASC, Figure 1) is a near-circumpolar, westward feature that forms a narrow, rapid flow across broad sections of the continental shelf. In the Weddell Sea, the ASC is thought to form the southern border of the WG before reaching waters north of the Antarctic Peninsula (Caccavo

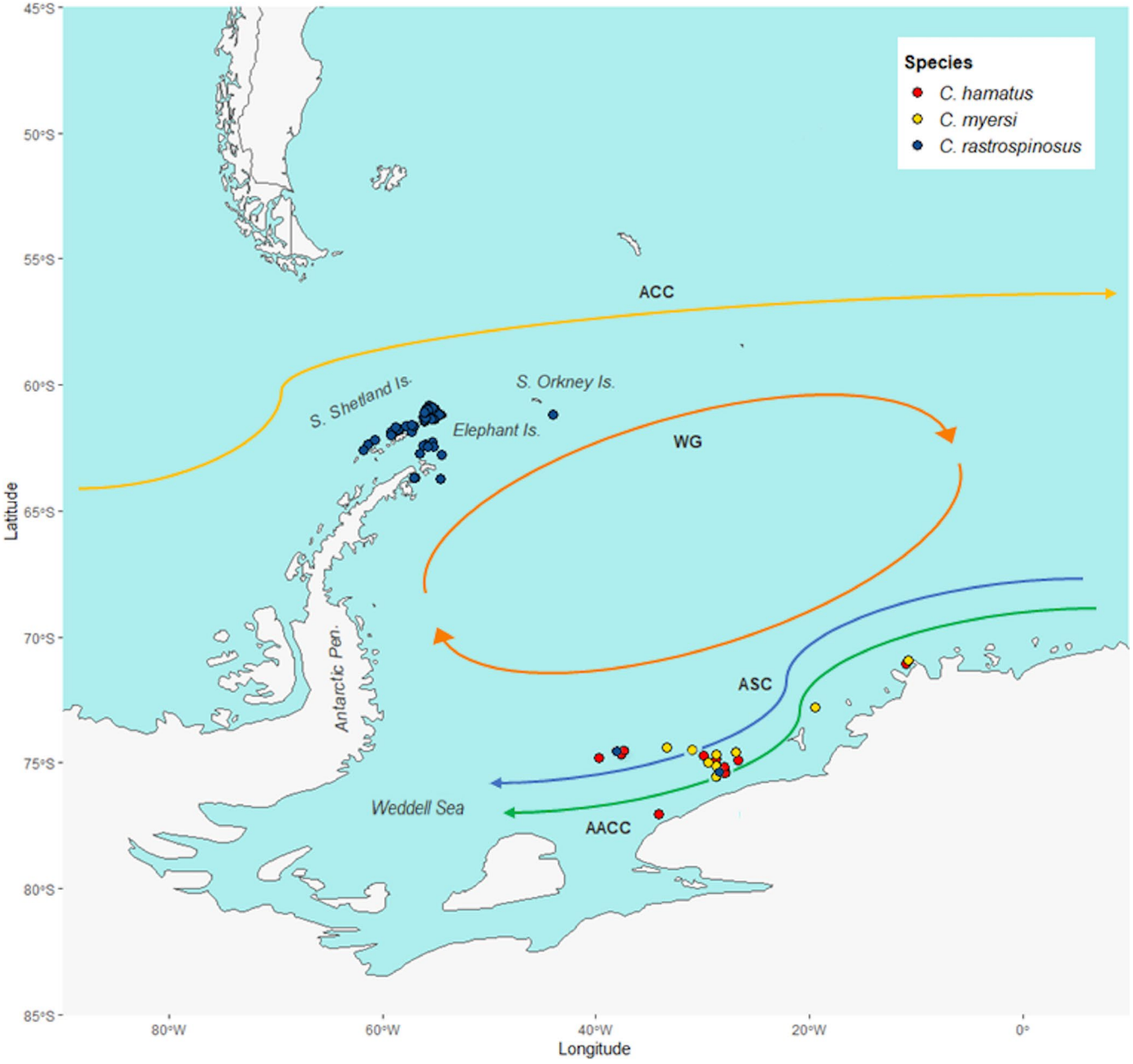

FIGURE 1 Map of sampling locations for the three *Chionodraco* species and schematic representation of the main marine currents occurring in the region considered in this study. The figure represents the regions of the Antarctic Peninsula and the Weddell Sea. The dots show the sampling locations of the specimens analysed in this study (red for *C. hamatus*, yellow for *C. myersi* and blue for *C. rastrospinosus*). The arrows indicate the main marine currents in the area: yellow for the Antarctic Circumpolar Current (ACC), orange for the Weddell Gyre (WG), blue for the Antarctic Slope Current (ASC) and green for the Antarctic Coastal Current (AACC). The map was initially generated with the software environment R ver. 4.0.0, using packages ggplot2 (Wickham, 2016) and sf (Pebesma, 2018) for plotting, and the package rnaturalearthdata (South, 2017) to access and download Natural Earth (http://www.naturalearthdata.com) map dataset, and refined by hand

TABLE 1 List of samples used in this study

Species	Cruise	Location	Acronym	Sample size (microsatellites)	Sample size (mtDNA)	References
Chionodraco hamatus	ANT-VII/4, 1988, AWI	Weddell Sea	Wedd–88	9	8	Marino et al. (2013)
	11th Italian Expedition, 1995, PNRA	Ross Sea (Terra Nova Bay)	Ross–95	23	20	Marino et al. (2013)
	PS82, 2014, AWI	Weddell Sea	H-Wedd–14	88	88	This study
	PS96, 2016, AWI	Weddell Sea	H-Wedd–16	1	1	This study
	Total			121	117	
Chionodraco myersi	5th Italian Expedition, 1995, PNRA	Ross Sea (Terra Nova Bay)	Ross–89	27	26	Marino et al. (2013)
	ANT-XXI/4, 2003 AWI	Weddell Sea	Wedd–03	10	10	Marino et al. (2013)
	PS82, 2014, AWI	Weddell Sea	M-Wedd–14	78	78	This study
	PS96, 2016 AWI	Weddell Sea	M-Wedd–16	1	1	This study
	Total			116	115	
Chionodraco rastrospinosus	ANT XIV/2, 1996, AWI	Elephant Island	EI–96	19	19	Marino et al. (2013)
	ANT XXIII/8, 2006, AWI	Joinville Island	JI–06	20	20	Marino et al. (2013)
	ANT XIX/3, 2002, AWI	Elephant Island	EI–02	40	40	This study
		South Shetland	SS–02	40	40	This study
		Joinville Island	JI–02	30	30	This study
	ANT XXVII/3, 2011, AWI	South Orkney	SO–11	52	53	This study
	ANT XXVIII/4, 2012, AWI	Elephant Island	EI–12	53	53	This study
		South Shetland	SS–12	15	15	This study
		Bransfield Strait	BS–12	9	9	This study
	PS82, 2014, AWI	Weddell Sea	R-Wedd–14	12	13	This study
	PS112, 2018, AWI	Elephant Island	EI–18	18	18	This study
		South Shetland	SS–18	10	10	This study
		Antarctic Sound	AS–18	9	8	This study
	Total			327	328	
Complete dataset				564[a]	560[a]	

Note: The table reports species names, sampling cruises, general area of sampling (location), sample acronyms, sample sizes for the microsatellite and mtDNA datasets and if the samples came from the previous study of Marino et al. (2013) or were newly analysed in this work. AWI: Alfred Wegner Institute Helmholtz Centre for Polar and Marine Research, PNRA: Italian National Program for Antarctic Research. The single individual from the sample H-Wedd-16 and the individual from the sample M-Wedd-16 were included in the samples H-Wedd-14 and M-Wedd-14, respectively, for the calculation of all statistics.

[a]The two samples differ in size as it was not possible to sequence the mtDNA D-loop for 4 individuals.

et al., 2018; Meredith & Brandon, 2017; Orsi et al., 1995; Ryan et al., 2016; Thompson et al., 2018). Inshore, the Antarctic Coastal Current (AACC, Figure 1) occupies the upper part of the water column, transports shelf waters between glacial trough systems of the Weddell Sea and merges with the ASC off the eastern Antarctic Peninsula (Graham et al., 2013).

Such transport pathways, coupled with a relatively long pelagic passive larval stage (of up to five months) (La Mesa et al., 2013), can be expected to contribute to the exchange of *Chionodraco* individuals by connecting habitats of the Antarctic Peninsula and Weddell Sea. The contribution of adult movement to dispersal is expected to be smaller than via passive larval dispersal as adults of the *Chionodraco* species are bentho-pelagic (Eastman & Lannoo, 2004; Eastman & Sidell, 2002). Therefore, the third and final aim of this study was to assess the hydrodynamic connectivity of the three species in terms of preferential routes of *Chionodraco* larval dispersal between the Antarctic Peninsula and the Weddell Sea. We wanted to test whether the dispersal of *C. rastrospinosus* occurs preferentially along

the ACC, with larvae that get entrained in the Weddell Gyre and are transported from the Antarctic Peninsula to the Weddell Sea. We wanted also to verify whether the pelagic passive larval dispersal of *C. hamatus* and *C. myersi* could occur along the shelf from the south-eastern Weddell Sea towards the Antarctic Peninsula driven by coastal currents (AACC and ASC) and the Weddell Gyre.

To meet the aims of this study, we genotyped a panel of 19 microsatellites and sequenced 326 bp of the mitochondrial D-loop of 560 individuals. For the three species, we simulated more than 3 million drifters and analysed the dispersal routes estimated by the model starting from different release areas in the Antarctic Peninsula and in the Weddell Sea.

2 | METHODS

2.1 | Study areas, sampling and DNA extraction

Specimens of *C. hamatus*, *C. myersi* and *C. rastrospinosus* were collected during different cruises on the continental shelf of the south-eastern Weddell Sea in 2014 and 2016, along the eastern and western Antarctic Peninsula and on the shelf of southern Scotia Arc islands between 2002 and 2018 (Table 1, Figure 1). Thirteen individuals of *C. rastrospinosus* were collected for the first time in the Weddell Sea in 2014 during an R/V *Polarstern* cruise (Table 1).

Specimens were collected by bottom trawl, weighted and measured and assigned to species according to morphological characteristics reported in Gon and Heemstra (1990) and Fisher & Hureau (1985). The total length range for sampled specimens was 14.5–49 cm for *C. hamatus*, 24.5–38 cm for *C. myersi* and 7–54 cm for *C. rastrospinosus*. A piece of muscle or fin clip was collected from each specimen and preserved in ethanol 95% for the subsequent molecular analysis. Sample collection conducted on specimens obtained during R/V *Polarstern* cruises and analysed for the first time in this study was approved by the competent national authority for Antarctic research (Umweltbundesamt, UBA, Germany).

Genomic DNA was extracted from 456 specimens following a standard salting out protocol (Patwary et al., 1994). For all samples, concentration and quality of the extracted DNA were estimated by NanoDrop Spectrophotometer 2000c (Thermo Scientific), while DNA integrity was checked on 1% agarose gel in TBE 1X stained with GelRed (Biotium; GelRed™ Nucleic Acid Stain 10,000 in water).

In this study, we also reanalysed 108 individuals from Marino et al. (2013) extending our dataset to a total number of 564 individuals (Table 1). Pools of specimens of the same species from each collection site and year (Table 1 for acronyms) were considered as population samples for population differentiation tests (see sections 2.3 and 2.5). Samples H-Wedd-16 and M-Wedd-16 included a single individual and were pooled with the population sample H-Wedd-14 and M-Wedd-14, respectively, for the calculation of all statistics.

2.2 | Microsatellite amplification and genotyping

A total of 456 individuals were newly genotyped in this study for a panel of 19 microsatellites. All loci were previously characterized for the three *Chionodraco* species by Agostini et al. (2013). Eleven loci were isolated from *C. hamatus*: Ch126, Ch623, Ch684, Ch1968, Ch2309, Ch2788, Ch3603, Ch3866, Ch5817, Ch8461 and Ch8501 (Coppe et al., 2013; Molecular Ecology Resources Primer Development Consortium et al., 2011). Three microsatellites were isolated from *C. rastrospinosus*: Cr15, Cr127 and Cr236 (Papetti et al., 2006), and four from *Chaenocephalus aceratus*: Ca21, Ca35, Ca48 and Ca86 (Susana et al., 2007). One locus was originally isolated from *Pleuragramma antarctica*: 221Pl (Papetti et al., 2011). Detailed amplification conditions follow Marino et al. (2013) and references therein (see also Table S1 in Supporting Information Appendix S1). Fragment lengths were estimated by Genoscreen (www.genoscreen.fr) on an ABI PRISM 3,700 DNA Analyzer (Applied Biosystems) (Table S1 in Supporting Information Appendix S1). Scoring and binning were performed by two operators, independently, with Peak Scanner ver. 1.0 (Applied Biosystems) and with the Excel macro Flexibin (Amos et al., 2007), respectively. The final dataset was refined by eye. We combined our new dataset with the previously published dataset from Marino et al. (2013). As microsatellite alleles can suffer from size shifts when genotyping is performed with different procedures or in different moments (Davison & Chiba, 2003; Lahood et al., 2002), we used the software Allelogram ver. 2.2 (Morin et al., 2009) to normalize allele size from multiple data sources. The input files for subsequent analyses were generated with CREATE ver. 1.38 (Coombs et al., 2008) or PGDSpider ver. 2.1.1.5 (Lischer & Excoffier, 2012).

2.3 | Microsatellite analyses

2.3.1 | Genetic diversity

Basic statistics, such as number of alleles (A), percentage of total observed alleles per locus per population sample (%), allelic richness (AR) and observed (H_O) and expected heterozygosity (H_E) under the Hardy–Weinberg equilibrium (HWE) assumptions, were calculated with the function *basicStats* of R package "diveRsity" ver. 1.9.90 (Keenan et al., 2013) (R version 3.4.3; R core team, 2019). Exact test for HWE for each locus per population sample and species and linkage disequilibrium (LD) between pairs of loci per species were performed with Genepop on the Web, online ver. 4.2 (Rousset, 2008), by setting 10,000 dememorization steps, 500 batches and 10,000 iterations per batch.

Correction for multiple testing was applied with the Benjamini and Hochberg method (Benjamini & Hochberg, 1995) with the software Myriads ver. 1.1 (Carvajal-Rodríguez, 2018). The significance threshold before correction was 0.05 for all the multiple tests except for the LD, for which we set the threshold at 0.01.

The software FreeNA (Chapuis & Estoup, 2007; Chapuis et al., 2008) was used to estimate the frequency of null alleles following the expectation–maximization (EM) algorithm and their impact on the genetic distances among species and population samples. Genetic distances were calculated with FreeNA as global F_{ST} values with 95% confidence intervals (CI) using 10,000 replicates over loci for the original dataset and from allelic frequencies taking into account the putative presence of null alleles.

2.3.2 | Identification of outlier loci

To detect possible outlier markers showing extremely low or high divergence among species, we used the approach implemented in the software BayeScan ver. 2.1 (Foll & Gaggiotti, 2008). The following parameters were used: burn-in = 100,000, thinning interval = 100, sample size = 10,000, number of pilot runs = 50, length of each pilot run = 10,000 and prior odds = 10.

2.3.3 | Population differentiation and detection of admixture

Pairwise and global F_{ST} values included their *p*-values were estimated with Arlequin ver. 3.5.2.2 (Excoffier & Lischer, 2010) by 10,000 permutations of the datasets.

To estimate the number of clusters in our dataset, and the extent of admixture among clusters, we used Structure ver. 2.3.4 (Pritchard et al., 2000). For each run, we set a burn-in of 100,000 steps followed by 1,000,000 MCMC iterations, using the admixture model and independent allele frequencies. We tested from 2 to 6 K putative clusters, with 10 runs for each K following indications from Evanno et al. (2005) (see also Porras-Hurtado et al., 2013 for a review). The most probable value of K was identified with the method described in Evanno et al. (2005) and implemented in Structure Harvester ver. 0.6.94 (Earl & vonHoldt, 2012). The different runs for each K were averaged with Clumpak (Kopelman et al., 2015) and the result plotted with the R package "Pophelper" ver. 2.3.0 (Francis, 2017).

2.3.4 | Detection of hybrids

We ran Structure with the USEPOPINFO model (burn-in of 100,000 steps, 1,000,000 MCMC iterations, independent allele frequencies) to identify hybrids or migrants and the extent of introgression when pre-defined groups (in our case the three *Chionodraco* species) correspond almost exactly to Structure clusters. To estimate the optimal threshold values to distinguish an individual as admixed and to test the power of our dataset to discriminate between pure and hybrid individuals, we followed a simulation approach as performed by Vähä and Primmer (2006). For each species, we selected the individuals with a Q-value > 0.99 (pure individuals) from the former Structure analysis and used these individuals as input for the software HybridLab ver. 1.0 (Nielsen et al., 2006). These pure individuals were virtually crossed randomly in a simulation with HybridLab to generate 810 simulated new pure individuals for each species and 30 simulated new admixed individuals for each of the following crosses (F1 hybrid groups): *C. hamatus* × *C. rastrospinosus*, *C. myersi* × *C. rastrospinosus*, and *C. hamatus* × *C. myersi*. We also generated six simulated new groups by backcrossing individuals of the three F1 hybrids with one of the two parental species. Sample sizes for every group followed indications by Vähä and Primmer (2006) and aimed to obtaining a 10% of admixed individuals. This simulated dataset was analysed with Structure applying the same settings as for the former run. The resulting Q-values for the real and simulated datasets were plotted in a box plot with BoxPlotR (http://shiny.chemgrid.org/boxplotr/), and the distributions were compared.

Admixture was analysed also with the software NewHybrids ver. 1.1 (Anderson & Thompson, 2002). Differently from Structure, which treats the Q-value as a continuous variable to assign individuals to a number of clusters specified by the user, NewHybrids calculates the probability that each individual belongs to these categories: pure, F1, F2 (F1 x F1) and the two types of backcross (F1 × parental pure species). In nature, many degrees of admixture are expected, but this approach aims to investigate the early stages of hybridization (F1, F2 and backcrosses). As NewHybrids works with two species at a time, we ran three pairwise comparisons. For each run, a burn-in of 100,000 steps, 1,000,000 MCMC iterations and uniform priors were set. We ran NewHybrids also with the simulated dataset generated with HybridLab. For each pairwise comparison, the simulated dataset was composed of 405 randomly selected pure individuals for each species and 30 individuals for each of the admixed categories (F1 and the two backcrosses F1 × parental pure species). The number of pure individuals was set to 405 to maintain a proportion of 10% of hybrids, while the number of admixed individuals remained the same.

2.4 | Mitochondrial DNA amplification and sequencing

A 326 bp D-loop sequence was obtained for 560 individuals, including 457 specimens newly analysed in this study and 103 originally used by Marino et al. (2013) but never sequenced for this mitochondrial marker (Table 1). The number of samples genotyped for microsatellites ($N = 564$) and sequenced for the mitochondrial marker ($N = 560$) differs as it was not possible to sequence the D-loop for 4 individuals (see Table 1 for more details). The mitochondrial D-loop was used in this study as previous studies (Damerau et al., 2012; Deli Antoni et al., 2019; Hüne et al., 2015; Matschiner et al., 2009; Patarnello et al., 2003; Zane et al., 2006) already demonstrated that this marker carries sufficient variability to resolve the population genetic structure and allows the species differentiation in notothenioids. Patarnello et al. (2003) clearly distinguished the three *Chionodraco* species and identified population clusters within the three species by means of a 249 bp D-loop marker.

Primers LPRO2 (5'-AACTCCCACCACTAACTCCCAAAGC-3') and HDL1 (5'-CCTGAAGTAGGAACCAGATGCCAG-3') were used for the amplification (Patarnello et al., 2003). The amplification was performed in a volume of 25 μl containing Buffer 1X (Solis BioDyne, Estonia), $MgCl_2$ 2 mM, dNTPs 0.16 mM, primers 0.2 μM each and Taq 0.5 U (Solis BioDyne). For each PCR, 50 ng of genomic DNA were used. The PCR profile consisted of denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 60 s, 52°C for 45 s and 72°C for 45 s, and a final single step at 72°C for 5 min. PCR products were purified with the EuroSAP PCR Enzymatic Clean-Up Kit (EuroClone). The sequencing reaction was performed by Eurofins Genomics (www.eurofinsgenomics.eu). All sequences are accessible at GenBank (Accession Nos.: MT571397-MT571447).

2.5 | Mitochondrial DNA variability

D-loop sequences were aligned with CLUSTAL OMEGA (Sievers et al., 2011), and descriptive statistics (segregating sites, number of haplotypes, haplotype diversity and nucleotide diversity) for each species and population sample were obtained with DNASP VER. 6.12.03 (Rozas et al., 2017). To identify possible patterns of genetic structure within each species, a haplotype network was built with POPART VER. 1.7 (Leigh & Bryant, 2015) using the TCS algorithm (Clement et al., 2002). Individuals were assigned to their maternal parental species according to their position in the network. Patterns of population structure and species differentiation were investigated by estimating F_{ST} and average number of pairwise differences (Pi) with ARLEQUIN.

2.6 | Connectivity analyses

To estimate the possible routes of dispersal of each *Chionodraco* spp. from the Weddell Sea and from the Antarctic Peninsula under the assumption that connectivity may be mainly driven by pelagic passive larval dispersal, we used the mathematical model COHERENS VER. 2 (Luyten, 2011). COHERENS includes a Lagrangian particle tracking module able to compute the trajectory of particles that are virtually released into the ocean. In this study, it was assumed that each particle (as a proxy for a virtual larvae) passively drifts following the surrounding water masses. The Lagrangian approach included in COHERENS uses the second-order Runge–Kutta scheme plus vertical and horizontal diffusion following a random-walk approach assuming isotropic diffusion with horizontal and vertical diffusion coefficients of 10 and 0.0001 m^2/s, respectively. The Lagrangian module has been validated (Dulière, Ovidio, & Legrand, 2013; Legrand & Dulière, 2014). Hydrodynamic forcing is produced based on results from the Operational Mercator global ocean analysis and forecast system and made available via the Copernicus data portal (http://marine.copernicus.eu/). Information on the quality of the hydrodynamic forcing can be found in Lellouche et al. (2019).

The model domain covers the Southern Ocean with northern and southern boundaries at 45° south and 80° south, respectively. The grid resolution is 1/12 degree in the horizontal and 50 vertical sigma levels (from 0 to 5,500 m). Once the particle leaves the model domain, it is lost and does not come back. The model time step is 5 min.

The dispersal pattern of the pelagic larval stage was modelled in the context of several life history traits of the three *Chionodraco* species. For every species, we simulated the sites of particle release (virtual larvae) from locations on the continental shelf where the specimens analysed in this study were caught (see Table 1 for corresponding sampling campaigns and Table S2 in Supporting Information Appendix S1 for the coordinates of the release locations) as a proxy for spawning areas because precise data on spawning areas for the three species are not known. As specimens analysed in the genetic section of this study were all big enough for not being passive dispersers, we did not distinguish between juvenile and adult stage in the definition of potential spawning areas for the hydrodynamic connectivity section. As a proxy for spawning areas, we also considered where some larvae of *C. rastrospinosus* were recently sampled (cruise PS112, M. La Mesa, personal communication, see Table S2 in Supporting Information Appendix S1 for details, samples not available for the genetic analyses performed in this study). Three locations were very close to the coast (see Table S2 in Supporting Information Appendix S1 for details) and were adapted according to the model land/sea mask. The drift lasted five months (observed time of passive pelagic larval stage; La Mesa et al., 2013). Virtual larvae (i.e. the particles) were released in the model at three different depths (100, 150 and 200 m; depths where larvae have been observed; La Mesa et al., 2013). Every day during the release (i.e. hatching) periods, 150 larvae were virtually released in the model at the defined dispersal starting locations. The release periods were the following: from 1st August to 30th November for *C. hamatus* and *C. rastrospinosus*; from 1st November to 31st December for *C. myersi*. These release periods were chosen according to observations reported in the literature (Ekau, 1991; Kock, 2005; La Mesa et al., 2013; Vacchi et al., 1996). For *C. hamatus*, duration of drift and release (i.e. hatching) period was not known, and as a proxy, we used the most reasonable data obtained for *C. rastrospinosus*.

Model simulations were performed for each year from 2008 to 2017 (years included in the COHERENS model set-up) to account for interannual variability. In this way, the model calculated the 5-month trajectories of 3.330.925 particles.

3 | RESULTS

3.1 | Microsatellite analyses

3.1.1 | Genetic diversity

A total of 564 individuals were genotyped at 19 microsatellites (Table 1 and Table S3 in Supporting Information Appendix S1). One hundred and twenty-eight individuals (22.7% of the dataset) had missing data for a number of loci between 1 and 4, with an average of 1.02. All loci were polymorphic in each *Chionodraco* spp. with the number of alleles per locus ranging from 2 to 26 in *C. hamatus*, from

3 to 28 in *C. myersi* and from 5 to 44 in *C. rastrospinosus*. Allelic richness ranged from 1.62 to 24.44 in *C. hamatus* (mean: 8.57), from 2.65 to 25.39 in *C. myersi* (mean: 9.07) and from 3.17 to 33.23 in *C. rastrospinosus* (mean: 9.88). Mean expected heterozygosity varied from 0.52 in *C. hamatus* to 0.56 in *C. myersi*. Mean observed heterozygosity varied from 0.43 in *C. hamatus* to 0.51 in *C. rastrospinosus*.

Out of 361 probability tests for HWE, 23 showed a significant departure from HWE after correction for multiple tests (Table S4 in Supporting Information Appendix S1). Five out 513 comparisons showed the presence of linkage disequilibrium after correction for multiple tests. None of these tests involved the same pair of loci in the three species, and none of these loci were in linkage in previous studies so these results were attributed to chance.

FreeNA indicated that null alleles do not significantly impact the dataset, as F_{ST} values, estimated with and without application of the ENA correction method, have largely overlapping 95% CI (Figure S1 in Supporting Information Appendix S2). For this reason, all subsequent comparisons were performed with the complete, original dataset.

3.1.2 | Identification of outlier loci

The analysis with BayeScan indicated five outlier loci: Ch8501 under putative directional selection and Ch3866, Cr127, Cr236 and Ca86 potentially under balancing selection. As the results from the Structure analysis are not impacted by these loci (see below and Figure 2), all loci were included in the subsequent analyses.

3.1.3 | Population differentiation and detection of admixture

At interspecific scale and based on microsatellite variability, the three species are clearly differentiated (Table S5 in Supporting Information Appendix S1, *p*-values < .001).

At intraspecific scale, the population sample of *C. rastrospinosus* found in the Weddell Sea is not statistically different from any of the other population samples of the same species. *Chionodraco rastrospinosus* from South Orkney (2011) were significantly different from South Shetland (2002) though with very low F_{ST} values (Table 2). *Chionodraco hamatus* from the Weddell Sea sampled in 2014 are significantly different from the population samples of the Weddell Sea (1988) and the Ross Sea (1995) (from the dataset of Marino et al., 2013), but F_{ST} values were low (Table S6 in Supporting Information Appendix S1). Population comparisons for *C. myersi* showed complete homogeneity (Table S7 in Supporting Information Appendix S1).

The genetic assignment with Structure attributed 12 specimens (out of 179 *Chionodraco* individuals collected during the same cruise in the Weddell Sea) to the species *C. rastrospinosus*. These individuals were also morphologically identified as *C. rastrospinosus* during the sampling cruise.

The most probable number of clusters inferred from Structure is three (Figure 2). These groups corresponded to the three species under study and confirmed the original morphological identification. Some individuals showed admixed ancestry.

3.1.4 | Detection of hybrids

Three putative hybrids, one with *C. hamatus* and *C. rastrospinosus* ancestry and two with *C. myersi* and *C. rastrospinosus* ancestry, were identified by running Structure with the USEPOPINFO option (indicated by the arrows in Figure 2). The putative hybrids were all collected in the Weddell Sea in 2014. Individuals previously considered hybrids in Marino et al. (2013) were classified as pure by the Structure analysis.

The distribution of the Q-values obtained with the Structure analysis of the dataset simulated with HybridLab (Figure 3) showed that pure and F1 hybrid individuals could be clearly separated. On the contrary, the distribution of the Q-values for simulated backcrosses overlapped with the other two categories.

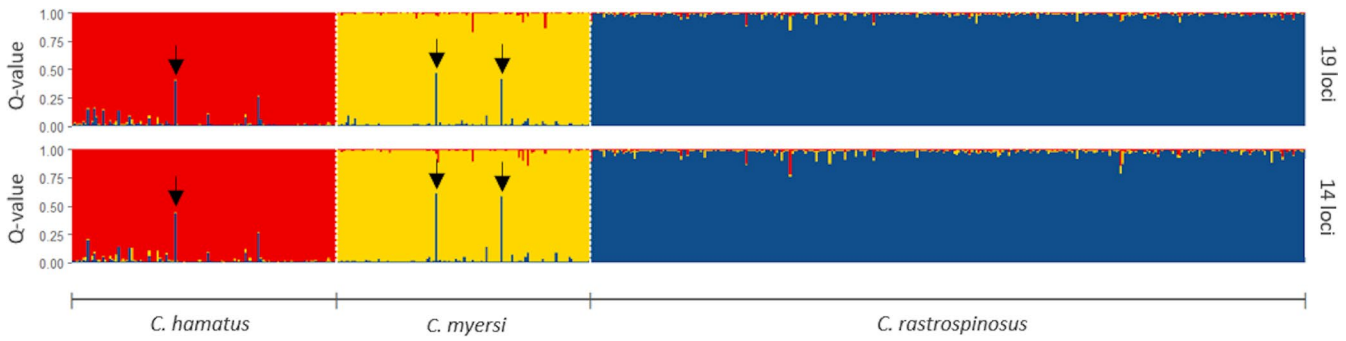

FIGURE 2 Clustering analysis of the three *Chionodraco* species. Structure results from the run without the USEPOPINFO option for 564 individuals obtained with the complete microsatellite dataset (19 loci, top) and without the five loci putatively under selection (14 loci, bottom). Each individual is represented by a column; each colour indicates a species. The portion of each colour within each column shows the percentage of ancestry for the individual (Q-value along the y-axis). The arrows indicate the putative hybrids identified by Structure applying the USEPOPINFO option

TABLE 2 Genetic distances (F_{ST}) calculated among population samples of *Chionodraco rastrospinosus* based on a panel of 19 microsatellite loci

	EI–96	SS–06	EI–02	SS–02	JI–02	SO–11	EI–12	SS–12	BS–12	R-Wedd–14	EI–18	SS–18	AS–18
EI–96	–	0.635	0.877	0.174	0.508	0.069	0.660	0.432	0.722	0.846	0.562	0.695	0.879
SS–06	0.000	–	0.058	0.046	0.701	0.043	0.059	0.140	0.072	0.894	0.136	0.428	0.812
EI–02	0.000	0.009	–	0.083	0.232	0.016	0.523	0.644	0.563	0.286	0.361	0.582	0.245
SS–02	0.005	0.009	0.005	–	0.511	**<0.001**	0.005	0.061	0.570	0.273	0.017	0.056	0.674
JI–02	0.000	0.000	0.003	0.000	–	0.075	0.293	0.120	0.368	0.368	0.383	0.588	0.776
SO–11	0.007	0.009	0.007	**0.015**	0.005	–	0.002	0.116	0.214	0.087	0.530	0.507	0.188
EI–12	0.000	0.007	0.000	0.008	0.001	0.008	–	0.232	0.363	0.036	0.929	0.644	0.313
SS–12	0.002	0.010	0.000	0.010	0.008	0.007	0.003	–	0.950	0.224	0.425	0.061	0.138
BS–12	0.000	0.018	0.000	0.000	0.002	0.006	0.001	0.000	–	0.070	0.566	0.207	0.295
R-Wedd–14	0.000	0.000	0.004	0.005	0.002	0.009	0.012	0.007	0.020	–	0.080	0.154	0.889
EI–18	0.000	0.008	0.002	0.011	0.001	0.000	0.000	0.000	0.000	0.013	–	0.856	0.304
SS–18	0.000	0.002	0.000	0.013	0.000	0.000	0.000	0.016	0.008	0.011	0.000	–	0.459
AS–18	0.000	0.000	0.006	0.000	0.000	0.007	0.002	0.012	0.008	0.000	0.004	0.000	–

Note: F_{ST} below the diagonal, *p*-values above the diagonal. Significant results after correction for multiple tests are in bold. Negative F_{ST} values have been changed to zero. Sample acronyms as in Table 1.

In the empirical dataset (i.e. the samples analysed in this study), most of the specimens showed a distribution of the Q-values that highly resembled that of pure simulated individuals. However, the Q-values of the three individuals identified as hybrids with the STRUCTURE USEPOPINFO option (indicated by the arrows in Figures 2 and 3) fall outside the range of Q-values for simulated pure individuals and can be considered admixed. Results obtained with NEWHYBRIDS suggest that these specimens have very low probability of pure ancestry (Figure S2 in Supporting Information Appendix S2) and are more likely F2 or a backcross.

3.2 | Mitochondrial DNA variability

A 326 bp D-loop fragment was successfully sequenced for 560 individuals (Tables 1 and S8 in Supporting Information Appendix S1). The fragment contained 17 polymorphic sites for *C. hamatus*, 8 for *C. myersi* and 16 for *C. rastrospinosus* (Table S8 in Supporting Information Appendix S1). Considering the whole dataset, the polymorphic sites defined 50 unique haplotypes (Table S8 in Supporting Information Appendix S1). The haplotype and nucleotide diversities were similar for *C. hamatus* and *C. rastrospinosus* (H_d = 0.747 and 0.735; π = 0.005 and 0.004, respectively) and lower for *C. myersi* (H_d = 0.407; π = 0.001; Table S8 in Supporting Information Appendix S1).

A network of D-loop haplotypes suggested the presence of three main groups of related haplotypes corresponding to the three *Chionodraco* species (Figure 4). The network confirmed what was found with the STRUCTURE analysis of the microsatellite dataset and assigned 13 individuals collected on the continental shelf of the south-eastern Weddell Sea to the species *C. rastrospinosus* (in light blue in Figure 4). Twelve of these individuals were assigned to the species *C. rastrospinosus* also with microsatellites. For the thirteenth individual, it was not possible to genotype the full panel of microsatellites because of low DNA quality. The network also showed that three individuals from the Antarctic Peninsula, indicated by STRUCTURE as pure *C. rastrospinosus*, have a *C. hamatus* D-loop haplotype (black arrows in Figure 4). Individuals classified as putative hybrids by STRUCTURE carry a mitochondrial D-loop haplotype of *C. hamatus* or *C. myersi*, the two species commonly present in the Weddell Sea (in green in Figure 4).

Interspecific differentiation tests based on mitochondrial data were all statistically significant (Tables S9 and S10 in Supporting Information Appendix S1). F_{ST} and Pi estimates indicated that *C. hamatus* and *C. rastrospinosus* were the two closest species (F_{ST} = 0.785, Pi = 6.947). According to F_{ST}, *C. myersi* and *C. rastrospinosus* were the most distant species (0.902), instead the highest Pi occurred between *C. hamatus* and *C. myersi* (10.585).

Intraspecific comparisons showed that the samples of *C. rastrospinosus* from the Weddell Sea were not significantly different from the population samples of the Antarctic Peninsula and the islands of the southern Scotia Arc. However, within *C. rastrospinosus*, five comparisons resulted significantly differentiated based on F_{ST} estimates (Table S11 in Supporting Information Appendix S1), while two pairs of population samples were significantly different based on Pi and overlapping with F_{ST} results (SO-11 versus EI-02 and SO-11 versus EI-12, Table S12 in Supporting Information Appendix S1). Detailed statistics of genetic variability and genetic distances among

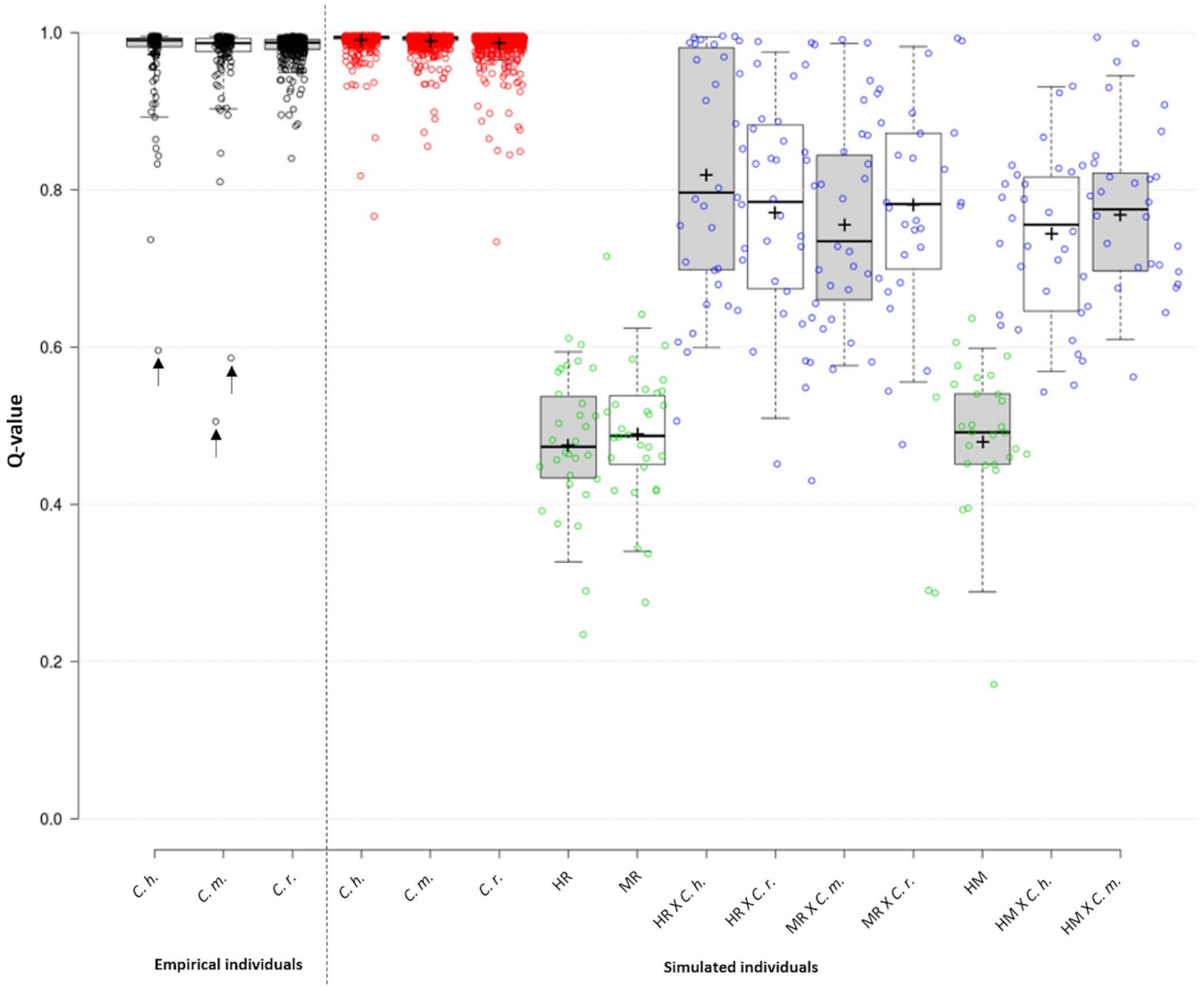

FIGURE 3 Distribution of the Q-values calculated by STRUCTURE from the empirical and simulated datasets. Central bold lines in the box plots show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the 5th and 95th percentiles; data points are plotted as open circles and crosses represent sample means. The empirical dataset is in black, and the other colours are used for the simulated dataset (red = pure, green = F1 hybrids, blue = backcrosses). The arrows indicate the putative hybrids found in the Weddell Sea. *C. h. C. hamatus*; *C. m. C. myersi*; *C. r. C. rastrospinosus*; HR first-generation hybrid between *C. hamatus* e *C. rastrospinosus*; MR first-generation hybrid between *C. myersi* e *C. rastrospinosus*; HM first-generation hybrid between *C. hamatus* and *C. myersi.* The remaining acronyms represent the backcrosses

population samples for the three species are reported in Tables S11–S16 in Supporting Information Appendix S1.

3.3 | Connectivity analyses

Particles simulating larvae of *C. rastrospinosus* were virtually released in the model from the Antarctic Peninsula (blue dots in Figure 5a) every day during the period from August to November. Individuals are assumed to passively follow the water masses and to drift during a 5-month period after which they die. Model simulations were run for a time range of 10 years to account for interannual variability. All simulations were combined, and resulting dispersal patterns of *C. rastrospinosus* are presented in Figure 5b. Most individuals of *C. rastrospinosus* consistently moved north-eastward, following the ACC and did not cross the sub-Antarctic front nor reach the Weddell Sea. Only a minority of individuals first drifted southward before being transported north-eastward.

Particles simulating larvae of *C. hamatus* and *C. myersi* were released from the south-eastern Weddell Sea (where specimens used in this study for genetic analysis were caught, see Table 1, and see red and yellow dots in Figure 5a). According to the simulations, both species remain in the Weddell Sea basin, south of 70°S, for the duration of the dispersal. Most of the larvae of *C. hamatus* and *C. myersi* entrained the southern boundary of the Weddell Gyre and drifted westward (Figure 5c and d). A minority

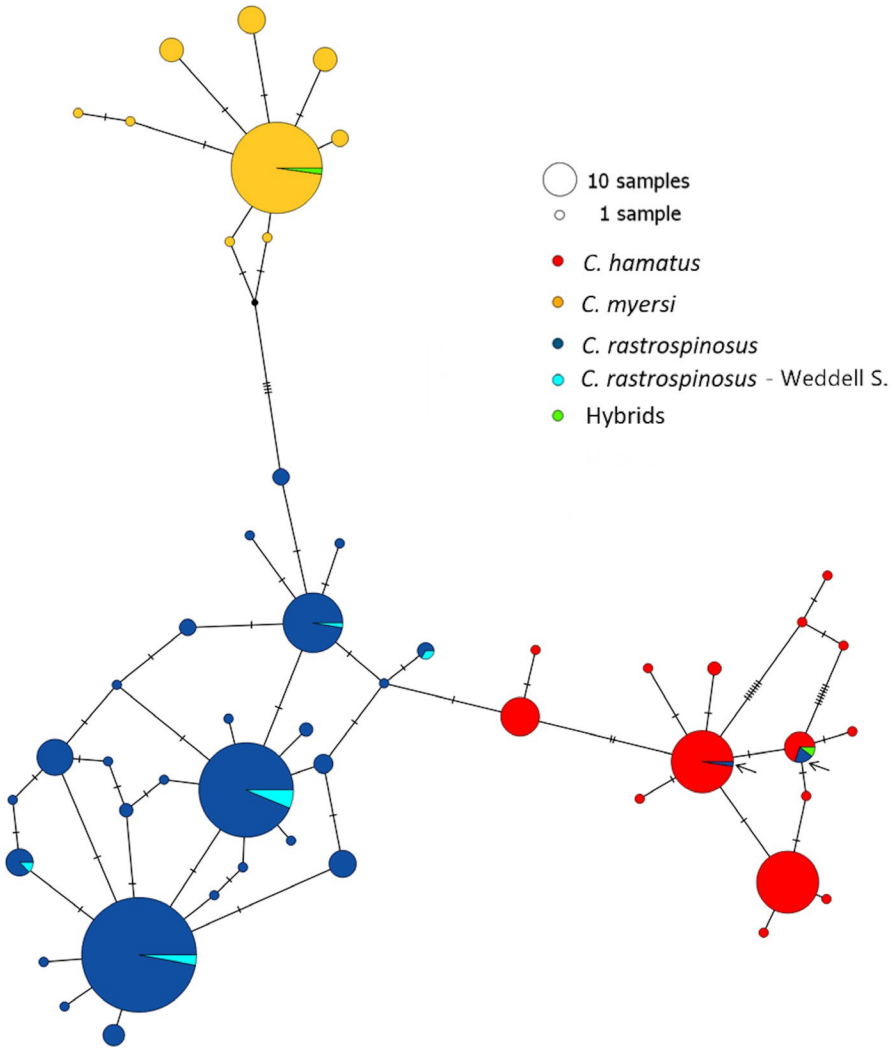

FIGURE 4 Mitochondrial haplotype network of three *Chionodraco* species. Haplotype network constructed by PopArt with the TCS algorithm and based on a 326 bp D-loop sequence for 560 specimens. Individuals are coloured based on the microsatellite genetic assignment. Circles in the legend are proportional to sample size. Black arrows indicate individuals identified as *C. rastrospinosus* by microsatellite analysis but carrying a *C. hamatus* mitochondrial haplotype. Hatch marks on the links between circles represent the number of mutations between haplotypes

of *C. myersi* reached the Antarctic Peninsula at the end of the dispersal period (Figure 5d).

4 | DISCUSSION

This study provides the first evidence of *C. rastrospinosus* on the continental shelf of the south-eastern Weddell Sea, where the occurrence of this species had never been formally documented by means of morphological and molecular methods. This study also suggests the possibility of interspecific hybridization among the three species of the notothenioid icefish genus *Chionodraco* in the Weddell Sea. We also demonstrated that, despite the suggestion of gene flow at interspecific scale and within the species *C. rastrospinosus*, our hypothesis of connectivity between the Antarctic Peninsula and south-eastern Weddell Sea afforded by oceanic currents and passive larval stages was not supported by Lagrangian modelling. This result generates alternative possible hypotheses that need to be tested in future studies about the mechanisms that maintain the interspecific connectivity among *Chionodraco* spp.

4.1 | The diversity of *Chionodraco* species in the Weddell Sea: species identification, population structure and connectivity

Chionodraco rastrospinosus was previously considered to inhabit only the Antarctic Peninsula and the southern Scotia Arc. However, considering our large sample of *Chionodraco* specimens as approximately representative of the abundance of the genus in the south-eastern Weddell Sea, we estimated that 7.2% (CI: 3.4%–10.9%) of the *Chionodraco* population is composed of *C. rastrospinosus* in that area (*C. hamatus* 49.2%, CI: 41.9%–56.4%; *C. myersi* 43.6%, CI: 36.4%–50.2%).

From a genetic point of view, the population sample of *C. rastrospinosus* is homogenous and undistinguishable from samples collected on both sides of the Antarctic Peninsula (Elephant Island, South Shetlands, Antarctic Sound) and at South Orkneys. This prompted us to hypothesize that the Weddell population, if separated, only recently split from the Antarctic Peninsula pool. In addition, the weak population structure observed in *C. rastrospinosus*, as also in *C. hamatus*, could be explained mainly by random fluctuations

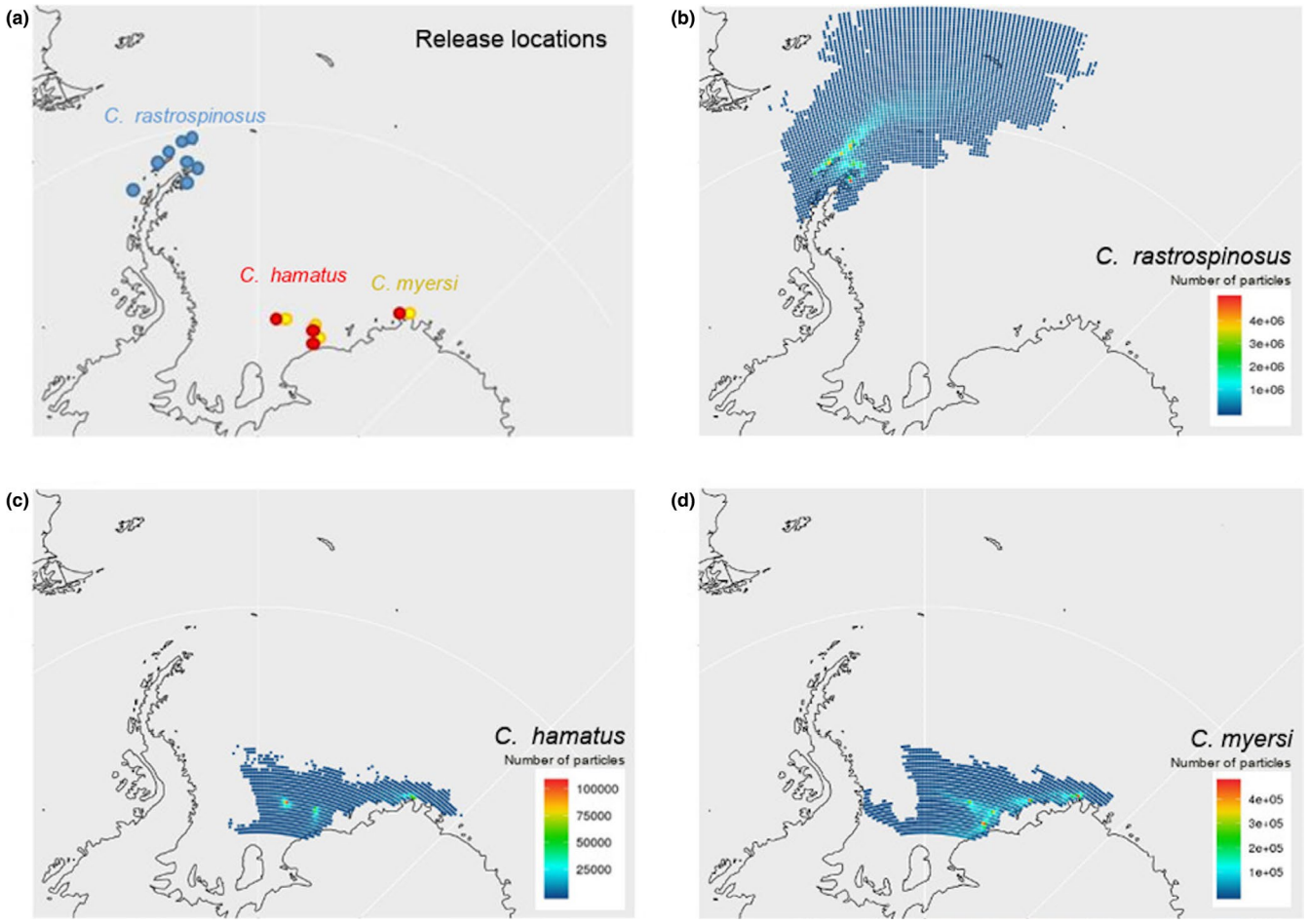

FIGURE 5 Passive larval dispersal patterns estimated for each *Chionodraco* species and based on COHERENS particle module. Panel a: location of spawning grounds from where the passive dispersal phase of the larvae starts (red for *C. hamatus*, yellow for *C. myersi*, blue for *C. rastrospinosus*) (see Table S2 in Supporting Information Appendix S1 for the exact coordinates); panels b, c and d: geographic dispersal patterns of the fish larvae during their pelagic phase as estimated from model results generated with the Lagrangian particle module of COHERENS. Panel b: model estimated dispersal pattern for *C. rastrospinosus*. Panel c: model estimated dispersal pattern for *C. hamatus*. Panel d: model estimated dispersal pattern for *C. myersi*. The colours of the dots in panels b, c and d represent the number of particles found per model grid cell on the 15th of each month of their dispersal journey and over the 10 years of the model simulation, as shown in the legend

of the allelic and haplotypic frequencies at the temporal and geographic scale. Genetic drift could act with different strength on the two markers used in this study justifying the stronger differences indicated by the mitochondrial marker (F_{ST} and Pi, Tables S11–S14 in Supporting Information Appendix S1) compared with the microsatellites (F_{ST}, Tables 2 and S6 in Supporting Information Appendix S1). It is also possible that *C. rastrospinosus* has always been present in the Weddell Sea and has gone undetected due to its low abundance and morphological similarity to the other *Chionodraco* species. Very scarce observations as in the Register of Antarctic Marine Species RAMS (http://www.marinespecies.org/rams/index.php) and in the Antarctic Biodiversity Information Facility (https://data.biodiversity.aq/) report the instances of no more than two specimens of *C. rastrospinosus* per location outside the Antarctic Peninsula region, in particular in the Indian Ocean sector of the Southern Ocean. It is likely that the original documented allopatric distributions of *C. rastrospinosus* versus *C. myersi* and *C. hamatus* have been used so far as the only diagnostic trait for the identification of *Chionodraco* spp. in the Weddell Sea and Antarctic Peninsula. Both explanations, recent separation and undetected presence, would hinge on the expectation of a well-connected Weddell Sea and Antarctic Peninsula, where exchange mainly occurs via larval dispersal driven by the ACC and Weddell Gyre. However, our Lagrangian simulations suggested that drifters released from sites selected at the Antarctic Peninsula do not reach the south-eastern Weddell Sea within the estimated time of larval stage duration. In addition, results from the Lagrangian simulation are at odds with our observation of individuals with *C. hamatus* D-loop haplotype sampled in the Antarctic Peninsula, indicating that an actual arrival of *C. hamatus* individuals from the Weddell Sea is possible.

We can, however, speculate that other mechanisms maintain the connectivity and species distribution pattern observed in this study. Some distance could be covered by actively swimming individuals (juveniles and adults), or larvae may be transported further

by occasional reinforcements of the currents due to weather conditions. The Lagrangian simulations also show that some *C. rastrospinosus* particles may drift south from the tip of the Antarctic Peninsula along the eastern coast of the peninsula inside the Weddell Sea. This observation would suggest that *C. rastrospinosus* may be distributed along the continental shelf from the Antarctic Peninsula to the south-eastern Weddell Sea. The sampling of *C. myersi* specimens on the shelf of Larsen A and Larsen B (south-eastern Antarctic Peninsula) in 2008 (Kattner & Koch, 2009) suggests indirectly that the east side of the Antarctic Peninsula may offer proper habitats for species of the genus *Chionodraco*, and therefore, it might be a possible area of connection with the *Chionodraco* populations of the Weddell Sea.

A possibly discontinuous presence of individuals of *C. rastrospinosus* could be hypothesized between the Antarctic Peninsula and the Weddell Sea. Such distribution could either result from a contraction of a past wider range or a recent range expansion driven by colonizers from the Antarctic Peninsula. Because the Antarctic Peninsula is warming up faster than other regions of Antarctica (Teschke et al., 2016) and given the high cold specialization of icefishes, we speculate that an expansion of *C. rastrospinosus* distribution towards higher and colder latitudes (i.e. the Weddell Sea) may indeed be induced by environmental changes and could be driven by active adult movement and by some larval transport along the eastern side of the Antarctic Peninsula.

4.2 | Incomplete reproductive isolation among *Chionodraco* species in the Weddell Sea

Besides documenting a potentially wider distribution than previously known for *C. rastrospinosus*, our study shows that the three *Chionodraco* species can be clearly separated and that pairwise interspecific distances based on microsatellites and D-loop reflected the classical phylogenetic relationships with *C. myersi* being generally more distant from the two sister species *C. hamatus* and *C. rastrospinosus* (Near et al., 2012). Nonetheless, the admixture analyses with STRUCTURE and NEWHYBRIDS showed instances of interspecific introgression and hybridization. These results are in agreement with previous observations by Marino et al. (2013) although the specimens described as hybrids in this study are different. This could be due to our five times larger and more recent sample set, which allowed a more precise estimation of allele frequencies. This result could also depend on the different criterion used by Marino et al. (2013) for the classification of the pure and introgressed individuals. In fact, Marino et al. (2013) identified 29 hybrid individuals based on the Q-value < 0.95 (Table S17 in Supporting Information Appendix S1) while we decided to apply a more conservative approach and suggested as putative hybrids only those individuals identified as hybrids by the USEPOPINFO option in STRUCTURE and with a Q-value non-overlapping with the pool of pure individuals based on our simulations (Figure 3). Therefore, considering our approach, none of the individuals identified by Marino et al. (2013) would be considered as putative hybrids and only seven would result as admixed (Table S17 in Supporting Information Appendix S1). In addition, previous observations by Marino et al. (2013) of hybridization between *C. rastrospinosus* and the other two *Chionodraco* species were mainly explained by past events of secondary contacts during interglacial periods and were less supported by the geographic distribution of the species as it was known before this study. In fact, the possibility of hybridization events and introgression also at present temporal scale finds additional support given our evidence of sympatric occurrence of the three *Chionodraco* species in the Weddell Sea.

Some theoretical and methodological caveats limit our power to identify hybrids and introgressed individuals with absolute certainty. In fact, in recently diverged species with a history of isolation during glacial periods and secondary contacts in post-glacial times (Near et al., 2012), it is often difficult to separate shared polymorphisms due to secondary admixture from those owing to common ancestry (incomplete lineage sorting—ILS), and because both processes frequently co-occur (Allendorf et al., 2001; Qu et al., 2012). In this study, we have attempted to solve this complexity by analysing two marker types with different evolutionary pace and inheritance (mtDNA versus microsatellites) and by applying simulations to define a Q-value threshold to separate the pure from the admixed individuals and numerically evaluate their relative abundance. The analysis with NEWHYBRIDS and the comparison between the simulation study conducted with STRUCTURE and NEWHYBRIDS showed a large overlap in the distributions of the Q-values between simulated pure and introgressed individuals. In particular, the analysis of the simulated dataset showed that, while pure and F1 individuals were generally correctly assigned, it was possible to misclassify some individuals as backcrosses and the backcrosses could be incorrectly assigned as pure (Figure S3 in Supporting Information Appendix S2). However, for pure individuals the probability of being correctly assigned was never smaller than ~ 34%. The backcrosses, instead, could wrongly be identified as pure with probabilities that can be almost of 100%. Our simulation suggested that, if we classified an individual as hybrid with a probability greater than 70%, we could reasonably assume that individual as having a true admixed ancestry. On the contrary, even if an individual had a nearly 100% probability of being pure, there would be the possibility that it actually were an introgressed individual. A clear-cut separation between pure and introgressed individuals was not possible and the use of a fixed threshold to classify hybrids and introgressed individuals would have resulted in a high assignment error rate. Therefore, the results of the admixture analyses with STRUCTURE and NEWHYBRIDS suggested the presence of both ILS and introgression occurred during secondary contacts. The two explanations can be justified by the recent time of divergence for this genus (Near et al., 2012). However, while our approach allowed us to survey many individuals and our markers clearly enabled us to assign individuals to their species of origin, future studies should target what are the genomic regions affected by introgression or hybridization to disentangle the relative role of secondary contacts and ILS.

Considering the above caveats, we decided to adopt a conservative approach and considered as hybrids only three individuals that showed a Q-value completely outside the range of pure individuals and that, at the same time, were identified as hybrids in STRUCTURE using the USEPOPINFO function. Although possibly underestimated, the percentage of individuals with admixed ancestry (three hybrids and three specimens with nuclear–mitochondrial discordance; see below) that we found (~1%) is in line with the level of abundance reported in literature for sympatric species (1/100 or 1/10,000; Mallet, 2005). Moreover, independently from the adopted marker, after few generations the signal of introgression fades away, becoming less than 1% after seven generations. Thus, in many situations only the discordance between nuclear DNA and mitochondrial DNA can detect an individual with admixed ancestry (Weisrock et al., 2005). In our dataset, we also identified three specimens from the Antarctic Peninsula, which, according to STRUCTURE, were assigned to *C. rastrospinosus* with ~ 100% probability but exhibited a mitochondrial D-loop haplotype from *C. hamatus*. This case of nuclear–mitochondrial discordance indicates that these specimens are descendants of hybrid individuals and that they or their ancestors must have migrated or been transported from the Weddell Sea to the Antarctic Peninsula. This conclusion suggests that, despite the indication of the Lagrangian simulation, opportunities of connection might occur between the two areas.

According to STRUCTURE, two hybrid individuals resulted from the cross between *C. myersi* and *C. rastrospinosus* and one derived from *C. hamatus* × *C. rastrospinosus*. Thus, a reproductively active *C. rastrospinosus* population in the Weddell Sea may be postulated. The specimens of *C. rastrospinosus* collected in the south-eastern Weddell Sea and analysed in this study have a heterogeneous composition with both males and females at different stages of maturity (but none of them were larval stages). Among these, a special instance of a gravid *C. rastrospinosus* female (gonadosomatic index of 24% of total weight and 39% of gutted weight, maturity stage 4 following the scale by Kock & Kellermann, 1991) indicates that this population may, indeed, be reproductively active. Despite the need to further test the origin and the distribution limits of *C. rastrospinosus* in the Weddell Sea, this region certainly represents an area of overlapping distribution of three different species capable of interbreeding and of producing viable hybrid offspring.

The hybridization among the *Chionodraco* species poses questions about what pre- or post-zygotic reproductive barriers partially maintain the species isolation, what biological conditions allow successful interspecific reproduction and whether any specific pattern of hybridization direction is favoured. Our observation, that crosses only occur between *Chionodraco* species previously considered allopatric, suggests that *C. hamatus* and *C. myersi* might have evolved stronger reproductive barriers through reinforcement after a long evolutionary time spent in sympatry. On the contrary, *C. rastrospinosus* may have had only limited opportunities of contact with the other *Chionodraco* species during interglacial times (Marino et al., 2013), which might explain more permeable reproductive barriers between *C. rastrospinosus* and *C. hamatus*/*C. myersi*.

In *Chionodraco* spp., pre-zygotic barriers can consist of differences in the reproductive period. *Chionodraco rastrospinosus* in the Antarctic Peninsula is reported to have a similar reproductive season compared with *C. hamatus* (February–April and January–March, respectively). Indeed, the gravid female indicative of adults of *C. rastrospinosus* reproductively active in the Weddell Sea was collected in February. The reproductive period of *C. myersi* is different, from July to September, and would not favour interspecific mating with *C. rastrospinosus*. However, the information about the precise reproductive period for *C. rastrospinosus* in the Weddell Sea is insufficient. For some notothenioid species in the Antarctic Peninsula, for example *Gobionotothen gibberifrons*, *Lepidonotothen larseni* and the icefish *Chaenocephalus aceratus*, the onset of spawning exhibits a latitudinal trend in which the reproductive season starts earlier at lower latitudes (Kock & Kellermann, 1991; Papetti et al., 2007; Ruzicka, 1996). Therefore, it could be hypothesized that some individuals of *C. rastrospinosus* have adopted a similar change in the reproductive time in the south-eastern Weddell Sea.

Another pre-zygotic barrier can consist of differences in reproductive behaviour that allow for intraspecific recognition. Males of *C. hamatus* display nesting behaviour: they prepare nests on the sea floor where females lay eggs subsequently guarded by males until hatching (Ferrando et al., 2014). Although nest construction has never been demonstrated conclusively in *C. rastrospinosus* and *C. myersi*, evidence of hybridization among *Chionodraco* species may represent an indirect proof of a similar nesting behaviour. Desvignes et al. (2019) also mentioned that the characteristics of icefish nests and the reproductive behaviour are usually species-specific. In our study, none of the three hybrid individuals carry a *C. rastrospinosus* genetic contribution as a maternal species and three individuals from the Antarctic Peninsula, indicated as pure *C. rastrospinosus* with microsatellite data, have a *C. hamatus* D-loop haplotype (black arrows in Figure 4). This preliminary observation leads to speculate that if interspecific mating occurred among *Chionodraco* spp., it might preferentially involve males of *C. rastrospinosus* with females of the other two species.

This remains a speculation owing to the very limited number of observations in our study, and an alternative explanation would imply post-zygotic barriers to hybridization as, for instance, differential embryonic survival rates, fertility and mating success of interspecific crosses (Desvignes et al., 2019). At the post-zygotic level, hybridization among icefish species is possible as recently demonstrated by Desvignes et al. (2019). The authors successfully performed an in vitro fertilization of *C. aceratus* eggs with sperm of *C. rastrospinosus* (thus two icefish species from different genera) obtaining viable larvae. The experiment was terminated artificially after five months, at the end of the Antarctic field campaign, leaving the survival rate, fertility and mating success of the hybrids unassessed (Desvignes et al., 2019).

4.3 | Conclusions

Connectivity among populations is fundamental to shape the genetic diversity and demography of species and the stability of ecological

communities (Clobert et al., 2012). In the case of *Chionodraco*, future sampling and a constant monitoring are needed to fully understand the pattern of connectivity and the evolution of the population of *C. rastrospinosus* and the hybrids recently discovered in the Weddell Sea. Similar studies should target also other icefishes to understand the extent of interspecific gene flow at the whole family scale and whether this is promoted by environmental changes. Experiments in vitro of interspecific crossing among *Chionodraco* spp. could also help testing survival of hybrid larvae and assessing fecundity of hybrid adults. Regular surveys may also help discover changes in species richness and early arrivals of allochthonous species. This becomes even more relevant when it comes to propose and support the implementation of marine protected areas.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ddi.13249.

DATA AVAILABILITY STATEMENT

The microsatellite datasets supporting the conclusions of this article are available in Genepop format as additional files in DRYAD at https://doi.org/10.5061/dryad.83bk3j9p1. Mitochondrial sequences are available in GenBank under the Accession Numbers MT571397-MT571447.

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BIOSKETCH

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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