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No evidence for hybridization between *Calanus finmarchicus* and *Calanus glacialis* in a subarctic area of sympatry

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Abstract

In the North Atlantic and the Arctic Ocean, four species of the copepod genus *Calanus* dominate the zooplankton biomass. Because of their morphological resemblance, knowledge of their respective distribution range has long been biased by misidentification, until the recent use of molecular tools uncovered numerous areas of sympatry. As hybridization between *Calanus finmarchicus* and *Calanus glacialis* has been claimed in the East-Canadian Arctic based on microsatellites, we investigated further the potential for interbreeding in newly uncovered areas of sympatry. *Calanus* species and stage composition were analyzed during winter in two Norwegian subarctic fjords, using molecular markers developed specifically for species identification and hybrid detection between *C. finmarchicus* and *C. glacialis*. Overall, *C. glacialis* were the most abundant throughout the winter, followed by *C. finmarchicus* and *Calanus hyperboreus* with only a few records of *Calanus helgolandicus*. The presence of *C. glacialis*, *C. hyperboreus*, and *C. finmarchicus'* nauplii was recorded, indicating that these species reproduce locally. In January and February, the simultaneous occurrence of males and females of both *C. finmarchicus* and *C. glacialis* suggested a potential for interspecies mating. However, genetic admixture tests performed on all 1126 individuals revealed no signal of hybridization, implying a strong reproductive isolation mechanism. We conclude that no evidence supports a potential for hybridization between *C. finmarchicus* and *C. glacialis*.

Calanoid copepods of the genus *Calanus* play a key role in marine food webs of the northern hemisphere as primary consumers and main source of food for many predators (Falk-Petersen et al. 2007). In the North Atlantic and the Arctic Ocean, four species prevail: the boreal species *Calanus helgolandicus*, the boreal-arctic *Calanus finmarchicus* with a preference for North Atlantic habitats, the circumpolar arctic neritic species *Calanus glacialis*, and the holarctic *Calanus hyperboreus* with its core distribution located in the Greenland Sea (Conover and Huntley 1991). Although the four species are similar morphologically and have comparable life histories (Conover 1988), including a dormant stage (diapause) at depth during winter, they differ greatly in their abundances depending on the environment. Because of their habitat preferences, *Calanus* species are often used as indicators for specific water masses and temperature regimes and are thus commonly used to investigate potential impacts of climate change on marine ecosystems (Beaugrand et al. 2003).

However, despite being among the most studied zooplankton organisms, identification of *Calanus* copepods to species level remains a challenge, and it is particularly difficult to discriminate between *C. glacialis* and *C. finmarchicus* in areas of sympatry (Lindeque et al. 2006; Parent et al. 2011; Gabrielsen et al. 2012; Choquet et al. 2018). Therefore, different molecular tools have been developed to facilitate the identification of these ecologically important species without bias due to their morphological plasticity (Lindeque et al. 1999; Hill et al. 2001; Provan et al. 2007; Parent et al. 2012; Smolina et al. 2014). Currently, the most cost-effective and easiest way to reliably identify the *Calanus* species involves the use of a combination of six molecular markers type insertion/deletion (InDel) (Smolina et al. 2014) following the optimized protocol described in Choquet et al. (2017). InDel markers are nucleotide sequences whose length varies from one species to another due to motifs of nucleotide insertion or deletion. The polymorphism used to distinguish between species is therefore a polymorphism of length, where each marker is sized to determine the species identity. These markers have proven useful to identify the four *Calanus* species living in the North

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Atlantic and the Arctic Ocean (Nielsen et al. 2014; Smolina et al. 2014; Choquet et al. 2017). Their performance was tested over thousands of individuals across the North Atlantic and the Arctic Ocean, and their validity was confirmed by comparison with traditional 16S mitochondrial DNA sequencing for species identification (Nielsen et al. 2014; Smolina et al. 2014; Choquet et al. 2017).

The initial motivation from Smolina et al. (2014) to develop this particular set of InDel markers was triggered not only by the need of a more straightforward species identification method for *Calanus* spp. but also because of the reporting of hybridization between *C. finmarchicus* and *C. glacialis*. Indeed, recent studies based on microsatellite molecular markers suggested that *C. finmarchicus* and *C. glacialis* are able to interbreed and produce fertile hybrids at high rates (up to 50% of the *Calanus* community) in the northwestern Atlantic and the Canadian Arctic (Parent et al. 2012, 2015). Microsatellite molecular markers are repetitive DNA sequences, with varying number of repetitions. Although microsatellite markers also display a length polymorphism, their mutation mechanism is very different from that of InDels and thus microsatellites are generally not the most suited molecular markers for species identification and hybrid detection because of frequent occurrences of null alleles (Dakin and Avise 2004), possible homoplasy when comparing two species (Chambers and MacAvoy 2000), high mutation rate, and difficulties to score alleles (Pompanon et al. 2005; Selkoe and Toonen 2006). In contrast, InDel markers (such as the ones used in the present study) have a low mutation rate due to single mutation event, thus resulting in a conserved phylogenetic signal (Liu and Cordes 2004) and alleles are easier to genotype with more reproducibility (Väli et al. 2008). Therefore, in their study, Smolina et al. (2014) selected InDel markers derived from partial sequences of genome and transcriptome from both *C. finmarchicus* and *C. glacialis*. These markers are nuclear, hence they are inherited by both parents, and they were chosen to be codominant (both alleles are expressed equally when cooccurring in an individual). As a result, these InDel markers are the most reliable tools currently available for detecting putative hybrids between *C. finmarchicus* and *C. glacialis* (Nielsen et al. 2014; Smolina et al. 2014). In the West and East of Greenland, the aforementioned microsatellites were combined with InDels to investigate potential hybridization between *C. finmarchicus* and *C. glacialis*, and no hybrids were detected then (Nielsen et al. 2014). In the same study, in silico simulations suggested that the use of microsatellites alone has less power to fully discriminate between introgressed individuals (introgression: incorporation of genetic material from one species in the genome of another) and parental species, in comparison with the InDels (Nielsen et al. 2014), casting the doubt on the validity of the "*Calanus* hybrids" hypothesis and calling for deeper investigations. Moreover, despite numerous examples of strong morphological resemblance between species in marine zooplankton (Aarbakke et al. 2011), no case of hybridization has been reported yet to our knowledge (apart from *Calanus*: Parent et al. 2012). There are, however, documented examples of hybridization occurring in freshwater zooplankton (e.g., *Daphnia*: Wolf 1987).

In Choquet et al. (2017), the InDel markers were used to analyze the *Calanus* species composition within 83 zooplankton samples taken from various locations across the North Atlantic and the Arctic Ocean. This large-scale investigation revealed the occurrence, sometimes in high proportions, of some of the species in areas where they had not been reported before (Choquet et al. 2017). In particular, several regions of cooccurrence between *C. finmarchicus* and *C. glacialis* were unveiled, or confirmed from previous molecular-based studies in boreal and subarctic fjords (Lindeque et al. 2004; Choquet et al. 2017). There, both species cooccur in similar proportions but they are so morphologically alike that only genetics can distinguish the two species (Choquet et al. 2018). Skjerstadfjord and Mistfjord (northern Norway) are striking examples of localities where not only *C. finmarchicus* and *C. glacialis* cooccurrence was reported, but where *C. hyperboreus* and *C. helgolandicus* were also found in lesser proportions (Lindeque et al. 2004; Choquet et al. 2017). These two deep fjords, separated from the Norwegian Sea shelf by shallow sills, represent optimal natural systems to investigate the question of hybridization between *C. finmarchicus* and *C. glacialis*.

In the present study, we combined classical field sampling of zooplankton with the use of molecular InDel markers to follow *Calanus* species composition during a season of potential interbreeding, from November to March, in Skjerstadfjord and Mistfjord. By monthly sampling, we investigated the ecological potential for interspecies mating by recording the presence of adult males together with adult females, simultaneously with molecular analyses (InDels) to search for signals of introgression in different developmental stages of *C. finmarchicus* and *C. glacialis*.

Materials and methods

Sample collection

Zooplankton samples were collected in Skjerstadfjord (67°16′39.3″N 14°53′52.5″E) and in Mistfjord (67°26′89.7″N 14°50′45.8″E) (northern Norway; Fig. 1) throughout the main mating season, in winter, by towing a Juday net vertically from 500 m depth in Skjerstadfjord (max. depth 535 m) and from 285 m in Mistfjord (max. depth 297 m) to the surface. The Juday net mesh size was 200 μm and the opening of the net 0.1 m^2.

Skjerstadfjord is a 535 m deep fjord with a shallow sill (23 m) that separates the fjord basin from Saltfjord basin. The sill area is narrow, forming a tidal channel that concentrates the tidal energy in a strong tidal jet that severely influences the water exchange between its basin and Saltfjord (Eliassen et al. 2001). Mistfjord has a shallower basin (285 m) and deeper sill (34 m), but the fjord is smaller in area compared to Skjerstadfjord (Fig. 1). The tidal current is weaker compared to that of Skjerstadfjord.

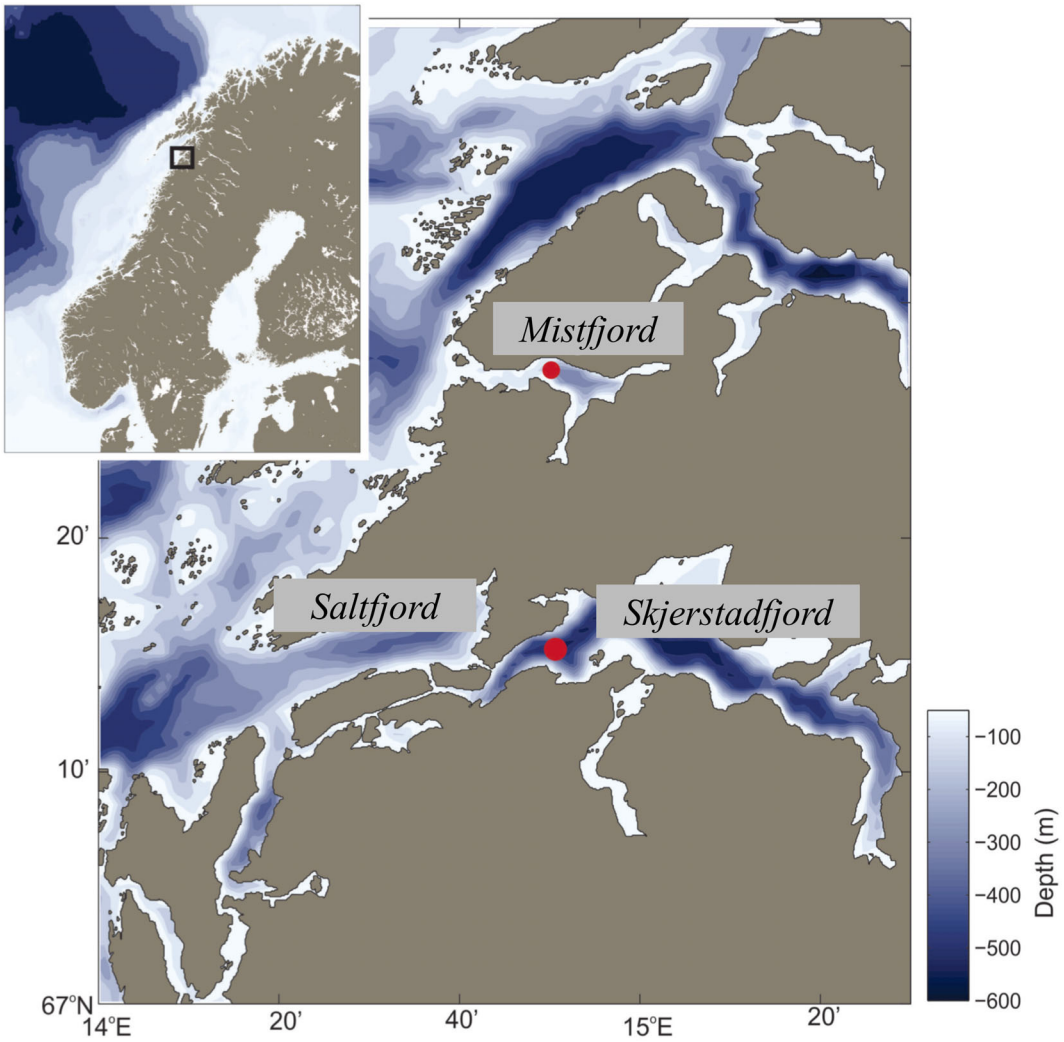

Fig 1. Sampling locations in Skjerstadfjord and Mistfjord in northern Norway.

Measurements of temperature, salinity, oxygen, and fluorescence were carried out at each sampling site by a CTD (model SD204) with a fluorometer attached. We assumed 100% filtration efficiency of the net. Sampling was done with a periodicity of approximately 1 month from November 2016 to March 2017 in Skjerstadfjord (16 November 2016, 21 December 2016, 30 January 2017, 28 February 2017, 30 March 2017) and Mistfjord (17 November 2016, 08 December 2016, 24 January 2017, 23 February 2017, 29 March 2017). An additional sampling was conducted in Skjerstadfjord on 11 May 2017. Five replicates were collected for each date and location. Samples were preserved in 70–80% undenatured ethanol, with subsequent change of ethanol after the first 24 h.

Calanus spp. stage composition

Zooplankton samples of the three first replicates of each month from November to March were divided in subsamples containing about 50 individuals of *Calanus* spp. from developmental stage CIII and older (CIV, CV, CVI) using a Folsom plankton splitter. Forty-eight of these individuals were identified to developmental stage and sex under a stereomicroscope (Leica 10× /23, ×4). They comprised almost exclusively the overwintering copepodite stages CIV, CV, and adults (CVI). The very few CIII present were discarded. We divided the remaining specimens into four Petri plates (one for each developmental stage and sex) containing nuclease-free water in order to remove the ethanol. Each individual was given a unique ID before proceeding to DNA extraction for molecular species identification.

Nauplii of *Calanus* spp.

To investigate whether the different species are reproducing locally, we tentatively sorted out nauplii of *Calanus* spp. (all stages confounded) randomly by successive subsampling from samples where they appeared to be more abundant (Table 1). We expected *C. finmarchicus* and *C. helgolandicus* to start

Table 1. Results of molecular species identification of *Calanus* spp. nauplii. *N* is the number of Calanoid nauplii picked from the different samples and analyzed genetically. The numbers of nauplii genetically identified as *Calanus* spp. are divided per species: *C. finmarchicus* (Cfin), *C. glacialis* (Cgla), *C. hyperboreus* (Chyp), and *C. helgolandicus* (Chelg).

		Molecular ID			
	N	Cfin	Cgla	Chyp	Chelg
Skjerstadfjord					
01 Mar 2017	44	0	0	12	0
30 Mar 2017	128	0	14	49	0
11 May 2017	48	2	16	0	0
Mistfjord					
23 Feb 2017	47	2	0	8	0
29 Mar 2017	29	0	1	0	0

reproducing later than *C. glacialis* and *C. hyperboreus*, since they have been described as income breeders relying on the spring phytoplankton bloom to fuel their egg production (Conover 1988; Bonnet et al. 2005; Falk-Petersen et al. 2009). Therefore, to ensure we would be able to find their nauplii in case they reproduced later, an additional sampling was completed in May. Size estimations proposed in Daase et al. (2011) were followed in order to select only (or mostly) *Calanus* spp. nauplii. The nauplii were rinsed in nuclease-free water to remove ethanol and each individual was given a unique ID before proceeding to DNA extraction and molecular species identification (*see* next section).

Calanus species composition

Calanus species composition was determined for each sample from November to March using molecular markers for species identification. InDel was used to identify each individual as *C. finmarchicus*, *C. glacialis*, *C. hyperboreus*, or *C. helgolandicus*. For each replicate, we removed the antennules of the 48 individuals randomly selected beforehand (*see* the *Calanus* spp. stage composition section) to extract the DNA of each specimen and used them for molecular species identification following the protocols described in Choquet et al. (2017) (for DNA extraction) and Smolina et al. (2014) (for InDel markers amplification and genotyping). The obtained number of each genetically identified species was used for estimation of species abundances in the corresponding fjords. The nauplii, selected in the previous section, were also identified genetically using DNA extracted from their whole body.

Hybridization between *C. finmarchicus* and *C. glacialis*

Putative hybridization between *C. finmarchicus* and *C. glacialis* was investigated within the *Calanus* spp. nauplii and copepodite individuals (i.e., CIV, CV, and CVI) by performing an admixture analysis with STRUCTURE (version 2.3.4; Pritchard et al. 2000) (parameters: ancestry model = admixture; frequency model = correlated; burn-in = 2,000,000; MCMC length = 1,000,000 after burn-in). The software uses a Bayesian algorithm to identify K (K = 2 for *C. finmarchicus* and *C. glacialis*) clusters of genetically homogenous individuals. Based on its multilocus genotype, each individual is then characterized by an admixture coefficient, defined as the probability of belonging to the *C. finmarchicus* or *C. glacialis* cluster.

Results

Physical and biological environment

Both fjords were dominated by local waters with warm (ca. 8°C) and relatively fresh (< 33 psu) surface waters in November that gradually cooled and became more saline as winter convection proceeded towards March (Fig. 2). In general, Skjerstadfjord was less saline than Mistfjord over the period studied. In Skjerstadfjord, the less saline (< 33 psu) surface layer extended up to 150 m depth, while in Mistfjord only the first 50 m appeared with lower salinity compared to the rest of the water column. Below 150 m depth, the salinity was temporally stable in Skjerstadfjord, while stability in salinity was reached at around 75 m depth in Mistfjord. The temperature was stable below 200 m depth in Skjerstadfjord with ca. 5°C. In Mistfjord, temperature records showed a stratification of the water column, not observed in Skjerstadfjord, indicating less mixing of the water column in Mistfjord vs. Skjerstadfjord. These different water layers of temperature decreasing with depth in Mistfjord were stable over the period studied below 75 m. Fluorescence measurements showed typically low (< 0.05 μg Chl *a* L^{-1}) winter Chl *a* values from December to February (no data recorded in November). Much higher values (up to 5 μg Chl *a* L^{-1}) were measured at the end of March, signaling the onset of the phytoplankton bloom.

Calanus species composition

Overall, the *Calanus* spp. abundances were slightly lower in Skjerstadfjord (1278–1309 ind. m^{-2}) than in Mistfjord (1673–2270 ind. m^{-2}) from November to February (Fig. 3; Supporting Tables 1, 2), but all four species were recorded in both fjords (Fig. 3). Occurrences of *C. helgolandicus*, however, were scarce (three individuals detected in Skjerstadfjord at the end of March and a few individuals in Mistfjord from the end of January to the end of March; Fig. 3; Supporting Tables 1, 2).

In Skjerstadfjord, *C. glacialis*, *C. finmarchicus*, and *C. hyperboreus* were almost equally present from November to January (Fig. 3; Supporting Table 1). In February, the numbers of *C. finmarchicus* started to decline and in March its abundance was low as that of *C. helgolandicus*. For *C. glacialis* and *C. hyperboreus* the population numbers were constant from November to February, but a marked decline was also seen for these two species in March (Fig. 3).

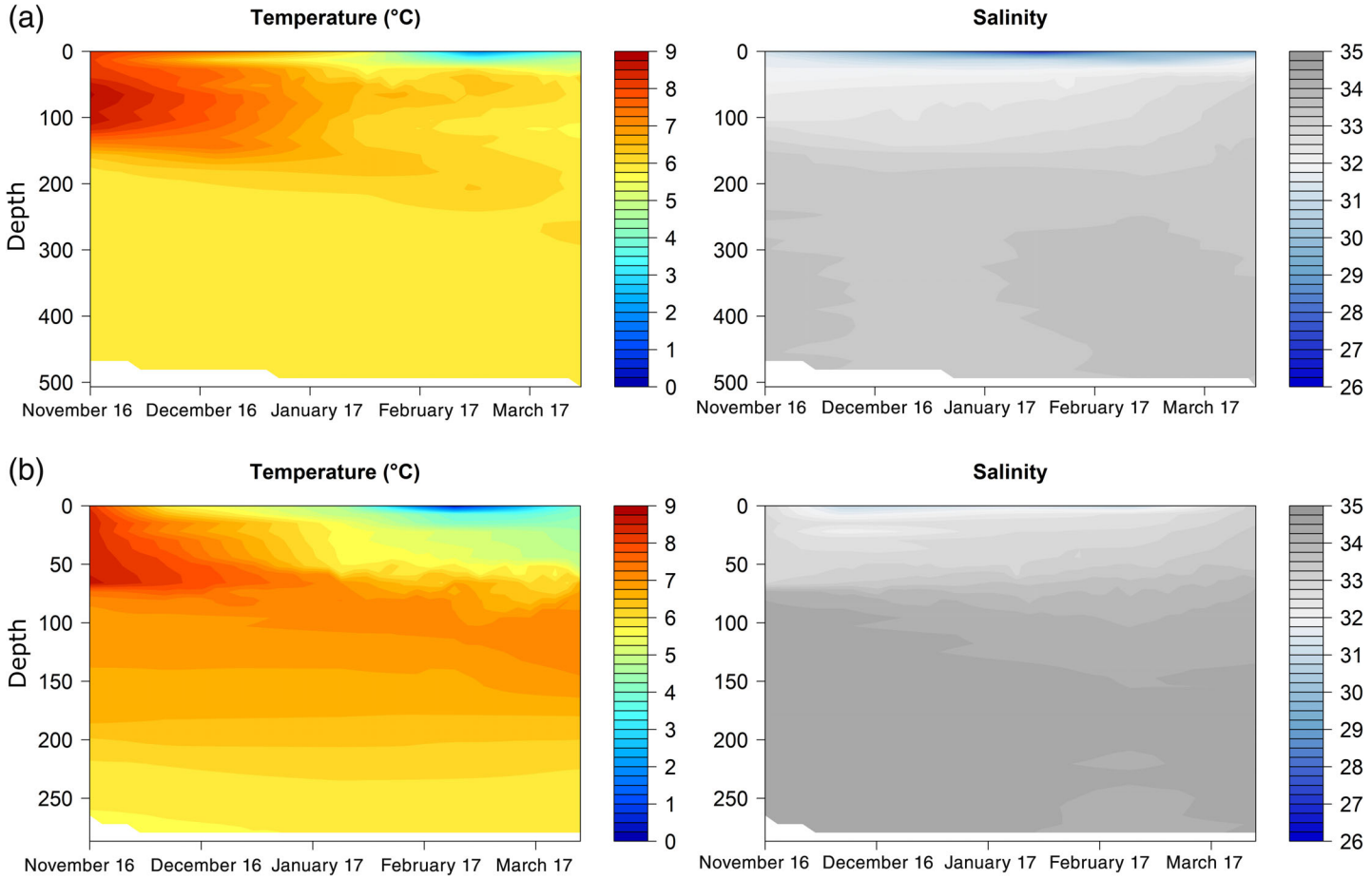

Fig 2. Temperature (°C) and salinity (psu) in (**a**) Skjerstadfjord and (**b**) Mistfjord from November 2016 to March 2017.

In Mistfjord, *C. glacialis* dominated the *Calanus* community in terms of abundance (Fig. 3), contributing from 47% to 72% of the total number of *Calanus* identified (Supporting Table 2). The numbers of *C. glacialis* started to decline from February and became very low in March (Fig. 3). In the period from November to February, *C. finmarchicus* was the second most abundant *Calanus* species (16–21%), followed by *C. hyperboreus* (5–16%) and *C. helgolandicus* (< 2%) (Fig. 3; Supporting. Table 2). A strong decline of *C. finmarchicus* numbers was also found in March, similar to *C. glacialis* (Fig. 3). The numbers of *C. hyperboreus* did not vary much and remained relatively low throughout the studied period.

Calanus spp. stage composition

Developmental stage CIV was the less abundant among all stages observed (Fig. 3) and > 81% of these CIV individuals were *C. hyperboreus*, present in both fjords in low abundance (< 129 ind. m^{-2}). *C. finmarchicus* and *C. glacialis* CIVs were seldom occurring and mainly found in Mistfjord from December to February (Fig. 3; Supporting Tables 1, 2). From November to January, the dominant developmental stage was CV for all species in both fjords (Fig. 3). In both fjords, adult females of *C. glacialis* and *C. hyperboreus* were found starting from November, while for *C. finmarchicus* and *C. helgolandicus* they were observed starting from January. Females peaked in abundance in all four species in February–March. Adult males of *C. glacialis*, *C. finmarchicus*, and *C. hyperboreus* appeared in January and were present until the end of February for *C. glacialis* and *C. hyperboreus*, and until March for *C. finmarchicus*. In March, one male of *C. helgolandicus* was recorded in Skjerstadfjord, otherwise not.

Nauplii of *Calanus* spp.

In Skjerstadfjord, 93 nauplii were successfully identified as *Calanus* spp., including 2 *C. finmarchicus*, 30 *C. glacialis*, and 61 *C. hyperboreus* from the different samples (Table 1). In Mistfjord, from the 11 nauplii successfully identified as *Calanus* spp., two were *C. finmarchicus*, one *C. glacialis*, and eight were *C. hyperboreus* (Table 1).

Hybridization between *C. finmarchicus* and *C. glacialis*

The timing of adult male and female occurrence revealed an overlap between *C. finmarchicus* and *C. glacialis*. However,

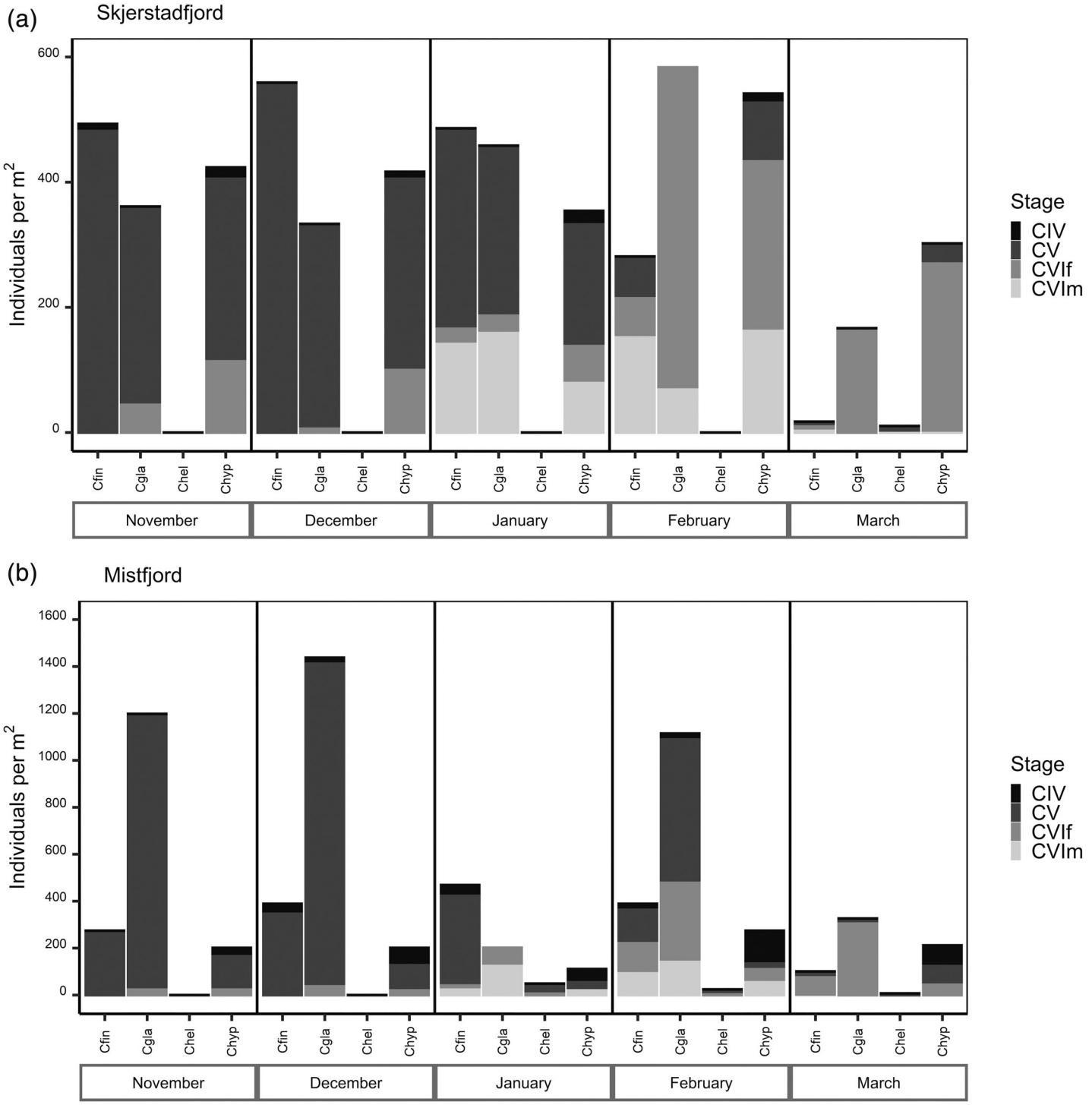

Fig 3. Abundances of four *Calanus* species calculated per developmental stage from CIV to adult individuals in (**a**) Skjerstadfjord and (**b**) Mistfjord from November 2016 to March 2017. Abundances are represented in number of individuals per m^2 for *C. finmarchicus* (Cfin), *C. glacialis* (Cgla), *C. helgolandicus* (Chel), and *C. hyperboreus* (Chyp).

the genetic admixture analysis based on the InDel genotypes of 503 and 588 individuals (*C. finmarchicus* and *C. glacialis* combined) from Skjerstadfjord and Mistfjord respectively did not detect any introgression (Fig. 4a,b). The same analysis performed on nauplii did not detect any introgression either (Fig. 4c).

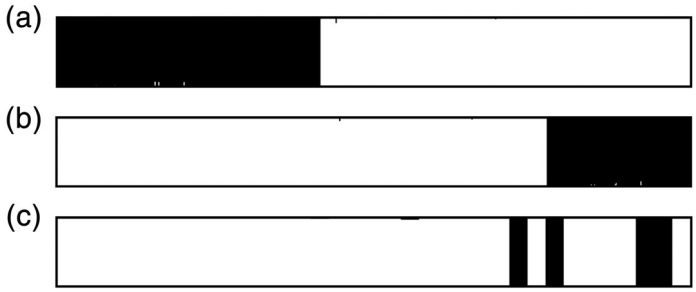

Fig 4. Genotype admixture analysis based on nuclear InDel markers shows no hybrids between *C. finmarchicus* (black) and *C. glacialis* (white). Tests of genotype admixture were performed for 503 individuals from developmental stages CIV, CV, and adult males and females in Skjerstadfjord (**a**), 588 individuals from stages CIV, CV and adult males and females in Mistfjord (**b**), and 35 nauplii from both fjords together (**c**). For each plot, one individual is represented by one vertical line, and the associated color (black or white) corresponds to the cluster to which each individual is assigned based on its genotype. Here, the lines are either black or white for each individual, suggesting no admixture between the two. A hybrid individual of first generation would appear as a vertical line half black and half white. A few individuals/lines contain a small portion of the second color, indicating a minor shared part of their genotype with the other species, but not big enough to be interpreted as an admixture signal resulting from hybridization. This figure was produced using the program CLUMPAK (Kopelman et al. 2015).

Discussion

Calanus species in Skjerstadfjord and Mistfjord

Skjerstadfjord and Mistfjord are both characterized by the presence of deep basins and sills that are shallower than 50 m, which distinguishes them from fjords with deeper sills (Ibrekk et al. 1993). Nonetheless, the environmental data recorded there from November 2016 to March 2017 depicted distinct patterns of hydrography associated to each fjord (Fig. 2), reminding us that fjord systems are very complex and influenced by many variables not always well understood. It is therefore important to consider each fjord as a unique distinct habitat. In these two fjords, *C. finmarchicus*, *C. glacialis*, *C. hyperboreus*, and *C. helgolandicus* were all genetically identified during the winter 2016–2017, confirming records from previous studies (Lindeque et al. 2004; Choquet et al. 2017). However, *C. glacialis* was the dominant species in Mistfjord throughout the entire study period, while *C. finmarchicus*, *C. glacialis*, and *C. hyperboreus* were found in approximately equal proportions in Skjerstadfjord. The differences in hydrography between fjords may explain this contrast. In Mistfjord, where *C. glacialis* abundances were more than twice higher than in Skjerstadfjord, the tides generate only weak turbulent diffusion in the upper part of the basin water. Consequently, the deepest part of Mistfjord basin water is regarded to be conserved over many years, close to a decade before a new major inflow (Skreslet et al. 2015). In contrast, Skjerstadfjord is connected to Saltfjord by Saltstraumen, a narrow channel triggering a forceful tidal jet onto Skjerstadfjord basin (Eliassen et al. 2001). On average, this channel transports about 3×10^{-8} m^3 of water from the upper 100 m depth from the inner part of Saltfjord to the outer part of Skjerstadfjord, and back, on every semidiurnal tide. The jet erodes on the basin water and the turbulent diffusion causes complete renewal of the bottom water every 4 to 5 months (Eliassen et al. 2001). Therefore, considering *C. glacialis* as a possible resident species of the fjords (Choquet et al. 2017) and apparently less successful outside on the shelves and open ocean in the sub-Arctic (Niehoff and Hirche 2005; Choquet et al. 2017), it seems likely that the relatively stable basin waters of Mistfjord constitute a more favorable habitat for this species compared to the strongly mixed waters of Skjerstadfjord.

Different types of molecular markers (microsatellites and single nucleotide polymorphisms) have indicated higher levels of population differentiation between fjords for *C. glacialis* when compared with *C. finmarchicus* (Choquet et al. 2017, 2019). However, it does not necessarily mean that the fjords are completely isolated systems. The very extensive tidal advection caused by Saltstraumen probably transports some reproducing *Calanus* spp. from the 0–100 m depth range from Skjerstadfjord to Saltfjord and back. That may also be the case in Mistfjord, although the wider sill generates no tidal jet and weaker turbulent diffusion in the basin. The lack of genetic structure reported for *C. finmarchicus* and *C. helgolandicus*, in opposition to *C. glacialis*, in a few Norwegian fjords (Bucklin et al. 2000; Choquet et al. 2017) including Skjerstadfjord, suggests that these species are drifting seasonally in and out of the fjords, transported by advective currents (Skreslet and Rød 1986; Choquet et al. 2017). In spite of the above, the environmental parameters measured in the present study together with the abundance data reported from November 2016 to March 2017 do not indicate any clear evidence of strong advection happening during these months. The very few specimens of *C. helgolandicus* appearing only from January in Mistfjord do not bring a strong evidence of an advective input since they were so seldom occurring. Likewise, the drop in *Calanus* spp. abundance observed from February to March in both fjords cannot be directly associated with an advective effect either because levels of mortality are expected to increase at that time (Skarðhamar et al. 2011). Therefore, it seems that the four *Calanus* species recorded in our seasonal study spend a large part of the winter together in sympatry in the fjords.

Calanus spp. reproduction

For each of the four species, the presence of both adult males and females was detected in both fjords, suggesting that local mating may take place. The presence of nauplii identified as *C. finmarchicus*, *C. glacialis*, and *C. hyperboreus* in Skjerstadfjord and in Mistfjord confirms that these three species do most likely reproduce locally. The very small number of nauplii identified for *C. finmarchicus* in May (Table 1) can be explained by the timing of the sampling. As an income breeder, *C. finmarchicus* relies entirely on the phytoplankton

bloom to fuel its egg production and therefore the first nauplii start to be present in the fjord a few days/weeks after the beginning of the bloom. In Skjerstadfjord, the phytoplankton bloom started already from the end of March, and by the 11th of May (date of our "extra" sampling) most of the nauplii had probably molted to copepodite stages (Tande 1982). Theoretically, the presence of nauplii may be an indication that either the studied fjords are the part of a core area where listed *Calanus* species maintain their own populations, or that the fjords are an immigration area where they have an advective origin but can still reproduce successfully (Beklemishev 1969). Considering the global genetic differentiation among fjords populations of *C. glacialis* reported in Choquet et al. (2017, 2019) and the new elements presented in this study, there are now several elements suggesting that *C. glacialis* may be a fjord resident with a core distribution in each fjord allowing the particular fjord population to maintain itself. This is somewhat contrasting with the drifting *C. finmarchicus* that is found in high abundances both in and outside fjords and that appears genetically undifferentiated from one fjord to another (Choquet et al. 2017, 2019). Based on this, the fjords probably represent an immigration area for that species, where it is still able to successfully reproduce but may not be self-sustaining (Beklemishev 1969). In the case of *C. helgolandicus*, no nauplii were detected and it is therefore unsure whether the species is able to reproduce in these fjords although the simultaneous occurrence of adult males and females suggests that mating could take place.

Hybridization between *C. finmarchicus* and *C. glacialis*

The ability of different *Calanus* species to successfully reproduce in sympatric areas calls for an investigation of their potential to hybridize. Therefore, we looked at the timing of potential mating in each species, when adult females and males cooccur, to determine whether these periods overlap among species. In both Skjerstadfjord and Mistfjord, adult males of *C. hyperboreus*, *C. glacialis*, and *C. finmarchicus* first appeared in January and were already mostly gone by the end of March. Monthly sampling may have been too coarse to detect species-specific differences in timing of male occurrence since *Calanus* spp. males are known to be present for a short period of time only (Kosobokova 1999; Daase et al. 2018). This is not the case, however, for females that persist for a much longer time in the population (Kosobokova 1999). Here, a distinct difference in the presence of adult females was found between the Arctic species *C. glacialis* and *C. hyperboreus* and the boreal *C. finmarchicus* and *C. helgolandicus*, with the Arctic species preceding the boreal ones by up to 2 months (November vs. January). In the high Arctic, a similar divergence in timing is seen but with a two-months delay (Arnkværn et al. 2005), which most likely is an adjustment to difference in timing of the spring bloom. In Skjerstadfjord and Mistfjord, the spring bloom in 2017 started at the end of March, while at higher latitudes it usually starts 1 to 2 months later (Zenkevitch 1963). Maturation of the female gonads is energy and time demanding and is associated with a strong decline in females lipid reserves (Jonasdottir 1999). In Skjerstadfjord and Mistfjord, the low fluorescence observed from November to end of February indicated poor feeding conditions. Thus, gonad maturation had to be primarily fueled by internal reserves, which may explain the strong decline in *Calanus* spp. abundances in both fjords from February to March, and especially for *C. finmarchicus*, considered as an income breeder (Falk-Petersen et al. 2009). Overall, from January to February, adult males and females of *C. finmarchicus*, *C. glacialis*, and *C. hyperboreus* were present simultaneously in Skjerstadfjord and Mistfjord, suggesting a potential for interspecies mating. Since our molecular markers (InDels) were developed based on genomic and transcriptomic information from *C. finmarchicus* and *C. glacialis*, we only tested for hybridization between these two species, as these markers would not be accurate to test for hybridization among other species. However, the results of genetic admixture analyses based on InDel genotypes of nauplii and copepodite stages CIV, CV, and adults CVI (males and females) showed no sign of hybridization nor genetic introgression between *C. finmarchicus* and *C. glacialis*.

The absence of hybrids in the two fjords presently investigated as well as in 85 samples across the North Atlantic and high-Arctic (Nielsen et al. 2014; Choquet et al. 2017) suggests that *C. finmarchicus* and *C. glacialis* do not hybridize, maybe unsurprisingly given the high genetic divergence and large genome size differences between the two species (McLaren et al. 1988; Bucklin et al. 1995). The only study reporting the presence of *Calanus* hybrids relied on a set of microsatellite markers developed specifically for *C. finmarchicus* (Provan et al. 2007; Parent et al. 2012). However, these microsatellites were initially developed for *C. finmarchicus* only, for studying genetic differentiation among populations and were therefore not reliable tools to be applied to *C. glacialis* or to characterize hybrids. Furthermore, Parent et al. (2012, 2015) reported up to 50% of hybrids, suggested to be fertile in the Northwest Atlantic and Canadian Arctic. If true, this should quickly lead to large-scale introgression and ultimately to the formation of hybrid swarms (Perry et al. 2001), especially given the short generation time of *Calanus* species. However, neither was found in the *Calanus* spp. analysis in the present nor in recent studies (Nielsen et al. 2014; Choquet et al. 2017). Moreover, considering the lack of genetic differentiation and consequently high dispersal potential of *C. finmarchicus* reported by Provan et al. (2009) using the same microsatellite markers as used in Parent et al. (2012) (see also the weak genetic differentiation revealed by 24 single nucleotide polymorphisms in Unal and Bucklin 2010), hybrid swarms would move relatively fast and ultimately lead to the global replacement of *C. finmarchicus* and *C. glacialis* by hybrids. This is clearly not the case. In addition, if we consider that hybridization has happened but only in the past, we may expect to detect a

cytonuclear disequilibrium signature, which would be characterized by conflicting signals for species identification between mitochondrial and nuclear markers. None was found in the 677 individuals genotyped both for nuclear (InDels) and mitochondrial (16S) markers in the West and East of Greenland (389 individuals in Nielsen et al. 2014) and in the North Atlantic Ocean (288 individuals in Choquet et al. 2017).

Reliability of InDel markers

The InDel markers used in the present and in earlier studies (Nielsen et al. 2014; Choquet et al. 2017) were originally developed as molecular tools to discriminate between the morphologically similar *C. finmarchicus* and *C. glacialis* (Smolina et al. 2014). Genome and transcriptome areas targeted to identify InDel markers were chosen so that they would exhibit species-specific differences. The fact that Smolina et al. (2014) used genomic and transcriptomic data from both species for the development of these markers made the InDels reliable for detecting putative hybrids. The individuals of *C. finmarchicus* and *C. glacialis* selected for generating genomic and transcriptomic data were collected in areas with limited sympatry (Smolina et al. 2014). However, since there was sympatry and thus a risk of hybridization in these areas, all precautions were taken by the authors of that study to ensure that the individuals used were pure species, and not hybrids. Firstly, morphological features were used so that only individuals with a prosome length far below/above the delimitation threshold would be assigned either as *C. finmarchicus* (for smaller individuals) or *C. glacialis* (for larger individuals). Pigmentation criteria (redness, *see* Nielsen et al. 2014) was used in addition for individuals collected in Disko Bay (West Greenland). Secondly, putative species IDs based on morphology were all confirmed by traditional mitochondrial 16S DNA sequencing and the set of microsatellites used in Parent et al. (2012) for hybrids detection was applied to all individuals to confirm their "purity." Although extremely unlikely, if some of these individuals were nonetheless introgressed, the InDels developed by Smolina et al. (2014) that were selected to be species-specific could thus underestimate putative introgression. However, even in that case, the InDel markers would still be able to detect at the very least hybrids of first generation (F1), because in a F1 hybrid the whole genome is introgressed and contains 50% of each parental species genome. But the presence of F1s has never been detected and can definitely be excluded, both in the present dataset (Fig. 4) and in the extensive genotyping carried out on *Calanus* communities in the North Atlantic and the Arctic Ocean (> 4400 individuals in Choquet et al. 2017). From a more general perspective, very rare or past introgression might not be detectable with a small number of markers (10s) and may require genome-wide datasets to be detected (Martin and Jiggins 2017).

Potential barriers to hybridization between *C. finmarchicus* and *C. glacialis*

There seems to be no temporal barrier to reproduction between the two species that could prevent them from interbreeding, and yet no hybrids were detected in our dataset. Other pre-zygotic mechanisms may explain the absence of hybridization between these species. Newly molted females signal their presence to males by depositing vertical pheromone trails that males search for and follow (Tsuda and Miller 1998). Hybridization may simply not take place due to very species-specific pheromones or too large distance between males and females of different species due to different depth preferences and/or timing in seasonal ascent. Furthermore, Bucklin et al. (1995) suggested that reproductive isolation in copepods may be reached by subtle variations in the structure of sexual characters (*see* also Fleminger and Hulsemann 1977). Morphological studies have documented on the similarity of sexually modified appendages and body segments of *Calanus* species (Brodskii 1967; Frost 1974) albeit potentially biased by morphologically based species identification (Choquet et al. 2018). However, strong species-specific differences in the ventral integumental organs of the female's urosome have also been reported and suggested to have a role in protecting the sibling species from hybridization (Fleminger and Hulsemann 1977—again potentially biased by morphologically based species identification). More recently, an investigation of genetically identified specimens of females *C. finmarchicus* and *C. glacialis* using different methods of microscopy found no clear morphological differences between the two species that could represent a barrier to intercopulation (K. Kosobokova, pers. comm.). Overall, we should keep in mind that very little is known on *Calanus* spp. pheromones and the actual mating with copulatory clasp and spermatophore transfer, thus more studies will be needed to identify potential species-specific patterns. Post-mating prezygotic reproductive barriers may also play a role as shown for example in *Drosophila montana*, where reproductive isolation can be explained by a failure in the fertilization process due to mismatches between male ejaculate–female reproductive tract interactions (Garlovsky and Snook 2018). Lastly, post-zygotic isolation mechanisms may prevent introgressive hybridization from happening (Mayr 1972), due to the production of sterile hybrids (Leary et al. 1993) or not-viable offspring unable to develop until adulthood. In the genus *Calanus*, most studies have focused on older developmental stages and adults, early stages usually being ignored due to technical challenges associated with identification of small individuals. Although distinct differences have been documented for nauplii identification within Calanoida (Lovegrove 1956), the simultaneous presence of *Paraeuchaeta norvegica* and *Metridia longa* (both Calanoid copepods) together with *Calanus* spp. nauplii in our samples, plus the fact that the samples were preserved in ethanol (shrinking animals) have led to difficulties in identification. Besides, traditional identification keys developed based on morphology for nauplii within Calanoida have so far not been confirmed with genetics. Assessment of the validity of identification methods exclusively based on morphology should be undertaken using molecular tools. In the small number of *Calanus* spp. nauplii

genetically identified from the two fjords, no hybrid nauplii were found, suggesting that prezygotic isolation may be prevalent.

Conclusion

Using specifically designed molecular tools, we did not identify any hybrids between *C. finmarchicus* and *C. glacialis* in two areas of sympatry where the species reproduce at the same time. The initial report of hybrids in the *Calanus* genus is likely the result of technical artifacts and therefore, there is today no solid evidence to assume that *Calanus* species can hybridize. Further investigations using genome-wide screening should reveal if such hybridization has happened in the past.

DATA AVAILABILITY STATEMENT

Genotypes of InDels have been deposited to DRYAD (https://doi.org/10.5061/dryad.stqjq2bzv).

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Conflict of Interest

The authors of this manuscript declare they have no conflict of interest.

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