Mar Biodiv (2018) 48:1027–1035
DOI 10.1007/s12526-017-0774-4

SENCKENBERG

ORIGINAL PAPER

Postglacial expansion of the Arctic keystone copepod *Calanus glacialis*

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Received: 22 February 2016 / Revised: 10 July 2017 / Accepted: 27 July 2017 / Published online: 15 August 2017

Abstract *Calanus glacialis*, a major contributor to zooplankton biomass in the Arctic shelf seas, is a key link between primary production and higher trophic levels that may be sensitive to climate warming. The aim of this study was to explore genetic variation in contemporary populations of this species to infer possible changes during the Quaternary period, and to assess its population structure in both space and time. *Calanus glacialis* was sampled in the fjords of Spitsbergen (Hornsund and Kongsfjorden) in 2003, 2004, 2006, 2009 and 2012. The sequence of a mitochondrial marker, belonging to the *ND5* gene, selected for the study was 1249 base pairs long and distinguished 75 unique haplotypes among 140 individuals that formed three main clades. There was no detectable pattern in the distribution of haplotypes by geographic distance or over time. Interestingly, a Bayesian skyline plot suggested that a 1000-fold increase in population size occurred approximately 10,000 years before present, suggesting a species expansion after the Last Glacial Maximum.

Keywords *Calanus* · Zooplankton · mtDNA · Population genetics · Genetic diversity

Communicated by R. R. Hopcroft

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Introduction

A large part of zooplankton biomass in the Arctic shelf seas is formed by *Calanus glacialis* (Fleminger and Hulseman, 1977; Blachowiak-Samolyk et al. 2008; Weydmann et al. 2013), a lipid-rich calanoid grazer. In the lipid-based Arctic food web, it is an essential link between the low-energy microalgae and higher trophic levels (Lee and Hirota 1973; Falk-Petersen et al. 2009). Its life cycle is between one (MacLellan 1967; Weydmann et al. 2013) and three years (Kosobokova 1999), depending on the region and environmental conditions, although *C. glacialis* typically has a 2-year life span (Hirche and Kwaśniewski 1997). The areas of its occurrence, including peripheral seas of the Arctic Ocean and adjacent regions of the North Atlantic and Pacific Oceans (Jashnov 1970; Conover 1988), are now facing intensive modifications from an unprecedented combination of environmental changes, such as increasing ocean temperatures and reduction in sea ice extent, caused by climate warming (IPCC 2014), with the record high Atlantic Water temperature and salinity in 2006 (Walczowski et al. 2012). With the Arctic region likely to continue warming more rapidly than the global mean (IPCC 2014), changes are expected to affect Arctic marine biota. For example, the loss of sea ice represents a loss of critical habitat for ice-related species, such as *C. glacialis*, that needs energy from the ice algal bloom to fuel its reproduction (Søreide et al. 2010). Major changes in the function of the Arctic marine ecosystem are now anticipated.

The capacity for populations to evolve in response to environmental changes is based on genetic diversity, which encompasses the variation among individuals within a population and the genetic variation among populations (Gray 1997; Kenchington et al. 2003; Reed and Frankham 2003). Climatic changes during the Quaternary period in Arctic regions, with repeated glacial and interglacial periods causing cyclical

expansions and contractions of species, have shaped their genetic variation and genealogies. During this time, some populations and lineages became extinct, while others underwent bottlenecks and founder events. Mitochondrial markers, which provide suitably variable sequences, are among the most favored for tracking such events during the Quaternary (Hewitt 2004).

To adequately assess patterns of genetic diversity at the population level, fast-evolving markers should be used, particularly for the study of animal populations that have expanded substantially since the Last Glacial Maximum 10,000–14,000 years ago (Baker 2000). To date, few Arctic species have been studied in detail. The lack of adequate polymorphic makers was one of the factors limiting genetic research in *Calanus* spp., although several microsatellite markers have recently been published (Provan et al. 2007; Provan et al. 2009; Parent et al. 2012; Weydmann et al. 2014). Provan et al. (2009) used microsatellite markers and mitochondrial cytochrome b gene (*CYTB*) in *Calanus finmarchicus*, revealing no significant genetic differentiation at the inter-population level or across the species' range, in either nuclear or mitochondrial data sets. The authors postulated that these results indicated high levels of dispersal and a constant effective population size over the period 359,000–566,000 years before present, and suggested that *C. finmarchicus* possessed the capacity to track changes in available habitat, a feature that may be of crucial importance for the species' ability to cope with the current period of global climate change. However, similar studies have not been conducted on its Arctic sibling, *C. glacialis*. Using the 16S ribosomal RNA gene, Nelson et al. (2009) defined two genetically distinct *C. glacialis* populations—an Arctic and a North Pacific (Bering Sea) population—although the latter was not reproductively established in the Arctic Ocean. The authors suggested that climate warming could increase opportunities for southern organisms to become established in the Arctic. In contrast, Weydmann et al. (2016), on the basis of microsatellite markers, reported a panmictic population of *C. glacialis* with large-scale gene flow around the Arctic.

Here we aimed to estimate genetic variation in contemporary populations of *Calanus glacialis* to examine possible changes during the Quaternary, especially after the Last Glacial Maximum, when major latitudinal species range shifts occurred. Additionally, we wished to assess the recent population structure of this key Arctic zooplankton species at both geographic and temporal scales. To this end, we chose two Spitsbergen fjords (Svalbard Archipelago) that are contrasting in terms of water masses, in addition to the availability of samples from a time series collected between 2003 and 2012. Finally, we based our study on a newly developed mitochondrial marker, chosen based on the length of mitochondrial genes, and intermediate intra- and interspecies polymorphism, which is greater than those used to date.

Material and methods

Study area

Our study area covered two fjords in the Atlantic sector of the Arctic Ocean. Hornsund is a medium-sized fjord located in the southwest part of Spitsbergen (Fig. 1). The fjord is under the influence of the cold coastal South Cape Current and warmer, more saline West Spitsbergen Current. The inner fjord basin, Brepollen, is isolated from the main basin by an underwater sill establishing a reservoir of winter cooled water throughout all seasons (Swerpel 1985), where a local population of *C. glacialis* was reported to exist (Weydmann and Kwaśniewski 2008).

Kongsfjorden is an open fjord situated on the west coast of Spitsbergen. Due to the absence of a sill at the entrance, the fjord faces strong pulsed influxes of relatively warm Atlantic water (Cottier et al. 2005). Despite the fjord's location at 79°N latitude, the fauna of Kongsfjorden is of a rather sub-arctic character due to the strong influence of the West Spitsbergen Current and advection processes (Kwaśniewski et al. 2003; Walkusz et al. 2009).

Sampling

Zooplankton samples were collected from the fjords during the summers of 2003 (Hornsund), 2004 (Kongsfjorden), 2006 (Hornsund), 2009 (Kongsfjorden), and 2012 (both fjords) (Table 1) during the Arctic cruises of the R/V *Oceania*, using a WP-2 mesozooplankton net (0.25 m^2 mouth opening; 180 μm mesh size), and were preserved in 96% ethanol, which was changed 24 h after sampling.

DNA extraction and amplification

In total, 140 *Calanus glacialis* individuals of the fifth copepodite stage and adult females were identified to the species level based on the prosome length (Weydmann and Kwasniewski 2008) and characteristic morphological features (Brodskii et al. 1983) and were retrieved from the mixed zooplankton samples. Their genomic DNA was extracted using the Sherlock AX kit (A&A Biotechnology).

Specific PCR primers (popF: 5'-AAGATACTTGGTATATTTCTGACACC-3', popR: 5'-ATATTTATGTTGATTCTCAGCCC-3') and a third sequencing primer (popR2 5'-TTCACAATATAAAAGATTACC-3') were designed using sequences available in the NCBI database of sequence read archives (SRA, accession numbers SRR1793125, SRR1791606, SRR1791605, SRR1791524, SRR1791525) (Ramos et al. 2015). The PCR product, covering 1465 base pairs (bp) of the mitochondrial *ND5* gene encoding the fifth subunit of the respiratory chain complex I (NADH dehydrogenase subunit 5), was obtained. This fragment was chosen

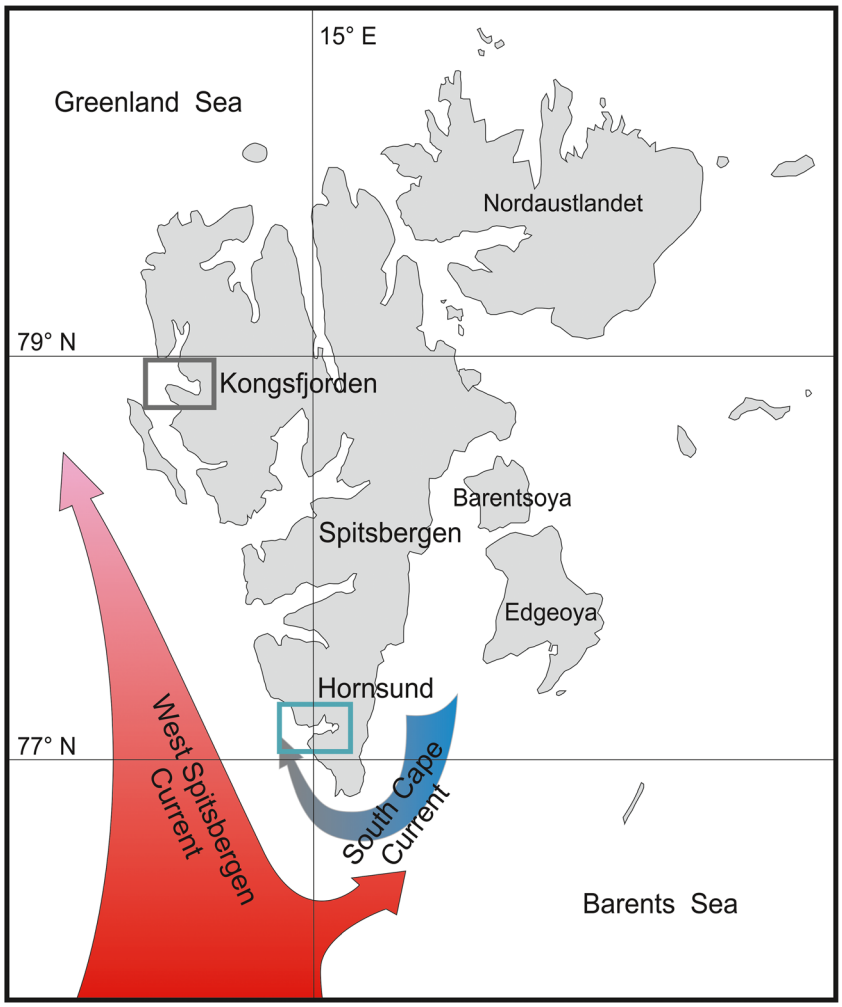

Fig. 1 The Svalbard Archipelago with a schematic circulation of the dominant ocean currents and locations of sampling stations

based on the length of mitochondrial genes, their intermediate intra- and interspecies polymorphism in comparison to conserved *CYTB*, cytochrome oxidase subunit I (*COI*) and highly variable NADH dehydrogenase subunits 3 and 4 (*ND3* and *ND4*) genes in the copepod subclass (Minxiao et al. 2011).

The final reaction volume for PCR amplification was 10 μl, with approximately 5 ng of total DNA, 0.5 μM of popF and popR primers, dNTPs at 200 μM each, 2 mM $MgCl_2$ and 0.5 U of DyNAzyme EXT DNA Polymerase (Thermo Fisher Scientific), in a buffer supplied by the manufacturer. The PCR amplification protocol was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. The final extension lasted 5 min (TProfessional Gradient Cycler from Biometra).

PCR products were separated by a 1% agarose gel electrophoresis in 0.5X TBE buffer and visualized with ethidium bromide in UV light. Products which showed a strong band of the correct size were selected for sequencing. DNA concentration was estimated based on gel images, and the

Table 1 Sampling details: stations' positions, sampling depths, dates and the number of *Calanus glacialis* individuals sequenced

Fjord	Sample	Latitude (°N)	Longitude (°E)	Date	Sampling depths (m)	Number of individuals
Kongsfjorden	K2004	78° 53.35	12° 27.62	22.07.2004	0–70	17
	K2009	78° 57.03	11° 50.16	01.08.2009	0–60	16
	K2012	78° 53.21	12° 27.43	07.08.2012	0–70	25
Hornsund	H2003	76° 58.63	15° 45.66	25.07.2003	85–140	31
	H2006	77° 00.47	16° 28.46	22.07.2006	50–120	21
	H2012	77° 00.54	16° 28.30	01.08.2012	30–100	30

products were cleaned using Exonuclease I and alkaline phosphatase treatment (Werle et al. 1994). Sequencing was performed by Sanger technology, in both directions, using all three primers (Macrogen, Inc.).

Bioinformatic analysis

The raw sequence reads were assembled using Staden Package software (Staden 1996). The resulting partial *ND5* sequences were aligned in MEGA6 (Tamura et al. 2013) using the ClustalW (Larkin et al. 2007) algorithm and trimmed to the same length of 1249 base pairs. The alignment was straightforward, as there were no indels in the sequences. All sequences were deposited in GenBank under accession numbers MF447532 - MF447671.

General diversity indices were calculated in DnaSP (Librado and Rozas 2009): haplotype diversity (Hd), which is expected to be high for organisms with large effective population sizes (Hd close to 1); nucleotide diversity (π), which is expected to be within 1% for intraspecies mitochondrial polymorphism; and Tajima's D statistics used to test the departure of haplotype distribution from neutral expectations. To check for genetic differentiation among samples, analysis of molecular variance (AMOVA), including population pairwise fixation indices (Φ_{ST}, with no. of permutations for significance = 1000) was calculated in Arlequin 3.5 (Excoffier and Lischer 2010). A minimum spanning network (MSN) of all observed haplotypes was built using a median-joining algorithm (Bandelt et al. 1999) implemented in Network software (fluxus-engineering.com). This type of analysis is more appropriate for population-level data than classic phylogenetic tree building, and allows quick visual inspection of existing relationships between genetic diversity and other factors (such as geographic or temporal scales). To elucidate the demographic history of the studied population of *C. glacialis*, analysis of population size changes was performed in BEAST 2.4.5 (Bouckaert et al. 2014) using a Bayesian skyline plot (BSP) reconstruction approach. The best-fit model of substitutions (HKY+G) as well as clock model (relaxed uncorrelated lognormal clock) was selected using Bayes factor comparison (Baele et al. 2012). No constrains were used; therefore, the obtained plots were scaled in mutational units. The Markov chain Monte Carlo (MCMC) was run for 10 million generations, in four replicates. The default 25% of initial (burn-in) generations was discarded after inspection of the results in Tracer v1.6 (Rambaut et al. 2014). All runs converged at the same solution; hence the resulting log and tree files were combined. The effective sample size (ESS) of all parameters exceeded 300, ensuring that the results of the analysis were meaningful. BSP was created in Tracer using combined tree and log files.

Results

A 1249-bp long fragment of mitochondrial DNA, encoding part of the *ND5* gene, was sequenced in six samples of *C. glacialis* (Table 1). The diversity indices showed overall high haplotype diversity (Hd), which was apparently associated with low nucleotide diversity (π) in the studied population of *C. glacialis* (Table 2). There were 75 haplotypes among 140 sequenced individuals (Hd = 0.892). Despite this appreciable number of haplotypes, the overall nucleotide diversity was very low, at the level of $\pi = 0.004$. There was no genetic differentiation between tested pairs of populations (population pairwise Φ_{ST} did not differ significantly from zero); hence there was no evidence of any population genetic structure among the compared samples ($p > 0.05$; Table 3). Various groupings were checked for possible higher-level structuring using AMOVA, but no significant fixation indices were recovered, regardless of the grouping tested (data not shown). The MSN of all haplotypes (Fig. 2) was relatively simple and well-resolved, with three closely related haplotypes surrounded by several minor-frequency variants. However, there was no visible trend in the distribution of haplotypes by geographic location (Fig. 2a) or year of sampling (Fig. 2b). Taking into account the lack of structuring, all subsequent analyses were run on a combined set of all 140 sequences obtained.

Tajima's D test statistic was significantly negative (D = −2.51, $p < 0.05$), indicating an excess of rare variants and hinting at possible recent population expansion. To test this interpretation and to elucidate the demographic history of the studied population of *C. glacialis*, BSP analysis was performed (Fig. 3). The resulting plot indicates a strong increase in population size occurring at approximately the time sufficient to accumulate between 6×10^{-4} and 9×10^{-4} substitutions per site, with a relatively wide CI of 4×10^{-4} substitutions.

Facing the complete lack of possible calibration points, the dating of this event can only be highly provisional. However, in order to fall within the postglacial limit, the expansion start (9×10^{-4} substitutions) would have to be inferred at no more than 2×10^{4} years before present (the Last Glacial Maximum). Accordingly, to fit our data within the confidence limits, the substitution rate would have to be in the range of 3.5–5.5% per MY ($[9\times10^{-4}– 2\times10^{-4}]/2\times10^{4}$ substitutions per site per year for the lower limit). Assuming a lower substitution rate would push the expansion event out of the interglacial.

Discussion

Our study revealed no evidence of genetic structure in *Calanus glacialis* among the fjords compared nor among different years of sampling, regardless of their classification as warm (2006) or cold (2003, 2004). The results are similar to those by Weydmann et al. (2016), who reported a

Table 2 Standard diversity indices for the sampled population of *C. glacialis*

Fjord Sample	n	Hd	sd	π	sd	π_s	π_a	D	*p*
K2004	12	0.934	0.046	0.0039	0.00033	0.01397	0.00063	−0.99000	>0.1
K2009	13	0.950	0.048	0.0040	0.00063	0.01426	0.00066	−1.12811	>0.1
K2012	13	0.807	0.079	0.0037	0.00039	0.01305	0.00060	−1.19952	>0.1
H2003	21	0.916	0.043	0.0037	0.00052	0.01165	0.00062	−1.99554	<0.05
H2006	16	0.929	0.051	0.0064	0.00134	0.01564	0.00256	−1.73006	>0.05
H2012	19	0.871	0.060	0.0033	0.00045	0.01148	0.00064	−1.57126	>0.1
All	75	0.892	0.024	0.0040	0.00031	0.01298	0.00092	−2.50683	<0.001

Number of unique haplotypes (n), haplotype diversity (Hd), nucleotide diversity (π) along with the estimate of its standard deviation (sd), nucleotide diversity at synonymous (π_s) and non-synonymous (π_a) sites as well as the results of Taijima's D test (D and *p*) calculated in DnaSP are shown. The last row (All) represents the indices calculated for the combined data set comprising all 140 sequences

lack of genetic structure in *C. glacialis* from seven locations distributed around the Arctic (Svalbard fjords, White Sea, and Amundsen Gulf), sampled in 2008 and 2009, in support of the hypothesis that large-scale effective dispersal and gene flow driven by ocean currents allows for the free exchange of planktonic copepods in the Arctic. Therefore, we believe the results would be similar even if we had sampled more sites around the Arctic. There is also numerous evidence of panmictic populations and/or high gene flow of planktonic copepods across extensive geographic ranges of the Northern Hemisphere, which has been reported for the Atlantic *Calanus finmarchicus* (Provan et al. 2009), Pacific *Calanus sinicus* (Huang et al. 2014), cosmopolitan *Clausocalanus arcuicornis* (Blanco-Bercial et al. 2011), and Arctic *Pseudocalanus minutus* (Aarbakke et al. 2014; Questel et al. 2016). At the same time, to our knowledge, there is only one study confirming the existence of two populations of *C. glacialis*, in the Arctic and the North Pacific (Bering Sea), although the latter was not reproductively established in the Arctic Ocean (Nelson et al. 2009).

Although there was no connection to locations or time in the distribution of *C. glacialis* haplotypes, one interesting feature of the observed topology was the existence of star-like elements in the MSN: single, dominant haplotypes connected by short branches with several low-frequency haplotypes (Fig. 2). Such structures usually indicate recent expansion of the clades represented by them (*Network* software documentation, www.fluxus-engineering.com). Recent population expansions are also known to leave certain traces in the observed diversity indices. The excess of rare polymorphism is expected in such situations, leading to significantly negative Tajima's D test statistic (Tajima 1989), which was the case in our study. This excess can also be caused by selection acting on the studied marker; however, in the case of a mitochondrial marker it is usually assumed that demographic processes are responsible for this phenomenon (Grant 2015).

Table 3 Genetic differentiation between pairs of samples. Above diagonal: pairwise *p*-values; below diagonal: fixation indices (Φ_{ST})

	H2003	K2004	H2006	K2009	H2012	K2012
H2003		0.324	0.153	0.919	0.982	0.207
K2004	−0.001		0.595	0.622	0.297	0.892
H2006	0.018	−0.007		0.279	0.189	0.315
K2009	−0.022	−0.021	0.005		0.856	0.559
H2012	−0.020	−0.004	0.016	−0.024		0.270
K2012	0.015	−0.029	0.011	−0.017	0.006	

Multilocus data are known to be better for inferring demographic histories, particularly when combined with ancient DNA sampling (Grant 2015). Unfortunately, for various technical reasons, such data are currently unavailable for *Calanus* species. Active marker development is ongoing (Smolina et al. 2014; Weydmann et al. 2014), but is hampered by the atypical genome organization in *Calanus*. Also, using a single mitochondrial gene has some advantages: as a maternally inherited, haploid genome, it has a smaller effective population size and is more prone to bottleneck effects (Hartl and Clark 2007). Therefore, the expansion seen in our data was not necessarily preceded by a very strong bottleneck.

The rapid change recorded on the BSP plot (Fig. 3) confirms that the studied population had undergone an expansion. The observed high haplotype diversity is indicative of a large effective population size, typically expected for a planktonic marine invertebrate. The causes of the observed pattern can be attributed to the bottleneck experienced by *C. glacialis* during the Pleistocene glaciation and the following rapid expansion of this species after the Last Glacial Maximum, when the Arctic was exposed to warming, resulting in a transition from full glacial conditions to widespread interglacial conditions attained approximately 10,000 years ago. Such expansion is common for many Arctic species, which survived in a few refugia and very rapidly recolonized their current ranges after deglaciation; their current genetic diversity depends largely on the number of refugia and effective population sizes of the surviving populations (Hewitt 2000; Hewitt 2004). Marine species like

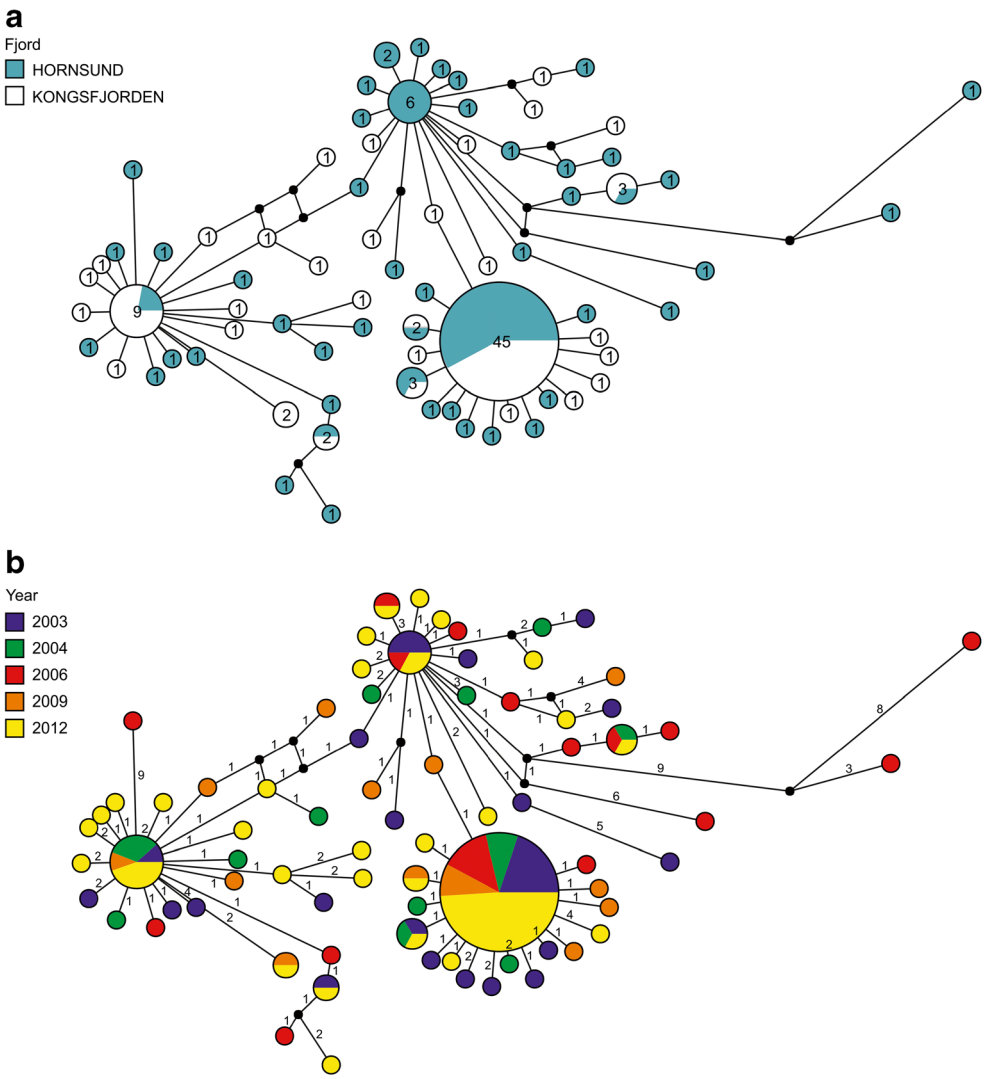

Fig. 2 The minimum spanning networks (MSN) of *ND5* haplotypes of *Calanus glacialis* from the fjords of Spitsbergen. Circle diameters are proportional to the number of individuals bearing each haplotype, and lines connecting circles are roughly proportional to the number of mutational steps connecting haplotypes. **a** Distribution of haplotypes between sampling locations; number of individuals is also shown here. **b** Distribution of haplotypes between sampling years; exact numbers of mutational steps are shown here

C. glacialis are additionally affected by ocean currents that contribute to mixing processes between their populations.

The question remains whether this explanation is plausible and the estimated substitution rates are acceptable. The typically assumed general mitochondrial substitution rates are based on separating pairs of shrimp species by the emerging Isthmus of Panama (Knowlton et al. 1993; Knowlton and Weigt 1998) and a relatively short fragment of the conserved *COX1* gene. It is assumed that other crustaceans, including *Calanus* species, accumulate mitochondrial substitutions at a similar pace, resulting in the 1.4–2.2% increase in overall divergence per million years (Papadopoulos et al. 2005). The substitution rate needed to attribute the observed expansion in *C. glacialis* to the last interglacial is 3.5% per million years, leading to the accumulation of divergence at the speed of at least 7% per million years, a value seemingly much higher but still in the same order of magnitude. At least two factors must be considered, each acting in favor of the increased substitution rate. First, the published data are dealing with the most conservative *COX1* gene, while the *ND5* sequence is most likely evolving much faster. Recent mitogenomic analysis of Metacrangonyctidae crustaceans (Pons et al. 2014) have shown that *ND5* is among the fastest-evolving mitochondrial genes and accumulates substitutions about twice as fast as *COX1*.

Direct estimates of the substitution rate are rare, but Haag-Liautard et al. (2008) measured the mitochondrial mutation rate in *Drosophila*. The obtained value of 6.2×10^{-8} per site per fly generation would correspond to 3.1×10^{-8} per site per year for *C. glacialis* (assuming the 2-year generation time). That would fit our requirement reasonably well, further indicating that the assumed substitution rate is quite plausible.

Another important consideration is the apparent time dependency of molecular rate estimates (Ho et al. 2005), frequently leading to large errors in calibrating recent events by using rate estimates derived from phylogenetic species separations (Grant 2015). These effects are difficult to measure, but they both act in the same direction: towards the

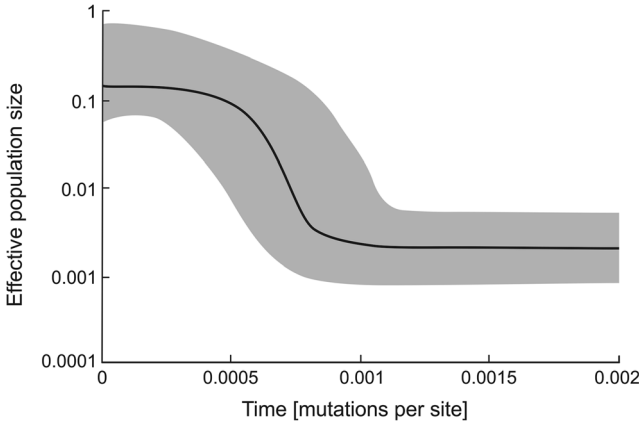

Fig. 3 The demographic history of the Arctic population of *Calanus glacialis* based on a Bayesian skyline plot (BSP) reconstruction approach. The black line depicts median values, and the shaded region represents 95% highest posterior density. Both axes are scaled in uncalibrated, mutational units; if divided by the true substitution rate, they would produce time (*x*-axis) or the effective population size (*y*-axis)

increase in an apparent substitution rate and the placement of the expansion event within the current interglacial. In line with this view is the notion that, when dealing with contemporary sequence data, only the last major expansion event can be noted after even a moderate bottleneck (Grant 2015); the information about the past demographic events is lost. It is difficult to imagine that the Last Glacial Maximum had no effect on the demography of *C. glacialis*, and only such an assumption would allow us to interpret the observed expansion as an earlier event.

Why would the Arctic ice-associated species benefit from the interglacial conditions? One possible answer is that the thinning of sea ice affected the primary production regime, allowing for the earlier ice algal bloom and subsequent phytoplankton bloom after the ice melts, which are required for the early maturation, reproduction and growth of this key Arctic grazer (Niehoff et al. 2002; Søreide et al. 2010). Another possible explanation is that during the glacial, the sea level was lower and shelves were mostly covered by grounded ice, so there was no optimal habitat for *C. glacialis*, which is primarily a shelf and shelf break species, probably not as successful in the basins (Ji et al. 2012). On the other hand, interglacial conditions enhance thermohaline circulation in the subpolar North Atlantic (Sarnthein et al. 1994; McManus et al. 2002), thus providing better conditions for transporting zooplankton. In conclusion, we hypothesize that the recent interglaciation opened up the Arctic Ocean's shelves, changed the circulation of ocean currents and accelerated the blooms, and the combination of these jointly enabled the postglacial expansion of this Arctic keystone copepod.

Acknowledgements This research project was supported by grants no. 2011/03/B/NZ8/02876 and GAME from the National Science Centre, the Polish Ministry of Science and Higher Education Iuventus Plus grant no. IP2014 050573 and by CCMAR/Multi/04326/2013 from FCT- PT.

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