Live discrimination of *Calanus glacialis* and *C. finmarchicus* females: can we trust phenological differences?

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Abstract:

Two key players in the Arctic and subarctic marine ecosystem are the calanoid copepods, *Calanus finmarchicus* and *C. glacialis*. Although morphologically very similar, these sibling species have different life cycles and roles in the Arctic pelagic marine ecosystem. Considering that the distribution of *C. glacialis* corresponds to Arctic water masses and *C. finmarchicus* to Atlantic water masses, the species are frequently used as climate indicators. Consequently, correct identification of the two species is essential if we want to understand climate-impacted changes on *Calanus*-dominated marine ecosystems such as the Arctic. Here, we present a novel morphological character (redness) to distinguish live females of *C. glacialis* and *C. finmarchicus* and compare it to morphological (prosome length) and genetic identification. The characters are tested on 300 live females of *C. glacialis* and *C. finmarchicus* from Disko Bay, western Greenland. Our analysis confirms that length cannot be used as a stand-alone criterion for separation. The results based on the new morphological character were verified genetically using a single mitochondrial marker (16S) and nuclear loci (six microsatellites and 12 InDels). The pigmentation criterion was also used on individuals ($n = 89$) from Young Sound fjord, northeast Greenland to determine whether the technique was viable in different geographical locations. Genetic markers based on mitochondrial and nuclear loci were corroborative in their identification of individuals and revealed no hybrids. Molecular identification confirmed that live females of the two species from Greenlandic waters, both East and West, can easily be separated by the red pigmentation of the antenna and somites of *C. glacialis* in contrast to the pale opaque antenna and somites of *C. finmarchicus*, confirming that the pigmentation criterion is valid for separation of the two species.

Introduction

Calanus dominate the zooplankton in the Arctic marine ecosystem and constitute the link between the primary producers and the rich stocks of fish, birds and marine mammals (reviewed by Falk-Petersen *et al*. 2009). In the subarctic North Atlantic and Arctic, three species of *Calanus* coexist: the Arctic *Calanus hyperboreus* and *C. glacialis* and the North Atlantic *C. finmarchicus*. All three species have life cycles adapted to Arctic conditions including seasonal ontogenetic migration and accumulation of lipids during spring and summer, as well as hibernation and arrested development during winter (Conover 1988; Madsen *et al*. 2001). Despite being morphologically very similar (Frost 1974), their life cycles, size and in particular lipid content are quite different (Swalethorp *et al.* 2011).

In spring, when the breakup of the sea ice triggers the formation of the spring bloom (Dünweber *et al.* 2010), the *Calanus* species ascend from the deep water. In Disko bay, western Greenland *C. finmarchicus* and *C. glacialis* ascend to the surface layer at the same time in spring (Madsen *et al.* 2001, Swalethorp *et al.* 2011). However, the two species represent different phenology"s; *C. glacialis* commences spawning before the onset of the spring bloom utilizing stored lipids (Hirche and Kattner 1993) (capital breeder sensu Varpe) while *C. finmarchicus,* which can be characterized as an income breeder, needs to feed on the bloom (Plourde & Runge 1993) in order to initiate spawning a few weeks after *C. glacialis* (Madsen *et al.* 2008, Swalethorp *et al.* 2011).

The core distribution of *Calanus glacialis* and *C. finmarchicus* corresponds to Arctic and Atlantic water masses (Van Aken *et al.* 1991, Falk-Petersen *et al.* 2009) respectively; therefore, they are used as climate indicators. Based on model simulations for the Arctic pelagic ecosystem it is predicted that climate change in particular will cause a northward shift of the Atlantic *C. finmarchicus* (Slagstad *et al.* 2011) and will increase the area where the sibling species *C. glacialis* and *C. finmarchicus* co-occur and reproduce.

The model results are experimentally supported by Kjellerup *et al*. 2012 who, based on extensive temperature experiments with *C. glacialis* and *C. finmarchicus*, found that a slightly warmer ocean will potentially cause a shift in the composition of the zooplankton community from a dominance of *C. glacialis* towards a more prominent role of the smaller less energy rich *C.*

finmarchicus. Such shifts will, according to Falk-Petersen *et al.* 2007, have major implications on the food web, since *C. finmarchicus* support an alternative pelagic food web than the more lipid rich *C. glacialis*. Therefore, if the energy flows through the Arctic marine ecosystem in a warmer future should be quantified and modeled realistically correct identification of C. *finmarchicus* and *C. glacialis* in field samples, and especially live collections used for experiments investigating phenology, is essential.

Recent publications have questioned the prosome length criterion used to separate *Calanus finmarchicus* and *C. glacialis* (Undstad and Tande 1991, Hirche *et al.* 1994) due to an overlap of the prosome length range of the two species (Lindeque *et al.* 2006, Parent *et al.* 2011). Separation of the two species have recently been additionally challenged by the documented hybridization between the two species in the northwest Atlantic (Parent *et al.* 2012)

However, in situations where morphological characters do not provide sufficient variation to identify to species level then taxonomic discrimination can be achieved using genetic characters. Such techniques have been developed for copepods of the genus *Calanus* using microsatellite markers (nuclear, bi-parentally inherited: Provan *et al.* 2009, Parent *et al.* 2012) and Restriction Fragment Length Polymorphism (RFLP) PCR of mitochondrial 16S rDNA (mtDNA, maternally inherited: Lindeque *et al.* 1999). This later technique has successfully been used in numerous past studies (Lindeque *et al.* 2004, 2006, Parent *et al.* 2012, Gabrielsen *et al.* 2012). Molecular techniques, relying on analysis of the DNA, quite obviously preclude identification of live individuals. It is possible that adult females are individually incubated and left to spawn such that the eggs are used for molecular identification, thereby confirming the identity of the mother. However, this is labour intensive, unsuitable for large studies, relies on the females spawning eggs, adds to the handling and stress of the animals and still doesn´t solve the problem of potential hybrids. It is therefore necessary to have a quick and reliable identification technique in order to sort live females for experimental procedures, thereby allowing investigation and documentation of phenological differences in response to environmental variables such as temperature and food (Kjellerup *et al.* 2012).

Accordingly the aim of this study is to present new morphological criterion for discrimination of *C. glacialis* and *C. finmarchicus* live females based on "redness index" verifying its applicability with molecular methods, and compare its performance with prosome length criterion.

Materials and methods

Sampling:

Western Greenland – Copepods were sampled on the 2nd, 8th and 13th of May 2011 during the spring bloom in Disko bay, western Greenland. Sampling was done from R/V Porsild (Arctic station, Copenhagen University) at a monitoring station used in previous studies (Levinsen *et al.* 2000, Madsen *et al.* 2001, Hansen et *al.* 2012) (69° 14" N, 53° 23" W) using a WP2 net with a non-filtering cod-end (200 μm mesh size). After sampling, the contents of the cod end were quickly transferred into buckets with seawater and placed in thermo boxes filled with seawater and ice.

East Greenland – Following the protocol described above copepods were sampled from the motor boat Åge V. Jensen I on the 10th August 2012 in Young Sound fjord, Daneborg in Northeast Greenland, at Sampling Station A (74° 18' N, 20° 15' W, Rysgaard *et al.* 1999) and in the Greenland Sea station GH 5 (74°10.35" N 19°41.98"W; ca. 2 km from the mouth of the fjord).

Morphological identification:

Within 5 h of collection, adult females collected in west Greenland were returned to the research station and sorted in seawater filled Petri dishes kept in ice-filled trays under a dissecting microscope. Based on size and redness approximately 50 *C. glacialis* and 50 *C. finmarchicus* (Fig. 1) females, including those with ambiguous characters, were picked from each sample i.e. the females were taken in the order they were encountered in the raw sample until 50 of one species (*C. finmarchicus*) were collected. Thereafter only the other species as well as females that demonstrated ambiguous pigmentation (FDAP) were picked until 100 individuals were collected in total from each sample (Fig. 2). The females were incubated for egg production and processed as described below. In East Greenland 89 females were sorted, taken in the order they were encountered in the raw sample. None of the east Greenland females were incubated for egg

size or morphology or analyzed for redness but individually stored in 75% non-denatured ethanol and 0.2 μm filtered seawater in 5 ml PCR-tubes at 5°C until molecular analysis. No females showing ambiguous morphological characters were observed in the east Greenland samples.

Egg morphology - Females picked from the west Greenland samples were placed individually in 24-well tissue culture trays (NUNC™Multi wells) containing 3 ml of 45 μm filtered seawater and incubated at constant temperature (5 °C) for 36-40 hours to allow the females to produce eggs. Eggs produced by each female were carefully collected directly from the 24-well culture tray by a Pasteur pipet and placed on a glass slide in a small volume of water. A cover slip with four Vaseline corners was place on top of the glass slide before being photographed using a Nikon 90D camera attached to an Olympus BX51 microscope by an eyepiece adaptor [NDPL-1(x2)]. A clear discriminator of live eggs is the outer membrane of *C. glacialis* eggs , which is absent in *C. finmarchicus (*Werner and Hirche 2001). Egg diameter was measured as the diameter between the inner membrane of the egg using the image analysis software ImageJ (v. 1.38w) (http://rsb.info.nih.gov/ij/) by calibrating the software using photos with known distances.

Prosome length - After incubation of the west Greenland samples each female was placed in a Petri dish with as little water as possible to position animals at the same level and photographed on an Olympus SZX12 microscope using a Nikon 90D camera with an eyepiece adaptor [NDPL-1(x2)]. Each female was then washed in GFF filtered seawater and stored in 75% non-denatured ethanol and 0.2 μm filtered seawater in 5 ml PCR-tubes at 5°C until molecular analysis. Prosome length was measured using the image analysis software ImageJ (v. 1.38w) (http://rsb.info.nih.gov/ij/) by calibrating the software using photos with known distances.

Redness index - To create an objective tool to evaluate the coloration of the females a redness index was defined and determined using ImageJ with the plug-in „Threshold Color" (http://www.dentistry.bham.ac.uk/landinig/software/software.html). For each female, the genital segment was cut out of the picture and the % of red pixels of the genital somites out of the entire genital segment was quantified. We chose the HSB color model and selected red areas using the following values: hue 0-23 and 0-255, saturation 75-255 and brightness 1-255. In a second step

we measured the complete area of the genital segment. The images were converted to 8-bit grey scale, and the selected areas were measured using the „Image/Adjust/Threshold" and the „Analyze/Measure" commands. This analysis was repeated for the antennae.

Genetic identification:

All samples from west and east Greenland were analysed „blind" without prior knowledge of either prosome length or pigmentation. Both mitochondrial (mtDNA) and nuclear (nDNA) markers were used to genetically characterize each individual

Mitochondrial marker – Individual *Calanus* were identified to species according to the RFLP signature of their mitochondrial 16S rDNA following PCR amplification, restriction digestion and agarose gel electrophoresis, using the molecular identification technique described by Lindeque *et al.* (1999, 2006) with minor modifications. The final technique is described below. Individual animals were removed from ethanol and cut in half with a sterile scalpel blade. Excess ethanol was removed from the half individual by dabbing on absorbent paper and rehydrated in 200 µL of MilliQ water in a 96-well plate for approximately 6 h at room temperature. Following rehydration the water was removed and replaced with 22.75 µL MilliQ water and 10 µL of 5x Flexi GoTAQ DNA polymerase buffer (Promega UK). The sample was homogenised by inserting a hyperdermic needle (19G) into a pellet pestle hand held homogeniser (Anachem Ltd.) and incubated overnight at 4 ^{o}C. The remaining PCR reaction components were then added: 5 µL 2 mM dNTPS (Promega UK Ltd.), 10 µM of primers 16SAR (50-CGCCTGTTTAACAAAAACAT-30; Palumbi and Benzie, 1991) and 16SB2R (50-ATTCAACATCGAGGTCACAAAC-30; Lindeque *et al.*, 1999), 2 µL 25 mM $MgCl_2$ and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd.). Amplifications were carried out in a G Storm or VWR thermocycler. The cycling parameters included an initial denaturation step at 94°C (5 min) followed by 40 cycles of 94°C (1 min), 45°C (2 min), 72°C (1 min). A final annealing phase at 45°C (2 min) was followed by an extension phase at 72°C (5 min) and storage at 10°C. Aliquots (5 µL) of the amplification reaction were analysed by gel electrophoresis (1%) to check amplification efficiency. Restriction digests were performed on a 7.5 µL aliquot of each amplification by the addition of 0.75 µL bovine serum albumin (1mg mL^{-1}), 0.25 µL 5M NaCl and 2.5 U of each restriction enzyme *Dde* I and *Vsp* I (Promega UK Ltd.). Incubations were

performed at 37°C for > 4h. The digestion products were separated by electrophoresis through a 2% agarose gel, and visualised by UV transillumination.

Nuclear markers – Individual *Calanus* were also genotyped using six microsatellite loci (EL696609, EL585922, EH666870: Provan *et al*. 2009; FK868270, FK670364, FK867682: Parent *et al*. 2012) and twelve recently developed insertion/deletion (InDel) nuclear markers (Smolina *et al*. submitted). These loci are diagnostic between *Calanus finmarchicus* and *C. glacialis* (Online Resource 1: Table S1) and as co-dominant nuclear markers they can allow the identification of hybrids (Online Resource 1: Fig S1, Table S2). DNA was extracted from the remaining half of each individual using the E.Z.N.A.® Insect DNA Kit (Omega Bio-Tek, USA) according to manufacturer´s instruction. PCR reactions were performed using a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, USA) in a total volume of 5 μL. PCR mix contained approximately 3 ng of DNA, reverse and fluorescently labelled forward primers with a final concentration of 0.25 μM/μL each and 1X AmpliTaq Gold® PCR Master Mix (Applied Biosystems, USA). Cycling parameters included an initial cycle of 95°C (60 s), 54°C (20 s), 72°C (25 s), followed by 5 cycles of 95°C (30 s), 54°C (20 s), 72°C (20 s), 30 cycles of 95°C (20 s), 54°C (20 s), 72°C (20 s) and a final extension at 72°C (10 min). All 18 loci were run on a 3500xL Genetic Analyzer (Applied Biosystems, USA) following the manufacturer´s protocol. Alleles were scored using GeneMapper 3.7 (Applied Biosystems, USA) and checked by eye.

Admixture of nuclear genotypes was analyzed using STRUCTURE (v. 2.3.4) (Pritchard et al., 2000). STRUCTURE uses a Bayesian algorithm to identify K (K=2 for *C. finmarchichus* and *C glacialis*) clusters of genetically homogenous individuals and to assign each individual to one of the two clusters or jointly to both clusters if their multilocus genotype indicates hybridization. We used the following parameters: ancestry model = admixture; frequency model = correlated; burn-in = 2 000 000; MCMC length = 1 000 000 after burn-in. The output of STRUCTURE is an admixture coefficient, defined as the proportion of membership to the *C. glacialis* or *C. finmarchichus* cluster for each individual.

The power of admixture analyses to detect hybridization in *Calanus* was evaluated by simulation following Coyer et al 2007. We used the genotypes from 30 individuals of each species for East

Greenland and 50 for West Greenland, to generate 100 artificial genotypes for F_1, F_2, and backcrosses using H Y B R I D L A B (v 1.0) (Nielsen et al., 2006). The analysis was done for the microsatellites, the InDels and both markers together and the admixture of artificial genotypes was analysed with STRUCTURE using the parameters presented above. Genetic differentiation between the two species was estimated with F_{st} using GENETIX 4.02 (Belkhir et al., 2001)

Results

Morphological identification:
Out of 300 females sampled in west Greenland, 288 were identified to species based on size and pigmentation by eye using a microscope (before analysis of prosome length and redness criteria from photographs). 139 of these were attributed to *C. glacialis* and 149 to *C. finmachicus*. There were 12 individuals that could not readily be ascribed to either species and appeared to demonstrate mixed characteristics, especially ambiguous pigmentation criteria (FDAP).

Of the 89 females from East Greenland 60 were identified as *C. glacialis* and 29 as *C. finmarchicus* based on size and pigmentation by eye using a microscope. No females showing ambiguous morphological characters were observed in the east Greenland samples.

Egg morphology –Analysis of egg size and morphology from the females collected and incubated from west Greenland showed the eggs of the two species to have different average diameters, 151±12 µm, n = 86 and 177 ±11 µm, n = 54 for *C. finmarchicus* and *C. glacialis*, respectively. In addition the surfaces of the eggs of the two species are very different. *C. finmarchicus* eggs have a smooth surface while *C. glacialis* eggs have spines on the outer membrane (Fig 1). Of the 12 females which demonstrated ambiguous pigmentation (FDAP) only 3 produced eggs, all eggs had the same size and morphological trait as *C. glacialis* eggs.

Prosome length - The prosome length distribution of females from west Greenland (n=288) showed a bimodal pattern with a mean length for *C. finmarchicus* and *C. glacialis* of 2.7 µm ± 0.16 µm and 3.5 µm ± 0.27 µm respectively (Fig 3). The 12 females that did not display the

defining pigmentation criteria (Fig. 1 and 2) had a prosome length representative of *C. glacialis* females 3.4 ±0.31 (Fig. 3, Table 1). Based on prosome length alone, there is an overlap between species in the range 2.79-3.07mm representing the smallest *C. glacialis* and longest *C. finmarchicus*, respectively. Within this range we find 47 of the 300 females sampled. 38 being *C. finmarchicus,* 8 *C. glacialis* and 1 FADP corresponding to 25%, 6% and 8% of the three groups respectively.

Redness – Since females analyzed were photographed alive and some females kept turning and folding their antenna along the prosome, redness could only be quantified on 218 and 219 females for genital somites and antenna, respectively. It was possible to ascertain the redness values for 109 *C. finmarchicus* from west Greenland using ImageJ analysis of pictures. The redness values for the genital somites of *C. finmarchicus* were 0.06%±0.19 for the somites and 0.013%±0.19 for the antennae. By contrast the redness values for *C. glacialis* (n= 100) from west Greenland were much higher; 25.51%±9.08 for the somites and 11.7%±9.23 for the antennae. The 12 females that demonstrated ambiguous pigmentation (FDAP) based on identification by eye under a microscope would not "normally" be identifiable to species level and consequently not used for experiments by our group. Redness analysis on the FDAP was possible for the genital somites in 10/12 females and for 9/12 females for the antenna.. The FDAP showed low redness % especially on the genital somites where most (7 females) had less than 1.2 % red-pixels. For the antenna pigmentation the FDAP were also at the low end of "normal" for *C. glacialis* (Table 1, Fig. 4). Consequently 4% of the females could have been misidentified, but on the other hand avoided in experiments due to their ambiguous pigmentation.

Genetic identification, mtDNA markers – Of the 300 individual females analyzed from west Greenland all but one were unambiguously identified to species level by RFLP-PCR. One of the samples appeared decomposed in the ethanol and could not be successfully amplified by PCR. The resulting DNA fingerprints or restriction profiles of the remaining samples assigned 149 individuals to be *C. finmarchicus* and 150 individuals to be *C. glacialis*. This identification matched exactly with the morphological identification.

Genetic analysis of the 89 individuals from east Greenland, based on the RFLP signature of their mitochondrial 16S rDNA, identified 60 to be *C. glacialis* and 29 to be *C. finmarchicus*. This also matched exactly with the morphological based identification.

Genetic identification, nuclear markers (microsatellites and InDels) - Genotypes were obtained at 18 nuclear diagnostic loci for 283/300 individuals from west Greenland and for 89/90 individuals from east Greenland. For the 18 missing individuals, not enough good quality tissue was left for DNA extraction.

Genetic differentiation between species was highest using the InDels (Table S1) and .Simulations showed that the microsatellites alone do not have sufficient power to fully discriminate between the introgressed individuals and parental species. The highest discrimination power was achieved using all the co-dominant nuclear markers together (microsatellites and InDels) (Fig S1, table S1). These nuclear markers confirmed species status assessed using morphological and mtDNA identification. All individuals were clearly (probability > 99%) assigned to either species, no hybrids were detected. Genetic analysis revealed all females with ambiguous characteristics (FDAP) to be *C. glacialis* (table 1), these individuals were also not significantly different in size (P>0.005, Tukey-test) or in antennae redness (P>0.005, Dunn‟s-test) to „nomal‟ *C. glacialis*.

Discussion

Identification and separation of formalin fixed, ethanol preserved or fresh *Calanus finmarchicus* from *C. glacialis,* in particular the younger stages, based on size criterion (Unsad & Tande 1991, Hirche *et al.* 1994) is questionable (Lindeque *et al.* 2006; Parent *et al.* 2011) due to an overlap in prosome length between the different species at any particular developmental stage. In recent studies the use of molecular identification techniques has confirmed that *Calanus* species are consistently misidentified to species level when using the morphological character of prosome length as a distinguishing characteristic. This has been proven for *Calanus* over a wide geographical range including the Irminger Basin (Lindeque *et al.* 2006), the Canadian Arctic and Atlantic coasts (Parent *et al.* 2011) and Svalbard (Gabrielsen *et al.* 2012). However the extent of overlap varies with in the area where the species co-occur, Parent *et al.* 2011 showed that the

extent of size overlap was primarily associated with the variable *C. finmarchicus* mean size which is dependent on temperature during development. Even for a single species population Lindeque *et al.* 2006 concluded that a bimodal distribution of size could be sampled, possibly as a result of *C. finmarchicus* mixing from different regions and hence developing under different temperatures as suggested by Parent *et al.* 2011. Our investigation corroborates that prosome length of adult females from Disko bay cannot be used as a stand-alone criterion for separating *Calanus finmarchicus* from *C. glacialis* due to an overlap in size (Fig. 3).

Recently, Parent *et al.* (2011) questioned to what extent the Atlantic *C. finmarchicus* and the Arctic *C. glacialis* can be separated to species. Though they overlap in length they do differ in size, pigmentation (Kjellerup *et al.* 2011), lipid content, lifecycle (Swalethorp *et al.* 2011) as well as overall distribution (Lindeque *et al.* 2004, Hirche and Kosobokova 2007). The present study has for the first time through molecular analysis proven that the pigmentation/ redness criterion documented in this study can successfully be used to identify and separate live adult females of the two sibling species *C. finmarchicus* and *C. glacialis* in both west and east Greenlandic waters. Verification of these field characters visible by eye greatly facilitates setting up experiments, e.g. to determining how the different species will react to an increase in temperature (Kjellerup *et al.* 2012), and thereby describe and model species-specific physiological response to climate change and evaluate the increase of prevalence of the smaller *C. finmarchicus* in a future warmer arctic.

During our previous investigations of phenology of *Calanus* species from Disko bay only *Calanus* females unambiguously fulfilling the pigmentation criteria were included in experiments, dubious females as those in Fig. 2 were ignored. In the present investigation 12 females, all found in West Greenland (4 %), showed ambiguous pigmentation criterion. According to the signature of their maternally inherited 16S mtDNA and egg morphology these females displayed *C. glacialis* characters. However, in a recent study on natural hybridization between *C. finmarchicus* and *C. glacialis* Parent *et al.* (2012) pin pointed Disko bay as an area of potential frequent hybridization due to the temporal and spatial overlap of the two reproducing females (Madsen *et al.* 2001, Madsen *et al.* 2008, Swalethorp *et al.* 2011). Our molecular identification relied on species-specific variation at restriction sites in the 16S mitochondrial

gene and on 18 diagnostic nuclear loci. The combination of molecular markers used in the present study give us unprecedented power to discriminate between parental species and hybrids (Online Resource 1). Interestingly, no hybrids were detected in either west or east Greenland samples.

To date the pigmentation criterion presented here for adult female *C. finmarchicus* and *C. glacialis* from Disko bay have been used and confirmed by differences in phenology i.e. the timing of egg production and the egg morphology of the two species in the southern part of Greenland (Arendt et al. 2011) and Eastern part of Greenland (Nielsen et al. 2007). Now these morphological characteristics have been confirmed by molecular analysis, we hope that the new pigmentation criteria presented here can be helpful, applied and confirmed in future studies covering the North Atlantic and Arctic to generate knowledge on the plasticity of the phenology of these two key species throughout their distribution area and in particular in the regions where they co-occur.

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Tables

Table1. Mean prosome length, percentage of reproducing females, redness index for genital somites and antennae of C. finmarchicus, C. glacialis and females that demonstrated ambiguous pigmentation (FDAP)

Species	Mean prosome length (mm) ± SD (n)	% of reproducing females	Redness index (% of red pixels) for genital somites ± SD (n)	Redness index (% of red pixels) for antennae ± SD (n)
C. finmarchicus	2.68 ± 0.16 (149)	57.3	0.06 ± 0.19 (109)	0.013 ± 0.06 (109)
C. glacialis	3.52 ± 0.27 (139)	24.5	25.51 ± 9.08 (100)	11.7 ± 9.23 (100)
FDAP	3.43 ± 0.31 (12)	27.3	4.91 ± 8.21 (10)	8.87 ± 12.36 (9)

Figures

Fig 1. Morphological and pigmentation criteria used to separate females and eggs of A) *Calanus finmarchicus* and B) *C. glacialis*. The black scalebar is 1 mm and 0.1 mm for females and eggs, respectively. Photo: Sanne Kjellerup.
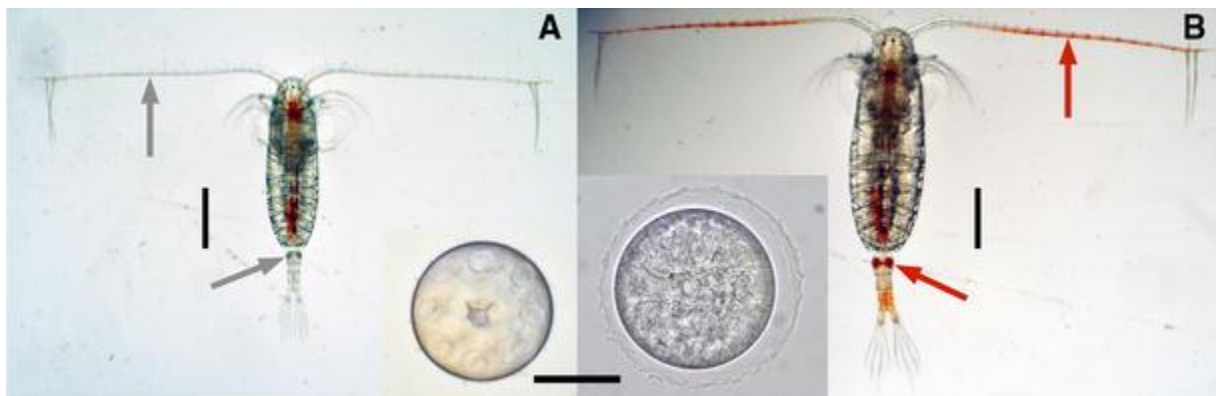
Fig 2. Example of two females that demonstrated ambiguous pigmentation (FDAP). 2A shows a female with two pale genital somites; 2B shows a female displaying one pale and one red genital somite. Besides the apperence of the somites both females display morphological traits similar to *C. glacialis* both in size and pigmentation of antennae. Photo: Sanne Kjellerup.
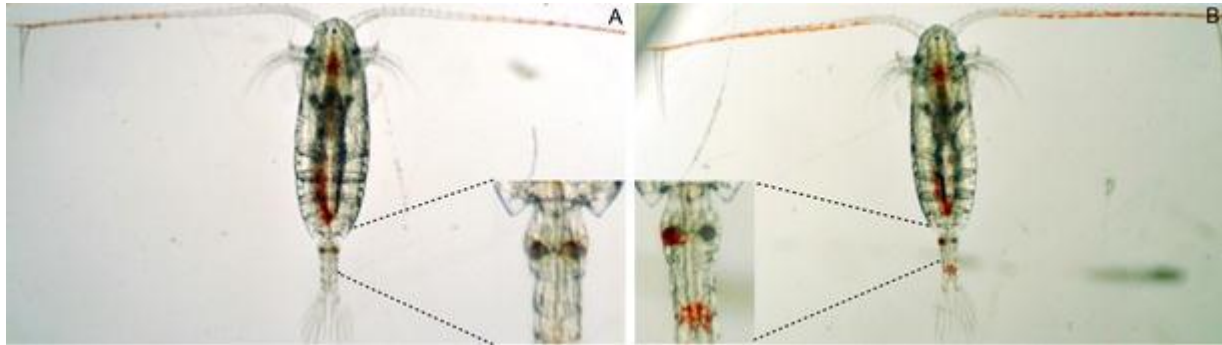

Fig 3. Bar chart of the prosome length distribution of females of *Calanus finmarchicus*, *C. glacialis* and females that demonstrated ambiguous pigmentation (FDAP). The scatter plot with error bars in the top of the figure represent mean length ± SD for each of the three groups.
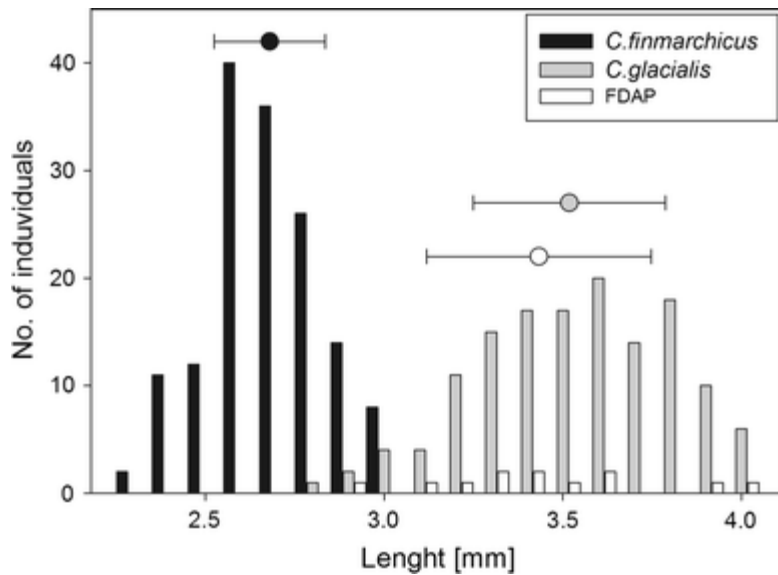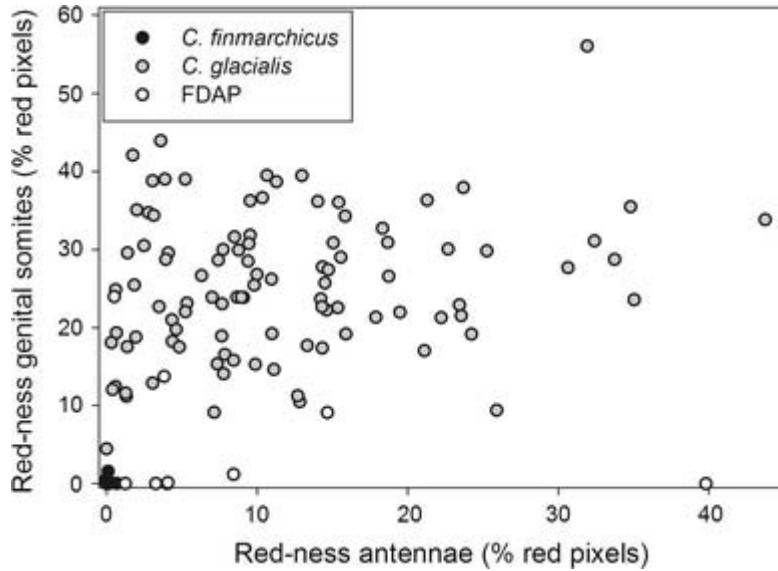
Fig 4. Scatterplot of the redness of genital somites and antennae (% red pixels) and size (mm) for each *Calanus finmarchicus* (n = 109), *C. glacialis* (n = 100) and female that demonstrated ambiguous pigmentation (FDAP) (n= 9).

Online Resource 1. Discriminative power of nuclear markers used for *Calanus* spp. identification: six microsatellites, 12 InDels, and their combination.

Table S1 Discrimination power of six microsatellites and 12 InDels in two Greenland populations performed as the fixation index (F_{ST})[1] (Weir & Cockerham 1984)

Location	Microsatellites	InDels
Western Greenland	0.43	0.99
Eastern Greenland	0.59	0.98

Fig S1 Power of six microsatellites, 12 InDels, and combined set of the markers to discriminate pure *Calanus finmarchicus*[1] and *C. glacialis*[1] and their simulated hybrids[2] (F1, F2 and backcrossed) in two Greenlandic populations using admixture coefficient inferred with STRUCTURE (v. 2.3.4) (Pritchard et al., 2000)
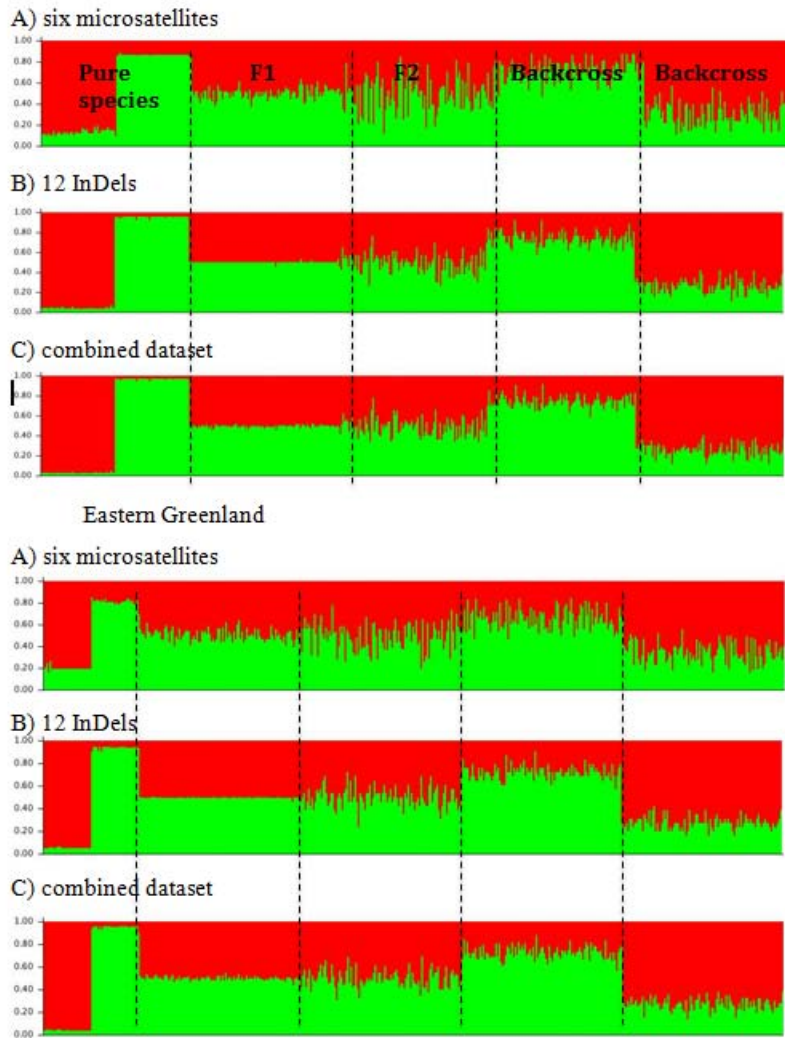

Eastern Greenland

Table S2 Admixture coefficient range (the proportion of membership to the *C. glacialis* or *C. finmarchichus* cluster) for pure species and simulated hybrid classes in two Greenlandic populations based on six microsatellites, 12 InDels, and combined set of the markers. Admixture coefficient inferred with STRUCTURE (v. 2.3.4) (Pritchard et al., 2000)

Identification class	Western Greenland			Eastern Greenland		
	Micro-satellites	InDels	Combined set	Micro-satellites	InDels	Combined set
C. finmarchicus	0.11-0.14	0.04-0.07	0.03-0.06	0.15-0.30	0.05-0.10	0.04-0.10
C. glacialis	0.80-0.90	0.93-0.96	0.95-0.98	0.73-0.84	0.93-0.95	0.94-0.96
Hybrid F1	0.36-0.62	0.46-0.54	0.47-0.54	0.36-0.60	0.50-0.54	0.46-0.56
Hybrid F2	0.15-0.88	0.22-0.73	0.22-0.66	0.22-0.80	0.28-0.76	0.30-0.69
F1 x *C. finmarchicus*	0.12-0.53	0.07-0.42	0.08-0.42	0.15-0.54	0.09-0.43	0.12-0.43
F1 x *C. glacialis*	0.42-0.89	0.58-0.89	0.59-0.89	0.44-0.84	0.58-0.84	0.62-0.86