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Can morphology reliably distinguish between the copepods *Calanus finmarchicus* and *C. glacialis*, or is DNA the only way?

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Abstract

Copepods of the genus *Calanus* play a key role in marine food webs as consumers of primary producers and as prey for many commercially important marine species. Within the genus, *Calanus glacialis* and *Calanus finmarchicus* are considered indicator species for Arctic and Atlantic waters, respectively, and changes in their distributions are frequently used as a tool to track climate change effects in the marine ecosystems of the northern hemisphere. Despite the extensive literature available, discrimination between these two species remains challenging. Using genetically identified individuals, we simultaneously checked the morphological characters in use for *C. glacialis* and *C. finmarchicus* identification to compare the results of molecular and morphological identification. We studied the prosome length (1); the antennules and the genital somite pigmentation (2); the morphology of the fifth pair of swimming legs and of the mandible (3). Our results show that none of these morphological criteria can reliably distinguish between *C. glacialis* and *C. finmarchicus*. This has severe implications for our current understanding of plankton ecology as a large part of our knowledge of *Calanus* may be biased due to species misidentification and may subsequently require reinvestigation with the systematic use of molecular tools.

Copepods of the genus *Calanus* are the dominant component of the zooplankton in the North Atlantic and the Arctic (Jaschnov 1972; Fleminger and Hulsemann 1977; Conover 1988; Kosobokova et al. 2011; Kosobokova 2012) and are by far the most studied zooplankton species, with ca. 100 scientific publications per year for the last 30 years (*Web of Science*). They play a key role in marine food webs as consumers of primary producers and microzooplankton and as prey for many commercially and non-commercially important species (Gislason and Astthorsson 2002; Beaugrand et al. 2003; Skjoldal 2004; Varpe et al. 2005; Michaud and Taggart 2007; Steen et al. 2007; Falk-Petersen et al. 2009). Furthermore, they are key drivers of the vertical export of material from the upper part of the water column due to the ability of packing organic material into large fast-sinking fecal pellets (Wilson et al. 2008). In marine food webs, *Calanus* spp. are essential agents of matter and energy transfer between phyto- and microzooplankton and higher trophic levels.

In the North Atlantic and Arctic regions, the Arctic species *Calanus glacialis* and the smaller north Atlantic *Calanus finmarchicus* account for most of the zooplankton biomass (Fleminger and Hulsemann 1977; Hassel 1986; Blachowiak-Samolyk 2008; Søreide et al. 2008; Kosobokova and Hirche 2009; Kosobokova 2012). The spatial distribution of these two copepods is linked to the distribution of Arctic and Atlantic waters, respectively, and they are thus considered indicator species for these water masses (Jaschnov 1966; Jaschnov 1970; Unstad and Tande 1991; Bonnet and Frid 2004; Daase and Eiane 2007; Helaouët and Beaugrand 2007; Blachowiak-Samolyk 2008; Broms et al. 2009). Recently, *C. glacialis* and *C. finmarchicus* have been regarded as beacons

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of climate change (Hays et al. 2005; Wassmann et al. 2015), as changes in their distribution are interpreted as changes in Atlantic water circulation and potential "Atlantification" of the Arctic (Wassmann et al. 2006; Falk-Petersen et al. 2007).

The ecological importance of *C. finmarchicus* and *C. glacialis* is unquestionable, but distinguishing between them in regions of co-occurrence has always been challenging (Unstad and Tande 1991; Hirche et al. 1994). Three main morphological characters have been used, (1) prosome length; (2) redness of antennules and genital somite (the two spermathecae); (3) structure of the fifth pair of swimming legs and the coxal endid of the mandible (in adults).

Because of convenience, the prosome length measurements (1) have been and remain the most commonly used method to separate the two species (see, for example: Unstad and Tande 1991; Kwasniewski et al. 2003; Arnkværn et al. 2005; Forest et al. 2011; Hirche and Kosobokova 2011; Kosobokova 2012) although several recent studies have demonstrated a size-overlap in specific regions (Lindeque et al. 2006; Parent et al. 2011; Gabrielsen et al. 2012).

Another trait that has been recently suggested to distinguish between *C. finmarchicus* and *C. glacialis* is the presence or absence of red pigmentation on their antennules and, in the case of adult females, on their genital somite (originally genital field) (2) (Nielsen et al. 2014). Examination of this character requires that individuals are alive, so the samples have to be sorted directly after collection, which is also a challenge.

The classical, but most complex and time-consuming approach to identify *C. finmarchicus* and *C. glacialis* is to examine their morphological characters (3) that have been suggested as diagnostic of the two species. Most common is the examination of the structure of the fifth pair of swimming legs in adult females and males (Jaschnov 1955), and the morphology of the coxal endid of the mandible (gnathobase) (Beklemishev 1959). Examination of both characters requires performing a fastidious and specific preparation on each specimen, and is therefore seldom applied during routine zooplankton samples analyses.

Although several diagnostic molecular markers have been developed for *Calanus*, from mtDNA RFLP (Lindeque et al. 1999) to nuclear InDels (Smolina et al. 2014), their use in the zooplankton research community has so far remained limited. A recent reappraisal of *Calanus* spp. distribution in the North Atlantic/Arctic Oceans relying on large scale sampling and molecular identification has suggested that misidentification is widespread and has led to erroneous conclusions regarding *Calanus* biogeography (Choquet et al. 2017).

Species misidentification may be less problematic in studies focusing on describing zooplankton assemblages based on higher taxonomic categories (e.g., Aßmus et al. 2009) or in trait-based studies, which aim at investigating ecological functions of assemblages (e.g., Brun et al. 2016). A correct species identification is however crucial for understanding species-specific life history strategies, species-specific productivity estimates and for studying distribution patterns, particularly if species are considered indicative for specific water masses and if changes in their distribution are assumed to have far reaching ecosystem impacts.

Both species differ in life strategies such as energy requirements for reproduction and growth, timing of reproduction, composition of overwintering populations, and seasonal vertical migration patterns. These differences reflect adaptations to the environmental conditions in their main areas of distribution (Falk-Petersen et al. 2009), with *C. glacialis* having adapted more flexible life history strategy to deal with the constrains of seasonally ice-covered seas (Daase et al. 2013) and low temperature leading to a larger body size and longer life span compared to *C. finmarchicus*. It is crucial to correctly identify them to understand their life history adaptations fully, how they have evolved differently in each species and how climate change will be affecting each species' productivity, population success, distribution and role in the food web. Using prosome length to discriminate between species has shown to underestimate smaller sized *C. glacialis* (Gabrielsen et al. 2012), which may bias species-specific biomass estimates and our understanding of energy allocations in that species.

In the present study, we use molecular tools to assess the reliability of the morphological characters used to discriminate between *C. finmarchicus* and *C. glacialis* across a large part of their distributional range.

Material and procedures

Samples collection and pre-sorting

Zooplankton were sampled in fjords along the Norwegian coast, in the White Sea, in Svalbard waters and in the Nansen Basin (Table 1) by vertically towed plankton nets (WP-2/Juday types) with mesh sizes between 150 μm and 200 μm. The whole water column was sampled for most of the locations, except for the White Sea (100–0 m) and the Svalbard fjords (20–0 m). The sampling locations were selected to represent a latitudinal gradient from the southernmost (Lurefjord) to the northernmost (Nansen Basin) areas of co-occurring of *C. finmarchicus* and *C. glacialis*. The White Sea, where only *C. glacialis* occurrence was reported historically (Jaschnov 1955; Jaschnov 1966) and recently confirmed genetically (Choquet et al. 2017), and the region of Raunefjord/Korsfjord where only *C. finmarchicus* occurrence was reported, were also sampled in order to have more elements of comparison. Directly after sampling, a Folsom plankton splitter was used to randomly subsample $\sim$ 100–200 live individuals of the older ($\geq$ CIV) copepodite stages. Prosome length measurements and examination of the redness of antennules and genital somite (for details, see below) were carried out right after sampling, on the subsampled individuals kept alive in seawater. These live individuals were subsequently preserved individually in

Table 1. Sampling locations with positions, sampling dates, and number of individuals used for each analysis.

Location		GPS	Date	N ind. analyzed			
					Redness		
				PL	Ant	Gen	Legs/Gnath
Nansen Basin		87°00′N 55°47′E	10/4/16	96	94	0	0
Svalbard	Isfjord	78°19′N 15°09′E	6/5/16	136	227	60	0
	Van Mijenfj. (VM)	77°46′N 15°02′E	6/3/16	90	0	0	16
White Sea		66°33′N 33°43′E	8/22/16	116	115	1	0
Sørfolda (Sorf)		67°35′N 14°50′E	4/20/16	0	0	0	7
Salten/Skjerstadfj.	Saltenfjord (SALT)	67°16′N 14°38′E	2/15/16	72	190	102	24
	Skjerstadfj. (SKJ)	67°15′N 14°50′E	7/12/16	109	0	0	2
Lurefjord (Lure)		60°41′N 05°09′E	6/22/16	188	189	5	22
Raune/Korsfj.	Raunefjord	60°17′N 05°08′E	6/4/16	43	88	0	0
	Korsfjord	60°11′N 05°12′E	6/6/16	45	0	0	0

Arctic locations are presented first, starting with the northernmost; the Atlantic locations are listed from North to South. Number of individuals analyzed is given ("N ind. analyzed"), with the precision for the three different analyses: PL, prosome length measurements; Redness—Ant/Gen, examination of redness of antennules/genital somite; Legs/Gnath, examination of morphology of the 5th pair of legs and mandibular gnathobase.

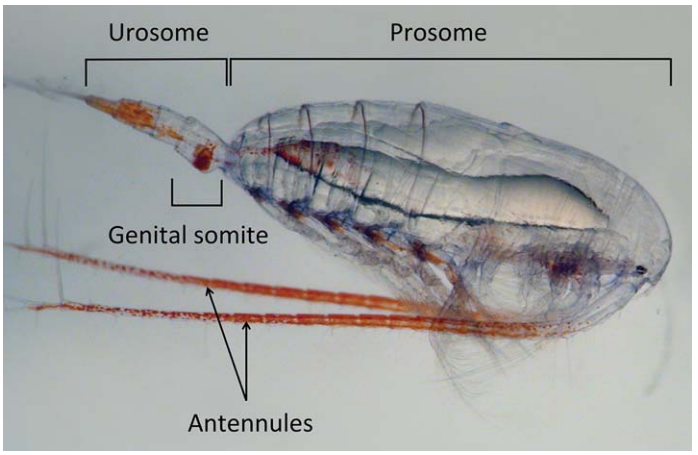

Fig. 1. *Calanus* sp. adult female, structure of the body.

70–80% undenatured ethanol for later molecular-based species identification and morphological examinations.

Prosome length measurements

We subsampled up to 200 live individuals of late copepodite stages IV, V, and CVI female (CIV, CV, and CVI F) of *Calanus* per sample from each of the nine locations (pooled into six geographically distant regions—895 specimens in total) (Table 1). For the sampling locations where it was possible, photographs of individuals were taken with a camera attached to a stereomicroscope. The prosome length of each specimen was measured from the tip of the cephalosome to the distal lateral end of the last thoracic somite (Fig. 1) either using the ruler in the eye-piece of a stereomicroscope to measure directly (resolution 1 μm), or by using cellSens Standard software (version 1.8.1—Olympus corporation© 2009–2013) to analyze the photograph taken (resolution 0.01 μm). All the 895 individuals were identified with molecular markers (see "Molecular species identification" section below). Correlation between latitude and body size (prosome length) was tested independently for *C. finmarchicus* and *C. glacialis*, and separately for each developmental stage (CIV, CV, and CVI female) with use of Pearson's correlation (in Microsoft® Excel® version 14.7.3).

Redness assessment

We evaluated the potential of red pigmentation ("redness") on antennules and genital somite to separate live *C. finmarchicus* and *C. glacialis*, as suggested in Nielsen et al. (2014). A total of 903 *Calanus* individuals of developmental stage CIV to CVI (adult female and male) from six distant populations in the North Atlantic and Arctic Oceans were investigated in regard to their antennule redness (Table 1). Additionally, pigmentation of the two spermathecae on the ventral surface of the genital somite (the first urosome somite) in adult females was examined for 168 individuals from the same populations. All the individuals examined for their redness were subsequently identified with molecular markers (see "Molecular species identification" section below).

The degree of antennule red pigmentation ("redness") was very heterogeneous among the studied individuals. We distinguished four different categories of individuals: antennules with more than 90% of redness; from 50% to 90% of redness; from 10% to 50% of redness; and less than 10% of redness. The percentage of redness used to distinguish different categories is based on the subjective evaluation of how much of the surface of antennules is red, and how dense this pigmentation is (*see* Fig. 2 for examples of each category). This choice is justified by our search for a parameter that could be easily and quickly used for routine species identification especially in the field. Objective quantification of redness using an image analysis software

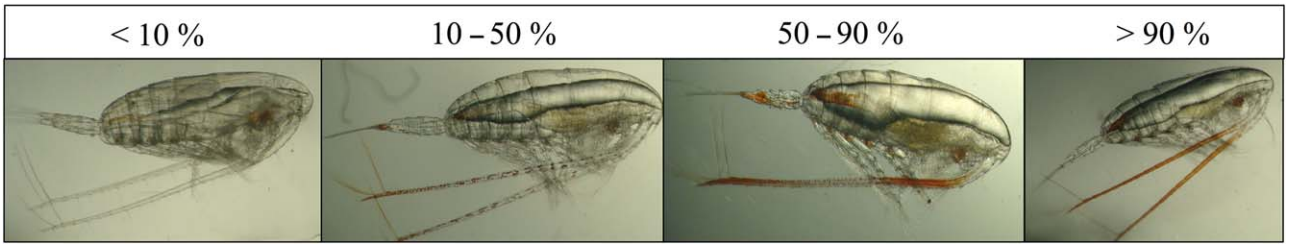

Fig. 2. Categories of red pigmentation of antennules in *Calanus*. Four photos are shown as examples of the four categories defined as follows: less than 10% of red pigmentation; between 10% and 50% pigmentation; between 50% and 90% of pigmentation; and more than 90% pigmentation.

Fig. 3. Pigmentation of genital somite in *Calanus*. Pigmentation is defined as red (left photo) or pale (right photo).

(as in Nielsen et al. 2014) was not performed, as the sorting of live individuals was mostly done in conditions precluding taking high-quality photographs, required for such analysis.

Statistical differences in antennule redness between the two species *C. finmarchicus* and *C. glacialis*, among the different developmental stages, and among the locations sampled were tested using the Kruskal-Wallis *H* test.

To evaluate the pigmentation of the genital somite, we considered only two categories: red or pale (Fig. 3). Individuals with any redness on one or two of the spermathecae were assigned to "red"; the individuals for which no redness at all on the genital somite was noticeable were reported as "pale."

Molecular species identification

Each *Calanus* individual used for this study was genetically identified (913 individuals in total—Table 1). Molecular species identification followed the procedure described in Choquet et al. (2017). In brief, DNA was extracted from animals' antennules using the HotSHOT DNA extraction method (Montero-Pau et al. 2008) and six nuclear molecular markers of the type InDels (Insertion or Deletion motifs—Smolina et al. 2014) were amplified by Polymerase Chain Reaction (PCR). PCR amplicons were sized using a 3500xL Genetic Analyzer (Applied Biosystems, U.S.A.), generating a species specific profile (Smolina et al. 2014). Together, these six markers allow the reliable identification of *Calanus* species in the North Atlantic and Arctic Oceans (Nielsen et al. 2014; Smolina et al. 2014; Choquet et al. 2017). This method allows the genotyping of each individual for species identification without using or destroying the animal's body. Once the antennules are removed, the rest of the body is intact and can still be examined for morphology.

Fifth pair of legs and gnathobase morphology examination

Seventy-one individuals from five different locations (Table 1) were examined (49 individuals of developmental stage CV and 22 individuals of CVI adult females), by following a specific procedure. M. Choquet selected the individuals among the genetically identified specimens preserved in ethanol, in order to have both species represented. The 71 selected ones were sent to S. Kwaśniewski for dissection (see procedure below), without giving any information about the molecular results of species ID for these particular individuals. After dissection, photographs of the dissected body parts were taken for each individual by S. Kwaśniewski, and shared with K. Kosobokova. Examination of the fifth thoracic leg (swimming leg P5—Figs. 4a,b, 5a,b) and the coxal endid of the mandible (gnathobase—Figs. 4c,d, 5c,d) were carried out by both S. Kwaśniewski and K. Kosobokova independently, based on the photographs only. Their species identification decisions, based on the pictures analysis, were then sent back to M. Choquet to compare with molecular results. We decided to follow this approach in order to avoid any bias in the expert interpretation of the pictures due to the prior knowledge of molecular ID.

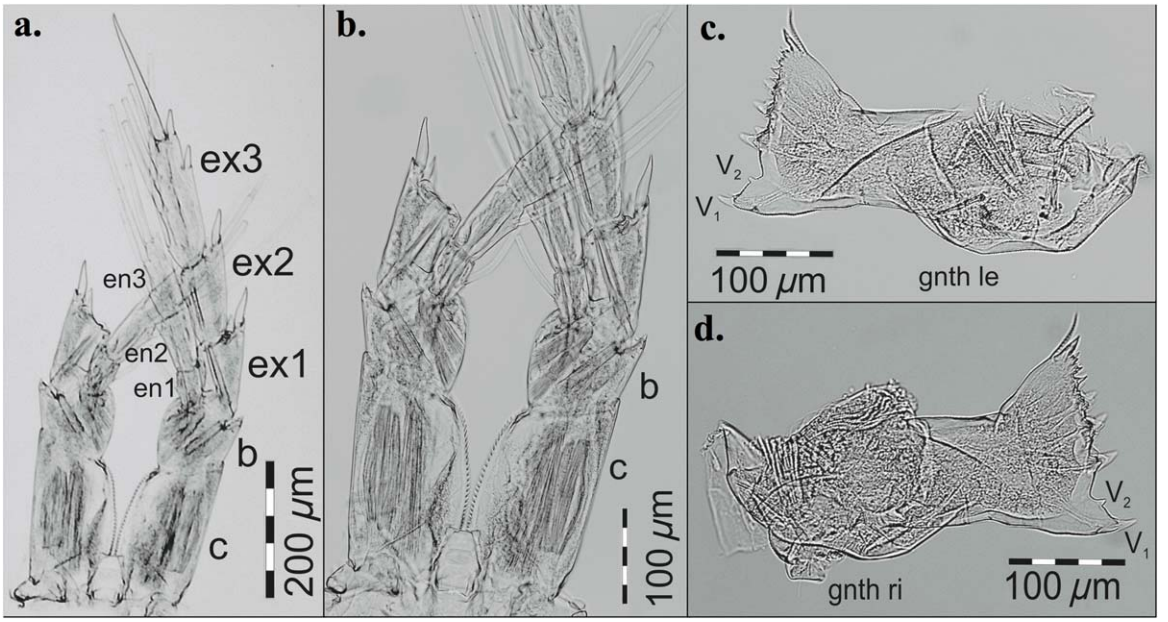

Fig. 4. Morphology of the fifth thoracic pair of legs and the gnathobase of an adult female *C. finmarchicus* (genetically confirmed) exhibiting the species-specific traits as described in the literature. Specimen collected from Van Mijenfjord (ID: VM41). (**a**, **b**) Anterior view of the fifth thoracic pair of legs (P5) with denticulated lamellae on the medial margin of the coxa, showing typical "straight form." Abbreviations: b, basis; c, coxa; en1–en3, endopods 1 to 3; ex1–ex3, exopods 1 to 3. (**c**, **d**) Anterior view of mandible gnathobases, with a typical small second ventral tooth on the cutting edge. Abbreviations: gnth le, left mandible gnathobase; gnth ri, right mandible gnathobase; V_1, first ventral tooth; V_2, second ventral tooth.

For the examination of the P5 morphology, descriptions of the leg structure provided in Jaschnov (1955); Frost (1974); and Brodskii et al. (1983) were used. The P5 in *Calanus* consists of a remnant of precoxa, well developed coxa (basipod 1) and basis (basipod 2), from which two 3-segmented rami (exopod and endopod) grow out (Huys and Boxshall 1991). Examination focused on the lamellar structure with denticulated edge, termed also the denticulated lamella, which is present on each of the two coxa (basipod 1) of P5. The denticulated lamella extends along the medial margin of the coxa from the intercoxal plate to nearly the distal medial corner of the segment. According to the species morphological descriptions (Jaschnov 1955; Frost 1974), in *C. finmarchicus*, this lamella is straight or almost straight, missing clearly expressed incurvation characteristic for *C. glacialis* (*see* Fig. 4a,b). In *C. glacialis*, the denticulated lamella is clearly concaved, with well-expressed curvature (deflection) slightly shifted to the posterior surface of the segment in its middle part (*see* Fig. 5a,b).

For examination of the gnathobase, descriptions provided in Beklemishev (1959), Vyshkvartzeva (1972), and Vyshkvartzeva (1976) were used. The gnathobase is the coxal endid (a medially directed process on the protopodal segment of the appendage), bearing the toothed cutting edge distally (Huys and Boxshall 1991). The cutting (masticatory) edge of the gnathobase bears several groups of teeth varying in form and structure. Some of these teeth (at least in sexually developed stages) are covered with silicate crowns. In adult females of *C. glacialis* and *C. finmarchicus*, the complete arrangement of gnathobase cutting edge includes ventral (V_1–V_2), central (C_1–C_4), and distal (D_1–D_3) teeth plus flexible setae with one or two rows of spines. Between groups of V and C teeth, there is a diastema (a gap between the teeth). Tooth V_2 does not have a crown and teeth of group D are often equipped on their lateral surfaces with small denticles. According to Beklemishev (1959) and Vyshkvartzeva (1972, 1976), species-specific differences in the form and arrangement of the teeth concern teeth V_1 and V_2. In *C. glacialis* adult females, the crown of the tooth V_1 is not very high, compressed in the anterior-posterior direction, and has 2–3 peaks. The tooth V_2, which does not have a crown, is well developed and placed on wide cuticular platform. Its size is close to the size of V_1 and it approximately equals the diameter of its base (Fig. 5c,d). In *C. finmarchicus* adult females, the tooth V_2 is smaller than V_1 and its height is larger than the diameter of its base, but its form and size varies (Fig. 4c,d). In comparison with *C. glacialis*, the tooth V_2 in *C. finmarchicus* presents as not completely formed.

The examination of the two structures was done after dissection and slide preparation. Each individual from the study collection was first immersed for 10 min in a drop of glycerol : ethanol 1 : 1 mixture placed on a microscope slide with cavity. In 10 min, each individual was photographed using Olympus SC50 CMOS Color Camera, mounted with a photo adapter U-TV0.5xc-3 on Olympus SZX12 Research Stereomicroscope, equipped with AXH1x and DFPL2x-3 objectives. The acquisition of the digital pictures was made with Olympus cellSense Imaging Software v.1.12. The pictures of

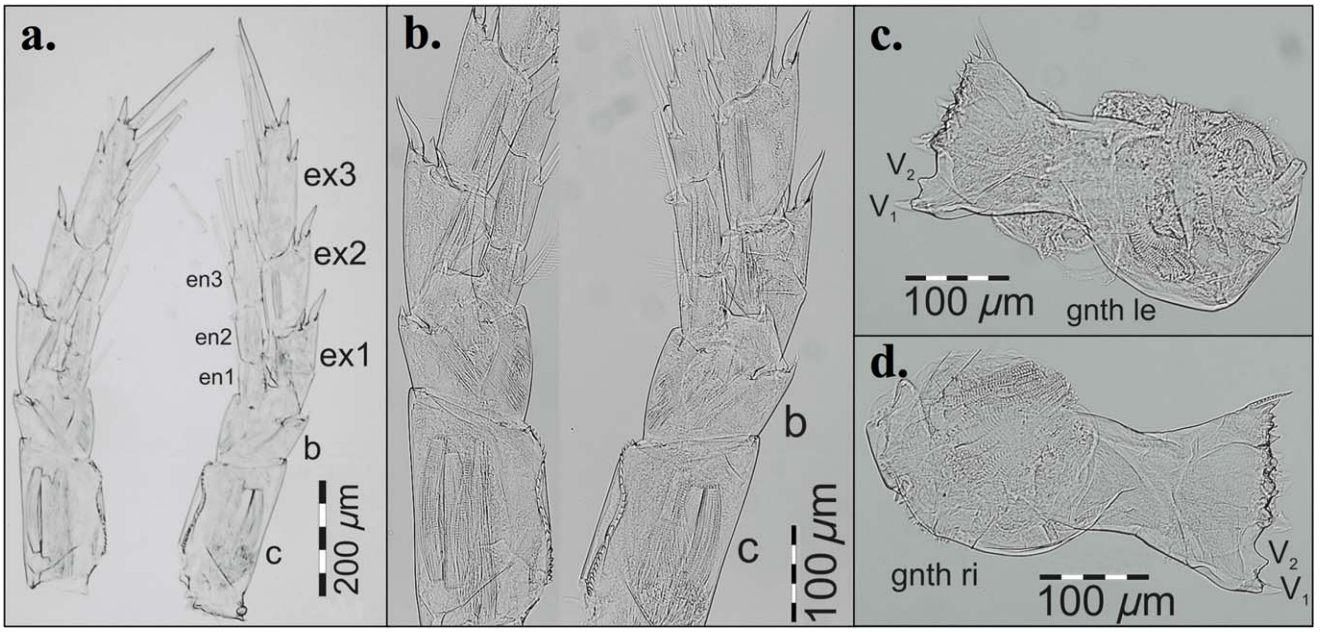

Fig. 5. Morphology of the fifth thoracic pair of legs and the gnathobase of an adult female *C. glacialis* (genetically confirmed) exhibiting the species-specific traits as described in literature. Specimen collected from Skjerstadjord (ID: SKJ25). (**a**, **b**) Anterior view of the fifth thoracic pair of legs (P5) showing denticulated lamellae on the medial margin of the coxa in a typical concave form, with well-expressed curvature. (**c**, **d**) Anterior view of mandible gnathobases with the cutting edge with a typical large second ventral tooth on a wide basis (*see* Fig. 4 legend for abbreviations meaning).

the body habitus of each individual were made at 10X total magnification, one picture with the use of an AXH1x objective and one with the use of a DFPL2x-3 objective. Then the two structures under consideration were dissected from the body. The P5 was cut off the thoracic somite and placed in a drop of the same glycerol : ethanol 1 : 1 mixture, on a regular microscope slide, anterior side upward.

The mandibles were also dissected one by one from the cephalosome. After removal of the mandible, the gnathobase was dissected from the appendage, and mounted in another drop of glycerol : ethanol 1 : 1 mixture, anterior side upward. The same procedure was repeated for the second mandible, and finally the pair of gnathobases belonging to one individual was covered with a glass coverslip. The dissection of the appendages and preparation of the microscope slides was done with use of Olympus SZX12 stereomicroscope, at magnifications ranging from 7X to 90X. In the following step, the investigated structures were photographed using Olympus SC30 CMOS Color Camera, mounted with a photo adapter U-TV1x-2 on Olympus BX51 system microscope, equipped with PlanN 4X and UPlanFLN 10X objectives. The acquisition of the digital pictures was made with use of Olympus cellB Imaging Software v.3.3.

Assessment

Prosome length measurements

Based on prosome length measurements of 895 genetically identified individuals from six regions, we confirm that this character shows a global overlap of size between *C. finmarchicus* and *C. glacialis* regardless of developmental stage (Fig. 6). Size frequency distributions, however, differed among different regions. In the Norwegian fjords (Saltenfjord/Skjerstadfjord; Lurefjord), *C. glacialis* showed a complete size overlap with *C. finmarchicus*, but these *C. glacialis* were significantly smaller (*t*-test, $p < 0.01$) than the *C. glacialis* captured in the White Sea and the high Arctic. Noteworthy, our data showed positive correlations between latitude and body size for both *C. glacialis* and *C. finmarchicus* (Table 2).

Thus, the prosome length cannot reliably discriminate between *C. finmarchicus* and *C. glacialis* in any of the investigated regions, and even less in the Norwegian fjords. However, in the Nansen Basin and Svalbard waters, the majority of the length values for *C. finmarchicus* and *C. glacialis* follow a dichotomy. Prosome length could therefore be used in those particular areas to approximate the overall *C. glacialis* and *C. finmarchicus* composition. It has to be kept in mind, however, the inaccuracy of the method leading to underestimation of *C. glacialis* (especially small-sized individuals), and overestimation of *C. finmarchicus* numbers (Gabrielsen et al. 2012).

Redness assessment

We tested if redness can be used to reliably separate between live *C. finmarchicus* and *C. glacialis*, at different developmental stages and across different regions of co-occurrence. According to Nielsen et al. (2014), the genital somite (originally genital field) and the antennules of *C.*

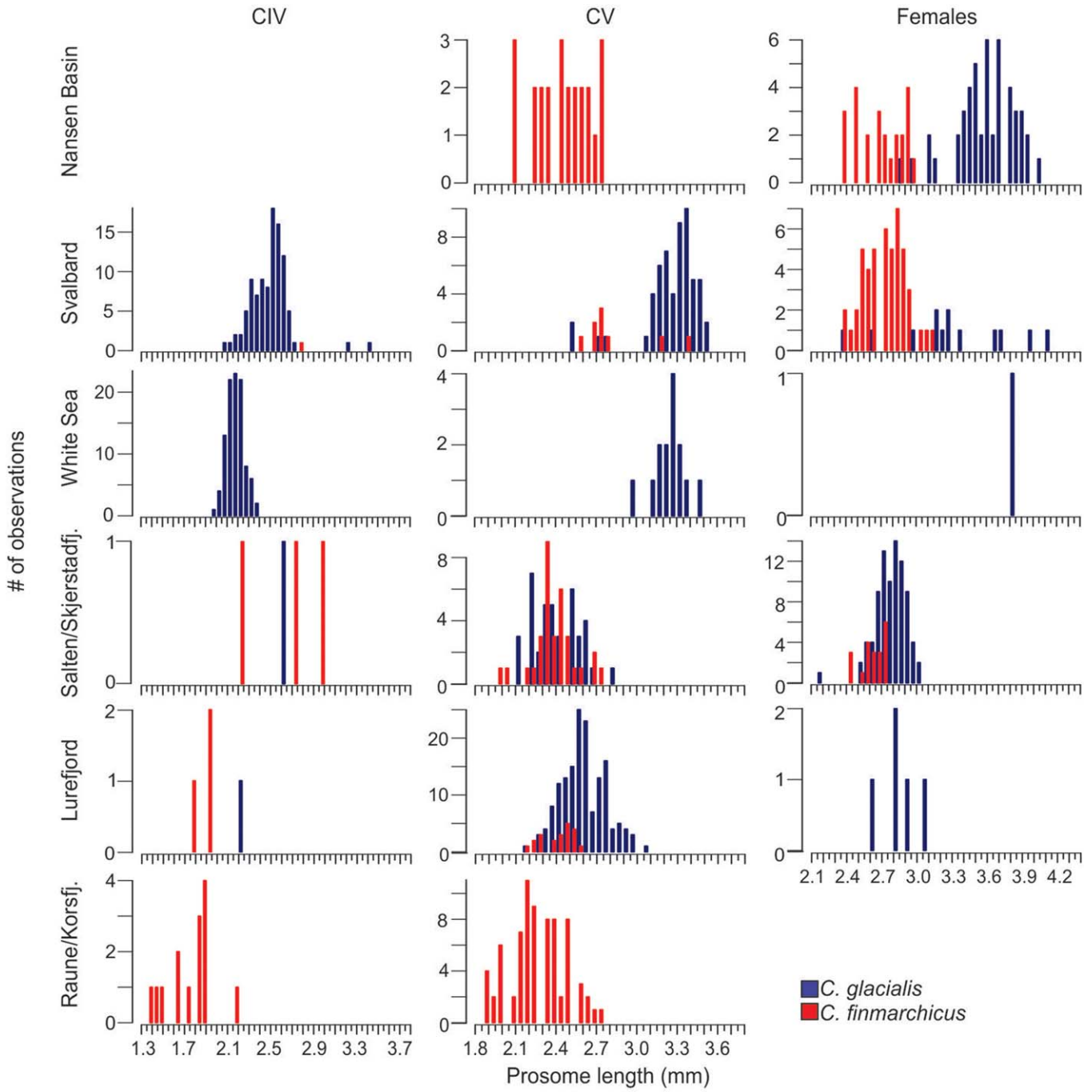

Fig. 6. Stage-specific length frequency distributions of prosome length (mm) for copepodites CIV, CV, and adult females of *C. glacialis* and *C. finmarchicus* in different regions. In total, 895 individuals were measured, from nine locations, pooled into six distant regions, in the North Atlantic and Arctic Oceans. Only *C. glacialis* (blue) occurred in the White Sea, and only *C. finmarchicus* (red) occurred in Raunefjord/Korsfjord area.

glacialis adult females had red pigmentation, while the pigmentation of female *C. finmarchicus* were mostly pale. However, the study focused only on adult females from a limited geographic location (Greenland) (Nielsen et al. 2014).

In our study, red pigmentation of antennules was variable (Figs. 7, 8, Supporting Information Fig. 2a,b), with significant differences in redness between the two species, among developmental stages, and locations sampled (Supporting Information Table 1). Antennule redness was assessed for 903 individuals, from copepodite stage CIV to adult females and males, at six different locations (Table 3a–f; Fig. 7). Molecular identification of these 903 individuals was performed consecutively.

Table 2. Pearson's *r* calculation for testing the correlation between *Calanus* body size (prosome length) and latitude.

Species	*C. finmarchicus*			*C. glacialis*		
Stage	CIV	CV	CVI F	CIV	CV	CVI F
n	21	161	92	201	269	151
Pearson's *r*	0.8**	0.39**	0.19	0.74**	0.65**	0.84**

Significance levels (*p* value) are indicated by: * $p < 0.05$ and ** $p < 0.01$.

At the northernmost location, the Nansen Basin (Table 3a), all genetically identified *C. glacialis* had > 10% redness on their antennules, and they were all adult females. Stages CV and adult females of genetically identified *C. finmarchicus* individuals collected in the same place were mainly pale except for three females with a slight redness (10–50%).

In Svalbard, the majority of *C. glacialis* identified genetically, including stages CIV, CV, and adult females, had also > 10% redness (except one CV and one adult female with < 10%—Table 3b). *C. finmarchicus* individuals, including stages CIV, CV, and CVI, tended to be paler compared to *C. glacialis* in Svalbard, but some *C. finmarchicus*, especially females, had > 10% redness. One male was detected there, identified as *C. finmarchicus* with pale antennules.

Only *C. glacialis* was detected in the White Sea sample (stages CIV, CV, and adult females—Table 3c). Individuals from stage CIV exhibited almost none, or very little (less than 50%) redness, but a stronger red pigmentation was observed for the older stages CV and adult females.

In the boreal fjords Saltenfjord and Skjerstadfjord (Table 3d), *C. glacialis* individuals (stages CIV–CVI) most often (88%) had red pigments. All males, 4% of the females and 20% of the CV *C. glacialis* were pale. The majority of *C. finmarchicus* individuals were pale in these two fjords, independently of the developmental stage, however, with three exceptions (one CIV and two adult females). Interestingly, males of both species were totally pale.

In Lurefjord (southern Norway—Table 3e), the majority (73%) of *C. glacialis* had red pigmentation, with 25% pale CV and 100% pale females. In comparison, the majority (84%) of *C. finmarchicus* (CVs) were pale there.

In the open southern fjords Raunefjord and Korsfjord (Table 3f), we only identified *C. finmarchicus* among the older stages (CIV and CV) in our samples and 56% of these were pale and another 16% had 10–50% redness.

Despite identifying significant differences (Kruskal-Wallis *H* test) in the redness of antennules between species, among stages for each species, and among locations for each species for every set of variables compared (Supporting Information Table 1), the general trend was that the majority of individuals of *C. finmarchicus* tends to have pale antennas whereas the majority of *C. glacialis* tends to have red ones (Figs. 7, 8). In both species, there were exceptions, especially for *C. glacialis* in the White Sea and *C. finmarchicus* in Raunefjord/Korsfjord (Supporting Information Fig. 2a). The tendencies in pigmentation were similar for the different developmental stages (Supporting Information Fig. 2b) except that males of both species were pale without exception (albeit only a few males were investigated) and *C. glacialis* CIV in general being less pigmented than *C. glacialis* CV and adult females. Antennule redness thus appears not to be a reliable diagnostic feature and is clearly not a species-specific trait. It was never 100% diagnostic for any of the six regions investigated. Assessment of pigmentation might be useful to get an overall impression of the species composition in the Arctic Ocean and in isolated fjords, taking into account the error threshold (region dependent), and the fact that investigations have to be done on live organisms.

Regarding the redness of the genital somite of *Calanus* females, all the *C. finmarchicus* examined had pale spermathecae, although we only found females of this species in Svalbard and Saltenfjord/Skjerstadfjord (Table 4). Most of the *C. glacialis* examined (from four regions) had red genital somite, but also a few individuals had pale genital somite in each region. Importantly, we noticed that all the individuals with red genital somite were *C. glacialis*. Furthermore, all the individuals displaying both red antennules and red genital somite were *C. glacialis* (Table 5). Although our results indicate that redness of the genital somite is also not 100% diagnostic for species identification, this character seems to be useful to get a global idea of species composition of a zooplankton sample, but using it may result in an underestimation of *C. glacialis* number of individuals.

Fifth pair of legs and gnathobase morphology examination

The curvature of the inner denticulated margin of P5 swimming legs and the shape of the mandibular cutting blade are morphological characters that have been described early in the literature as species-specific (Jaschnov 1955; Beklemishev 1959; Frost 1974; Vyshkvartzeva 1976; Brodskii et al. 1983). However, due to the arduousness of their examination, they remain rarely used to identify *Calanus* species.

Only 23 individuals out of the 71 examined exhibited the species-specific features typical for the species they belong to (verified by genetics), according to the literature (Supporting Information Table 3). For the other individuals, the morphological characteristics examined were different from that of the species according to the literature (Figs. 9, 10). Furthermore, no geographic coherence was found in the deviations of the characteristics (Supporting Information Table 3). This resulted in an error rate of 30% and 31% in the identification decisions made by the experts in *Calanus* morphology, after comparing their decision with results of genetic identification. Identification decisions of both experts matched only 36 times, and of these only 32 individuals (45% of the total) were confirmed to be correct by genetic identification. More specifically, experts' decision and genetics matched at

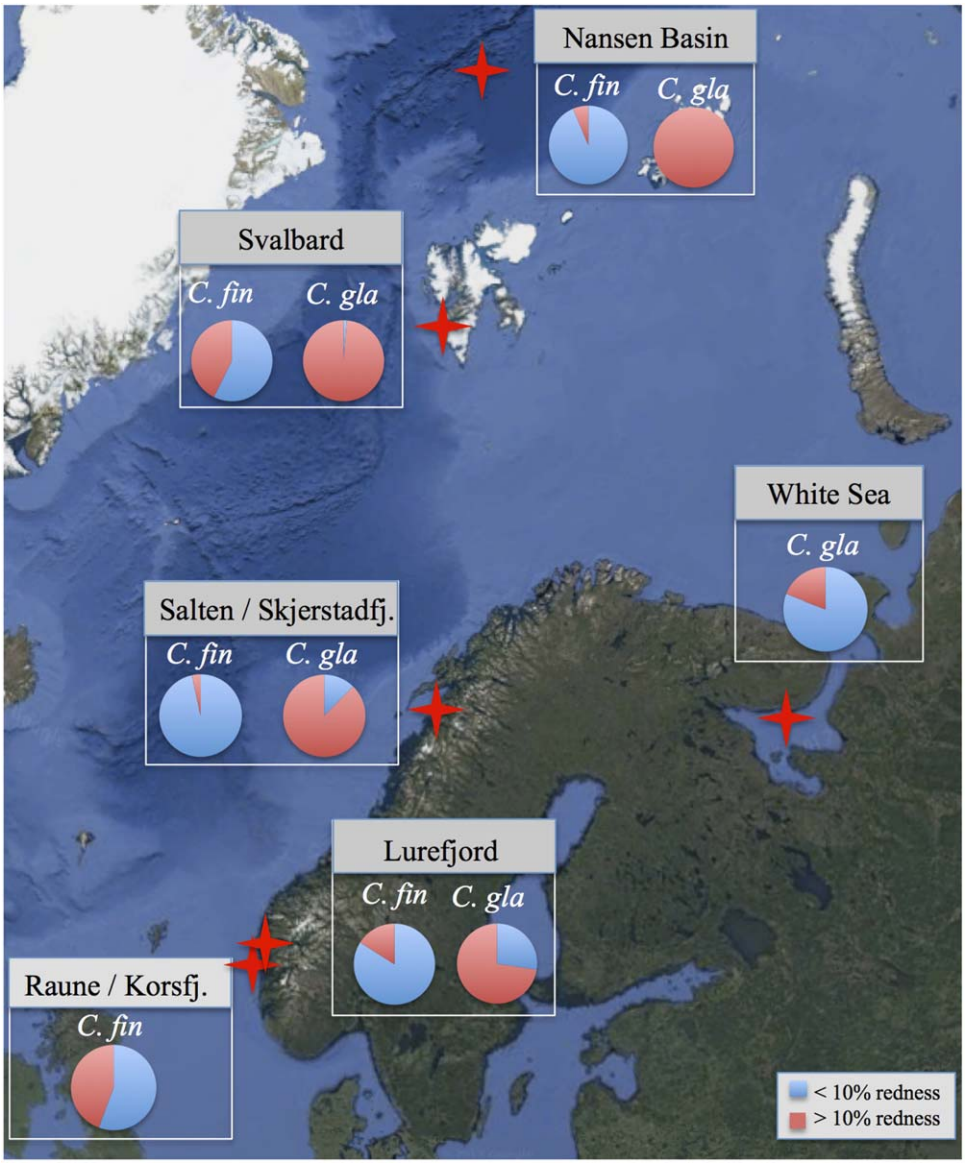

Fig. 7. Red pigmentation on *Calanus finmarchicus* (*C. fin*) and *Calanus glacialis* (*C. gla*) antennules in different regions. Species-related redness from four regions where both species co-occur: the Nansen Basin, Svalbard, Saltenfjord/Skjerstadfjord, and Lurefjord; and from the White Sea where only *C. glacialis* occurs, and Raunefjord/Korsfjord where only *C. finmarchicus* occurs. Blue color of the pie charts indicates proportion of individuals for which less than 10% of the surface of their antennules was red; red color indicates proportion of individuals for which more than 10% of red pigmentation was noticed.

51% for the individuals at stage CV, while experts' decision and genetics only matched at 32% for the adult female individuals. It has to be kept in mind that the morphological features described in literature to discriminate between *Calanus* species are typically described and can be applied directly for identification of adult females (or males) only, while we tested them on both adult females and CVs. They may not work for distinguishing copepodids at pre-adult CV stage, as some morphological structures are still not fully developed or expressed. However, the misidentification of 68% of adult females and disagreement between two experts is striking. In a few cases, the characteristics observed in genetically identified species had appearance theoretically typical of the opposite species. Part of the problem may result from the fact that the characteristics are at the moment predominantly of a descriptive type and they have been portrayed based on "typical" individuals from only a few sites over the species distribution range.

To conclude, the morphological characters involving the 5th pair of legs and the gnathobase were not consistent enough to be used for species identification. Therefore, we cannot recommend using these characteristics to reliably identify *C. finmarchicus* and *C. glacialis* without additional investigations.

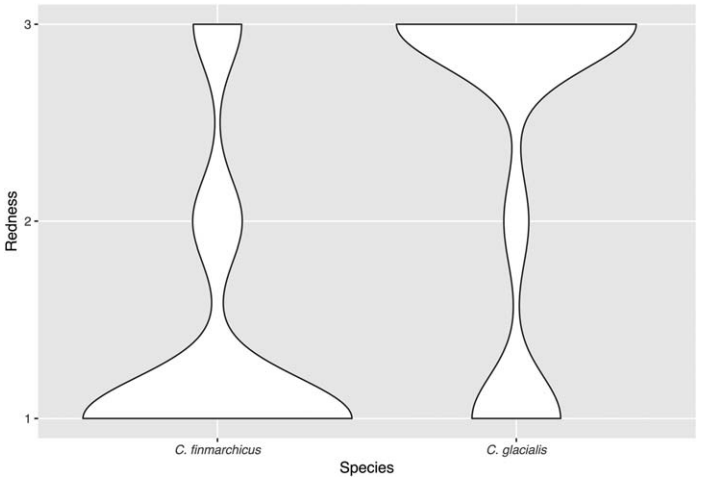

Fig. 8. Antennules redness frequency distribution per *Calanus* species. This violin graph was realized under RStudio v.1.0.143 with the package ggplot2 (Wickham 2009). The graph shows the distribution of each species individuals on the following three ranks scale of redness: 1 = less than 10% of redness; 2 = 10–50% redness; 3 = more than 50% redness.

Discussion

Characters variability

The smaller size of *C. glacialis* in the Norwegian fjord populations, compared to high Arctic populations, largely explains why the species wide boreal occurrence (Choquet et al. 2017) has not been detected before. For instance, occurrence of *C. glacialis* in the southern Lurefjord was not detected before molecular markers were applied (Bucklin et al. 2000). In the context of climate change and ocean warming, it is to be expected that more and more *C. glacialis* individuals will be able to complete their life-cycle within a year, and then have a body size comparable to that of *C. finmarchicus*. The decrease in body size with decreasing latitude is likely a direct effect of temperature (Atkinson and Sibly 1997), but variation in the duration of the productive season and predation pressure by visual predators (Brooks and Dodson 1965) may also play an important role. Copepods are ectothermic, they primarily rely on external sources to regulate their body heat. The temperature-size-rule refers to the widely observed phenomenon that ectotherms reared at lower temperatures usually grow more slowly, but become larger as adults compared to individuals reared at higher temperatures (Atkinson 1994; Atkinson and Sibly 1997). Calanoid copepods appear especially sensitive to temperature by having a fourfold greater reduction in adult body mass per degree Celsius compared to Cyclopoid copepods (Horne et al. 2016). Increasing latitude and mean temperature are strongly correlated (Sunday et al. 2011), and distinguishing separate effects may not be straight forward. However, oxygen demand and supply has been suggested as a driver of both processes (Horne et al. 2015), as the metabolic demand increases with increasing temperature, while the oxygen

Table 3. Antennules red pigmentation in copepodite stages CIV, CV, and adult females (CVI F) and males (CVI M) of *Calanus finmarchicus* (*C. fin*) and *Calanus glacialis* (*C. gla*) from different geographical locations.

		Antennules redness				
Species	**Stage**	**< 10%**	**10–50%**	**50–90%**	**> 90%**	**Total**
a. Nansen Basin						
C. fin	CV	100%	0%	0%	0%	23
	CVI F	88%	12%	0%	0%	24
	Total	94%	6%	0%	0%	47
C. gla	CVI F	0%	4%	85%	11%	47
b. Svalbard						
C. fin	*CIV*	*100%*	*0%*	*0%*	*0%*	*1*
	CV	*40%*	*20%*	*20%*	*20%*	*10*
	CVI F	*59%*	*35%*	*6%*	*0%*	*49*
	CVI M	*100%*	*0%*	*0%*	*0%*	*1*
	Total	58%	31%	8%	3%	61
C. gla	CIV	0%	1%	9%	90%	98
	CV	2%	4%	21%	73%	56
	CVI F	8%	25%	33.5%	33.5%	12
	Total	1%	4%	15%	80%	166
c. White Sea						
C. gla	CIV	93%	7%	0%	0%	100
	CV	0%	57%	36%	7%	14
	CVI F	0%	0%	100%	0%	1
	Total	81%	13%	5%	1%	115
d. Salten/Skjerstadfj.						
C. fin	CIV	67%	0%	0%	33%	3
	CV	100%	0%	0%	0%	33
	CVI F	90%	10%	0%	0%	20
	CVI M	100%	0%	0%	0%	4
	Total	95%	3%	0%	2%	60
C. gla	CIV	0%	0%	0%	1%	1
	CV	20%	20%	38%	22%	40
	CVI F	4%	16%	40%	40%	84
	CVI M	100%	0%	0%	0%	5
	Total	12%	16%	38%	34%	130
e. Lurefjord (in 3 categories)						
C. fin	CIV	0%	0%	100%		3
	CV	95%	0	5%		22
	Total	84%	0	16%		25
C. gla	CIV	0	0	100%		1
	CV	25%	0	75%		158
	CVI F	100%	0	0		5
	Total	27%	0	73%		164
f. Raune/Korsfj.						
C. fin	CIV	14%	8%	64%	14%	14
	CV	63%	18%	18%	1%	74
	Total	56%	16%	25%	3%	88

Table 4. Redness of *C. finmarchicus* and *C. glacialis* female genital somite. Any red pigmentation observed on one or both spermathecae (= genital field) was reported as "Red". No redness at all was reported as "Pale."

Species	*C. finmarchicus*			*C. glacialis*		
Genital somite	Red	Pale	Total	Red	Pale	Total
Svalbard	0	100%	49	73%	27%	11
White Sea	0	0	0	100%	0	1
Salten/Skjerstadfj.	0	100%	20	90%	10%	82
Lurefjord	0	0	0	80%	20%	5
Total	0	100%	69	88%	12%	99

availability in the water decreases (Verberk et al. 2011). In addition, on-going climate change that is impacting the temperature of *Calanus* habitat brings another unpredictable variable affecting body size of *Calanus* species. Predation by visual predators, such as fish, may also induce a change in body-size composition in zooplankton communities. In the classical study by Brooks and Dodson (1965), the zooplankton community shifted from dominance of large- to dominance of small species in a freshwater lake after a fish-predator was introduced. According to optimal foraging theory, predators should target larger sized prey when handling time is a restriction. Both modeling studies and field investigation confirm that lesser sandeel (*Ammodytes marinus*) in the North Sea actively target large copepods, such as *C. finmarchicus*, over smaller copepod taxa when these are available (van Deurs et al. 2014; van Deurs et al. 2015). On a longer timescale, adaptive responses to predation pressure on the larger species may result in a dominance of species with shorter lifespans and smaller body-size (Stearns 1992; Berge et al. 2012). However, to the best of our knowledge, there are no studies showing that predation may cause intraspecific changes in body size within populations of *Calanus* spp.

It has been proposed that the pigment involved in redness of Calanoid copepods is astaxanthin, a form of ketocarotenoid (Mojib et al. 2014). This pigment has a role in the protection against UVR irradiance, and usually appears red in copepods. Copepods can adjust their level of astaxanthin pigment quickly, even within a season, depending on the prevailing threat, UVR, or predators (Hansson 2000). Given such variability, it is thus not surprising that redness cannot be used reliably as a species diagnostic tool. Examination of more samples for each developmental stage, from different depths, and seasonal observations may help to better understand the reasons for variability of red pigmentation in *Calanus* and its relation to environmental parameters.

Biological implications

Copepod species of the genus *Calanus* are the most studied among the zooplankton. They are often used as biological indicators of water masses and to follow the effects of climate change on the marine ecosystems. However, in the majority of past studies, species identification has been based on morphometric and morphological characteristics. We found that none of the morphometric and morphological characteristics used in literature allow for unequivocal identification and separation of species. Therefore, it is likely that our knowledge of *Calanus* geographical distribution is plagued by species misidentification. Predictions on climate change effects and ecological models based on the present view of *Calanus* distribution and stocks dynamics are thus likely to be at least partially erroneous, especially in the areas of sympatry. Furthermore, as *Calanus* species distributions are expected to change and overlap even more in response to global warming (Slagstad et al. 2011), the systematic use of molecular identification is required to document these changes. Considering the importance of *Calanus* range shifts

Table 5. Redness of antennules vs. genital somite for *C. finmarchicus* and *C. glacialis* females in distinct locations. Numbers of individuals with different combinations of pigmentation (antennules vs. genital somite) are reported. For the antennules, every individual with > 10% redness was considered "Red". For the genital somite, every individual with any distinguishable red pigmentation was considered "Red."

Redness		**Svalbard**	
Antennules	Genital somite	*C. finmarchicus*	*C. glacialis*
Pale	Pale	19	1
Red	Red	0	8
Pale	Red	0	0
Red	Pale	20	2
Redness		**White Sea**	
Antennules	Genital somite	*C. finmarchicus*	*C. glacialis*
Pale	Pale	0	0
Red	Red	0	1
Pale	Red	0	0
Red	Pale	0	0
Redness		**Salten/Skjerstadfj.**	
Antennules	Genital somite	*C. finmarchicus*	*C. glacialis*
Pale	Pale	18	0
Red	Red	0	71
Pale	Red	0	3
Red	Pale	2	8
Redness		**Lurefjord**	
Antennules	Genital somite	*C. finmarchicus*	*C. glacialis*
Pale	Pale	0	1
Red	Red	0	0
Pale	Red	0	4
Red	Pale	0	0

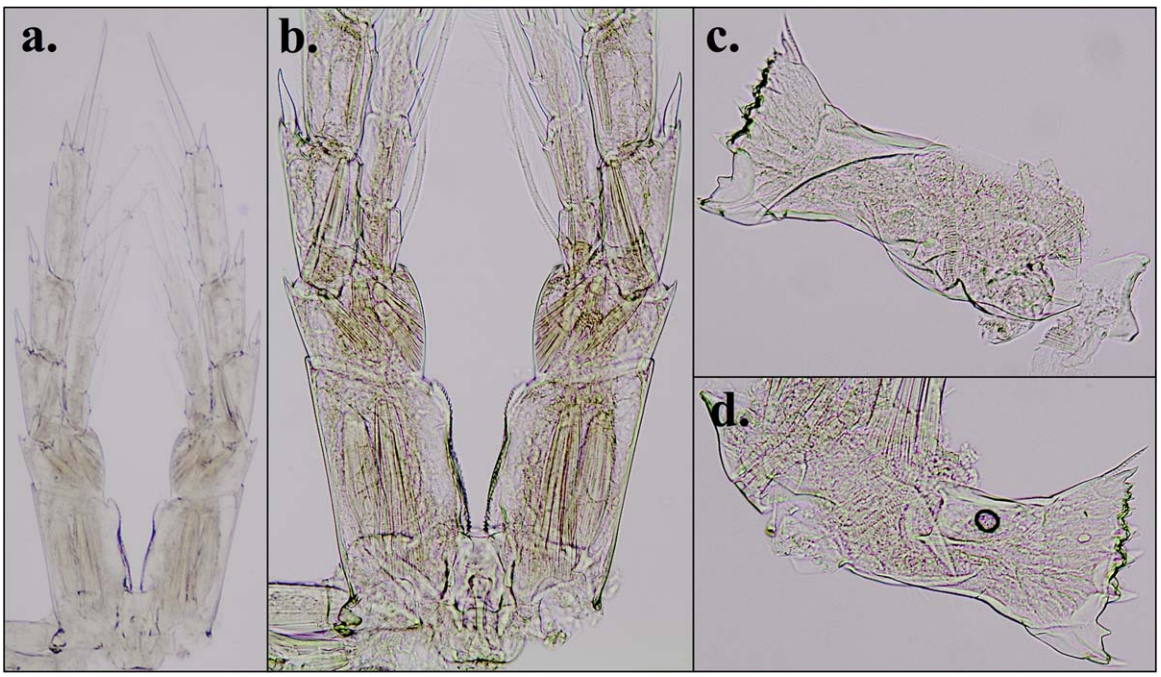

Fig. 9. Morphology of the fifth thoracic pair of legs and the gnathobase of an adult female *C. finmarchicus* exhibiting traits theoretically assigned to *C. glacialis*. The specimen from Saltenfjord (ID: SALT27) exhibits concave denticulated lamellae with a well-expressed curvature, and a wide basis of the second ventral tooth, typical of *C. glacialis* according to the literature.

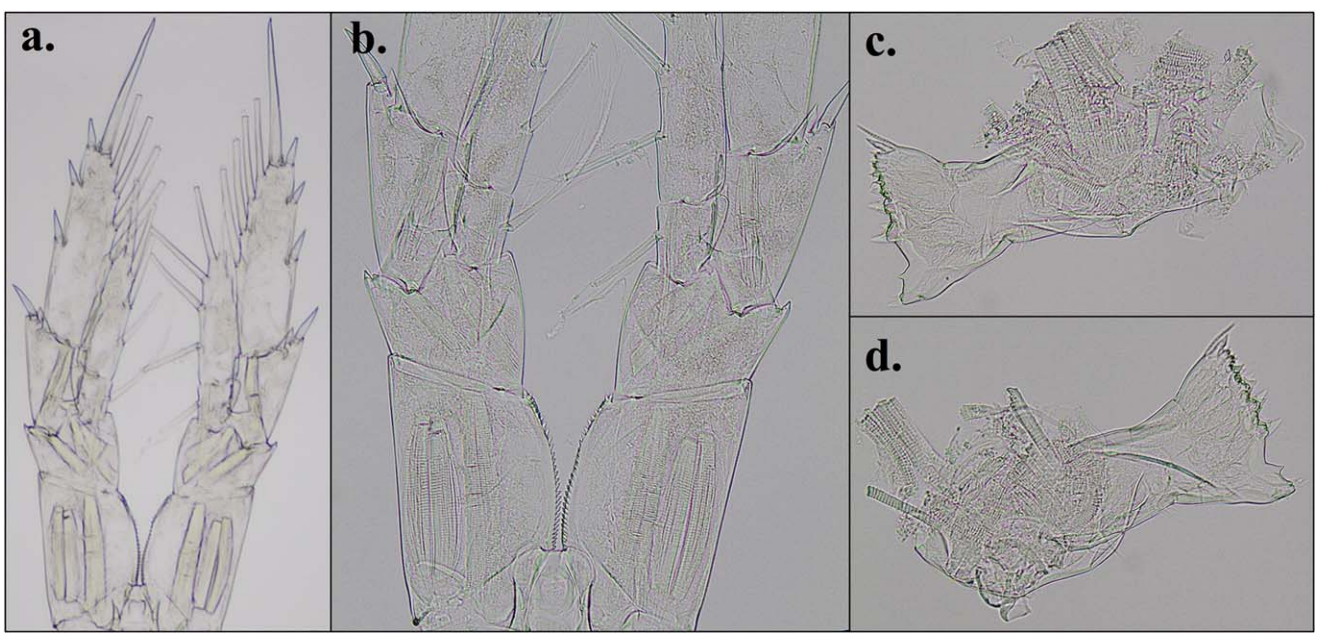

Fig. 10. Morphology of the fifth thoracic pair of legs and the gnathobase of an adult female *C. glacialis* exhibiting traits theoretically assigned to *C. finmarchicus*. The specimen from Saltenfjord (ID: SALT14) exhibits straight shaped denticulated lamellae, and a small second ventral tooth on the cutting edge of the coxa, typical of *C. finmarchicus* according to the literature.

for our understanding of climate change impact on pelagic ecosystems (Beaugrand and Kirby 2010; Søreide et al. 2010), it is critical to tease apart the respective effects of morphological misidentification from on-going range shifts and this will require a thorough reassessment of historical distribution using molecular tools.

Misidentification of *Calanus* species is also detrimental for our understanding of marine ecosystems. For example, a large part of the distribution range of *C. glacialis* has only been recently identified along the Norwegian coast (Choquet et al. 2017) questioning our understanding of fjords dynamics. In other words, life cycles, phenology and the exact role of each species within fjord ecosystems, potential for adaptability/resilience to climate variability, as well as response to environmental variations and population dynamics are not fully understood.

Comments and recommendations

None of the morphological characters described in the literature and re-assessed in the present study can reliably identify *C. finmarchicus* and *C. glacialis* with 100% confidence. There are some global trends that can bring information about the species composition though, but certainly not equally everywhere. Prosome length may be useful to approximate the species composition in the Nansen Basin and in Svalbard waters, and likely in the Arctic Ocean. However, it is critical to keep in mind the underestimation of *C. glacialis*. In fjords along the Norwegian coast, prosome length is clearly not usable, as the size range of both species overlaps completely. Regarding the redness of antennules/genital somite of *Calanus*, it seems to be a useful indicator of species in the Arctic and in relatively closed fjords (with a sill—e.g., Saltenfjord, Skjerstadfjord, Lurefjord), but not in open fjords (without sill—e.g., Raunefjord, Korsfjord). Again, by using this character, it is critical to keep in mind the variable error rates associated (Fig. 7). However, our data suggest that individuals with both red antennules and red genital somite can be identified as *C. glacialis* (but the opposite is not true, leading to an underestimation of *C. glacialis)*. We recommend not using the curvature of the inner denticulated margin of the P5 swimming legs and the shape of the mandibular cutting blade to discriminate between species, until the variability of these characters in all parts of the species distribution range is thoroughly investigated simultaneously with molecular identification.

The use of molecular tools is thus the only reliable method for discriminating between the two species. It is likely that the problems of identification encountered with *Calanus* also exist in other taxa in pelagic zooplankton (e.g., Aarbakke et al. 2011). Therefore, it is critical to start using molecular tools routinely for reliable species identification, especially for ecologically important organisms such as *Calanus*. Equipment, time, competences needed, and cost related to molecular identification of *Calanus* are today a much lesser issue than it used to be. Indeed, as described in Smolina et al. (2014), the set of InDels markers that we used in the present study can be run on agarose gels and therefore used in a low-cost setting on board a research vessel. We also simplified the method of DNA extraction, which now consists of only removing the antennules of each individuals and incubating them 30 min in a buffer at no cost. With these simplifications, genotyping 96 individuals of *Calanus* can be done in 5 h for less than 2 USD per individual.

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Conflict of Interest

None declared.

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