

Master Thesis in Biology (60 ECTS)
Field of study Marine Ecology

***Pseudocalanus* in Svalbard waters: identification and distribution patterns of two sibling copepod species**

Ragnhildur Guðmundsdóttir

Department of Aquatic Bio Sciences
Norwegian College of Fishery Science
University of Tromsø
University Centre in Svalbard

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Abstract

Marine copepods of the genus *Pseudocalanus* (Calanoida) are common in Svalbard waters as well as throughout the northern hemisphere. They contribute considerably to plankton biomass in addition to *Calanus* spp. and play an important role in the marine food web as a link between planktivores, such as fish larvae, and microalgae. *Pseudocalanus* consists of several sibling species, and two of them, *Pseudocalanus minutus* and *P. acuspes*, are known to inhabit the Svalbard area. A third species, *P. major*, is listed as potentially present in this area, but has rarely been reported. As for the other members of the genus, discrimination between these species is time consuming and requires detailed microscopy. Most studies thus lump them into *Pseudocalanus* spp., ignoring differences in life history strategies and behaviour. In the current master's project, a molecular species-specific polymerase chain reaction (PCR) was developed, using the mitochondrial gene cytochrome oxidase subunit I (mtCOI) to aid morphological and morphometrical discrimination. The DNA sequence variation confirmed the presence of both species in Svalbard waters. The statistical comparison of morphological identification with the PCR results gave ambiguous results: a Chi-square test yielded a significant difference between the groups, but with low power due to a low sample size. However, this indicates that microscopic species determination may be wrong. Moreover, morphometrical analysis revealed deviations from expected length ratios in both species, and the PCR sometimes failed to produce results. This may suggest the presence of a third species, presumably *P. major*, for which no molecular information is available.

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Introduction

Marine copepods of the genus *Pseudocalanus* Boeck 1872 are widespread throughout the Northern Hemisphere and they are among the most abundant copepods in these waters (Corkett and McLaren 1978). Copepods of this genus play a significant role as a secondary producers, feeding mainly on microalgae at the base of the marine food web (Corkett and McLaren 1978). Under post-bloom conditions they are also capable of participating in the trophic web in close association with the microbial loop, where they may feed on heterotrophic protozoans (Wassmann et al. 2006). *Pseudocalanus* spp. therefore provide an important food source for larval stages of many fish species (Corkett and McLaren 1978; Hinrichsen et al. 2002) and also for adult pelagic planktivorous fish like sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) (Mollmann and Koster 2002).

The Arctic Ocean is probably the least studied of the world oceans. It is inaccessible due to ice coverage and therefore the marine ecology of the Arctic Ocean is poorly known (Blix 2005). The key factors affecting Arctic Ocean ecosystems are low temperatures and seasonal variability of ice cover, light regime and primary production. For primarily herbivorous zooplankton like *Pseudocalanus* this means life cycle adaptations such as energy storing in the form of lipids for overwintering and makes reproduction possible before the major phytoplankton bloom outburst in the spring (Hagen 1999).

The genus *Pseudocalanus* consists of seven sibling species, some of which co-occur in certain habitats, for example *P. newmani* and *P. moultoni* in Georges Bank (Bucklin et al. 2001) and *P. minutus* and *P. acuspes* in the Barents Sea (Falk-Petersen et al. 1999) and in an Arctic fjord of Svalbard (Lischka and Hagen 2005). Some attempt has been made to describe the morphology of the different species and the most comprehensive one is written by Frost (1989) where he distinguishes and describes seven species through out the Northern hemisphere. Earlier six species had been described, those are reviewed in Corkett and McLaren (1978) where they noted various proposed synonymies, inconsistencies and uncertainties concerning the six species. To distinguish between these

sibling species morphologically is time consuming and the procedure requires detailed microscopy. In many ecological studies on species distribution patterns they are referred to as *Pseudocalanus* spp. (Walkusz 2003; Gislason and Astthorsson 2004) or even grouped together with the genus *Paracalanus* (Heath and Lough 2007). By merging different species and even genera, the biodiversity of the zooplankton community is underestimated and furthermore the ecology of co-existing species combined ignoring the different preferences for habitat, food and time of reproduction. In order to understand the function of the ecosystem, both species richness and functional diversity need to be mapped in details. Therefore methods need to be developed to correctly identify species that formerly have been lumped together.

According to Frost (1989) two *Pseudocalanus* species, *P. acuspes* (Giesbrecht, 1881) and *P. minutus* (Krøyer, 1845) are found in Svalbard waters. Frost (1989) also suggested that a third species, *P. major*, might be found in the fjords of Svalbard. *P. major* has occasionally been found in Arctic locations together with *P. minutus* and *P. acuspes* in samples from near shore waters and where the water is strongly influenced by freshwater runoff (Frost 1989). The species might thus be expected in Svalbard fjords especially during the melting season, when a lot of freshwater is mixed into the fjords.

Size alone is not a good identification factor for the genus *Pseudocalanus* as the species differ only in minor details of morphology (Frost 1989). Size of individual copepods has been related to temperature, where as they tend to grow larger at lower temperatures but be smaller at higher temperatures (Saiz and Calbet 2007). Another factor of size is a variance in DNA content of cells, as more DNA means larger cells and therefore larger individuals. Studies on *P. major* showed that they had greater DNA content per cells than any other species of *Pseudocalanus* (Frost 1989; McLaren et al. 1989).

P. acuspes is distributed through out the Arctic region with southern limits in the Baltic Sea and the Bedford Basin (Nova Scotia, Canada) in the North Atlantic Ocean. In the North Pacific Ocean, the distribution ranges south into the eastern Bering Sea (Frost 1989). The life cycle of *P. acuspes* varies depending on location. In the southern parts of

the Barents Sea two-three generations per year are produced, while there are likely to be fewer generations further north (Norrbin 1991). The overwintering stages are CIII-CV (Norrbin 1991). *P. minutus* is distributed throughout the Arctic Ocean with a southward extension into the western North Atlantic Ocean and western North Pacific Ocean (Frost 1989). A life history study from a high Arctic fjord (Kongsfjorden, Svalbard) revealed a one year life cycle of *P. minutus* also with the overwintering stages CIII-CV (Lischka and Hagen 2005).

The genus *Pseudocalanus* is generally considered to comprise neritic species (Corkett and McLaren 1978), but *P. minutus* is regarded as more oceanic (Norrbin 1991). Therefore it would be expected that *P. acuspes* could be prevalent in more isolated fjords, while *P. minutus* should be more common in fjords that have regular exchange of water masses with the open ocean.

The seas around Svalbard are influenced by different water masses and are therefore divided into biogeographically different zones. The West Spitsbergen Current, with water of Atlantic origin, flows along the west and north coasts, while along the east coast there is water of Arctic origin (Loeng 1991). These environmental settings provide the opportunity to investigate possible differences in the presence, abundance and distribution of *Pseudocalanus minutus* and *P. acuspes* at locations influenced by different water masses.

One way to achieve accurate identification of sibling species is to apply a molecular method, such as species-specific polymerase chain reaction (PCR) (Bucklin et al. 1999). The PCR technique involves three main steps (Figure 1). The first step is a denaturisation of the double-stranded DNA by heating. The strands then separate and the second step takes place as the annealing of the primers to the edges of the amplification sites takes place. This occurs at slightly lower temperature. For the final step the temperature is raised somewhat again and primer elongation takes place. That is where the strands between the bordering primers are synthesized with a thermostable DNA polymerase. These steps are repeated 20 times or more, depending on optimization (Avisé 2004). A

primer is a short sequence of DNA, usually 20 to 30 base pairs (bp). It has high sequence similarity to regions bordering the target sequence (Avice 2004). Primers are used in pairs. One is a common primer that recognises sequence sites for both species, while the other primer recognises only a species-specific site. The length of the sequence between the common primer and the species-specific primer has to be different to make it possible to identify the differently sized bands (also termed amplification products) that are produced (Bucklin 2000). The primers usually are named after the base pair number site on the sequence they attach to and those names can be used to find the size of the amplification product by subtracting the higher number from the lower one and then add one (e.g. the primer pair HCO-2198 and LCO-1490 would yield a amplification product of the size 709 bp). The bands are usually visualised on agarose gel electrophoresis. Species-specific PCR can be carried out simultaneously and competitively for both species at once in the same tube, as the species-specific primers recognise only their specific sequence site (Bucklin 2000).

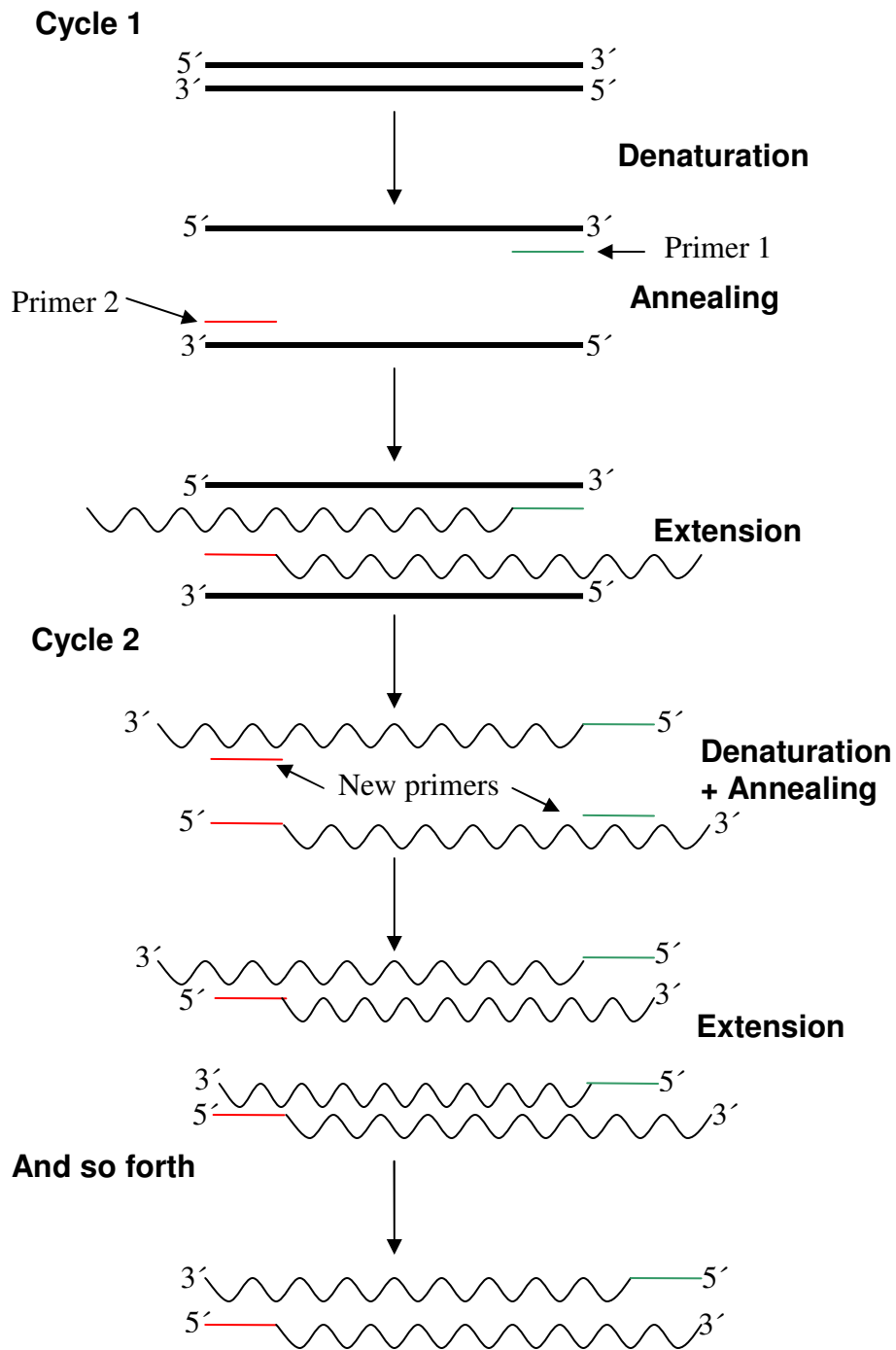


Figure 1: Polymerase chain reaction, PCR. The three steps of DNA amplification.

To establish a species-specific PCR the first step is to obtain the DNA sequence of a selected region of interest (Bucklin 2000). Universal primers have been described to amplify a 709 bp fragment of the mitochondrial gene cytochrome c oxidase subunit I

(mtCOI gene) (Folmer et al. 1994). Based on the sequence of this fragment, primers can be designed that are specific to a given species (Bucklin 2000). Historically, the mtCOI gene has been chosen to distinguish species with PCR methods, because it has proved to be diagnostic at species level (Bucklin et al. 2003). Amplification sites within the mitochondrial COI gene have been used with good results for the identification of other *Pseudocalanus* species, such as *P. moultoni* and *P. newmani* (Bucklin et al. 2001).

The objectives of this study are fourfold: 1) to determine the distribution of the two *Pseudocalanus* species, *P. minutus* and *P. acuspes*, in the Svalbard area in order to find out if they co-occur and where; 2) to determine if differential distribution patterns of both species relate to Atlantic/Arctic water influences; 3) to determine how useful morphometrics could be for identification; and 4) to develop a molecular method to discriminate the species using their DNA. The methodological comparison of microscopic and molecular identification will reveal the accuracy and suitability for future ecological studies on the species involved.

In order to achieve the objectives of the study, we collected samples of *Pseudocalanus* females from several fjords around Svalbard. They were then identified morphologically and a number of morphometrical features were measured. Furthermore a species-specific PCR protocol was developed and then applied on the already morphologically identified individuals. According to morphology identification *P. minutus* and *P. acuspes* co-occur in all sampling locations but due to low sample size for the PCR analysis further studies are needed to conclude on the difference between the morphological and molecular identification.

Materials and methods

The study area

Svalbard is an archipelago situated in the north-western Barents Sea. It ranges from 76°-81° N and 10°-35° E. The largest island, Spitsbergen, is the location of all sampling sites except for one located on the north-central shore of Nordaustlandet (Figure 2). The samples were taken in spring and summer 2007 (Table 1).

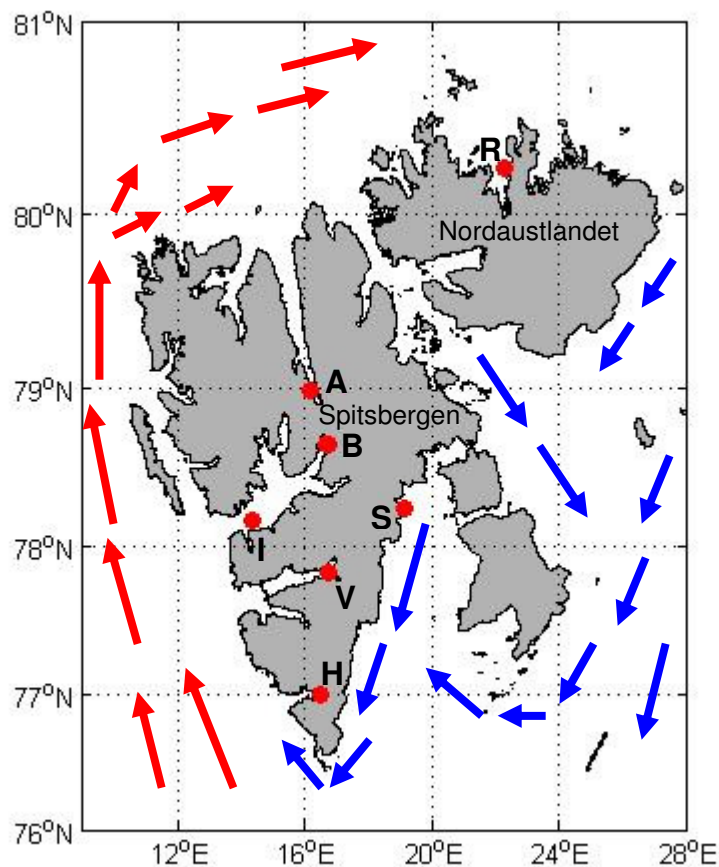


Figure 2: The archipelago of Svalbard. Arrows indicate the main current systems, with red arrows for water of Atlantic origin (The West Spitsbergen Current) and blue arrows for water of Arctic origin (The East Spitsbergen Current). Sampling locations are A = Austfjorden, B = Billefjorden, I = Isfjorden, H = Hornsund, R = Rijpfjorden, S = Storfjorden and V = Van Mijenfjorden.

The west coast of Spitsbergen is exposed to water transported with the West Spitsbergen Current (Figure 2), which is a continuation of the Norwegian Atlantic Current that carries

warm and saline Atlantic water from the Norwegian Sea to the Arctic Ocean (Piechura et al. 2001). The current flows along the continental slope, but onshore of the continental shelf another current carries water of Arctic origin (Saloranta and Svendsen 2001). The latter is originated in the East Spitsbergen Current and is called South Cape Current as it passes the south cape of Spitsbergen. The two water masses are usually separated by a hydrological front outside the west coast of Spitsbergen, but since the current speed and net volume transport are likely much larger in the West Spitsbergen Current than in the shelf current, it would be more likely to find remnants of the slope water on the shelf than the other way around (Saloranta and Svendsen 2001). This hypothesis is supported by the relatively light ice conditions in the open (no sill or a low sill) fjords along the west coast of Spitsbergen (Saloranta and Svendsen 2001).

The fjords chosen for the present study differs in location and degree of Atlantic water mass input, with some fjords strongly influenced by Atlantic inflow, and others only mildly influenced or uninfluenced. Four of the fjords are located on the west coast of Spitsbergen: Isfjorden, Billefjorden, Van Mijenfjorden and Hornsund (Figure 2). Isfjorden is a broad fjord with no sill at its mouth and is therefore exposed to the Atlantic water coming in with the West Spitsbergen Current (Berge et al. 2005). Billefjorden, a branch-fjord of Isfjorden, is a threshold fjord with very little or no Atlantic inflow (Walkusz 2003). Van Mijenfjorden is located on the south west coast of Spitsbergen and its opening is partly closed by the island Akseløya and a sill which restrict, to some extent, the water flow of the coastal water, including the West Spitsbergen Current into the fjord (Renaud et al. 2007). Hornsund is an open fjord without a sill. It is the southernmost fjord on the west coast of Spitsbergen and is under the influence of both the coastal South Cape Current that carries Arctic Water and the West Spitsbergen Current (Weydmann and Kwasniewski 2008).

Additional sampling sites in the north and east of Svalbard are Austfjorden, Rijpfjorden and Storfjorden (Figure 2). Austfjorden is the innermost part of a longer fjord called Wijdefjorden. Wijdefjorden branches in from the north coast of Spitsbergen and some Atlantic inflow is expected as a result of the West Spitsbergen current flow along the

north coast of Spitsbergen. The inner part, Austfjorden, is rather isolated from the rest of Wijdefjorden by a shallow sill (Dale et al. 2006), so Atlantic influences are expected to be limited there, but its oceanography is little studied. Rijpfjorden branches from the north coast of Nordaustlandet, a large island northeast of Spitsbergen. It opens to a broad shallow shelf, resulting in little warm, Atlantic subsurface water entering the fjord from the north (Ambrose et al. 2006). Storfjorden is a large fjord separating Barentsøya and Edgeøya from the east coast of Spitsbergen, where the East Spitsbergen current flows with relatively cold and fresh Arctic water from north to south (Loeng 1991).

Collection and preservation of samples

Zooplankton samples were collected in March and April through the sea ice and in ice free conditions in May, June and July, by vertical tows from the bottom to the surface. In a few cases, samples taken in Austfjorden and Billefjorden, vertically stratified tows were performed with two and three depth intervals, respectively (Table 1). The depth intervals for Billefjorden for the two different months were not the same but were considered to be equivalent. The sampling position in the fjords varied: in Isfjorden and Rijpfjorden sampling was carried out in the fjord mouth, whereas in for the other fjords sampling positions were located in the fjord head. The plankton net used was a standard WP-2 net, mesh size 200 μm , except for Hornsund, where a net of 180 μm mesh size was deployed.

Table 1: Sampling details for zooplankton samples and environmental data. Temperature is given as average temperature in the water column for a given month. Temperature references and corresponding month: 1. Daase, April (unpublished data) (Appendix A, Figure C); 2. Arnkvaern et al. (2005), March and April, 3. Weydmann and Kwasniewski (2008), July; 4. Steen et al. (2007), July; 5. Nygård (unpublished data), April and June, (Appendix A, Figure A and B); 6. Skogseth et al. (2005), April; 7. Fer and Widell (2007), March.

| Location | Latitude (N) | Longitude (E) | Date/Month | Sample depth intervals (m) | Bottom depth (m) | Nr of repl. | Temp ^{ref} °C |
|------------------|--------------|---------------|------------|----------------------------|------------------|-------------|------------------------|
| Austfjorden | 78°59.932' | 16°11.430' | 28/3 | 0-50-150 | 170 | 3 | -1.7 ¹ |
| Billefjorden | 78°39.683' | 16°44.268' | 29/3 | 0-50-100-150 | 170 | 3 | -1.0 ² |
| Billefjorden | 78°39.566' | 16°42.274' | 8/5 | 0-25-75-180 | 196 | 2 | -1.0 ² |
| Hornsund | 77°00.494' | 16°29.218' | 28/7 | 0-180 | 180 | 1 | -0.3 ³ |
| Isfjorden | 78°10.990' | 14°20.451' | 8/5 | 0-100 | 100 | 1 | 1.0 ⁴ |
| Rijpfjorden | 80°15.954' | 22°17.397' | 23/4, 5/6 | 0-145 | 148 | 1, 1 | -1.8 ⁵ |
| Storfjorden | 78°15.703' | 19°07.440' | 17/4 | 0-28 | 30 | 5 | -1.7 ⁶ |
| Van Mijenfjorden | 77°50.942' | 16°43.233' | 12/3, 16/4 | 0-50 | 50 | 3, 3 | -1.8 ⁷ |

All samples were preserved in 96% ethanol, except for samples from Rijpfjorden and Austfjorden which were preserved in 99% ethanol, and then stored until further analysis.

Abundance

The abundance of each species was estimated by counting the females from each sample after microscopic identification. For replicated samples abundance was estimated as the average of abundance in all individual replicates. Usually whole samples were counted but for very dense ones females were counted from subsamples with a split of ½ to ¼ of the total. In those cases the samples were divided with a Motoda splitter (Motoda 1959) and at least 100 individuals total were counted. The abundance as individuals per m³ was calculated using the diameter of the net opening (0.25 m²) and the sampling depth, assuming full filter efficiency (Eq. 1 and 2).

$$\text{Opening of the net (m}^2\text{) x tow depth m = filtered volume (V)} \quad \text{Eq. 1}$$

$$\text{Number of individuals x aliquot / filtered volume (V) = abundance per m}^3 \quad \text{Eq. 2}$$

Morphological identification

Adult *Pseudocalanus* females were sorted from the samples for morphological identification and morphometric measurements. Adult females were targeted in this study because their morphological features are best known and more extensively described than for younger stages. Main body parts are outlined in Figure 3.

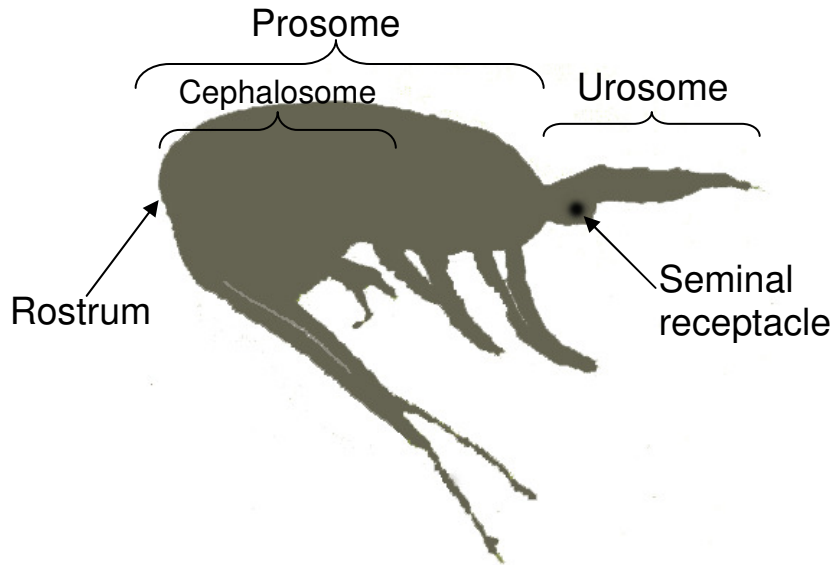


Figure 3: A schematic figure of a copepod labelled with body parts mentioned in the text.

To distinguish between *Pseudocalanus acuspes* and *P. minutus* the difference in the shape of the prosome was used (Frost 1989). The prosome of *P. acuspes* looks stocky in lateral view (Figure 4), although this is somewhat variable, and the cephalosome is usually rounded, barely extending anterior of the rostrum (Frost 1989). The prosome of *P. minutus*, on the other hand, looks slender (Figure 4), and the cephalosome protrudes anterior of the rostrum, often somewhat angularly (Frost 1989).



Figure 4: Photograph from the stereo microscope. *P. acuspes* (the three individuals on the left) and *P. minutus* (the three to the right). Both species have prosome length of about 1000 μm .

Morphometrics

An image analyzer (Motic Images Plus 2.0 ML) was used to measure the length of the prosome, the cephalosome, the urosome and the seminal receptacle (Figure 3) for all samples except from Hornsund where only prosome and urosome was measured (using Leica Application Suite, Version 3.1.0). The measurements were used to estimate body ratios as those have been used as potential identifiers in addition to morphology. Frost (1989) studied ratios of lengths of various body parts, such as prosome length vs. urosome length, and concluded that such ratios, along with body shape, are well suited to identify the *Pseudocalanus* species. According to his results the distinguishing feature between *P. minutus* and *P. acuspes* in Arctic locations is the ratio of prosome to urosome, being larger for *P. minutus*, as it has a shorter urosome, than for *P. acuspes*. The ratio of the length of the seminal receptacle to cephalosome length, separates *P. minutus* and *P. acuspes* from a third species, *P. major* (Frost 1989).

Molecular identification

Development of species-specific PCR protocol

A species-specific PCR protocol was designed in order to identify *P. minutus* and *P. acuspes*. DNA was extracted with QIAGEN DNeasy Blood & Tissue kit from 10 *P. minutus* and 10 *P. acuspes*, from Billefjorden, that had previously been identified by morphological traits as described above. A 709 bp region of the mtCOI gene was amplified for *P. minutus* using general invertebrate primers, LCO-1490 and HCO-2198 (Folmer *et al.* 1994) that have been utilized to amplify DNA from other *Pseudocalanus* species (Bucklin *et al.* 2001). For *P. acuspes* a 1118 bp region of the same gene was amplified using LCO-1490 (Folmer *et al.* 1994) and COI – 2607 (Bucklin, unpublished).

LCO-1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' (forward)

HCO-2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (reverse)

COI - 2607 5'- ACA TAG TGG AAA TGT GCT ACA TA-3' (reverse)

The amplification protocol for *P. minutus* was: 95°C (1 min), 45°C (2 min), 72°C (3 min) carried out for 40 cycles in a Perkin Elmer 480 thermal cycler. The same protocol was used to amplify *P. acuspes* mtCOI, with the exception that the annealing temperature was 37°C instead of 45°C.

PCR products were purified with the Qiagen PCR Purification Kit and processed with the BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on an Applied Biosystems 3130 Genetic Analyzer. The sequencing protocol was 96°C (10 sec), 50°C (5 sec) and 60°C (4 min) with an initial denaturation step of 96°C (1 min) carried out for 35 cycles. Sequences were edited using Sequencer (Gene Codes corp) and entered into BLAST (Altschul et al. 1997) to confirm molecular species identification. The obtained sequences (Appendix B) were used to design species-specific primers.

Species-Specific PCR

179 individuals of each species from 5 locations (Austfjorden, Rjipfjorden, Storfjorden, Billefjorden and Van Mijenfjorden) were processed. They were chosen based on the total number in the samples, samples with numbers lower than 30 of each species were not considered (Bucklin et al. 2001). For the processing of the 358 individual copepods the following protocol was performed: each individual copepod was transferred to a microcentrifuge tube with 32.75 µl sterile distilled water and heated in a microwave oven for one minute to allow the ethanol to evaporate. The copepods were then crushed with a pipette tip against the tube in order to aid the release of DNA. Each 50 µl reaction consisted of 10 µl of 5 x PCR buffer (Promega) and 3 µl of 25 mmol l⁻¹ MgCl₂ solution, 1 µl of a 10 mmol l⁻¹ dNTP solution (equimolar mix dATP, dCTP, dGTP, dCTP), 1 µl of a 10 µmol l⁻¹ solution of Primer A (common Primer), 1 µl of a 10 µmol l⁻¹ solution of Primer B (*P. minutus*-species-specific), 1 µl of a 10 µmol l⁻¹ solution of Primer C (*P. acuspes*-species-specific) and 0.25 µl (1,25 units) of GoTaq Flexi DNA polymerase. The primers used in the protocol were:

Primer A: PsCOI_1561F 5'-GCA GGW ATR ATT GGG ACA GG-3', (forward)

Primer B: COI_1932R 5'-AAC ACC TGC TAA A T GTA AA-3', (reverse)

Primer C: COI_2060R 5'-TGA CAG CAG TAG AAG AAT AG-3', (reverse)

PCR amplification with PsCOI 1561 and *P. minutus* SS-primer COI 1931R produced a product of 372 bp while PsCOI 1561 and *P. acuspes* SS-primer COI 2060R produced a 500 bp product. A negative control, containing only mastermix but no copepod, was run with every procession to control for contamination.

The amplification protocol was 40 cycles of: 45 sec at 94°C for the denaturation step, 1 min at 47°C for the annealing step and 1 min and 30 sec at 72°C for the elongation step and a final elongation step of 72°C for 3 min. This was performed on an Applied Biosystems 2720 thermal cycler.

The buffer used to make the gels was a 1x TBE buffer and the agarose was measured precisely to make the gel concentration 2%. After heating the gel mixture, 5 µl of Ethidium Bromid was added to allow visualisation of the DNA. The gels were left to stiffen for 10 minutes with two rows of gel combs for making the DNA loading wells. The gel system was High Speed/ conventional submarine gel system from Biokey American Instruments Inc. Before loading the PCR amplification products into the gel, 1x TBE buffer solution (~450 ml) was poured into the chamber surrounding the gel, distilled water (~50 ml) was loaded on top of the gel and the gel combs were removed. Then 10 µl of PCR amplification products were loaded into the wells of the gel. This was electrophorized with a programmed power system (Bio Rad, Power Pac 300) for exactly 10 minutes at 220 volts. After the electrophoresis the gels were captured on photos using the program GeneSnap from SynGene. The camera system was Gene Genius, Bio Imaging System from SynGene.

The number of individuals of each species was then determined by migration distance of the DNA fragments through the gel from the loading well. Larger products (*P. acuspes*, 500 bp) migrate for shorter distance than do smaller ones (*P. minutus*, 372 bp) and therefore produce identifiable bands on the gel.

Some problems occurred throughout the processing of the PCR samples due to various technical problems. For example frequent contamination problem which made some of the gel results unusable. Contamination is displayed as a positive result in the negative control. The buffer that was used to make the gels might also have been getting to old by the time when the last gels were processed, so the different sized bands were not separating well enough to make a clear distinction between the two species. Furthermore in some cases there was no amplification produced from individual copepods.

Data analysis

Morphometrics

The difference of prosome length for each species was tested between sampling locations. First the data was tested for normality by calculating the skewness and kurtosis of the distribution and plotting a qq plot. As the prosome length data were not normally distributed, a nonparametric test for difference between in ranked data, Kruskal – Wallis test, was performed. This was carried out in S-PLUS 8.0 for Windows. The difference was then analysed with a graph of mean prosome length \pm 95% confidence limits.

Molecular data

The number of morphological identified individuals (the expected numbers) were compared with the number of molecularly identified individuals (the observed numbers) by using the frequency chi square test for goodness of fit. This was performed in Microsoft Office Excel 2003. The results of the chi square were then tested with a Binomial test of Power and Sample Size. This was performed in S-PLUS 8.0 for Windows.

Graphs

The graphs were drawn using S-PLUS 8.0 and Microsoft Office Excel 2003. The map was made with Matlab 7.0.4.

Results

Abundance

Females of both species were found at all sampling locations except in the sample from Isfjorden in May, where only *P. acuspes* was found. The abundance of *P. minutus* ranged from 0.2 females m⁻³ in Hornsund in July, not replicated sample, to 14 females m⁻³ in Austfjorden in March, three replicates of all depth intervals. The abundance of *P. acuspes* ranged from 0.5 females m⁻³ in Van Mijenfjorden in April to 15 females m⁻³ in Billefjorden in May. *P. minutus* dominated in samples from March and April, while *P. acuspes* was more abundant in the samples from May, June and July (Figure 5).

The west coast samples were from Van Mijenfjorden, Billefjorden, Isfjorden and Hornsund. In Van Mijenfjorden, *P. minutus* was dominating in both the sample from March and April with 3 females m⁻³ and 1 female m⁻³ respectively. For Billefjorden *P. minutus* was dominating in the March sample with 4 females m⁻³ while *P. acuspes* was far more abundant in the May sample with 15 females m⁻³. The Isfjorden sample, from May, like already stated, contained only *P. acuspes* with 1.5 females m⁻³. In the Hornsund sample, from July, *P. acuspes* was dominating with 3 females m⁻³.

The north coast samples were from Austfjorden and Rjipfjorden. The Austfjorden sample, from March, had *P. minutus* dominating with 14 females m⁻³. In the Rjipfjorden sample from April, *P. minutus* was more abundant (3.5 females m⁻³) but in the June sample it had switched to *P. acuspes* being more numerous with 2 females m⁻³. The only east coast sample was from Storfjorden in April. There *P. minutus* had 5 females m⁻³ while *P. acuspes* had 2.5 females m⁻³.

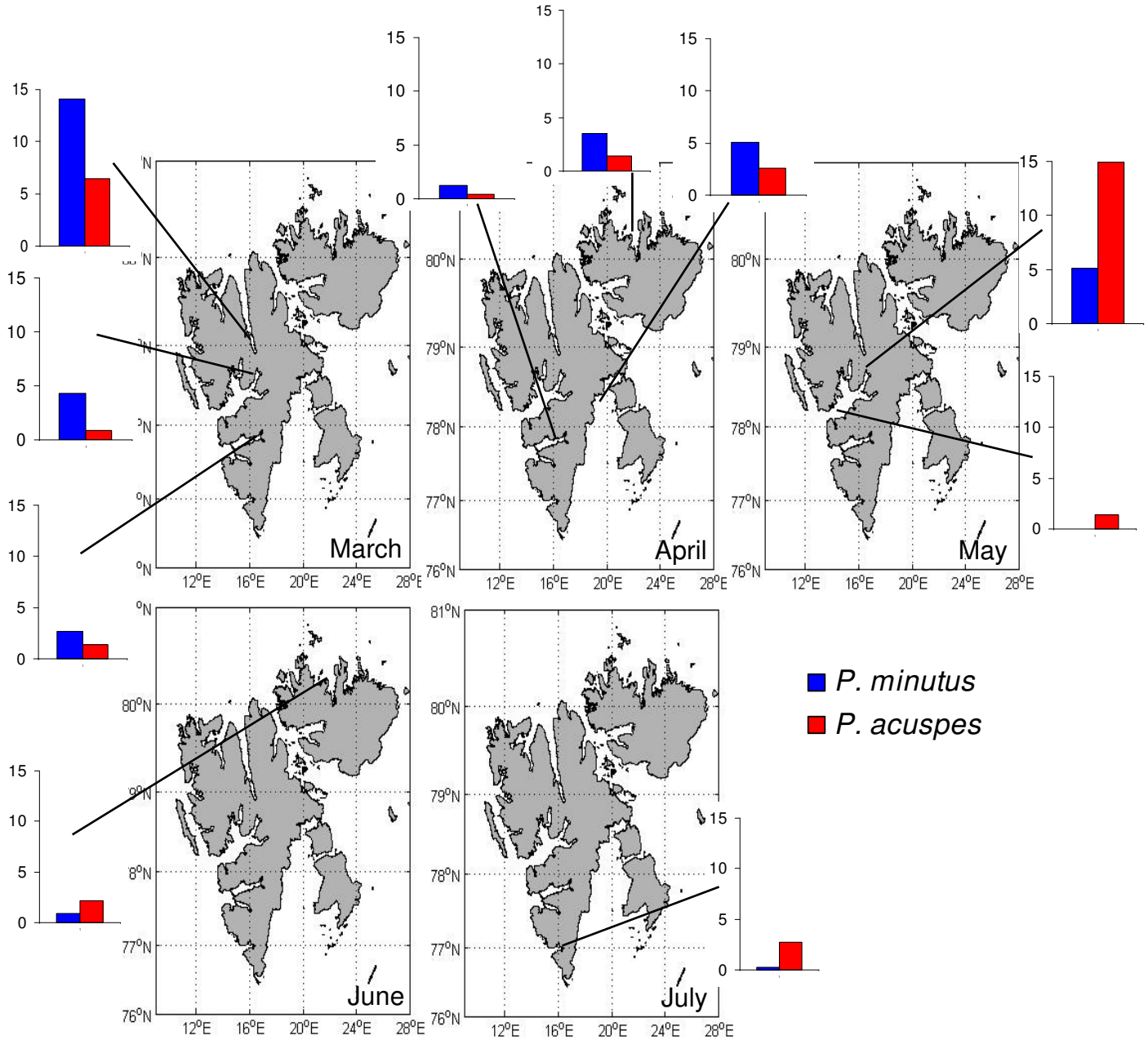


Figure 5: *P. minutus* and *P. acuspes* females. Abundance per m^3 for sampling locations around Svalbard from March to July 2007.

Vertical distribution

In Billefjorden *P. minutus* dominated in all sampling depths in March. Its abundance was 3 females m^{-3} in the deepest layer (100 - 150 m), increased to 6 females m^{-3} in the middle layer (50 – 100 m) and decreased again to 4 females m^{-3} in the surface layer (0 – 50 m). The maxima was thus in the middle layer. In the May sample *P. minutus* had the

abundance of 1 female m^{-3} in the deepest layer (75 – 180 m). In the middle layer (25 – 75 m) it was 6 females m^{-3} and in the surface layer (0 – 25 m) 8 females m^{-3} (Figure 4). The maximum of *P. minutus* had thus shifted to the surface layer in May.

The abundance of *P. acuspes* in Billefjorden, March, was 0.5 females m^{-3} in the deepest layer (100 – 150 m). In the middle layer (50 – 100 m) the abundance was 0.7 females m^{-3} and in the surface layer it was 2 females m^{-3} . In the May sample the abundance was 1.5 females m^{-3} in the deepest layer (75 – 180 m), in the middle layer it was 5 females m^{-3} and in the surface layer it was 38 females m^{-3} (Figure 6). The maximum number of *P. acuspes* was thus in the surface layer for both months.

The abundance of *P. acuspes* in Billefjorden increased extensively between March and May in all three depth intervals (Figure 6). However the depth intervals are not the same but are considered to be more or less equivalent in terms of numbers of copepods. The highest increase was in the surface layer where in March it was 1.5 females m^{-3} but in May it had raised to 38 females m^{-3} . The increase was less pronounced in the two deeper layers; with 0.7 females m^{-3} in the 50 - 100 m layer and 0.5 females m^{-3} in the layer from 100 – 150 m in March rising to 5 females m^{-3} at intermediate depth (25 – 75 m) and 1.5 females m^{-3} close to the bottom (75 – 180 m).

The changes were not as distinct in *P. minutus*. In the top layer the abundance was 4 females m^{-3} in March but 8 females m^{-3} in May. For the two deeper intervals the number of females was 6 m^{-3} from 50 – 100 m and 3 females m^{-3} at depths of 100 – 150 m in March and increased to 6 females m^{-3} (25 – 75 m) and 1 female m^{-3} (75 – 180 m).

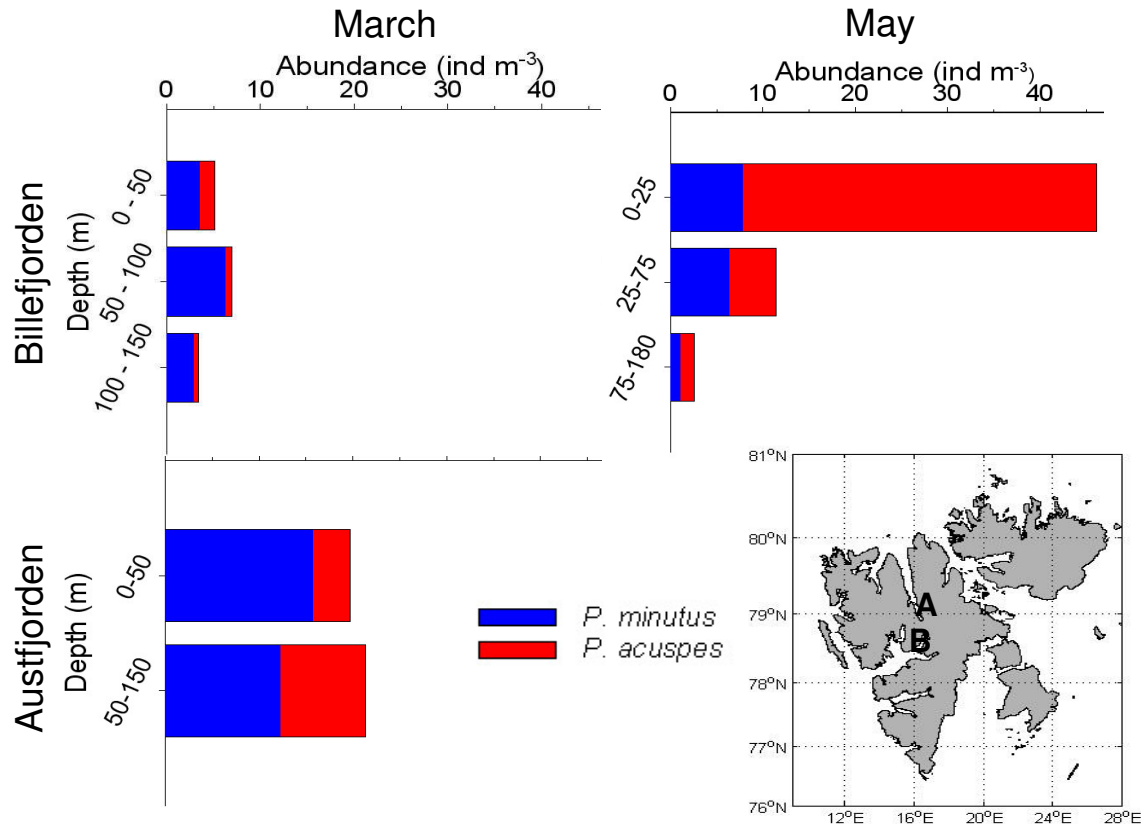


Figure 6: *P. minutus* and *P. acuspes* females. Abundance per m³ in different depth layers for Billefjorden (B) in March and May, and Austfjorden (A) in March, 2007. Note the different depth intervals for Billefjorden between the two months.

In Austfjorden, *P. minutus* dominated in both sampling intervals in March (Figure 6). In the surface layer (0 – 50 m) the abundance of *P. minutus* was 16 females m⁻³ while *P. acuspes* was 4 females m⁻³. In the lower layer (50 – 150 m) the abundance of *P. minutus* was 12 females m⁻³ and *P. acuspes* 9 females m⁻³. Within each species *P. minutus* was more numerous in the surface layer while *P. acuspes* had more representatives in the deeper layer (Figure 6).

Morphometrics

The prosome length of *P. minutus* ranged from 742 µm to 1296 µm with an average of 1073 ± 76 µm. The prosome length of *P. acuspes* ranged from 786 µm to 1315 µm with an average of 1044 ± 107 µm. There was almost a complete overlap in the prosome length of the two species (Figure 7).

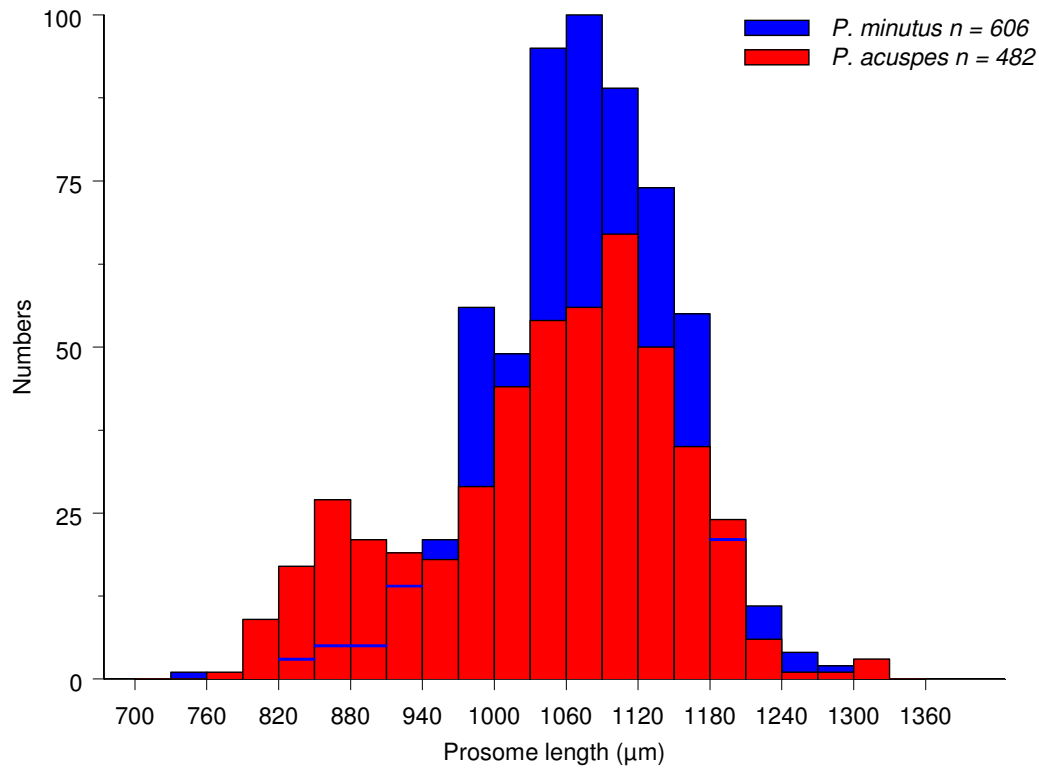


Figure 7: The prosome length (μm) of the two species. All sampling locations included.

However the length frequency distribution for *P. acuspes* was bi-modal with modes at $\sim 860 \mu\text{m}$ and $\sim 1100 \mu\text{m}$ (Figure 7). Individuals of the smaller group were found at all sampling locations (Figure 8).

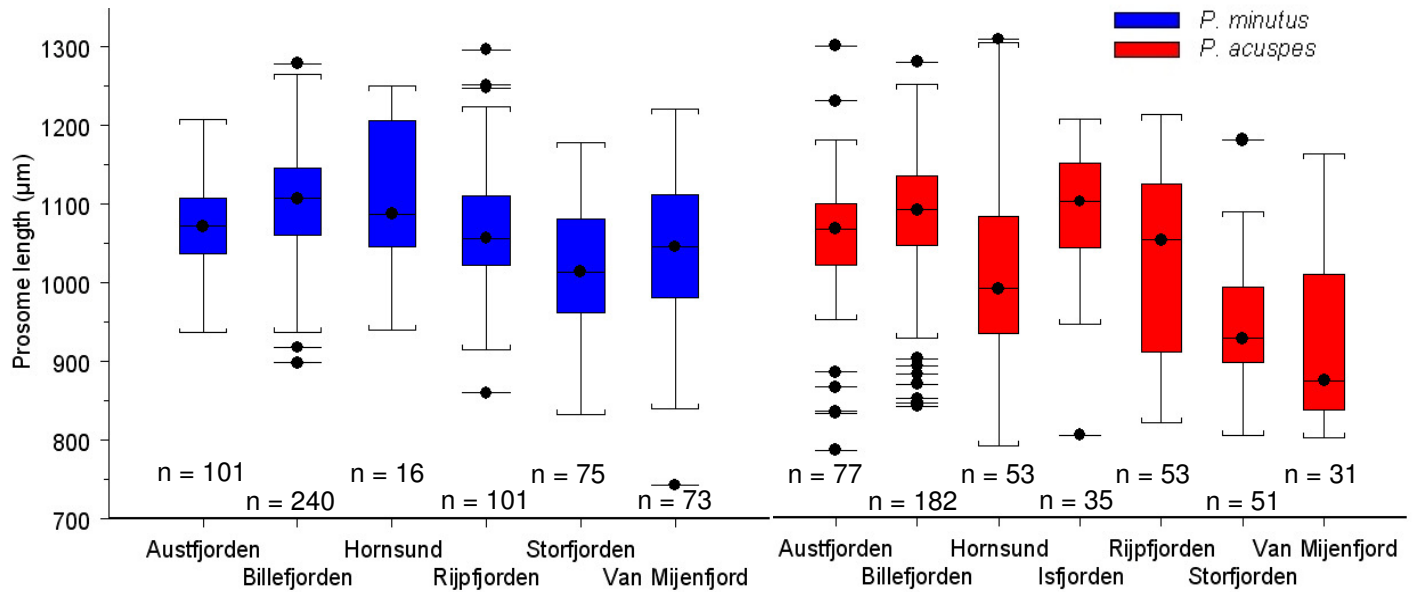


Figure 8: Prosome length (μm) of *P. acuspes* and *P. minutus* for each sampling location. For each box the line in the middle represent the median of the data set. The lower limit of the box is equal to the first quartile and the upper limit is equal to third quartile. The whiskers are equal to 1.5 x interquartile distance (the difference between the third and the first quartiles). Data points plotted outside the whiskers are outliers.

Prosome length differed significantly between locations for both *P. minutus* and *P. acuspes*. For both species the significance value p was much lower than 0.01 (*P. minutus*: Kruskal-Wallis $\chi_{[5]}^2 = 83.64$ and *P. acuspes* Kruskal Wallis $\chi_{[6]}^2 = 121.26$). The locations that differed in prosome length for *P. acuspes* are Storfjorden and Van Mijenfjorden where they were smaller (Figure 9). At the other locations there is some degree of overlap in 95% confidence limits of the mean.

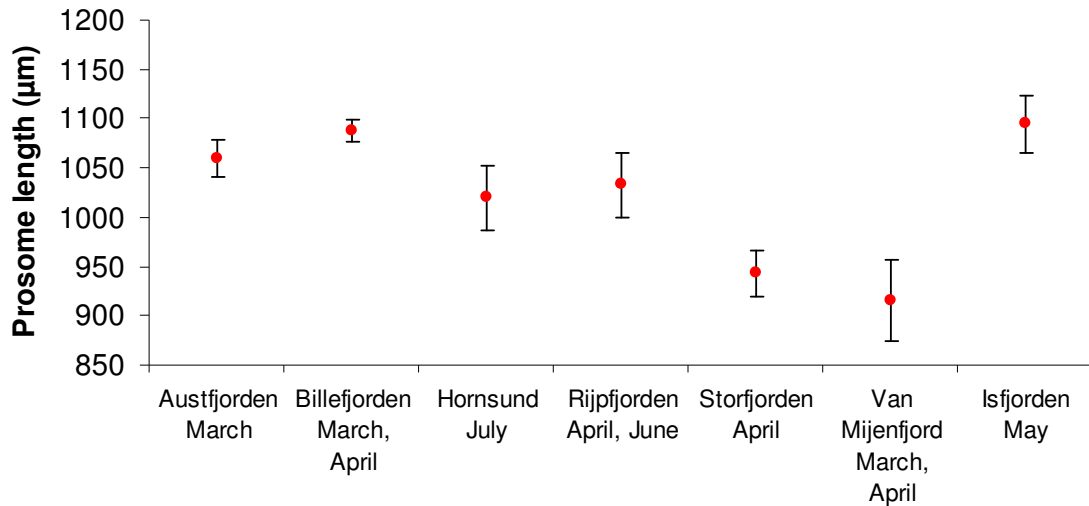


Figure 9: *P. acuspes*. Mean prosome length \pm 95% confidence limits for different locations.

For *P. minutus* there are no locations that differ drastically from the others but like for *P. acuspes* there is a similarity between Storfjorden and Van Mijenfjorden, and those two fjords differ somewhat from the rest of the locations whereas *P. minutus* also is a bit smaller than for the other locations (Figure 10).

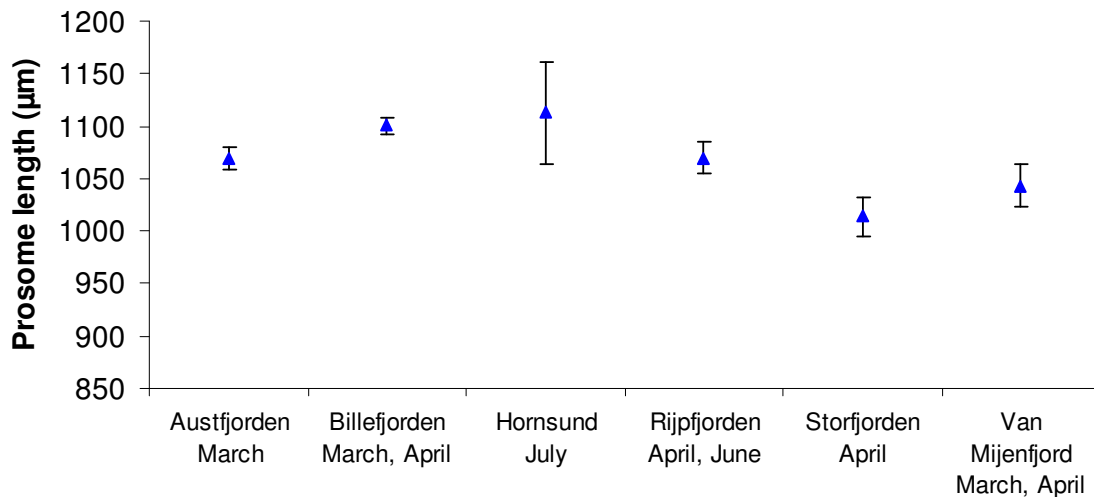


Figure 10: *P. minutus*. Mean prosome length \pm 95% confidence limits for different locations.

The mean prosome length \pm 95% confidence limits for the two species in different locations (Figure 9 and 10) indicate that in Storfjorden and Van Mijenfjorden where *P. acuspes* is smaller the *P. minutus* also is smaller, while in Austfjorden, Billefjorden, Hornsund and Rijpfjorden both species are of the larger form.

The mean prosome:urosoma length ratio ($\pm 95\%$ confidence limits of mean) for the two species was 2.75 (± 0.02) for *P. minutus* and 2.54 (± 0.03) for *P. acuspes*. Although the ratio for *P. minutus* is slightly higher, its cluster (Figure 11) falls within the cluster formed by the data for *P. acuspes*. The regression equation for *P. minutus* is $77.12 + 0.29PL$ and for *P. acuspes* $57.89 + 0.34PL$ (Figure 12). The slope values are 0.33 R^2 for both species.

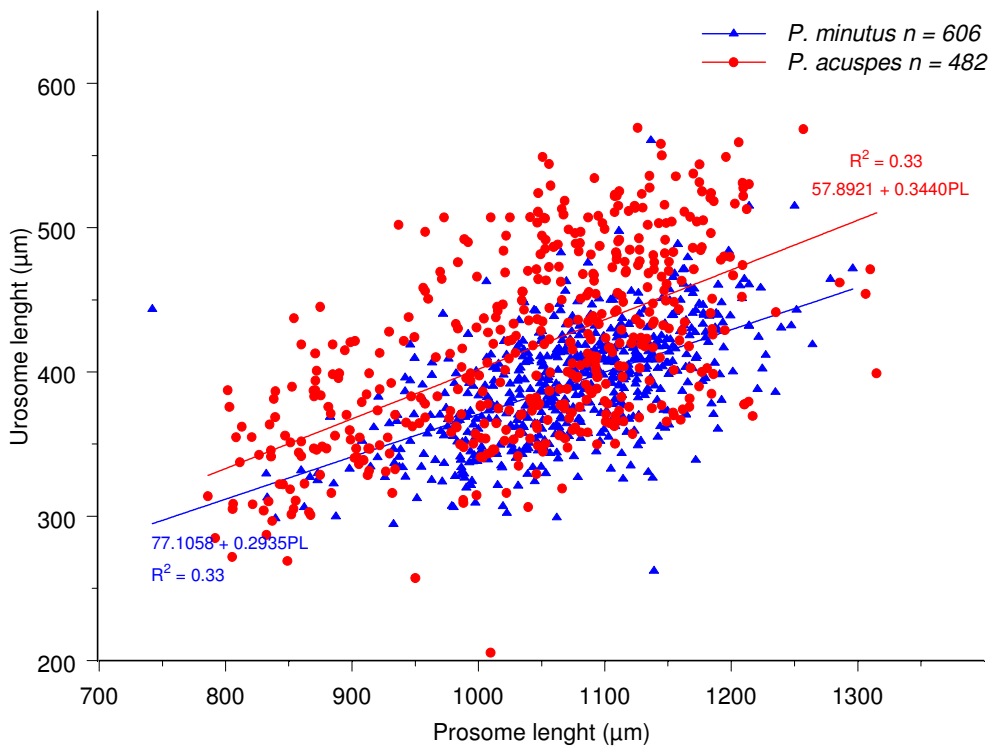


Figure 11: Prosoma length in relation to urosoma length for adult females of *P. acuspes* and *P. minutus* pooled for all sampling locations. Note axes range in relation to equations intercepts, the axes are not drawn from zero in order to display the data better.

The data points for the relationship between the cephalosoma length and the length of the seminal receptacle overlap for *P. minutus* and *P. acuspes* (Figure 12). However the regression lines show that there is a slight difference between the two data clusters. The black line ($SRL = 0.027CL + 21.5$) in figure 12, Frost (1989), indicates the separation line for the species *P. major* (above the line) which could potentially co-occur with *P. minutus* and *P. acuspes* (below the line).

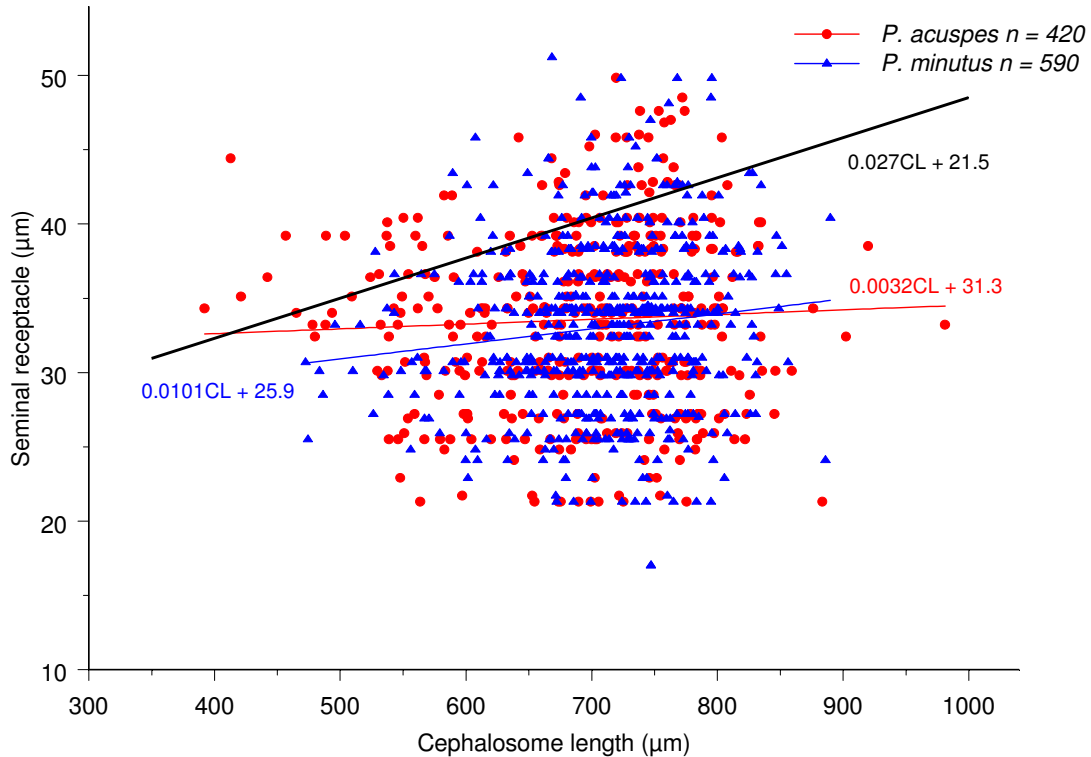


Figure 12: Length of seminal receptacle (SRL) in relation to cephalosome length (CL) for adult females of *P. minutus* and *P. acuspes* with regression lines. No data points for Hornsund. For the black line see text for details. Note axes range in relation to equations intercepts, the axes are not drawn from zero in order to display the data better.

Molecular analyses

Development of species-specific PCR

Approximately 400 base pair (bp) long sequences were obtained from 9 individuals of *P. minutus* and ~200 bp sequences from 4 individuals of *P. acuspes* (Appendix B). These were used to develop species-specific primers for each species, *P. minutus*-SS: COI_1932R and *P. acuspes*-SS: COI_2060R (see primer sequences in Materials and methods).

The PCR amplification products for *P. minutus* yield bands of 372 bp and *P. acuspes* gives bands of 500 bp (Figure 13). The bands were found to migrate for 12 mm (*P. acuspes*) and 10 mm (*P. minutus*) on a 2% agarose gels run for 10 minutes at 220 volts (Gel photo 1 in Appendix C).

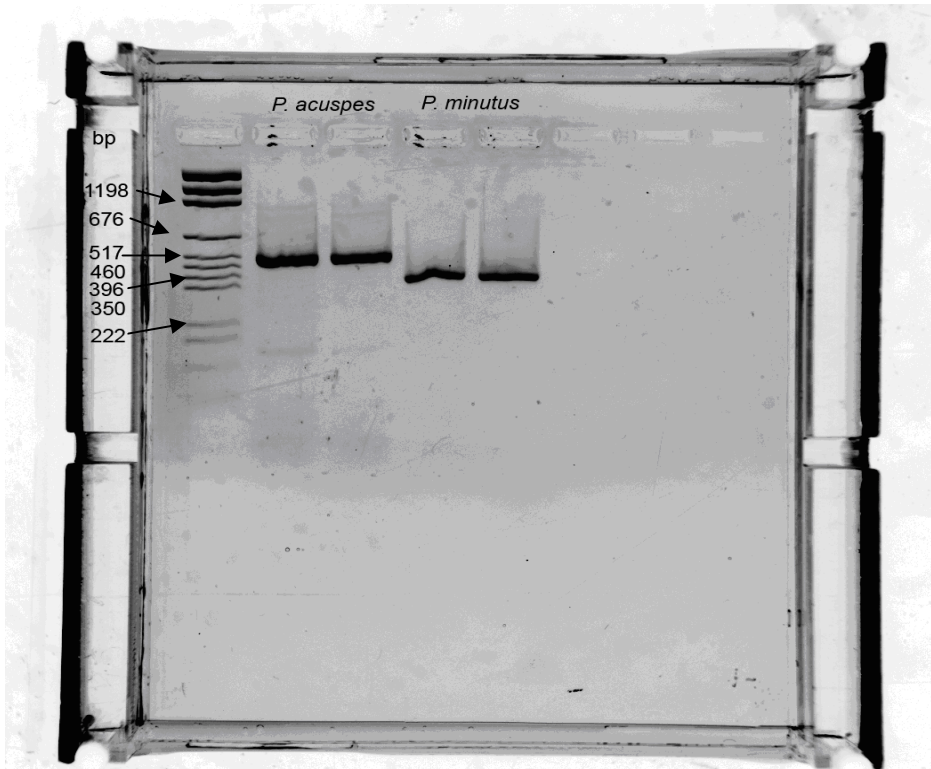


Figure 13: Gel photo showing species-specific PCR products for individual *Pseudocalanus* spp. The first lane is a molecular size marker, lanes 2 and 3 represent *P. acuspes* (500 bp) and lanes 4 and 5 *P. minutus* (372 bp).

Comparison of morphological identification and species-specific PCR

A total of 358 individuals of both species were processed on 14 gels but only 95 individuals on 5 gels were usable due to contamination and other technical problems, listed in 'Materials and methods' (see appendix C for successful gel photos). These 95 individuals came from 3 different locations (Figure 14 and 15), Rjipfjorden, Austfjorden (both species) and Billefjorden (only *P. acuspes*). Of the 96 (only 95 yielded products) of each species identified morphologically 58 turned out to be *P. minutus* and 37 *P. acuspes* (Table 2).

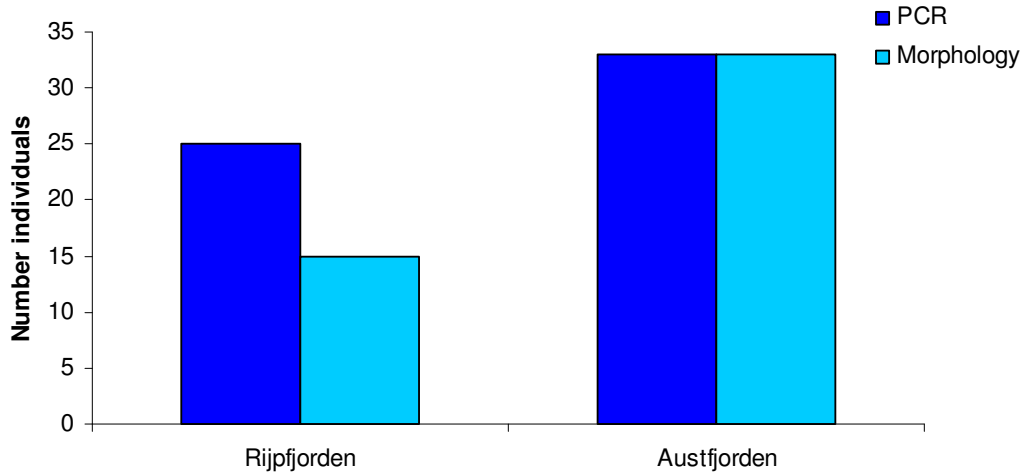


Figure 14: *P. minutus* Numbers of individuals identified with both methods.

Of the 15 *P. minutus* identified with morphology from Rijpfjorden came out as *P. minutus* (Figure 14) and in addition 9 of those identified as *P. acuspes* (Figure 15) according to the PCR analysis. All of the 30 individuals from Austfjorden were equally identified by both methods.

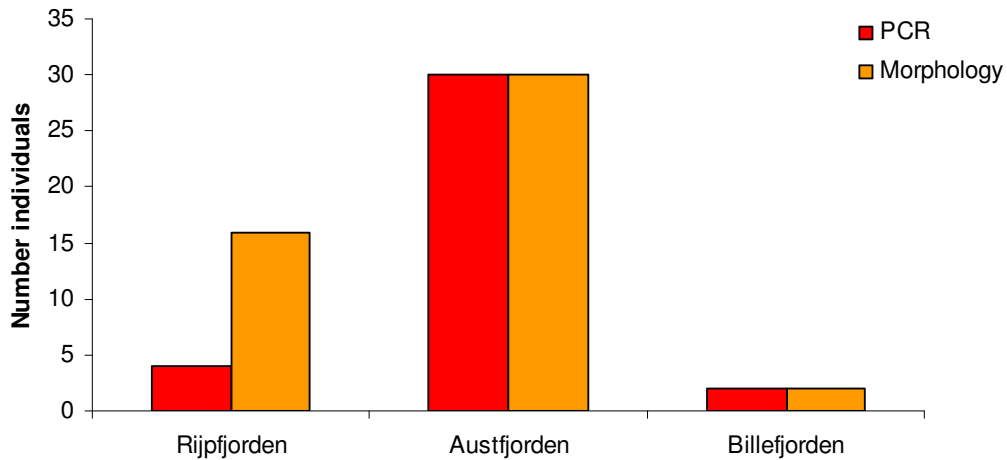


Figure 15: *P. acuspes*. Numbers of individuals identified with both methods.

Only 2 *P. acuspes* were successfully processed from Billefjorden and both of them were *P. acuspes* according to PCR (Figure 15).

The successful agarose gel electrophoresis was tested with chi square test of goodness of fit. The H_0 hypothesis for the chi-square is that there is no difference between the expected (those identified with morphology) and observed (those identified with PCR) frequency of *P. minutus* and *P. acuspes*. The p value was less than 0.05 so the H_0 hypothesis was rejected (Table 2). Thus there was a difference between what was identified morphological and molecular identification of the species.

Table 2: Results from successful gel PCR amplifications (See gel photos in appendix C)

| | Expected | Observed | Failed |
|-------------------|--|----------|--------|
| <i>P. minutus</i> | 48 | 58 | 0 |
| <i>P. acuspes</i> | 48 | 37 | 1 |
| Sample size n | 96 | 95 | 1 |
| Chi test | p value < 0.05 | | |
| H_0 | Expected values = Observed values | | |
| H_1 | Expected values \neq Observed values | | |

However, a power analysis indicates that the power of this test with a sample size of $n = 95$ is only 50% so we might be incorrectly rejecting the H_0 hypothesis. For a power of 80% the ideal sample size should be $n = 178$ (Table 3). The null hypothesis (H_0) for the power analysis is based on the expected ratio of the two species from the gel electrophoresis and the alternative hypothesis (H_1) is based on the observed ratio (Table 2).

Table 3: Power analysis based on successful outcome of gel electrophoresis

| H_0 | H_1 | Alpha | Power | Sample size n |
|-------|-------|-------|-------|---------------|
| 0.50 | 0.39 | 0.05 | 0.50 | 95 |
| 0.50 | 0.39 | 0.05 | 0.80 | 178 |

Discussion

The genus *Pseudocalanus* was found all around the archipelago of Svalbard. The genus was represented by two identified species, *P. minutus* and *P. acuspes*. Those were found to co-occur in the fjords studied for this thesis. Which species dominated represented by the abundance of adult females was found to vary with location and season. As morphology of the two species is very similar, a molecular method was developed and applied to verify the microscopy identification. A potential difference was revealed between morphological species identification and discrimination between the species using the molecular method. These points will be discussed below.

Distribution of Pseudocalanus species around Svalbard

There appeared to be no geographical difference in occurrence between the two species around the study area of Svalbard according to morphological identification and thus the different water masses do not seem to affect the occurrence of the species. Both species were present at all locations except one, Isfjorden where only *P. acuspes* was found. However, the Isfjorden sample was not replicated and since *P. minutus* was found in Billefjorden (a branch fjord of Isfjorden), and has been reported in the Barents Sea (Falk-Petersen et al. 1999) and Kongsfjorden (Lischka and Hagen 2005) its absence from the Isfjorden sample may have be attributed to a low number of samples (only one).

P. minutus dominated numerically in all samples from March and April (Figure 5). Those samples are from all around Svalbard, or the west, east and north coast (Figure 5). In the late spring/summer samples *P. acuspes* is dominating. Those samples are from the west and the north coast. Explanations for that might lay in the life history of the two species as *P. acuspes* has been shown to be able to grow very quickly when food is abundant and be more of an opportunistic species than *P. minutus* (Norrbin 1991). Furthermore it might have higher reproductive rates, or differ in predation mortality compared to *P. minutus*.

The vertically stratified tows from Billefjorden in March and May showed that the abundance of *P. acuspes* increases many times more than did *P. minutus*, especially in the surface layer (Figure 6). That might indicate that *P. acuspes* is quicker in responding to increased food abundance than *P. minutus* and utilise the resource to grow from overwintering stages to adults. In March both species were in similar numbers in all three depth layers. In the May sample, however, most were found in the middle and in the surface layers while almost none were in the deepest interval. That might indicate that the copepods migrate upwards later in the spring when food is probably more abundant when the ice breaks up and spring bloom starts.

Morphometrics and distribution

The bimodal length distribution of *P. acuspes* might indicate advection of the species from further south. The smaller group with average length of ~ 860 µm fits well with the length reported for the species from northern Norwegian fjord, ranging from ~ 700 µm to ~900 µm depending on season (Norrbin 1994). Another reason for this bimodal length distribution could be overlap of generations, but since *Pseudocalanus* is a copepod with a long generation time and potentially short adult life that is an unlikely explanation (Frost 1989). However, the smaller form of *P. acuspes* was found mainly to be in Storfjorden and Van Mijenfjorden. Those locations are rather isolated from Atlantic water inflow and therefore these animals are not likely to originate from further south but rather from locally formed populations. These two locations also showed to be separated (Figure 10, prosome length difference) from others for *P. minutus* though it was not as distinct as for *P. acuspes*.

These two locations, Storfjorden and Van Mijenfjorden, where both species were somewhat smaller than for the other locations, might thus be perhaps more oligotrophic in terms of food than the other locations. Less food would mean that juveniles of both species would not have enough resources to add up to extra size growth for the last copepodite stages.

The usefulness of morphometrics

The prosome to urosome length ratio was found to be 2.54 for *P. acuspes* and 2.75 for *P. minutus*. This ratio is quite a bit larger than what Frost (1989) reported from Baffin Bay or 2.14 for *P. acuspes* and 2.39 for *P. minutus*. His data formed two distinct clusters *P. minutus* having a significantly smaller ratio than *P. acuspes*. In this study the two data clusters overlap although the data for *P. minutus* seem to be in the lower range. That might indicate some misidentification of *P. acuspes* as *P. minutus* or perhaps the presence of the third species *P. major*, that according to Frost (1989), had prosome to urosome ratio (2.20) somewhat between the *P. minutus* and *P. acuspes* and looks fairly similar to *P. acuspes* with a stocky prosome and rounded cephalosome. One might speculate if *P. major* could possibly be a large form of *P. acuspes* due to increased amount of DNA content of cells.

The data on the seminal receptacle to cephalosome ratio also indicates a possibility that individuals of *P. major* have been inadvertently included in the data. Frost's (1989) separation line for *P. major* plotted with the data from this study (Figure 13) fell within the upper section of the data cluster. This species has been associated with conditions of extensive melt water runoff (Frost 1989). Most of the samples were taken before this time, but there could nevertheless be small numbers of *P. major* at these locations. No data points were obtained for the Hornsund sample that was the only one taken in July, at a time of brackish conditions, and thus the most likely to contain *P. major* in great numbers.

Molecular method for species discrimination

The species-specific PCR method developed for *P. minutus* and *P. acuspes* indicated that it is possible to discriminate between the two species based on different sizes of the amplification products (500 bp for *P. acuspes* and 372 bp for *P. minutus*; Figure 13).

The results from the gel electrophoresis revealed that there is a difference between what is identified with morphological methods and the species-specific PCR method. From the

three locations, Rjipfjorden, Austfjorden and Billefjorden, that successful samples were identified with both methods (Figures 14 and 15) only Rjipfjorden showed difference between methods. However some difficulties occurred throughout the testing of the samples and the final sample size of 95 identified individuals were only enough to reject the H_0 hypothesis with an accuracy of 50%. It might thus be incorrectly rejected and H_0 could be correct as in there is no difference between morphologically identified individuals and molecularly identified individuals. Furthermore it is hard to draw some conclusion from if there is a true difference between the three locations in terms of what identification method is used as more processed individuals might indicate a different pattern.

The difficulties that occurred throughout the process can be divided into three categories. First there was a frequently occurring contamination, possibly in stock solution of the PCR reagents like buffers, primers etc. Another problem was that the gel buffer might have been too old at the end of the process (the time interval between the first gels and the last gels is 5 months) so the bands were not separating well enough making it hard to discriminate between them, even though the running time of the electrophoresis was extended and the buffer had been stored in a sealed container. The third problem was that of non amplifying copepods which occurred in all processed samples. That might be due to failure in releasing DNA from the copepods or possibly in the existence of the third species, *P. major*, in which case the primers would not recognise its DNA. Another factor that might be worth looking into before continuing with PCR analysis on these two species is a further optimization of the protocol with focus on primer design. It is especially important to amplify more sequences from *P. acuspes*, as only four sequences were obtained from those ten processed in this study. More sequences from various locations, also for *P. minutus*, could give an idea on subpopulations as well.

Conclusions and further studies

This study revealed that *P. minutus* and *P. acuspes* co-occurred in all sampling locations except for one where only *P. acuspes* was found, Isfjorden, but that might be due to

chance as that sample was not replicated and *P. minutus* has been recorded in locations close to Isfjorden and in comparable other locations, such as Kongsfjorden (Lischka and Hagen 2007). The morphometrical data indicated an overlap for the two species and thus alone did not make up for a good identification but instead revealed patterns for prosome length difference for both species, although more apparent for *P. acuspes*. The species-specific PCR method made it possible to discriminate between the two species using their DNA although the final sample size of successfully identified individuals were not enough to conclude comprehensively on the overall distribution of the two species.

This study raises some interesting questions as the morphometrics and the species-specific PCR indicated the possibility of existence of the third species *P. major* but also on if it is a good species or a larger form of the other species due to genome size.

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Appendix A

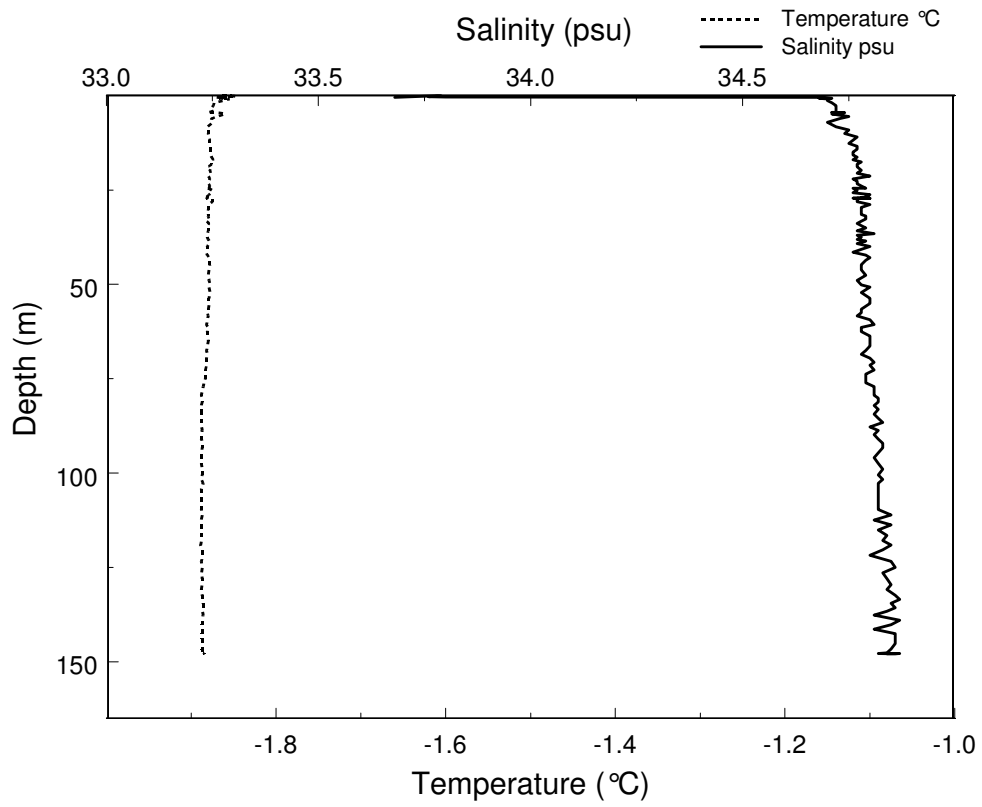


Figure A: Rijpfjorden, April 2007. Temperature and salinity profiles.

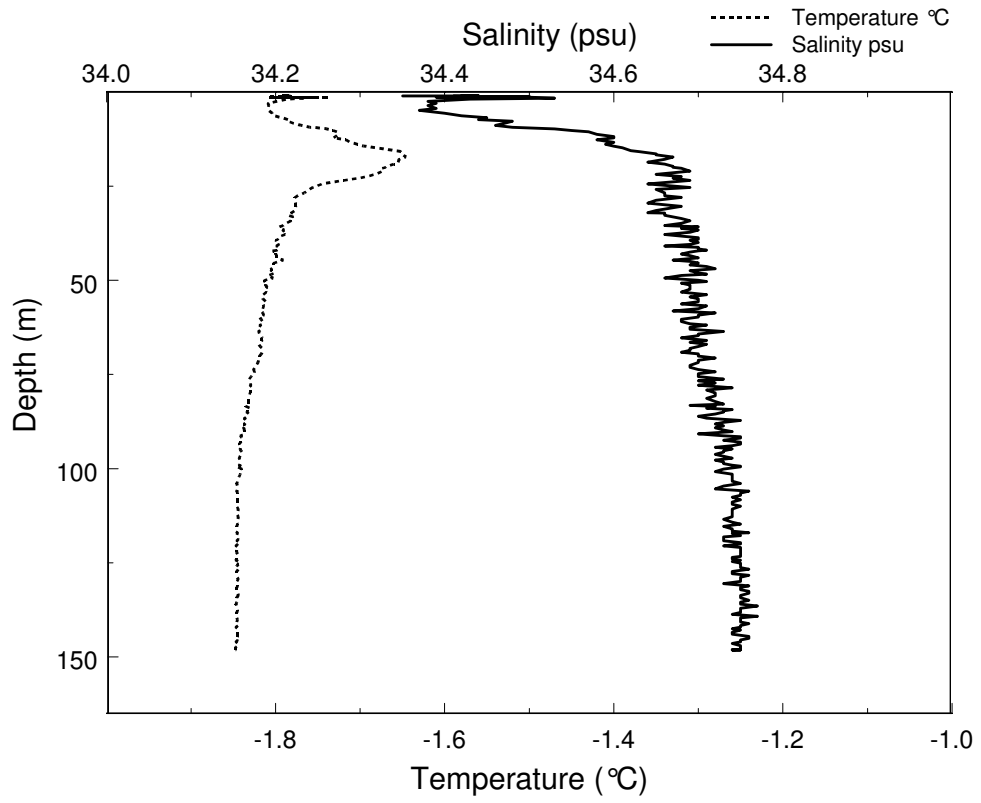


Figure B: Rijpfjorden, June 2007. Temperature and salinity profiles.

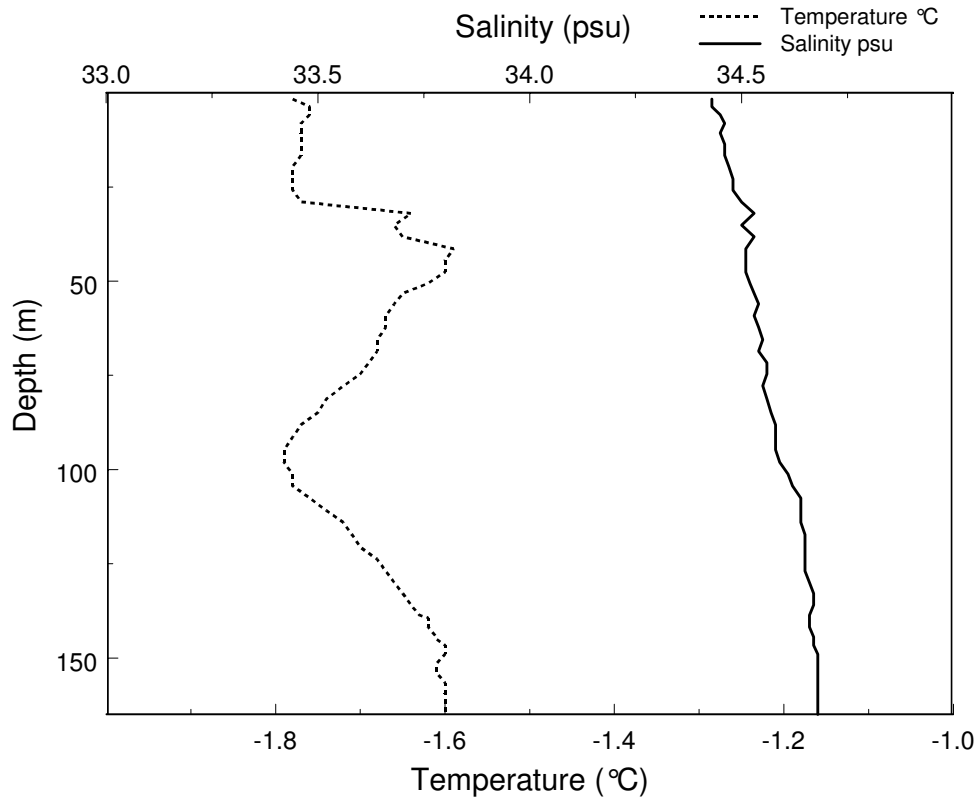


Figure C: Austfjorden, April 2004. Temperature and salinity profiles.

Appendix B

Sequences obtained from *P. minutus*:

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AATTTTTTTTATAGTTATAACCATCTTAATTGGGGGCTTTGGTAATTGA
CTAGTACCCTTAATATTAGGTGCGGCAGATATAGCTTTTCCACGTATA
AATAATATGAGATTCTGATTTTTTAATACCGGCTTTAATCATACTTCTTT
CAAGATCCTTAGTTGAAAGGGGGGCAGGTACAGGATGAACTGTTTACC
CCCCATTATCCAAAAATATTGCTCATGCAGGAGGGTCAGTAGATTTTG
CTATTTTTTCTTTACATTTAGCAGGTGTTAGATCTATTTAGGGGCTGT
AAATTTTATTAGCACATTAGGTAATTTACGAGTATTTGGTATACTCCTA
GACCAAATACCTTTGTTT-5'
2. 3'GATGACCAAATTTATAATGTAGTTGTGACAGCTCATGCATTTATCAT
AATTTTTTTTATAGTTATAACCATCTTAATTGGGGGCTTTGGTAATTGA
CTAGTACCCTTAATATTAGGTGCGGCAGATATAGCTTTTCCACGTATA
AATAATATGAGATTCTGATTTTTTAATACCGGCTTTAATCATACTTCTTT
CAAGATCCTTAGTTGAAAGGGGGGCAGGTACAGGATGAACTGTTTACC
CCCCATTATCCAAAAATATTGCTCATGCAGGAGGGTCAGTAGATTTTG
CTATTTTTTCTTTACATTT-5'
3. 3'CAAATTTATAATGTAGTTGTGACAGCTCATGCATTTATCATAATTTTT
TTTATAGTTATAACCATCTTAATTGGGGGCTTTGGTAATTGACTAGTAC
CCTTAATATTAGGTGCGGCAGATATAGCTTTTCCACGTATAAATAATA
TGAGATTCTGATTTTTTAATACCGGCTTTAATCATACTTCTTTCAAGATC
CTTAGTTGAAAGGGGGGCAGGTACAGGATGAACTGTTTACCCCCATT
ATCCAAAAATATTGCTCATGCAGGAGGGTCAGTAGATTTTGCTATTTTT
TCTTTACATTTAGCAGGTGTTAGATCTATTTTAGGTGCTGTAAATTTTA
TTAGCACATTAGGTAATTTACGAGTATTTGGTATACTCCTAGACCAA
TACCTTTGTTTGCG-5'
4. 3'CACTAATTGGAGATGACCAAATTTATAATGTAGTTGTGACAGCTCAT
GCATTTATCATAATTTTTTTTATAGTTATAACCATCTTAATTGGGGGCT
TTGGTAATTGACTAGTACCCTTAATATTAGGTGCGGCAGATATAGCTTT
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ATACTTCTTTCAAGATCCTTAGTTGAAAGGGGGGCAGGTACAGGATGA
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GTAGATTTTGCTATTTTTTCTTTACATTTAGCAGGTGTTAGATCTATTTT
AGGTGCTGTAAATTTTATTAGCACATTAGGTAATTTACGAGTATTTGGT
ATACTCCTAGACCAAATACCTTTGTTTGCGTGGTCTGTATTAGTCACAG
CCATCCTTTTATTACTATCCT-5'
5. 3'GCAGGGTCACTAATTGGAGATGACCAAATTTATAATGTAGTTGTGAC
AGCTCATGCATTTATTATAATTTTTTTTATAGTTATAACCATCTTAATTG

GGGGCTTTGGTAATTGACTAGTACCCTTAATATTAGGTGCGGCAGATA
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TTAATCATACTTCTTTCAAGATCCTTAGTTGAAAGGGGGGCAGGTAC
AGGATGAACTGTTACCCCCATTATCCAAAATATTGCTCATGCAGG
AGGGTCAGTAGATTTTGCTATTTTTCTTTACATTTAGCAGGTGTTAGA
TCTATTTTAGGTGCTGTAAATTTTATTAGCACATTAGGTAATTTACGAG
TATTTGGTATACTCCTAGACCAA-5'

6. 3' TCACTAATTGGAGATGACCAAATTTATAATGTAGTTGTGACAGCTCA
TGCATTTATCATAATTTTTTTTATAGTTATACCCATCTTAATTGGGGGC
TTTGGTAATTGACTAGTACCCTTAATATTAGGTGCGGCAGATATAGCTT
TTCCACGTATAAATAATATGAGATTCTGATTTTTAATACCGGCTTTAAT
CATACTTCTTTCAAGATCCTTAGTTGAAAGGGGGGCAGGTACAGGATG
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AGTAGATTTTGCTATTTTTCTTTACATTTAGCAGGTGTTAGATCTATTT
TAGGTGCTGTAAATTTTATTAGCACATTAGGTAATTTACGAGTATTTGG
TA-5'
7. 3' ATGACCAAATTTATAATGTAGTTGTGACAGCTCATGCATTTATCATA
ATTTTTTTTATAGTTATACCCATCTTAATTGGGGGCTTTGGTAATTGAC
TAGTACCCTTAATATTAGGTGCGGCAGATATAGCTTTTCCACGTATAA
ATAATATGAGATTCTGATTTTTAATACCGGCTTTAATCATACTTCTTT
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CCCATTATCCAAAATATTGCTCATGCAGGAGGGTCAGTAGATTTTGC
TATTTTTCTTTACATTTAGCAGGTGTTAGATCTATTTTAGGTGCTGTA
AATTTTATTAGCACATTAGGTAATTTACGAGTATTTGGTATACTCCTAG
ACCAAATACCTTTGTTTG-5'
8. 3' TTTATAATGTAGTTGTGACAGCTCATGCATTTATTATAATTTTTTTTA
TAGTTATACCCATCTTAATTGGGGGCTTTGGTAATTGACTAGTACCCTT
AATATTAGGTGCGGCAGATATAGCTTTTCCACGTATAAATAATATGAG
ATTCTGATTTTTAATACCGGCTTTAATCATACTTCTTTCAAGATCCTTA
GTTGAAAGGGGGGCAGGTACAGGATGAACTGTTTACCCCCATTATCC
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CACATTAGGTAATTTACGAGTATTTGGTATACTCCTAGACCAAATACC
TTTGTGCGTGGTCTGT-5'
9. 3' AATTGGAGATGACCAAATTTATAATGTAGTTGTGACAGCTCATGCAT
TTATCATAATTTTTTTTATAGTTATACCCATCTTAATTGGGGGCTTTGGT
AATTGACTAGTACCCTTAATATTAGGTGCGGCAGATATAGCTTTTCCA
CGTATAAATAATATGAGATTCTGATTTTTAATACCGGCTTTAATCATA
TTCTTTCAAGATCCTTAGTTGAAAGGGGGGCAGGTACAGGATGAACTG
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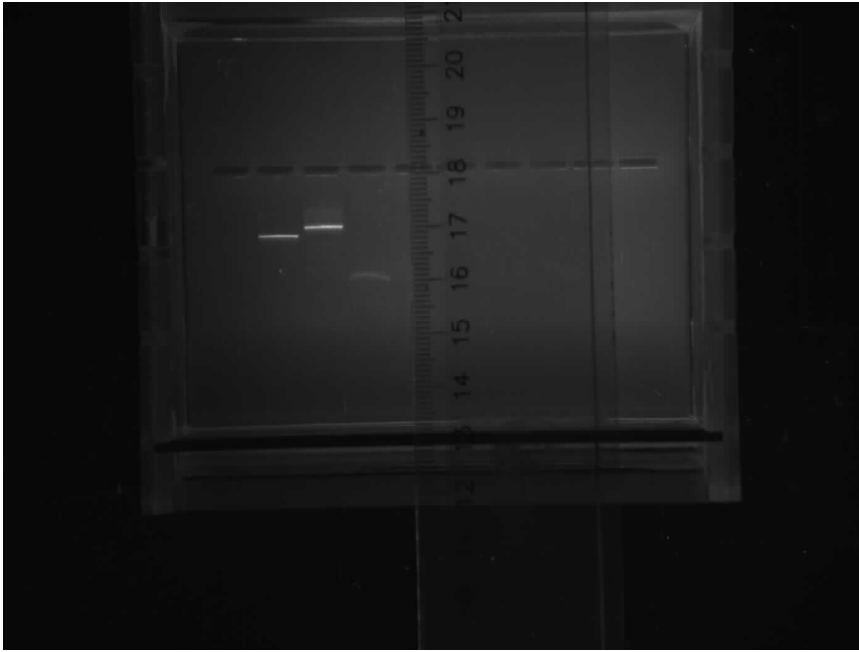
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Sequences obtained from *P. acuspes*

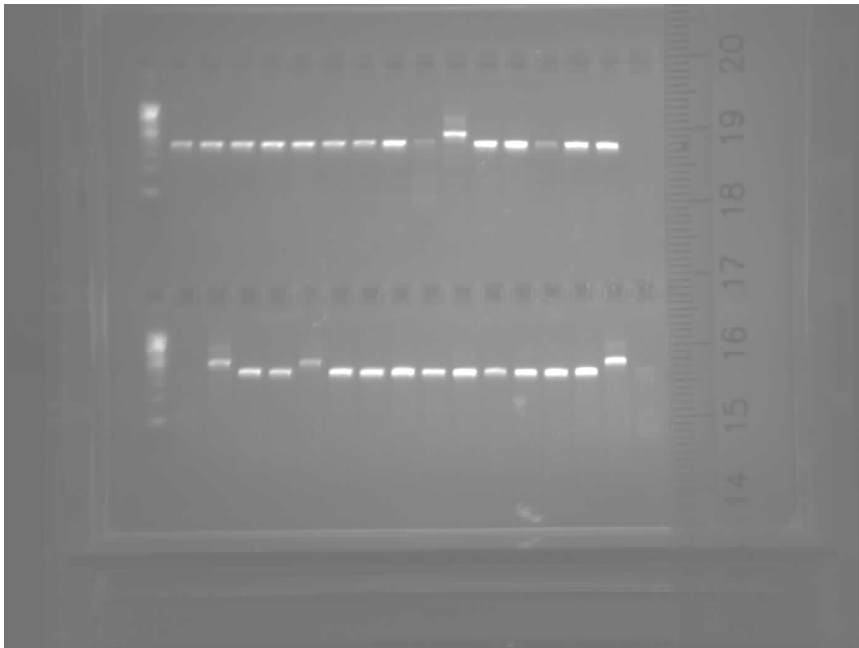
1. 3'GGAGATGACCAAATTTATAATGTAGTCGTTACTGCGCATGCATTCAT
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CTCCAGGTCTCTAGTGGAGAGAGGTGCAGGTACAGGGTGAACGTATA
CCCTCCTCTATCAAGAAATATTGCTCATGCTGGAGGTTCTGTAAATTTT
GCTATTTTTTCCCTGCACCTCGCAGGAGTAAGATCTATCTTA
2. 3'CCTGGATCATTAATTGGAAATGACCTATTTTTTAATGGAGTCGTTACT
GCGCAAGCATTTCATCATAATTTTTTTTATAGTTATGCCAATTTTAATTG
GGGGATTTGGTAACCTGGTAAAACCTTTGATATTTGGTGCGGGAAAAA
TAGCTTTTCCTCGTATAAAATAAAATAAGGTTCTGATTTTTTAAAACCAGC
CCTAATTATACTCCTCTCTAGGTCTCTAGTGGAAAGAGGGGCAGGAAC
AGGGGGAAAAGAATACCCTCCTTTCAAGAAAATTGCTCATGCTGG
AAGAAGTGTAAATTTTGCTATTTTTTTC-5'
3. 3'TTCATAAAAAATTATTCCAATAGAGGTAGGGCAAGCTGGATCACTCC
TTGGAGATGACCAATTTTATAATGTTGTCGTTAGTGCTCATGCATTCAT
CATAATTTTTTTTATAGTTATGCCAATTTTAATTGGGGGATTTGGTAAC
TGGTTAAAACCTTTGGA-5'
4. 3'ATAGAGGGGGGGCAACCTGGATCGCCCCTTGGAAACGACCTATTTTT
TAATCCCTCTATAGGGCTCCAGTATTCAACATTATTTTTTTTATAGTT
ATGCCAATTTTAAGTGGGGGATTTGGTAACCTGGTAGAACCTTTGATA
TTAGGTGCGGCAGATTTAGCTTTTCCTCAATAAATAATATAAGGTTCTG
ATTTTTAATACCAGGCCTAATTATACTCCTCTCCAGGTCTCTTGGGGAA
AGAGGTGCAGGTAAAAGGCCAAGTGAACCCCTCCTCTATCAAGAA
TATTG-5'

Appendix C

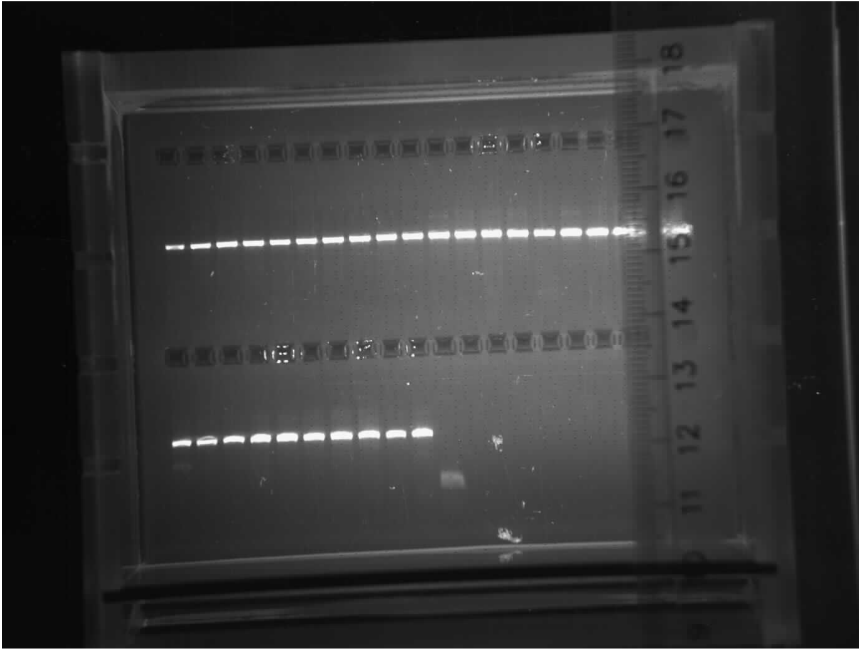
Successful gel analysis



Gel photo 1: Two individuals. One from Austfjorden, identified as *P. acuspes*, and one from Rjipfjorden, identified as *P. minutus*.



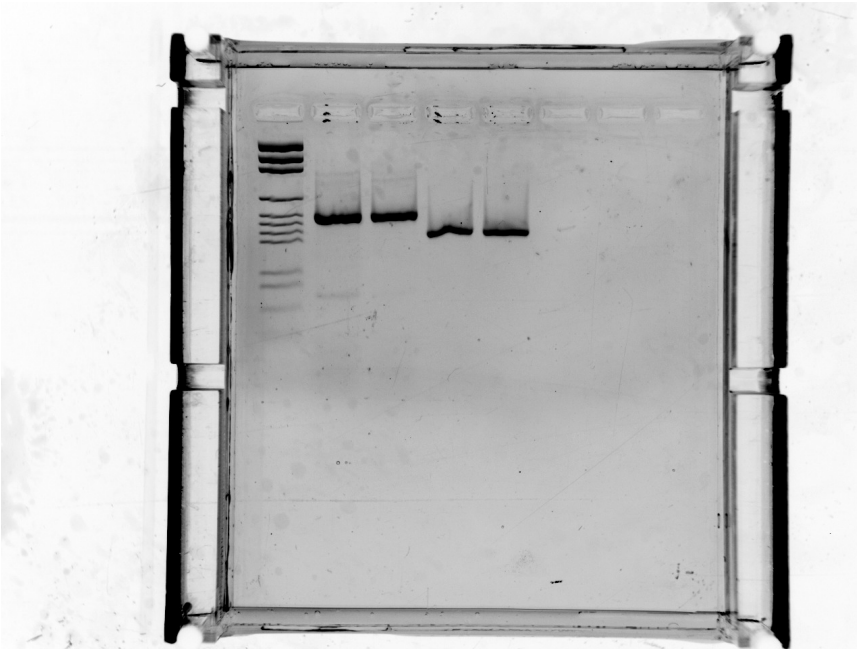
Gel photo 2: 30 individuals from Rjipfjorden. 25 identified as *P. minutus*, 4 as *P. acuspes* and 1 failed (second lane in lower row).



Gel photo 3: 30 individuals from Austfjorden. 30 out of 30 identified as *P. minutus*.



Gel photo 4: 30 individuals from Austfjorden. 30 out of 30 identified as *P. acuspes*.



Gel photo 5: 4 individuals. Two from Billefjorden, identified as *P. acuspes* and two from Austfjorden, identified as *P. minutus*.