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Faculty of Biosciences, Fisheries and Economy
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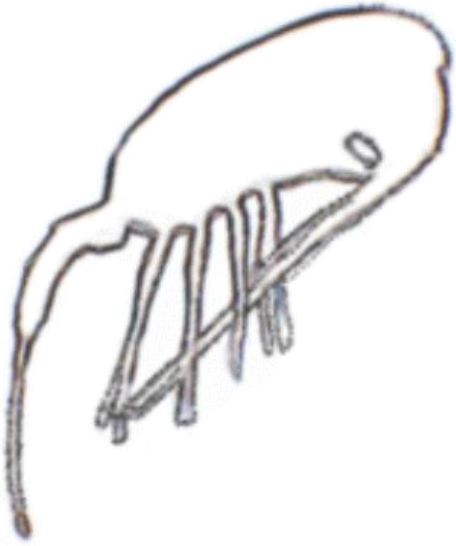
Pseudocalanus (Copepoda: Calanoida) of the North Atlantic Ocean

Species composition, environmental preferences and phylogeography



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Ole Nicolai Staurland Aarbakke, Tromsø 15. May 2013

Summary

The seven species of the genus *Pseudocalanus* (Copepoda: Calanoida) are difficult to identify because of very small interspecific, and comparatively large intraspecific, divergence of morphologic and morphometric traits. Thus, despite the fact that *Pseudocalanus* spp. are among the most abundant metazoans in the world, our knowledge of them at the species level is limited. The main objective of this thesis was to lay the groundwork for future studies of *Pseudocalanus* spp. in the Northeast Atlantic Ocean and Euro-Arctic by determining which species are present. Through a combined DNA barcoding and traditional identification approach, this work reveals the presence of at least four species of *Pseudocalanus,* one of which was previously not believed to occur in the East Atlantic Ocean. Furthermore, the thesis reveals differences in environmental preferences and distributions of *Pseudocalanus elongatus*, *P. minutus*, *P. moultoni* and *P. acuspes* in the Northeast Atlantic Ocean and Euro-Arctic, and discusses implications of these differences in relation to rising sea temperatures. Finally, this work examines the phylogeography and demographic history of *Pseudocalanus minutus*, *P. moultoni*, *P. elongatus*, *P. acuspes* and *P. newmani* in the North Atlantic Ocean and based on these results suggests an evolutionary hypothesis for the divergence of *Pseudocalanus* spp. into one oceanic and one coastal clade.

Hopefully, this thesis has achieved its main goal of providing a basis from which the biology and ecology of these fascinating copepod species can be further explored.

Sammendrag

De syv artene som til sammen utgjør slekten *Pseudocalanus* (Copepoda: Calanoida) er vanskelige å skille fra hverandre på grunn av veldig liten interspesifikk og til sammenligning stor intraspesifikk forskjell i morfologi og morfometri. Derfor, er vår kunnskap om disse hoppekrepsene på artsnivå begrenset, til tross for at *Pseudocalanus* spp. er blant de mest tallrike dyrene på jorden. Hovedmålet med denne avhandlingen var å legge et grunnlag for fremtidige studier av *Pseudocalanus* spp. i det Nordøstlige Atlanterhavet og Europeisk-Arktiske områder ved å fastslå hvilke arter som finnes der. Ved å kombinere DNA barcoding og tradisjonelle identifikasjonsprosedyrer avslører denne avhandlingen tilstedeværelse av minst fire arter *Pseudocalanus*, blant dem en art som tidligere ikke var kjent i det østlige Atlanterhavet. Videre avslører avhandlingen forskjeller i miljøpreferanser mellom *Pseudocalanus elongatus*, *P. minutus*, *P. moultoni* og *P. acuspes* og diskuterer implikasjonene av dette relatert til økende havtemperatur.
Til slutt utforskes fylogeografi og demografi hos *Pseudocalanus minutus*, *P. moultoni*, *P. elongatus*, *P. acuspes* and *P. newmani* i det nordlige Atlanterhavet. Resultatene tyder på at *Pseudocalanus* spp. representerer to evolusjonære grupper, en oseanisk og en som er begrenset til kysthabitat.

Jeg håper at denne avhandlingen har oppfylt sitt hovedmål; å legge et grunnlag for fremtidige studier av biologien og økologien til disse fascinerende hoppekrepsartene.

1. Introduction

1.1. Difficult identification.

Taxonomy forms the basis of all biological research for two important reasons. Firstly, it provides a classification of all living things and secondly, the scientific nomenclature provides a unique definition for each taxon. Thanks to philosophers, physicians, clerics, trained taxonomists and amateur naturalists through the ages, we have an ever increasing record of unique and uniquely named taxa. Anyone describing a new taxon must provide a unique scientific name (binomial for new species; uninomial for other ranks) and a description of a vouchered name bearing type specimen with sufficient attributes to differentiate the taxon from all other taxa (*ICZN Code*, Article 13.1.1; *ICN*, Article 38.2).

However, as pointed out by Hebert *et al*. (2003) there are four major pitfalls in traditional species identification. 1) The phenotypic plasticity and genetic variability of the organism under investigation can result in misidentification. 2) Cryptic taxa (i.e. previously unidentified sibling species) may be overlooked. 3) Many taxa lack identification keys for certain stages and sexes (e.g. Genus *Pseudocalanus*) and 4) Using identification keys correctly requires extensive experience, without which misidentifications are common (Hebert *et al.*, 2003). To this I will add that fragile species (e.g. gelatinous taxa) are often damaged beyond recognition upon capture, rendering morphological identification impossible.

On the topic of difficult identification, marine calanoid copepods (Copepoda: Calanoida) are a case in point. At present 2056 species have been described (CMarZ.org; Razouls *et al*., online) and to the untrained eye many copepod taxa appear identical, but closer inspection reveals a wonderful magnitude of shapes and sizes. Still, even the most studied copepod taxon, *Calanus finmarchicus* Gunnerus 1770 (Mauchline, 1998) causes confusion because of subtle morphological traits and great intraspecific variability in body size (Gabrielsen *et al.*, 2012, Parent *et al.*, 2011).

1.2. DNA barcoding

Using DNA sequences to recognize and discriminate species overcomes some of the challenges outlined above. With the development of universal invertebrate primers (Folmer *et al.,* 1994) for the mitochondrial cytochrome c oxidase subunit I (COI) gene, the number of taxa identified using DNA barcodes (i.e. short DNA sequence used for species recognition and discrimination; Hebert *et al*., 2003) is ever increasing (Bucklin *et al.*, 2011). Disadvantages to DNA barcoding are that it requires some relatively sophisticated equipment, and laboratory protocols are time consuming and expensive. Furthermore, the method requires proper preservation to ensure good DNA quality. Another issue is that DNA identification of new taxa may challenge other species concepts (e.g. the biological species concept, Mayr, 1942).

DNA barcoding has been applied extensively for identification of various zooplankton taxa and libraries have been established for several groups. Examples include euphausiids (Bucklin *et al.*, 2007), chaetognaths (Jennings *et al.*, 2010b), medusozoans (Ortman *et al.*, 2010), pelagic gastropods (Jennings *et al.*, 2010a) and copepods (Bucklin *et al.*, 2003, Bucklin *et al.*, 2010, Bucklin *et al.*, 2011).

1.3. *Pseudocalanus*, the need for data

Calanoid copepods of the genus *Pseudocalanus* often dominate the zooplankton in the Northern Hemisphere (Corkett and McLaren, 1978) and are therefore among the most abundant metazoans on Earth (Mauchline, 1998). However, species level identification is made difficult by very subtle interspecific divergence of morphological and morphometric traits (Frost, 1989). Moreover, size differences between the species are not diagnostic and growth is influenced by temperature (Corkett and McLaren, 1978). In fact, their study has since before the establishment of the genus by Boeck in 1872, been hampered by confusing taxonomy. For an excellent review of 133 years of confusion, from 1845 to 1978, I recommend Corkett and McLaren (1978). Corkett and McLaren themselves make no attempt at guessing which species is in question and consistently refer to all records as *Pseudocalanus*. Their expressed wish for a taxonomic revision of the genus was, as we shall see, fulfilled.

Pseudocalanus were held as an example of a “successful marriage” of DNA barcoding and morphological identification methods by McManus *et al.* (2009). The facilitators of this happy union were Bruce W. Frost and Jean Marie Sévigny and co-workers. Frost (1989) undertook a complete reevaluation of the taxonomy and biogeography of the genus *Pseudocalanus*. The result was the removal of two species names, *Pseudocalanus clausii* Brady 1865 and *P. gracilis* G. O. Sars 1903, as they were found to be synonymous with *P. acuspes* and *P. minutus*, respectively. As well as sorting out the confusion, Frost found evidence of three additional species, thereby expanding the genus and bringing the total number of species to seven. The seven species comprising the genus today are:

Pseudocalanus minutus (Krøyer 1845)
Pseudocalanus elongatus Boeck 1865
Pseudocalanus acuspes Giesbrecht 1881
Pseudocalanus major G. O. Sars 1900
Pseudocalanus moultoni Frost 1989
Pseudocalanus newmani Frost 1989
Pseudocalanus mimus Frost 1989

Sévigny *et al.* (1989) investigated patterns of allozyme variation at the glucose phosphate isomerase (GPI) locus and verified *P. minutus*, *P. acuspes*, *P. moultoni*, *P. newmani* and *P. elongatus* as genetically isolated from one another and concluded that they were good species. Their conclusions were later confirmed through sequencing of COI by Bucklin *et al*. (2003) who also obtained a sequence for *P. mimus*. As of today, no COI or other DNA sequence exists for *P. major*, which proved the most elusive of the *Pseudocalanus* species and probably occurs primarily in estuarine arctic fjords (Frost, 1989).

Despite Frost, Sévigny *et al*. and Bucklin *et al*.`s., efforts, the number of studies that accurately identify *Pseudocalanus* species is dwarfed by the number of studies pooling them together. Occasionally they are merged with *Paracalanus* sp. The level of taxonomic resolution required of course depends on your research question, but the inherent ecological significance of so numerous organisms warrants investigation.

At the genus level there is considerable knowledge regarding *Pseudocalanus* sp. They are widely distributed and very abundant, a high quality food source for higher trophic levels (Parrish *et al.*, 2012), and a preferred prey for Atlantic cod *Gadus morhua*, Atlantic mackerel *Scomber scombrus* (Robert *et al.*, 2009; 2011; 2013) and Northern right whales *Eubalaena glacialis* (Waring *et al.*, 2009). But which *Pseudocalanus* species are the studies cited above (and others) concerned with? Correct identification is paramount if these and other ecologically important questions are to be answered. What's more, some studies describing the presence of specific *Pseudocalanus* species do so without sufficient taxonomic justification (e.g. Dvoretsky and Dvoretsky, 2010).

Because most studies do not identify *Pseudocalanus* species and some apply species names that may not be completely correct, there is a need for knowledge about *Pseudocalanus* species that can only be revealed if the species are unambiguously identified.

1.4. Objectives

The main objectives of this study were:

1) To establish which *Pseudocalanus* species are present in Northeast Atlantic waters and thereby facilitate future research.

2) To assess the environmental preferences of *Pseudocalanus* spp. and thereby determine if the species are useful as bio-indicators of environmental change.

3) To investigate the phylogeographic relationships between different populations of the species to determine their dispersal potential and make predictions for the future.

2. Materials and Methods

2.1. Study Area

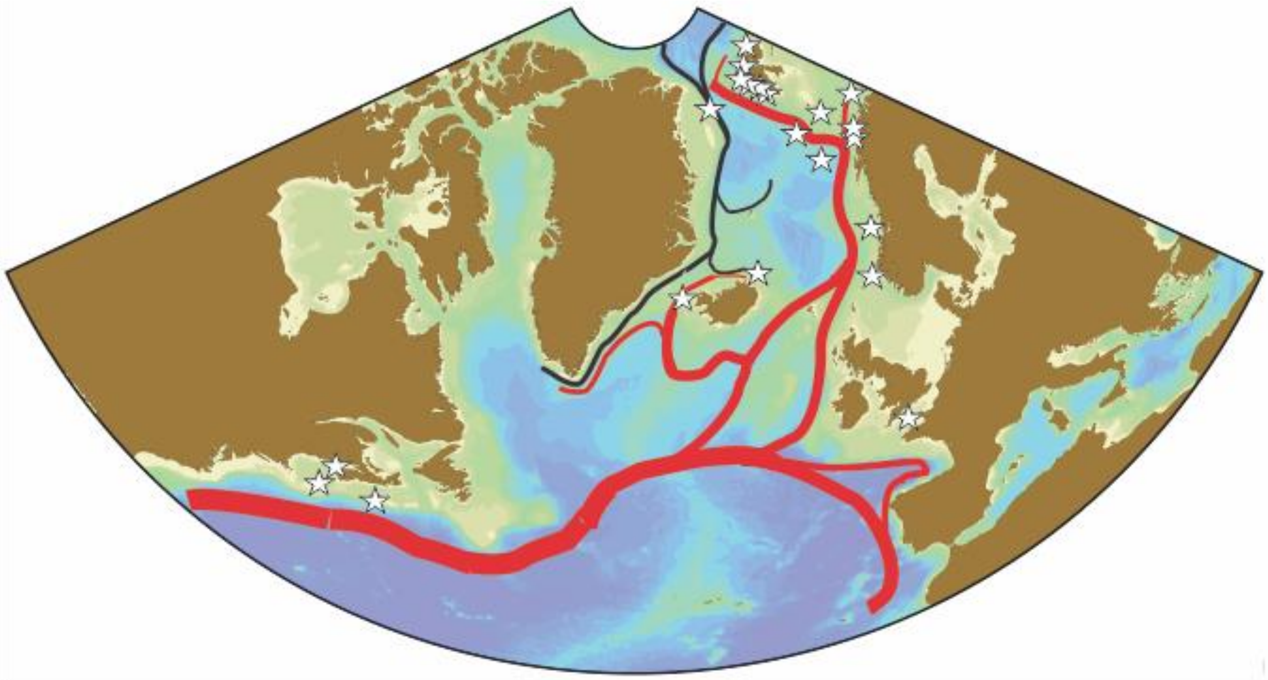

Fig. 1. Map of the North Atlantic Ocean showing all sampling locations (indicated by white stars) and the major ocean currents. Red = The North Atlantic current and its extensions. Black = Arctic water.

2.2. Samples

Zooplankton samples from the Northeast Atlantic and Euro-Arctic were collected during cruises with RV Jan Mayen (now RV Helmer Hanssen, University of Tromsø, UiT, Norway), RV Hyas (UiT), SY Oceania (Institute of Oceanology Polish Academy of Sciences, IOPAS) and through the ice in Rijpfjorden. Samples from the West and Central

Atlantic were obtained from the Census of Marine Zooplankton (CMarZ) archives located at the Department of Marine Sciences, University of Connecticut, USA. All samples were preserved in 95 – 100 % ethanol. Details of station data and equipment used are given in papers I, II and III.

2.3. Species identification procedure

All three studies focused on adult female copepods. If adult females were sparse, males or individuals in the fifth copepodite stage (CV) were selected. A high resolution picture was taken of each copepod with a Leica TCS SP8 STED camera (magnification; 50 x). The camera software incorporates an adjustable scale bar into the picture, enabling calibration at any time. After being photographed, the specimens were submerged in a large Petri dish filled with MilliQ purified water to remove the alcohol prior to DNA extraction (see papers I, II and III for details on DNA extraction, polymerase chain reaction (PCR) and sequencing).

Morphometric measurements and morphologic assessments (e.g. lengths of prosome and urosomal segments; shape of cephalosome) were made from the pictures using the Java-based software ImageJ. The “Straight lines” and “segmented lines” tools were used to measure prosome and urosome lengths, respectively (Fig. 2.) The use of image J expedites morphometric measurements because it requires less manipulation of the animal under the stereo microscope.

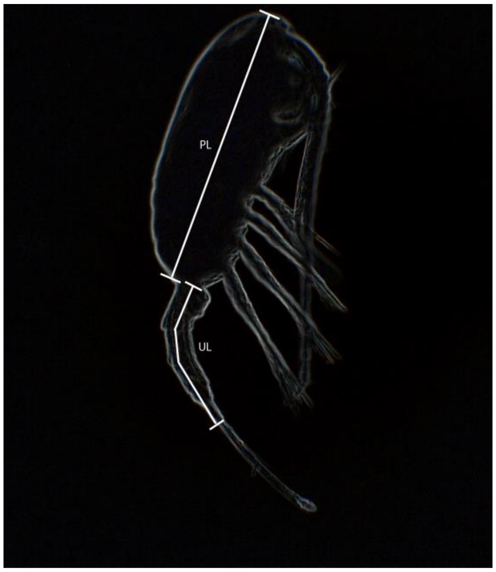

Fig. 2. Silhouette of adult female *Pseudocalanus* sp. showing the two measurements prosome length (PL) and urosome length (UL)

In instances where visual identification was ambiguous, COI sequences were used to identify the species through BLAST searches of the GenBank database (Altschul *et al.*, 1997). When sample preservation prevented sequencing of COI, sequences of the nuclear internal transcribed spacer region 1 (ITS 1) were compared to (COI confirmed) reference sequences for *P. elongatus, P. acuspes, P. minutus, P. moultoni* and *P. newmani* (Paper III). To our knowledge, no ITS 1 sequences are available for *P. mimus* or *P. major*.

3. Results

3.1. Summary of the main results

The main results of the manuscripts constituting this thesis are as follows:

3.1.1. *Paper I*

Aarbakke, O. N. S., Bucklin, A., Halsband, C. and Norrbin, F. (2011). Discovery of *Pseudocalanus moultoni* Frost 1989 in Northeast Atlantic waters based on mitochondrial COI sequence variation. *Journal of Plankton Research*. 33, 1487-1495.

The main result of the first paper was the detection of a widespread occurrence of *Pseudocalanus moultoni* in European arctic waters. The species previously thought to occur only on the East and West coasts of North America was found in Balsfjorden, North Norway, and in several fjords of the Spitsbergen archipelago. Furthermore, individuals from Georges Bank, U.S.A., were genetically similar to individuals from Balsfjorden, and there were indications that the presence of *P. moultoni* could be associated with the North Atlantic Current because all locations in which it was found receive inflow from that body of water.

3.1.2. *Paper II*

Aarbakke, O. N. S., Weydmann, A. and Fevolden, S-E. (submitted). *Pseudocalanus* (Copepoda: Calanoida) species distribution and relative abundance as indicators of changing sea temperature. Submitted to *Journal of Plankton Research*

The second paper established that *Pseudocalanus* species differ markedly with respect to their environmental preferences and as such are suitable for use as bio-indicators of different water masses and environmental change. *Pseudocalanus elongatus* was found further North than previously reported and it was confirmed that, compared to its siblings, this species is associated with higher temperatures. Although no correlation was found between *P. minutus* presence or absence and temperature, *P. minutus* is more northerly distributed and it appeared that *P. minutus* shared domain with *P. elongatus* at their southern and northern range limits, respectively. The species most associated with cold and less saline waters was found to be *P. acuspes*, while *P. moultoni* was present in all locations sampled which led to the conclusion that *P. moultoni* has the highest tolerance of different environmental conditions of these four *Pseudocalanus* species.

3.1.3. *Paper III*

Aarbakke, O., Bucklin, A., Halsband, C. and Norrbin, F. (manuscript) Comparative phylogeography and demographic history of five sibling copepod species in the North Atlantic Ocean. Manuscript formatted to the standard of *Journal of Experimental Marine Biology and Ecology*.

In the last manuscript we investigated the phylogeographic relationships between populations of *Pseudocalanus minutus, P. moultoni, P. elongatus, P. acuspes* and *P. newmani* in the North Atlantic Ocean. Sequence analysis of mitochondrial COI and Cytochrome B (CytB) revealed marked differences in genetic structuring between, and phylogeographic structuring within, the species. The three species, *P. minutus*, *P. moultoni* and *P. elongatus*, displayed the lowest levels of genetic structuring, while *P. acuspes* and *P. newmani* showed comparatively higher levels of genetic structuring, and also higher intraspecific genetic variability. This was surprising because the populations of respectively *P. acuspes* and *P. newmani* were separated by a much smaller geographic distance compared to the three other species. All three genetic markers used in Paper III displayed the same basic evolutionary relationship between the five *Pseudocalanus* species. Furthermore, the phylogenetic trees for COI and ITS 1 formed two clades with *P. minutus*, *P. moultoni* and *P. elongatus* in one and *P. acuspes* and *P. newmani* in the other.

A Bayesian demographic analysis showed that the populations of two species, *P. minutus* and *P. moultoni*, increased by one order of magnitude approximately 20.000 and 65.000 years ago, respectively. The populations of *P. acuspes* and *P. newmani* have been stable for a longer time period (1.000.000 and 250.000 years, respectively) but the former recently experienced a bottleneck with a subsequent population increase. The similarities of *P. minutus*, *P. moultoni* and *P. elongatus* and *P. acuspes* and *P. newmani* led us to hypothesize that the division of the five species into two clades represents an evolutionary divergence of oceanic versus coastal species.

3.2. *Pseudocalanus* species in the North Atlantic

Because the discussion in some parts will concern the appearance of the different *Pseudocalanus* species, a photograph along with Frost`s plates for each of the six species are given below.

Pseudocalanus minutus (Krøyer 1845)

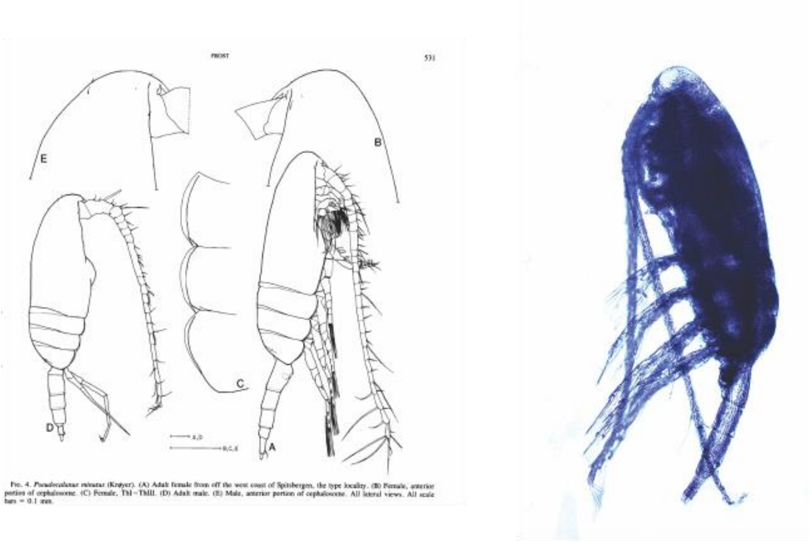

FIG. 4. *Pseudocalanus minutus* (Krøyer). (A) Adult female from off the west coast of Spitsbergen, the type locality. (B) Female, anterior portion of cephalosome. (C) Female, ThI–ThIII. (D) Adult male. (E) Male, anterior portion of cephalosome. All lateral views. All scale bars = 0.1 mm.

Fig. 3. Adult female of *Pseudocalanus minutus*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author.

Pseudocalanus elongatus Boeck 1865

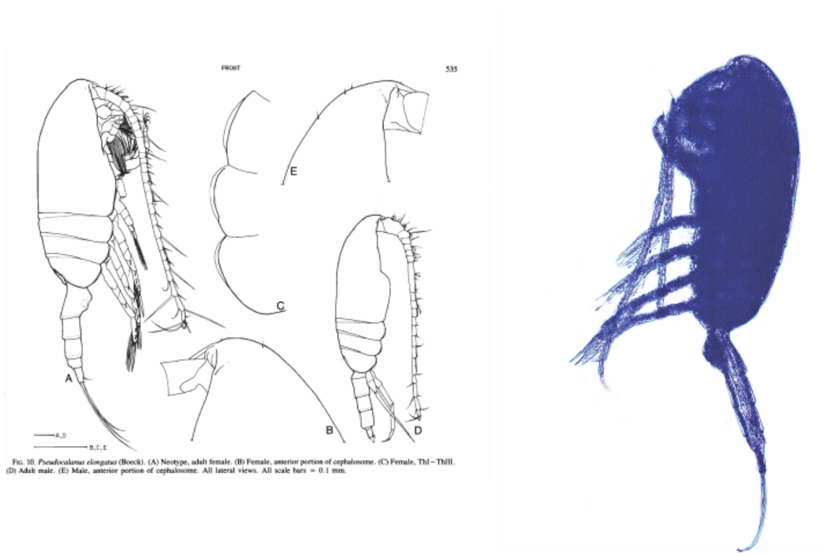

FIG. 10. *Pseudocalanus elongatus* (Boeck). (A) Neotype, adult female. (B) Female, anterior portion of cephalosome. (C) Female, ThI–ThIII. (D) Adult male. (E) Male, anterior portion of cephalosome. All lateral views. All scale bars = 0.1 mm.

Fig. 4. Adult female of *Pseudocalanus elongatus*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author.

Pseudocalanus acuspes Giesbrecht 1881

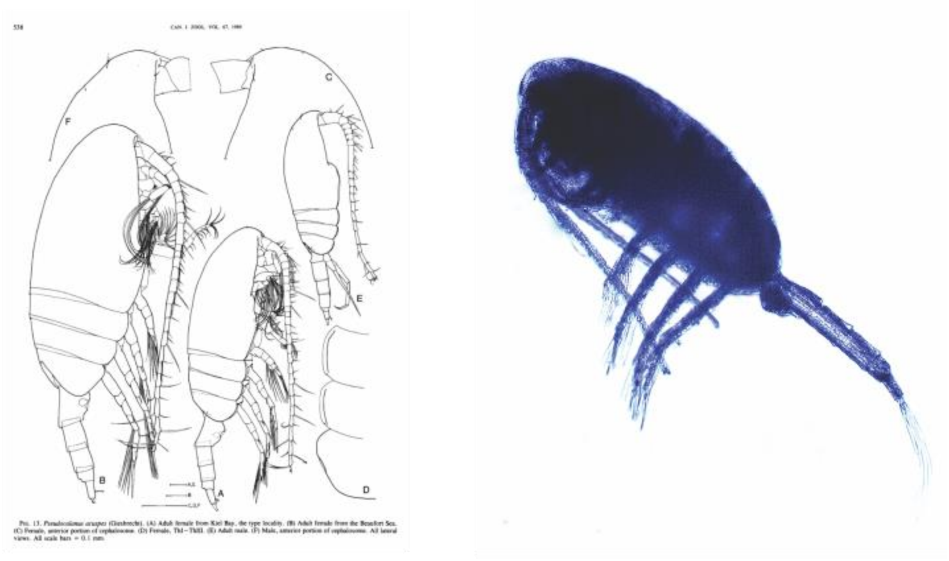

Fig. 5. Adult female of *Pseudocalanus acuspes*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author.

Pseudocalanus major G. O. Sars 1900

Fig. 6. Adult female of *Pseudocalanus major*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author. Note, the specimen is the lectotype selected by B. Frost, Cat. No. 2479a, Natural history museum, Oslo, Norway.

Pseudocalanus moultoni Frost 1989

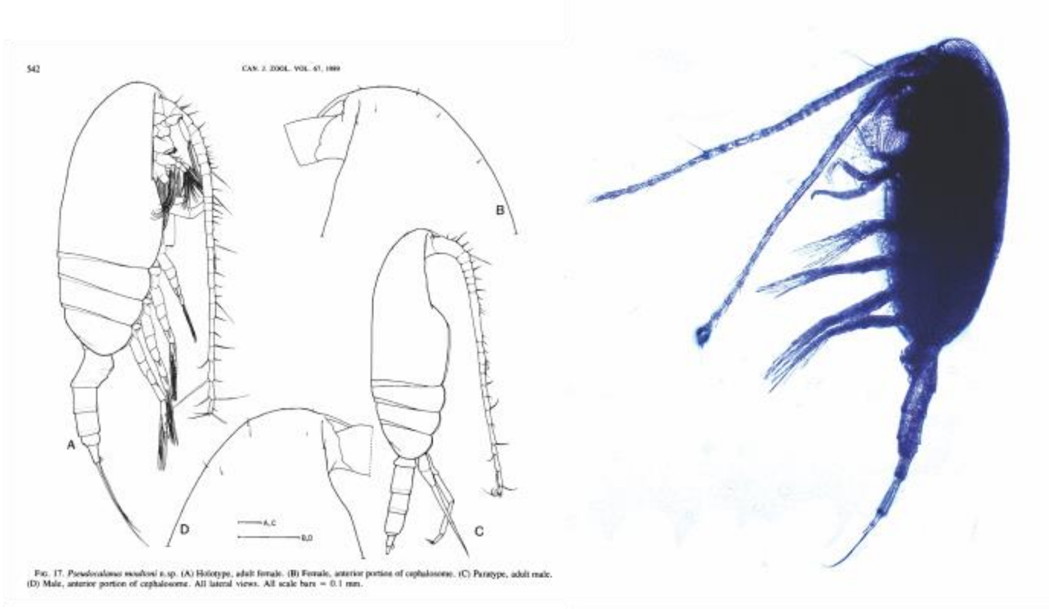
542 CAN. J. ZOOL. VOL. 67, 1989

FIG. 17. *Pseudocalanus moultoni* n.sp. (A) Holotype, adult female. (B) Female, anterior portion of cephalosome. (C) Paratype, adult male. (D) Male, anterior portion of cephalosome. All lateral views. All scale bars = 0.1 mm.

Fig. 7. Adult female of *Pseudocalanus moultoni*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author.

Pseudocalanus newmani Frost 1989

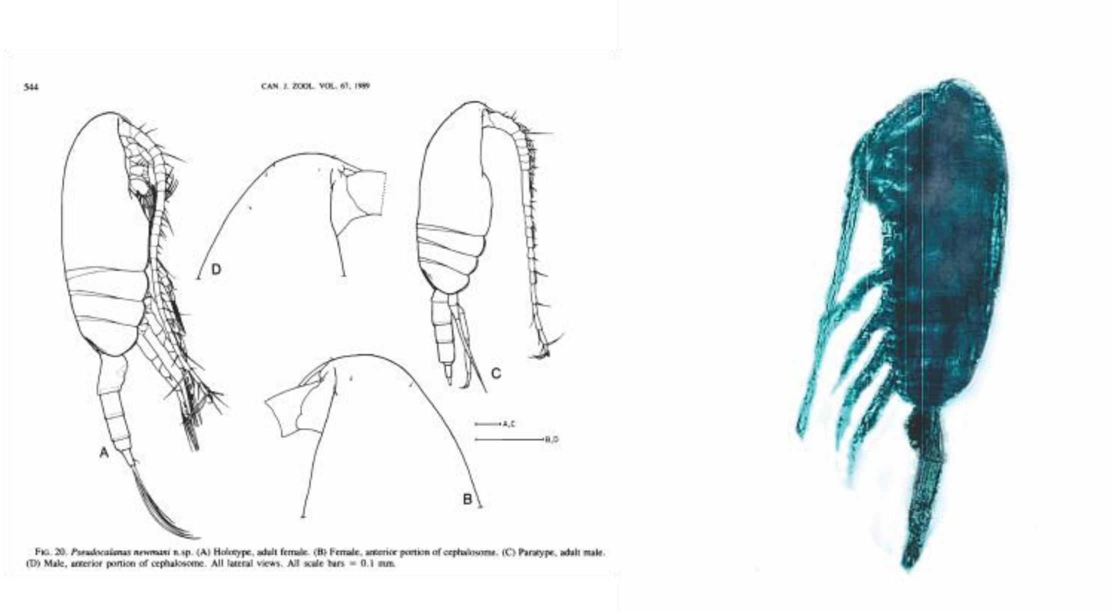
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FIG. 20. *Pseudocalanus newmani* n.sp. (A) Holotype, adult female. (B) Female, anterior portion of cephalosome. (C) Paratype, adult male. (D) Male, anterior portion of cephalosome. All lateral views. All scale bars = 0.1 mm.

Fig. 8. Adult female of *Pseudocalanus newmani*. Left, plate by Bruce Frost (*in* Can. J. Zool., 1989, **67**, 525-551). Right, picture by the author.

4. Discussion

4.1. The importance of accurate identification

“*The beginning of wisdom is to call things by their proper name*”

(Confusius 551 - 479 BC)

The importance of unambiguous identification is exemplified by all papers in this thesis. Each article expands the known ranges of one or two *Pseudocalanus* species. Papers I, II and III exposed that *P. moultoni* is widespread in the Northeast Atlantic and the Arctic. The number of locations in which *P. moultoni* has been reported was further expanded in Paper III, by the finding of this species in Northwest Icelandic waters (n = 1). The range of *P. elongatus* was, prior to this work, confined to waters south of 60 ° N (Frost, 1989; Renz *et al.*, 2008). It is now confirmed in Northeast Icelandic waters (67°19 N, 23°39 W, Paper III) and off the coast of Mid Norway (65°45 N, 10°04 E, Paper II). Twenty-five individuals in Paper II could not be unambiguously identified and were designated *Pseudocalanus* sp. However, based solely on morphological identification, I would say that the seven unidentified *Pseudocalanus* from Kongsfjorden 2009 (78°53 N, 12°27 E, Paper II) were *P. elongatus* as they had strongly tapered prosomes. Because this could not be confirmed with neither COI nor ITS 1, the nomen sp. was the correct designation.

4.2. Environmental preferences of *Pseudocalanus* spp.

The results from Paper II established that the distribution of *Pseudocalanus* species is strongly related to temperature, and in the case of *P. acuspes* also salinity, and thus water mass distribution.

Pseudocalanus elongatus was confirmed as the most thermophile of the *Pseudocalanus* species and with continued increasing sea temperatures, this species is likely to extend or move its range northward, thereby intruding on the domain of *P. minutus*.

Pseudocalanus minutus did in turn not show any temperature preference, though there might be an upper limit not detected in our study. This species was the overall most abundant in all three papers of this thesis and is a species that clearly tolerates a wide range of temperatures.

The most widespread species in the combined sampling locations from all three papers was *P. moultoni*. Like *P. minutus*, this species did not show any relationship with temperature and I therefore conclude that *P. moultoni* has the ability to endure the widest range of environmental conditions of all *Pseudocalanus* species.

Pseudocalanus acuspes prefers cold, arctic and less saline waters which were noted in Kongsfjorden, and in particular in Hornsund (Paper II). Our results imply that increasing sea temperatures will most severely affect this species. A study of the life cycle of *P. acuspes* in the Baltic Sea supports this conclusion, as Renz and Hagen (2006) found a significant negative correlation between all developmental stages and temperature. The copepods avoided the high temperatures by staying below the summer thermocline.

4.3. Advances in the biogeography of *Pseudocalanus* spp.

The work in this thesis has extended the known geographical distribution of *P. moultoni* and *P. elongatus*. Prior knowledge held that *P. moultoni* consisted of two geographically isolated populations on the East and West coasts of North America (Frost, 1989). We now know that this species is also present in Iceland, the southern and northern Norwegian Sea, Balsfjorden (Norway), Håkøybotn (Norway), Hornsund, Van Mijenfjorden, Billefjorden, Kongsfjorden, and Rijpfjorden (Paper I, II and III). In short, it is everywhere.

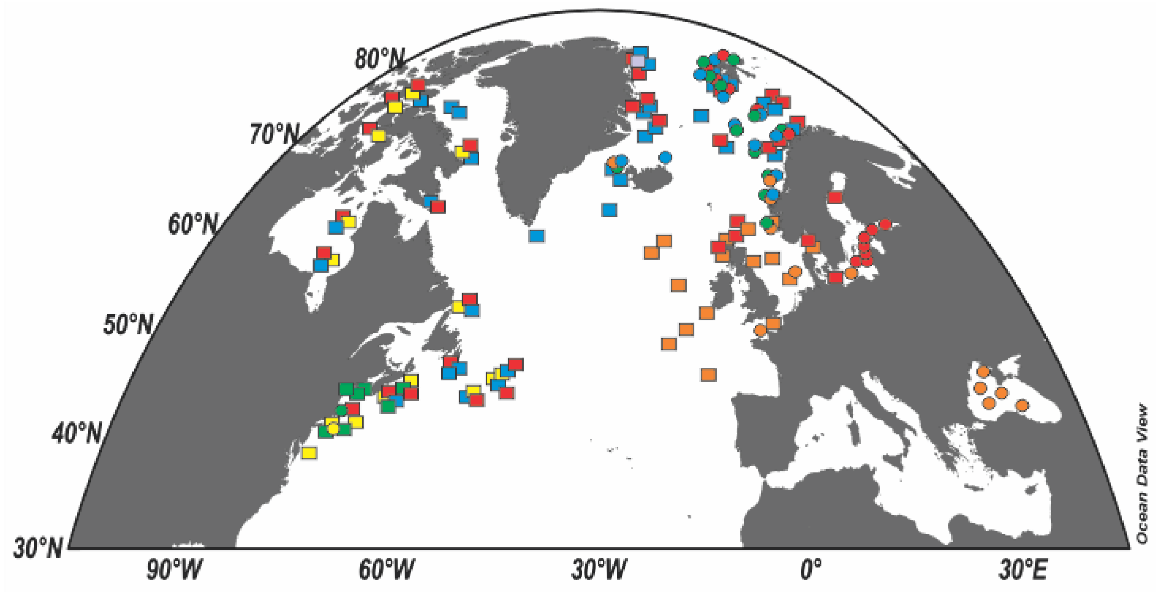

Fig. 9. Map of the North Atlantic Ocean showing the distribution of *Pseudocalanus* spp. Squares = Frost (1989). Circles = other studies (Aarbakke *et al.*, 2011; Paper II; Paper III; Bucklin *et al.*, 1998; 2001; Grabbert *et al.*, 2010; Holmborn *et al.*, 2011; Unal *et al.*, 2006). Blue = *P. minutus*, green = *P. moultoni*, orange = *P. elongatus*, red = *P. acuspes*, yellow = *P. newmani*, purple = *P. major*.

The presence and absence of *P. moultoni* can be nicely explained by influence of the North Atlantic Current and its extensions. Two sampled locations that did not have *P. moultoni* were the East coast of Iceland (Paper III) and Austfjord (Paper I), which is the innermost part of Wijdefjorden on Spitsbergen. The ocean circulation around Iceland is well studied and the Iceland station without *P. moultoni* is at an oceanographically complex spot that is primarily influenced by Arctic water (Jónsson, 2007), as opposed to the station northwest of Iceland where *P. moultoni* (n = 1) was found (Orvik and Niiler, 2002; Fig. 1). The absence of *P. moultoni* in Austfjorden, as discussed in Paper I, could be due to the fact that Austfjorden is separated from the Northern part of the fjord by a shallow sill that probably reduces the inflow of Atlantic water (Dale *et al.*, 2006). If *P. moultoni* is indeed an oceanic form (chapter 4.4), it is feasible that this species is readily transported throughout the North Atlantic Ocean by the North Atlantic Current.

The northern edge of *P. elongatus*` distributional range was believed to be ~ 60 ° N prior to the studies reported in Papers II and III (Frost, 1989; Renz *et al.*, 2008). The 2007 South (Paper II) and Northwest Iceland (Paper III) samples were taken at 65.45 and 67°19 N ° N, respectively. These records represent a 5.5 and 7.2 degree northward movement compared to published (Frost 1989) records. As discussed in Paper II, we suggest that the 80 % decrease in the biomass of *Para-Pseudocalanus* spp. from 2003 to 2011 in the North Sea (Fossum *et al.*, 2012) may represent a northward movement of this species` range. Furthermore, *P. elongatus* should be sought in Kongsfjorden, where I suspect it may be present at least occasionally. As hypothesised for *P. moultoni*, *P. elongatus*, if my assumption that it is oceanic (chapter 4.4) holds true, will also drift with the North Atlantic Current. Under favourable circumstances it could be advected all the way up to Kongsfjorden. This fjord is at times heavily influenced by Atlantic water, which is known to affect the zooplankton community (Cottier *et al.*, 2010; Kwasniewski *et al.*, 2010; Walkusz *et al.*, 2009; Willis *et al.*, 2008).

Pseudocalanus minutus was prior to this work known to occur extensively throughout the northern hemisphere (Frost, 1989; Fig. 9) and this still holds true. The results from Papers I, II and III show that *P. minutus* is the second most widespread *Pseudocalanus* species in the North Atlantic and the Arctic. It, like *P. moultoni* and *P. elongatus*, appears to be oceanic (chapter 4.4.) and associated with Atlantic, but also Arctic water and has its main distribution area north of 65 ˚ N in the East Atlantic.

4.4. Phylogeography of *Pseudocalanus* spp. in the North Atlantic Ocean.

The phylogeography of *Pseudocalanus* was, and still is, not adequately described, but paper III probes both this and the demographic history of five species of *Pseudocalanus* in the North Atlantic.

Owing to phylogenetic results for COI and ITS 1, similarities in intraspecific genetic structuring, interspecific phylogeography and demographic history of *P. minutus, P. moultoni, P. elongatus* and *P. acuspes* and *P. newmani*, we propose that the five species constitute two evolutionary clades. An oceanic clade consisting of *P. minutus*, *P. moultoni* and *P. elongatus* and a coastal clade constituted by *P. acuspes* and *P. newmani*. (Fig. 10). The biogeography outlined in chapter 4.3. supports this hypothesis.

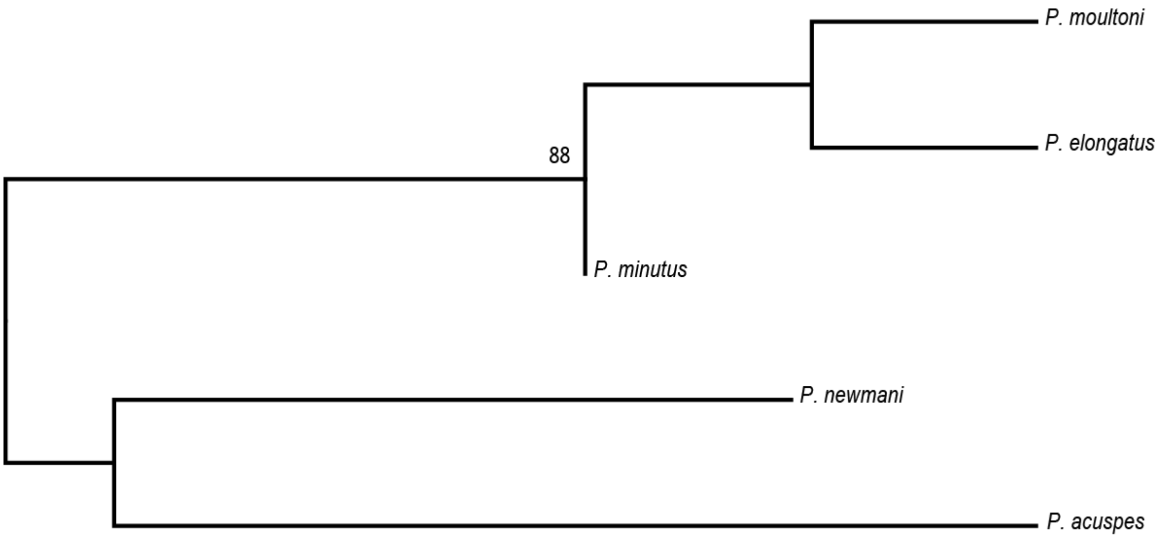

Fig. 10. Maximum likelihood tree based on ITS 1 sequence variation showing relationships between ITS 1 consensus sequences from five species of *Pseudocalanus*. Analysis used HKY (Hasegawa et al., 1985) gamma corrected. Bootstrap values (< 50, not shown) among 1000 subreplicates are shown at branchpoints (Felsenstein, 1985).

The distribution of *P. minutus* shows that this species is capable of surviving the open ocean environment (Fig. 9). The same could to some degree be said for *P. elongatus* (Fig. 9). Frost (1989) describes *P. moultoni* as a temperate coastal species, but paper I, II and III shows that this is clearly not the case. Furthermore, McLaren *et al*. (1989) describes the species as offshore, comparing it to the more coastal *P. newmani*.

We speculate that the driving force of this proposed divergence was differences in behavior between the ancestors of these two clades. In order to assess this hypothesis, investigations of species specific depth preferences and the diel and seasonal movements of *Pseudocalanus* spp. must be conducted.

4.5. Identifying *Pseudocalanus* in the Northeast Atlantic

"*Real discovery is by nature unpredictable*"

(Corkett and McLaren, 1978)

The initial objective of this thesis was to investigate the biology and ecology of *Pseudocalanus acuspes* and *P. minutus*, the two *Psedudocalanus* species at the time known to occur in fjords of northern Norway and the Svalbard archipelago. Because these species are difficult to distinguish from one another, the first step was the development of a multiplexed species-specific polymerase chain reaction (ssPCR) protocol for rapid and reliable identification of *P. acuspes* and *P. minutus*. However, this effort was abandoned for two important reasons. The first became evident upon realizing that *P. moultoni* was present in four out of five sampled fjords (Paper I). This discovery not only necessitated the inclusion of this species in the protocol, but also *P. elongatus*, *P. newmani* and possibly *P. major* if a reference sequence for the latter species could be obtained. Attempts at expanding the ssPCR protocol to include five species were made, but this exposed an important limitation of the 708 base pair (bp) fragment of the COI locus. In order for DNA fragments to be readily separated on an agarose gel, the fragments need to be of different size, preferably 100 bp difference. It follows that for a five taxa ssPCR using COI there must be a unique primer site every 162 bases, and in the case of *Pseudocalanus* there is not. Secondly, as I started to use samples collected at different points in time, preserved by various people and stored in a variety of ways it became apparent that the mitochondrial genome is rapidly degraded by both time and more critically, suboptimal preservation and storage. I experienced comparatively more success with 10 year old samples treated in accordance with Bucklin (2000) and stored away from light than with two month old samples not properly preserved.

The low success rate with amplification of COI in some samples required additional approaches to species identification. The nuclear internal transcribed spacer region 1 (ITS 1) of ribosomal DNA proved easier to amplify, but required more careful examination of the sequences to assign a species name. This applied in particular to distinguish *P. elongatus* from its close relative *P. moultoni* (Fig. 10).

The morphological approach by Frost (1989) is in my opinion sufficient, in the majority of cases, to separate female *P. minutus* from females of all other *Pseudocalanus*, particularly if it is combined with morphometric measurement of prosome/urosome length ratio (PUR). The long spiniform processes on the posteroventral margins of thoracic segments I and II are diagnostic of *P. minutus* and a feature only partially shared by *P. mimus*, in which these spiniform processes are shorter or absent. Because the detection of the spiniform processes requires high magnification, I suggest that for higher throughput the anteriorly protruding cephalosome relative to the rostrum and often slender body shape, combined with a relatively high PUR will be more than a strong indication that the species is *P. minutus*.

Distinguishing the three other species that co-occur with *P. minutus* in the Northeast Atlantic from one another is more difficult. Of *P. acuspes*, *P. elongatus* and *P. moultoni*, Frost (1989) writes:

> "*Adults have exceedingly similar morphology. Were it not for evidence of genetic isolation (...) that parallels the seemingly slight morphological differences I observed, I would be inclined to ascribe all three populations to a single, widely distributed and morphologically variable species*".

However, certain morphologic and morphometric features are indicative of each of these three species. The cephalosome of *P. elongatus* is strongly tapered anteriorly (Frost, 1989) and the body shape is often somewhat less stocky in lateral view compared to *P. acuspes* and *P. moultoni*. *Pseudocalanus acuspes* is typically stocky with a rounded cephalosome, particularly in large individuals. *Pseudocalanus moultoni* is in my opinion the most morphologically variable of all *Pseudocalanus* species. It can be very stocky, intermediately stocky or even slender, but individuals from the same sample tend to look similar. If a zooplankton sample containing the typical *P. acuspes/ P. moultoni* body type exhibits two or more morphotypes, plotting the PUR of the individuals can indicate the presence of *P. moultoni*. Nevertheless, as shown in Fig. 11 there is considerable overlap and the only way to be certain is through DNA barcoding. Consequently, published literature of in particular *P. acuspes* from this geographical area should be cautiously interpreted.

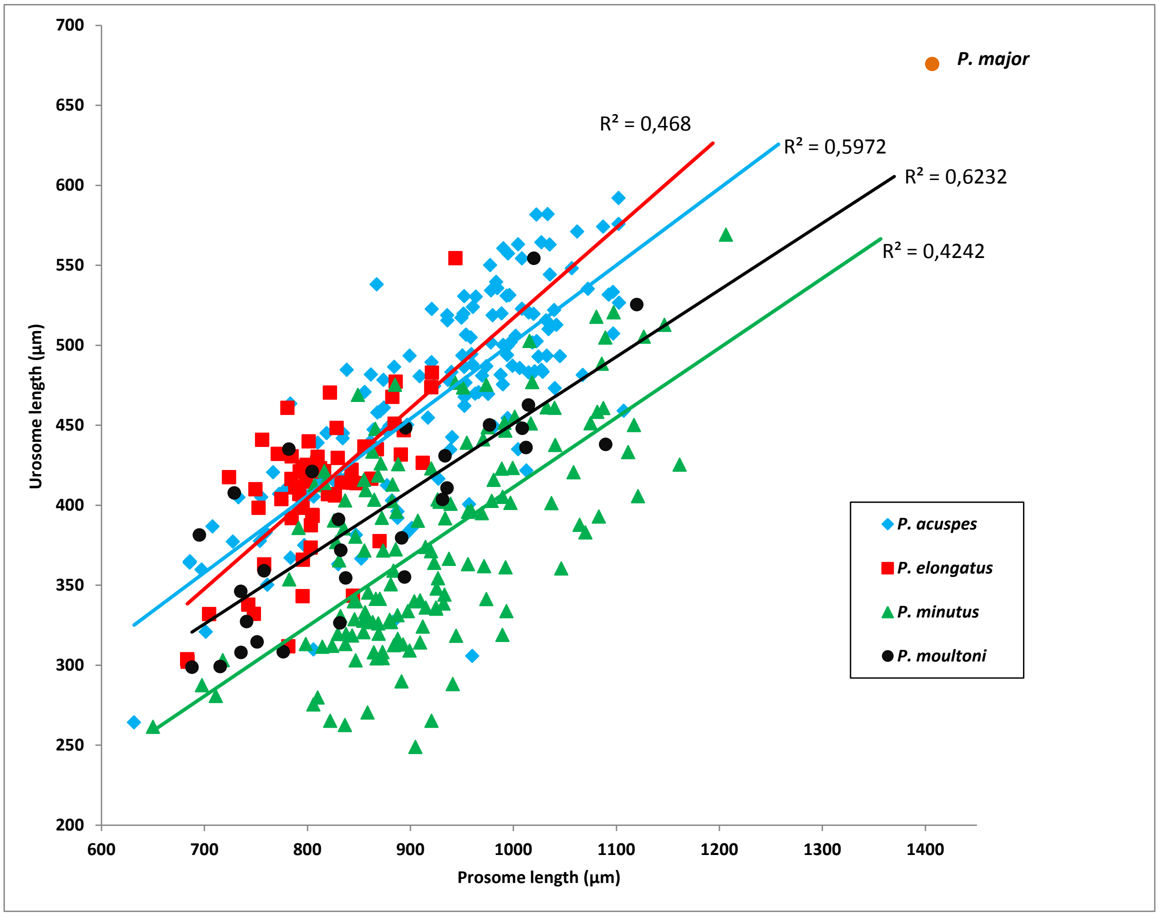

Fig. 11. Scatter plot showing the relationship between prosome and urosome length for 387 *Pseudocalanus* spp.

4.6. How many species do we need to look for in the Northeast Atlantic?

After the above discussion it is evident that the scientist working with *Pseudocalanus* at the species level in the Northeast Atlantic and Euro-Arctic, need to be aware of and look for *Pseudocalanus minutus*, *P. acuspes*, *P. moultoni* and *P. elongatus*. But what of *P. newmani*, *P. mimus* and *P. major*? The total number of *Pseudocalanus* spp. identified during my thesis work is 1446 collected from 31 samples, the vast majority from the Northeast Atlantic and Euro-Arctic. None of these three species were among them. That is not to say that they cannot occur in the Northeast Atlantic. However, my best guess for

P. newmani and *P. mimus* would be no, unless they are transported by ballast water, a scenario increasingly plausible with increased shipping in the Arctic. *Pseudocalanus newmani* appears to be, and is described by McLaren *et al.* (1989) as, a coastal species and will therefore be confined to the East and West coasts of North America. *Pseudocalanus mimus* is “essentially restricted to the eastern North Pacific Ocean and Bering Sea” (Frost, 1989) and would as such have undertaken a very long and cold journey if it were to appear in the Northeast Atlantic.

Pseudocalanus major was and still is a mystery. Frost (1989) had very little *P. major* material, considered records of this species not accompanied by illustrations or specific descriptions unreliable, and suggested that the species should be sought in fjords of Spitsbergen. During my PhD work, I have not seen anything resembling the animal in Fig. 6. Apart from the obvious difference in size, this lectotype is strikingly similar morphologically to G.O. Sars` syntype of *P. elongatus* (collected by Boeck in Oslofjord; Fig. 11). *Pseudocalanus major* was, in the words of G.O. Sars:

"*so very resembling the type species* [meaning *P. elongatus*] *that I should have been very much inclined to regard it as only a large variety if both forms were not found together in the very same samples, without exhibiting any transitions*". (Sars, 1900)

The prosome lengths of *P. major* (Fig. 6; Fig. 11) and *P. elongatus* (Fig. 12) are 1417 and 909 µm, respectively. This makes *P. majors* prosome 56 % longer than that of *P. elongatus*. Temperature is known to influence growth, and within sample size variability is a hallmark of *Pseudocalanus* species. The largest within sample difference in prosome length I have found was 43 % for *P. minutus* in an Iceland sample. The sample G. O. Sars was referring to was collected at ~78 ° N. Was this indeed *P. elongatus* on an extreme northern journey, or was G.O. Sars inadvertently looking at a *P. moultoni* which at the time was not described? The small size indicates that whichever the species, it was an expatriate from warmer waters.

Fig. 12. Adult female *Pseudocalanus elongatus* Boeck 1865 (reexamined and relabeled by G.O. Sars). Syntype, filed under Cat. No. F16550, Natural History Museum, Oslo, Norway.

Because the chances of acquiring a DNA sequence from 116 year old museum specimens are less than slim (“my best guess would be no”, Ann Bucklin; personal communication), the validity of *P. major* is still an unresolved question, but if my time as a PhD candidate has taught me anything it is that *Pseudocalanus* are, borrowing the words of Ann Bucklin, “tricky beasts” and that their identity and distributions should never be taken for granted.

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Paper 1

Paper 2

Paper 3

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