Discovery of *Pseudocalanus moultoni* (Frost, 1989) in Northeast Atlantic waters based on mitochondrial COI sequence variation

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The genus *Pseudocalanus* (Copepoda, Calanoida) consists of seven species, all of which are known to co-occur with two or more sibling species in some areas of their geographic ranges. Despite the ecological importance of this abundant genus, there is no available method that can reliably and accurately identify *Pseudocalanus* species without knowledge of origin. We present evidence of several observations of *Pseudocalanus moultoni* [Frost (1989) *Can. J. Zool.*, 67, 525–551] in fjords of Svalbard and northern Norway; this species has previously been known to occur only on the east and west coasts of North America. Patterns of DNA sequence variation of the mitochondrial cytochrome oxidase subunit I (COI) gene allow us to confidently identify the species, discriminate it from co-occurring sibling species and infer relationships among the newly discovered and previously sampled *P. moultoni* populations. Our observations suggest that NE Atlantic populations of *P. moultoni* are self-sustaining and we discuss potential source populations and pathways of transport. In light of recent reports of climate-driven shifts in distributional ranges of marine zooplankton, accurate species identification is essential for monitoring and understanding marine ecosystems.

KEYWORDS: *Pseudocalanus moultoni*; COI; species identification; biogeography

INTRODUCTION

Marine copepods of the genus *Pseudocalanus* Boeck 1872 are very common throughout the northern hemisphere (Pendleton et al., 2009; Beaugrand and Kirby, 2010; Beaugrand et al., 2010). These species are of major importance for marine food webs in temperate and Arctic oceanic domains (Heath and Lough, 2007). At present, seven species are known, all of which co-occur with two or more sibling species in some areas of their geographic ranges (Frost, 1989). Species level identification of *Pseudocalanus* is especially challenging due to subtle interspecific divergence of morphological and morphometric traits (Frost, 1989). Prosome length, which has been used to separate sibling species of other calanoid genera (e.g. *Calanus finmarchicus* and *C. glacialis*; Kwasniewski et al., 2003; Arnværn et al., 2005), is not useful for *Pseudocalanus* species. Size differences between the species are not diagnostic and growth is influenced by temperature (Corkett and McLaren, 1978) and food conditions (Ban, 1994; Lee et al., 2003). Owing to their difficult identification, these species are often classified as *Pseudocalanus* spp. (e.g. McGillicuddy et al., 1998;
Sell et al., 2001; Beaugrand et al., 2003; Liu and Hopcroft, 2008) and on occasion pooled together with Paracalanus (e.g. Heath and Lough, 2007; Fileman et al., 2007). Consequently, we lack information regarding species-specific differences in distribution, habitat preferences, life history and population dynamics. Accurate characterization of species-level zooplankton diversity for ecologically important genera, such as Pseudocalanus, will significantly improve the accuracy and forecasting power for monitoring and modeling marine ecosystems.

The confusing taxonomy of Pseudocalanus prevents accurate description of the geographic distributions of the seven species. Based on current understanding, their biogeography in the Atlantic Ocean and adjacent seas can be summarized as follows: in the NW Atlantic Ocean (i.e. Gulf of Maine and Georges Bank), P. newmani (Frost, 1989) and P. moultoni (Frost, 1989) co-occur (Frost, 1989; Bucklin et al., 1998, 2001). Further north at Chebucto Head and in the Bedford Basin, P. acuspes Giesbrecht 1881 is present and may merge with the northern distributional boundary of P. moultoni (Sévigny et al., 1989). The genus is represented by two species in the North Sea (Frost, 1989), with P. acuspes being most common in the northern part, while P. elongatus Boeck 1865 predominates farther south (Renz et al., 2008). Pseudocalanus acuspes is considered the sole species in the Baltic Sea (Peters et al., 2006; Renz and Hirche, 2006; Grabbert et al., 2010; Holmborn et al., 2010). Svalbard and North Norwegian coastal waters are inhabited by P. acuspes and P. minutus Kroyer 1845 (Frost, 1989; Lischka and Hagen, 2005). Frost (Frost, 1989) also suggested the presence of P. major Sars, G.O. 1900 in estuarine arctic fjords. In the White Sea, P. minutus is most abundant (Lukashin et al., 2003; Dvoretsky and Dvoretsky, 2011) and in the Kara Sea, P. major and P. acuspes co-occur (Fetzer et al., 2002). Correct identification of sibling species is paramount if the above description of the biogeographical distribution of Pseudocalanus spp. is to be confirmed (McManus and Katz, 2009).

The advent of molecular methods, such as DNA barcoding (i.e. use of DNA sequences to recognize and discriminate species; Hebert et al., 2003), has made accurate identification of copepod species achievable, despite the subtle morphological differences within sibling species groups. A number of studies using DNA sequence information have confirmed large divergences and diagnostic genetic characteristics among sibling species of calanoid copepod genera (e.g. Bucklin et al., 2003, 2010a, b). Molecular methods of species identification have been applied to distinguish between Pseudocalanus species for about two decades. Sévigny et al. (Sévigny et al., 1989) found allozymic variation at the glucose phosphate isomerase (GPI) locus which supported the taxonomy proposed by Frost (Frost, 1989) for P. minutus, P. newmani, P. moultoni and P. acuspes. Bucklin et al. (Bucklin et al., 1998) investigated distribution and relative abundance of P. moultoni and P. newmani on Georges Bank through sequencing of mitochondrial cytochrome oxidase subunit I (COI) and 16s ribosomal RNA genes. Holmborn et al. (Holmborn et al., 2010) used restriction fragment length polymorphism and found P. acuspes to be the sole species throughout the Baltic Sea. Species-specific polymerase chain reaction (ssPCR) has proved both reliable and effective to distinguish between P. moultoni and P. newmani (Bucklin et al., 2001) and P. acuspes and P. elongatus (Grabbert et al., 2010). However, these studies targeted only 1–2 species that were expected to be found in the study area based on morphological identification or historical data. There is no approach available to date that is fully successful in identifying and discriminating Pseudocalanus species without knowledge of origin.

While working to resolve the relative abundances and distribution of P. acuspes and P. minutus in Svalbard and Norwegian waters through sequencing of COI genes, we discovered a species not normally found in Norwegian waters and the European Arctic. We present evidence here of several observations of P. moultoni in fjords of Svalbard and northern Norway, a species that is abundant on the east and west coasts of North America. We suggest potential pathways for the arrival of P. moultoni in the NE Atlantic and discuss the relationship among the different populations.

METHOD

Samples were collected in Balsfjord, northern Norway in December, 2008 and in four fjords of the Svalbard archipelago from March through May, 2007 (Fig. 1; Table 1). At all stations, a WP-2 net was hauled vertically at 0.2 ms⁻¹ (UNESCO, 1968) and samples were preserved immediately in 95% ethanol and treated according to published protocols (Bucklin, 2000).

DNA extraction and sequencing

A total of 307 specimens of Pseudocalanus spp. was randomly selected (i.e. first encountered under a dissecting microscope) from zooplankton samples collected from Balsfjord, Norway and four fjords of Svalbard (Fig. 1; Table 1). Sample sizes differed due to variation in catch. With the exception of 12 (of 20) individuals from Balsfjord, which were juveniles in the fifth copepodite stage (CV), all Pseudocalanus spp. analyzed were adult
DNA was extracted from individual copepods using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). A 709 bp region of the COI gene was amplified using the consensus invertebrate primers LCO-1490: 5'-GGTCAACAAATCATCAAAGATATTG G-3' and HCO-2198: 5'-TAAACTTCAGGGTGACCAAAAAATCAA-3' (Folmer et al., 1994). The PCR was run for 35 cycles. Total reaction volume was 50 µL and the PCR protocol was 95°C for 1 min, 50°C for 2 min and 72°C for 3 min. The products were purified with the PGR Purification Kit (Qiagen) and cycle sequenced using the BigDye Ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., ABI). Sequencing was performed on an ABI 3130 Genetic Analyzer. The sequencing protocol was 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The sequences were edited manually using Sequencher (Gene Codes Corp.).

Genetic analysis

Sequence alignment and analysis was done using ClustalW (Thompson et al., 1994). The sequences were manually edited, carefully scrutinized for intraspecific variation and ambiguous bases were kept as unknowns. DNA sequences were identified to species based on BLAST searches of the NCBI GenBank database (Altschul et al., 1997). Multiple alignments were done including our sequences of *P. moultoni* collected from the NE Atlantic, previously unpublished sequence data for *P. moultoni* from Georges Bank in the NW Atlantic and Puget Sound in the NE Pacific (GenBank Acc. Nos. HQ219011-HQ29033), as well as sequences for five other *Pseudocalanus* species (Bucklin et al., 2003). Owing to use of various sequencing primers, the comparison was based on an aligned region of 278 bp in length. PCR and sequencing success rates varied among the samples analyzed (Table I). Low success rates in some samples were attributed to poor sample preservation and the frequencies of COI haplotypes detected were considered to accurately reflect the genetic diversity within and between species of *Pseudocalanus* in the areas sampled. Analysis of molecular variance (AMOVA; Excoffier et al., 1992) and population pairwise genetic distances were calculated in Arlequin Ver. 3.5.1.2. (Excoffier and Lischer, 2010). Wright’s $F_{ST}$ (Wright, 1951) was calculated using algorithms detailed in Weir and Cockerham (Weir and Cockerham, 1984) as an index of genetic differentiation among six populations of *P. moultoni* (Balsfjord, Van Mijenfjord, Billefjord, Fig. 1. (Colour) Map of the Svalbard archipelago and the northern coast of Norway showing the sampling sites.

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Lat (N)</th>
<th>Long (E)</th>
<th>Date</th>
<th>Depth (m)</th>
<th>Individuals sequenced</th>
<th><em>P. moultoni</em></th>
<th><em>P. acuspes</em></th>
<th><em>P. minutus</em></th>
<th>Failed sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rijpfjord</td>
<td>80.15954</td>
<td>22°17.397</td>
<td>06.05.2007</td>
<td>50–0</td>
<td>79</td>
<td>3</td>
<td>14</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Van Mijenfjord</td>
<td>77°50.942</td>
<td>16°43.233</td>
<td>12.03.2007</td>
<td>50–0</td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Austfjord</td>
<td>78°59.302</td>
<td>16°11.430</td>
<td>28.03.2007</td>
<td>50–0</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>54</td>
<td>36</td>
</tr>
<tr>
<td>Billefjord</td>
<td>78°39.683</td>
<td>16°44.268</td>
<td>29.03.2007</td>
<td>150–0</td>
<td>110</td>
<td>2</td>
<td>0</td>
<td>92</td>
<td>16</td>
</tr>
<tr>
<td>Totals</td>
<td>307</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18</td>
<td>21</td>
<td>179</td>
<td>89</td>
</tr>
</tbody>
</table>
Rijpfjord, Georges Bank, Puget Sound) and the published sequences for *P. acuspes, P. minutus, P. newmani* and *P. mimus* (Bucklin et al., 2003). Transformations were applied to linearize distance with population divergence time (Reynolds et al., 1983; Slatkin, 1995). Under the hypothesis of no difference between the populations, the null distribution of $F_{ST}$ values was obtained from 50 000 haplotype permutations among the populations. The $P$-value is the proportion of permutations leading to a $F_{ST}$ value larger or equal to the observed one (Excoffier and Lischer, 2010). To correct for multiple tests, a sequential Bonferroni correction was done (Holm, 1979) at 0.0001, 0.01 and 0.05 significance levels. Phylogenetic analyses were performed in Molecular Evolutionary Genetic Analysis (MEGA) Ver. 4 (Tamura et al., 2007). Phylogenetic trees were constructed using parsimony, maximum likelihood, and distance-based methods. The distance-based tree used the Neighbour Joining algorithm (Saitou and Nei, 1987) to analyze evolutionary relationships based on COI variation within *P. moultoni*, including the five haplotypes found in this study, and the most common haplotypes each of the Georges Bank and Puget Sound collections (Bucklin et al., 2003; unpublished data). To test the reliability of inferred trees, we used Felsenstein’s (Felsenstein, 1985) bootstrap test with 1000 replicates. A gene genealogy analysis was done using the TCS software (Clement et al., 2000), which uses the TCS (Templeton, Crandall and Sing) (Templeton et al., 1992) method for inferring population level genealogies when genetic divergence is low.

### RESULTS

Based on COI sequence variation for a total of 307 individuals of *Pseudocalanus* spp., 18 individuals were identified as *P. moultoni* (6 CV; 12 females); 21 were shown to be *P. acuspes* (3 CV; 18 females); 179 were identified as *P. minutus* (all female; Table I). A total of 89 specimens did not yield sequences; low success rates were attributed to poor preservation of some samples. In the only Norwegian fjord sampled, Balsford, 6 of 20 individuals were shown to be *P. moultoni* (all CV) and 7 were *P. acuspes* (three CV; four females). In all, *P. moultoni* was found in three Svalbard fjords, but not found in Austfjord (Fig. 1; Table I). *Pseudocalanus moultoni* co-occurred with *P. minutus* in Billefjord and with both *P. acuspes* and *P. minutus* in Rijpfjord. In Van Mijenfjord, *P. moultoni* was the only *Pseudocalanus* species found ($n = 7$), while the sample from Austfjord contained only *P. minutus* ($n = 54$).

### Table II: Outline of five *P. moultoni* haplotypes found in North Norwegian (H1–H2) and Svalbard (H3–H5) fjords, the base substitutions distinguishing the haplotypes and their respective GenBank accession numbers.

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Position number/ haplotype number</th>
<th>10</th>
<th>119</th>
<th>244</th>
<th>278</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ706659</td>
<td>H1</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>HQ706660</td>
<td>H2</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HQ706661</td>
<td>H3</td>
<td>C</td>
<td></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>HQ706662</td>
<td>H4</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>HQ706663</td>
<td>H5</td>
<td>C</td>
<td>A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Among 18 *P. moultoni* sequences obtained in this study, there were four polymorphic sites along the 278 bp analyzed region, and five haplotypes were identified (H1–H5; Genbank Acc. Nos. HQ706659-HQ706663; Table II). Two sets of haplotypes were distinguished. Haplotypes 1 ($n = 3$) and 2 ($n = 1$) were unique to Balsfjord. Haplotypes 3, 4 and 5 were only present in Svalbard fjords. Haplotype 3 was found in Van Mijenfjord ($n = 4$) and Rijpfjord ($n = 1$), as was haplotype 4 with one occurrence in each location. Haplotype 5 was unique to Rijpfjord ($n = 1$). Six sequences had ambiguous bases and were omitted from this analysis.

The genetic relationship among the six *P. moultoni* populations was examined by calculating population pairwise genetic distances. The resulting $F_{ST}$ values indicated that the NE and NW Atlantic populations of *P. moultoni* are isolated from the NE Pacific population ($F_{ST}$ ranged from 0.7020, $P = 0.000–0.9484$, $P = 0.0022$; Table III). With the exception of individuals from Balsfjord and Billefjord, all *P. moultoni* from the Atlantic Ocean were more genetically similar to each other than they were to individuals in the Pacific Ocean (Table III). The closest genetic relationship with Pacific populations (Puget Sound) was found for individuals from Balsfjord ($F_{ST} = 0.7020$, $P = 0.000$). In turn, individuals from Georges Bank are genetically more similar to the North Norwegian animals (Balsfjord, $F_{ST} = -0.0381$, $P = 0.7178$) than to specimens from Svalbard ($F_{ST}$ ranging from 0.6632, $P = 0.2701–0.2804$, $P = 0.000$). The smallest genetic distances were found between the Svalbard populations (Table III).

A hierarchical AMOVA was done by dividing the *P. moultoni* populations into four groups: Norway ($n = 6$ samples), Svalbard ($n = 12$), Georges Bank ($n = 18$) and Puget Sound ($n = 6$). A large portion of the variance was attributed to differences among the groups (51.30 %, $P < 0.0001$), with similar level among populations.
within groups (45.97%, \( P < 0.0001 \)). The within population variance was 2.73%, \( P < 0.0001 \).

Molecular phylogenetic evaluation of *Pseudocalanus* species based on COI sequences supported the taxonomy suggested by Frost (Frost, 1989) and was in concordance with the phylogeny reported by Bucklin et al. (Bucklin et al., 2003; Fig. 2). The phylogenetic trees resulting from all analyses separated the NE Pacific sample of *P. moultoni* (>95% bootstrap support) from both the NE and NW Atlantic samples. The Neighbour Joining tree closely grouped the NW Atlantic and NE Atlantic haplotypes (86% bootstrap support; Fig. 2). The haplotype network (Fig. 3) indicated a closer relationship among NW and NE Atlantic haplotypes compared with NE Pacific haplotypes. The Puget Sound individuals were separated from all Atlantic individuals by 6 \(( n = 4 \) ) and 7 \(( n = 2 \) ) substitutions. Furthermore, the network indicated a close connection between the northern Norway and NW Atlantic haplotypes (Fig. 3).

**DISCUSSION**

The importance of unambiguous identification of copepod species, as exemplified by these findings, is
occurrence of *P. moultoni* observations. Patterns of DNA sequence variation of a coasts of North America are.

Previous records of this species on the east and west of Rijpfjorden, which is considered a true Arctic fjord. in three fjords of the Svalbard archipelago, including 6 from Balsfjorden, a Norwegian fjord, and another 12 samples from Norwegian and European Arctic waters: Atlantic Ocean. A total of 18 individuals were found in plankton taxa (Hays et al., 2005; Edwards, 2009). Zooplankton are put forward as “beacons of climate change” (Richardson, 2008) because populations respond quickly to very slight changes in temperature, thereby amplifying weak environmental signals (Taylor et al., 2002). Shifts in abundance and distribution of these species have been interpreted as indicators of a system either in transition (Hays et al., 2005) or having crossed a threshold (Beaugrand et al., 2008, 2009).

Our results confirm the widespread and frequent occurrence of *P. moultoni* in coastal locations in the NE Atlantic Ocean. A total of 18 individuals were found in samples from Norwegian and European Arctic waters: 6 from Balsfjorden, a Norwegian fjord, and another 12 in three fjords of the Svalbard archipelago, including Rijpfjorden, which is considered a true Arctic fjord. Previous records of this species on the east and west coasts of North America are >5000 km from these observations. Patterns of DNA sequence variation of a short portion of the COI gene allow us to confidently identify the species, discriminate it from a co-occurring sibling species and infer relationships among the newly discovered NE Atlantic specimens and previously sampled *P. moultoni* populations in the NW Atlantic and NE Pacific Oceans.

It is possible that *P. moultoni* is a native species of NE Atlantic waters that prior to this study has been misidentified as *P. acuspes*. Owing to their remarkably similar morphology, defending *P. moultoni* and *P. acuspes* as reproductively isolated populations was the biggest challenge for Frost (Frost, 1989), and he found support for his conclusion in strong and consistent differences at the GPI locus (Sévigny et al., 1989). There are no diagnostic morphological criteria to distinguish adult females of *P. acuspes* and *P. moultoni*, but these are among the most genetically divergent of *Pseudocalanus* species (Sévigny et al., 1989; Bucklin et al. 2003; Fig. 2). Because studies identifying *Pseudocalanus* species have frequently relied on sparse and patchy descriptions of distribution and disregarded other possibilities, we cannot know when *P. moultoni* may have first arrived in the NE Atlantic. The species may have dispersed both east and west in the Atlantic during the ice retreat following the last glacial maximum some 8000 years ago, or *P. moultoni* may be relatively new to the eastern Atlantic. In the latter case, anthropogenic pressure through ballast water introduction or a warming ocean may have aided the dispersal.

The limited number of sequences available for this study prevents us from drawing definitive conclusions regarding timing of introduction and transport pathway. However, based on the data we analyzed here, it seems likely that *P. moultoni* may have been advected across the Atlantic Ocean from the Gulf of Maine with the North Atlantic Current (NAC). Our genetic results, showing a closer relationship between NE and NW Atlantic individuals than between Atlantic and NE Pacific ones (Table III) support this transport pathway. Furthermore, the smaller genetic distance between Norway and Georges Bank individuals compared with Norway and Svalbard individuals (Table III) suggests the possibility of regular and recurring input of *P. moultoni* into the Norwegian Sea and the Arctic via the NAC. Physical oceanographic data from Kongsfjorden, Svalbard, showed that winter and spring of both 2005 and 2006 were characterized by inflow of unusually warm Atlantic Water (Cottier et al., 2007). *Pseudocalanus moultoni* could have drifted with the NAC into Norwegian and Svalbard fjords during these anomalous years. Of the four areas sampled, the one lacking *P. moultoni*, Austfjord, is the innermost part of Wijdefjord, the longest fjord on Svalbard. The oceanography of these fjords in not well studied, but Austfjord and Wijdefjord
are separated by a shallow sill that probably reduces the inflow of Atlantic water (Dale et al., 2006). It is possible that the absence of *P. moultoni* in Austfjord could be due to limited Atlantic water inflow.

Another hypothesis, although less likely based on our genetic data, is that *P. moultoni* came to the NE Atlantic from the NE Pacific via the Arctic Ocean. This route is now possible due to diminishing summer sea ice and has been proposed for *Calanus marshallae* (Sundt and Melle, 1998) and the Pacific diatom *Neodenticula seminae* (Reid et al., 2007). It is also possible that *P. moultoni* arrived in northern Norway and Svalbard by means of ballast water, which is a known dispersal mediator of copepods (e.g. Williams et al., 1988; Cordell et al., 1992). The North Atlantic route linking Western Europe and the USA and Canada is one of the busiest international shipping routes (Slack, 1999).

Despite the uncertainty regarding mechanism and timing of transport, our discovery of *P. moultoni* in two different years and in several locations spanning a large geographical area suggests that local populations are presently maintained. *Pseudocalanus* sp. have a life span of maximum one year (Norrbin, 1991). Thus, finding *P. moultoni* in two successive years implies either repeated introduction or successful reproduction. Because the sampling locations where the species were found are separated by large distances, we propose that local populations are maintained. Moreover, all Svalbard *P. moultoni* were mature females, suggesting ongoing or recent reproduction (Norrbin, 1991). The species could now be established in the NE Atlantic and may co-occur extensively with *P. minuta* and *P. acuspes*, and possibly with *P. elongatus*, the dominant *Pseudocalanus* species in the southern N. Atlantic (Renz et al., 2008; Eloire et al., 2010).

The genus *Pseudocalanus*, comprising seven species with overlapping distributions covering the northern hemisphere, including five in the N. Atlantic, is potentially valuable as a set of indicator species for ecosystem monitoring. Toward this end, a rapid, inexpensive and reliable molecular protocol for identification and discrimination of all *Pseudocalanus* species is needed. Existing PCR-based protocols can discriminate only two or three species (Bucklin et al., 1998, 2001; Grabbert et al., 2010). A single species-specific multiplexed PCR reaction for all species would be useful for regular assessments of *Pseudocalanus* species and would allow detection of the full complex of species throughout the North Atlantic Ocean and adjacent seas. For a comprehensive phylogeographic assessment of *Pseudocalanus* spp., including disentangling the timing and introduction pathway of *P. moultoni* to the NE Atlantic, additional genes should be included in the analysis. Integrated morphological, molecular, ecological and biogeographical analyses are needed to ensure the accurate discrimination and identification of all *Pseudocalanus* species, even those that are unexpected, previously unreported or introduced, or whose distributional patterns may have been altered by human activities and/or climatic variation.

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