

A first report on coexistence and hybridization of *Mytilus trossulus* and *M. edulis* mussels in Greenland

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Abstract Semi-sessile *Mytilus* mussels are used as indicators of climate changes, but their geographic distribution is not sufficiently known in the Arctic. The aim of this study was to investigate the taxonomic status and genetic differentiation of *Mytilus* populations in a Northwest Greenlandic fjord at Maarmorilik, impacted by contaminations from a former mine. In this study, mussels were collected at three sites differing in exposure to environmental factors. A total of 54 polymorphic SNPs found in the *Mytilus* EST and DNA sequences analyzed were successfully applied to 256 individuals. The results provided the first evidence for the existence of *M. trossulus* in Greenland. The mussel from *M. trossulus* and *M. edulis* taxa are shown to coexist and hybridize in the fjord. The three studied sites were found to differ significantly in the distribution of taxa with a higher prevalence of *M. trossulus* in the inner fjord. The identified *M. edulis* × *M. trossulus* hybrids mostly had a hybrid index score of about 0.5, indicating a similar number of alleles characteristic for *M. trossulus* and *M. edulis*. There was a low number of backcrosses between ‘pure’ taxa and hybrids. This newly

discovered hybrid zone between the two taxa is unique in comparison with the Canadian populations. As *Mytilus* mussels in Greenland hitherto have been regarded as the one taxon *M. edulis*, the results have importance for biogeography and future monitoring and environmental studies.

Keywords *Mytilus edulis* · *M. trossulus* · Population structure · Hybridization · Greenland · SNPs · Hybrid index

Introduction

The blue mussel, *Mytilus*, is known to inhabit subarctic and arctic regions of Norway, Russia, Alaska, Canada and Greenland in addition to temperate areas of both the Northern and Southern hemispheres (Riginos and Henzler 2008; Väinölä and Strelkov 2011). In Greenland, populations of blue mussels are widely found in fjords in West and South Greenland, but they have not been found north of central east Greenland (Versteegh et al. 2012; Høgslund et al. 2014). The *M. edulis* species complex consists of three morphologically similar congeners: *M. edulis*, *M. trossulus* and *M. galloprovincialis*. Recently research has been intensified on the distribution of the species complex (e.g., Moreau et al. 2005; Shields et al. 2008; Riginos and Henzler 2008; Smietanka et al. 2009; Stuckas et al. 2009; Zbawicka et al. 2012; Smietanka et al. 2013, 2014; Zbawicka et al. 2014), which evidently reflects a history of inter- and transoceanic dispersal, secondary contact and hybridization, besides the effect brought about by human activities (Väinölä and Strelkov 2011). It is thought that the complex originated from *M. trossulus* in the Pacific followed by colonization in the Northern Atlantic giving rise to both *M. edulis* and *M. galloprovincialis* (Vermeij 1992; Riginos and

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Cunningham 2005). While until recently it has been believed that the distribution of *M. trossulus* was restricted to the North Pacific, eastern Canada and the Baltic Sea (Riginos and Cunningham 2005), more recent research has documented its occurrence on the coasts of Scotland, Iceland, the Barents Sea, the White Sea and Norway (Beaumont et al. 2008; Zbawicka et al. 2010; Kijewski et al. 2011; Väinölä and Strelkov 2011). The presence of *M. edulis* has been reported in the northern part of the Atlantic and European seas from the White and Barents Sea to the Atlantic coast of southern France and *M. galloprovincialis* in the Mediterranean Sea, Black Sea and along the Atlantic coastline of Western Europe including the British northern islands (e.g., Väinölä and Hvilsom 1991; Borsa et al. 1999; Bierne et al. 2003; Riginos and Cunningham 2005; Riginos and Henzler 2008; Zbawicka et al. 2012, 2014).

It is known that there is an overlap between *M. edulis* and *M. trossulus* throughout the North Atlantic with both co-occurrence and interbreeding (Riginos and Cunningham 2005; Gardner and Thompson 2009; Väinölä and Strelkov 2011; Zbawicka et al. 2012). It has been suggested that the colonization of Greenland by blue mussels occurred recently and exclusively from North America (Riginos and Henzler 2008). The occurrence of *Mytilus* in Greenland has until now been considered to consist of a single taxon, *M. edulis*. Few genetic studies have been performed on Greenlandic mussels. Versteegh et al. (2012) performed RFLP fingerprinting on mussels collected in an area restricted to Godthaabsfjord, central west Greenland. Wanamaker et al. (2007) sampled outside Sisimiut in open water and used genetic PCR-based markers, and Riginos and Henzler (2008), who applied mtDNA analysis of populations in the very south of Greenland, found that all sampled mussels belonged to *M. edulis*.

Mytilus edulis is important in the coastal areas in Greenland and has been applied in a range of ecology studies (e.g., Theisen 1973; Strand and Asmund 2003; Wanamaker et al. 2007; Blicher et al. 2013; Høgslund et al. 2014; Thyrring et al. 2015). In the mid-intertidal zone, blue mussel beds are found to dominate at wave-exposed steep sites. Such beds are highly productive, and besides being the primary food source for birds, fish and invertebrates, they provide habitat and refuge for numerous organisms (Blicher et al. 2013). Further, *Mytilus* has been used extensively in environmental monitoring studies, for example around mining-related activities to monitor for element dispersal (e.g., Riget et al. 1997, 2000; Søndergaard 2013; Søndergaard et al. 2014; Bach et al. 2014). Whether related to monitoring or to basic ecology, populations have been assumed to belong to this single taxon *M. edulis* only.

In Greenland, mussels must conform to extremely harsh environmental conditions. *Mytilus* is part of the intertidal and subtidal hard-bottom communities, where adults are

attached by byssus threads to rocks or stones in shallow waters, from the tidal zone down to depths of several meters. In Greenland, the mussels may experience long periods during winter where the sea freezes and the mussels thus are enclosed by ice. They can survive long dormant periods and the shift between freezing and thaw in spring and autumn, but do not survive if the summer period is too short (Bennike and Wagner 2013). The fjord water forms in early summer after the ice break and disappears when the sea ice begins to form. Due to the influence of melt water, the salinity is reduced and temperature is elevated because of the solar insolation. Thus, temperatures in inner fjords may reach 5–8 °C in surface waters, while the temperature at a few meters depth is 2–4 °C. While *M. edulis* for example requires summer temperatures of a minimum of 4 °C (Bennike and Wagner 2013), studies on larval development have shown that *M. trossulus* is less tolerant to temperatures at 10 °C and above than *M. edulis* (Hayhurst and Rawson 2009). Further, *M. trossulus* has been found to inhabit lower salinity areas (Väinölä and Hvilsom 1991; Ridgway and Naevdal 2004; Zbawicka et al. 2010, 2014; Väinölä and Strelkov 2011). Experimental studies have shown that this taxon is less sensitive to lower salinities in the early ontogenetic stages than its congeners (Qiu et al. 2002). It is thus hypothesized that the distribution of *M. edulis* and *M. trossulus* is influenced greatly by temperature and salinity, with an expectation of a higher frequency of *M. trossulus* in cold and low salinity waters. However, no research has yet reported the presence of *M. trossulus* in Greenland.

The aim of this study was to investigate the taxonomic status and genetic differentiation of *Mytilus* populations in a Northwest Greenlandic fjord at Maarmorilik. The fjord system was impacted by elevated concentrations of contaminants, especially lead (Pb) and zinc (Zn), in seawater, sediments and surrounding biota discharged from a mine in the period 1973–1990. *Mytilus* mussels have been extensively used for monitoring studies in the fjord system, which is also influenced by changes in salinity during spring and summer as well as ice cover during winter time. This main aim is achieved by comparison of the Greenland *Mytilus* populations with European and North American *M. edulis* and *M. trossulus* populations using SNP markers. As *Mytilus* mussels in Greenland hitherto have been regarded as one taxon *M. edulis*, the results can thus have importance for biogeography, monitoring and environmental studies.

Methods

DNA sampling

Mytilus spp. samples, consisting of 256 individuals in total, were collected from nine localities (Fig. 1; Table 1). Adult



Fig. 1 Geographic location of ten *Mytilis* spp. sampling sites. See Table 1 for definition of site abbreviations

mussels of mixed age, sexes and size 15–50 mm were collected from two different geographic scales—from three localities in a fjord in Northwest Greenland in an area of a former lead-zinc mine, the Black Angel at Maarmorilik (GLL, GLS and GLD), and from reference samples from both sides of the Atlantic. Three reference samples of *M. edulis* were obtained, one from the Atlantic coast of North America at Indian River, Delaware (IRD), and two from the Atlantic coast of North Europe, at Loire, France (LOI), and Loch Etive, Scotland (LEE), and Loch Etive, Scotland (LET). Two reference samples of *M. trossulus* were obtained, one from the Atlantic coast of North America at Placentia Bay, Newfoundland (PBAT), and one from the Atlantic coast of North Europe at Loch Etive, Scotland (LET). Two reference samples of *M. galloprovincialis* were obtained, one from the Atlantic coast of North Europe at Bidasoa, Spain (BID), and one from the Adriatic Sea at Lido, Italy (LID). Most of the samples were collected in 2012, with four in the years 2006–2008 (Table 1), and stored frozen at $-20\text{ }^{\circ}\text{C}$ until the SNP

analyses. Seven samples consisted of about 30 individuals each, and three reference samples from Scotland and Newfoundland had from 9 to 15 individuals. DNA was isolated from the mantle tissue using a modified CTAB method according to Hoarau et al. (2002) and suspended in sterile, filtered, distilled water.

Identification and genotyping of SNPs

The EST sequences (Table 2) were aligned with appropriate GenBank EST sequences (mainly from *M. galloprovincialis* and *M. edulis*) using the ClustalX program version 1.83 (Thompson et al. 1997) with default settings. Multiple sequence alignments were used for SNP discovery using the Staden software programs (Staden et al. 2001). SNP polymorphisms were detected by visual inspection of aligned sequences. SNPs were scored as heterozygous when two peaks occurring in the sequence trace differed in intensity by $<25\%$. The possible effect of the SNPs,

Table 1 Location and sample size used for 54 SNP genotyping

Sample name	No. of individuals	Sampling site	Location	<i>Mytilus</i> spp.	Coordinates	Sample collection
GLD	33	Greenland	North-west Greenland, Maarmorilik, st 17	<i>M. edulis/M. trossulus</i>	71°8'42.96"N 51°16'31.99"W	2012
GLL	30	Greenland	North-west Greenland, Maarmorilik, st L	<i>M. edulis/M. trossulus</i>	70°59'42.42"N 52°16'41.37"W	2012
GLS	33	Greenland	North-west Greenland, Maarmorilik, st 12SW	<i>M. edulis/M. trossulus</i>	71°7'56.63"N 51°14'58.81"W	2012
IRD	30	North America	USA, Delaware, Indian River Inlet	<i>M. edulis</i> North America	36°52'6.19"N 75°58'2.16"W	2012
LOI	30	Europe	France, Loire, Atlantic Sea	<i>M. edulis</i> Europe	47°14'43.83"N 2°13'48.88"W	2006
LEE	12	Europe	Great Britain, Scotland, Loch Etive	<i>M. edulis</i> Europe	56°27'21.35"N 5°18'26.62"W	2008
PBAT	9	North America	Canada, Newfoundland, Placentia Bay	<i>M. trossulus</i> North America	47°2'40.05"N 54°11'34.72"W	2012
LET	15	Europe	Great Britain, Scotland, Loch Etive	<i>M. trossulus</i> Europe	56°27'21.35"N 5°18'26.62"W	2008
BID	30	Europe	Spain, Bidasoa, Atlantic Sea	<i>M. galloprovincialis</i>	43°21'38.71"N 1°51'11.15"W	2006
LID	34	Europe	Italy, Lido, Adriatic Sea	<i>M. galloprovincialis</i>	45°30'25.42"N 12°38'30.04"E	2006

resulting in change or no change in amino acid sequence (non-synonymous or synonymous changes, respectively), was predicted on the basis of the open reading frames (ORF) identified (Table 2). An average of 21 EST sequences were used to characterize each SNP, with a minimum of four sequences. With a sufficient number of sequences, sequencing errors were disregarded by Staden software; other sequencing errors were identified during the validation. Additional SNPs were identified in Zbawicka et al. (2012, 2014). Genotyping of SNPs was performed using the Sequenom MassARRAY iPLEX platform (Gabriel et al. 2009). PCR and extension primers were designed using the Assay Design 3.1 program (Sequenom). Analysis and scoring were performed using Typer 3.4 software (Sequenom). SNPs were classified, based on manual inspection, as ‘failed assays’ (meaning that the majority of genotypes could not be scored and/or the samples did not cluster well according to genotype), ‘monomorphic SNPs’ (all studied individuals had the same genotype) or ‘polymorphic SNPs.’ Assays were designed for 79 candidate SNPs including 69 based on EST sequences and 10 identified by DNA sequences, selected from 385 putative SNPs (Zbawicka et al. 2012, 2014).

Analysis of genotype data

SNP markers and populations were analyzed for genetic diversity, proportion of polymorphic SNP (P_O), observed (H_O) and expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}) using Arlequin v3.5 (Excoffier and Lischer 2010). The statistical significance of F_{IS} (>0) was tested by

10,000 permutations of alleles between individuals. Departures from Hardy-Weinberg equilibrium (HWE) were tested by exact test and significance was determined by Markov chain Monte Carlo simulations. Pairwise analysis in Arlequin was used to calculate mean pairwise F_{ST} values defining population differentiation. F_{ST} values at individual SNPs were calculated using the AMOVA function of the same program. Permutation testing with 1000 iterations was used to calculate p values for mean and locus-by-locus F_{ST} values. The program PopTree2 (Takezaki et al. 2010) was used to produce a neighbor-joining tree based on F_{ST} distances from allele frequencies of the SNP loci. The robustness of the phylogenetic relationship calculated by the NJ tree was assessed using 1000 replication bootstrap analysis. Finally the NJ tree was constructed in MEGA version 4 (Tamura et al. 2007).

Correction for multiple tests of overall significance values was carried out using Benjamini-Yekutieli correction for multiple comparisons, a less conservative modified false discovery rate (FDR) method (Narum 2006). A list of loci with $F_{ST} \geq 0.5$, having the alleles that distinguish *M. trossulus* from *M. edulis* and from *M. galloprovincialis*, was prepared. The “*M. trossulus* characteristic allele” was defined as the allele characteristic for pure *M. trossulus* (about 90–100 %) for each marker. Fourteen SNP markers met the above condition (Table 2). The number of markers showing the “*M. trossulus* characteristic allele” was counted for each individual. The hybrid index score (HI) for each individual was calculated as the number of markers showing the “*M. trossulus* characteristic allele” divided by the number of selected markers (14).

Table 2 SNP properties, genome location, references, GenBank annotation and substitution

SNP	Location	GenBank annotation	Region	Substitution	Allele	Obs. Het. BP	Obs. F_{ST}	Reference
BM101A	Ribosomal protein L7a	KT713378	coding	synon	A/T	0.4133	0.9726*	Present study
BM102A	Proteasome subunit beta type-6-like	KT713379	coding	synon	C/T	0.4386	0.0886*	Present study
BM103B ^{HI}	Proteasome subunit beta type-5-like	KT713380	coding	nonsyn	A/G	0.3760	0.5113	Present study
BM105A	UnKnown	KT713381	NA	NA	A/G	0.2301	0.3435	Present study
BM106B	UnKnown	KT713382	NA	NA	A/G	0.0000	0.0000	Present study
BM10B	Ribosomal protein S20	KJ871040	Coding	Synon	A/C	0.3927	0.4555	Zbawicka et al. (2014)
BM113A ^{HI}	Protein BTG1	KT713385	coding	synon	A/T	0.4380	0.5661	Present study
BM115B ^{HI}	UnKnown	KT713384	NA	NA	A/G	0.5455	0.7218*	Present study
BM118A	UnKnown	KT713388	NA	NA	A/G	0.0986	0.1458	Present study
BM11A ^{HI}	Ribosomal protein L22	KJ871041	Coding	Synon	A/G	0.4485	0.5747	Zbawicka et al. (2014)
BM12A	Ribosomal protein L23a	KJ871042	Coding	Synon	C/T	0.4609	0.4936	Zbawicka et al. (2014)
<u>BM12C</u>	Ribosomal protein L23a	KJ871042	Coding	Synon	C/T	0.4895	0.2014*	Present study
BM147A ^{HI}	UnKnown	KT713383	NA	NA	C/T	0.4627	0.5477	Present study
BM16B	Ribosomal protein L21	KJ871044	Coding	Synon	C/T	0.2894	0.0637*	Present study
BM17B	Ribosomal protein L7a	KJ871045	Coding	Synon	A/G	0.5102	0.4065	Zbawicka et al. (2014)
BM201B	<i>H4 histone</i> gene	AY267750.1	Noncoding (intron)	NA	A/C	0.3044	0.4226	Zbawicka et al. (2012)
<u>BM201C</u>	<i>H4 histone</i> gene	AY267750.1	Coding	Synon	G/T	0.3080	0.6332	Zbawicka et al. (2012)
<u>BM202A</u>	<i>H3 histone</i> gene	AY267749.1	Noncoding (intron)	NA	A/C	0.4555	0.5972	Zbawicka et al. (2012)
BM202B ^{HI}	<i>H3 histone</i> gene	AY267749.1	Coding	Synon	A/T	0.4589	0.6149	Zbawicka et al. (2012)
<u>BM203B</u>	<i>H2B histone</i> gene	AY267742.1	Noncoding (intron)	NA	C/T	0.2853	0.5596	Zbawicka et al. (2012)
BM203C	<i>H2B histone</i> gene	AY267742.1	Noncoding (intron)	NA	C/T	0.4089	0.3089	Zbawicka et al. (2012)
BM203D ^{HI}	<i>H2A histone</i> gene	AY267757.1	Noncoding (intron)	NA	A/T	0.4539	0.6143	Zbawicka et al. (2012)
BM204A	<i>p53</i>	DQ865151	Coding	Synon	C/T	0.2863	0.3693	Zbawicka et al. (2012)
BM21B	qm-like protein	KJ871047	Coding	Nonsyn	C/G	0.5519	0.8238*	Zbawicka et al. (2014)
<u>BM21C</u>	qm-like protein	KJ871047	Coding	Synon	A/C/T	0.4466	0.5524	Zbawicka et al. (2014)
BM26B ^{HI}	UnKnown13	KJ871050	NA	NA	A/T	0.4538	0.4958	Zbawicka et al. (2014)
BM2G ^{HI}	UnKnown05	KJ871032	Coding	Synon	G/T	0.4335	0.5691	Zbawicka et al. (2014)
BM30A	Ribosomal protein I17	KJ871052	Coding	Synon	A/G	0.4057	0.4891	Zbawicka et al. (2014)
<u>BM30C</u>	Ribosomal protein I17	KJ871052	Coding	Synon	A/T	0.2843	0.294	Zbawicka et al. (2014)
BM32A	Ubiquinol-cytochrome c reductase subunit 6	KT713371	Coding	Synon	A/G	0.4338	0.1003*	Present study
BM33B	Cytochrome c oxidase subunit IV	KJ871054	Coding	Synon	A/T	0.3022	0.3367	Zbawicka et al. (2014)

Table 2 continued

SNP	Location	GenBank annotation	Region	Substitution	Allele	Obs. Het. BP	Obs. F_{ST}	Reference
<u>BM35C</u>	Ribosomal protein L7	KJ871055	Coding	Synon	A/T	0.4962	0.295	Present study
BM35D	Ribosomal protein L7	KJ871055	Coding	Synon	A/G	0.3768	0.3487	Zbawicka et al. (2014)
BM36F	Ribosomal protein S3a	KT713373	Coding	Synon	A/C	0.4773	0.4486	Present study
BM38B	Ribosomal protein S8e	KT713368	Coding	Synon	A/G	0.4436	0.3156	Present study
BM44B	Ubiquitin/ribosomal protein S27a	KJ871057	Coding	Synon	A/G	0.2340	0.2237	Zbawicka et al. (2014)
BM50B	CoA-binding protein	KJ871059	Coding	Synon	A/G	0.0556	0.1227	Zbawicka et al. (2014)
BM54A ^{HI}	ETC_C1_NDUFA4	KJ871060	Coding	Synon	A/G	0.4544	0.5886	Zbawicka et al. (2014)
BM57A	NADH-ubiquinone oxidoreductase	KT713374	Coding	Nonsyn	C/T	0.2629	0.4975	Present study
<u>BM57D</u>	NADH-ubiquinone oxidoreductase	KT713374	Coding	Synon	A/C	0.2435	0.4714	Present study
<u>BM5B</u>	Ribosomal protein S6e	KJ871035	Coding	Synon	A/G	0.0646	0.0403*	Present study
BM5D	Ribosomal protein S6e	KJ871035	Coding	Synon	C/T	0.3850	0.096*	Zbawicka et al. (2014)
BM60A	UnKnown08	KJ871063	Coding	Synon	A/G	0.1192	0.1039*	Zbawicka et al. (2014)
BM61A	Ribosomal_L1	KT713375	Coding	Synon	C/T	0.5166	0.2777	Present study
BM62A ^{HI}	Ribosomal L13e	KJ871064	Coding	Synon	A/G	0.5168	0.7326*	Zbawicka et al. (2014)
BM64A ^{HI}	Ribosomal protein L35	KJ871065	Coding	Synon	C/T	0.4507	0.6096	Zbawicka et al. (2014)
BM67C	Ribosomal protein S6e	KJ871066	Coding	Synon	A/T	0.5142	0.337	Present study
BM6C	EFG_N	KJ871036	Coding	Synon	C/T	0.3673	0.1478*	Zbawicka et al. (2014)
BM75C	UBA_UBA-TS-N_domain	KT713370	Coding	Synon	C/G	0.0192	0.0805	Present study
BM78B	UnKnown12	KJ871069	Coding	Synon	A/G	0.3652	0.4783	Zbawicka et al. (2014)
BM8E ^{HI}	Ribosomal protein L3	KJ871038	Coding	Synon	A/G	0.4552	0.5588	Zbawicka et al. (2014)
BM92B ^{HI}	UnKnown06	KJ871074	Coding	Synon	A/T	0.4525	0.6163	Zbawicka et al. (2014)
BM9B	Ribosomal protein S2	KJ871039	Coding	Synon	A/G	0.5069	0.1367*	Zbawicka et al. (2014)
<u>BM9C</u>	Ribosomal protein S2	KJ871039	Coding	Synon	A/C/T	0.5453	0.2343*	Zbawicka et al. (2014)

Values with $p < 0.05$ after Benjamini-Yekutieli correction are marked in bold; * $p < 0.05$; F_{ST} , fixation index; underlined, 10 SNPs excluded from the LD analysis; NA, not applicable; HI, *M. trossulus* characteristic alleles, included in the hybrid index analysis

A score of zero is a ‘pure’ *M. edulis*, whereas a score of one is a ‘pure’ *M. trossulus*. HI was calculated for all individuals and averaged for each population to assess the degree of introgression between *M. edulis* and *M. trossulus*. Contingency table analyses were used to compare inner and outer fjord groups for significant differences in species distribution.

GENEPOP 4.1 (Rousset 2008) was used to test for linkage disequilibrium (LD) between all pairwise combinations of 44 loci [one, the most differentiated SNP per

fragment, marked in Table 2 for three groups of putatively ‘pure’ populations (3 *M. edulis*, 2 *M. galloprovincialis* and 2 *M. trossulus*)]. For a pair of diploid loci, no assumption was made about the gametic phase in double heterozygotes.

Correspondence analysis (CA; Benzécri 1992), implemented in GENETIX (Belkhir et al. 2003), was used for visualizing the genetic substructure at population and individual levels. The results have been presented as a scatter plot, with the axes representing the contribution of inertia of the data matrix in a way that can be considered

analogous to the total variance in allelic frequency (Benzécri 1992).

Results

SNP validation

About 2300 Expressed Sequence Tags (ESTs) obtained from the Baltic *M. trossulus* × *M. edulis* hybrid were aligned with appropriate GenBank EST sequences and used to study SNPs. Three hundred eighty-five putative SNPs were found, distributed across 151 contigs.

SNP multiplexes were designed in silico and tested on a panel of 30 mussels from the reference samples. Finally, 79 candidate SNPs identified in 59 contigs and 5 DNA fragments (Zbawicka et al. 2012, 2014) were used to genotype 256 individual mussels from 9 localities. For 16 SNPs, the assay design failed, 6 did not provide an acceptable quality score, and 3 were monomorphic in all samples. The remaining 54 SNPs, found in 4 DNA fragments and 39 EST contigs, with one to three SNPs per fragment, were polymorphic with a high quality score in most of the sampled individuals. SNP annotation is presented in Table 2. Eight SNPs localized in DNA fragments and 25 SNPs identified in EST sequences (Table 2) have already been used (had been previously discovered and described) in the study of the European populations of the *Mytilus* mussels (Zbawicka et al. 2012, 2014). The remaining 21 SNPs were newly characterized in the present study.

Open reading frames were identified in 38 fragments; only 6 SNPs (marked NA in Table 2) were in fragments without ORFs. Of the 54 SNPs, 43 (79.6 %) were located in coding regions of which 40 (93 %) were synonymous and 3 (7 %) nonsynonymous. Of the SNPs used, five (9.3 %) were located in noncoding regions and six (11.1 %) had unknown location. Of the 54 SNPs, only two (BM21C and BM9C) had more than two alleles each.

Very little linkage disequilibrium between pairs of 44 markers in the three groups of *Mytilus* spp. populations was found. Only one pair of loci (BM201C vs. BM203B) out of a total of 1892 were in highly significant linkage disequilibrium ($p < 0.0001$) and concerned only *M. galloprovincialis* populations.

Hybrid index score and taxonomic status of mussels

Most individuals from Greenland were “pure” *M. edulis* (hybrid index HI near 0), “pure” *M. trossulus* (HI near 1) or hybrids with HI near 0.5 (Fig. 2). Only two individuals with HI between 0.7 and 0.9 were observed in one of the Greenlandic populations (GLS). The greatest variance of

HI was observed for the population GLL located outside the fjord towards open waters, which was characterized by the highest number of loci with departures from HWE (Table 3). The two populations from the inner Maarmorilik Fjord (GLS and GLD) showed a significantly higher percentage of *M. trossulus* characteristic alleles (contingency table analysis, $p < 0.05$), and consequently up to 50 % of individuals had a ‘pure’ *M. trossulus* genotype. The ‘pure’ *M. edulis* genotype only was identified in 21 % of the mussels from those two sites.

An estimated 30 and 39 % of individuals were hybrids in the GLD and GLS populations, respectively. Most hybrids (25 individuals) had the hybrid index 0.42–0.59. HI for ten hybrids from GLD populations ranged from 0.50–0.57, for ten hybrids from GLS ranged from 0.42–0.59 and for five hybrids found in GLL ranged from 0.47–0.53. An average of 85 % of the loci in each of the 25 described hybrids (range 64–100 %) was heterozygous for one *M. trossulus* allele and one *M. edulis* allele. The presence of a large number of multiple heterozygotes indicates the existence of both the F1 hybrids as well as a certain number of F2 hybrids, which are backcrosses between ‘pure’ taxa and F1 hybrids.

In locus-specific analyses performed for the Greenland populations, different levels of introgressions were observed at the same loci. Fifteen SNPs characteristic of *M. trossulus* populations were observed in the inner fjord populations with at least 59 % frequency, including three SNPs with at least 75 % frequency (Online Resource 1). However, in the GLL population the frequency of the same loci was about 40 %. A neighbor-joining tree was constructed using only pure individuals identified by HI (near 0 and 1). The Greenland samples grouped respectively within the reference samples of pure *M. edulis* and *M. trossulus* from North America (Fig. 3).

Genetic diversity and Hardy-Weinberg equilibrium

The highest percentage of polymorphic SNP (35–40) was identified in Greenland populations compared to the reference samples of *Mytilus*. The most heterogeneous of the Greenlandic samples was the GLL population (Table 3). H_O for 54 loci among all populations was in general lower than H_E . The greatest difference can be seen in the Greenland populations, especially in GLL where H_O is two times lower than H_E . Most loci were in HWE in the reference samples from both sides of the Atlantic, while many loci were not in HWE in Greenland populations. The biggest fraction of SNPs that were not in HWE ($p < 0.01$) was observed in the outer fjord population GLL (43 %). Twenty-three loci showed significant departures from HWE after a Benjamini-Yekutieli correction. BM115B, BM147A (unknown location), BM54A (ETC complex) and

Fig. 2 The frequency distribution of the score for a hybrid index giving the percentage of *M. trossulus* characteristic alleles (marked in Table) in three populations from Greenland. A score of zero is a pure *M. edulis*, whereas a score of one is a pure *M. trossulus*. Data are presented for three populations from Greenland and reference populations of *M. trossulus* and *M. edulis*

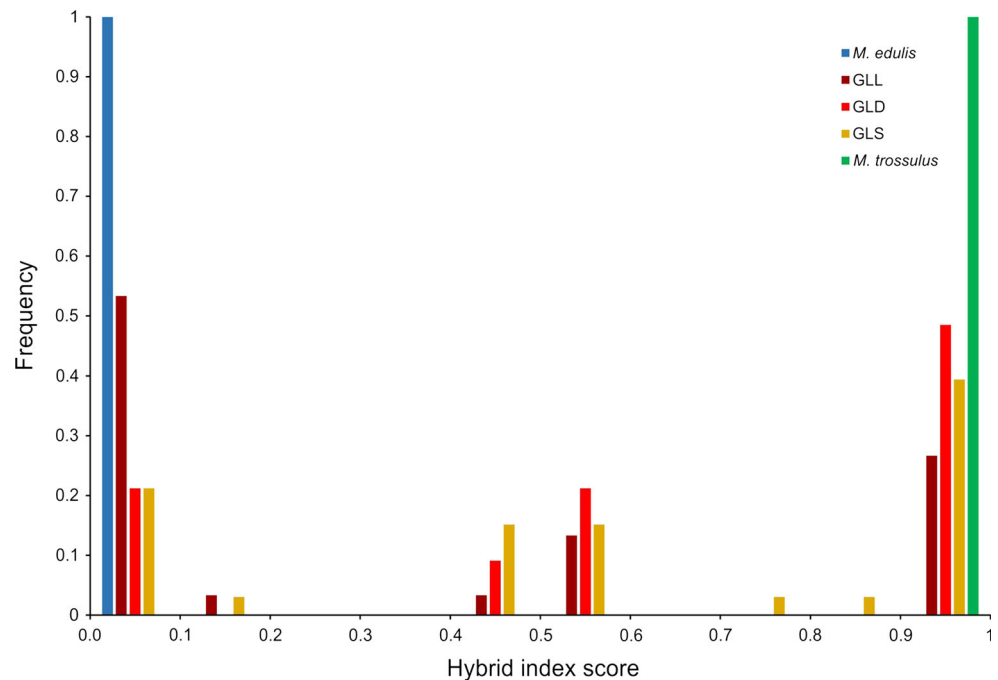


Table 3 Genetic parameters of the ten samples of Mytilus mussels

Sample	No. of polymorphic loci	F_{IS}	HI	V_{HI} with HWE departure ($p < 0.01$)	No. of loci	H_O	H_E	MAF	Average gene diversity over loci	Average no. of pairwise differences within population
GLD	35	0.3381*	0.6610	0.3799	12	0.2255	0.3712	0.2175	0.2805	13.6625
GLL	40	0.503*	0.3926	0.4088	23	0.1875	0.3873	0.2235	0.3061	14.4966
GLS	36	0.3507*	0.6085	0.3732	11	0.2265	0.3859	0.2583	0.2796	14.0173
IRD	24	0.1222*	0.0060	0.0166	0	0.2385	0.2706	0.0958	0.1204	5.3808
LEE	19	0.0743	0.0062	0.0144	1	0.2986	0.3345	0.0860	0.1250	5.3841
LOI	23	0.0958*	0.0013	0.0070	0	0.2292	0.2505	0.0920	0.1129	5.3735
LET	24	0.1788*	0.9679	0.0369	2	0.1928	0.2669	0.1011	0.1276	6.7770
PBAT	21	0.2955*	0.9841	0.0188	1	0.2017	0.3160	0.0951	0.1336	6.9739
BID	27	0.031	n.d.		1	0.3207	0.3368	0.1328	0.1608	7.9424
LID	25	0.0166	n.d.		2	0.2628	0.2955	0.1214	0.1384	6.8472

Values with $p < 0.05$ after Benjamini-Yekutieli correction are marked in bold; * $p < 0.05$; F_{IS} , inbreeding coefficient; HWE, Hardy-Weinberg equilibrium; H_O , observed heterozygosity; H_E , expected heterozygosity; MAF, minor allele frequency; HI, hybrid index; V_{HI} , variance of hybrid index; n.d., not defined

BM17B, BM44B, BM67C (ribosomal proteins) were not in HWE in the three Greenland populations.

The positive values of the within-population fixation index F_{IS} (averaged over all polymorphic loci in each population) indicate the most significant excess of homozygotes (deficiency of heterozygotes) in three populations from Greenland and in a Newfoundland sample of *M. trossulus* (Table 3).

In the Greenland populations, 16 % (GLD, GLS) to 37 % (GLL) of the loci had very large (>0.6) F_{IS} values (Online

Resource 2). Most of these loci were nearly homozygous for one allele in a population, with the absence of any heterozygotes or a single heterozygote only, and a few individuals homozygous for the alternative allele. In a few cases, F_{IS} had values of 1, indicating the existence of two alternative homozygotes without any heterozygotes (e.g., BM17B, BM203C and BM44B). ‘Pure’ *M. edulis* populations were mostly homozygous for this alternative allele, and the *M. trossulus* sample from Scotland and Newfoundland was predominately homozygous for the second allele.

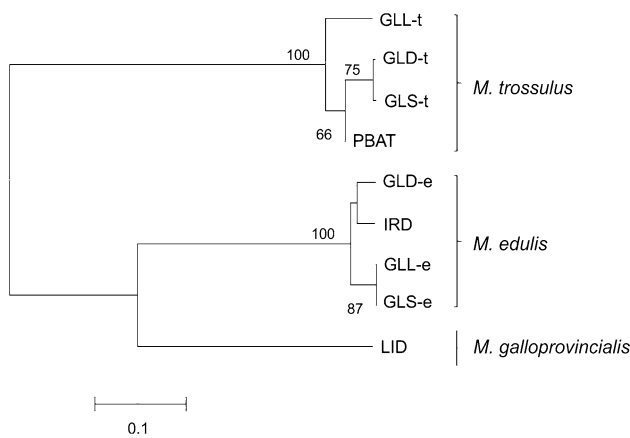


Fig. 3 Neighbor-joining tree of *Mytilus* spp. samples from Greenland constructed using only pure individuals identified by HI and reference samples of *M. edulis*, *M. trossulus* and *M. galloprovincialis*, based on the F_{ST} distance from allele frequencies of the SNP loci. The numbers at the nodes represent bootstrap values estimated from 1000 replications. *e*, individuals with “pure” *M. edulis* genotype (HI near 0); *t*, individuals with “pure” *M. trossulus* genotype (HI near 1)

Genetic differentiation among populations

The neighbor-joining tree (Fig. 4) revealed the three main groups representing *M. edulis*, *M. trossulus* and *M. galloprovincialis* and the Greenland samples, which contained mixed *Mytilus* populations that are located between the *M. trossulus* and a group of *M. edulis* and *M. galloprovincialis*. Further, it can be observed that the populations from the inner fjord (GLD and GLS) are grouped more with *M. trossulus* samples than the more open water Greenland (GLL) population.

The three Greenlandic sites were found to differ in the taxa distribution with a significantly higher proportion of *M. trossulus* ($p < 0.05$, 2×2 contingency table analysis) in the inner fjord (GLD and GLS). *M. edulis* and *M. trossulus* numbers of specimens at both stations were

respectively 7 and 16, 7 and 13 compared to the outer fjord (GLL) with 16 *M. edulis* and 8 *M. trossulus*. The average genetic level of differentiation among all studied populations was very high ($F_{ST} = 0.426$) (Table 4). F_{ST} values were significantly greater than zero between most of the pairs of samples (reaching values as high as 0.787 for *M. trossulus* and *M. edulis* populations). All this means is that the inner fjord populations had more *M. trossulus* specimens and consequently fewer *M. edulis* specimens than the outer (GLL) population.

Population structure

Correspondence analysis was carried out for individuals to characterize the Greenlandic mixed *Mytilus* populations (Fig. 5). The first two axes accounted for 90 % of the total variation, with axis 1 accounting for 69.43 % showing a clear separation of *M. edulis* and *M. trossulus* populations. Individuals from Greenland either grouped with *M. edulis* or *M. trossulus* samples or formed a separate group between them. Hardly any individuals were observed between the group of hybrids and ‘pure’ taxa (either *M. edulis* or *M. trossulus*).

Discussion

Characteristics of Greenland Mytilus populations and interpopulation differentiation

This study is the first presenting evidence for existence of *M. trossulus* in Greenland. Knowledge of the life-history differences between the *M. edulis* and *M. trossulus* taxa is needed to understand the dynamics of mussel taxa coexistence and hybrid zones as well as the environmental and genetic factors that can explain not only the occurrence of hybrids but also the mechanisms that keep the species

Fig. 4 Neighbor-joining tree of three *Mytilus* spp. populations from Greenland and reference samples of *M. edulis*, *M. trossulus* and *M. galloprovincialis* from North America and Europe based on the F_{ST} distance from allele frequencies of the SNP loci. The numbers at the nodes represent bootstrap values estimated from 1000 replications. The population abbreviations and descriptions are given in Table 1

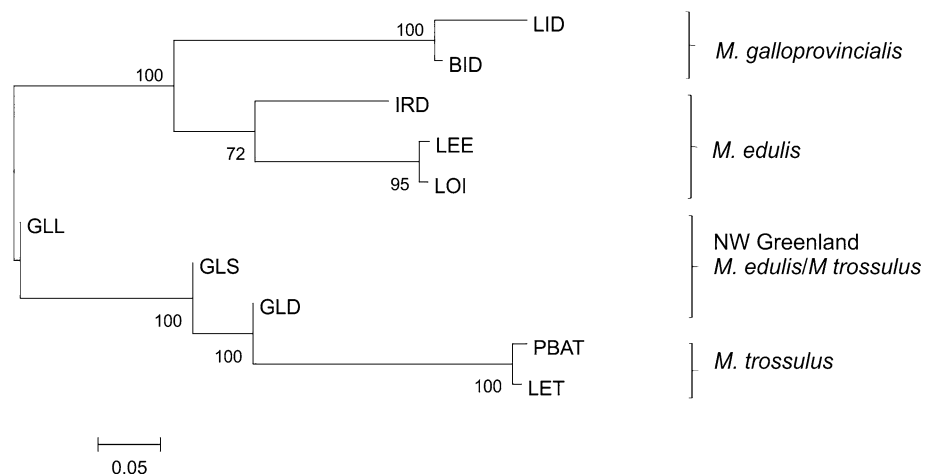
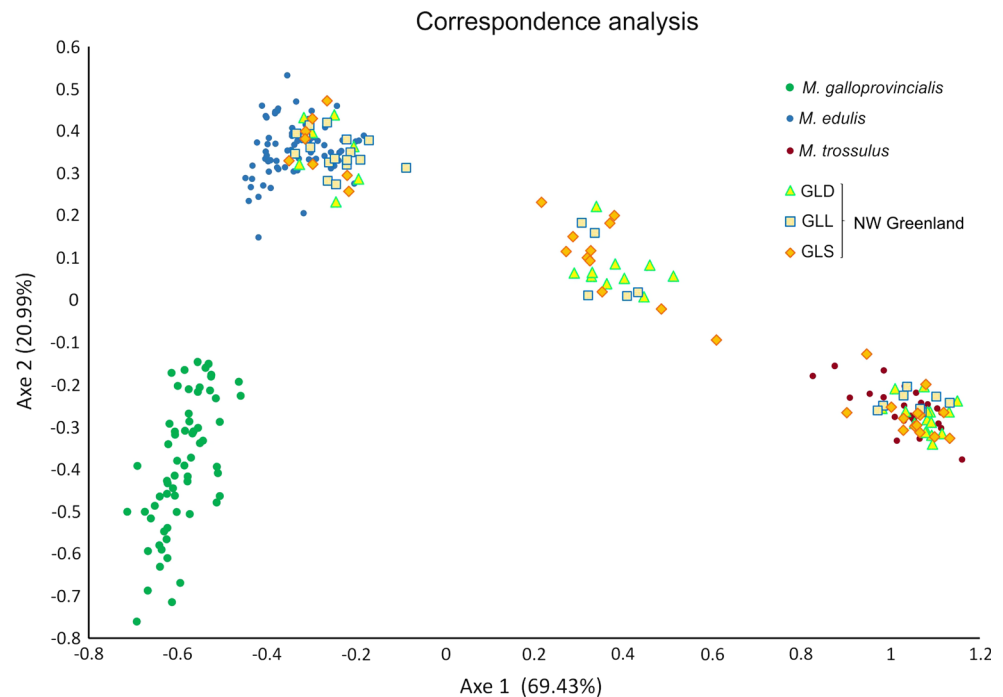


Table 4 Pairwise molecular variance (F_{ST} distance matrix) for 54 SNP

	GLD	GLL	GLS	IRD	LEE	LOI	LET	PBAT	BID
GLL	0.0965								
GLS	−0.0021	0.0690							
IRD	0.4572	0.2104	0.4172						
LEE	0.4729	0.2563	0.4272	0.2862					
LOI	0.5188	0.3061	0.4766	0.2925	0.0165				
LET	0.1599	0.3849	0.1961	0.7617	0.7678	0.7778			
PBAT	0.1494	0.3756	0.1838	0.7705	0.7768	0.7870	0.0082		
BID	0.5299	0.3831	0.4988	0.4358	0.3780	0.3689	0.7432	0.7456	
LID	0.5664	0.4450	0.5391	0.5345	0.4964	0.4791	0.7643	0.7720	0.0775

$p < 0.05$ after Benjamini-Yekutieli correction is marked in bold

Fig. 5 The first two axes of the correspondence analysis (CA) computed from the SNP data on three populations of *M. edulis*/*M. trossulus* from Greenland and reference samples of *M. edulis*, *M. trossulus* and *M. galloprovincialis* from North America and Europe. Each dot (point) is an individual

distinct despite hybridization (McDonald et al. 1991; Toro et al. 2004). The two taxa, *M. edulis* and *M. trossulus*, hold the same habitat, where individuals are attached to the substrate by byssus threads and filter food particles from the water column. They are almost morphologically indistinguishable, and results of ongoing studies debate whether small shell differences are caused by environmental impacts or genetic differences (e.g., McDonald et al. 1991; Innes and Bates 1999).

The mussels *M. trossulus* and *M. edulis* are here shown to coexist and hybridize in a fjord in the Maarmorilik area, West Greenland. This finding is further documented by comparison of F_{ST} values between *M. edulis* and *M. trossulus* populations, which revealed that the GLD and GLS samples were found more introgressed than the GLL population. While the GLL (outer fjord site) was mostly

composed of *M. edulis* mussels (53 %), and to a lesser degree *M. trossulus* (27 %) and hybrids (20 %), the GLS and GLD (inner fjord sites) both had a much higher occurrence of both *M. trossulus* (about 40 and 50 %, respectively) and hybrids (39 and 30 %) than of *M. edulis* (both 21 %).

All Greenlandic mussels were sampled in the intertidal zone, where the mussels were collected during low tide from underneath or in crevices in the rocks. While the open water site (GLL) was a true marine site, the GLS and GLD were collected in a fjord that can be described as a stratified estuary during summer. The stratification is caused by melt water from the rivers and glacier runoff of approximately $110 \times 10^6 \text{ m}^3$ freshwater, which flows on top of the heavier seawater, creating a stagnant water mass in the bottom of the fiords and thus salinities from 34 down to

26 psu (Møller 1984). Based on findings of *M. trossulus* in areas of lower salinities, studies relate this to a higher tolerance to lower salinities compared to *M. edulis*, a theory that is further backed up by experiments documenting a higher tolerance for *M. trossulus* at early life history stages (e.g., Qiu et al. 2002; Ridgway and Naevdal 2004; Przytarska et al. 2010; Zbawicka et al. 2014). While the two taxa in most studies are suggested to be physiologically differentiated with respect to salinity, one must bear in mind that areas characterized by lower salinity are also often subjected to significant variation in other environmental factors such as pH, temperature, dissolved oxygen and particulate loadings (Gardner and Thompson 2001), and the proportion of *M. edulis* vs. *M. trossulus* may not be solely attributed to a single environmental factor such as salinity.

In Greenland, the harsh Arctic climate challenges the intertidal mussels as in winter they may be exposed to extreme cold air temperatures (down to -25°C) twice daily if not covered by a stable ice foot (Scrosati and Eckersley 2007). The taxa distribution with a tendency to more *M. trossulus* in the inner fjord may be affected by the fact that the ice breaks up later in the spring in the inner fjord than at open sea, possibly affecting the time set for mussel reproduction and settlement of larvae. While *M. edulis* has been shown to start their spawning in late spring, *M. trossulus* generally starts later in the summer (Toro et al. 2002, 2006), paving a possibility for *M. trossulus* to dominate areas with longer-lasting sea ice. *M. trossulus* is more robust compared to its congener, *M. edulis*, not only in relation to salinity, but also temperature (Hayhurst and Rawson 2009). While this study documented occurrences of *M. trossulus* in Greenland, other studies in other parts of Greenland by Wanamaker et al. (2007), Riginos and Henzler (2008) and Versteegh et al. (2012) failed to document the existence of other *Mytilus* taxa than *M. edulis*. *Mytilus* mussels are spawners with an extended pelagic larval phase lasting up to several months (Bayne 1965), and with the short distance across the David Strait between Greenland and Canada, the introduction could have occurred after the end of the last glacial period approximately 10,000 years ago.

Hybridization

The genetic structure of Greenland populations can be compared with Scotland, Baltic and North American populations where *M. trossulus* and *M. edulis* individuals coexist and hybridize (Riginos and Cunningham 2005; Zbawicka et al. 2010, 2012, 2014). However, while European populations are characterized by large numbers of hybrids, including some with intermediate hybrid index values (backcrosses), Riginos and Cunningham (2005) and

Toro et al. (2002) described North American populations as having a low frequency of hybrid mussels.

This study found low numbers of hybrids between ‘pure’ species and F1 hybrids, and such backcrossing could only be assigned to one individual from GLL and three from GLS biased toward both *M. trossulus* and *M. edulis*. This situation is completely different from that at the Danish strait (Zbawicka et al. 2014) and Scotland (Comesaña et al. 1999; Zbawicka et al. 2010, 2012) where hybridization between ‘pure’ taxa and hybrids is frequent.

The deficiency of heterozygotes observed in Greenland populations was observed also in the Scottish and Norwegian populations of *Mytilus* spp. (European populations from hybridization regions), where 25–30 % of SNP loci also had very large (>0.6) F_{IS} values compared to the reference population of *Mytilus*. The possible impact of recent mixing of *M. trossulus* with *M. edulis* was discussed by Zbawicka et al. (2012). Structures in hybrid zones have been studied with respect to reproductive barriers, and results have indicated higher conspecific than heterospecific fertilization rates and greater survival of larvae from conspecific than heterospecific crosses (e.g., Miranda et al. 2010; Toro et al. 2004; Klibansky and McCartney 2014). There are also indications of differences in life-history traits, where for example *M. edulis* have been found to initiate spawning before the time of *M. trossulus*. Thus, the maintenance of the hybrid zone may at least partly be caused by reproductive fertilization isolating mechanisms in addition to differences in life history traits, limiting the production of hybrids. Unfitness/fitness of hybrids compared to parent taxa is an ongoing discussion, and for the *M. edulis*-*M. trossulus* hybrid zones, examples of both decreased fitness and no differences exist in the literature when adult physiological fitness is compared (Gardner and Thompson 2001).

Implications related to monitoring programs

The finding of *M. trossulus* might have implications not only for the ecological understanding of the Greenlandic ecosystem, but indeed also in the context of biomonitoring of marine impacts. Mussels are commonly used across Greenland (and worldwide) as bioindicators for industries such as mining, where a sample usually consists of approximately 20–30 pooled individuals to represent a specific site (e.g., Riget et al. 1997, 2000; Søndergaard et al. 2014; Bach et al. 2014). As there are indications that individuals of *M. trossulus* accumulate some elements to a higher extent than *M. edulis* (Lobel et al. 1990; Blackmore and Wang 2003), the taxa composition in a sample could thus have an impact on elemental monitoring results. As a result of this study, it clearly calls for further research on the distribution of *Mytilus* taxa across Greenland and

studies of differences among them and hybrids in uptake, accumulation and effects of heavy metals among other contaminant stressors. Such research would be relevant for monitoring perspectives within both national and international frameworks.

Conclusion

This study is the first presenting evidence for the existence of *M. trossulus* in Greenland. The mussels *M. trossulus* and *M. edulis* taxa are here shown to coexist and hybridize in a fjord in the Maarmorilik area, West Greenland. The discovered hybrid zone is characterized by particular features that are different from those of the hybrid zones of both taxa found in the North American Atlantic areas. This finding has implications for future monitoring programs related to climate changes and understanding of the evolution of marine benthic communities in Greenland.

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