Estuarine, Coastal and Shelf Science 271 (2022) 107863

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Ultra-conserved elements provide insights to the biogeographic patterns of three benthic macroinvertebrate species in the Baltic Sea

H. Cecilie Petersen [a,b,*], K. Emily Knott [b], Gary T. Banta [c], Benni W. Hansen [a]

[a] Department of Science and Environment, Roskilde University, DK-4000, Roskilde, Denmark
[b] Department of Biological and Environmental Science, University of Jyväskylä, FI-40014, Jyväskylä, Finland
[c] Department of Biology, University of Southern Denmark, DK-5238, Odense M, Denmark

ARTICLE INFO

Keywords:
Population genetics
Biogeography
Baltic sea
Benthic invertebrates
Ultra-conserved elements

ABSTRACT

The Baltic Sea, with its steep salinity gradient, high water retention time, and relatively young age, represents a marginal ecosystem between marine and freshwater extremes. Due to differing invasion history and dispersal capabilities of Baltic species, there are large differences in species distributions, species-specific genetic structure and variation, and edge populations that may represent both a subset of the original population, as well as unique genetic lineages. We used a phylogenomic approach to investigate relationships between populations of three benthic macroinvertebrate species: *Pygospio elegans*, *Corophium volutator*, and *Mya arenaria*, providing new insight into evolutionary dynamics among populations in the Baltic Sea and the adjacent North Sea. We found little relation among the populations of *P. elegans* and *C. volutator*, in contrast to *M. arenaria,* which exhibits a higher degree of resemblance between populations. We also found low relation within sites sampled at different times of the year for all species. Each species exhibited unique phylogenetic patterns, suggesting the North Sea populations of *P. elegans* and *M. arenaria* are closely related to populations within the Baltic Sea, and with only *C. volutator* showing trends resembling isolation by distance. These differences could be explained by both their different invasion histories and dispersal capabilities of the individual species.

1. Introduction

The Baltic Sea is one of the largest brackish water seas in the world (Leppäkoski et al., 2009). As the opening into the North Sea formed after the Weichsel-ice age, the Baltic Sea is also a relatively young sea. Due to its seafloor topology, the Baltic Sea is divided into several sub-basins with a reduced exchange between the waterbodies, which has resulted in a high water retention time (Meier, 2007) and reduced connectivity between populations in the different basins. The current salinity regime was established only 3000 years ago (Snoeijs, 1999), and is marked by a significant freshwater influx from both land runoff and precipitation. This, together with the high retention time, results in a steep decline in salinity from the entrance (35) to the Danish Belt Seas (12), and a gradual salinity decline towards the Bothnian Bay (2), a gradient which influences the species distribution in the Baltic Sea to a high degree (Zettler et al., 2014). The distinctive environmental conditions and young geological history indicates that it is a marginal ecosystem (Johannesson and André, 2006), which together with the dispersal capabilities and invasion history of the species can create a bottleneck for the populations (Johannesson and André, 2006; Wennerström et al., 2013). As a result, populations in the Baltic Sea exhibit unique patterns of genetic diversity.

The invasion of marine fauna in the Baltic has taken place in multiple waves, from different origins (Väinölä, 2003; Nikula et al., 2007; Virgilio et al., 2009). This has resulted in the individual species living in the Baltic Sea having distinctive invasion histories and evolutionary patterns (Wennerström et al., 2013). Together with the physicochemical characteristics of the Baltic this has led to some subpopulations or lineages being both genetically and reproductively isolated from each other (Audzijonyte et al., 2008), whereas others have various degrees of overlap between populations (Väinölä and Hvilsom, 1991; Riginos and Cunningham, 2005; Nikula et al., 2008; Väinölä and Strelkov, 2011; Luttikhuizen et al., 2012). As a consequence of these phenomena, marine fauna in the Baltic show species-specific variation in the genetic structure. Their populations not only support a subset of the genetic structure in the original populations, but due to the complexity of invasion patterns, gene flow and adaptation to the Baltic environment, they also include unique evolutionary lineages (Johannesson and André,

* Corresponding author. Universitetsvej 1, DK-4000, Roskilde, Denmark.
E-mail address: ceciliep@ruc.dk (H.C. Petersen).

https://doi.org/10.1016/j.ecss.2022.107863
Received 5 February 2022; Received in revised form 15 April 2022; Accepted 19 April 2022
Available online 28 April 2022

2006; Wennerström et al., 2017). Some populations, in e.g. cod (*Gadus morhua*) and baltic clam (*Macoma balthica*), exhibit a decline in diversity along the Baltic gradient, compared to Atlantic populations from which they originated (Johannesson and André, 2006). However, other species, such as herring (*Clupea harengus*) have shown opposite trends with higher genetic diversity in the innermost parts of the Baltic Sea (Wennerström et al., 2013). While the trends in genetic diversity for some species in the Baltic Sea (e.g., *Mytilus* spp.) have been known for decades (Väinölä and Hvilsom, 1991), the evolutionary history and genetic structure of many species in the Baltic Sea still remain unknown.

Pygospio elegans, Corophium volutator, and *Mya arenaria* are all benthic macrofauna species common over a large range of the Baltic Sea (HELCOM, 2020), and all play an important role in the communities they inhabit (Strasser, 1998; Bolam and Fernandes, 2003; Mermillod--Blondin and Rosenberg, 2006). While they have well-known life history and ecological functions, little is known about their population phylogeography in the Baltic Sea. These three species represent different taxonomic groups and have different invasion histories and dispersal strategies that could affect their population genetic patterns. The annelid worm *P. elegans* is poecilogonous, thus exhibiting both benthic and planktonic developmental modes with different dispersal potential, and the genetic diversity of this species in the Baltic Sea has previously been studied in relation to larval developmental mode. Although a connection was found between genetic diversity and larval developmental mode, the studies found little divergence between populations in the Baltic Sea (Kesäniemi et al., 2012a; Kesäniemi Jenni et al., 2012b). The crustacean *C. volutator,* on the other hand, has only benthic larval development, which may lead to a smaller dispersal range. Both juveniles and adults do, however, occasionally swim (Hughes, 1988), which may increase their potential to disperse over larger ranges. Genetic studies of populations of *C. volutator* in the Atlantic have shown that this species is well established in the eastern part of the North Atlantic, and populations in the south-eastern Baltic Sea have close genetic resemblance to Atlantic populations (Einfeldt and Addison, 2015). Unlike the other two species that are considered indigenous to the Baltic Sea (Leppäkoski et al., 2002; Janas and Kendzierska, 2014), the bivalve *M. arenaria* has recolonised and settled in the Baltic Sea more recently, within the last 300–800 years (Strasser, 1998). The life history of this species is also different from the other two species, since *M. arenaria* has exclusively pelagic reproduction and larval development (Loosanoff and Davis, 1963) and also exhibits some post-larval dispersal (Jennings and Hunt, 2009). The species, therefore, has a large dispersal potential. Studies of the genetic diversity of *M. arenaria* have shown that North European populations represent a fraction of the genetic diversity of populations from North America (Lasota et al., 2004; Cross et al., 2016) supporting the hypothesised invasion history of *M. arenaria* from the North Atlantic. Another study showed that populations of *M. arenaria* in the southern part of the Baltic, Kiel, had little divergence from populations on the Atlantic coast (De Noia et al., 2020), however, the genetic connection could also reflect influx of individuals from the Atlantic mediated by ship traffic in the Kiel Canal.

Using data from ultra-conserved elements (UCEs), we reconstructed phylogenetic networks of populations of these three species in the Baltic Sea and adjacent North Sea, aiming to provide new insight into the phylogeography of the species. If the species invaded the Baltic from the Atlantic, we hypothesise that obstacles to connectivity and salinity decline in the Baltic Sea will represent barriers to gene flow, and result in patterns of isolation by distance for all species. So, we expected that populations in the North Sea represent the populations of origin, and populations inhabiting the Baltic Sea will be more similar genetically than they are to North Sea populations. We also expected that populations from the same site at different times over the year will be more similar genetically than they are with populations from other sites. Since the three species represent different taxonomic groups, invasion histories and dispersal strategies, differences in their phylogenies were expected. The two species with older invasion history in the Baltic Sea and less dispersal potential, *P. elegans* and *C. volutator*, were expected to show more genetically distinct populations at each site, in comparison to *M. arenaria,* which has a more recent invasion and higher dispersal potential.

2. Materials and methods

2.1. Field collection

Sampling on a spatial scale was carried out during August 2018, at six study sites (List, Saltö, Herslev, Gollwitz, Öland, Tvärminne) in the North Sea and Baltic Sea (Fig. 1). Samples on a temporal scale were collected at four times during a year (August 2018, November 2018, April 2019, August 2019) at three of the sampling sites (Saltö, Herslev, Öland) in the Baltic Sea (Fig. 1, stations indicated with bold and underline) when the species were present (see Supplementary Table 1). All sites were sampled from the coast at 0–0.80 m water depth, except for Tvärminne, where sampling was also performed by SCUBA at 3.8–5 m depth. Twenty specimens of each species (when present) were collected on the same day at one sampling site for assessing population genetic diversity (see Supplementary Table 1). Specimens were stored in 99% ethanol for later analysis. Species ID were confirmed in the laboratory post collection based on morphology as described in Kirkegaard (1992), and Hayward and Ryland (2017), using a dissecting microscope (20× magnification) and/or COI molecular barcoding tools.

2.2. UCE baits design

Identifying orthologous loci and comparing genetic variation across a wide taxonomic range of different species is challenging due to their genetic differences (Smith et al., 2014). Ultra-conserved elements (UCEs) are regions of high conservation which can be found throughout genomes across taxonomically divergent species (Faircloth et al., 2012). The method uses UCE baits, or probes, designed to bind to the ultra-conserved sequences, which may be affected by selection, as

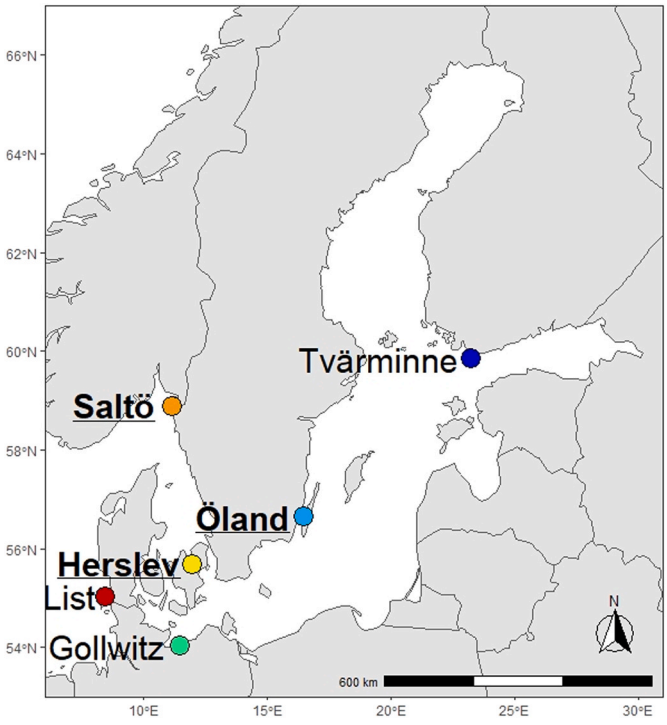

Fig. 1. Map of the Baltic Sea, including sampling sites: List, Saltö, Herslev, Gollwitz, Öland, Tvärminne, Pori. Temporal sample sites Saltö, Herslev, Öland, indicated with bold and underlined.

allowing sequencing of the flanking regions, which are assumed to be neutrally evolving and exhibiting non-selective variation. Because the conserved UCE loci are flanked by sequences of higher variability (Faircloth et al., 2012), they provide a good tool for building phylogenies based on numerous loci among a wide range of species (Faircloth et al., 2012; Quattrini et al., 2018; Winker et al., 2018).

UCEs are located by aligning several genomes to a base genome, searching for highly conserved areas that overlap, and extracting the sequences of a preferred minimum length (Bejerano et al., 2004). Baits for Annelida, Bivalvia, Gastropoda and Crustacea, were designed for each taxonomic group separately, based on conserved loci shared by a minimum of five species. UCE baits for annelids were based on *Amynthas corticis, Capitella teleta, Hydroides elegans,* and *Lamellibranchia luymesi,* using *Eisenia fetida* as base genome and *Mytilus galloprovincialis* as outgroup. The bivalve baits were based on *Dreissena rostriformis, Lutraria rhynchaena, Mytilus galloprovincialis,* and *Sinonovacula constricta,* using *Modiolus philippinarum* as the base genome, and *Lamellibranchia luymesi* as outgroup. The gastropod baits were based on *Cumia reticulata*, *Lanistes nyassanus*, *Marisa cornuarietis*, and *Pomacea maculata*, using *Babylonia areolata* as the base genome, and *Lamellibranchia luymesi* as outgroup. The crustacean baits were designed using genomes of *Platorchestia hallaensis*, *Trinorchestia longiramus*, *Ligia exotica*, and *Hyalella azteca*, using *Parhyale hawaiensis* as the base genome and *Lamellibranchia luymesi* as outgroup. All genomes were assessed from NCBI Genbank (for GenBank assembly accession numbers see Supplementary Table 2), and they were chosen on basis of size, assembly, GC % (guanine-cytosine content), as well as taxonomic relation to our target species. For each taxonomic group, genome sequence reads of 100 bp were simulated for each species except the base species using ART-20160605-3 (Huang et al., 2012). Simulated reads were aligned to the base genome using Stampy v. 1.0.32 (Lunter and Goodson, 2011), with a substitution rate of 0.1. UCE probes were designed using phyluce v. 1.6.6 (Faircloth et al., 2012; Faircloth, 2016) (https://github.com/faircloth-lab/phyluce), using a screening coverage of 4 × . The analysis generated 3316 baits for 590 conserved loci in annelids, 4110 baits for 519 loci in bivalves, 25547 baits for 4367 loci in gastropods, and 27545 probes for 3755 conserved loci in crustaceans. The final set of 3000 baits per taxonomic group (12000 baits in total) was created by Arbor Biosciences (Ann Arbor, MI, USA, arborbiosci.com) using their myBaits probe design support. This was done by BLASTing the designed probes against the respective base genome, and filtering at moderate settings (at most 10 hits 62.5–65 °C and 2 hits above 65 °C, and fewer than 2 passing baits on each flank) to an estimated hybridisation melting temperature (temperature at which 50% of molecules are hybridized). Furthermore, the probes were filtered to a GC content closest to 42.5% GC. The UCE baits included in the final probe set are available via the JYX data repository (Petersen et al., 2022).

2.3. DNA extraction and library preparation

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). For small specimens (*P. elegans, M. arenaria, C. volutator*) DNA was extracted from whole complete specimens; for large individuals (*M. arenaria*) DNA was extracted from foot tissue. DNA concentration was quantified using a Qubit 4.0 fluorometer with 1X dsDNA HS Assay Kit (Thermo Fisher, Cambridge, UK). For library preparations and sequencing, DNA from 20 individuals were pooled with equal concentration in populations (one species at one sampling point for each sampling site), and the pools were purified using QIAquick Nucleotide Removal Kit (Qiagen). Library preparations, amplification and sequencing were performed at Arbor Biosciences (Ann Arbor, MI, USA, arborbiosci.com).

At Arbor Biosciences, samples were sonicated and size selected to an average length of approximately 500 nucleotides. For targeted capture with the designed baits, up to 200 ng DNA of each pool was used for library preparation, and unique dual-index combinations were added to each sample via 9 cycles of PCR amplification. Indexed libraries were quantified with both a spectrofluorometric assay and quantitative PCR. Up to 1 μg (80% of the library volume if 1 μg was not available) was dried down to 7 μL by vacuum centrifugation. Captures on individual libraries were performed following myBaits v5 protocol with overnight hybridization at 65 °C and washes at 65 °C. For each sample, half of the volume of beads in the elution buffer were amplified for 8 cycles and the second half of the beads were amplified for 14 cycles. The two halves were combined and quantified with both spectrofluorometric assay and a quantitative PCR assay. The two captures were pooled in approximately equimolar ratios, but some captures were underrepresented due to lack of DNA availability. A screen using a MiSeq Nano PE150 run was performed to check pooling equilibration. Samples were sequenced on the Illumina NovaSeq 6000 platform on a partial S4 PE150 lane with v1.5 chemistry. Due to a low number of reads in the initial sequencing, a second sequencing was performed, and demultiplexed reads for each sample from both runs were concatenated.

2.4. Bioinformatics

Raw demultiplexed reads were length trimmed to a minimum length 40 bp, adapter trimmed and quality trimmed to a phred score of 33, using illumiprocessor v. 2.10 with trimmomatic v. 0.39 (Lohse et al., 2012; Del Fabbro et al., 2013; Faircloth, 2013) (https://github.com/faircloth-lab/illumiprocessor). Following trimming, the reference UCE loci for each population sample were prepared. Firstly, each sample pool was assembled into contigs using ABySS in phyluce v. 1.7.0 (Faircloth et al., 2012; Faircloth, 2016; Jackman et al., 2017) (https://github.com/faircloth-lab/phyluce) with a kmer of 35. Contigs were then aligned to our probe set with a minimum coverage of 80, and minimum identity 80, and duplicates removed in phyluce integrating LASTZ v. 1.04.00 (Harris, 2007). A complete matrix for each taxon with shared loci per taxon was made, and a concatenated and aligned NEXUS file including shared UCE loci sequences for all samples was made using phyluce with RAxML (Stamatakis, 2014). From this file, single FASTA files including a concatenated string of all shared UCE loci sequences for each sample was extracted. To include variation within populations, a consensus sequence for each sample was constructed by mapping the trimmed Illumina reads of each sample to their respective FASTA file separately, and subsequently paired using BWA v. 0.7.17 (Li and Durbin, 2009) (https://github.com/lh3/bwa). The mapped paired read files were filtered to keep only proper pairs, removing unmapped reads and reads with unmapped mates, and formatted to a BAM-file. These were sorted and a consensus FASTQ file for each sample separately was made with mpileup using samtools v. 1.10 (Li et al., 2009) (https://github.com/samtools/samtools).

To assess the genetic variation within each population, average Tajima's Pi was calculated for each sample by mapping and pairing trimmed reads using BWA. This was done for each sample to the corresponding FASTA file of shared UCE loci in each sample, extracted from the complete matrix using phyluce. Mapped paired read files were subsequently filtered keeping only proper pairs, and removing unmapped reads, and reads with unmapped mates. The file was then formatted to BAM-format, sorted and converted to pileup files using samtools, and indels removed using PoPoolation 1.2.2 (Kofler et al., 2011) (https://sourceforge.net/projects/popoolation). Mapping of single-nucleotide polymorphisms (SNPs) and calculation of Tajima's Pi in each sample pool was done using PoPoolation, with parameters: window and step size 1000, min. covered fraction 0,1, min. count 1, min. coverage 2, max. coverage 20, and a pool size of 24–40, according to the original pool size multiplied by two to correct for diploidy. Due to technical difficulties in alignment, the *P. elegans* sample from List, and *C. volutator* samples from Öland Apr18, Öland Aug19 and Tvärminne, were excluded from further analyses. Due to the same technical issues, F_{ST} were not recoverable from the pooled samples.

2.5. Construction of phylogenetic trees and statistical analysis

FASTQ files were converted to FASTA format using EMBOSS v. 6.5.7 (Rice et al., 2000) (http://emboss.open-bio.org/), and separate sample files were converted to FASTA format, concatenated, and aligned using MAFFT v. 7.429 using the L–INS–I algorithm (Katoh et al., 2005). The concatenated datasets for each species were analysed using the Bayesian method, of MrBayes v. 3.2.7 (Ronquist and Huelsenbeck, 2003). To identify the most appropriate substitution model to use in the analysis, a model test was run for each taxon using ModelTest-NG v. 0.1.7 (Darriba et al., 2020), using Bayesian information criterion. Models selected were HKY with gamma distribution (Hasegawa et al., 1985) for *M. arenaria* and *C. volutator* and F81 (Felsenstein, 1981) for *P. elegans*. The phylogenetic analyses were run with two independent analyses (setting nruns = 2, to allow convergence diagnostics), each using four MCMC (Markov Chain Monte Carlo) chains (three heated, one cold). Number of generations was set to 10 million, sampling every 1,000 generations, and burn-in was set to 1 million generations. For visualising genetic relationship between populations, Kruskal's nonmetric multidimensional scaling (nMDS) plots were constructed using R packages MASS v. 7.3–54 (Venables and Ripley, 2002). The nMDS plots were based on a distance matrix (distances expressed as number of substitutions per 100 bases) constructed using Jukes-Cantor algorithm extracted using distmat in EMBOSS v. 6.5.7 (Rice et al., 2000). Plots were made using ggplot2, v. 3.3.0 (Wickham, 2016).

3. Results

The number of UCE loci amplified for *P. elegans* ranged between 359 and 437 for each sample, of which 14 were shared among samples. The number of UCE loci for *C. volutator* ranged between 362 and 609 per sample, of which 19 were shared among all samples, and for *M. arenaria* we sequenced between 431 and 540 loci for each sample, with 29 loci shared among all samples. Our analyses were limited to only the loci shared among all samples to allow comparison. Tajima's Pi for *P. elegans* varied between 0.0032 and 0.0065 lowest in sample Saltö November 2018 and highest in Saltö August 2019. For *C. volutator,* Tajima's Pi varied between 0.0017 and 0.0031 lowest in sample Öland August 2018 and highest in List. And for *M. arenaria,* Tajima's Pi varied between 0.0022 and 0.0038, lowest in sample List and Saltö August 2018 and highest in Herslev August 2018 (see Table 1).

3.1. Phylogenetic trees

A consensus of the posterior distribution of phylogenetic trees resulting from our Bayesian Analysis was made for each species, combining results from the replicate analyses and using a burnin of 10% to yield a single topology. For all three species, the phylogenetic trees are rooted to List, and thus the remaining samples form a separate clade with maximum support (Bayesian posterior probability (BPP) = 1). For *P. elegans* (Fig. 2A) all Herslev samples are placed together in a separate clade (BPP = 0.586) and the relationships among the remaining samples are unresolved (BPP = 1). The phylogenetic tree for *C. volutator* (Fig. 2B) showed more structure, with majority of samples forming a clade (BPP = 0.756) with the Gollwitz sample grouping with a clade consisting of all Öland samples together with samples from Herslev collected in April and August 2019 (BPP = 0.908). The other samples from Herslev collected in August and November 2018, and the sample from Tvärminne showed unresolved relationships with the other group. In the phylogenetic tree of *M. arenaria* (Fig. 2C) most samples were grouped together (BPP = 0.623) except for the sample from Saltö collected in August 2018. However, the larger group consists mostly of unresolved relationships, except for samples from Herslev in November 2018 and August 2019 which group together (BPP = 0.877), and samples from Öland in April and August 2019 and from Saltö in November 2018 and April 2019 which group together (BPP = 0.549).

Table 1
Tajima's Pi, nucleotide diversity, for each population. – indicates samples not included in the study, NA indicates samples with missing value due to alignment difficulties.

Sample	*P. elegans*	*C. volutator*	*M. arenaria*
List	NA	0.0031	0.0022
Saltö Aug18	0.0034	–	0.0022
Saltö Nov18	0.0032	–	0.0036
Saltö Apr19	0.0056	–	0.0027
Saltö Aug18	0.0065	–	0.0035
Herslev Aug18	0.0063	0.0019	0.0038
Herslev Nov18	0.0036	0.0021	0.0034
Herslev Apr19	0.0046	0.0026	0.0030
Herslev Aug19	0.0048	0.0023	0.0032
Gollwitz	0.0037	0.0018	0.0028
Öland Aug18	–	0.0017	0.0031
Öland Nov18	–	0.0024	0.0037
Öland Apr19	–	NA	0.0037
Öland Aug19	–	NA	0.0037
Tvärminne	–	NA	–

3.2. nMDS plots

Visualization of genetic relationships with nMDS plots reflected similar patterns identified in the phylogenetic analyses, but showed that samples from List (used to root our phylogenies) were not significantly different than the other samples. Our rooting makes List appear distinct in the phylogenetic trees, but genetic variation within the samples are similar according to the distances/nMDS plot. The samples of *P. elegans* (Fig. 3A) were placed in the nMDS plot with a stress of 0.05 and generally did not form any clusters except for Saltö April 2019 and Herslev April 2019 which overlap and are placed closely to the List sample. The other samples are more widely spread, indicating genetic differences. The samples in the nMDS plot for *C. volutator* (Fig. 3B) were resolved with a stress of 0.07. All samples from Öland cluster together with the sample from Herslev April 2019, the samples from Gollwitz and Herslev August 2018 also cluster together, and the remaining samples List, Herslev November 2018, Herslev August 2019, and Tvärminne are placed separately, not forming clusters. For *M. arenaria* (Fig. 3C) the nMDS plot was resolved with a stress of 0.07. The samples from List, Herslev August 2019, Saltö April and August 2019, and Öland November 2019 and August 2019 cluster together. The samples from Saltö November 2018 and Öland August 2018 form a discrete cluster, and samples from Saltö August 2018 and Öland April 2019 are placed somewhat close together. The remaining samples of Herslev (August and November 2018 and April 2019) and Gollwitz, are each placed separately, none of which form clusters with other samples. In particular, Gollwitz is placed far from the other samples.

4. Discussion

We studied phylogeographic relationships among the populations of three benthic macroinvertebrate species, *P. elegans*, *C. volutator*, and *M. arenaria* in the Baltic Sea and adjacent North Sea, to investigate how their different invasions of the Baltic and barriers to gene flow have affected population genetic structure. We constructed phylogenies using Bayesian inference and examined genetic relationship among populations with nMDS based on DNA sequence data from ultra-conserved elements (UCEs). Our focus is on relationships between the populations, since the pooled sequencing data does not allow for analysis of relationships among individuals within populations. Contrary to our expectation, the North Sea population (List) was closely related to the populations within the Baltic Sea for both *P. elegans* and *M. arenaria.* In contrast, the North Sea population for *C. volutator* could be distinguished from the remaining Baltic populations. Furthermore, we did not observe any trends of resemblance of populations related to distance between

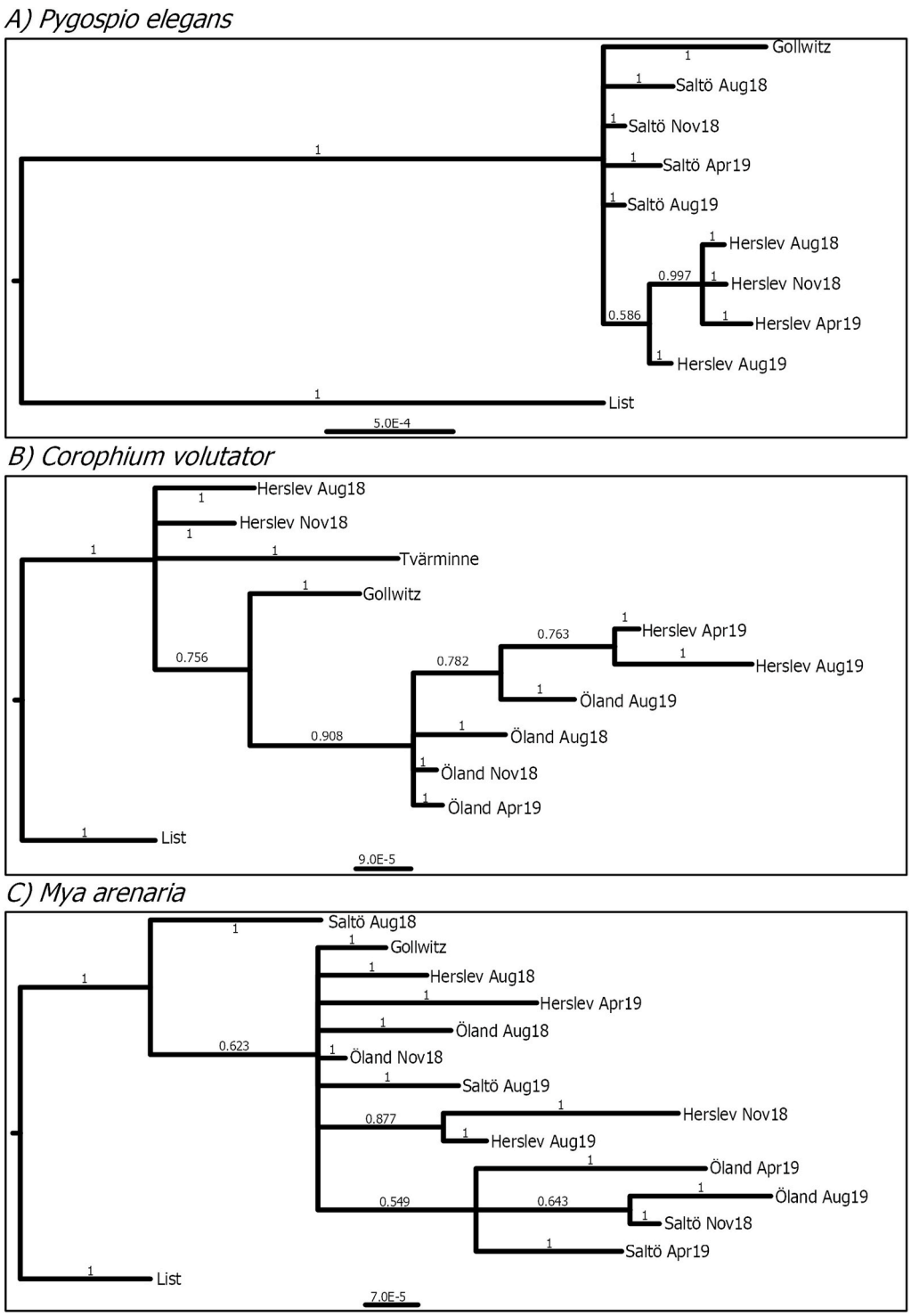

Fig. 2. Phylogenetic tree of (A) *Pygospio elegans*, (B) *Corophium volutator,* (C) *Mya arenaria*, based on Bayesian analysis. Nodal support (Bayesian posterior probability) is displayed at each branch.

sites for either *P. elegans* or *M. arenaria*. However, in the nMDS plots for *P. elegans,* samples from List, Saltö and Herslev showed closer relation compared to the remaining samples from the inner Baltic basins (Fig. 3). For *C. volutator,* we found that samples with the largest geographical distance were less related to the remaining samples, and populations with closer proximity to each other were more closely related, suggesting isolation by distance. In contrast to our expectation, only few of the temporal samples from the same site were clustered more closely together than they were with samples from other sites in the phylogenetic analysis (Fig. 2), and when assessing the nMDS plots (Fig. 3), none of the temporal samples from the same location formed distinct clusters. We did find unique phylogeographic patterns for each of the species in both the phylogenetic- and nMDS analysis. Most populations were relatively different from each other; although samples for *M. arenaria* exhibited closer relations between populations compared to the two other species (Fig. 3).

All three study species originate from the Atlantic from where they have invaded the Baltic Sea at different times (Strasser, 1998;

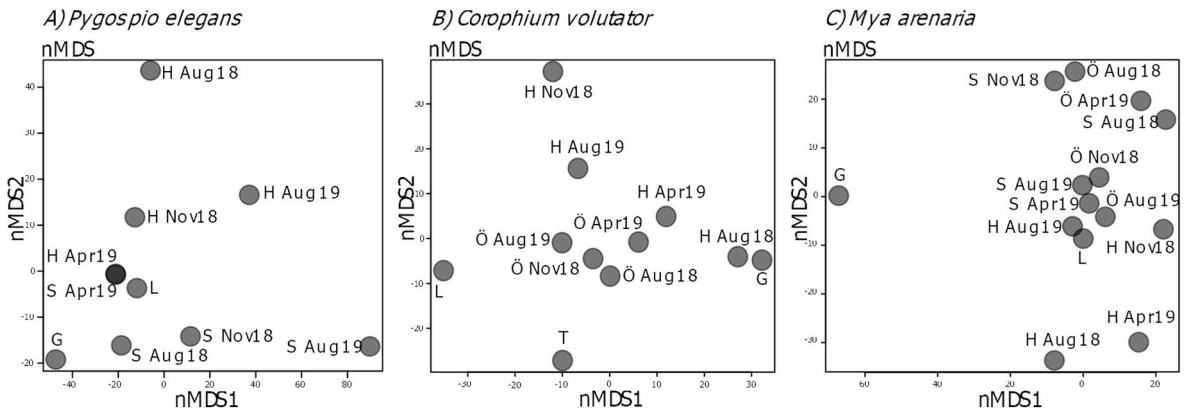

Fig. 3. nMDS of (A) *Pygospio elegans*, (B) *Corophium volutator,* (C) *Mya arenaria*, based on Jukes-Cantor distance matrix. Stress values for the nMDS analyses were (A) 0.05, (B) 0.07, and (C) 0.07, respectively.

Leppäkoski et al., 2002; Kesäniemi et al., 2012a; Einfeldt and Addison, 2015). We therefore expected the populations in the North Sea (List) to be genetically diverged from populations in the Baltic Sea, due to barriers of gene flow. This expectation seemed to hold for the crustacean *C. volutator*, as revealed in both analyses. The nMDS analysis of *C. volutator* also showed that the population from the innermost part of the Baltic Sea, Tvärminne, was separated from all other populations, and that the populations in the inner parts of the Baltic Sea (Herslev, Öland and Gollwitz) were more similar, a pattern that could reflect isolation by distance. For both *P. elegans* and *M. arenaria* on the other hand, the populations from the North Sea (List) are similar genetically to populations within the Baltic Sea, grouping with them in both phylogenetic and nMDS analyses, although rooting of the phylogenetic trees to the North Sea sample List makes their similarity to the Baltic populations harder to discern. A previous population study of *P. elegans* also reported little divergence between populations in the North Sea and the Baltic Sea (Kesäniemi et al., 2012a), but did show some degree of isolation by distance. Although our data did not allow for a test of isolation by distance in this study, the nMDS plots do give an insight into the relationship between populations, and for these we did not observe populations of closer proximity to resemble each other more, thus no patterns related to isolation by distance. The present study, however, did not include as many populations and covered a smaller geographical range of the Baltic Sea compared to the Kesäniemi et al. (2012a) study. The observed chaotic pattern in our study could therefore be a consequence of low sample sizes combined with high variation of the genetic markers (Table 1). The populations of *M. arenaria* from List, Saltö, Herslev and Öland showed no sign of isolation by distance, and the nucleotide diversity showed less variation between sites compared to e.g., *P. elegans* (Table 1). This could be explained by *M. arenaria* having dispersal by pelagic larvae, thus sustaining high connectivity between populations or a more thoroughly mixed gene pool due to the overlap of generations in this very long-lived species. However, we did observe one outlier, Gollwitz, which was markedly different from the other samples in the nMDS analysis. This could reflect an isolated population, though previous studies have found *M. arenaria* from the German coast in the Baltic to be genetically close to populations in the North Sea (De Noia et al., 2020).

We expected populations from the same site at different times of year to be more like each other genetically than like populations from other sites, since mixing of the different populations should be restricted by the long distances between sites. For *P. elegans*, this was the case for the samples from Herslev (Fig. 2A), but not for the samples from Saltö. The same pattern was observed for *C. volutator,* where the Öland samples formed groups in both analyses, but only two of the Herslev samples grouped together in the phylogenetic analysis. For *M. arenaria* only a few of the samples from the same site grouped together. The high genetic variation among temporal samples within sites could be due to differences in connectivity, if recruitment from genetically different populations potentially contributes new cohorts with a distinct genetic makeup at the different times. Seasonal variation in genetic diversity sustained by immigration of genetically different cohorts were found earlier for *P. elegans* (Thonig et al., 2017). This is likely also the case for *C. volutator*, which due to its reproductive patterns (Fish and Mills, 1979), has potential for introduction of genetically different cohorts twice a year. Both species are also known to fluctuate temporally in population size (Bick, 1994; Kesäniemi et al., 2012a), also observed here, and fluctuations in abundance and density could lead to a change in genetic diversity (Bay et al., 2008). However, the pattern could also be due to chance, if populations are so large that the different samples only capture a fraction of the present genotypes. In contrast to the other two species, *M. arenaria* is long-lived, and our samples were taken from individuals of different age classes. Due to this, the samples represent several generations of recruitment, and the observed temporal differences within sites for *M. arenaria* are thus not likely due to seasonal recruitment, but more likely reflect a general high diversity within the different, long-lived cohorts in these populations.

Though we expected some common patterns shared by all three species, we also expected the species to exhibit differences in phylogenetic patterns, not only due to differences in dispersal capability, but also related to their different invasion histories in the Baltic Sea (Leppäkoski et al., 2002). Rapid invasion and establishment of new species in a geographical area may be based on closely related individuals, and consequently exhibit low diversity and genetically homogenous populations (Lasota et al., 2004), if additional invasions from other cohorts or genetically different source populations have not provided more diversity. We therefore expected *P. elegans* and *C. volutator* to have more genetically diverged populations, and show chaotic genetic patchiness compared to *M. arenaria* since the latter species has a more recent invasion in the Baltic Sea. Most populations of *M. arenaria*, except for Gollwitz, showed little genetic differences in comparison to populations of the two other species. *M. arenaria* in the eastern Atlantic and Baltic Sea were previously shown to exhibit homogenous populations with low diversity (Lasota et al., 2004; Cross et al., 2016; De Noia et al., 2020). This could be explained by this species having a more recent invasion, as well as a high dispersal potential via its pelagic planktotrophic larvae.

Our phylogenetic analysis often resulted in unresolved clades, and the nMDS plots also resulted in unexpected clustering. For example, samples from Saltö April 2019 and Herslev April 2019 of *P. elegans* are placed closely in the nMDS plot, but separately in the phylogeny, albeit as sister clades. While this could reflect true relationships, this could also be an artefact of a low sample number, few loci shared among all studied populations (Branstetter et al., 2017), low diversity at the UCEs, or technical problems in obtaining the UCE data using pool-seq (pooled

sequencing of 20 individuals from each population, likely representing multiple genotypes). Our analyses are based on estimates of the genetic variation from a consensus sequence generated by calculating the most frequent nucleotide among all individuals, but does not take into account possible different haplotypes in the same population, and so, are conservative estimates of the genetic variation. Though UCEs previously have been used successfully in phylogenomic studies (Faircloth et al., 2012; Quattrini et al., 2018; Winker et al., 2018), the low number of amplified loci in this study may reflect the absence of several potential loci (poor matches to the designed UCE baits). Furthermore, pooling of samples could have resulted in low read depth, resulting in the variable number of loci obtained in each sample and few loci shared among all samples. These technical problems could not be avoided in the current study, since genomes of closely related species were not available to improve design of the UCE baits and financial constraints limited our sequencing analysis to pools of individuals. Consequently, our analysis of the diversity and differences among populations could be underestimated and should be interpreted cautiously and represent a first attempt at assessing the genetic patterns with UCE loci for these three species in the greater Baltic region.

5. Conclusion

This study provides a comparison of genetic similarities and phylogeographic relationships among the populations of three macrobenthic invertebrate species, *P. elegans*, *C. volutator*, and *M. arenaria,* in the Baltic Sea. We found the phylogeographic patterns to be different for the three species: *P. elegans* displayed chaotic diversity, *C. volutator* showed evidence of isolation by distance and for *M. arenaria* the populations exhibited a more uniform diversity. Furthermore, there was large genetic difference between populations at the same sampling site collected at different times of the year for all species. This high within-site variation is possibly due to different reproductive patterns and recruitment events, or to a general high diversity within the cohorts in these populations and limited sampling. Populations in the North Sea (List) for both *P. elegans* and *M. arenaria* were not genetically divergent from populations within the Baltic Sea, and only the populations of *C. volutator* exhibited a trend towards isolation as a function of distance. These differences could both be explained by differences in invasion history or dispersal potential of the individual species. However, these patterns must be interpreted with caution due to the technical difficulties and limitations imposed by our study design. The use of ultraconserved elements provided a way to assay similar loci among species from very different taxonomic groups, but the number of obtained loci were few and our results are based on pooled samples, which could have led to underestimates of the genetic variation and population differences of these species. This could be due to difficulties of designing baits for multiple divergent taxa with high genetic variation. To gain the full potential of UCEs, more focused baits design would be needed, which requires more sequenced genomes from within these taxonomic groups. Applying the UCE approach to the individual level, rather than pools, as a next step will enable a fuller understanding of the biogeographic patterns of these species in the Baltic Sea. Additionally, to better understand the potential of UCEs relative to other genetic markers, such as microsatellites, a comparison of methods is needed.

CRediT authorship contribution statement

H. Cecilie Petersen: Writing – original draft. **K. Emily Knott:** Writing – review & editing. **Gary T. Banta:** Writing – review & editing. **Benni W. Hansen:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully thank Karsten Reise for help with field sampling and Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research in List, Germany, for use of facilities. Stefan Förster for field assistance and Rostock University, Germany, for use of facilities. Kerstin Johannesson and Per Jonsson for help and guidance with collecting field samples, and Tjärnö field station, Gothenburg University, Sweden, for use of facilities. Laura Kauppi for assistance in field sampling, and Tvärminne field station, University of Helsinki, Finland, for use of facilities. We also thank Anna-Lotta Hiillos, Theophilus Alale, and Anne Busk Faarborg for assisting with the field sampling and CSC–IT Center for Science, Finland for providing computing facilities. Field sampling and laboratory work for this study was funded by Roskilde University (Department of Science and Environment), University of Jyväskylä (Department of Biological and Environmental Science), University of Southern Denmark (Department of Biology), Elite Research travel grant, Ministry of Higher Education and Science, Denmark (to HCP), Faxe Fonden (to HCP), H. C. Wegges Mindelegat for Zoologer (to HCP).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2022.107863.

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