Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2015) **81**: 495–501. doi:10.1093/mollus/eyv022
Advance Access publication date: 6 July 2015

Environmental DNA evidence of transfer of North Sea molluscs across tropical waters through ballast water

Alba Ardura[1,2†], Anastasija Zaiko[2†], Jose L. Martinez[3], Aurelija Samuiloviene[2,4], Yaisel Borrell[1] and Eva Garcia-Vazquez[1]

[1]*Department of Functional Biology, University of Oviedo, C/ Julian Claveria s/n, Oviedo 33006, Spain;*
[2]*Marine Science and Technology Centre, Klaipeda University, H. Manto 84, Klaipeda LT 92294, Lithuania;*
[3]*Unit of DNA Analysis, Scientific-Technical Services, University of Oviedo, Edificio Severo Ochoa, Campus del Cristo, Oviedo 33006, Spain; and*
[4]*Department of Biology, Klaipeda University, H. Manto 84, Klaipeda LT 92294, Lithuania*

Correspondence: A. Ardura; e-mail: alarguti@hotmail.com

(Received 23 September 2014; accepted 2 May 2015)

ABSTRACT

Maritime transport, in particular of ballast water, is considered to be one of the most important pathways of marine biological invasions worldwide. Here we provide the first molecular evidence of potential survival of the European mudsnail, *Peringia ulvae*, in ballast water on cross-latitudinal voyages. Ballast water from the RV *Polarstern* was sampled at its departure from the North Sea and again in tropical latitudes; DNA was extracted and amplicon-sequenced employing high-throughput sequencing methodology. Mollusc species were detected by cytochrome oxidase subunit I DNA barcode sequences. The increasing proportion of operational taxonomic units that were identified as *P. ulvae* after 2 weeks of navigation suggests that this species withstands the harsh conditions in the ballast tank. As such, *P. ulvae* has the potential to reach very distant, new marine areas where it eventually might establish itself as a nonindigenous species. We also discuss the potential of environmental DNA analysis for en-route biodiversity screening and species-specific risk assessments, as well as some current limitations of the approach.

INTRODUCTION

Shipping is believed to be one of the most important pathways for transfer of indigenous species across marine regions (Leppäkoski, Gollasch & Olenin, 2002). This pathway involves several potential vectors—transport of organisms in ballast water and ballast tank sediments, and fouling of hull, sea chests, anchors and anchor chains, etc. (Hewitt, Gollasch & Minchin, 2009). Ballast water (BW) is recognized as the most significant of these vectors (Molnar *et al.*, 2008). Approximately 2.2 to 12 billion tons of BW are transported across the world oceans annually (Endresen *et al.*, 2004), transferring daily some 7,000 species (Gollasch & David, 2011). In a summary of 15 European BW surveys, living specimens of more than 1,000 taxa were found in ballast tanks of vessels arriving in European ports (Gollasch *et al.*, 2002).

Due to the extremely harsh conditions (darkness, temperature changes, salinity pulses, variable turbidity, turbulence and oxygen depletion), the numbers of living organisms in ballast tanks decline rapidly after ballasting (Gollasch *et al.*, 2000; Hewitt, Gollasch & Minchin, 2009). Nevertheless, there are examples of organisms surviving long intercontinental transfers (Gollasch *et al.*, 2000).

A golden rule for successful invaders is 'the more tolerant are the more dangerous' (Sakai *et al.*, 2001; Lee, 2002; Madariaga *et al.*, 2014). Therefore, migrants that survive long cross-latitudinal voyages within ballast tanks should be of particular concern as potential invaders. Identifying such species is crucial for conducting reliable risk analyses, preventing expansions and developing efficient control methods (Tsolaki & Diamadopoulos, 2010). However, many species transported in BW as eggs or larvae are often very difficult to identify. This is especially true in en-route surveys, when samples are collected and analysed on board without taxonomic expertise. DNA methodologies are very useful complementary tools to identify organisms in BW (Darling & Blum, 2007; Harvey, Hoy & Rodriguez, 2009; Darling & Mahon, 2011; Briski *et al.*, 2012). Recently, the development of next generation sequencing (NGS) technologies has simplified and speeded-up the process by allowing the identification of entire communities in water samples, using bulk or environmental DNA (eDNA) analysis. eDNA is extracted directly from environmental samples (e.g. soil or water) (Ficetola *et al.*, 2008). This allows the detection of species from single cells in a sample, such as gametes, secreted faeces or mucus, and is particularly advantageous for small, rare and cryptic species or life stages that are difficult to detect otherwise (Ficetola *et al.*, 2008; Valentini, Pompanon & Taberlet, 2009; Taberlet *et al.*, 2012; Thomsen *et al.*, 2012). The eDNA approach in combination with NGS is increasingly exploited in metabarcoding/metagenetic studies aimed at biodiversity research (Hajibabaei *et al.*, 2011; Wood *et al.*, 2013). Many mollusc invasions have been associated

[†]Contributed equally.

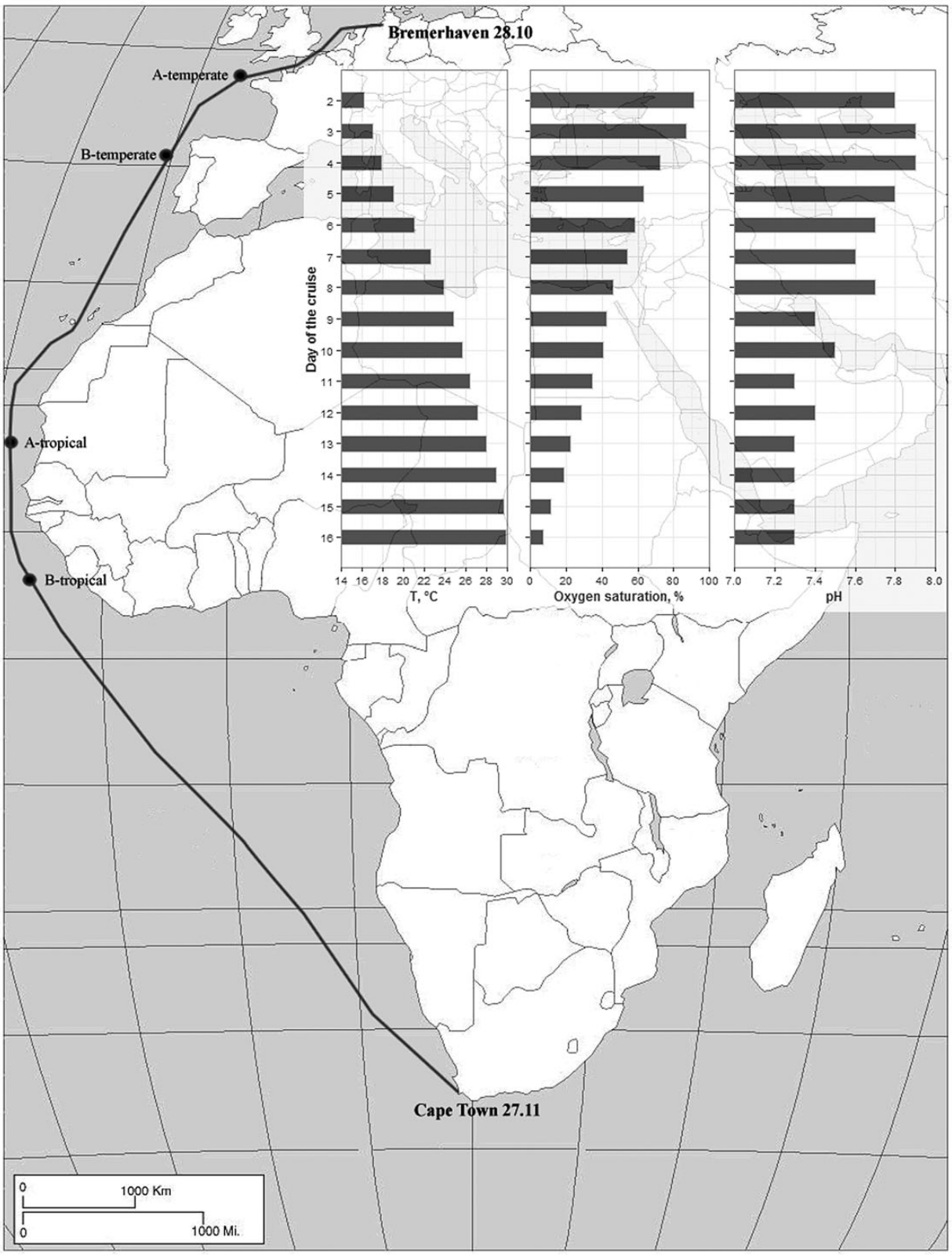

Figure 1. Approximate geographical position of RV *Polarstern* on the sampling days and dynamics of the physical-chemical conditions (temperature, oxygen saturation and pH) in the ballast tank over the 16 d of the cruise.

with the unintentional transport of planktonic life stages (e.g. Strayer, 2010). Examples are the bivalves *Dreissena* species (Benson, 2013), *Corbicula* species (Grigorovich *et al.*, 2003), *Limnoperna fortunei* (Ricciardi, 1998), *Corbula amurensis* (Carlton *et al.*, 1990) and the gastropods *Crepidula fornicata* (Elliot, 2003) and *Potamopyrgus antipodarum* (Alonso & Castro-Diez, 2008).

In this study we apply metabarcoding (eDNA) for species identification in BW from the RV *Polarstern* during the expedition

Table 1. Summary of the NGS results and taxonomic assignment.

	A		B	
	Temperate	Tropical	Temperate	Tropical
Reads	27,497	14,304	22,341	11,536
Assigned reads	16,989	3,032	22,242	11,525
Chromista	423	33	1,707	1,447
Plantae	2	1	4,935	2,767
Animalia	16,562	2,998	15,600	7,311
Mollusca	2	1,102	750	353
Peringia ulvae	0	1,100	748	353

A and B are Ion Torrent and 454 platforms, respectively. Totals are, respectively, the numbers of reads obtained for each sample after sequence-quality check; total numbers of sequences assigned to marine taxa OTUs, numbers of Chromista, Plantae and Animalia, and within the last the number of mollusc and *Peringia ulvae* sequences.

ANT-XXIX/1 in October–December 2012 (from Bremerhaven, Germany to Cape Town, South Africa). We focused on the detection of molluscs that could have survived the harsh BW conditions over the cross-latitudinal transfer, and which hence could become nonindigenous or invasive species. Metabarcoding has been successfully applied for studying the development of general biodiversity in BW during this expedition (Zaiko *et al.*, 2015) and we here explore the applicability of eDNA for the taxonomic screening of BW and species-specific risk assessments.

MATERIAL AND METHODS

Collection of water samples and environmental metadata

The aft ballast tank (70 m^3) of the vessel was filled with North Sea water on 28 October 2012, off Bremerhaven. At the time of the BW upload, water temperature and salinity were 13.1 °C and 34 ppt, respectively. Four samples of BW were collected via the water pipe on days 2 and 4 (in temperate latitudes) and days 12 and 16 (in tropical latitudes) of the cruise (Fig. 1).

For each sample, 100 l of BW were pumped through a plankton net (30 cm diameter, 55 μm mesh size). The concentrated material (*c.* 50 ml) was then vacuum-filtered through a 0.2 μm Nuclepore™ membrane, which was thereafter preserved in 96% ethanol until eDNA extraction. Changes in temperature, pH and oxygen saturation of the BW were measured using an Ysi Professional Plus Multimeter.

DNA and bioinformatics analyses

DNA was extracted from the filters using the QIAamp DNA Mini Kit (Qiagen) and was quantified with a fluorescence-based quantification method (Picogreen, Invitrogen). In order to validate the findings of mollusc eDNA, the extracted bulk DNA was analysed with two different NGS platforms: days 2 and 12 samples: the Ion Personal Genome Machine system (PGM Life Technologies) at the Sequencing Unit of Oviedo University (Spain); days 4 and 16 samples: the Genome Sequencer FLX (Roche 454) at Macrogen (Korea). Universal minibarcode primers (Meusnier *et al.*, 2008) were used to amplify and sequence *c.* 140 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. For the 454 sequencing, 1/40 of the 454 plate was used for each BW sample. The GS FLX data processing was performed using the Roche GS FLX software (v. 2.9). The software used tag (barcode) sequences to segregate the reads from each sample, by matching the initial and final bases of the reads to the known tag sequences used in the preparation of the libraries. Zero base errors were allowed in this sorting by tag step. Raw data were then processed using PRINSEQ v. 0.20.4 (Schmieder & Edwards, 2011) for filtering too short and/or too long reads (mode ±2 SD) and also to eliminate low-quality reads (mean ≥20). Ambiguous sequences were discarded.

The sequencing and data processing with Ion Torrent were performed as explained by Zaiko *et al.* (2015). Briefly, libraries were constructed using the kit Ion Plus Fragment Library Kit (Life Technologies) and templates were obtained using the Ion PGM™ Template OT2 200 Kit (Life Technologies). The templates were loaded in a 314 chip and sequenced using the Ion PGM Sequencing 200 Kit v. 2 (Life Technologies). The resulting sequences were filtered by length (between 130 and 200 bp) and quality (+20). The expected length of the target miniCOI region falls within this length range, so further contig analysis was not necessary for operational taxonomic unit (OTU) assignment.

Taxonomic classification of the obtained datasets was done by BLAST-aligning sequences against the NCBI nucleotides database (http://www.ncbi.nlm.nih.gov/) using the QIIME platform (Caporaso *et al.*, 2010). The same software was used for prior de-noising and detection of chimeras. Taxonomic criteria were: best hit, max *E*-value = 0.001, min percent identity = 90.0, which are not sufficiently strict for species assignation, but which allow retention of class-, order- or family-level taxa.

After initial analysis, the dataset of OTUs with their closest reference matches was curated, i.e. species taxonomic information was verified and checked against the World Register of Marine Species (http://www.marinespecies.org/), AlgaeBase (http://www.algaebase.org/) and Encyclopedia of Life (http://eol.org/) databases. OTUs involving nonmarine organisms were eliminated. The curated sequence dataset was employed for the subsequent analyses.

Since this study did not aim at analysing biodiversity, we only assigned the sequences of interest to kingdoms and, within Animalia, we calculated the frequency of putative molluscs in temperate and tropical samples. Percentages were employed for this quantification.

Phylogenetic analysis

Sequences identified as mollusc DNA were manually extracted from the NGS output files identified as molluscs in the OTU list and aligned using the BioEdit software (Hall, 1999). Haplotypes were determined with the program DnaSP (Librado & Rozas, 2009). Distinguishing between the results from different NGS platforms, we called sequences A or B, according to whether they were obtained from Ion Torrent or 454 respectively.

A reference database of invasive molluscs was constructed from COI gene sequences obtained from GenBank (Supplementary material, Table S1). The species selection was made based on recognized invasive capacity of different mollusc taxa from the sequences available in GenBank. The species were included if classified as dangerous or globally invasive for marine habitats in the IUCN ISSG database.

Phylogenetic analyses were conducted using MEGA v. 6 (Tamura *et al.*, 2013). Phylogenetic trees containing the reference and BW sequences obtained in this work were inferred with maximum likelihood (ML) with the following settings: Tamura Nei model (Tamura & Nei, 1993) for nucleotides and Jones-Taylor-Thornton (JTT) Matrix model (Jones, Taylor & Thornton, 1992) for amino acids. Robustness of the tree topology was assessed using 1,000 bootstrap replicates.

The chi-square statistic was employed to assess the significance of the shift in proportions of the particular haplotypes.

RESULTS

The environmental conditions of the *Polarstern* BW changed dramatically over the sampling period (Fig. 1). The temperature

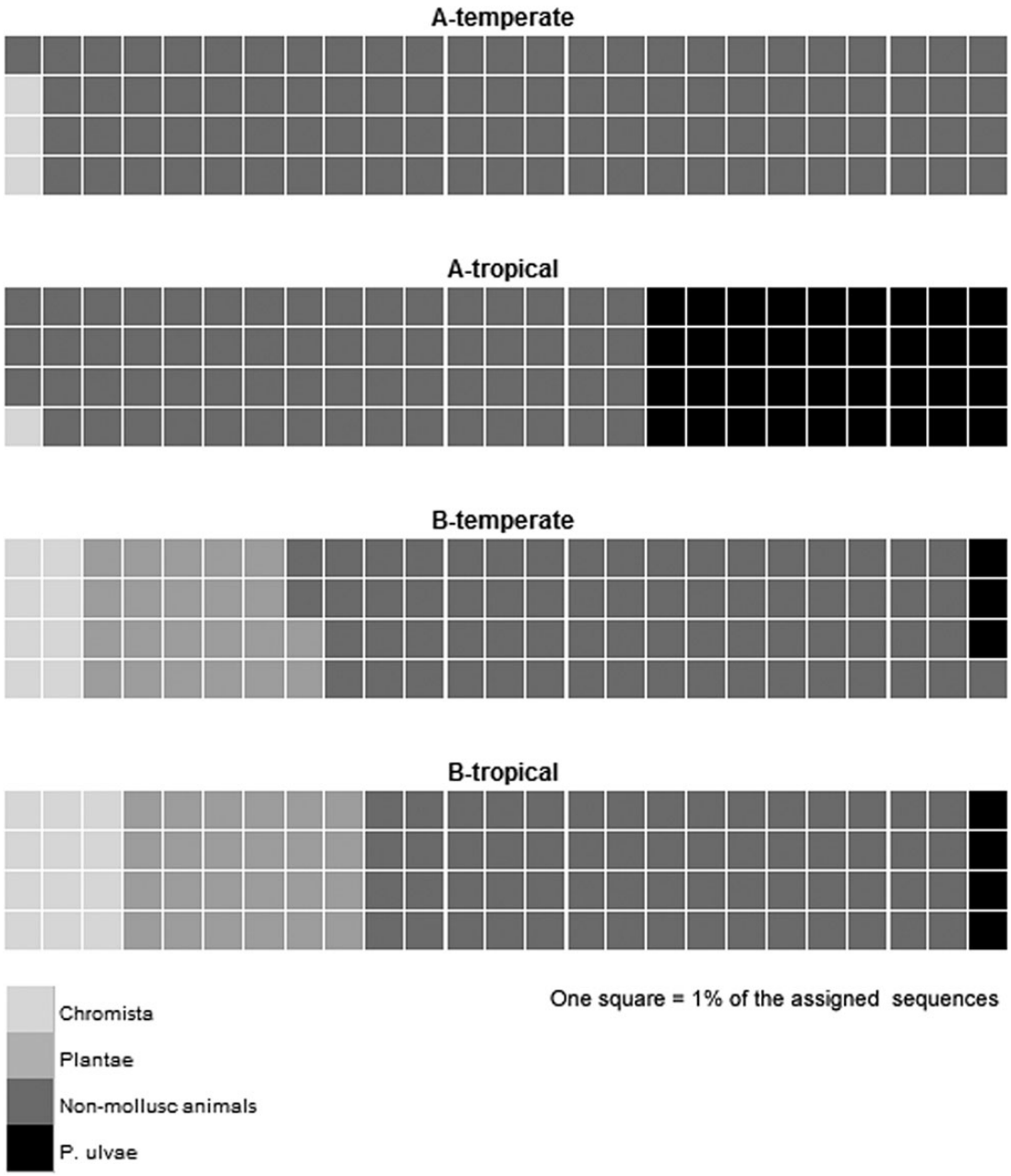

Figure 2. Proportion of DNA sequences assigned to three eukaryote kingdoms (Chromista, Plantae and Animalia, the last with the proportion of *Peringia ulvae* sequence shown) found in the ballast water sampled in temperate and tropical latitudes. A and B are datasets obtained from Ion Torrent and 454 NGS platforms, respectively.

increased by nearly 14 °C, while oxygen saturation and pH decreased by 84% and 0.5, respectively, between days 2 and 16 of the voyage.

The sequences and putative marine taxa obtained from the analysed samples using 454 and Ion Torrent platforms are summarized in Table 1. A total number of 16,989 and 22,242 sequences of the expected size (around 150 bp, Meusnier *et al.*, 2008) that BLASTed to marine taxa were obtained from the two temperate samples with the Ion Torrent and 454 platforms respectively. From the tropical samples, 3,032 and 11,525 sequences were assigned to marine taxa in datasets A and B, respectively, demonstrating a substantial reduction in NGS reads (Table 1). In general, the share of assigned sequences was higher (nearly 100%) in 454 datasets comparing to Ion Torrent ones (62 and 21% in temperate and tropical samples respectively). Animalia were clearly the dominant domain in all analysed datasets, while Plantae and Chromista were underrepresented in the Ion Torrent datasets. The absolute majority of Mollusca sequences obtained from the samples were BLASTed to *Peringia ulvae* (formerly *Hydrobia ulvae*). The closest match for the OTUs found here from both tropical samples and the B-temperate sample was the GenBank reference AF118308 followed by AF118290. In addition two OTUs with the closest match with *Lophiotoma leucotropis* (GenBank HQ834093) were found in the B-temperate sample, while four were BLASTed to cephalopods (*Sepia* spp.) in B-temperate and tropical samples.

The BW biota composition shifted between the temperate and the tropical samples, with a particular increase in the proportion of Chromista in the B dataset (Fig. 2). The overall proportion of *P. ulvae* within the total number of assigned sequences increased from 3 to 4% in the B dataset and from 0 to 36% in the A dataset, being by far the most abundant molluscan OTU.

In the manually extracted sequences from the NGS data files, the mollusc sequences found in the B-temperate sample and BLASTed to *P. ulvae* and *L. leucotropis* corresponded, respectively, to eight and one different haplotypes (BWTemperate01-08B, EMBL references HG963478-85 and BWTemperate09; Fig. 3). The eight haplotypes BWTemperate 1 to 8 were found approximately in the same proportion within the 748 OTUs BLASTed to *P. ulvae*.

In the B-tropical sample, the 353 mollusc-BLASTed sequences corresponded to one unique haplotype (BWTropical01B, EMBL reference HG963486) with the closest match to *P. ulvae*. Exactly the same haplotype was retrieved from the *Peringia*-BLASTed OTUs A tropical sample (BWTropical01A, EMBL reference HG963486). This haplotype was also present in the B-temperate sample, named there as BWTemperate02 (EMBL reference HG963479) (Fig. 3). The other haplotypes in the B-temperate sample did not appear in the tropical sample. A rough quantitative analysis demonstrated an increase of the proportion of this haplotype from approximately 12.6% to 100% of all the *Peringia*-BLASTed OTUs in the B-temperate and B-tropical samples, respectively. This increase was statistically significant (contingency chi-square = 760.3, $P \ll 0.001$, for 1 degree of freedom). On the other hand, the proportion of this haplotype over the total number of the BW OTUs increased from 0.42% in the B-temperate to 3.03% in the B-tropical sample (contingency chi-square = 397.5, $P \ll 0.001$, for 1 degree of freedom). In the A-tropical sample from the Ion Torrent platform its proportion was much higher (36%), being the unique *Peringia* haplotype as noted above.

To confirm BLAST species identification, the mollusc-like sequences from the B dataset were aligned with the reference GenBank COI sequences of invasive molluscs (Supplementary material, Table S1) plus the GenBank sequences with the closest match (two *P. ulvae* and one *L. leucotropis*). The resulting phylogenetic trees showed similar topologies (e.g. Fig. 3) in which gastropods and bivalves were clustered in separated branches. All the BW sequences that BLASTed as *P. ulvae* clustered closely with the reference *P. ulvae* sequences. The other mollusc-like sequence found in the B-temperate sample, BWTemperate09, clustered in the branch of gastropods, but clearly separated from the *L. leucotropis* reference. A closer examination of this sequence revealed that the haplotype contained stop codons (Fig. 4). This means that it does not correspond to the true mitochondrial COI coding sequence and could be considered a pseudogene. On the other hand, the eight haplotypes assigned to *P. ulvae* (EMBL accession numbers HG963478-85) code for amino acid sequences compatible with the standard COI proteins.

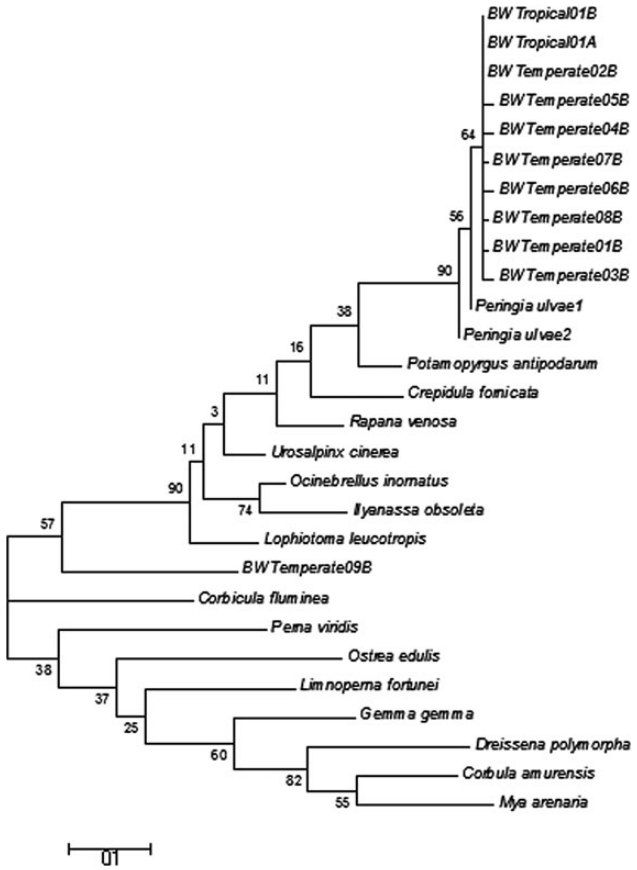

Figure 3. Maximum likelihood tree based on NCBI retrieved sequences (with reference numbers) and ballast water COI gene sequences. Bootstrap values as percentages.

DISCUSSION

The results of this study suggest that the European mudsnail *Peringia ulvae* can successfully cross the oceans in BW. We detected the presence of sequences most closely matching with this species in BW samples, with the proportion of a particular haplotype increasing over time. This could be explained if this haplotype was less degraded than the rest. Of course, the mere presence of a species-specific DNA does not ensure that the species has been sampled alive. Previous studies have demonstrated that extracellular eDNA molecules can persist in water for several days to weeks (Dejean *et al.*, 2011; Barnes et al. 2014), even if degraded by the action of environmental factors such as UV, pH and microbial activity (Hall & Ballantyne, 2004; Thacker *et al.*, 2006; Pilliod *et al.*, 2013; Barnes *et al.*, 2014). Hence, decay is expected if DNA molecules are not inside living cells (Levy-Booth *et al.*, 2007; Dejean *et al.*, 2011). So, after 16 days of navigation under increasing temperatures, low oxygen and slightly decreasing pH, it is expected that only living organisms will increase their relative DNA contribution to the BW eDNA pool. In the temperate sample we found eight different haplotypes and only one of them was maintained (and even increased its relative proportion) in the tropical sample (Fig. 3). These observations indicate that at least one *P. ulvae* haplotype persisted longer than other organisms during the cross-latitudinal BW transfer. Alternatively, the apparent increase of haplotype BWTemperate02 (=BWTropical01) could be attributed to a difference in sequencing success between the two samples. Nevertheless, its very high proportion in the A-tropical data rather points to our first hypothesis.

To our knowledge there are no reports of *P. ulvae* out of its native range (northeastern Atlantic Ocean and Mediterranean Sea) so far. However, due to its biological traits it could exhibit invasive behaviour if introduced to other marine ecosystems. Within its native range (e.g. Danish waters) it is known to compete with the sympatric hydrobiid *Ecrobia ventrosa* (Gorbushin, 1996). In other European regions, *P. ulvae* appears to be tolerant of diverse ecological conditions, inhabiting intertidal zones, whereas its

Figure 4. Alignment between inferred amino acid sequences of Temperate09 ballast water sample and a *Lophiotoma leucotropis* reference. Asterisk represents stop codons.

hydrobiid competitors *E. ventrosa* and *Hydrobia neglecta* are confined to the nontidal lagoons (Barnes, 1999). On the other hand, in similar salinity conditions *P. ulvae* would adapt to warm temperatures (up to 30 °C) better than other Hydrobiidae (Pascual & Drake, 2008). These examples indicate the capacity of the species to survive in diverse environments, including the BW conditions. There are some well-known examples of Hydrobiidae being extremely aggressive invaders, e.g. the New Zealand mudsnail *Potamopyrgus antipodarum*, that have been introduced into many aquatic ecosystems worldwide and have induced numerous adverse impacts to the local habitats and communities (Snoeijs, 1989; Alonso & Castro-Diez, 2008). Hence, further investigation and risk assessment of the potential invasive capacity of *P. ulvae* are recommended in order to set an adequate management strategy to prevent its spread overseas with BW.

The NGS methodology applied here is a promising tool for biodiversity screening and detection of potentially invasive taxa in BW. It meets the efficiency, consistency and comprehensiveness prescribed for BW surveillance and risk-assessment procedures (Helcom, 2010; Zaiko *et al*., 2015). However, there are still potential limitations that need to be taken into account, and ideally ruled out in the future, to ensure the robustness of the approach.

For instance, the universal primers employed in our study might not amplify equally well in all taxa present in a sample (Meusnier *et al*., 2008) or in all samples, so that certain taxa may be overlooked in certain samples. This might explain the apparent lack of *P. ulvae* in the A-temperate sample. Therefore eDNA analyses need to be validated by taking samples on consecutive days and by using different platforms. The discrepancy between platforms is one of the problems that must be solved for a generalized use of NGS data for routine monitoring of biological invasions. The DNA fragment targeted here was comparatively short (Meusnier *et al*., 2008). Although this is an advantage for detecting DNA traces in environmental samples, longer fragments will discriminate better between closely related species and increase the robustness of taxonomic assignments. Additional confirmation of taxonomic assignments from other markers would be desirable when higher taxonomical resolution and identification confidence are required (Kelly *et al*., 2014).

Another important issue that can potentially compromise NGS results is the availability, taxonomic coverage and reliability of the reference sequence databases (Ardura, Planes & Garcia-Vazquez, 2013; Pochon *et al*., 2015). In order to at least partly overcome this limitation, the results of the present study were confirmed by NCBI-derived reference sequences of selected invasive species and their phylogenetic analysis, for the taxonomic assignment of eDNA derived sequences. Phylogenetic analyses have been successfully applied in diversity studies to confirm the taxonomic status of unknown sequences (e.g. Moon-van der Staay, De Wachter & Vaulot, 2001). However, there is no reference sequence collection specifically for invasive species. For this study, we have compiled a reference database of invasive mollusc species sequences from publically available sources. By relying on this database we could reasonably reject the idea that mollusc eDNA sequences found in our samples belonged to any of the already recognized invasive species, although they were somewhat similar to the New Zealand mudsnail *Potamopyrgus antipodarum*.

The results of this study indicate the high likelihood of species survival in BW or ballast sediments on cross-regional voyages. It can therefore be used for the species-specific risk assessments required by the Ballast Water Management Convention (IMO, 2004) and for prioritizing species of greatest management concern (Lehtiniemi *et al*., 2015).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

ACKNOWLEDGEMENTS

We thank the crew of the RV *Polarstern* for their collaboration and Thierry Backeljau for his valuable comments on the manuscript. This study has been supported by the Erasmus Mundus EMBC for BW sampling, the Spanish National Project MINECO CGL2013-42415-R, the Campus of Excellence of the University of Oviedo, the Principality of Asturias Grant GRUPIN14-93 and the Research Council of Lithuania project BALMAN (development of ships' ballast water management system to reduce biological invasions, project no. TAP-LLT-14-013). This is a contribution from the Marine Observatory of Asturias. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

ALONSO, A. & CASTRO-DIEZ, P. 2008. What explains the invading success of the aquatic mud snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca)? *Hydrobiologia*, **614**: 107–116.

ARDURA, A., PLANES, S. & GARCIA-VAZQUEZ, E. 2013. Applications of DNA barcoding to fish landings: authentication and diversity assessment. *ZooKeys*, **365**: 49–65.

BARNES, M.A., TURNER, C.R., JERDE, C.L., RENSHAW, M.A., CHADDERTON, W.L. & LODGE, D.M. 2014. Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science Technology*, **48**: 1819–1827.

BARNES, R.S.K. 1999. What determines the distribution of coastal Hydrobiid mud snails within north-western Europe? *Marine Ecology*, **20**: 97–110.

BENSON, A.J. 2013. Chronological history of Zebra and Quagga Mussels (Dreissenidae) in North America, 1988–2010. In: *Quagga and zebra mussels: biology, impacts and control*. Edn 2 (T. Nalepa & D. Schlosser, eds), pp. 9–32. CRC Press, Boca Raton, FL.

BRISKI, E., GHABOOLI, S., BAILEY, S.A. & MACISAAC, H.J. 2012. Invasion risk posed by macroinvertebrates transported in ships' ballast tanks. *Biological Invasions*, **14**: 1843–1850.

CAPORASO, J.G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F.D., COSTELLO, E.K., FIERER, N., GONZALEZ PEÑA, A., GOODRICH, J.K., GORDON, J.I., HUTTLEY, G.A., KELLEY, S.T., KNIGHTS, D., KOENIG, J.E., LEY, R.E., LOZUPONE, C.A., McDONALD, D., MUEGGE, B.D., PIRRUNG, M., REEDER, J., SEVINSKY, J.R., TURNBAUGH, P.J., WALTERS, W.A., WIDMANN, J., YATSUNENKO, T., ZANEVELD, J. & KNIGHT, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**: 335–336.

CARLTON, J.T., THOMPSON, J.K., SCHEMEL, L.E. & NICHOLS, F.H. 1990. Remarkable invasion of San Francisco Bay (California, USA) by the Asian clam *Potamocorbula amurensis*. I. Introduction and dispersal. *Marine Ecology Progress Series*, **66**: 81–94.

DARLING, J.A. & BLUM, M.J. 2007. DNA-based methods for monitoring invasive species: a review and prospectus. *Biological Invasions*, **9**: 751–765.

DARLING, J.A. & MAHON, A.R. 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research*, **111**: 978–988.

DEJEAN, T., VALENTINI, A., DUPARC, A., PELLIER-CUIT, S., POMPANON, F., TABERLET, P. & MIAUD, C. 2011. Persistence of environmental DNA in freshwater ecosystems. *PLoS One*, **6**: e23398.

ELLIOT, M. 2003. Biological pollutants and biological pollution—an increasing cause for concern. *Marine Pollution Bulletin*, **46**: 275–280.

ENDRESEN, Ø., BEHRENS, H.L., BRYNESTAD, S., ANDERSEN, A.B. & SKJONG, R. 2004. Challenges in global ballast water management. *Marine Pollution Bulletin*, **48**: 615–623.

FICETOLA, G.F., MIAUD, C., POMPANON, F. & TABERLET, P. 2008. Species detection using environmental DNA from water samples. *Biology Letters*, **4**: 423–425.

GOLLASCH, S. & DAVID, M. 2011. Sampling methodologies and approaches for ballast water management compliance monitoring. *Traffic and Environment*, **33**: 397–405.

GOLLASCH, S., LENZ, J., DAMMER, M. & ANDRES, H.G. 2000. Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. *Journal of Plankton Research*, **22**: 923–937.

GOLLASCH, S., MACDONALD, E., BELSON, S., BOTNEN, H., CHRISTENSEN, J., HAMER, J.P., HOUVENAGEL, G., JELMERT, A., LUCAS, I., MASSON, D., McCOLLIN, T., OLENIN, S., PERSSON, A., WALLENTINUS, I., WETSTEYN, L.P.M.J. & WITTLING, T. 2002. Life in ballast tanks. In: *Invasive aquatic species of Europe: distribution, impacts and management* (Springer Science & Business Media, eds), pp. 217–231. Kluwer Academic Publishers, Dordrecht, The Netherlands.

GORBUSHIN, A.M. 1996. The enigma of mud snail shell growth: asymmetrical competition or character displacement? *Oikos*, **77**: 85–92.

GRIGOROVICH, I.A., COLAUTTI, R.I., MILLS, E.L., HOLECK, K., BALLERT, A.G. & MACISAAC, H.J. 2003. Ballast-mediated animal introductions in the Laurentian Great Lakes: retrospective and prospective analyses. *Canadian Journal of Fisheries and Aquatic Science*, **60**: 740–756.

HAJIBABAEI, M., SHOKRALLA, S., ZHOU, X., SINGER, G.A. & BAIRD, D.J. 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS One*, **6**: e17497.

HALL, A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**: 95–98.

HALL, A. & BALLANTYNE, J. 2004. Characterization of UVC-induced DNA damage in bloodstains: forensic implications. *Analytical and Bioanalytical Chemistry*, **380**: 72–83.

HARVEY, J.B.J., HOY, M.S. & RODRIGUEZ, R.J. 2009. Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. *Journal of Experimental Marine Biology and Ecology*, **369**: 93–99.

HELCOM. 2010. *HELCOM Ministerial Declaration on the implementation of the HELCOM Baltic Sea Action Plan*. Moscow. http://www.helcom.fi/.

HEWITT, C.L., GOLLASCH, S. & MINCHIN, D. 2009. The vessel as a vector—biofouling, ballast water and sediments. In: *Biological invasions in marine ecosystems* (G. Rilov & J.A. Crooks, eds), pp. 117–129. *Ecological Studies* 204. Springer-Verlag, Berlin.

IMO. 2004. *International Convention for the Control and Management of Ships' Ballast Water and Sediments*. IMO. www.imo.org.

JONES, D.T., TAYLOR, W.R. & THORNTON, J.M. 1992. The rapid generation of mutation data matrices from protein sequences. *Computer Applications in the Biosciences*, **8**: 275–282.

KELLY, R.P., PORT, J.A., YAMAHARA, K.M., MARTONE, R.G., LOWELL, N., THOMSEN, P.F., MACH, M.E., BENNET, M., PRAHLER, E., CALDWELL, M.R. & CROWDER, L.B. 2014. Harnessing DNA to improve environmental management. *Science*, **344**: 1455–1456.

LEE, C.E. 2002. Evolutionary genetics of invasive species. *Trends in Ecology and Evolution*, **17**: 386–391.

LEHTINIEMI, M., OJAVEER, H., DAVID, M., GALIL, B., GOLLASCH, S., McKENZIE, C., MINCHIN, D., OCCHIPINTI-AMBROGI, A., OLENIN, S. & PEDERSON, J. 2015. Dose of truth—monitoring marine non-indigenous species to serve legislative requirements. *Marine Policy*, **54**: 26–35.

LEPPÄKOSKI, E., GOLLASCH, S. & OLENIN, S. (eds) 2002. *Invasive aquatic species of Europe—distribution, impact and management*. Kluwer Academic Publishers, Dordrecht.

LEVY-BOOTH, D.J., CAMPBELL, R.G., GULDEN, R.H., HART, M.M., POWELL, J.R., KLIRONOMOS, J.N., PAULS, K.P., SWANTON, C.J., TREVORS, J.T. & DUNFIELD, K.E. 2007. Cycling of extracellular DNA in the soil environment. *Soil Biology & Biochemistry*, **39**: 2977–2991.

LIBRADO, P. & ROZAS, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**: 1451–1452.

MADARIAGA, D.J., RIVADENEIRA, M.M., TALA, F. & THIEL, M. 2014. Environmental tolerance of the two invasive species *Ciona intestinalis* and *Codium fragile*: their invasion potential along a temperate coast. *Biological Invasions*, **16**: 2507–2527.

MEUSNIER, I., SINGER, G.A.C., LANDRY, J.F., HICKEY, D.A., HEBERT, P.D.N. & HAJIBABEI, M. 2008. A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics*, **9**: 214.

MOLNAR, J.L., GAMBOA, R.L., REVENGA, C. & SPALDING, M.D. 2008. Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment*, **6**: 485–492.

MOON-VAN DER STAAY, S.Y., DE WACHTER, R. & VAULOT, D. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature*, **409**: 607–610.

PASCUAL, E. & DRAKE, P. 2008. Physiological and behavioral responses of the mud snails *Hydrobia glyca* and *Hydrobia ulvae* to extreme water temperatures and salinities: implications for their spatial distribution within a system of temperate lagoons. *Physiological and Biochemical Zoology*, **81**: 594–604.

PILLIOD, D.S., GOLDBERG, C.S., ARKLE, R.S. & WAITS, L.P. 2013. Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, **14**: 109–116.

POCHON, X., ZAIKO, A., HOPKINS, G.A., BANKS, J.C. & WOOD, S.A. 2015. Early detection of eukaryotic communities from marine biofilm using high-throughput sequencing: an assessment of different sampling devices. *Biofouling*, **31**: 241–251.

RICCIARDI, A. 1998. Global range expansion of the Asian Mussel *Limnoperna fortunei* (Mytilidae): another fouling threat to freshwater systems. *Biofouling*, **13**: 97–106.

SAKAI, A.K., ALLENDORF, F.W., HOLT, J.S., LODGE, D.M., MOLOFSKY, J., WITH, K.A., BAUGHMAN, S., CABIN, R.J., COHEN, J.E., ELLSTRAND, N.C., McCAULEY, D.E., O'NEIL, P., PARKER, I.M., THOMPSON, J.N. & WELLER, S.G. 2001. The population biology of invasive species. *Annual Review of Ecology, Evolution and Systematics*, **32**: 305–332.

SCHMIEDER, R. & EDWARDS, R. 2011. Quality control and preprocessing of metagenomic datasets. *Bioinformatics*, **27**: 863–864.

SNOEIJS, P. 1989. Effects of increasing water temperatures and flow rates on epilithic fauna in a cooling-water discharge basin. *Journal of Applied Ecology*, **26**: 935–956.

STRAYER, D.L. 2010. Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. *Freshwater Biology*, **55**: 152–174.

TABERLET, P., COISSAC, E., HAJIBABEI, M. & RIESEBERG, L.H. 2012. Environmental DNA. *Molecular Ecology*, **21**: 1789–1793.

TAMURA, K. & NEI, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**: 512–526.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. & KUMAR, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, **30**: 2725–2729.

THACKER, C.R., OGUZTURUN, C., BALL, K.M. & SYNDERCOMBE COURT, D. 2006. An investigation into methods to produce artificially degraded DNA. *International Congress Series*, **1288**: 592–594.

THOMSEN, P.F., KIELGAST, J., IVERSEN, L.L., MOLLER, P.R., RASMUSSEN, M. & WILLERSLEV, E. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One*, **7**: e41732.

TSOLAKI, E. & DIAMADOPOULOS, E. 2010. Technologies for ballast water treatment: a review. *Journal of Chemical Technology and Biotechnology*, **85**: 19–32.

VALENTINI, A., POMPANON, F. & TABERLET, P. 2009. DNA Barcoding for ecologists. *Trends in Ecology and Evolution*, **24**: 110–117.

WOOD, S.A., SMITH, K.F., BANKS, J.C., TREMBLAY, L.A., RHODES, L., MOUNTFORT, D., CARY, S.C. & POCHON, X. 2013. Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. *New Zealand Journal of Marine and Freshwater Research*, **47**: 90–119.

ZAIKO, A., MARTINEZ, J.L., SCHMIDT-PETERSEN, J., RIBICIC, D., SAMUILOVIENE, A. & GARCIA-VAZQUEZ, E. 2015. Metabarcoding approach for the ballast water surveillance—an advantageous solution or an awkward challenge? *Marine Pollution Bulletin*, **92**: 25–34.