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NOTE

Evidence of horizontal transmission of the cancer-associated *Steamer* retrotransposon among ecological cohort bivalve species

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ABSTRACT: Bivalve specimens from legacy frozen tissue collections, and others freshly obtained, were surveyed for the presence of the *Steamer* long terminal repeat (LTR)-retrotransposon associated with disseminated hemic neoplasia of the soft-shelled clam *Mya areneria.* Of 22 species investigated using primers for the *pol* region, only Atlantic *M. arenaria*, Atlantic and North Sea razor clams *Ensis directus*, and Baltic clams *Macoma balthica* from the North Sea were found to possess copies of *Steamer* in their genomes. Notably, close relatives like *Mya truncata* and *Siliqua patula* did not exhibit evidence of *Steamer*. Amplified *Steamer* sequences were uniformly identical in all *M. areneria* specimens, and were highly variable across specimens of *E. directus*. Variation in the latter included nucleotide polymorphisms among and within individuals as well as length variation in 2 specimens corresponding to the deletion of a predicted stable hairpin structure. Results implicate Atlantic razor clams as the proximal source for horizontal transmission of *Steamer* among ecologically similar yet markedly distantly related bivalves. The consequences of cross-species transmission of the *Steamer* retrotransposon are unknown, and the finding of *Steamer* in 3 bivalve species suggests that further spread is possible.

KEY WORDS: Disseminated neoplasia · Hemic neoplasia · Retrotransposon · *Steamer* · Bivalvia · Cancer

INTRODUCTION

Disseminated, or hemic, neoplasias are leukemia-like conditions that affect circulating hemocytes of a variety of bivalve mollusks (Carballal et al. 2015). Advanced stages of the disease, with tissue infiltration by neoplastic hemocytes, are fatal in most affected bivalves (Barber 2004, Metzger et al. 2015). Since first described in oysters from the eastern coast of the USA (Farley 1969), similar pathologies have been found globally in at least 23 bivalve species (Barber 2004, Carballal et al. 2015). Although both prevalence and mortality vary by species and by locality, particularly susceptible populations include northwest Atlantic soft-shelled clam *Mya arenaria* (Barber 2004, Carballal et al. 2015), northeast Atlantic common cockle *Cerastoderma edule* (Barber 2004, Carballal et al. 2015), northeast Pacific bay mussel *Mytilus trossulus* (Barber 2004, Carballal et al. 2015), and several populations of Baltic clam *Macoma balthica* (Christensen et al. 1974, Pekkarinen 1993, Thiriot-Quiévreux & Wolowicz 1996).

Whereas a variety of biotic and abiotic factors have been proposed (Barber 2004, Walker et al. 2011, Carballal et al. 2015), the etiology of disseminated neoplasia in *M. arenaria* is now well understood to involve transmission of a clonal lineage of neoplastic cells from one clam to another (Metzger et al. 2015, Mateo et al. 2016). In moribund *M. arenaria*, neoplastic hemocytes exhibit high genomic copy number and high RNA ex-

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pression of the *Steamer* retrotransposon. *Steamer* is a long terminal repeat (LTR)-retrotransposon in the Mag family of Ty3/Gypsy elements, complete with a single open reading frame encoding *gag* and *pol* genes. Retrotransposons are present in genomic DNA and replicate through reverse transcription of an mRNA transcript into DNA, followed by integration into a new location within the genomic DNA of a cell. As they are notably lacking a retroviral *env* gene, they are not expected to be able to transfer from cell to cell, as retroviruses do. Similar retrotransposons have been identified in the genomes of 3 other bivalve species known to have transmissible neoplasias, one of which seems to cross the species boundary (Metzger et al. 2016).

To date, the *Steamer* retrotransposon itself is unique to *M. arenaria.* Here we examined legacy biodiversity collections and freshly acquired bivalve tissues for the presence of a *Steamer* signature in their genomes in a manner that might (1) better elucidate whether disseminated neoplasias are ecologically or phylogenetically determined and (2) shed light on the potential for horizontal spread and genomic modification by *Steamer*-like retrotransposons in the marine environment.

MATERIALS AND METHODS

Bivalve tissue samples were obtained from the American Museum of Natural History's Ambrose Monell Cryo Collection (AMCC), and included 22 species from the North Atlantic (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d124p165_supp.pdf). Fresh specimens of steamer softshelled clams *Mya arenaria* were collected in June 2015 from Lattingtown Harbor Beach, New York (NY). Fresh specimens of Atlantic razor clams *Ensis directus* from Maine were obtained from Citarella (New York, NY). Fresh specimens of Pacific razor clams *Siliqua patula* were collected from Taylor Shellfish Farms (Bow, Washington). Fresh specimens of quagga mussels *Dreissena rostriformis* were collected from the Hudson River (New York State Museum). Fresh specimens of zebra mussels *D. polymorpha* were collected from the Seneca River (Baldwinsville, NY). Ethanol-preserved tissue samples of truncate softshells *Mya truncata* from Nunavut, Canada, were provided by the Canadian Centre for DNA Barcoding (University of Guelph, Ontario).

DNA isolates from all frozen and fresh samples were obtained using the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-tek). All DNA concentrations were quantified using a Qubit fluorometer. Successful isolation was also confirmed through gel electrophoresis.

Attempts to amplify *Steamer* retrotransposon sequences from bivalve genomic DNA employed 2 primer pairs designed to amplify *pol* fragments specific to this retrotransposon (Stmr2988F ACT CCA AGC CGT CAA GAG AA with Stmr3340R TGC TTT CTG GCA AAT GAC TG; and Stmr2765F GCA TAA AGC GCC AAA GAG AC also with Stmr3340R) using 15.5 µl of water, 2.5 µl of 10× PCR Gold Buffer (Thermo Fisher Scientific), 2.5 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 0.5 µl of each 10 µM primer, 0.5 µl AmpliTaq Gold® (Thermo Fisher Scientific), and finally 1 µl of DNA isolate. Amplification proceeded by way of a preliminary 5 min at 94°C followed by 55 cycles of 94°C for 15 s, 53°C or 54°C for 25 s, and 72°C for 45 s. In addition, and in order to positively identify host species, the cytochrome *c* oxidase I barcoding locus (*cox1*) was amplified with LCO and HCO primers of Folmer et al. (1994), using Ready2Go PCR beads (GE Healthcare Life Sciences), to which 1 µl of DNA isolate was added, along with 0.5 µl both of the 10 µM forward and the 10 µM reverse primers and 23 µl of distilled deionized water.

PCR amplification products were purified with AMPure (Agencourt Bioscience). Samples were cycle-sequenced in both directions on an Eppendorf Mastercycler using 1 µl ABI Big Dye Terminator v.3.1 (Applied BioSystems), 1 µl Big Dye Extender Buffer v.3.1 (Applied BioSystems), 1 µl of 1 µM primer (above), and 3 to 7 µl of cleaned PCR template, depending on the concentration of PCR product estimated by eye on electrophoretic gels. Sequences were purified by ethanol precipitation and analyzed with an ABI PRISM 3730 sequencer. Sequences were edited and reconciled using CodonCode Aligner. Sequences were aligned with MUSCLE (Edgar 2004), and alignments were distinguished by parsimony analysis using Mesquite (Maddison & Maddison 2015) and confirmed via BLAST (Altschul et al. 1990) to the nr database at NCBI.

RESULTS

Amplification products were obtained from primers Stmr2988F and Stmr3340R for all *Mya arenaria* samples, for all samples of *Ensis directus* (including those from AMCC), and from 1 of 2 *Macoma balthica* in AMCC. The remainder of the sampled bivalve taxa from AMCC, as well as specimens of *Siliqua patula, Mya truncata*, and the 2 *Dreissena* species yielded no product for *Steamer*, although all yielded *cox*1 amplification products for the Folmer et al. (1994) primers, confirming viability of the DNA isolates.

Output from BLAST querying each product from primers Stmr2988F and Stmr3340R to the nr database at NCBI returned high scoring matches only to the canonical *Steamer* retrotransposon sequence KF319019 (Arriagada et al. 2014). Pairwise alignments from BLAST, and multiple sequence alignment (see Fig. S1 in the Supplement), revealed 100% sequence identity for amplified sequence products from all 10 specimens of *M. arenaria* with the canonical retrotransposon sequence KF319019, while revealing nucleotide variation and sequence length variation for amplicons obtained from all other taxa. Parsimony analysis of the aligned data revealed that all isolates grouped by taxon, and that the frozen specimens of *E. directus* and *M. balthica* possessed the most divergent sequences (Fig. 1). All sequences from *M. arenaria* were distinct from all other sequences obtained by the presence of a silent transitional point mutation (G2958 in *M. arenaria*; A2958 in all others). The sequence obtained from *M. balthica* included 2 transitions and 2 transversions compared to all other taxa. Nucleotide variation was observed among *E. directus* samples at 22 nucleotide positions, 10 of which were variable within a single individual (evidenced by equal chromatogram heights for 2 nucleotides in forward and reverse strands for *E. directus* sample numbers 8R, 3R, 13F, 5R, and 9R).

In addition to nucleotide site variation, alignment of the retrotransposon sequences (Fig. S1) revealed a homozygous shortened length variant for 2 specimens of *E. directus.* Relative to all other isolates, these 2 sequences were missing a region 109 nucleotides long. The 5′ region flanking the missing region comprised nucleotides (...ag AAA ACA aca) that were complementary to the 3′ flanking region (gt gtg TGT TTT gg…), and internally, 62 of 109 nucleotides were self-complimentary as an inverted doublet.

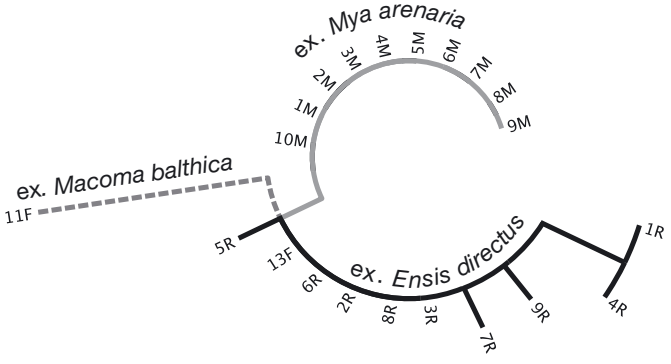

Fig. 1. Parsimony minimum spanning tree of *Steamer* retrotransposons isolates (also see the Supplement at www.int-res.com/articles/suppl/d124p165_supp.pdf) obtained from surveyed bivalves. Branch lengths are proportional to the amount of change

DISCUSSION

Examination of a variety of bivalves demonstrates that the *Steamer* retrotransposon, which is known to be present in high copy numbers and is highly expressed in disseminated hemic neoplasia in *Mya arenaria* (Arriagada et al. 2014, Metzger et al. 2015, 2016), is also present in the genome of other relatively phylogenetically unrelated bivalves including *Ensis directus* and *Macoma balthica*. Furthermore, that *Steamer* is absent from *Mya truncata* (closely related to, but geographically isolated from, *M. arenaria*), and from *Siliqua patula* (closely related to, but geographically isolated from, *E. directus*) suggests that the distribution of this retrotransposon is not due to ancient common ancestry, but rather due to more recent cross-species horizontal transmission. Despite their phylogenetic relatedness to retroviruses, retrotransposons do not normally move from cell to cell or species to species. The finding of sequences of nearly identical retrotransposons in 3 species suggests that horizontal transmission of the *Steamer* retrotransposon has occurred multiple times relatively recently. That the genera *Mya*, *Ensis*, and *Macoma* are representative of distinct and distantly related bivalve families (Myidae, Pharidae, and Tellinidae, respectively) suggests that phylogenetic constraints present little, if any, barrier to the spread of the *Steamer* retrotransposon. Notably, all 3 species now found to genomically harbor *Steamer* are infaunal filter-feeding bivalves with closely matched ecological and habitat distributions.

Horizontal transmission of transposons is rare (Schaack et al. 2010). Other comparatively rare transmissible cancers include devil facial tumor disease (DFTD), transmitted by bite among Tasmanian devils *Sarcophilus harrisii* (Pearse & Swift 2006), and canine transmissible venereal tumor (CTVT) in dogs (Murgia et al. 2006); the proliferative cells of each down-regulate expression of MHC class-I and class-II antigens allowing escape from immune detection (Rebbeck et al. 2009, Siddle & Kaufman 2013). Other than what is implied for these disseminated neoplasia of bivalves, the only other known cross-species transmission of cancerous cells concerns the establishment of a tapeworm malignancy in the lungs of a highly immunocompromised patient (Muehlenbachs et al. 2015). Mechanisms similar to DFTD and CVTV may be at play in bivalves insofar as the putative infective cells are hemocytes responsible for non-self recognition via agglutination and aggregation factors (Moreira et al. 2012).

It is evident that there is markedly greater genetic diversity for *Steamer* among *E. directus* specimens

(Fig. 1 and see Fig. S1), which is manifest in terms of length variation, as well as nucleotide polymorphism both among and within individual genomes of this species of razor clam. In comparison to the homogeneity of *Steamer* sequences from *M. arenaria*, this level of variation across coincident geographic ranges for soft-shell clams and Atlantic razor clams is consistent with *E. directus* as the ancestral source for what would appear to be a very recent horizontal transmission event to *M. arenaria*. The consequences of the introduction of a retrotransposon such as *Steamer* to the genomes of naïve organisms are unknown, but amplification of a retrotransposon does cause genomic changes which have the potential to lead to oncogenesis or other pathogenic outcomes. The massive amplification of *Steamer* in the transmissible neoplastic cell lineage in *M. arenaria* does suggest that their activity may be involved in oncogenesis. Whether *M. balthica* of the North Sea also are recently exposed by virtue of geographically and ecologically coincident invasive populations of *E. directus* should become evident with broader sampling of the Baltic clam.

The invasive Atlantic razor clam *E. directus* is now well established in the North Sea, where it is outcompeting the endemic razor clam, *E. ensis* (see Raybaud et al. 2015). In addition to its presence in *M. arenaria* in the northwest Atlantic, here we have shown the presence of *Steamer* in the North Sea invasive *E. directus* and in the North Sea endemic *M. balthica*. The presence or absence of this retrotransposon in the genome of *E. ensis*, and other mud-dwelling bivalves, deserves scrutiny, particularly as the distribution of *E. directus* suggests near-term expansion into the Adriatic (Raybaud et al. 2015).

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