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Differentiation of sympatric zebra and quagga mussels in ecotoxicological studies: A comparison of morphometric data, gene expression, and body metal concentrations

E. Kerambrun*, L. Delahaut, A. Geffard, E. David

Université de Reims Champagne-Ardenne, UMR-I 02 SEBIO (Stress Environnementaux et BIOsurveillance des milieux aquatiques), INERIS-URCA-ULH, France

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ABSTRACT

The zebra mussel is among the best studied freshwater molluscs in ecotoxicology, but information on the quagga mussel is lacking. Considering its potential spread, we selected a river in France in which zebra and quagga mussels coexisted, and then we used genetic markers to differentiate the two species and compared morphological parameters. cDNA sequencing assays of ten genes already used in zebra mussels were performed on quagga mussels to obtain functional specific primers. Then we analyzed the expression of genes involved in cellular metabolic activities (Cytochrome-c-oxidase – *cox*, and ATP synthase – *atp*), detoxification processes (Glutathione-S-Transferase – *gst*), oxidative stress (Catalase – *cat*), and digestive functions (Amylase – *amy*) on the two species. Whereas morphometric analysis underlined similarities in shape between the two species, relative gene expression profiles and metal concentrations evidenced strong differences. Quagga mussels notably presented half as high concentrations in Cd and Pb, two particularly toxic elements, as zebra mussels. These results imply that i) particular attention should be paid to properly distinguish the two species considering their similar external appearance, and ii) zebra mussels cannot be replaced by quagga mussels in ecotoxicological studies without preliminary investigations on biomarker response patterns. To our knowledge, this study is the first to have undertaken such an approach in gene expression analysis in quagga mussels, and more generally to have compared such biomarker responses of zebra and quagga mussels in the field.

1. Introduction

Over the last decades, the zebra mussel (*Dreissena polymorpha*) has been extensively used as a model organism to assess and biomonitor the quality of freshwater environments in Europe (Binelli et al., 2001; Guerlet et al., 2007; Bacchetta and Mantecca, 2009; Bourgeault et al., 2010). This species is an invasive bivalve that has largely colonized freshwaters and allows a fast intake of environmental pollutants due to high filtration rates (Marescaux and Van Doninck, 2013; Baldwin et al., 2002). Another species of dreissenid was recently found in western Europe, i.e. the quagga mussel (*Dreissena rostriformis bugensis*). Similarly to the zebra mussel, the quagga mussel is native from the Ponto-Caspian Region (Son, 2007), but its first record in western Europe dates back to 2006 in the Rhine delta in the Netherlands (Molloy et al., 2007). The species migrated rapidly within the Rhine and the Meuse Rivers (van der Velde and Platvoet, 2007; Haybach and Christmann, 2009; Marescaux et al., 2012; Matthews et al., 2014). The first record of the species in France was made in 2011 in the Moselle River, which is connected to the Meuse River by canals (Bij de Vaate and Beisel, 2011).

Dreissenids display a high diversity of shell morphology. This considerably hinders the study of mixed zebra and quagga mussel populations because they frequently contain individuals with similar shapes but belonging to different species (Voroshilova et al., 2010). For example, the invasion of the Meuse River in Belgium first by quagga mussels remained undetected because Belgian national agencies never differentiated them from zebra mussels. However, zebra mussels diverged from quagga mussels approximately 13–18 million years ago, according to the rRNA gene (Stepien et al., 1999) and cytochrome oxidase I (Gelembiuk et al., 2006) molecular clocks, respectively (Ram et al., 2012). Genetic methods (DNA and isozyme analysis) have demonstrated a considerable genetic distance between these species and characterized them as valid species (Spidle et al., 1994; Stepien et al., 1999).

Even if several studies have proposed morphological keys to differentiate the two dreissenids, visual identification and morphometric analyses are not always sufficient to differentiate zebra mussels from quagga mussels probably because of phenotypic plasticity (Marescaux et al., 2012). This lack of differentiation between the two species

* Corresponding author.
E-mail address: elodie.kerambrun@wanadoo.fr (E. Kerambrun).

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represents a considerable risk in ecotoxicological studies because several authors have shown that the two species present numerous metabolic and physiological differences (Claxton and Mackie, 1998; Diggins, 2001; Nalepa et al., 2010; Peyer et al., 2009). Genetic markers were subsequently developed to identify the two species and help managers and national agencies (Voroshilova et al., 2010; Marescaux and Van Doninck, 2013).

The zebra mussel is among the best studied freshwater molluscs, but comparative information about the quagga mussel is lacking considering the potential spread of this important freshwater invader. Eighty-seven percent of all *Dreissena*-related papers published between 1989 and 2014 dealt with *D. polymorpha* (Karatayev et al., 2014). In particular, only few studies undertook an ecotoxicological approach on quagga mussels. Some authors compared bioaccumulation levels (Johns and Timmerman, 1998; Rutzke et al., 2000; Richman and Somers, 2005; Matthews et al., 2015), genotoxicity, HSP70 induction (Schäfer et al., 2012), or the responses of physiological and biochemical biomarkers (Potet et al., 2016; Farkas et al., 2017) between the two species. To our knowledge no study has examined gene expression in *D. r. bugensis* although an increasing number of ecotoxicological studies has been employing molecular tools to assess the impacts of chemical contaminants. Gene expression studies represent powerful tools to identify metabolic pathways impaired by environmental disturbances, their modes of action, and their consequences on growth, survival, and reproduction (Contardo-Jara and Wiegand, 2008; Lacroix et al., 2014). These gene expression biomarkers have already been applied on zebra mussels for ecotoxicological analyses (Faria et al., 2009; Navarro et al., 2011; Contardo-Jara et al., 2010) and to field monitoring schemes (Contardo-Jara and Wiegand, 2008; Kerambrun et al., 2016). Information about gene expression in quagga mussels is needed to carry out similar risk assessment in freshwater colonized by this species.

In order to compare biological responses between the two dreissenid species, we selected a river in France in which zebra and quagga mussels coexist. We used genetic markers to differentiate the two species, and compared morphological parameters. Gene sequencing assays of ten genes already used in zebra mussels were first performed on quagga mussels to obtain functional primers for qPCR. Then we analyzed the expression of genes involved in cellular metabolic activities (Cytochrome-c-oxidase – *cox*, and ATP synthase – *atp*), detoxification processes (Glutathione-S-Transferase – *gst*), oxidative stress (Catalase – *cat*), and digestive functions (Amylase – *amy*) on the two species. We also measured metal concentrations in the two dreissenid species.

2. Materials and methods

2.1. Preliminary cDNA sequencing assays

2.1.1. Mussel collection

Mussels were collected in the Oise River (Verneuil en Halatte, 2°30′36 E, 49°16′50 N), a tributary of the Seine River, where populations of zebra and quagga mussels were found in sympatry but could not be identified by visual inspection (Fig. 1). In September 2014, ten mussels were randomly picked by cutting the byssal thread. The mantle was sampled and preserved in ethanol (70%) to identify the species by RFLP (Restriction Fragment Length Polymorphism). Each digestive gland was dissected on site, frozen in liquid nitrogen and stored at − 80 °C until cDNA sequencing.

2.1.2. Species identification by Restriction Fragment Length Polymorphism (RFLP) analysis

The mitochondrial COI gene was used to identify the two species by the RFLP method adapted from Voroshilova et al. (2010). Briefly, total genomic DNA was isolated from mussel mantle using phenol/chloroform/isoamyl alcohol (25:24:1). The fragment of COI mitochondrial DNA containing the RSA I restriction site was amplified using the following COI universal primers from Folmer et al. (1994): 5′-GGTCAACAAATCATAAAGATATTGG-3′ and 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′. PCR amplifications were performed in a volume of 25 µL containing 1 × Taq polymerase buffer, 2 mM $MgCl_2$, 80 µM deoxynucleotides (dNTPs), 10 pmol of each primer, 0.5 units of Taq Uptitherm DNA polymerase (Interchim, Montluçon, France) and ca. 20 ng of total genomic DNA. The following steps were applied: denaturation at 94 °C for 4 min, then 35 cycles at 94 °C for 50 s, 56 °C for 50 s, 72 °C for 1 min, and a final step at 72 °C for 10 min. The resulting fragments were hydrolyzed using *Rsa I* restriction endonuclease (Euromedex, France), following the manufacturer's instructions. The restriction fragments were visualized under UV light after electrophoresis on 2% agarose gel in Tris Borate EDTA 0.5% buffer, and staining with ethidium bromide.

2.1.3. cDNA sequencing in quagga mussels

A total of eight genes was selected for partial cDNA sequencing in *D. r. bugensis*: genes involved in cellular metabolic activities (ATP synthase – *atp*), detoxification processes (Metallothionein – *mt* and Glutathione-S-Transferase – *gst*), oxidative stress (Catalase – *cat*, Superoxide Dismutase – *sod* and Glutathione peroxidase – *gpx*) and digestive functions (Amylase – *amy* and Cellulase – *ghf*) using existing primers for *D. polymorpha* (Kerambrun et al., 2016). Two housekeeping genes were also selected to be sequenced (Ribosomol protein S3 –*s3* and actin – *act*) according to Navarro et al. (2011).

Total RNA was extracted from a pool of mussel (genetically identified as quagga) digestive glands using TriReagent (MRC-Research, USA) following the manufacturer's instructions. Reverse transcription was performed on 400 ng of total RNA using a Verso cDNA Synthesis Kit (Thermo Scientific). The reaction was conducted at 42 °C for 30 min using a PCR Mastercycler (Eppendorf). PCR amplifications were carried out with primers used for *Dreissena polymorpha,* summarized in Table 1. A volume of 1 µL of 10-fold diluted RT product was added to 0.1 µL of Taq Polymerase (Interchim), 1 µL of each primer (10 µM), 1 µL of $MgCl_2$ (50 mM), 1 µL of dNTPs (2 mM) and 17 µL of distilled water. After an initial five-min denaturation step at 94 °C, samples were submitted to amplification, using denaturation at 94 °C for 5 min, then 60 °C for 2 min, 72 °C for 90 s, and then 40 cycles at 94 °C for 30 s, 60 °C for 40 s, 72 °C for 90 s, and a final step at 72 °C for 10 min.

The amplified cDNA fragments were purified with a Wizard DNA Clean Up system, inserted into pGEM-T vectors (Promega), cloned, and then sequenced by Biofidal-DTAMB Company (France). Sequences were subjected to a homology search using the BLASTX algorithm of the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Out of the 10 genes we tested, we successfully sequenced 6 *D. r. bugensis* ones: *s3*, *act*, *atp*, *gst*, *cat* and *amy*. Sequences were recorded in the NCBI database (Table 1).

2.2. Comparison of biomarker responses between the two species

2.2.1. Mussel collection

Sixty mussels between 18 and 25 mm in length were randomly picked from the Oise River (Parmain, 2°12′32 E, 49°6′32 N) by cutting the byssal thread in September 2014 for morphometric analysis. After species identification, twenty individuals of each species were measured for total length (the longest anteroposterior distance), height (the longest dorsoventral distance) and width (the longest distance between the left and right valves) (Table 2).

Twenty other mussels were sampled in September and December 2014, and then in March and June 2015 in Verneuil en Halatte (2°30′36 E, 49°16′50 N) nearby Parmain. They were measured for total length, weighed with their shell (total weight) and without it (body weight) to calculate their condition index (CI) using the following formula: CI = body weight / total weight. Each digestive gland was dissected on site, frozen in liquid nitrogen, and stored at − 80 °C until analysis of gene expression levels.

In June 2015, twenty other mussels were carried to the laboratory in water taken from the site, transferred to clean freshwater (spring water