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SHORT COMMUNICATION

Wolbachia and *Cardinium* infection found in threatened unionid species: a new concern for conservation of freshwater mussels?

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Abstract

Endosymbiotic bacterial species that manipulate host biology, reproduction and mitochondrial genetic diversity have been identified in many metazoans, especially terrestrial arthropods. Until now, the hypothesis that *Wolbachia* or other bacterial endosymbiont might be absent in mollusks has remained unexplored. We present here preliminary data on bacterial communities in a freshwater mussel *Unio crassus*—species with doubly uniparental inheritance of mtDNA (DUI). Next generation sequencing of 16S rRNA bacterial gene fragment allowed to identify endosymbiotic *Cardinium* and sequences that were classified to the order Rickettsiales. Finally, we discovered *Wolbachia* and confirmed *Cardinium* infection of *Unio crassus* using bacterial species-specific primers. Discovering *Wolbachia* and *Cardinium* infections in *Unio crassus* opens new opportunities of further investigations in the second largest animal phylum on Earth, very diversified phylogenetically, widespread geographically and inhabiting many environs, including freshwater, inhabited by the most threatened molluscan species. Considering the problems caused by endosymbionts identified in arthropods, the presence of endosymbiotic factor implies possibility of their influence on taxonomy of threatened unionids, on the results of studies of genetic diversity and proper conservation planning.

Keywords *Unio crassus* · Bacterial endosymbiont · Next generation sequencing · Microbiome · 16S rRNA

Introduction

Bacterial endosymbiont species exert significant impact on the microevolution and reproductive ecology of their hosts (Ma and Schwander 2017) and often appear to be retained in populations without conferring any apparent physiological benefit to the bearer. Endosymbiotic infections can also have profound effects on parthenogenesis induction and female-biased sex ratios through feminization, male killing, and cytoplasmic incompatibility (Engelstädter and Hurst 2009). Interestingly, bacterial endosymbionts can shape patterns of host mitochondrial genetic diversity (Hurst and Jiggins 2005) by linking infection patterns with phylogenetic clades (Sun et al. 2011) as well as certain haplotypes (Kambhampati et al. 1992). Moreover, these bacteria can contribute to the loss of species mitochondrial genetic diversity through selective sweeps (Jäckel et al. 2013).

The impact that endosymbionts can exert on the host species can be very detrimental, thus they attract the attention of conservation biologists, foreseeing negative influence of certain endosymbionts on the critical features of declining species populations (e.g. sex ratio distortion—Jiggins et al. 2000) and/or conservation measures (e.g. source populations for reintroductions—Dinca et al. 2018). Moreover, the interactions of endosymbionts with taxonomy have conservation importance (Ritter et al. 2013), because taxonomy is the basis for biodiversity estimation and foreordains taxons for conservation actions and legislation (Mace 2004).

Infections of endosymbiotic microorganisms have been detected in many invertebrates, but they are most widespread in the arthropods, the largest of the Animal phyla on Earth, especially in terrestrial insects (Ma and Schwander 2017) and mites (Zhang et al. 2016). The obvious question arises

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about their presence in molluscs, the second largest phylum among Animalia. In 1998, Schilthuizen and Gittenberger (1998) used *Wolbachia*-specific PCR primers that were found in arthropods and reported no evidence of infection by *Wolbachia*—a symbiotic bacterium that alters host reproduction—in 38 mollusks species. In consequence, the question about the presence of *Wolbachia* or other bacterial endosymbionts in mollusks has remained not answered for 20 years.

Interestingly, Whelan and Strong (2016) demonstrated that high mitochondrial heterogeneity produced polyphyletic species on mitochondrial gene trees of doubly uniparental inheritance of mtDNA (DUI) species of freshwater Pleuroceridae, freshwater snails, which also showed female-biased sex ratios (Ciparis et al. 2012). The indicated incongruence in molecular and morphological taxonomy was puzzling. The authors hypothesized that the observed pattern of mitochondrial genetic diversity was similar to the one the caused linkage disequilibrium with the mitochondrial genome of hosts by inherited *Wolbachia* endosymbiont infections. Indeed, *Neorickettsia*, an endosymbiont related to *Wolbachia*, was observed in pleurocerids and semisulcospirids, also freshwater snails (Fredricks 2006). Moreover, there is a lot of data on transovarially inherited harbor obligate bacterial endosymbionts in vesicomyid clams (Cary and Giovannoni 1993). In turn, the common presence of chemosynthetic symbiont in vent marine mussels inahibing extreme environments (e.g. Ikuta et al. 2016) is the evidence for the support of host fitness benefits associated with the presence of symbionts (Zug and Hammerstein 2018).

Considering above results, our aim was to perform a simple test for the presence of bacterial endosymbionts in highly threatened European freshwater mussel, *Unio crassus*. We used high throughput sequencing of 16S rRNA to detect endosymbionts and subsequently we applied the Sanger sequencing method with available species-specific primers to confirm its presence in males and females. We chose sexual species with doubly uniparental inheritance of mitochondrial DNA, low mitochondrial genetic diversity and female-biased sex ratios (see Mioduchowska et al. 2016) that offer a wide range of opportunities for endosymbiotic manipulation.

Materials and methods

In total 30 individuals of the thick-shelled river mussel *Unio crassus*, highly threatened European freshwater bivalve, were collected with the permission of the General Directorate for Environmental Protection in Poland from Czarna Hancza located in the Northern Poland (coordinates: 53°58′253″ N 23°18′217″ E). To test the bacterial endosymbiont presence, we selected foot tissues (approximately 3 mm^3), since mtDNA M-type and F-type were observed there. DUI phenomenon allowed also non-invasive molecular sex identification according to Mioduchowska et al. (2016). Total DNAs were extracted using silica membranes of the commercial Genomic Mini kit for universal genomic DNA isolation (A&A Biotechnology). Samples of both sexes gave positive results in the form of visible PCR products of F*cox1* gene fragment in 1% agarose gel electrophoresis. The M*cox1* marker was detected only in 12 of 30 individuals that were classified as males.

Identification of bacterial endosymbiont species is difficult by the fact that they cannot be cultured in the laboratory, there is no data concerning bacterial endosymbiont infection in male or female, and around 1% of the whole host microbiome can be amplified using broad-range universal primers and Sanger sequencing (Muyzer et al. 1996). Metagenomic approach seems to be the best solution to overcome this problem and allow to estimate relative abundance of sequences originated from bacterial endosymbiont in microorganism community. So that, the first step of our pilot study involved mixed isolates from one *Unio crassus* male and female for microbiome analyses—next generation sequencing of the V3–V4 hypervariable region of bacterial 16S rRNA sequences. PCR reactions were performed using universal primers (341F and 785R) and Q5 Hot Start High-Fidelity 2X Master Mix, according to procedures given by producer. Sequencing was carried out on the MiSeq sequencer, in paired-end (PE) technology, 2 × 250 nt, using Illumina v2 putty. Classification of readings to the bacterial species level was carried out based on the GreenGenes v13_8 reference sequence databases using the QIIME software package.

Second, we applied PCR screening and the Sanger sequencing to test for the presence of *Cardinium* and *Wolbachia* in both sexes of *Unio crassus*. We used all available species-specific primers described by Simões et al. (2011), Singh et al. (2013) and Mains et al. (2016). PCR reactions were performed in 20 µL volume containing 0.8 × Jump-Start Taq ReadyMix (Sigma-Aldrich, Germany), 0.4 µM of forward and reverse primers and about 100 ng of DNA. The 16S rRNA sequences were amplified under the following conditions: initial denaturation at 94 °C for 5 min followed by 44 cycles of 94 °C for 30 s, gradient PCR amplification ranging from 42 up to 62 °C was applied to determine the optimal annealing temperatures (for 40 s), and 72 °C for 1 min and ending with 72 °C for 5 min. All PCR products were purified by alkaline phosphatase and exonuclease I and sequenced with BigDyeTM terminator cycle sequencing method. Sequences were aligned manually using BIOEDIT 5.0.9 and haplotypes were retrieved using DNASP v.5.10.01 software. Species identification of obtained bacterial sequences were performed on homology searching using the GenBank records—megablast algorithm and nucleotide database were applied. We also used a positive control for

DNA extraction using isolates of freshwater crustacean hosts for which we previously discovered *Wolbachia* and *Cardinium* infections (see Mioduchowska et al. 2018): *Branchipus schaefferi* isolate, for which *Wolbachia* infection was detected and *Heterocypris incongruens* isolate, for which we identified *Cardinium*. In addition, we used negative control (blank control probes) – PCR amplification of an ultra-pure water sample that allows detection of contaminant DNA and no PCR products were obtained (data not shown).

Results and discussion

High-throughput 16S amplicon sequencing identified in total 331,933 sequences as OTUs (at 97% identity) across the rarefied dataset (supplementary material, S1). We detected the order Ricketssiales whithin the most common phylum, Proteobacteria, but no bacteria were classified to the species level as *Wolbachia*. In turn, the order Cythopagales and 1082 sequences of *Cardinium* endosymbiont were found within the phylum Bacteroidetes (Fig. 1).

Using WF 5′-CGGGGGAAAATTTATTGCT-3′ and WR 5′AGCTGTAATACAGAAAGGAAA-3′ (Singh et al. 2013) species-specific primers to amplify *Wolbachia* (annealing temperature 52 °C) and CF 5′-GCGGTGTAAAATGAGCTTG-3′ and CR 5′-ACCTCTTCTTTAACTCAAGCCT3′ (Singh et al. 2013) primers for detection *Cardinium* (annealing temperature 50 °C), we obtained sequences classified with almost 100% similarity to other 16S rDNA sequences of endosymbionts *Wolbachia* or *Cardinium* (Table 1), thereby confirming that *Unio crassus* was a new host species. In total, only 4 sequences of *Cardinium* and 2 sequences of *Wolbachia* were obtained and no coinfection was detected. We identified 18 females, out of which 6 were infected by endosymbionts. Nevertheless, given the fact that also for the rest individuals tested, the PCR products were obtained but unspecific (originated from another bacterial species) or weak (most likely due to genome mixing among several bacterial species) sequences were observed, more detailed study is needed (including design species-specific primers; see also Mioduchowska et al. 2018). So far, coinfection of *Cardinium* and *Wolbachia* was detected in one planthopper species (Nakamura et al. 2012), one parasitoid wasp species (e.g. White et al. 2011) and in a few mite species (e.g. Zhao et al. 2013).

Endosymbiotic *Cardinium* and *Wolbachia* bacteria are mostly maternally transmitted; however, occasional horizontal transmission has been also described (Morrow et al.

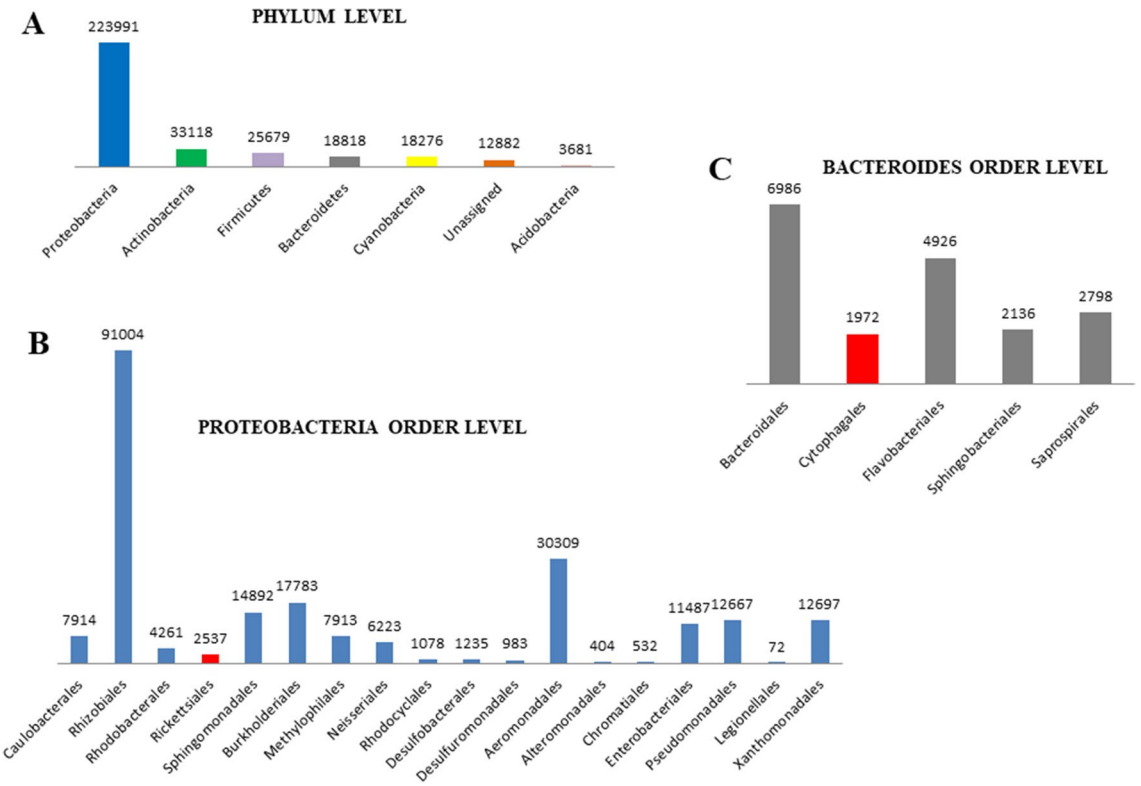

Fig. 1 Microbial communities detected in *Unio crassus*: **a** overview of grouped OTUs; red columns in **b** and **c** present order level of identified bacterial endosymbionts—Rickettsiales (in which no sequences were classified as *Wolbachia*) and Cytophagales (in which 1082 sequences of *Cardinium* were found)

Table 1 Identity of the obtained bacterial sequences confirming by BLASTN searches

Cardinium bacterial endosymbiont of *Unio crassus* identified bacterial 4 sequences length of 437 bp (3 haplotypes): GenBank accession numbers: MK040418–MK040421				
Sequences producing significant alignments from three first species	Accession	Query coverage (%)	Identity (%)	E value
Cardinium endosymbiont of *Culicoides ohmorii*	AB506778.1	100	96	0.0
Cardinium endosymbiont of *Culicoides arakawae*	AB506776.1	100	96	0.0
Cardinium endosymbiont of *Culicoides peregrinus*	AB506779.1	100	96	0.0
Wolbachia bacterial endosymbiont of *Unio crassus* identified bacterial 2 sequences length of 458 bp (2 haplotypes): GenBank accession numbers: MK040422 and MK040423				
Sequences producing significant alignments from three first species	Accession	Query coverage (%)	Identity (%)	E value
Wolbachia endosymbiont of *Agelastica alni*	AM180550.1	100	99	0.0
Wolbachia endosymbiont of *Polytremis gigantea*	MG872979.1	100	99	0.0
Wolbachia endosymbiont of *Citellophilus tesquorum deztysuensis*	MF045777.1	100	99	0.0

2014). These coinfected endosymbionts induce reproductive manipulations, including cytoplasmic incompatibility (CI) in hosts. Until now, there has been no data on how the endosymbiont density in hosts defines the expression of CI. Low *Wolbachia* density seems to be associated with a male development (MD) phenomenon. In turn, higher density of this bacteria MD may contribute to female mortality (FM) (Vavre et al. 2003). In view of facts that intracellular endosymbiotic bacteria, *Wolbachia*, influence host mtDNA variation, we hypothesize that it could affects detected by Mioduchowska et al. (2016) low genetic diversity of *Unio crassus*. Moreover, the presence of *Wolbachia* could be associated with a reduction in the effective population size, which might lead to a lower mitochondrial diversity (e.g. silent mitochondrial polymorphism as well as increase in non-synonymous substitution rates). In addition, it can fuel red-queen-like cytonuclear coevolution of coinfected endosymbionts and its host through the fixation of deleterious mitochondrial alleles.

The discovery of *Wolbachia* and *Cardinium* infections in freshwater mussel species with DUI opens new opportunities for further investigations. Over the last three decades, modern molecular techniques have been increasingly used in this group in several distinct research studies, but were mainly related to taxonomy, phylogeny and phylogeography (Lopes-Lima et al. 2014b), however, many taxonomical problems still remain. Until now, genetic diversity patterns and phylogeography of most freshwater bivalve species are not well characterized and the high-order phylogeny is still uncertain. The genetic techniques are leading approach in defining their biodiversity and determining species for conservation. Finally, this emerging genetic program needs to focus on the possible effects of endosymbionts.

In actual conservation actions, the biological and ecological effects of bacterial endosymbiont biology should be studied in endangered species, such as *Unio crassus*, to confirm or exclude its negative role in the species demography. Many species of freshwater mussels are threatened globally (Lydeard et al. 2004), also Europaean species including *Unio crassus* (Lopes-Lima et al. 2014a, 2017). Hence, they are the subject of large-scale active conservation projects, mainly based mainly on captive breeding (Gum et al. 2011). Conservation measures involving over 60 million of euro have been taken for native freshwater mussels in Europe (Lopes-Lima et al. 2017); however, as the rule conservation projects are based on limited number of individuals which progeny are spread over large areas (e.g. Zając et al. 2018 for *Unio crassus*, Gum et al. 2011 for *Margeritifera margaritifera*). Endosymbiotic *Cardinium* and *Wolbachia* bacteria are mostly maternally transmitted. Massive propagation of juvenile mussels, bred in captivity (including EU *U. crassus* conservation programs, e.g. Lundberg and Österling 2016; Schneider and Österling 2018), is usually based on a fairly small number of maternal individuals brooding large numbers of larvae. When they are released on host fish in artificial conditions, it results in an efficient production of millions of young individuals; however, massive propagation is associated with several problems, like mixing genetic stocks and reducting of genetic variation (Haag and Williams 2014). We want to emphasize that breeding programs usually employ a low number of maternal individuals: if any of them is infected, the entire line of thousands or millions of juveniles produced will also be infected. Freshwater mussels are usually sexed with invasive procedures (gonad puncturing and suctioning the contents with a syringe, but see non-invasive molecular sex identification in Mioduchowska et al. 2016), which may be the reason that any sex ratio distortion has never been reported and even if it actually occurs, it will not be easily detected without the use of genetic techniques.

Further screening of the presence of *Wolbachia* and *Cardinium* in other mollusks, its transmission routes, distribution in tissues and life history studies of infected mollusks with infection patterns shaping the genetic diversity of its hosts also need to be conducted. We also recommend testing the density of endosymbionts at the CI level. Our findings also warrant future phylogenetic as well as phylogeographic analysis of freshwater mussels, given the presence of endosymbiotic factor that induces genetic diversity of its host, including the identification of 'management units' and 'evolutionary significant units' for proper conservation and host-endosymbiont coevolution studies.

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