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16S rRNA gene-based metagenomic analysis of the gut microbial community associated with the DUI species *Unio crassus* (Bivalvia: Unionidae)

A short running title: Metagenomic analysis of bivalve gut microbiome

Monika Mioduchowska¹, Katarzyna Zając² (Corresponding Author, e-mail: kzajac@iop.krakow.pl), Krzysztof Bartoszek³, Piotr Madanecki⁴, Jarosław Kur⁵, Tadeusz Zając²

¹Department of Genetics and Biosystematics, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdańsk, Poland

² Institute of Nature Conservation, Polish Academy of Sciences, Adama Mickiewicza 33, 31-120 Krakow, Poland

³ Department of Computer and Information Science, Division of Statistics and Machine Learning, Linköping University, Linköping 581 83, Sweden

⁴ Department of Biology and Pharmaceutical Botany, Faculty of Pharmacy, Medical University of Gdansk, Hallera 107, 80-416 Gdansk, Poland

⁵ Empty Spaces Research, Miłosza 14b/3, 83-000 Pruszcz Gdański, Poland

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Abstract

What factors determine biome richness: genetic or environmental? Sex, phylogeny, tolerance indicated by other symbionts (e.g. endosymbionts) or simply is it related to local habitat, especially if the gut biome is considered? To answer these questions, we investigated the gut microbial profile of both sexes of three *Unio crassus* populations, species with unique system of mitochondrial DNA inheritance called doubly uniparental inheritance (DUI), living in different ecological conditions. High throughput sequencing of the V3-V4 hypervariable regions in the bacterial 16S rRNA gene fragment was performed, which resulted in a total of 1051647 reads, with 58424 reads / 65 OTUs (Operational Taxonomic Units) per sample on average. We identified a core microbiome, with all individual mussels sharing 69 OTUs (representing 23% of the total number of OTUs). Proteobacteria was the dominant phylum in all samples, followed by Firmicutes, Actinobacteria and Bacteroidetes. There were no significant differences in gut microbiome compositions between the two sexes of this species; however, we observed different phyla in geographically isolated populations. A non-metric multidimensional-scaling plot and dendrogram showed that the bacterial profile complies with the genetic structure of populations. Although we found differences in microbiomes between populations, their genetic structure suggests that the microbiome is weakly related to habitat, and more strongly to phylogeography (on both F and M mitotypes). We found no significant differences in beta diversity between the individuals of the bacterial communities measured using the Bray-Curtis index. Finally, we also examined whether OTUs were represented by symbiotic bacteria that enable cellulose digestion and by endosymbiotic bacteria that play important functions in the biology of their hosts and also affect micro-evolutionary processes and population phenomena. With regard to the endosymbionts, however, there was no relation to sex of the studied individuals, which suggests that there are no straightforward relations between DUI and microbiome.

Introduction

In most of the Metazoa, the mitochondria, the fundamental component of the eukaryotic cell, are devoid of genetic material inherited from the male. However, in some Bivalvia there is a mechanism by which male mitochondrial DNA (M-mtDNA) can also be inherited: although both gametes supply mtDNA to the zygote, the M-mtDNA is retained only in male somatic tissues, whereas in female soma, the transferred mitochondria disappear (Guerra et al., 2017). This mechanism indicates that life with mitochondria of mixed origin is possible; however, M-mtDNA is removed in most organisms.

If for some unknown reason, a standard metazoan organism has evolved a mechanism for removing external M-mtDNA, why does it tolerate endosymbionts, despite their strong influence on host fitness by cytoplasmic incompatibility (Tram, Fredrick, Werren, & Sullivan, 2006)? This question implies that some sort of mutual benefits for an organism and its endosymbiont may exist, the best example being the mitochondria themselves (Archibald, 2015). But the term "mutual benefits" evokes another example of widespread bacterial symbionts, their complicated interplays being under the influence of the environment (Aschenbrenner, Cernava, Berg, & Grube, 2016). Considering that in contrast to most metazoans, DUI organisms tolerate external mtDNA, the question arises whether they are more likely to host endosymbionts. And a further question should be asked about the whole biome of such an organism: is a tolerant organism likely to have a microbiome consisting large number of bacterial taxons?

In seeking answers to these questions, one would need to study the whole bacterial biome of these organisms to see whether in organisms with DUI there are any interactions between sex (assuming males are more tolerant due to DUI), endosymbionts, microbiome and the environment. The relations with the environment would imply that possible tolerance mechanisms are subject to environmental selection and that they should indicate where to search for the relevant selection factors. As a first step one should look at the DUI-organism's microbiome and its environment.

Micro-organisms play important functions in the biology of all animals and also their micro-evolutionary processes and population phenomena. Bacteria that form animal microbiome communities ("microbiota") are ubiquitous and there is virtually no animal completely free from them. At present, studies on the microbiome profile of molluscs are still in progress, and the mechanisms that determine host-microbial associations are largely unknown, also with regard to endosymbionts (but see Mioduchowska, Zając, Zając, & Sell

2019). Owing to the crucial ecological and economic roles of bivalves, their health-regulating core microbial composition, which ranges from nutrient processing to protection against diseases, is of particular interest (Sweet & Bulling, 2017). There are examples of the enormous evolutionary potential of the "mollusc - microbiome" coevolution, like bacterial chemosymbioses enabling mussels to inhabit extreme ecosystems (e.g. hydrothermal vents and cold seeps on the ocean floor) (Taylor & Glover, 2010) or to possess unusual adaptations, like a xylophagous ability, very rare among animals (wood consumption; Distel et al. 2011).

The thick-shelled river mussel, *Unio crassus* Philipsson, 1788, is a large-bodied and formerly widespread freshwater bivalve species, now threatened with extinction (Lopes-Lima et al. 2017). Results from molecular analyses suggest the existence two clades that correspond to *Unio crassus crassus* and to *Unio crassus* cf. *courtillieri* Hattemann, 1859, following the nomenclature applied by Prié & Puillandre (2014). Both were found in Poland (including the studied populations; Mioduchowska, Kaczmarczyk, Zajac, Zajac, & Sell, 2016) as well as in several European countries (Klishko et al. 2017). The mussel has an obligatory parasitic phase during its life cycle, when larvae parasitise a fish host to complete their development; its populations are strongly diversified phylogenetically (Mioduchowska, Kaczmarczyk, Zajac, Zajac, & Sell, 2016). It is also colonised by endosymbionts (Mioduchowska et al. 2019); it feeds on organic matter and microorganisms suspended in the water, indicating as yet little studied relationships with bacteria associated with habitat conditions (Aceves, Johnson, Bullard, Lafrentz, & Arias, 2018).

Currently, next-generation sequencing (NGS) has led to rapid improvements in microbiome research, enabling an expansion of its breadth and scope over the past 20 years. NGS technology allows one to gain insight into relationships within microbial communities, including uncultured bacterial endosymbionts, that were previously unclear. Finally, metagenomic approaches have allowed us to discover and characterize a number of bacterial symbiotic relationships – from marine invertebrates to the human gut (Kennedy, Marchesi, & Dobson 2007; Simon & Daniel, 2011). These associations between hosts and their gut-associated bacteria are particularly underexplored in the case of freshwater mussels and in terms of potential pathogen identification (Starliper Neves, Hanlon, & Whittington, 2008; Grizzle & Bruners, 2009). However, a direct connection has been demonstrated with bivalve molluscs, consuming and digesting microbes (Christian, Smith, Berg, Smoot, & Findley, 2004), and the gut microbiome could be transient and/or commensal (Harris, 1993). The relations suggesting a strong coevolution between food digestion and gut microbiome, observed in so many groups, seems to be very important. They become even more interesting

in mussels if we consider that the gut as somatic tissue differs between the sexes with regard to DUI (Mioduchowska et al. 2016).

This study is the first report on the gut microbiome profile of *U. crassus* that uses metagenomics analysis of the V3-V4 fragment of the bacterial 16S rRNA gene. We focused on showing up differences in the hosts' microbiome composition that would indicate niche selection by bacteria for which *U. crassus* represents a favourable environment (Lokmer & Wegner, 2015). Based on previous results of phylogeographical relationships among *U. crassus* populations from rivers in Poland which showed independent maternally (F-type) and paternally (M-type) inherited mitochondrial DNA lineages, we decided to analyse the microbiome profile of populations from very different habitats and representing different evolutionary lineages (see Mioduchowska et al. 2016).

Materials and Methods

Sample Collection

Three males and three females of *U. crassus* were sampled in 2018 from each of three rivers, differing considerably in ecological character: the Czarna Hańcza (53°58'15.34"N, 23°18'12.39"E; northern Poland; an oligotrophic, cold, lowland river flowing through lakes in a post-glacial landscape), the Pilica (50°56'36.98"N, 19°50'27.81"E; central Poland; a typical, eutrophic, lowland river flowing through an agricultural landscape), and the San (49°11'57.71"N, 22°40'35.99"E; southern Poland; the headwater section of a large mountain river in the Carpathians, with a short period of favourable nutrition conditions for mussels). The mussels represented two lineages described by Prié & Puillandre (2014) as well as two haplogroups observed by Mioduchowska, Kaczmarczyk, Zając, Zając, & Sell (2016): the first clade represented by individuals from northern Poland (*Unio crassus* cf. *courtilieri* / haplogroup I) and the second clade consisted by individuals from southern Poland (*Unio crassus* / haplogroup II). Haplotypes from Central Poland were intermingled in both clades. Probes were collected and transported to the laboratory.

U. crassus is a dioecious species (Bauer, 2001). The sex of each individual was determined by gonad biopsy. Each mussel's gonad was punctured with a needle and a very small sample of gonad tissue was sucked out using a syringe. The tissue samples were examined under a microscope in order to identify the sex. The procedure enabled animals of the required sex (3 ♂ and 3 ♀) to be collected.

In the lab the somatic tissues of the mussels were dissected and stored at -80°C . Mussel tissues including the guts and hepatopancreas were used for metagenomic analysis.

DNA extraction and amplicon library generation

DNA was extracted from approximately 25 mg of gut using silica membranes from the commercial Genomic Mini kit for universal genomic DNA isolation (A&A Biotechnology). In addition to the protocol, we added 100 μl of lysis solution, 0.66 $\mu\text{g}/\mu\text{l}$ proteinase K and 0.16 $\mu\text{g}/\mu\text{l}$ RNase for efficient lysis of the microbial community. The incubation was performed overnight at 37°C and then for 2 h at 50°C . To avoid cross contamination of the sample, all the laboratory procedures were conducted with sterile equipment. The extracted DNA was quantified by photometry using a NanoDrop ND-1000 UV-vis (Thermo Fisher Scientific).

Next-generation sequencing of V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the following universal bacterial primer set: 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') (Eiler, Heinrich, & Bertilsson, 2012). Each library was prepared with a two-step PCR protocol based on Illumina's "16S metagenomic library prep guide" using Q5 HotStart High-Fidelity DNA Polymerase (NEBNext) and the Nextera Index kit (2×250 bp) according to the manufacturer's protocol. Pooled amplicons were sequenced using a MiSeq sequencer. Paired-end (PE) technology using Illumina v2 chemistry (Genomed, Poland) with the manufacturer's run protocols (Illumina, Inc., San Diego, CA, USA) was applied and paired-end reads were quality trimmed and joined with a quality threshold of 0.9 with PANDAseq v. 2.8.123. All sequences that did not meet this quality criteria were removed.

Taxonomic classification of the bacterial 16S rRNA gene and bioinformatic analysis

We used the MiSeq Reporter (MSR) v. 2.6 (16S Metagenomics Protocol) for automatic de-multiplexing of raw reads and primary sequences analysis. The hclust method, v. 1.2.22q125 (Edgar, 2010) was applied to subsequently clustered next-generation reads into operational taxonomic units (OTUs), at 97% similarity. Taxonomic classifications for the next-generation reads were conducted using the Greengenes release 13.5 database as a reference (DeSantis et al., 2006). However, it is difficult to identify interspecific interactions between microbiota and parasites. Nevertheless infections have been previously linked to higher diversity of microbiome community, but the impact on

bacterial OTUs have been parasite species specific (e.g. Baxter et al. 2015, Newbold et al. 2017).

Each probe was described by a vector that calculates the abundance of the microbiome community. This is quantified as an integer count of the number of sequences obtained for different bacterial species within the guts of *U. crassus*. Beta diversity (β diversity) index, i.e. Bray–Curtis dissimilarity matrix (Yan et al. 2016), was estimated using the Explicit software package (Robertson et al., 2013). To visualize the similarity between probes we reduced the data to a 2-dimensional space through non-metric multidimensional scaling (Chapter 4.5 Everitt & Hothorn, 2011). This was done using the isoMDS() function from the MASS (Venables & Ripley, 2002) R package (R Core Team 2017). Then, the proximity between the microbiome composition from all the individuals tested was also presented in the form of a dendrogram, using R's hclust() function that is in the package stats found in R. Our custom PHP script (PHP Group, <https://www.php.net>) was applied to determine specific and shared OTUs.

The raw NGS reads were deposited under the study accession number PRJNA530367 in NCBI BioSample. All scripts are available on request.

Results and Discussion

Diet is one of the main components determining the community structure of an organism, and hosts are known to select their gut microbes selectively and/or functionally (Turnbaugh et al., 2007, Zilber-Rosenberg & Rosenberg, 2008). To date, the microbial profiles of bivalve tissues have been analysed by means of culture-dependent methods (Garnier et al. 2007, Wendling et al. 2014); only a few studies on bivalve microbiome communities have been conducted using NGS technology (e.g. King, Judd, Kuske, & Smith, 2012; Trabal Fernandez, Mazon-Suastegui, Vazquez-Juarez, Ascencio-Valle, & Romero, 2014; Wegner, Volkenborn, Peter, & Eiler, 2013; Lokmer, Kuenzel, Baines, Wegner, 2016). Here, we investigated the gut microbial composition of both sexes of six individuals from the three *U. crassus* populations living in different ecological conditions. Sequencing of the V3-V4 amplicon of the 16S rRNA gene resulted in 1051647 reads, with 58424 reads / 65 OTUs per sample on average. For details, see Table 1 and Supporting Information S1.

Previous research has suggested that the host has an influence on its bacterial community structure, which helps to understand the persistence of widespread core

microbiomes across diverse phylogenies (Fraune & Bosch, 2007). The core microbiome of the host is defined as the microbial community present in all individuals of the species, regardless of the environment (Turnbaugh et al., 2007). Moreover, it has been observed that the bacterial phylum level is seasonally stable in mollusc gut bacterial communities, with some shifts in abundance but not diversity. Proteobacteria and its classes have been described as the most abundant members of the microbiome, regardless of the host species (e.g. Trabal Fernández et al. 2014; Cleary et al. 2015; Vezzulli et al. 2018), and this is consistent with our research. The abundance of core bacteria phyla is presented in Figure 1.

The next most common phyla were Firmicutes and Actinobacteria, although Bacteroides was also widespread – observed in all populations, though less abundant. A total of 13 bacterial phyla were identified as unique or less abundant in single individuals/populations from the three rivers (Figure 2, Supporting Information S1). There were no significant differences in gut microbiome composition between the two sexes of the target species. However, we did observe different phyla in geographically isolated populations, which is consistent with previous research on the gut microbiome of bivalves (e.g. Trabal Fernandez et al. 2014). Although both extrinsic (environmental) and intrinsic (host) factors are crucial in shaping the microbiome profile of aquatic organisms, it seems that host genetic factors may be the driving force in bacterial colonization control (Dishaw et al. 2012).

Analysis of OTU distribution in all three populations revealed that they shared a total of 69 OTUs, representing 23% of the total number of OTUs (Figure 3). The population from the River Pilica has the most OTUs in common with the other populations investigated. In addition, each juxtaposition showed distinct differences between the microbiomes from the San and the Czarna Hańcza (Figure 3).

We generated a non-metric multidimensional-scaling plot and dendrogram to visualize the clustering patterns related to the origin of *U. crassus* based on gut OTU abundances (Figure 4). This analysis showed that the bacterial profile was consistent with the genetic structure of the populations: populations from the San (blue marks) and the Czarna Hańcza (red marks) formed two dense clusters but separate from that of the Pilica (green marks in Fig. 4). This observation is consistent with the phylogenetic relationships of these populations at mitochondrial molecular markers that clearly identified two groups of haplotypes according to F and M mitotypes. These groups comprised haplotypes found in the Czarna Hańcza (haplogroup I) and San (haplogroup II) populations, respectively. We also observed that haplotypes from the Pilica population were intermingled in both clades (Mioduchowska et al.

2016). This is surprising from the ecological point of view, because the Pilica, a eutrophic, lowland river, is quite distinct from the other two, which are fast-flowing and oligotrophic. Trabal et al. (2012) pointed out that geographical location was the primary driver of the gut microbiome composition of bivalves. Nevertheless, the influence of random events could be another factor affecting microbiome diversity, which helps to explain differences in the microbiome profile among geographically separated organisms and may be attributed to random host- bacterial interactions (Lankau Hong, & Mackie, 2012). In turn, beta diversity allowed for analyzing the diversity between particular assemblages. Here, the Bray-Curtis index, that measures the dissimilarity between the individuals, was applied and no significant differences was found within species (Figure 5). This is not surprising because a species-specific core microbiome was observed in all the samples and the microbial composition diversity was analyzed based on taxonomic abundance profiles.

It has also been shown that molluscs have a microbiota specialized in various functions, e.g. the degradation of native cellulose (Flari & Charrier, 1992, Charrier & Rouland, 1999). Comprehensive analysis of the *U. crassus* microbiome profile allowed us to find a symbiotic bacteria enabling cellulose digestion: uncultured *Flavobacterium* sp. (Dar, Pawar, Jadhav, & Pandit, 2015), *Stenotrophomonas* sp. (Pinheiro Correa, R. F., Cunha, R. S., Cardoso, & Chaia 2015), *Bacillus* sp. (Pinheiro, Correa, Cunha, Cardoso, & Chaia, 2015) and *Pseudomonas* sp. (Charrier, 1990, Watkins & Simkiss, 1990). Moreover, *Chryseobacterium* was found in all individuals from the River San, in two males from the Czarna Hańcza and one male from the Pilica (Van Horn et al. 2011). Evidently, considering the ubiquity of native cellulose degrading bacteria, this microbiome could be species-specific. Nevertheless, our findings were surprising because we discovered the presence of an endosymbiotic bacterium, *Candidatus Xiphinematobacter*, in both sexes in the Pilica population. *C. Xiphinematobacter* has hitherto been identified in Nematoda, in which host-endosymbiont co-evolution has been demonstrated, and the induction of parthenogenesis in their hosts also observed (Vandekerckhove, Willems, Gillis, & Coomans, 2000). Since the mechanism of *C. Xiphinematobacter* transmission is unknown, horizontal transfer to its host cannot be ruled out. A more in-depth distribution analysis of this putative bacterial endosymbiont in *U. crassus* tissues is therefore required.

Although we have found differences in gut microbiomes between populations, their genetic structure suggests that the microbiome is weakly related to habitat, and more strongly to phylogeography. However, we did not find any relation of the endosymbionts to the sex of the individuals studied, which suggests that there are no straightforward relations between

DUI and microbiome. Although complex relationships between the bivalves, their habitats and microbiome profiles have been studied for 30 years, the mechanisms mediating mussel-bacterial symbioses are still far from being explained.

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Figures

Figure 1 Composition of the *Unio crassus* gut microbiome – relative abundances of the core microbiome; P - Pilica river, C - Czarna Hańcza river, S - San river, m – male, f – female, W – gut, 1 - 6 - numbers of individuals.

Figure 2 Composition of the *Unio crassus* gut microbiome – relative abundance of the microbiome that was unique or relatively less abundant; P - Pilica river, C - Czarna Hańcza river, S - San river, m – male, f – female, W – gut, 1 - 6 - numbers of individuals.

Figure 3 Diagram showing the shared and unique identified OTUs in the microbiome from the gut of *Unio crassus*.

Figure 4 Phylogenetic relationships of *Unio crassus* based on OTUs in microbiome compositions: A – R's hclust() dendrogram, B – non-metric multidimensional scaling of guts of *U. crassus* from Polish rivers, based on percentage similarity in OTU abundances; P - Pilica river, C - Czarna Hańcza river, S - San river, m – male, f – female, W – gut, 1 - 6 - numbers of individuals.

Figure 5 Beta-diversity of the bacterial communities measured using the Bray-Curtis index.

Tables:

Table 1 Characteristics of the 16S rRNA gene metagenomic library.

Rivers	Sample ID (abbreviation: f – female, m – male)	Raw read pairs	Reads that passed quality controls(%)	Numer of OTUs (at 97% identity)
Czarna Hańcza	C1Wf	68596	98.93	47
	C2Wf	43894	98.72	59
	C3Wf	37493	99.03	44
	C4Wm	60182	98.99	68
	C5Wm	57955	98.53	67
	C6Wm	44146	98.90	68
Pilica	P1Wf	65623	98.99	65
	P2Wf	59700	98.83	42
	P3Wf	57941	98.94	48
	P4Wm	46659	98.66	65
	P5Wm	57576	98.82	120
	P6Wm	60649	98.68	56
San	S1Wf	66581	98.86	57
	S2Wf	72029	98.36	83
	S3Wf	56009	98.80	70
	S4Wm	63884	98.64	71
	S5Wm	69321	99.03	72
	S6Wm	63409	98.46	67

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533 List of Supporting Information:

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535 Supplementary data 1 _ OTU table