High-diversity microbiomes in the guts of bryophagous beetles (Coleoptera: Byrrhidae)

PETR PYSZKO 1, MARTIN ŠIGUT 1,2, MARTIN KOSTOVČÍK 3,4, PAVEL DROZD 1,* and JIŘÍ HULCR 2,5,6

1 Department of Biology and Ecology/Institute of Environmental Technologies, Faculty of Science, University of Ostrava, Chittussiho 10, 710 00 Ostrava, Czech Republic; e-mails: pyszko.petr@gmail.com, marton.sigut@gmail.com, pavel.drozd@osu.cz
2 Microbiology Institute, Academy of Science of the Czech Republic, Vídeňská 1083, 142 20 Praha 4, Czech Republic
3 BIOCEV, Institute of Microbiology, Academy of Sciences of the Czech Republic, Průmyslová 595, 252 42 Vestec, Czech Republic; e-mail: kostovci@gmail.com
4 Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Viničná 5, 128 44 Praha, Czech Republic
5 School of Forest Resources and Conservation, University of Florida – IFAS, 1745 McCarty Drive, Gainesville, FL 32611-0410, USA; e-mail: hulcr@ufl.edu
6 Entomology and Nematology Department, University of Florida – IFAS, 1881 Natural Area Drive, Gainesville, FL 32611, USA

Key words. Coleoptera, Byrrhidae, bryophagy, gut microbiota, Illumina MiSeq, richness

Abstract. The diversity and role of the gut microbiota of insects is a rapidly growing field of entomology, primarily fueled by new metagenomic techniques. Whereas endosymbionts in the guts of xylophagous or herbivorous insects are well studied, the microbiomes in moss-eating (bryophagous) insects remain uncharacterized. Using the Illumina MiSeq platform, we determined the composition of microbiomes in the gut, abdomen and on the body surface of two bryophagous species: Simplocaria semistriata (Fabricius, 1794) and Curimopsis paleata (Erichson, 1846) (Coleoptera: Byrrhidae). Gut microbiomes differed substantially from abdominal microbiomes in the same individuals, which indicates the need to separate them during dissection. Microbiomes in the gut and abdomen differed markedly from surface microbial assemblages. Gut microbiomes in bryophages had the highest MOTU richness, diversity and relative rarity. The eudominant bacteria in the guts and abdomens of bryophages were Novosphingobium, Bradyrhizobium, Ralstonia and Caulobacter, which are responsible for the detoxification of secondary metabolites or nitrogen fixation. These are less common in the surface samples and, therefore, likely to be associated with the specific ability of bryophages to feed on mosses.

INTRODUCTION

Many insects establish symbiotic interactions with microorganisms in their gut, body cavities, or cells (Dillon & Dillon, 2004). Protista have been identified in the digestive tract of lower termites and wood roaches (Hongoh, 2010); whereas fungi and methanogenic archaea are frequent in the gut of xylophagous and detritivorous beetles and termites (Egert et al., 2005; Brune, 2010). Bacteria are found in the gut community of most insects, including herbivores. Most gut microbes are commensals or parasites; however, some are known to provide beneficial services to their hosts (Engel & Moran, 2013). They can affect resistance against pathogens or parasites (Hedges et al., 2008; Oliver et al., 2010), intestinal cell renewal and systemic growth (Buchon et al., 2009), production of pheromones (Dillon et al., 2002) and kairomones (Leroy et al., 2011), or mating attractiveness (Sharon et al., 2010). Their beneficial role in diet processing through degradation of complex food components (Warnecke et al., 2007) and toxins (Kikuchi et al., 2012), nitrogen fixation (Nikoh et al., 2011), ammonia recycling (Hongoh et al., 2008), and nutrient supplementation via the synthesis of vitamins (Akman et al., 2002) and essential amino acids (Douglas, 1998) is also crucial.

Whereas the microbiota of xylophagous and herbivorous insects has been extensively studied, the microbiome of bryophagous (moss-feeding) insects has remained poorly characterized, except for an obligate endosymbiotic taxon in the bacteriomes of moss bugs (Hemiptera: Peloridiidae; Kuechler et al., 2013). At present, nothing is known about microbial assemblages in the guts or abdomens of
bryophagous insects, even though the utilization of mosses as a source of food is a unique phenomenon due to the specific properties of mosses. Bryophytes contain several compounds that reinforce the cell wall and hinder its digestion by inhibiting potential symbiotic organisms (Van derpoorten & Goffinet, 2009). Moreover, mosses have evolved effective defenses against herbivores by producing antimycotics (Frahm, 2004) and antibiotics (McCleary et al., 1960), organically soluble fractions (Parker et al., 2007) and high levels of water-soluble phenolic compounds (Davidson et al., 1989; Gline, 2006). It is assumed that all these mechanisms strongly affect the gut microbiota of their occasional consumers, and, as a result, deter the majority of herbivores from feeding on mosses (Gerson, 1969).

The aim of the present study was to determine the molecular operational taxonomic units (MOTUs) of bacteria in the gut and/or abdominal microbiomes in two bryophagous species Curimopsis paleata (Erichson, 1846) and Simplocaria semistriata (Fabricius, 1794) (Coleoptera: Byrrhidae). Adults of S. semistriata are recorded feeding, mating and ovipositing on mats of the moss Dicranella heteromalla (Hedw.) Schimp. and also feeding on the moss Mnium hornum Hedw. (Johnson, 1990). In the genus Curimopsis, all species are thought to be strictly bryophagous based on collecting, rearing or dissections. The dissections revealed fragments of undetermined moss in the alimentary tract of C. paleata (Johnson, 1986). Interestingly, Curimopsis nordensis Tshernyshev, 2013, a new species that occurs in Russian alpine tundra and steppes, also feeds on carrion, so at least some adults of the genus Curimopsis can feed also on non-vegetable matter (Tshernyshev, 2013).

We aim to determine, whether the gut microbiomes in both species, which are in permanent contact with a moss diet, are less diverse and distinctly different in their composition from that of their surface microbiota, which are in permanent contact with bacteria-rich soil. We compare the bacterial assemblages associated with both species and subsequently in different parts of their bodies in terms of their composition, species richness and diversity, overlap and relative rarity. Finally, we aimed to determine the MOTUs significantly associated with gut and/or abdominal microbiomes and discuss their potential role in moss digestion in both of the bryophagous species studied. In addition to the principal analysis, we compare the gut and abdominal microbiomes of the same bryophagous individuals to determine whether it is necessary to separate body parts before metagenomic analyses. This aspect is often neglected in studies on small insects because it is difficult, but its omission could confound the results.

**MATERIALS AND METHODS**

**Sampling and dissection of beetles**

Beetles were collected from November to December 2013 in deciduous forests near Ostrava, Czech Republic (49°52′04″N, 18°14′17″E). We focused on two species of the family Byrrhidae, a unique group of moss-feeding beetles: *C. paleata* and *S. semistriata*. Both are widespread Palearctic species with minute bodies and perennial activity. The beetles were captured individually on the surface and inside cushions of the moss *Dicranella heteromalla*. Individuals were placed in separate plastic boxes together with moss using sterilized tweezers to avoid contamination. The boxes were stored in a refrigerator until dissection (for a maximum of three days after collection to avoid considerable changes in the composition of bacterial assemblages). In order to obtain surface samples, each beetle was washed by vortexing in a 1.5-mL micro centrifuge tube with 1 mL sterile solution of 1% Tween 80 (Sigma-Aldrich, Saint Louis, USA) and phosphate-buffered saline (PBS) for 30 s at 2100 rpm. The procedure was repeated so that the beetle was completely clean and the second wash was discarded (Bateman et al., 2016). Gut contents and abdominal tissues were separated on paraffin wax, which was previously sterilized by pouring ethanol on it and igniting it. For further metagenomic analysis, surface washes, gut contents and abdominal tissues of five *C. paleata* and five *S. semistriata* individuals were obtained. In addition, we verified the bryophagy of both species by analyzing the gut contents of five individuals of *C. paleata* and five of *S. semistriata* that were dissected under a binocular microscope. This revealed that fragments of phyllids and rhizoids formed >95% of the gut contents and the remaining material probably consisted of soil particles.

**DNA isolation, PCR amplification and library preparation**

Microbial DNA from the surface wash, gut contents and abdominal tissues of each beetle was isolated using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer’s standard protocol and then subjected to sequencing library preparation. To ensure the recovery of broad spectra of bacterial and archaeal diversity, we used the universal F515/R806 bacterial ribosomal primers for the MiSeq platform to amplify the V4 region of 16S rDNA (Caporaso et al., 2010b). A two-step PCR was used during library preparation. First-step amplifications were done in quintuple reactions using only gene-specific primers to avoid PCR artifacts caused by long primers with at least some adults of the genus *Curimopsis* can feed also on non-vegetable matter (Tshernyshev, 2013).

We aim to determine, whether the gut microbiomes in both species, which are in permanent contact with a moss diet, are less diverse and distinctly different in their composition from that of their surface microbiota, which are in permanent contact with bacteria-rich soil. We compare the bacterial assemblages associated with both species and subsequently in different parts of their bodies in terms of their composition, species richness and diversity, overlap and relative rarity. Finally, we aimed to determine the MOTUs significantly associated with gut and/or abdominal microbiomes and discuss their potential role in moss digestion in both of the bryophagous species studied. In addition to the principal analysis, we compare the gut and abdominal microbiomes of the same bryophagous individuals to determine whether it is necessary to separate body parts before metagenomic analyses. This aspect is often neglected in studies on small insects because it is difficult, but its omission could confound the results.

**MATERIALS AND METHODS**

**Sampling and dissection of beetles**

Beetles were collected from November to December 2013 in deciduous forests near Ostrava, Czech Republic (49°52′04″N, 18°14′17″E). We focused on two species of the family Byrrhidae, a unique group of moss-feeding beetles: *C. paleata* and *S. semistriata*. Both are widespread Palearctic species with minute bodies and perennial activity. The beetles were captured individually on the surface and inside cushions of the moss *Dicranella heteromalla*. Individuals were placed in separate plastic boxes together with moss using sterilized tweezers to avoid contamination. The boxes were stored in a refrigerator until dissection (for a maximum of three days after collection to avoid considerable changes in the composition of bacterial assemblages). In order to obtain surface samples, each beetle was washed by vortexing in a 1.5-mL micro centrifuge tube with 1 mL sterile solution of 1% Tween 80 (Sigma-Aldrich, Saint Louis, USA) and phosphate-buffered saline (PBS) for 30 s at 2100 rpm. The procedure was repeated so that the beetle was completely clean and the second wash was discarded (Bateman et al., 2016). Gut contents and abdominal tissues were separated on paraffin wax, which was previously sterilized by pouring ethanol on it and igniting it. For further metagenomic analysis, surface washes, gut contents and abdominal tissues of five *C. paleata* and five *S. semistriata* individuals were obtained. In addition, we verified the bryophagy of both species by analyzing the gut contents of five individuals of *C. paleata* and five of *S. semistriata* that were dissected under a binocular microscope. This revealed that fragments of phyllids and rhizoids formed >95% of the gut contents and the remaining material probably consisted of soil particles.

**DNA isolation, PCR amplification and library preparation**

Microbial DNA from the surface wash, gut contents and abdominal tissues of each beetle was isolated using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer’s standard protocol and then subjected to sequencing library preparation. To ensure the recovery of broad spectra of bacterial and archaeal diversity, we used the universal F515/R806 bacterial ribosomal primers for the MiSeq platform to amplify the V4 region of 16S rDNA (Caporaso et al., 2010b). A two-step PCR was used during library preparation. First-step amplifications were done in quintuple reactions using only gene-specific primers to avoid PCR artifacts caused by long primers with attached sequencing adapters and identifiers, as well as the stochasticity in the PCR amplification (Berry et al., 2011; Schmidt et al., 2013). The first-step PCR was performed according to Caporaso et al. (2010b) with minor modifications consisting of initial denaturation at 94°C for 3 min; 25 cycles at 94°C for 45 s, 50°C for 60 s, 72°C for 90 s; and a final extension at 72°C for 10 min. The number of cycles was kept low to prevent potential depletion of specific primers from the degenerate mixture. Quintuple PCR reactions were pooled, purified (UltraClean® PCR Clean-Up Kit; MoBio) and subjected to a second-step PCR. The latter included 15 cycles of PCR amplification (following the same cycling profile as in the first-step PCR) with fused Illumina primers containing sequencing adapters and sample-unique multiplex identifiers necessary for demultiplexing the reads from each sample. Each PCR reaction volume (25 μL) contained 14.25 μL of molecular biology-grade water, 2.5 μL ExTaq 10× buffer, 0.2 mM dNTPs, 0.8 μM of each primer, 1.25 U ExTaq polymerase (all Clonetech, Mountain View, CA) and 20 ng extracted DNA or 2 μL cleaned PCR product in the second-step PCR. All PCR products were purified and checked using an agarose gel and quantified with the Quant-iT kit (Life Technologies, Carlsbad, CA). Subsequently, equimolar proportions of all samples were pooled to create a final sequencing library at 7.5 ng/μL and submitted for sequencing on the MiSeq platform (Illumina Technologies, San Diego, CA) at the Interdisciplinary Center for Biotechnology Research of the University of Florida, USA. Raw demultiplexed sequencing data with sample annotations are available at the Short Read Archive database (http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession number SRP055203 and further details can be found under the BioProject accession number PRJNA275854.
Processing of sequencing data and statistical analysis

Sequencing data were processed using QIIME 1.8.0 (Caporaso et al., 2010a), including quality checking, demultiplexing, read clustering and taxonomic assignments. Forward and reverse reads were joined to create contigs. Afterward, reads were demultiplexed in a parallel way with quality filtering that included a maximum unacceptable Phred quality score of 20 and a maximum number of consecutive poor quality base calls of 12 due to lower-quality overlaps of paired-end reads. Resulting reads were clustered into MOTUs using UCLUST (Edgar, 2010) with 97% similarity threshold against the bacterial 16S rRNA reference database Greengenes gg_13.8 release (DeSantis et al., 2006). Finally, we compiled information on read counts for all MOTU clusters from all samples together with taxonomic information into a MOTU table, which was used for comparing and describing the diversity of the samples. To enable comparison of beta diversity at the same sequencing depth rarefaction of resample datasets from all samples was carried out to the lowest observed read count. Resampling was done at a depth of 13,400 sequence reads to allow the inclusion of all samples. Singletons (clusters with only one read in individual samples) were discarded before producing the final dataset.

We analyzed data in R 3.4.3 (R Development Core Team, 2017) and Canoco 5.03 (Ter Braak & Smilauer, 2012). We used the “Rarity” library (Leroy, 2016) to calculate rarity indices for assemblages of bacterial MOTUs in individual samples. First, we calculated rarity weights for each MOTU using the weighting function “W” (Leroy et al., 2012) and the improvements proposed by Leroy et al. (2013). Rarity cut-off points were counted using the Leroy method on a set of actual species assemblages. The obtained rarity weights were used to calculate the index of relative rarity. Extrapolated MOTU richness (estimation of unobserved MOTUs) was based on abundances in subsamples using the abundance-based coverage estimator (ACE) in the “vegan” library (Oksanen et al., 2017). The diversity of assemblages was calculated using Fisher’s alpha diversity (Fisher et al., 1943).

We calculated rarity weights for each MOTU using the weight function “W” (Leroy et al., 2012) and the improvements proposed by Leroy et al. (2013). Rarity cut-off points were counted using the Leroy method on a set of actual species assemblages. The obtained rarity weights were used to calculate the index of relative rarity. Extrapolated MOTU richness (estimation of unobserved MOTUs) was based on abundances in subsamples using the abundance-based coverage estimator (ACE) in the “vegan” library (Oksanen et al., 2017). The diversity of assemblages was calculated using Fisher’s alpha diversity (Fisher et al., 1943).

At the order level, the most abundant MOTUs on the surface of beetles were the Enterobacteriales for S. semistriata (34%) and Pseudomonadales for C. paleata (28%). The surface assemblages of bacteria on both species of beetles were similar with the most dominant MOTUs Pseudomonas (24%), Burkholderiaceae (16%) or Pedobacter (12.5%). In the abdomen, the most abundant order was Sphingomonadales in both of the species of beetles (26.5%, 21% respectively). The bacterial assemblages in the abdomens of these beetles were also similar in composition and dominated by the MOTUS Novosphingobium (24%), Bradyrhizobium (20%), Ralstonia (14%) or Caulobacter (12.5%). In contrast, the bacterial assemblages in the guts of the two species differed. That in the gut of S. semistriata was dominated by Enterobacteriales (34.5%) and Rickettsiales, namely Rickettsia (14.5%), while that in C. paleata was dominated by another Rickettsiales, Wolbachia (17%) and by Entomoplasmatales (23%). A detailed composition at the order level is provided in Fig. 1 and the genera most strongly associated with both species of beetles are depicted in Fig. 2. The bacterial assemblages associated with Curimopsis paleata and Simplocaria semistriata did not differ either in terms of Fisher’s alpha diversity (df = 22, F = 0.36, P = 0.519), species richness (df = 22, F = 0.42, P = 0.481) or relative rarity (df = 22, F = 0.20, P = 0.623).

Diversity of bacterial assemblages in guts and abdomens of bryophages

As indicated by the first two axes of the PCoA, accounting for 58% of the variability in the data, assemblages on the body surfaces differed strongly from those in their abdomens and guts, which occur in a separate and distant cluster. The microbiota in the gut and abdomen was quite
similar in both species (Fig. 3) generally, but there was a substantial difference between the assemblages in the guts and abdomens at the level of individuals (df = 16; F = 5.63; P < 0.001), with J = 21.8% average MOTU overlap and mean P = 0.437 (Fig. 4). Fisher’s alpha diversity index was significantly dependent on the origin of the sample (df = 23; F = 10.98; P < 0.001). The highest overall (gamma) diversity was recorded in the gut microbiome (n = 241 MOTUs for S. semistriata, n = 215 MOTUs for C. paleata). As shown in Fig. 5a, the gut microbiome also had the highest average richness (96.22 MOTUs per sample) and the abdomen the lowest (63.89 MOTUs per sample). Fisher’s alpha diversity also reflects this pattern (Fig. 5b). Rarefaction curves showed that the species richness recorded in gut samples was higher (i.e., in terms of the number of MOTUs) than recorded for other samples. Estimated species richness differed significantly also based on the origin of the sample (df = 23; F = 6.82; P = 0.002). The species richness of the gut microbiota of beetles was higher than that recorded for the surface and abdomen microbiota (Fig. 6a). In addition, the species richness recorded in the gut of bryophagous beetles was significantly higher than that recorded for herbivorous beetles (df = 16; LR = 12.96; P < 0.001; Fig. 6b).
The highest proportion of origin-specific MOTUs was recorded in the gut (20.10%), followed by on the surface (13.76%) and the lowest in the abdomen (10.32%) (Fig. 7a). Consequently, index of relative rarity of assemblages differed significantly (df = 23; F = 9.88; P < 0.001), with the highest index values (many MOTUs with low frequency in the dataset) recorded for gut microbial assemblages (Fig. 7b). The eudominant taxa, *Bradyrhizobium, Caulobacter, Novosphingobium* and *Ralstonia*, were not only significantly more abundant in the abdomen than the gut, but also significantly more abundant in the gut than on the surface (for all: \( U_{\text{abd/gut}} = 36, P_{\text{abd/gut}} = 0.012; U_{\text{gut/sur}} = 36, P_{\text{gut/sur}} = 0.012 \)).

**DISCUSSION**

**High richness and low similarity of gut bacterial assemblages**

Comparable metagenomic studies report relatively few microbial species in most insect guts (Engel & Moran, 2013) and many previous identifications based on 16S rRNA gene sequences reveal fewer than 20–30 bacterial MOTUs per insect taxon (Dillon & Dillon, 2004; Robinson et al., 2010; Wong et al., 2011). We recorded great overall richness in bacterial MOTUs, particularly in the guts of bryophages. The overall high number of MOTUs detected may reflect the greater accuracy of the metagenomic approach used. For studies comparing bacterial richness, culture-independent DNA metabarcoding is more appropriate than conventional cultivation methods with subsequent taxonomic and/or molecular identifications (Broderick et al., 2004; Vaz-Moreira et al., 2011). A comparison with the results reported for herbivorous beetles (Yun et al., 2014) revealed that in bryophagous beetles there were more MOTUs per individual. For this comparison, we used only those MOTUs recorded in the gut of bryophages. A comparable metagenomic study of herbivorous beetles by Kel- ley & Dobler (2011) indicate that the gut of *Cryptocephalus* spp. (Chrysomelidae) harbours only 15–30 bacterial MOTUs per species. In contrast, Montagna et al. (2014) report that *Cryptocephalus* sp. averaged 86.3 MOTUs per individual, which is consistent with our results. A higher MOTU richness is reported for some non-herbivorous
beetles: 164 MOTUs for *Melolontha melolontha* (Scarabaeidae; Egert et al., 2005), 140 MOTUs for *Megetra cancellata* and 177 MOTUs for *Epicauta longicollis* (both Meloidae; Colman et al., 2012). The high MOTU richness recorded here in the gut of bryophagous beetles could be associated with the very specific diet and ecology of these insects.

Both seasonality and the substrate from which the beetles were sampled may substantially influence richness and diversity of bacterial assemblages. We collected our specimens at the beginning of winter. Although the effect of timing remains to be determined, bacterial diversity in the phyllosphere increases in response to drought and heat (Peñuelas et al., 2012). Similarly, insect gut microbiota changes with the season (Behar et al., 2008). We collected samples from the moss *D. heteromalla*, which forms low cushions barely a few millimeters above the soil. Soil bacterial assemblages are very complex (Borneman et al., 1996) and determine the composition and structure of gut microbial assemblages in insects living in direct contact with the soil (Huang & Zhang, 2013). Consistently, high bacterial richness of up to 695 MOTUs, are reported in the detritivorous beetles *Onthophagus* sp. (Yun et al., 2014).

The bacterial microbiome in the guts of beetles has the greatest proportion of specific MOTUs and the highest index of relative rarity compared to that recorded in other body parts, indicating that many bacterial MOTUs in the guts of bryophagous beetles differed among individuals. In some insects, gut bacterial communities vary among individuals within a species and consist mainly of bacteria without specific adaptation(s) to life in the gut of their host species (Cariveau et al., 2014), which indicates that the diet of the host might have some influence (Broderick et al., 2004). Nevertheless, a relatively static community is also documented (Tang et al., 2012). Physicochemical conditions in gut compartments, such as pH, redox potential, or availability of particular substrates, may select for particular species. Thus, even when acquired independently during each generation, gut communities are not expected to be random assemblages of bacteria derived from the food or local environment (Engel & Moran, 2013). Instead, the high abundance and ubiquitous presence of soil bacteria, such as *Variovorax*, *Pedobacter*, *Bacillus*, *Pseudomonas* and *Erwinia* on the surface of the bodies of *S. semistriata* and *C. paleata* could be explained by the proximity to the soil of mosses such as *D. heteromalla*. Indeed, the bacteria that are predominant on the surface of the beetles are also widespread in the rhizosphere of plants (Mahaffee & Kloepper, 1997) and, in the case of *Pseudomonas* and *Erwinia* are also endophytic bacteria of many species of plants (Cankar et al., 2005).

**Little overlap in the microbiomes in the gut and abdomen**

The bacterial assemblages in the guts and abdomens of individual bryophagous beetles differed substantially. This indicates the possibility of confounding two distinct microbial niches if gut and abdomen are not separated during dissection, as is the case of most studies on such small insects. Despite the careful dissection of the guts and abdomens of beetles, we cannot completely exclude the possibility of cross-contamination. Thus, the recorded dissimilarity of the assemblages in the gut and abdomen could be even more significant, further emphasizing the importance of their segregation during dissection.

**Ecology and function of potential symbionts**

Although symbiotic bacteria are often acquired via the diet or soil, they can be involved in digestion and other processes (Kelley & Dobler, 2011). Proteobacteria and Firmicutes are often the predominant bacterial phyla in the majority of insect guts (Vasanthakumar et al., 2008; Douglas, 2011; Colman et al., 2012), which is consistent with our findings. The eudominant bacterial genera significantly more associated with the gut and abdomen of both byrrhids were *Bradyrhizobium*, *Caulobacter*, *Novosphingobium* and *Ralstonia*. These bacteria were not only more abundant in the gut than on the surface of their body, but also more abundant in the abdomen than in the gut, indi-
cating a more intimate association with the organisms and possible involvement in the metabolic processing of bryophytes. \textit{Bradyrhizobium}, the commonest soil bacterium fixing nitrogen in legumes (Klimaszewski et al., 2013), has been repeatedly associated with \textit{Sphagnum} moss (Bragina et al., 2012) and reported in the guts of Cerambycidae (Grünwald et al., 2010), Staphylinidae (Klimaszewski et al., 2013) and Tortricidae fed on artificial diets (Landry et al., 2015). Many other MOTUs recorded in the gut and abdomen belong to Bradyrhizobiaceae, which are typical rhizosphere bacteria fixing nitrogen (De Jesús-Laboy et al., 2011). They have been also isolated from bryophytic mites (Moquin et al., 2012) and \textit{Sphagnum} mosses (Bragina et al., 2012). \textit{Caulobacter} are predominant in the mycorhiza of \textit{Xyleborus glabratus} (Curculionidae; Hulcr et al., 2012), the gut of \textit{Acromyrmex} ants (Van Borm et al., 2002), in Cicadellidae (Rogers & Backus, 2014), Noctuidae (Snyman et al., 2017) and the midgut of \textit{Pyrrhocoris} bugs and in their diet, lime seeds (Sudakaran et al., 2012), and in symbiotic interactions with \textit{Sphagnum} mosses (Bragina et al., 2012). \textit{Novosphingobium} is reported associated with the gut of \textit{Cryptocephalus} spp. (Chrysomelidae; Montagna et al., 2014), \textit{Anoplophora glabripennis} (Cerambycidae; Geib et al., 2009), oral secretions of Curculionidae (Cardoza et al., 2009), herbivorous Cicadellidae (Rogers, 2014), gall midges (Ojha et al., 2017) and also mosses (Bragina et al., 2012; Graham et al., 2017). \textit{Ralstonia} is a plant-associated genus abundant in the microbiota of Cicadellidae (Rogers & Backus, 2014), \textit{Cryptocephalus} spp. (Chrysomelidae; Montagna et al., 2014) and associated with ciliates in the rumen (Irvis & Ushida, 2004).

Some of these microorganisms can provide their host with nitrogen, which is typically deficient in plant materials (Benemann, 1973). Rapid passage through the midgut may reduce the ability to extract nitrogenous compounds from food. Therefore, an association with nitrogen-fixing bacteria may be especially beneficial. Some genera present in the microbiota of bryophytes, such as \textit{Bradyrhizobium}, fix nitrogen. Closely related to the nutritional role of symbiotic microorganisms is their ability to detoxify (Engel \\& Moran, 2013). Bryophytes contain a broad spectrum of secondary metabolites that protect them from being eaten (Gerson, 1982). For example, symbionts of the bryophagous Peloridae are thought to play a role in excretion or detoxification of ingested moss (Kuechler et al., 2013). Similarly, the microorganisms found in the bryophagous beetles studied, such as \textit{Novosphingobium} and \textit{Ralstonia} can degrade phenols and aromatics.

**CONCLUSION**

The recorded MOTU diversity in the microbiomes in the gastrointestinal tract of bryophagous beetles was high. Substantial differences in the composition of the assemblages recorded in the gut and abdomen indicate the importance of separating body parts before any metagenomic analysis. High bacterial diversity may stem from the particular diet of bryophagous beetles, as the most abundant MOTUs recorded are involved in metabolic processes associated with digestion; alternatively, they may reflect the greater possibilities of metagenomics. Further studies are required to distinguish bacteria, which are either incorporated only transiently through the diet or are ingested accidentally with soil particles, from those involved in bryophyte digestion. This vast bacterial assemblage could potentially serve as a source of enzymes degrading a variety of chemical compounds present in mosses. Moreover, further research is likely to help us understand the unique ability of bryophagous beetles to digest such a specific and generally deleterious source of food, moss.

**ACKNOWLEDGEMENTS.** This research was funded by the Ministry of Education, Youth and Sports of the Czech Republic (LO1208), the Institute of Environmental Technologies (CZ.1.05/2.1.00/03.0100), NextGenProject (CZ.1.07/2.3.00/20.0303), Extension and Appreciation of Instruments IET (CZ.1.05/2.1.00/19.0388), University of Ostrava (SGS28/PrF/2014, SGS20/PrF/2015, SGS23/PrF/2016), European Regional Development Fund BIOCEV (CZ.1.05/1.1.00/02.0109) and Czech Science Foundation (GA18-08803S). We would like to thank Editage (www.editage.com) for English language editing.

**REFERENCES**

Akman L., Yamashita A., Watanabe H., Oshima K., Shiba T., Hattori M. \\& Aksoy S. 2002: Genome sequence of the endocellular obligate symbiont of tssetse flies, \textit{Wigglesworthia glossinidia}. — Nat. Genet. 32: 402–407.

Bateman C., Sigut M., Skelton J., Smith K.E. \\& Hulcr J. 2016: Fungal associates of the \textit{Xyllosandrus compactus} (Coleoptera: Curculionidae, Scolytinae) are spatially segregated in the insect body. — Environ. Entomol. 45: 883–893.

Bates D., Mächler M., Bolker B. \\& Walker S. 2014: Fitting linear mixed-effects models using \textit{lme4}. — J. Stat. Softw. 67: 1–48.

Benemann J.R. 1973: Nitrogen fixation in termites. — Science 181: 164–165.

Berry D., Mahfoudhi K.B., Wagner M. \\& Løy A. 2011: Bar-coded primers used in multiplex ampiclon pyrosequencing bias amplification. — Appl. Environ. Microbiol. 77: 7846–7849.

Borremans J., Skroch P.W., O’Sullivan K.M., Palus J.A., Rumiansk N.G., Jansen J.L., Niethuis J. \\& Triplett E.W. 1996: Molecular microbial diversity of an agricultural soil in Wisconsin. — Appl. Environ. Microbiol. 62: 1935–1943.

Bragina A., Berg C., Cardinale M., Sicherrjakov A., Chebotar V. \\& Berg G. 2012: \textit{Sphagnum} mosses harbour highly specific bacterial diversity during their whole lifecycle. — ISME J. 6: 802–813.

Broderick N.A., Raffa K.F., Goodman R.M. \\& Handelms J. 2004: Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. — Appl. Environ. Microbiol. 70: 293–300.

Brune A. 2010: Methanogens in the digestive tract of termites. In Hackstein J.H.P. (ed.): \textit{Endosymbiotic Methanogenic Archaea, Microbiology Monographs}. Springer, Berlin, Heidelberg. pp. 81–100.

Buchon N., Broderick N.A., Pordevin M., Pradervand S. \\& Le Maytre B. 2009: \textit{Drosophila} intestinal response to bacterial in-
LEROY B., PETILLON J., GALLON R., CANARD A. & YSNEL F. 2012: R Development Core Team 2017: R: A Language and Environment for Statistical Computing. R Package Ver. 3.0.1. R Foundation for Statistical Computing. R Dev. Core Team R Lang. Environ. Stat. Comput. Vienna R Found. Stat. Comput. 1–2.

LEROY P.D., SABRI A., HEUSKIN S., THONART P., LOGNAY G., VERTAUX P., FELTON G.W. & HAFNUDE E. 2011: Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. — Nat. Commun. 2: 348.

LEROY B., PETILLON J., GALLON R., CANARD A. & YSNEL F. 2012: Improving occurrence-based rarity metrics in conservation studies by including multiple rarity cut-off points. — Insect Conserv. Divers. 5: 159–168.

LEROY B., CANARD A. & YSNEL F. 2013: Integrating multiple scales in rarity assessments of invertebrate taxa. — Divers. Distrib. 19: 794–803.

MAHAFEE W.F. & KLOEPPEL J.W. 1997: Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (Cucumis sativus L.). — Microb. Ecol. 34: 210–223.

MCCLEARY J.A., SYPHERD P.S. & WALKINGTON D.L. 1960: Mosses as possible sources of antibiotics. — Science 131: 108–108.

MONTAGNA M., GÓMEZ-ZURITA J., GIORGI A., EPIS., LOZZIA G., NIKOH N., HOSOKAWA T., OSHIMA K., HATTORI M. & FUKATSU T. 2014: Metamicrobiomics in herbivore beetles of the genus Chrysomelidae: toward the understanding of ecological determinants in insect symbiosis. — Insect Sci. 22: 340–352.

MOQUIN S.A., GARCIA J.R., BRANTLEY S.L., TAKACS-VESBACH C. & SHEPPARD U.L. 2012: Bacterial diversity of bryophyte-dominant biological soil crusts and associated mites. — J. Arid Environ. 87: 110–117.

MORALES M. 2017: Scientific Graphing Functions for Factorial Designs. R Package Ver. 1.0-7.

NIKO I., HOSOKAWA T., OSHIMA K., HATTORI M. & FUKATSU T. 2011: Reductive evolution of bacterial genome in insect gut environment. — Genome Biol. Evol. 3: 702–714.

OBIA A., SINHA D.K., PADMUKAMARI A.P., BENTUR J.S. & NAIR S. 2017: Bacterial community structure in the asian rice gall midge reveals a varied microbiont rich in Proteobacteria. — Sci. Rep. 7: 9424, 13 pp.

OKSANEN J., BLANCHET F.G., KINDT R., LEGENDRE P., O’HARA R.B., SIMPSON G.L., SOLYMOS P., STEVENS M.H.H. & WAGNER H. 2017: vegan: Community Ecology Package. R package version 2.4-5. R Dev. Core Team R Lang. Environ. Stat. Comput. Vienna R Found. Stat. Comput.

OLIVER K.M., DEIGAN P.H., BURKE G.R. & MORA N.A. 2010: Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. — Annu. Rev. Entomol. 55: 247–266.

PARKER J.D., BURKEPIE D.E., COLLINS D.O., KUBANEK J. & HAY M.E. 2007: Stream mussels as chemically-defended refugia for freshwater macroinvertebrates. — Ecos 116: 302–312.

PEÑUELAS J., RICO I., OGUIA R., JUMP A.S. & TERRADAS J. 2012: Summer season and long-term drought increase the richness of bacteria and fungi in the foliar phyllosphere of Quercus ilex in a mixed Mediterranean forest. — Plant Biol. 14: 565–575.

R DEVELOPMENT CORE TEAM 2017: A Language and Environment for Statistical Computing. R Dev. Core Team R Lang. Environ. Stat. Comput. Vienna R Found. Stat. Comput.

RENKENEN O. 1938: Statistisch-ökologische Untersuchungen über die Terrestri schen Käferwelt der Finnischen Bruchmoore. So cietas zoologica-botanica Fenncia, Vanamo, 231 pp.

ROBINSON C.J., SCHLOSS P., RAMOS Y., RAYA K. & HANDELSMAN J. 2010: Robustness of the bacterial community in the cabbage white butterfly larval midgut. — Microb. Ecol. 59: 199–211.

ROGERS E.E. 2016: Deep 16S rRNA gene sequencing of anterior foregut microbiota from the blue-green sharpshooter (Grapho cephal a atropunctata). — J. Appl. Entomol. 140: 801–805.

ROGERS E.E. & BACKUS E.A. 2014: Anterior foregut microbiota of the glassy-winged sharpshooter explored using deep 16S rRNA gene sequencing from individual insects. — PLoS ONE 9: e106215, 9 pp.

SCHMIDT P.-A., BAUMT M., GRESHAKE B., BANDOW C., RÖMBKE J. & SCHMITT I. 2013: Illumina metabarcoding of a soil fungal community. — Soil Biol. Biochem. 65: 128–132.

SCHMITT I. 2013: Illumina metabarcoding of a soil fungal community. — J. Appl. Entomol. 140: 801–805.

SUDAKARAN S., SALEM H., KOST C. & KALTONEPOTH M. 2012: Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, Pyrrhocoris apterus (Hemiptera, Pyrrhocoridae). — Mol. Ecol. 21: 6134–6151.

TANG X., FREITAK D., VOGEL H., PING L., SHAO Y., CORDERO E.A., ANDERSEN G., WESTERMANN M., HEGEL D.G. & BOLAND W. 2012: Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. — PLoS ONE 7: e36978, 9 pp.

TERRBAAK C.J.F. & SMALERU P. 2012: CANOCO Reference Manual and CanoDraw for Windows User’s Guide: Software for Canonical Community Ordination (version 4.5). www.canoco.com, Ithaca, NY, 500 pp.

THIENHESK S.E. 2013: A review of the species of the genus Carinopsis Ganglbauer, 1902 (Coleoptera, Byrrhidae) in North Asia. — Entomol. Rev. 92: 534–544.

VAN BORM S., BILLIEN J. & BOOMSAJA J.J. 2002: The diversity of microorganisms associated with Acromyrmex leaffcutter ants. — BMC Evol. Biol. 2: 9, 11 pp.

VANDERPOORTEN A. & GOFFINET B. 2009: Introduction to Bryophytes. Cambridge University Press, New York, 303 pp.

VÁSAANTAHKUMAR A., HANDELSMAN J.O., SCHLOSS P.D., BAUER L.S. & RAFFA K.F. 2008: Gut microbiota of an invasive subcortical beetle, Aprillus planipennis Fairmaire, across various life stages. — Environ. Entomol. 37: 1344–1353.

VAZMOREIRA I., EGAS C., NUNES O.C. & MANAIA C.M. 2011: Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. — Antonie Van Leeuwenhoek 100: 245–257.

WARNECKE F., LUGINBUHL P., IVANova N., GHAASEMAN M., RICHARDSON T.H., STIEE J.T., CAYOUETTE M., McHARDY A.C., DIORIEVIG C., ABOU-SHADI N. ET AL. 2007: Metagenomic and functional analysis of hindgut microbiota of a wood-feeding, leafcutter ant, Agylus planipennis Fairmaire, across various life stages. — Environ. Entomol. 37: 1344–1353.

WARNEKE F., LUGINBUHL P., IVANova N., GHAASEMAN M., RICHARDSON T.H., STIEE J.T., CAYOUETTE M., McHARDY A.C., DIORIEVIG C., ABOU-SHADI N. ET AL. 2007: Metagenomic and functional analysis of hindgut microbiota of a wood-feeding, leafcutter ant, Agylus planipennis Fairmaire, across various life stages. — Environ. Entomol. 37: 1344–1353.

WHITAKER R.H. 1965: Dominance and diversity in land plant communities: Numerical relations of species express the im-
The importance of competition in community function and evolution. — *Science* **147**: 250–260.

Wong C.N.A., Ng P. & Douglas A.E. 2011: Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. — *Environ. Microbiol.* **13**: 1889–1900.

Yun J.-H., Roh S.W., Whon T.W., Jung M.-J., Kim M.-S., Park D.-S., Yoon C., Nam Y.-D., Kim Y.-J., Choi J.-H. et al. 2014: Insects gut bacterial diversity determined by host environmental habitat, diet, developmental stage and phylogeny. — *Appl. Environ. Microbiol.* **80**: 5254–5264.

Received January 21, 2019; revised and accepted August 22, 2019
Published online November 26, 2019