The core gut microbiome changes throughout life cycle and season of bark beetle Ips typographus

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December 20, 2022

Abstract

Ips typographus, the most serious pest of spruce forests in Europe, is associated with microorganisms facilitating its invasion and development inside spruce tissues. Despite the importance of I. typographus, little is known about its core gut microbiome. Hereby, we describe the composition of bacterial and fungal microbiomes throughout I. typographus life cycle in spring and summer generations. We used cultivation technique and molecular identification in combination with DNA and RNA metabarcoding to achieve deep inside into the beetle's microbiome composition and structure. As it is not known whether microbiome forms stable communities inside the beetle's gut, we observed gut epithelium for biofilm formation with Transmission Electron Microscopy. Cultivation technique together with DNA and RNA metabarcoding indicated similar dominant taxa. The bacterial community belongs almost exclusively to the phylum Proteobacteria (newly Pseudomonadota) and the most common orders and genera are Enterobacteriales (Erwinia and Serratia), Pseudomonadales (Pseudomonas) and Xanthomonadales (Pseudoxanthomonas, Stenotrophomonas). Yeasts (Saccharomycetes) highly dominated the fungal microbiome, followed by Sordariomycetes represented mainly by Ophiostoma bicolor and Endoconidiophora polonica. The most common yeasts were Wickerhamomyces bisporus, Kuraishia molischiana, Nakazawaea ambrosiae, Yamadazyma spp. and Cyberlindnera sp. The proportions of the dominant taxa belonging to the core microbiome of I. typographus change throughout its life cycle and generations. We did not observe any biofilm formation on gut epithelium, which suggests that microbial cells pass through the beetle's gut with chyme. We propose that species belonging to the core microbiome has similar functions and alternate in the I. typographus ecosystem depending on environmental conditions.

INTRODUCTION

Bark beetles (Curculionidae: Scolytinae) is an ubiquitous, taxonomically and ecologically highly diversified group of insects. They include more than 6,000 species that feed on various plant tissues (Kirkendall et al., 2015). Bark beetles are an integral part of forest ecosystems as they promote forest heterogeneity and resilience across multiple scales (Kulakowski et al., 2016). However, they cause increasingly severe outbreaks worldwide as a result of global climate change, insufficient forest management and introductions of new invasive species often linked to international trade (Hlasny et al., 2021). Thus, bark beetles are considered the most important threat to agricultural forests (Kirkendall & Faccoli, 2010; Hicke et al., 2013; 2016; Mezei et al., 2017).

The evolutionary success of bark beetles has been undoubtedly facilitated by a wide range of associations with microbial symbionts (Sun et al., 2013; Douglas, 2015; García-Fraile, 2018; Chakrabotry 2020; Dinkins-Bookwalter et al., 2015). Nature of the symbiosis is diverse, ranging from parasitism to mutualistic relationship (Six, 2012; Su et al. 2013) and microbiome fulfills multiple proposed functions. Firstly, these microorga-

nisms enrich beetles' diet with sterols, vitamins, essential amino acids, and nitrogenous compounds (Rivera et al., 2009; Hernandez-Garcia et al., 2018; Ibarra-Juarez et al., 2020; Fabryová et al., 2018; Veselská et al., 2018; García-Fraile, 2018; Bentz & Six 2006; Ayres, Wilkens et al., 2000). Secondly, they ease plant tissue colonization by detoxification of tree defense compounds (Hammerbacher et al., 2013; Giron et al., 2020), or by production of small molecules defending against pathogens, parasites, and predators (Conord, 2008; Berasategui et al., 2016; Giron et al., 2020). Thirdly, they ferment sugars in the tree phloem and convert them into beetle´s pheromones (Zhao et al., 2019). For that purpose, insects' symbionts are a promising source of new bioactive compounds (VanMoll et al., 2021; Saati-Santamaría et al., 2018). Finally, some of the symbionts are phytopathogens that can necrotize healthy plant tissue, thereby not only increasing insects' fitness but causing tree mortality (Li et al., 2022; Hofestetter, 2015).

Previous studies of the microbiome have studied a very different set of substrates, such as galleries or whole larvae or adults, or their mixture, not allowing to distinguish between ecto- and endosymbiotic microbial communities. When looking at the gallery system in more detail, it is clear that it is composed of a number of different niches. The life cycle of the beetle begins with the maternal beetles laying eggs and actively or passively introducing microorganisms into the system, which then grow into the surrounding plant tissues and are further consumed by larvae, which build their tunnels. Ectosymbiotic microorganisms proliferating around and inside galleries are the most conspicuous and studied, because they include tree-killing ophiostomatoid fungi. However, bark beetles, like other herbivores, also have endosymbiotic communities in their guts, which are then in direct interactions (e.g. detoxification, nutrient supply, protection, pheromone production) with ingested plant matter (Douglas 2015; Engel & Moran 2013; Giron et al. 2017). Several studies, mostly on *Dendroctonus* beetles and bacteria (e.g. Hou et al. 2022), have investigated the structure and composition of bark beetle gut microbial communities. More work is needed to understand how specific or labile gut communities are, and how their structure and composition relates to functions required by the beetle host (reviewed in Six et al. 2013; Engel, P. & Moran 2013).

European bark beetle, *Ips typographus* is currently the most serious pest of spruce forests (Biedermann et al., 2019). Its distribution follows in its entirety the area of its host tree*Picea abies* which has a continuous range in Scandinavia, north-eastern Europe and western Russia, and central Europe.*Ips typographus* has one to three generations annually depending on temperature. Predictions suggest that the number of complete generations will increase as a result of global climate change (Jakoby, et al. 2019; Biedermann, et al. 2019). Despite the economic significance of *I. typographus* and the known importance of microorganisms on bark beetles´ ecology, we have only limited knowledge of the composition, ecological functions and seasonality of the microorganismal community associated with *I. typographu* s. Current knowledge is based almost exclusively on cultivation approaches, often focused on ophiostomatoid fungi only. Thus, baseline data on the total spectrum of associated microorganisms is missing. *Ips typographus* transmits microorganisms via gut and body surface, as it lacks any specialized phoretic structure (Bentz, et al. 2019). Ophiostomatoid fungi such as*Grosmannia penicillata* , *Ophiostoma bicolor* and*Endoconidiophora polonica* , which participate in the detoxification of spruce defense compounds (Hammerbacher et al., 2013; Zhao et al., 2019), are reported as dominant and stable associates. They are followed by a variety of other filamentous fungi and yeasts such as*Ogataea* , *Pichia* , *Candida* , *Kuraishia* and*Cryptococcus* (see Linnakoski et al., 2012 for review). Bacteriome has so far been studied sporadically and only few taxa such as*Serratia liquefaciens* (Muratoglu et al., 2009), *Erwinia typographi* (Skrodenytė-Arbačiauskienė et al., 2012),*Staphylococcus* , and *Pseudomonas* (Berasategui et al., 2016, Peral-Aranega et al., 2020, Saati-Santamaria et al., 2021) were reported. So far, only two studies based on fungal and bacterial DNA metabarcode sequencing respectively have been published (Chakraborty et al., 2020a, 2020b). However, these studies were focused only on large scale comparison of gut-associated microbiome among several bark beetle species, including *I. typographus* . Thus, our study is the first which brings detailed analyses of *I. typographus* microbiome throughout its life cycle and seasons. It is surprising that for such a fundamental forest pest, we do not have a clear idea of the complete structure of microbial communities (both intestinal and ectosymbiotic) and their changes during the life cycle and seasons (i.e. in different generations during the year). Due to sampling design biases, there is also a lack of information about which organisms the bark beetle vectors and which it acquires from the environment, via

environmental filtering.

Here we report the first rigorous description of the core microbiome of *I. typographus* throughout its life cycle and two generations using a combination of cultivation technique, DNA and RNA metabarcode sequencing and transmission electron microscopy. For that purpose, we collected parental adults (beetle-finding galleries), larvae, pupae and young adults (teneral adults) from the two generations of the same year (spring and summer generation). Precise taxonomic identification allowed us to distinguish taxa mostly at the species level, which is essential for the interpretation of the ecology and biology of the particular symbiont. According to the current state of art, there are multiple definitions of core microbiome (Berg et al., 2020; Risely, 2020). In the present study, dominant species from the DNA and RNA metabarcode sequencing were assigned to belong among the core microbiota. We found that even though the taxonomic composition of core microbiota is rather similar, the proportions of the dominant taxa varies throughout the life cycle and seasons. Fungal microbiome is dominated by yeasts (mainly *Wickerhamomyces bisporus* , *Nakazawaea ambrosiae* and *Kuraishia molischiana*). Bacterial microbiome is dominated by orders Enterobacteriales, mainly by *Erwinia typographi* , Pseudomonadales and Pseudoxanthomonadales.

MATERIAL AND METHODS

Sample collection

During May and August 2020, four and five trunks (dbh 20-25 cm), respectively, were sampled in the surroundings of Nižbor (Czechia, 49°59'09.9"N 13°56'47.5"E, 390 m.a.s.l.). The sampling site is situated in a continuously forested area belonging to Protected Landscape Area Křivoklátsko. The average annual temperature is 9 °C and the average annual precipitation is 494.9 mm (Lány observatory, Czech Hydrometeorological Institute). Spruce trees infested by *I. typographu* s were randomly selected, felled, cut into logs and transported to the Institute of Microbiology of the CAS, Prague. Logs were incubated in the exterior in a shaded place simulating the original forest site and successively sampled for various bark beetle life stages regardless of the gender (parental adults, larvae, pupae, young (teneral) adults, and infested pigmented and intact phloem as a control, see sampling scheme, Fig. 1). We also sampled freshly laid eggs; however, we were not able to ensure their proper surface sterilization. Thus, the eggs were omitted from the analysis. Beetle species determination was based on their macromorphology under a binocular magnifier (Nikon SZ30, Minato, Japan) using determination literature (Pfeffer, 1955). Beetle samples were taken out of the galleries and surface sterilized by subsequent washing with 70% ethanol, 2% Tween 80 (Avantor, USA) and sterile distilled water. Randomly selected 5 galleries from each log were sampled and pooled into one representative sample containing 10 individuals. Suspicious or parasitized galleries or individuals were excluded from the study. In the summer season, the parts of the infested phloem (2x5 mm) from active galleries and parts of intact phloem (2x5 mm) were collected. The single sample consisted of five phloem pieces collected on the same log, and pooled. Fresh intact phloem was sampled at the beginning of the beetle development at least 10 cm from the young maternal gallery where no colonization by microorganisms was visible. Infested phloem was sampled at the end of the development of teneral adults and was distinct by the colorization of plant tissues. Pooled samples were frozen at -80°C in Eppendorf tubes till DNA/RNA extraction, if necessary.

Fungal cultivation and identification

Surface sterilized larvae (described above) were dissected straight after collection, their guts were stored in 200 µl of 25% glycerol until further manipulation. Once we obtained enough guts, we crushed and homogenized them by sterile plastic pestle in 1.5 ml Eppendorf tube with 1 ml of sterile 1% Tween 80 in dH_2O, and vortexed for approximately 10 s on bench vortex (IKA MS3 vortexer). The vortexed inoculum was spread on 9 cm agar plates with 2% YES medium with antibiotics (5 g/l of yeast extract, 30 g/l of glucose, 15g/l of agar, 60 mg/l of streptomycin and 60 mg/l of chloramphenicol all from Sigma-Aldrich, St. Louis, Missouri, USA). The method of serial dilutions (1, 10, 100 ×) was used to determine the abundance of individual taxa. Agar plates were cultivated at 25 °C for one week in the dark and after this period colonies were morpho-typed and morphologically unique cultures were taken for further identification. In the present study, we describe cultivation and identification only for fungal microbiome as the bacterial part is described

elsewhere (Peral-Aranega et al., in press). For the identification of fungal isolates, the DNA was extracted from the fresh pure cultures using a DNeasy PowerSoil Pro Kit (QIAGEN GmbH, Hilden, Germany). Fungi were identified by ITS-LSU rDNA barcode which was amplified using the ITS4 primer (O'Donnell 1992, White et al., 1990). SAP-Exo kit (Jena Bioscience GmbH, Germany) was used for purification of PCR amplicons and sequencing was done at Macrogen Inc. (Seoul, Korea). Obtained sequences were manually aligned in Bioedit v.7.2.5. The obtained sequences were blasted (Altschul et al., 1990) to those of type strains of described species available in public databases to identify the isolates.

DNA metabarcode analysis

DNA extraction

DNA was extracted from the surface sterilized beetle samples (parental adults, larvae, pupae, and teneral adults) and infested and intact phloem. Although we were focused on intestinal microbiota, we isolated DNA from the whole individuals not directly from guts as the softness of pupal bodies impede such manipulation. DNA was extracted following the phenol-chloroform protocol (Sagova-Mareckova et al., 2008) using the phenol-chloroform–isoamyl alcohol (25:24:1) premixed solution (Sigma-Aldrich, St. Louis, Missouri, USA). The initial homogenization was done on FastPrep-24 5G Instrument (Irvine, California, USA) using wolfram beads in combination with glass beads (BioSpec Products, Inc., Bartlesville USA). DNA yield was quantified on Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using Qubit dsDNA BR Assay Kit and DNA quality was checked on Nanodrop (NanoDrop 2000c, ThermoFisher scientific).

Amplicon sequencing

PCR amplification was done by kit KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) using an input DNA concentration of 50 ng/µl. We used ITS2 and 16S markers for the identification of fungi and bacteria, respectively. Genetic markers were amplified using labeled pairs of primers ITS3_KYO2 and ITS4_KYO3 (Toju et al., 2012) for ITS2 marker and primer pair 799F (Chelius and Triplett 2001) and 1115R (Redford et a. 2010) for 16S marker V5-V6 region. Each DNA sample was amplified in triplicates in separate 96-microtiter plates, which were subsequently pooled into one sample. Each 96-microtiter plate also included three negative controls (PCR grade water used as a template) and one positive control for fungal/bacterial species (a random DNA sample of one of our pure bacterial/fungal cultures was used as a template). Amplicons were then purified from oligonucleotides by SAP-Exo kit (Jena Bioscience GmbH, Germany). 1 µg of purified amplicon served as a template for library construction using KAPA HyperPlus Kit in combination with KAPA UDI primer mixes (Kapa Biosystems, Massachusetts, USA). Amplicon's size selection of the final libraries was done by KAPA Pure Beads (Kapa Biosystems) and its effectiveness was checked on 1% agarose gel (SeaKem® LE Agarose, Lonza Group Ltd, Basel Switzerland). The amplicons size for ITS and 16S were around 450 bp and 300 bp, respectively. The quality of the ligated library was quantified using the EliZyme Library Quantification Kit (Elisabeth Pharmacon, Brno, Czechia). Library sequencing was done on the Illumina MiSeq platform (San Diego, California, USA) on a 2x300 bp paired-end reads run performed at CEITEC institute (Brno, Czechia).

DNA metabarcoding data processing

Sequencing data were processed using QIIME 2.0 2021.8 (Bolyen, et al. 2019). Raw reads were demultiplexed and quality filtered using the q2-demux plugin, and in the case of fungal datasets, the ITS region was extracted using the q2-ITSxpress plugin (Rivers et al. 2018). Afterwards, reads were denoised using the DADA2 algorithm (Callahan, et al. 2016) and a feature table with counts of amplicon sequence variants (ASVs) per sample was produced. Taxonomy was assigned using the q2-feature-classifier classify-sklearn (Bokulich, et al.2018) using a trained naive Bayes classifier against the SILVA_138_SSURef_Nr99 bacterial reference database and UNITE QIIME release for Fungi version 8.0. Rarefaction analysis of final ASV tables was performed to assess the completeness of the dataset and the admissible data resampling level for statistical analysis.

Metatranscriptome analysis

RNA extraction

For RNA extraction, the whole ventriculus, foregut, midgut and hindgut of each specimen was eviscerated under the binocular magnifier from the parental adults (11 samples), larvae (7 samples) and teneral adults (8 samples) of *Ips typographus* . Pupae were not analyzed as the softness of their bodies impeded such manipulation. RNA was extracted using the Nucleospin RNA plant kit (Macherey-Nagel). The intestines were eviscerated directly into lysis buffer RA1 supplemented with 1% β-mercaptoethanol and then kept in the freezer at -80°C until needed. Samples in lysis buffer were then homogenized using Lysing Matrix A (MP Biomedicals) on FastPrep-24 5G Instrument (Irvine, California, USA) for 30 s at 5.5 m/s. The DNA digestion step was omitted from the protocol. The DNA digestion was then performed with TURBO DNA free kit (Invitrogen). The absence of residual DNA in the samples was verified by PCR amplification of ITS and 16S regions and visualization of the reaction products on the agarose gel. The purity of isolated RNA was checked by Nanodrop (NanoDrop 2000c, ThermoFisher scientific). Samples with A260/A280 and A260/A230 lower than 1.8 were repurified by isopropanol precipitation with 3 M sodium acetate, pH 5.2. RNA concentration was measured on Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using Qubit dsRNA BR Assay Kit. Quality of isolated RNA was examined on Bioanalyzer using RNA 6000 Pico Kit (Agilent).

Library construction and sequencing

Isolated RNA was separated into two fractions. The first fraction included eukaryotic mRNA that was extracted from total RNA by NEBNext(r) Poly(A) mRNA Magnetic Isolation Module (BioLabs) following manufacturers' protocol. The second fraction (bacterial RNA and eukaryotic rRNA) was extracted from the supernatant after mRNA separation by RNAClean XP beads (Beckman Coulter). Both fractions were used for library constructions. cDNA libraries were built by Zymo-Seq RiboFree Total RNA-Seq Library Kit (Zymo Research) following manufacturers' protocol. The quality of cDNA libraries was then visualized on Bioanalyzer by High Sensitivity DNA Kit (Agilent). Libraries were sequenced at CEITEC institute (Brno, Czechia) on the Illumina novaseq platform on a 2x 150 bp paired-end reads run.

Metatranscriptome analysis

Raw data were processed using SqueezeMeta v1.4.0 pipeline (Tamames & Puente-Sanchez 2019). Assembly was done using Megahit (Li et al 2015). Short contigs (<150 bps) were removed using prinseq (Schmieder et al., 2011). Contig statistics were done using prinseq (Schmieder et al., 2011). RNAs were predicted using Barrnap (Seeman 2014). 16S rRNA sequences were taxonomically classified using the RDP classifier (Wang et al., 2007). tRNA/tmRNA sequences were predicted using Aragorn (Laslett & Canback 2004). ORFs were predicted using Prodigal (Hyatt et al., 2010). Similarity searches for GenBank (Clark et al 2016), eggNOG (Huerta-Cepas et al., 2016), KEGG (Kanehisa and Goto, 2000), were done using Diamond (Buchfink et al., 2015). HMM homology searches were done by HMMER3 (Eddy, 2009) for the Pfam database (Finn et al., 2016). Read mapping against contigs was performed using Bowtie2 (Langmead and Salzberg, 2012). Pathway prediction for KEGG (Kanehisa and Goto, 2000) and MetaCyc (Caspi et al., 2018, Nucleic Acid Res 46(D1), D633-D639) databases was done using MinPath (Ye and Doak, 2009).

Transmission electron microscopy (TEM)

Twenty-four larvae were left to develop in felled spruce longs up to their 2nd or 3rd instar. They were then removed from the logs, surface sterilized by rinsing in distilled water and 40% ethanol, which also killed them gently. The larvae were then dissected under a binocular magnifier in a drop of sterile buffer (10x PBS). The digestive tract of each individual was then separated into 3 subsamples (foregut, midgut and hindgut) to facilitate orientation in the sample and fixed by following protocol. For TEM analysis, pieces of the digestive tract were fixed for 24 h in a solution of 2.5% glutaraldehyde in 0,1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO4 in the same cacodylate buffer. Fixed samples were dehydrated through a standard ascending ethanol and acetone series and embedded in Araldite - Poly/Bed(r) 812 resin mixture. Thin sections were cut on an ultramicrotome (Reichert-Jung Ultracut E) and stained using uranyl acetate and lead citrate following the Hayat (2000). Sections were examined and photographed using JEOL JEM-

1011 (JEOL Ltd., Japan) transmission electron microscope. Fine structure measurements were performed using a Veleta camera and iTEM 5.1 software (both Olympus Soft Imaging Solution GmbH, Germany). Photos were made under accelerating voltage from 100 to 300 keV.

Statistical analyses

Statistical analyses were done either using plugins from Qiime 2.0 v.2021.8 (Bolyen, et al. 2019) environment or in R Statistical Software (v4.2.1; R Core Team 2021), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/., using packages Phyloseq (McMurdie and Susan Holmes 2013), Vegan (Oksanen et al. 2022), Qiime2R (Bisanz 2018), Microbiomeutilities (v1.0.16, Shetty et Lahti 2022), R package version 1.00.16., microViz (v0.9.7, Barnett et al. 2021), MicrobiomeStat (v1.1, Zhang et Chen 2022), MicrobiomeMarker (v1.2.2, Cao et al. 2022) and ANCOMBC (v1.6.3, Lin et Peddada 2020). Alpha diversity indices including Chao1, observed ASVs, shannon and simpson as well as beta diversity indices including bray-curtis, jaccard, weighted and unweighted unifrac were calculated using plugin diversity and corresponding pipelines from Qiime 2 and significant differences between groups were assessed for both groups of indices. Significance in beta diversity differences among groups was assessed by adonis PERMANOVA test. Furthermore, dispersion of beta diversity differences within groups was assessed by the permdisp method. All of the mentioned analyses were performed in Qiime 2. Ancom analysis to identify differentially abundant features was performed in the R environment with the help of aforementioned R packages.

RESULTS

Diversity of fungal isolates revealed by cultivation

Metabarcoding using the relatively short reads often does not allow accurate identification at species level. Thus, to determine the exact identity of ASVs identified by metabarcoding, we cultured and identified the dominant fungi, (bacteria were identified elsewhere by Peral-Aranega et al. in press and are deposited in GenBank with accession numbers OP935778-OP935900). In total, we isolated 108 fungal strains belonging to 12 species (8 yeasts, 2 filamentous fungi). The most frequently isolated species were *Ogataea ramenticola* , *Meyerozyma guilliermondii* , *Wickerhamomyces bisporus* , *Nakazawaea ambrosiae* , and *Ophiostoma bicolor* (Figure 2, Supplementary Table 1).

DNA metabarcode analysis

A total of 2, 231, 208 and 2, 796, 381 pair-end reads were obtained after the quality control test for ITS and 16S markers, respectively. Rarefaction curves for both genetic markers (Supplementary Figure 1) showed that our sampling was sufficient to cover species diversity present in the guts and galleries of *I. typographus* . A count of assigned ASVs in respect to season and sample type is summered in Table 1. The reads were grouped into 201 fungal and 239 bacterial ASVs in the spring season and 226 fungal and 560 bacterial ASVs in the summer season. The total number of assigned ASVs in the summer season is increased compared to spring due to the additional sampling of phloem samples.

Richness and diversity of the bacterial and fungal microbiome in seasons and throughout the life cycle

The bacterial ASVs richness and diversities were similar between the two seasons ($p>0.05$, Kruskal-Wallis, Fig. 3C); however, they change throughout the beetle's life cycle. In the spring season, Simpson and Shannon's bacterial diversities were significantly higher for larval and pupal samples than diversities of parental adult and teneral adult samples ($p<0.05$; Kruskal-Wallis). In the summer season, the bacterial richness and diversity indexes were rather similar throughout the beetle's life cycle; however, ASVs richness (Ace and Chao1 index) was much lesser compared to intact phloem ($p<0.02$; Kruskal-Wallis). See also Supplementary Figure 2.

Overall fungal ASVs richness and Shannon diversity were higher in the spring season compared to the summer season ($p < 0.05$, Kruskal-Wallis, Fig. 3C). Fungal richness and diversities fluctuated more throughout the life cycle than that of the bacterial microbiome (see Supplementary Figure 3). In both seasons, the richness of

the fungal microbiome was higher in parental and teneral adults compared to larval and pupal samples, where pupae have the lowest ASVs richness. Simpson and Shannon's indexes had a similar course. In the spring, they achieved the highest value in parental adult samples, then the values decreased in larval samples and were successively restored during pupal and teneral adult's development. In summer, the highest diversity was achieved in parental samples. The diversities in larval, pupal and teneral adult samples were more or less similar. The Chao1 index had a similar course in both seasons. The parental adult and teneral adult samples had significantly higher values than larval and pupal samples. Species richness (ACE and Chao1) was the highest in intact phloem ($p<0.05$, Kruskal-Wallis).

The core microbiome of Ips typographus in respect to season

Only ASVs that accounted for at least 0.5 % of total ASVs number and were present in at least in five samples were considered part of*I. typographus* bacterial or fungal core microbiome in the respective season. Bacterial and fungal communities were significantly affected by seasonality (Fig. 3B, $p < 0.005$, permanova Jaccard and Bray-Curtis distance).

In both seasons (Fig. 2, 3A), most of the bacterial ASVs belonged to the phylum Proteobacteria (98 % in spring and 96 % in summer season), other phyla were presented only marginally (Bacteroidetes up to 2 %, Actinobacteria up to 1 %, Patescibacteria up to 0.2 and Firmicutes up to 2 %). Proteobacteria were represented by the classes Alphaproteobacteria and Gammaproteobacteria. The last one dominated in both seasons as it accounted for more than 95 % of bacterial ASVs. Gammaproteobacteria were further represented mostly by the orders Enterobacteriales and Xanthomonadales in summer and additionally with Pseudomonadales (represented by *Pseudomonas bohemica*) in the spring season. The family Enterobacteriaceae largely dominated in both seasons and was mostly represented by species of *Erwinia* (around 70 % of the total bacterial reads) and *Serratia* (6.4 % of reads in spring and 2 % in summer). *Pseudoxanthomonas*(Xanthomonadales, Xanthomonadaceae) was the second most abundant genus in spring (5.5 %) and summer season (19.2%). Bacterial spring community further differed from the summer by presence of the genus*Stenotrophomonas* (Xanthomonadales) and by almost absence of the genus *Taibaiella* (Chitinophagales) and the family Lachnospiraceae (Clostridiales).

Ascomycetous fungi dominated in both seasons (Fig. 2, 3A). Saccharomycetes represented 81 % of diversity in the spring and 91 % in the summer season. The second most common class was Sordariomycetes which took almost the rest of the reads. The taxonomic representation was strongly biased toward several species that largely dominated the samples. In both seasons, the most abundant yeasts were*Wickerhamomyces bisporus* , *Kuraishia molischiana* and*Nakazawaea ambrosiae* ; however, their proportions differ between seasons. Spring season was characterized by high incidence of yeasts*Wickerhamomyces bisporus* and *Yamadazyma* species (Saccharomycetales) and filamentous fungi *Endoconidiophora polonica* and *Graphium fimbriasporum* (both Microascales). The proportion of these fungi largely decreases in the summer season. The proportion of the yeasts *Kuraishia molischiana* and*Nakazawaea ambrosiae* increased as the proportion of *W. bisporus* decreased. A similar course was found for *E. polonica*that was almost absent in the summer seasons and was replaced by*O. bicolor* (Ophiostomatales).

The microbiome of Ips typographus throughout its life cycle

Bacterial and fungal communities significantly change throughout the life cycle of *I. typographus* ($p=0.001$). We found significant differences between the individual beetle's developmental stages; however only part of them was kept between seasons. In both seasons, the fungal microbiome of parental adults significantly differs from pupal samples ($p<0.04$ in Bray-Curtis permanova test) and pupal samples differ from teneral adult samples ($p<0.02$ in Jaccard permanova test). Ancom analysis shows that in both seasons larval and pupal samples differ from parental and teneral adults by higher proportion of ASVs belonging to yeasts *Kuraishia molischiana* and*Nakazawaea ambrosiae* and almost absence of filamentous fungi*Ophiostoma brunneolum* and *Morchella importuna* . Pupae and parental adults differ from larvae and teneral adults by lower abundance of ASV belonging to yeast *Wickerhamomyces bisporus* . In the summer season, yeast *Saccharomycopsis lassenensis* and ASV belonging to closely undefined family Enterobacteriaceae was found to be specific for

parental adult samples. We are limited by a low number of parental adult samples in the spring season; however, our data suggest similar findings. In the summer season, we also analyzed the microbial community of intact and infested phloem. Intact phloem hosted much higher microbial diversity than infested phloem (Supplementary Figure 2 and 3), which was largely dominated by *Ophiostoma bicolor* , a filamentous fungus that is known to be vectored by *I. typographus* , and bacteria belonging to the genus *Pseudoxanthomonas* . All taxa of the gut microbiome, except of *E. polonica* and *O. bicolor*were presented also in uninfested phloem.

RNA metabarcode

The total number of reads processed was 848,460,028. The final number of contigs obtained was 457,282 totaling 301,840,624 bp with N50 of 711 bp. The number of ORFs identified reached 411,387. Results obtained from metatranscriptome analysis were in accordance with the results from DNA metabarcode analysis. In the Bacteria domain, the phylum Gammaproteobacteria dominated (48% of reads, Fig. 4); however, not so strongly as in DNA metabarcode analysis and other classes, mainly Betaproteobacteria (21% of reads) and Actinomycetia (6.6% of reads), were also abundant (Fig. 4). The most abundant bacterial genera revealed by DNA metabarcode were also detected in RNA metabarcode, especially genus *Erwinia* , which belongs among the most abundant genera with almost 5% of reads. Another highly represented genera were*Klebsiella* and *Enterobacter* (both belonging to Enterobacteriaceae) (Fig. 4).

Although RNA was isolated from the insect's gut, 91% of eukaryotic reads belong to Arthropoda. The second most abundant eukaryotic phylum was Nematoda with 1.9% of reads. Fungal domain took only 0.35% of reads which almost all belong to Ascomycota. Similarly to DNA metabarcode analysis, the most abundant fungi in the metatranscriptome dataset were yeasts from the class Saccharomycetes, followed by Sordariomycetes (Fig. 4). Other presented classes include Pezizomycetes, Eurotiomycetes, Leotiomycetes, Lecanoromycetes and Orbilliomycetes. However, these classes have minor representation.

Transmission electron microscopy (TEM)

In some insect specific structures for vertical transport of endosymbionts were formed during evolution (e.g. Xue et al. 2014, Matsuura et al. 2018). It is not known whether *Ips typographus*gut microbiome is transmitted vertically or is acquired horizontally from the environment. We used TEM for direct observation of *Ips typographus* gut in purpose to examine possible formation of biofilm on intestinal wall. We examined all three parts of the beetle's gut (foregut, midgut, and hindgut); however, we did not find any sign of polymicrobial biofilm formation on the beetle's tissues. We found bacterial and fungal cells as a part of chyme together with clean epithelia and microvilli without any signs of formed residual biofilms (Fig. 5).

DISCUSSION

Ips typographus is a serious spruce pest that largely impacts the European landscape. Albeit known importance of microorganisms on bark beetle fitness, sparse information is available about the microorganismal community associated with *I. typographus* (Chakraborty et al. 2020a, 2020b). Our study is the first that describes the fungal and bacterial intestinal microbiome of the spruce bark beetle *I. typographus* throughout its whole life cycle in two subsequent generations. Combination of DNA metabarcode analyses with cultivation and subsequent molecular identification of pure cultures enables us to identify the dominant fungal and bacterial taxa mostly up to the species level. As the DNA metabarcode analysis is biased by capturing total persisting environmental DNA, even DNA from already dead cells eaten by insects (Gifford at al.,2014), their functional dominance was further confirmed by RNA metaborcode analysis. The intestinal microbiome was also observed directly in the gut by Transmission Electron Microscopy (TEM) to examine potential formation of biofilm structure.

In some herbivorous insects as well as bark beetles, gut communities are fairly specific and highly resistant to perturbation (e.g.*Dendroctonus ponderosae,* Adams et al. 2013). In other cases, the bacterial community appears to be more dynamic, mostly food derived and differing between host populations (*D. valens* , Adams et al. 2010). It is known that host plants fundamentally influence gut microbiome of insect herbivores

(Jones et al. 2019, Šigut et al. 2022) and intestinal microorganisms could be acquired horizontally from the environment (Kikuchi et al. 2007). On the other hand, specific internal structures like bacteriocytes, mycetocytes (Douglas 1998), or fat body cells (Xue, Zhou et al. 2014) allowing vertical transmission have also been described in insects.*I. typographus* belongs among bark beetles that lack any specific external morphological structures for transmission of associated microorganisms and visualization of its gut epithelium is scarce (Takov et al., 2012). Thus, it is not clear to which extent are microorganisms transferred vertically via gut and body surface or recruited de novo in a new host.

Overall microbial intestinal α-diversity was low, which is typical for bark beetle associated communities (e.g. Briones-Roblero et., 2017; Barcoto et al., 2020), with a few dominant species. At the same time, the intestinal microbiome of *I. typographus* represents a subset of species endophytically residing in uninfested spruce phloem. Lower microbial diversity in gut compared to body surface of bark beetle*Dendroctonus valens* (Lou et al., 2014) also indicates that only a subset of species from the environment enters into the insect gut. In addition, we did not observe any specific structure or biofilm formation in the beetle gut by TEM analysis. This suggests non-specific distribution of microorganisms in gut lumens and their attachment to the digested plant residuals. We thus propose that the intestinal microbiome of *I. typographus* is mostly recruited from the plant tissue and formed by environmental filtering which selects for species that can cope with the stressful environment of insect gut (Appel & Maines 1995; Douglas, 2015; Engel &Moran 2013). Future feeding experiments may answer this hypothesis. Only exceptions for above mentioned observations were mutualistic filamentous fungi *Ophiostoma bicolor* and*Endoconidiophora polonica*(Jankowiak and Hilszczanski 2005; Linnakoski et al. 2016; Repe et al. 2013). These fungi were found in very low numbers in fresh uncolonized spruce phloem; however, their proportions increase throughout life cycle and finally they dominate in phloem adjected to bark beetles' galleries at the end of *I. typographus* development. These fungi create sticky conidia as an adaptation for transmission on beetles' body surface (Harrington, 2005) and our data support vertical transmission of these fungi into a new host.

The cultivation technic captured only around 11% of fungal and 9% of bacterial species identified by DNA metabarcoding as many species are hard or still impossible to cultivate (Six, 2003; Lou e al., 2014; Hiergeist et al., 2015; Wang et al., 2020). However, these species belong to the dominantly present species in DNA metabarcode analysis. We were able to identify functionally active microorganisms from RNA metabarcode analysis up to genera level in Bacteria and mostly to family level in Fungi. Functional analysis revealed similar dominant taxa; however, the proportions of some rare bacterial and fungal classes in DNA metabarcode analysis were increased, see below.

Fungal microbiome was dominated by yeasts (Ascomycota: Saccharomycetales), which indicates their important function in *I. typographus* gut. The most dominant yeasts are *Wickerhamomyces bisporus, Nakazawaea ambrosiae, Kuraishia molischiana, Ogataea ramenticola, Cyberlindnera, Yamadazyma scolyti* and *Meyerozyma guilliermondii. Meyerozyma guilliermondii* was captured by us only from cultivation and it was also recorded in the microbiome of ambrosia beetle *Platypus koryoensis* (Yun et al. 2015), where also its enzymatic capability of plant tissue degradation was detected. *Wickerhamomyces bisporus* was previously found in association with*I. typographus* (Giordano et al. 2012) and is also the dominant yeast species found on phoretic mites of the same beetle species (Linnakoski et al., 2021). Species of *Wickerhamomyces* have been also reported from galleries and guts of wood-boring insects (Hui et al. 2013; Ninomiya et al. 2013), indicating their common association with beetles. The importance of yeasts in bark beetles' ecosystem was proposed earlier based on the cultivation technic (Beck, 1922; Siemaszko, 1929; Shifrine & Phaff, 1956) and also emphasized in recent molecular studies (Chakraborty et al. 2020, Ibarra-Juarez et al., 2020). Some gut yeasts can convert host tree defensive chemicals to beetle pheromones; however, the insect is not dependent upon them for this function (Hunt and Borden 1990). Yeasts provide a variety of benefits in several insect systems (Ganter, 2006; Rohlfs & Kurschner, 2010). Nevertheless, the roles yeasts play in bark beetle systems remain unclear (Six, 2013). The second most abundant order was Sordariomycetes, which was mainly represented by symbiotic *Ophiostoma bicolor* and*Endoconodiophora polonica*(Jankowiak & Hilszczanski 2005, Linnakoski et al., 2016; Repe et al., 2013). Their abundance in the gut microbiome gradually increases throughout *I. typographus* life cycle. This may be due to the fact that their frequency also increases over

time in infested phloem, which in turn affects the composition of the intestinal biota. Another filamentous fungi, *Morchella importuna* (Pezizomycetes), was also significantly more abundant at the end of the beetle development. The significance of Pezizomycetes was further highlighted in RNA metabarcode analysis in which this class took 2.9 % of total fungal reads. That may also point to succession in beetle's galleries which are at first dominated by yeasts which are only after some time accompanied by filamentous fungi.

Bacterial class Gammaproteobacteria was found to dominate the bacterial microbiome of fungus growing insects (Barcoto et al., 2020). This predominance was also described in bark beetles (Hernandez-Garcia et al., 2018; Chakraborty et al., 2020) and confirmed in the present study. However, our functional analysis highlighted the importance of other bacterial classes: Betaproteobacteria (22% of reads), Actinomycetia (8% of reads) and Alphaproteobacteria (7% of reads). Interestingly, some of the most active microbes (higher abundance in metatranscriptomic samples) are not among the most abundant taxa identified in the metabarcoding analyses. For instance, *Klebsiella* ,*Enterobacter* , and *Phyllobacterium* , have been found as the three most active genera in *I . typographus.* It has been suggested that *Klebsiella* spp. may fix nitrogen within other bark beetle species and insects (Yaman et al., 2010). *Enterobacter*species have been isolated from other bark beetles, such as the great spruce bark beetle (*Dendroctonus micans*) (Morales-Jiménez et al., 2012). Finally, as far as we know, there is not any report that suggests the presence nor any function of *Phyllobacterium* spp. within bark beetles; however, it is a common plant endophytic genus, which harbors strains with the ability to promote the growth of spruce trees (Anand et al., 2006). The activity of these microbes should be further investigated to understand their roles within the *I. typographus*holobiont. The microbiome was dominated by order Enterobacteriales, especially by species *Erwinia typographi,* which is common in intestinal communities as it is able to grow in almost anaerobic conditions in the gut and is resistant against high concentrations of monoterpene myrcene (Skrodenytė-Arbačiauskienė et al., 2012). Other abundant taxa were *Pseudomonas bohemica* (Pseudomonadales) and Pseudoxanthomonas (Pseudoxanthomonadales). These genera were previously isolated from *I. typographus* and seem to be also common associates of other bark beetles (Briones-Roblero et., 2017; García-Fraile, 2018; Hernandez-Garcia et al., 2018; Saati-Santamaría et al., 2018; Barcoto et al., 2020; Chakraborty et al., 2020; Peral-Aranega et al., 2020; Saati-Santamaría et al., 2021). *Micrococcus luteus*isolated by us from *I. typographus* gut, was previously isolated from oral secretion of bark beetle *Dendroctonus rufipenis* where it helps with inhibition of growth of antagonistic filamentous fungi (Cardoza et al., 2006). Overall, inferred functions of these bacteria are degradation of lignocellulose, detoxification of plant secondary metabolites, protection against fungal pathogens and metabolism of diverse nutrients (García-Fraile et al., 2018; Ibarra-Juarez et al., 2020; Barcoto et al., 2020).

We found significant change in microbial communities throughout the life cycle of *I. typographus* . Some fungal species are more abundant in certain developmental stages, e.g. *Kuraishia molischiana* and*Nakazawaea ambrosiae* in larval and pupal stages or*Saccharomycopsis lassenensis* in parental adult stage. Developmental stages also differ in the number of fungal ASVs, concretely we observed a drop in fungal ASVs number in the larval and pupal life stages compared to parental and teneral stages. This phenomenon was also observed by Lou et al. (2014) in yeast communities of bark beetle *Dendroctonus valens* and by González-Serrano et al. (2020) in bacterial communities of moth *Brithys crini* . The first drop in fungal diversity in larval stage may be caused by loss of species transmitted in guts of parental adults from the previous hosts and the second drop in pupal stage may be linked with metamorphosis and construction of a new intestinal system (González-Serrano et al., 2020).

Season has a statistically significant effect on microbial communities. Communities differ in proportion of dominantly associated microbes rather than in change in species composition. Similarly to Jankowiak and Hilszczanski (2005), Louca et al. (2016), and Bang-Andreasen et al. (2020), we suppose that environmental conditions shape the composition and structure of microbiomes. Filamentous fungi associated with*Ips typographus* have similar capability in detoxification of plant secondary metabolites and thus have interchangeable functions (Zhao et al., 2019) and similar situation can take place in the yeast and bacterial community, when individual species have interchangeable functions and their proportion are driven by environmental conditions.

To conclude, this study is the first to show the composition of the core gut microbiome of spruce pest *I. typographus* based on cultivation and DNA/RNA metabarcode sequencing. We found it is changing throughout the life cycle of the beetle with the drop in fungal diversity in larval and pupal stages. Saccharomycetous yeasts and bacteria from the family Enterobacteriaceae dominate the intestinal microbiome. We also detected changes in proportions of the dominant taxa in respect to season. We propose that these species have interchangeable functions in bark beetles' habitat and their proportions are driven by environmental conditions. Future studies may focus on functional analyses of the core microbiome of *I. typographus* to address our hypothesis.

Acknowledgements

This research was funded by Czech Science Foundation (GAČR), grant number 19-09072S. We are grateful to Dr. M. Hiliš (Faculty of Sciences, Charles University, Prague) for technical assistance during TEM analyses. E.P-A, P.G-F and Z.S-S acknowledge funds from the Regional Government of Castilla y León, Escalera de Excelencia CLU-2018-04, and co-funded by the P.O. FEDER of Castilla y León 2014 to 2020. E.P-A received a grant from the Regional Government of Castilla y León. Z.S-S acknowledge a grant co-funded by the European NextGenerationEU, Spanish "Plan de Recuperación, Transformación y Resiliencia," Spanish Ministry of Universities, and the University of Salamanca ("Ayudas para la recualificación del sistema universitario español 2021-2022").

References

Adams, A. S., Adams, S. M., Currie, C. R., Gillette, N. E., & Raffa, K. F. (2010). Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae).*Environmental Entomology* , *39* (2), 406–414. doi: 10.1603/EN09221

Adams, A. S., Aylward, F. O., Adams, S. M., Erbilgin, N., Aukema, B. H., Currie, C. R., ... Raffa, K. F. (2013). Mountain pine beetles colonizing historical and naïve host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology* ,*79* (11), 3468–3475. doi: 10.1128/AEM.00068-13

Adams, A. S., Six, D. L., Adams, S. M., & Holben, W. E. (2008). In vitro interactions between yeasts and bacteria and the fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*).*Microbial Ecology* , *56* (3), 460–466. doi: 10.1007/s00248-008-9364-0

Anand, R., Paul, L., & Chanway, C. (2007). Research on Endophytic Bacteria: Recent Advances with Forest Trees. *Microbial Root Endophytes* , *9* , 89–106. doi: 10.1007/3-540-33526-9_6

Appel, H. M., & Maines, L. W. (1995). The influence of host plant on gut conditions of gypsy moth (Lymantria dispar) caterpillars. Journal of Insect Physiology, 41(3), 241–246. doi: 10.1016/0022-1910(94)00106-Q

Ayres, M. P., Wilkens, R. T., Ruel, J. J., Lombardero, M. J., & Vallery, E. (2000). Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology* , *81* (8), 2198. doi: 10.2307/177108

Bang-Andreasen, T., Anwar, M. Z., Lanzén, A., Kjøller, R., Rønn, R., Ekelund, F., & Jacobsen, C. S. (2019). Total RNA sequencing reveals multilevel microbial community changes and functional responses to wood ash application in agricultural and forest soil. *FEMS Microbiology Ecology* , *96* (3), 1–13. doi: 10.1093/femsec/fiaa016

Barcoto, M. O., Carlos-Shanley, C., Fan, H., Ferro, M., Nagamoto, N. S., Bacci, M., ... Rodrigues, A. (2020). Fungus-growing insects host a distinctive microbiota apparently adapted to the fungiculture environment. Scientific Reports, 10(1), 1–13. doi: 10.1038/s41598-020-68448-7

Beck, O. (1922). Eine neue Endomyces-Art, Endomyces bisporus. Annales Mycologici 20: 219-227

Bentz, B. J., & Six, D. L. (2006). Ergosterol content of fungi associated with Dendroctonus ponderosae and Dendroctonus rufipennis (Coleoptera: Curculionidae, Scolytinae). *Annals of the Entomological Society of America* , *99* (2), 189–194. doi: 10.1603/0013-8746(2006)099[0189:ECOFAW]2.0.CO;2

Bentz, B. J., Jönsson, A. M., Schroeder, M., Weed, A., Wilcke, R. A. I., Larsson, K., ... Parker, J. H. (2019). Adaptive traits of bark and ambrosia beetle-associated fungi. *PLoS ONE* , *41* (1), 1–14. doi: 10.1007/978-1-62703-712-9

Berasategui, A., Axelsson, K., Nordlander, G., Schmidt, A., Borg-Karlson, A. K., Gershenzon, J., ... Kaltenpoth, M. (2016). The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles. *Molecular Ecology* ,*25* (16), 4014–4031. doi: 10.1111/mec.13702

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., ... Schloter, M. (2020). Correction to: Microbiome definition re-visited: old concepts and new challenges.*Microbiome* , *8* (1), 1–22. doi: 10.1186/s40168-020-00905-x

Biedermann, P. H. W., Müller, J., Grégoire, J. C., Gruppe, A., Hagge, J., Hammerbacher, A., ... Bässler, C. (2019). Bark Beetle Population Dynamics in the Anthropocene: Challenges and Solutions. *Trends in Ecology and Evolution* , *34* (10), 914–924. doi: 10.1016/j.tree.2019.06.002

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., ... Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* , *6* (1), 1–17. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* , *37* (8), 852–857. doi: 10.1038/s41587-019-0209-9

Briones-Roblero, C. I., Rodríguez-Díaz, R., Santiago-Cruz, J. A., Zúñiga, G., & Rivera-Orduña, F. N. (2017). Degradation capacities of bacteria and yeasts isolated from the gut of Dendroctonus rhizophagus (Curculionidae: Scolytinae). *Folia Microbiologica* , *62* (1), 1–9. doi: 10.1007/s12223-016-0469-4

Buchfink, B., Xie, C., & Huson, D. H. (2014). Fast and sensitive protein alignment using DIAMOND. *Nature Methods* , *12* (1), 59–60. doi: 10.1038/nmeth.3176

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* , *13* (7), 581–583. doi: 10.1038/nmeth.3869

Cardoza, Y. J., Klepzig, K. D., & Raffa, K. F. (2006). Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi.*Ecological Entomology* , *31* (6), 636–645. doi: 10.1111/j.1365-2311.2006.00829.x

Carey, H. V., Walters, W. A., & Knight, R. (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* , *304* (1). doi: 10.1152/ajpregu.00387.2012

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., ... Karp, P. D. (2020). The MetaCyc database of metabolic pathways and enzymes-a 2019 update. Nucleic Acids Research, 48(D1), D455–D453. doi: 10.1093/nar/gkz862

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2016). GenBank. *Nucleic Acids Research* , *44* (D1), D67–D72. doi: 10.1093/nar/gkv1276

Conord, C., Despres, L., Vallier, A., Balmand, S., Miquel, C., Zundel, S., ... Heddi, A. (2008). Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: Additional evidence of symbiont replacement in the Dryophthoridae family. *Molecular Biology and Evolution* , *25* (5), 859–868. doi: 10.1093/molbev/msn027

Doug Hyatt, Gwo-Liang Chen, Philip F LoCascio, Miriam L Land, , Frank W Larimer, L. J. H. (2010). Integrated nr database in protein annotation system and its localization. *Nature Communications* , *6* (1), 1–8. doi: /10.1016/B978-0-12-407863-5.00023-X

Douglas, A. E. (1998). Aphids and their symbiotic bacteria Buchnera. Annual Reviews in Entomology, 43, 17–37.

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. *Annual Review of Entomology* ,*60* (1), 17–34. doi: 10.1146/annurev-ento-010814-020822.Multiorganismal

Eddy, S. R. (2009). A new generation of homology search tools based on probabilistic inference. Genome Informatics. International Conference on Genome Informatics, 23(1), 205–211. doi: 10.1142/9781848165632_-0019

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Reviews* ,*37* (5), 699–735. doi: 10.1111/1574-6976.12025

Fabryová, A., Kostovčík, M., Díez-Méndez, A., Jiménez-Gómez, A., Celador-Lera, L., Saati-Santamaría, Z., ... García-Fraile, P. (2018). On the bright side of a forest pest-the metabolic potential of bark beetles' bacterial associates. *Science of the Total Environment* , *619 –620* , 9–17. doi: 10.1016/j.scitotenv.2017.11.074

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., ... Bateman, A. (2016). The Pfam protein families database: Towards a more sustainable future. Nucleic Acids Research, 44(D1), D279–D285. doi: 10.1093/nar/gkv1344

François LIEUTIERa, b*, Annie YARTb, Hui YEc, Daniel SAUVARDb, V. Gallois. (2007). Variations in growth and virulence of*Leptographium wingfieldii* Morelet, a fungus associated with the bark beetle *Tomicus piniperda* L. *Annals of Forest Science* ,*64* , 219–228. doi: 10.1051/forest

Ganter, P. F. (2006). Yeast and invertebrate associations.*Biodiversity and Ecophysiology of Yeasts* , 303–370. doi: 10.1007/3-540-30985-3_14

García-Fraile, P. (2018). Roles of bacteria in the bark beetle holobiont – how do they shape this forest pest? *Annals of Applied Biology* ,*172* (2), 111–125. doi: 10.1111/aab.12406

Gifford, S. M., Sharma, S., Booth, M., & Moran, M. A. (2013). Expression patterns reveal niche diversification in a marine microbial assemblage. *ISME Journal* , *7* (2), 281–298. doi: 10.1038/ismej.2012.96

Giordano, L., Garbelotto, M., Nicolotti, G., & Gonthier, P. (2013). Characterization of fungal communities associated with the bark beetle*Ips typographus* varies depending on detection method, location, and beetle population levels. *Mycological Progress* , 12(1), 127–140. doi: 10.1007/s11557-012-0822-1

Giron, D., Dedeine, F., Dubreuil, G., Huguet, E., Mouton, L., Outreman, Y., ... Simon, J. C. (2017). Influence of microbial symbionts on plant–insect interactions. In *Advances in Botanical Research*(Vol. 81). Elsevier Ltd. doi: 10.1016/bs.abr.2016.09.007

González-Serrano, F., Pérez-Cobas, A. E., Rosas, T., Baixeras, J., Latorre, A., & Moya, A. (2020). The gut microbiota composition of the moth *Brithys crini* reflects insect metamorphosis. *Microbial Ecology* , *79* (4), 960–970. doi: 10.1007/s00248-019-01460-1

Grosmann, H. (1930). Beiträge zur Kenntnis der Lebensgemeinschaft zwischen Borkenkäfern und Pilzen. *Zeitschrift für Parasitenkunde*3 (1): 56-102.

Hammerbacher, A., Schmidt, A., Wadke, N., Wright, L. P., Schneider, B., Bohlmann, J., ... Paetz, C. (2013). A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Physiology* , *162* (3), 1324–1336. doi: 10.1104/pp.113.218610

Harrington, T. C. (2005). Ecology and evolution of mycophagous bark beetles and their fungal partners. Insect-Fungal Associations: Ecology and Evolution, (Norris 1979), 257–291.

Hayat, M. (2000). Principles and techniques of electron microscopy: biological applications. 4th edn. 543pp. Cambridge: Cambridge University Press. £65 (hardback). Annals of Botany, 87(4), 546–548. doi: 10.1006/anbo.2001.1367

Hernández-García, M., Pérez-Viso, B., Carmen Turrientes, M., Díaz-Agero, C., López-Fresneña, N., Bonten, M., ... Cantón, R. (2018). Characterization of carbapenemase-producing Enterobacteriaceae from colonized patients in a university hospital in Madrid, Spain, during the R-GNOSIS project depicts increased clonal diversity over time with maintenance of high-risk clones. *Journal of Antimicrobial Chemotherapy* , *73* (11), 3039–3043. doi: 10.1093/jac/dky284

Hicke, J. A., Meddens, A. J. H., & Kolden, C. A. (2016). Recent tree mortality in the Western United States from bark beetles and forest fires. Forest Science, 62(2), 141–153. doi: 10.5849/forsci.15-086

Hicke, J. A., Meddens, A. J. H., Allen, C. D., & Kolden, C. A. (2013). Carbon stocks of trees killed by bark beetles and wildfire in the western United States. *Environmental Research Letters* ,*8* (3). doi: 10.1088/1748-9326/8/3/035032

Hiergeist, A., Gläsner, J., Reischl, U., & Gessner, A. (2015). Analyses of intestinal microbiota: culture versus sequencing. ILAR Journal, 56(2), 228–240. doi: 10.1093/ilar/ilv017

Hlásny, T., König, L., Krokene, P., Lindner, M., Montagné-Huck, C., Müller, J., ... Seidl, R. (2021). Bark beetle outbreaks in europe: state of knowledge and ways forward for management. *Current Forestry Reports* , *7* (3), 138–165. doi: 10.1007/s40725-021-00142-x

Hoang, K. L., Morran, L. T., Gerardo, N. M., Lou, Q. Z., Lu, M., Sun, J. H., ... Zúñiga, G. (2020). Yeasts in natural ecosystems: Diversity.*PLoS ONE* , *10* (2), 1–499. doi: 10.1371/journal.pone.0175470

Hofstetter, R. W., Dinkins-Bookwalter, J., Davis, T. S., & Klepzig, K. D. (2015). Symbiotic associations of bark beetles. In bark beetles: biology and ecology of native and invasive species. Elsevier Inc. doi: 10.1016/B978-0-12-417156-5.00006-X

Hou, X. Q., Zhang, D. D., Powell, D., Wang, H. L., Andersson, M. N., & Löfstedt, C. (2022). Ionotropic receptors in the turnip moth Agrotis segetum respond to repellent medium-chain fatty acids. *BMC Biology* , *20* (1). doi: 10.1186/s12915-022-01235-0

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., ... Bork, P. (2016). EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research* ,*44* (D1), D286–D293. doi: 10.1093/nar/gkv1248

Hui, F. L., Chen, L., Chu, X. Y., Niu, Q. H., & Ke, T. (2013). *Wickerhamomyces mori* sp. nov., an anamorphic yeast species found in the guts of wood-boring insect larvae. *International Journal of Systematic and Evolutionary Microbiology, 63* (PART3), 1174–1178. doi: 10.1099/ijs.0.048637-0

Hunt, D. W. A., & Borden, J. H. (1990). Conversion of verbenols to verbenone by yeasts isolated from Dendroctonus ponderosae (Coleoptera: Scolytidae). *Journal of Chemical Ecology* , *16* (4), 1385–1397. doi: 10.1007/BF01021034

Chakraborty, A., Ashraf, M. Z., Modlinger, R., Synek, J., Schlyter, F., & Roy, A. (2020). Unravelling the gut bacteriome of *Ips*(Coleoptera: Curculionidae: Scolytinae): identifying core bacterial assemblage and their ecological relevance. *Scientific Reports* ,*10* (1), 1–17. doi: 10.1038/s41598-020-75203-5

Chakraborty, A., Modlinger, R., Ashraf, M. Z., Synek, J., Schlyter, F., & Roy, A. (2020). Core mycobiome and their ecological relevance in the gut of five *Ips* bark beetles (Coleoptera: Curculionidae: Scolytinae). *Frontiers in Microbiology* , *11* (September), 1–16. doi: 10.3389/fmicb.2020.568853

Chelius, M. K., & Triplett, E. W. (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microbial Ecology, 41(3), 252–263. doi: 10.1007/s002480000087

Ibarra-Juarez, L. A., Burton, M. A. J., Biedermann, P. H. W., Cruz, L., Desgarennes, D., Ibarra-Laclette, E., ... Lamelas, A. (2020). Evidence for succession and putative metabolic roles of fungi and bacteria in the farming mutualism of the ambrosia beetle *Xyleborus affinis* . *MSystems* , *5* (5). doi: 10.1128/msystems.00541-20

Jakoby, O., Lischke, H., & Wermelinger, B. (2019). Climate change alters elevational phenology patterns of the European spruce bark beetle (*Ips typographus*). *Global Change Biology* , *25* (12), 4048–4063. doi: 10.1111/gcb.14766

Jankowiak, R. (2005). Fungi associated with *Ips typographus* on*Picea abies* in southern Poland and their succession into the phloem and sapwood of beetle-infested trees and logs. *Forest Pathology* , *35* (1), 37–55. doi: 10.1111/j.1439-0329.2004.00395.x

Jankowiak, R., Kacprzyk, M., & Młynarczyk, M. (2009). Diversity of ophiostomatoid fungi associated with bark beetles (Coleoptera: Scolytidae) colonizing branches of Norway spruce (picea abies) in southern Poland. *Biologia* , *64* (6), 1170–1177. doi: 10.2478/s11756-009-0188-2

Jones, A. G., Mason, C. J., Felton, G. W., & Hoover, K. (2019). Host plant and population source drive diversity of microbial gut communities in two polyphagous insects. *Scientific Reports* , *9* (1), 1–11. doi: 10.1038/s41598-019-39163-9

Kikuchi, Y., Hosokawa, T., & Fukatsu, T. (2007). Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation.*Applied and environmental microbiology* , *73(13*), 4308-4316.

Kikuchi, Y. (2009). Endosymbiotic bacteria in insects: Their diversity and culturability. *Microbes and Environments* , *24* (3), 195–204. doi: 10.1264/jsme2.ME09140S

Kirisits, T. (2004). Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. *Bark and Wood Boring Insects in Living Trees in Europe.* , 181–235. doi: 10.1007/s13398-014-0173-7.2

Kirkendall, L. R., & Faccoli, M. (2010). Bark beetles and pinhole borers (Curculionidae, Scolytinae, Platypodinae) alien to Europe.*ZooKeys* , *56* (SPEC. ISSUE), 227–251. doi: 10.3897/zookeys.56.529

Kirkendall, L. R., Biedermann, P. H. W., & Jordal, B. H. (2015). Evolution and diversity of bark and ambrosia beetles. In *Bark Beetles: Biology and Ecology of Native and Invasive Species* . doi: 10.1016/B978-0-12-417156-5.00003-4

Kulakowski, D. (2016). Managing bark beetle outbreaks (*Ips typographus* , *Dendroctonus* spp.) in conservation areas in the 21st century. *Forest Research Papers* , *77* (4), 352–357. doi: 10.1515/frp-2016-0036

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods, 9(4), 357–359. doi: 10.1038/nmeth.1923

Laslett, D., & Canback, B. (2004). ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. *Nucleic Acids Research* , *32* (1), 11–16. doi: 10.1093/nar/gkh152

Leufvén, A., Bergström, G., & Falsen, E. (1984). Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus* . *Journal of Chemical Ecology* ,*10* (9), 1349–1361. doi: 10.1007/BF00988116

Li, D., Liu, C. M., Luo, R., Sadakane, K., & Lam, T. W. (2015). MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.*Bioinformatics* , *31* (10), 1674–1676. doi: 10.1093/bioinformatics/btv033

Li, H., Young, S. E., Poulsen, M., & Currie, C. R. (2021). Symbiont-Mediated Digestion of Plant Biomass in Fungus-Farming Insects.*Annual Review of Entomology* , *66* , 297–316. doi: 10.1146/annurev-ento-040920-061140

Linnakoski, R., Jankowiak, R., Villari, C., Kirisits, T., Solheim, H., de Beer, Z. W., & Wingfield, M. J. (2016). The Ophiostoma clavatum species complex: a newly defined group in the Ophiostomatales including three novel taxa. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* , *109* (7), 987–1018. doi: 10.1007/s10482-016-0700-y

Linnakoski, R., Lasarov, I., Veteli, P., Tikkanen, O. P., Viiri, H., Jyske, T., ... Wingfield, M. J. (2021). Filamentous fungi and yeasts associated with mites phoretic on *Ips typographus* in Eastern Finland. *Forests* , Vol. 12. doi: 10.3390/f12060743

Linnakoski, R., Mahilainen, S., Harrington, A., Vanhanen, H., Eriksson, M., Mehtatalo, L., ... Wingfield, M. J. (2016). Seasonal succession of fungi associated with *Ips typographus* beetles and their Phoretic mites in an outbreak region of Finland. *PLoS ONE ,11* (5). doi: 10.1371/journal.pone.0155622

Linnakoski, R., Wilhelm de Beer, Z. B., Niemelä, P., & Wingfield, M. J. (2012). Associations of conifer-infesting bark beetles and fungi in Fennoscandia. *Insects* , *3* (1), 200–227. doi: 10.3390/insects3010200

Linnakoski, R., Wilhelm de Beer, Z. B., Niemelä, P., & Wingfield, M. J. (2012). Associations of conifer-infesting bark beetles and fungi in Fennoscandia. *Insects* , *3* (1), 200–227. doi: 10.3390/insects3010200

López-García, D. M. and P., Jaillon, O., Massana, R., Sebastián, M., Vaqué, D., Labarre, A., ... Worden, A. Z. (2020). Review TRENDSin Microbiology Vol.10 No.1 January 2002 The molecular ecology of microbial eukaryotes unveils a hidden world. *ISME Journal* , *8* (1), 1–14. doi: 10.1186/s13059-016-0960

Lou, Q. Z., Lu, M., & Sun, J. H. (2014). Yeast diversity associated with invasive *Dendroctonus valens* killing *Pinus tabuliformis* in China using culturing and molecular methods.*Microbial Ecology* , *68* (2), 397–415. doi: 10.1007/s00248-014-0413-6

Louca, S., Jacques, S. M. S., Pires, A. P. F., Leal, J. S., Srivastava, D. S., Parfrey, L. W., ... Doebeli, M. (2017). High taxonomic variability despite stable functional structure across microbial communities. *Nature Ecology & Evolution* , *1* (1), 1–12. doi: 10.1038/s41559-016-0015

Matsuura, Y., Moriyama, M., Łukasik, P., Vanderpool, D., Tanahashi, M., Meng, X. Y., ... Fukatsu, T. (2018). Recurrent symbiont recruitment from fungal parasites in cicadas. *Proceedings of the National Academy of Sciences of the United States of America* , *115* (26), E5970–E5979. doi: 10.1073/pnas.1803245115

McCutcheon, J., & Southam, G. (2018). Advanced biofilm staining techniques for TEM and SEM in geomicrobiology: Implications for visualizing EPS architecture, mineral nucleation, and microfossil generation. *Chemical Geology* , *498* , 115–127. doi: 10.1016/j.chemgeo.2018.09.016

McMurdie PJ, Holmes S (2013). phyloseq: An R Package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE, 8* , e61217.*https://doi.org/10.1371/journal.pone.0061217*

Mezei, P., Blaženec, M., Grodzki, W., Škvarenina, J., & Jakuš, R. (2017). Influence of different forest protection strategies on spruce tree mortality during a bark beetle outbreak. *Annals of Forest Science* , *74* (4). doi: 10.1007/s13595-017-0663-9

Mikkelson, K. M., Bearup, L. A., Maxwell, R. M., Stednick, J. D., McCray, J. E., & Sharp, J. O. (2013). Bark beetle infestation impacts on nutrient cycling, water quality and interdependent hydrological effects. *Biogeochemistry* , *115* (1–3), 1–21. doi: 10.1007/s10533-013-9875-8

Miller, K. E., Inward, D. J., Gomez-Rodriguez, C., Baselga, A., & Vogler, A. P. (2019). Predicting the unpredictable: How host specific is the mycobiota of bark and ambrosia beetles? *Fungal Ecology ,42* , 100854. doi: 10.1016/j.funeco.2019.07.008

Morales-Jiménez, J., Zúñiga, G., Ramírez-Saad, H. C., & Hernández-Rodríguez, C. (2012). Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and Their Cellulolytic Activities.*Microbial Ecology* , *64* (1), 268–278. doi: 10.1007/s00248-011-9999-0

Moran, N. A. (n.d.). *Symbiosis . 16* (20), 866–871. doi: doi:10.1016/j.cub.2006.09.019

Motta, J. P., Wallace, J. L., Buret, A. G., Deraison, C., & Vergnolle, N. (2021). Gastrointestinal biofilms in

health and disease. *Nature Reviews Gastroenterology and Hepatology* , *18* (5), 314–334. doi: 10.1038/s41575-020-00397-y

Ninomiya, S., Mikata, K., Kajimura, H., & Kawasaki, H. (2013). Two novel ascomycetous yeast species, Wickerhamomyces scolytoplatypi sp. nov. and Cyberlindnera xylebori sp. nov., isolated from ambrosia beetle galleries. International Journal of Systematic and Evolutionary Microbiology, 63(PART7), 2706–2711. doi: 10.1099/ijs.0.050195-0

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro, Cunha, E., Smith, T., Stier, A., Ter Braak, C. & Weedon, J. (2022). Vegan: Community Ecology Package, <https://CRAN.R-project.org/package=vegan>.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* , *276* (5313), 734–740. doi: 10.1126/science.276.5313.734

Parfrey, L. W., Moreau, C. S., & Russell, J. A. (2018). Introduction: The host-associated microbiome: Pattern, process and function.*Molecular Ecology* , *27* (8), 1749–1765. doi: 10.1111/mec.14706

Pepori, A. L., Bettini, P. P., Comparini, C., Sarrocco, S., Bonini, A., Frascella, A., ... Santini, A. (2018). Geosmithia-Ophiostoma: a New Fungus-Fungus Association. *Microbial Ecology* , *75* (3), 632–646. doi: 10.1007/s00248-017-1062-3

Peral-Aranega, E., Saati-Santamaría, Z., Kolařik, M., Rivas, R., & García-Fraile, P. (2020). Bacteria belonging to *Pseudomonas typographi* sp. Nov. from the bark beetle *Ips typographus* have genomic potential to aid in the host ecology. *Insects* ,*11* (9), 1–22. doi: 10.3390/insects11090593

Persson, Y., Vasaitis, R., Långström, B., Öhrn, P., Ihrmark, K., & Stenlid, J. (2009). Fungi vectored by the bark beetle *Ips typographus* following hibernation under the bark of standing trees and in the forest litter. *Microbial Ecology* , *58* (3), 651–659. doi: 10.1007/s00248-009-9520-1

Redford, A. J., Bowers, R. M., Knight, R., Linhart, Y., & Fierer, N. (2010). The ecology of the phyllosphere: Geographic and phylogenetic variability in the distribution of bacteria on tree leaves.*Environmental Microbiology* , *12* (11), 2885–2893. doi: 10.1111/j.1462-2920.2010.02258.x

Repe, A., Kirisits, T., Piškur, B., De Groot, M., Kump, B., & Jurc, M. (2013). Ophiostomatoid fungi associated with three spruce-infesting bark beetles in Slovenia. *Annals of Forest Science* , *70* (7), 717–727. doi: 10.1007/s13595-013-0311-y

Repe, A., Kirisits, T., Piškur, B., De Groot, M., Kump, B., & Jurc, M. (2013). Ophiostomatoid fungi associated with three spruce-infesting bark beetles in Slovenia. *Annals of Forest Science* , *70* (7), 717–727. doi: 10.1007/s13595-013-0311-y

Risely, A. (2020). Applying the core microbiome to understand host–microbe systems. *Journal of Animal Ecology* , *89* (7), 1549–1558. doi: 10.1111/1365-2656.13229

Risely, A. (2020). Applying the core microbiome to understand host–microbe systems. *Journal of Animal Ecology* , *89* (7), 1549–1558. doi: 10.1111/1365-2656.13229

Rivera, F. N., GonzÁlez, E., GÓmez, Z., LÓpez, N., Hernández-Rodríguez, C., Berkov, A., & Zúñiga, G. (2009). Gut-associated yeast in bark beetles of the genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae). Biological Journal of the Linnean Society, 98(2), 325–342. doi: 10.1111/j.1095-8312.2009.01289.x

Rivers, A. R., Weber, K. C., Gardner, T. G., Liu, S., & Armstrong, S. D. (2018). ITSxpress: Software to rapidly trim internally transcribed spacer sequences with quality scores for marker gene analysis [version 1; peer review: 2 approved]. *F1000Research* , *7* (0). doi: 10.12688/F1000RESEARCH.15704.1

Rohlfs, M., & Kürschner, L. (2010). Saprophagous insect larvae,*Drosophila melanogaster* , profit from increased species richness in beneficial microbes. *Journal of Applied Entomology* ,*134* (8), 667–671. doi: 10.1111/j.1439-0418.2009.01458.x

Saati-Santamaría, Z., López-Mondéjar, R., Jiménez-Gómez, A., Díez-Méndez, A., Vetrovský, T., Igual, J. M., ... García-Fraile, P. (2018). Discovery of phloeophagus beetles as a source of pseudomonas strains that produce potentially new bioactive substances and description of *pseudomonas bohemica* sp. nov. *Frontiers in Microbiology* , *9* (MAY). doi: 10.3389/fmicb.2018.00913

Saati-Santamaría, Z., Peral-Aranega, E., Velázquez, E., Rivas, R., & García-Fraile, P. (2021). Phylogenomic analyses of the genus pseudomonas lead to the rearrangement of several species and the definition of new genera. Biology, 10(8). doi: 10.3390/biology10080782

Sagova-Mareckova, M., Cermak, L., Novotna, J., Plhackova, K., Forstova, J., & Kopecky, J. (2008). Innovative methods for soil DNA purification tested in soils with widely differing characteristics. *Applied and Environmental Microbiology* , *74* (9), 2902–2907. doi: 10.1128/AEM.02161-07

Sallé, A., Monclus, R., Yart, A., Garcia, J., Romary, P., & Lieutier, F. (2005). Fungal flora associated with Ips typographus: Frequency, virulence, and ability to stimulate the host defence reaction in relation to insect population levels. *Canadian Journal of Forest Research* , *35* (2), 365–373. doi: 10.1139/x04-186

Seemann T. 2013. Barrnap 0.7: rapid ribosomal RNA prediction

Shifrine, A. M., & Phaff, H. J. (1956). The association of yeasts with certain bark beetles. *Mycologia* , *48* (1), 41–55.

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* , *27* (6), 863–864. doi: 10.1093/bioinformatics/btr026

Siemaszko, W. (1939). Zepoly grzybow towarzyszacych kornikom polskim [Fungi associated with barkbeetles in Poland]. Pianta polonica, 7, I-54

Six, D. L. (2003). Bark beetle-fungus symbiosis. In K. Bourtzis & T. A. Miller (Eds.), *Insect Symbiosis* (pp. 99-116). CRC Press.

Six, D. L. (2012). Ecological and evolutionary determinants of bark beetle - Fungus symbioses. *Insects* , *3* (1), 339–366. doi: 10.3390/insects3010339

Six, D. L. (2013). *The Bark Beetle Holobiont: Why Microbes Matter* . 989–1002. doi: 10.1007/s10886-013-0318-8

Six, D. L. (2020). Niche construction theory can link bark beetle-fungus symbiosis type and colonization behavior to large scale causal chain-effects. *Current Opinion in Insect Science* , *39* , 27–34. doi: 10.1016/j.cois.2019.12.005

Skrodenytee-Arbaciauskiene, V., Radziute, S., Stunzenas, V., & Buda, V. (2012). Erwinia typographi sp. nov., isolated from bark beetle (*Ips typographus*) gut. *International Journal of Systematic and Evolutionary Microbiology* , *62* (4), 942–948. doi: 10.1099/ijs.0.030304-0

Solheim, H. (1992). Fungal succession in sapwood of Norway spruce infested by the bark beetle Ips typographus. *European Journal of Forest Pathology* , *22* (3), 136–148. doi: 10.1111/j.1439-0329.1992.tb01440.x

Strid, Y. M. E. (2012). *Bark beetles facilitate the establishment of wood decay fungi* .

Sun, B. F., Xiao, J. H., He, S. M., Liu, L., Murphy, R. W., & Huang, D. W. (2013). Multiple ancient horizontal gene transfers and duplications in lepidopteran species. *Insect Molecular Biology* , *22* (1), 72–87. doi: 10.1111/imb.12004

Šigut, M., Pyszko, P., Šigutová, H., Višňovská, D., Kostovčík, M., Kotásková, N., ... Drozd, P. (2022). Fungi are more transient than bacteria in caterpillar gut microbiomes. Scientific Reports, 12(1), 1–12. doi: 10.1038/s41598-022-19855-5

Takov, D. I., Doychev, D. D., Linde, A., Atanasova Draganova, S., & Kirilova Pilarska, D. (2012). Pathogens of bark beetles (Curculionidae: Scolytinae) and other beetles in Bulgaria. *Biologia* ,*67* (5), 966–972. doi: 10.2478/s11756-012-0086-x

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* , *7* (7). doi: 10.1371/journal.pone.0040863

Van Moll, L., De Smet, J., Cos, P., & Van Campenhout, L. (2021). Microbial symbionts of insects as a source of new antimicrobials: a review. *Critical Reviews in Microbiology* , *47* (5), 562–579. doi: 10.1080/1040841X.2021.1907302

Vega, F. E., Hofstetter, R. W. (2015) Bark beetles: biology and ecology of native and invasive species. Elsevier Academic Press ISBN: 9780124171565

Veselská, T., Skelton, J., Kostovčík, M., Hulcr, J., Baldrian, P., Chudíčková, M., ... Kolařík, M. (2019). Adaptive traits of bark and ambrosia beetle-associated fungi. *Fungal Ecology* ,*41* (July), 165–176. doi: 10.1016/j.funeco.2019.06.005

Višňovska, D., Pyszko, P., Šigut, M., Kostovčik, M., Kolařik, M., Kotaskova, N., & Drozd, P. (2020). Caterpillar gut and host plant phylloplane mycobiomes differ: A new perspective on fungal involvement in insect guts. *FEMS Microbiology Ecology* , *96* (9). doi: 10.1093/femsec/fiaa116

von Dohlen, C. D., Spaulding, U., Patch, K. B., Weglarz, K. M., Foottit, R. G., Havill, N. P., & Burke, G. R. (2017). Dynamic acquisition and loss of dual-obligate symbionts in the plant-sap-feeding adelgidae (Hemiptera: Sternorrhyncha: Aphidoidea). *Frontiers in Microbiology* , *8* (JUN), 1–15. doi: 10.3389/fmicb.2017.01037

Wang, F., Li, M., Huang, L., & Zhang, X. H. (2021). Cultivation of uncultured marine microorganisms. *Marine Life Science and Technology* , *3* (2), 117–120. doi: 10.1007/s42995-021-00093-z

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* ,*73* (16), 5261–5267. doi: 10.1128/AEM.00062-07

Wermelinger, B. (2004). Ecology and management of the spruce bark beetle Ips typographus — a review of recent research. *Forest Ecology and Management 202* , 67–82. doi: 10.1016/j.foreco.2004.07.018

Xu, L., Shi, Z., Wang, B., Lu, M., & Sun, J. (2016). Pine defensive monoterpene α-pinene influences the feeding behavior of*Dendroctonus valens* and its gut bacterial community structure.*International Journal of Molecular Sciences* , *17* (11). doi: 10.3390/ijms17111734

Xue, J., Zhou, X., Zhang, C. X., Yu, L. L., Fan, H. W., Wang, Z., ... Cheng, J. A. (2014). Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation. *Genome Biology* , *15* (12). doi: 10.1186/s13059-014-0521-0

Yaman, M., Ertürk, Ö., & Aslan, I. (2010). Isolation of some pathogenic bacteria from the great spruce bark beetle, *Dendroctonus micans*and its specific predator, *Rhizophagus grandis* . *Folia Microbiologica* , *55* (1), 35–38. doi: 10.1007/s12223-010-0006-9

Yamaoka, Y., Wingfield, M. J., Takahashi, I., & Solheim, H. (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Japan. *Mycological Research* ,*101* (10), 1215–1227. doi: 10.1017/S0953756297003924

Ye, Y., & Doak, T. G. (2009). A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Computational Biology* , *5* (8), 1–8. doi: 10.1371/journal.pcbi.1000465

Yi, Y., Fang, Y., Wu, K., Liu, Y., & Zhang, W. (2020). Comprehensive gene and pathway analysis of cervical cancer progression. *Oncology Letters* , *19* (4), 3316–3332. doi: 10.3892/ol.2020.11439

Yun, Y. H., Suh, D. Y., Yoo, H. D., Oh, M. H., & Kim, S. H. (2015). Yeast associated with the ambrosia beetle, *Platypus koryoensis* , the pest of oak trees in Korea. *Mycobiology* , 43(4), 458–466. doi: 10.5941/MYCO.2015.43.4.458

Zhao, T., Kandasamy, D., Krokene, P., Chen, J., Gershenzon, J., & Hammerbacher, A. (2019). Fungal associates of the tree-killing bark beetle, *Ips typographus* , vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior. Fungal Ecology, 38, 71-79. doi: 10.1016/j.chom.2019.03.007

Zhao, T., Kandasamy, D., Krokene, P., Chen, J., Gershenzon, J., & Hammerbacher, A. (2018). Fungal associates of the tree-killing bark beetle, *Ips typographus* , vary in virulence, ability to degrade conifer phenolics and in fluence bark beetle tunneling behavior.*Fungal Ecology* , 1–9. doi: 10.1016/j.funeco.2018.06.003

Zhao, T., Ganji, S., Schiebe, C., Bohman, B., Weinstein, P., Krokene, P., Borg-Karlson, A.-K., & Unelius, C. R. (2019). Convergent evolution of semiochemicals across Kingdoms: bark beetles and their fungal symbionts. *The ISME journal, 13* (6), 1535-1545. doi: 10.1038/s41396-019-0370-7

Data Accessibility Statement

The sequence reads have been deposited in the NCBI Sequence Read Archive database under the BioProject no. PRJNA913789. ITS rDNA barcode sequences are deposited in the NCBI Genbank under the codes OP874898- OP874916.

Author Contributions

Tereza Veselská: wrote the paper, analyzed data, performed research

Karel Švec: wrote the paper, performed research

Martin Kostovčík: analyzed data

Ezequiel Peral-Aranega: isolation of yeasts

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Miroslav Kolařík: designed research, wrote paper

Figure to Tables and Legends

Table 1. A number of assigned ASVs to a particular sample with respect to season.

Fig. 1 **Work flow of the present study.** Parental adults, larvae, pupae and teneral adults were sampled in two seasons (spring and summer). Intestinal microbiome of individuals was analyzed using a combination of several approaches (classical cultivation, DNA and RNA metabarcoding and TEM). Core microbiome in respect to developmental stage and season was assessed based on species abundances. Figure adapted from Six (2011).

Fig. 2. **Proportions of the dominant fungal and bacterial genera change with season, higher taxonomic ranks are similar.** Dominance of Saccharomycetes followed by Sordariomycetes in the fungal microbiome assessed by cultivation technique (only summer season) and DNA metabarcode in the spring season (left column) and the summer season (right column). Dominance of Gammaproteobacteria and order

Enterabacteriales in bacterial microbiome assessed by DNA metabarcode in the spring season (lift column) and the summer season (column). *Multiple species were found to have the same similarity based on blastn search. We chose *P. spadix* (99.33% of similarity) and *E. billingiae* (99.66% of similarity) based on our data from cultivation techniques (see Peral-Aranega et al., in press).

Fig. 3. **Gut microbiome is strongly affected by season.** A – heatmap representing 15 most abundant bacterial and fungal species. B – NMDS analysis of bacterial microbiome in respect to season. C – NMDS analysis of fungal microbiome in respect to season. Seasonality has a strong effect on bacterial and fungal species distribution ($p < 0.001$, Bray-Curtis Permanova, number of permutations 999).*Multiple species were found to have the same similarity based on blastn search. We chose*P. spadix* (99.33% of similarity) and *E. billingiae*(99.66% of similarity) based on our data from cultivation techniques (see Peral-Aranega et al., in press).

Fig. 4. **RNA metabarcode analysis revealed similar dominant taxa as DNA metabarcode analysis.** Bacterial core microbiome assessed by A – RNA metabarcode, B – DNA metabarcode. Fungal core microbiome assessed by C – RNA metabarcode, B – DNA metabarcode. *,**Multiple species were found to have the same similarity based on blastn search. We chose *P. spadix* (99.33% of similarity) and *E. billingiae* (99.66% of similarity) based on our data from cultivation techniques (see Peral-Aranega et al., in press).

Fig. 5. **TEM photograph of Ips typographus gut.** A – foregut, B – microvilli epithelium of midgut. Examples of bacterial cells are highlighted with red arrows, C -microvilli epithelium of hindgut, D - hindgut detail, Examples of bacterial cells are highlighted with red arrows. Scale bar = 2 μm.

Supplementary information

Supplementary Figure 1. **Rarefaction curve for DNA metabarcode markers** . A – 16S marker, after normalization, we obtained 4,760 reads per sample. B – ITS marker, after normalization, we obtained 5,700 reads per sample.

Supplementary Figure 2. **Βαςτεριαλ ΑΣ″ς ριςηνεσς ανδ α-διερσιτψ ινδιςες ιν ρεσπεςτ το βεετλε δεελοπμενταλ σταγε ανδ σεασον** . A - spring season, B - summer season. Different letters indicate significant differences between samples.

Supplementary Figure 3. **Φυνγαλ ΑΣ″ς ριςηνεσς ανδ α-διερσιτψ ινδιςες ιν ρεσπεςτ το βεετλε δεελοπμενταλ σταγε ανδ σεασον.** A - spring season, B - summer season. Different letters indicate significant differences between samples.

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