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The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles

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Abstract

The pine weevil (*Hylobius abietis*, Coleoptera: Curculionidae) is an important pest of conifer seedlings in Europe. Despite its economic importance, little is known about the composition of its gut microbial community and the role it plays in mediating the weevil's ability to utilize conifers as a food source. Here, we characterized the gut bacterial communities of different populations of *H. abietis* across Europe and compared them to those of other beetles that occupy similar ecological niches. We demonstrate that the microbial community of *H. abietis* is similar at higher taxonomic levels (family and genus) across locations in Europe, with *Wolbachia* as the dominant microbe, followed by Enterobacteria and Firmicutes. Despite this similarity, we observed consistent differences between countries and locations, but not sexes. Our meta-analysis demonstrates that the gut bacterial community of the pine weevil is very similar to that of bark beetles that also exploit conifers as a food source. The Enterobacteriaceae symbionts of both host taxa are especially closely related phylogenetically. Conversely, the microbiota of *H. abietis* is distinct from that of closely related weevils feeding on nonconifer food sources, suggesting that the microbial community of the pine weevil is determined by the environment and may be relevant to host ecology. Furthermore, several *H. abietis*-associated members of the Enterobacteriaceae family are known to contain genes involved in terpenoid degradation. As such, we hypothesize that the gut microbial community is important for the utilization of conifer seedlings as a food source, either through the detoxification of plant secondary metabolites or through the supplementation of essential nutrients.

Keywords: bark beetle, conifer, *Hylobius abietis*, insect symbiosis, microbiota, terpenes

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Introduction

Conifers represent a challenging resource for herbivorous insects. These trees contain high amounts of both constitutive and inducible chemical defence compounds, such as phenolics and terpenoids (Keeling & Bohlmann 2006; Li *et al.* 2012) that are toxic or deterrent to herbivores. In addition, many parts such as bark and wood have a high C:N ratio and are poor in essential amino acids,

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phosphorous, vitamins and sterols (Thornber & Northcote 1961a,b, 1962; Warren & Adams 2002).

Despite the poor nutritional value of conifer tissues, many insects across different orders (e.g. Lepidoptera, Coleoptera, Hemiptera) are able to exploit this food source using different mechanisms. Most of the research effort has so far focused on bark beetles (Coleoptera: Curculionidae: Scolytinae), due to their economic and ecological importance. For example, the bark beetle *Ips grandicollis* overcomes the poor nutritional quality of conifers by increasing its phloem consumption rate compared to that of mycangial bark beetles (i.e. those that harbour symbiotic fungi in a cavity called mycangium) (Ayres *et al.* 2000). In some other bark beetles, cooperative behaviour can mitigate the effects of conifer defences. For example, the larvae of *Dendroctonus micans, Dendroctonus terebrans* and *Dendroctonus valens* feed in cavities under the bark as groups in one continuous front, thus probably outrunning the tree-induced chemical defences (Grégoire *et al.* 1981; Deneubourg *et al.* 1990). Other bark beetle species colonize healthy trees by attacking in large numbers, thereby exhausting the tree defences and ultimately killing their host (Berryman 1976). Sawflies (Diprionidae) damage the resin ducts of their conifer host prior to feeding, to release part of the chemical defences, or feed gregariously to consume the needles before defences are induced (McCullough & Wagner 1993). These insects can also sequester conifer resins and use them against predators with an efficiency that is correlated with the host tree chemotype (Codella & Raffa 1995).

While some herbivores rely on their own capabilities to cope with the low nutrient content and toxic defences of their host plants, others engage in symbiotic relationships with microbes that supplement limiting nutrients or aid in degradation of toxic compounds (Douglas 2009). For conifer-feeding insects, most of the available information on symbionts concerns fungi that provide nutritional benefits or detoxify plant secondary metabolites. Three different bark beetle species (*Drendoctonus frontalis, Drendoctonus ponderosae* and *Drendoctonus brevicomis*) harbour symbiotic fungi in their mycangia that supplement the insect diet with assimilated nitrogen (Six & Paine 1998; Ayres *et al.* 2000; Bleiker & Six 2007). Specifically, after inoculation into the host tree by the beetle, the fungi assimilate sapwood nitrogen and transport it to the bark and phloem, where the beetle larvae feed, thereby increasing the available nitrogen content by as much as 40% (Bleiker & Six 2007). In addition, *D. ponderosae* acquires sterols from ophiostomatoid fungi during the larval stage, which are important for the beetle's fecundity (Six & Paine 1998; Bentz & Six 2006). Lack of vitamins in coniferous trees is overcome by bark beetles through association with symbiotic yeasts (Strongman 1987; Pignal *et al.* 1988). Furthermore, symbiotic fungi are also involved in insect resistance to conifer chemical defences. *Grossmania clavigera*, a bark beetle symbiont, is able to cope with terpenoids by mediating their transport outside the fungal cell via ABC transporters (Wang *et al.* 2013), and it can use monoterpenes as a carbon source (DiGuistini *et al.* 2011). Additionally, putative detoxification genes including *O*-methyltransferases and CYP450s are upregulated in *G. clavigera* following exposure to terpenoids (DiGuistini *et al.* 2011), with *O*-methyltransferases known to degrade phenolic compounds (Feltrer *et al.* 2010), and CYP450s to detoxify a number of plant secondary metabolites (Wöll *et al.* 2013). In comparison with fungal symbionts, the contributions of bacterial associates towards conifer-feeding in insects remain poorly known (Grossmann 1930; Craighead & George 1940; Barras 1967; Whitney & Cobb 1972; Paine *et al.* 1997). Gut bacteria in *D. ponderosae* beetles have been found to supplement their host's diet with nitrogen (Morales-Jimenez *et al.* 2009), bacteria isolated from the gut of *Dendroctonus rhizophagous* showed cellulolytic activity on plate (Morales-Jimenez *et al.* 2012), and gut bacteria from *D. valens* were able to degrade mono- and diterpenes in vitro (Boone *et al.* 2013). However, how this affects the insects' fitness remains to be determined. Likewise, the mechanistic basis of the degradation of these compounds remains unclear, although a complete diterpene degradation gene cluster (DDGC) has been found in the bacterial metagenome of *D. ponderosae* (Adams *et al.* 2013).

The pine weevil, *Hylobius abietis* (Coleptera: Curculionidae: Molytinae), feeds primarily on the phloem tissue of several conifers (mainly Scots pine, *Pinus sylvestris,* and Norway spruce, *Picea abies*), where the bark is thin. In some instances, especially when feeding on the stem bark of newly planted conifer seedlings, the pine weevil can cause over 80% mortality (Petersson & Orlander 2003). Thus, *H. abietis* is considered the most important pest of European conifer forests that are managed by clear-cutting followed by replanting (Leather *et al.* 1999; Nordlander *et al.* 2011). Because of its economic importance, the biology and ecology of the pine weevil have received considerable attention over the last decades (Wallertz *et al.* 2006; Wainhouse *et al.* 2014). In spring, after hibernation in the soil, adult pine weevils disperse by flight and may migrate very long distances (up to about 100 km) in their search for suitable reproduction sites (Solbreck 1980). In managed forest landscapes, pine weevils land mainly on newly clear-cut areas, to which they are attracted by volatiles released from fresh conifer stumps (Solbreck & Gyldberg 1979). During summer, weevils remain on the clear-cut where they feed and

mate, and females lay their eggs in the bark of stump roots or in the soil nearby (Nordlander *et al.* 1997). The larvae feed underground, tunnelling in the bark of the stump roots, whereas adults feed on conifer bark both below and above the surface of the soil (Nordlander *et al.* 2005; Wallertz *et al.* 2006). Thus, pine weevils are closely associated with forest soil and conifer bark, encountering high concentrations of terpenoids in their diet throughout their life. As these compounds are toxic to a wide range of insects (Keeling & Bohlmann 2006), it seems imperative that the weevil must have evolved mechanisms to detoxify or tolerate them. However, whether they do this on their own or in association with symbiotic microbes remains unknown.

In this study, we investigated whether *H. abietis* harbours a consistent microbial community across distant geographical locations and explored whether symbiotic microbes may be involved in terpenoid detoxification. To this aim, we characterized the bacterial gut community of pine weevils from six different locations across Europe using 454 pyrosequencing of bacterial 16S rRNA amplicons and oligotyping, to assess their composition and overall stability. To gain first insights into possible roles of the microbiota in pine weevil host ecology, we predicted the bacterial metagenome function based on the 16S rRNA gene, and we performed a meta-analysis to compare the gut bacterial community of beetle species feeding on conifer- vs. non-conifer-based food sources. Due to the importance of bacterial symbionts for digestive processes of insects, we expect beetle species feeding on similar (coniferous) food sources to exhibit convergence in their microbial community structure. Our results provide an ecological perspective on gut microbial community composition in conifer-feeding beetles, highlighting the potential role of microbial symbionts in exploitation of conifers as a food source.

Materials and methods

Insect collection and sample preparation

Pine weevils were collected from fresh clear cuts in different locations in Europe (Fig. S1, Supporting information). We sampled six locations following a North–South transect: Umeå, Uppsala, Asa and the isolated island of Gotland in the Baltic Sea are all located in Sweden, while locations in Hannover in Germany and Galicia in Spain were also sampled. Weevils were captured alive with clean pitfall traps baited with α-pinene and ethanol (Nordlander 1987). Vials without lids were filled with ethanol, and the entrance was blocked with bait-impregnated paper towels or cotton and placed leaning towards a pine branch. Once collected, the weevils were placed in boxes with holes for ventilation. They were sent to the laboratory in styrofoam boxes with ice packs wrapped with paper to avoid insect freezing.

DNA extraction, amplification and sequencing

Insects were dissected using dissecting scopes under sterile conditions. Only guts from insects free of apparent nematode infections were used for further analyses. An average of five isolated guts from weevils caught in the same trap were pooled (Table S1, Supporting information). Isolated guts were placed in Eppendorf tubes, flash frozen with liquid nitrogen and homogenized with a pestle. Then, samples were pretreated with 180 μl of enzymatic lysis buffer for Gram-positive bacteria (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA, 1.2% Triton X-100 and addition of lysozyme to 20 mg/ml). Total DNA was extracted by a Qiacube automated extraction robot (Qiagen) using the QIAamp DNA mini kit (Qiagen).

Emulsion PCR was performed with PCR beads (0.2-ml PuReTaq Ready-To-Go PCR beads; GE Healthcare) and sample-identifying tags, known as Multiplex Identifier Adaptors for Rapid Library Preparations (Technical Bulletin No. 2010-010; Roche) (Swanson *et al.* 2011) connected to the general eubacterial primers 8f (5′-AGAGTTTGATIITGGCTCAG-3′) and 1501r (5′-CGGITACCTTGTTACGAC-3′) (Lindh *et al.* 2005), targeting the V1–V9 region of the 16S rRNA gene. The DNA amplification program was as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58–48 °C for 30 s (the temperature was decreased by 1 °C every cycle for 10 cycles and then held at 48 °C for 20 cycles) and 72 °C for 1 min 30 s, followed by a final extension step at 72 °C for 25 min (Lindh *et al.* 2005). Size and quantity of PCR products were determined by a MultiNA Microchip Electrophoresis System (Shimadzu). Amplicons from each sample were diluted in equimolar amounts and sequenced on a 454-FLX system using Titanium chemistry (454 Life Sciences, Brandford, CT) at the SNP/SEQ platform hosted by SciLife Lab, Uppsala, Sweden.

454 sequencing analysis

Processing of high-quality reads was performed using QIIME (Caporaso *et al.* 2010). We retained sequences between 200 and 600 bp in length, allowing no errors in the barcode but one mismatch in the primer and one ambiguous base. The minimum average quality score per read was set to 25, and reads that were not assigned to any barcode were discarded. Potential

chimeric sequences were identified with USEARCH61 (QIIME) and removed from the data set. The resulting sequences were subjected to both open and closed-reference operational taxonomic unit picking strategies. Open-reference OTU picking was performed with the algorithm *cdhit* (QIIME) using 97% similarity as a threshold to cluster the sequences into OTUs. The most abundant sequence for each OTU was selected as a representative sequence. Taxonomy was assigned using RDP classifier (Wang *et al.* 2007), and remaining sequences with <0.8 confidence in their assignment were removed. Closed-reference OTU picking was also performed at 97% similarity against the GREENGENES database released in May 2013 (http://greengenes.lbl.gov/). OTU tables were generated describing the abundance of bacterial phylotypes within each sample. Results for both methods were compared after manually filtering chloroplast and mitochondrial sequences as well as singletons.

For downstream analyses, the raw OTU table obtained after open-reference OTU picking strategy was used. The OTU table was manually filtered, that is chloroplast and mitochondrial sequences and OTUs with <0.1% abundance in all samples were removed, and samples with <500 reads were eliminated. As the high abundance of *Wolbachia* overshadowed the underlying microbial gut community in most samples, we removed *Wolbachia* reads after initial analysis. Samples with <400 reads were eliminated, and OTU tables were rarified to the minimum depth of 481 reads per sample. Representative sequences were blasted against the NCBI database to identify their top hit using BLAST2GO (Conesa *et al.* 2005) to confirm taxonomic assignment. OTU abundance within samples was visualized in a heat map constructed with the MULTIEXPERIMENT VIEWER (MEV) software (Saeed *et al.* 2003).

Alpha-diversity estimates such as observed species richness and Chao1 (reported for 3% difference between sequences) were calculated. Rarefaction curves were obtained in QIIME by subsampling the OTU table with step increments of 10 sequences and 100 iterations at each step to see the adequacy of our sampling. Beta-diversity metrics (including abundance-weighted and unweighted UniFrac distances, binary Jaccard and Bray–Curtis dissimilarities, and Sorensen) were calculated using the same OTU table as above. The pylogenetic tree needed for beta-diversity analyses was produced with FASTTREE (Price *et al.* 2009) by aligning the representative sequences to the Greengenes core set (http://greengenes.lbl.gov/) using PYNAST, with a minimum sequence identity of 75%. All beta-diversity metrics and principal coordinate analysis (PCoA) plots were generated in QIIME. We statistically tested whether the association of OTUs with the weevils was dependent on site and sex, respectively, by independently analysing the matrixes with one-way analyses of similarities (ANOSIM) and Adonis. In parallel, we performed discriminant analyses (DA) in SPSS 17.0 on the OTU table containing the whole community, and also on an OTU table containing only the 15 most abundant OTUs. In both cases, we performed a principal component analysis (PCA) prior to the DA and retained only the first four principal components, in order to avoid excessive numbers of variables in the DA.

We also used QIIME to calculate the 'core' microbiome (using the script core_microbiome.py), defined as the OTUs present in ≥50% of all samples, for samples belonging to each location and each country. We visualized OTUs shared by the three countries using a Venn diagram with VENNY online software (Oliveros 2007).

Strain diversity analysis by oligotyping

To study strain diversity within the members of the most prevalent taxa, we carried out an oligotyping analysis (version 2.0) (Eren *et al.* 2013). We extracted all sequences belonging to the taxa of interest and kept sequences of 350 bp of length, excluding all sequences that were shorter. Sequences were aligned with PYNAST against the GREENGENE database (http://greengenes.lbl.gov/), and uninformative gaps were eliminated with the script o-trim-uninformative-columns-from-alignment (http://github.com/meren/oligotyping/blob/master/bin/). The ends of the sequences were trimmed to a common length with the script o-trim (same source as above). After the initial entropy analyses with the entropy-analysis script of the oligotyping pipeline, we ran oligotyping, selecting as many high entropy positions as necessary for oligotypes to converge. We considered an oligotype to have converged when all nucleotide positions had entropy values below 0.2. To reduce noise, each oligotype was required to have a minimum of 40 copies of the most abundant unique sequence. Oligotypes that did not meet this criterion were excluded from the analysis. We statistically tested whether the distribution of the different oligotypes was associated with geography or sex by calculating a Bray–Curtis dissimilarity matrix on the oligotyping output table and analysing the matrix with ANOSIM.

Functional inference on the bacterial metagenome

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST, version 1.0.0) to predict metagenome function based on 16S rRNA sequences using the GREENGENES database of reference genomes (Langille *et al.* 2013). For this purpose, we used the OTU table obtained after closed-reference

OTU picking strategy. We rarefied the OTU table to 497 reads, and OTUs were normalized by 16S rRNA gene copy number. The predicted abundance of different gene families was calculated using Kyoto Encyclopaedia of Genes and Genomes (KEGG; Kanehisa *et al.* 2012). Nearest Sequenced Taxon Index (NSTI) was calculated to assess the average similarity between the sequences of an OTU and those of the nearest sequenced genome present in the database. This value gives estimation on the accuracy of the PICRUST analysis. We used the PICRUST output table to build a heat map with MEV (Saeed *et al.* 2003).

The KEGG database does not include some genes of interest for this study, particularly diterpene degradation genes. Therefore, we additionally searched homologues of the DDGC described in Smith *et al.* 2004 using BLASTP (2.2.25+) in the NCBI NR database (downloaded in February 2014). The cut-off for BLAST was E-value $\leq 1e^{-5}$. The species ID of all identified DDGC protein homologues were retrieved from NCBI using TAXA database. Subsequently, they were compared to the identified taxa from our representative set. Only OTUs with genus-level taxonomic assignment that matched one of the DDGC-containing species from NCBI were scored as positive for DDGC. Given that some OTUs (especially those within the Enterobacteria) in our study were not taxonomically assigned beyond family, we performed a second homologue search against a custom database. This database comprised fully sequenced genomes of close relatives to the pine weevil's core microbiota. The cut-off for BLAST was E-value $\leq 1e^{-5}$.

Meta-analysis: phylogenetic placement of Hylobius abietis gut microbiota

To interpret the pine weevil microbial community composition in an ecological context, we tested whether it was consistent with that of other conifer-feeding beetles. Our data set on *H. abietis*-associated bacteria was expanded by the addition of 308 bacterial sequences from GenBank associated with 10 different insect host species belonging to four genera. These sequences included bacterial taxa found in the gut microbiota of five species of bark beetles belonging to two genera that feed on conifers, as well as five weevil species belonging to two genera that feed on red palm trees and ornamental plants, respectively (Table S2, Supporting information). We only selected culture-independent studies. Database sequences were aligned with those obtained in this study using SINA (Pruesse *et al.* 2012). The alignment was imported into ARB (Ludwig *et al.* 2004) together with the SILVA 115 database (http://arb-silva.de/projects/living-tree/) and a phylogenetic tree was constructed. We refined the alignment by selecting all *H. abietis*-associated sequences and those of closely related bacterial type strains present in the SILVA database. Due to the large number of sequences that were selected, the final alignment was produced by filtering some sequences: we only retained those sequences from the pine weevil that corresponded to the 50 most prevalent OTUs (i.e. those that are more often found across samples) and, in addition, those of minor OTUs that clustered together with other bark beetle or weevil-associated bacterial sequences. We also eliminated those bacterial sequences from bark beetles and weevils (other than *H. abietis*) that did not cluster together with any other insect-associated bacteria in this study. The final phylogeny was reconstructed with FASTTREE using the GTR model (Price *et al.* 2010) and edited in MEGA (Tamura *et al.* 2011).

Results

The microbial community of Hylobius abietis guts

The gut bacterial community of the pine weevil was characterized using tag-encoded FLX amplicon pyrosequencing of the bacterial 16S rRNA gene. A total of 234 055 high-quality sequences were generated across 51 samples from three different countries. The sequences were binned to 359 OTUs, of which 96 were removed because they were of likely chimeric origin. Both raw OTU tables obtained with open and closed-reference OTU picking methods were very similar, containing 263 and 257 OTUs, respectively. After filtering singletons as well as chloroplast and mitochondrial sequences, each OTU table contained 249 and 255 for open- and closed-reference methods, respectively. Taxonomical assignment was similar regardless of the OTU picking strategy used (Fig. S2 and S3, Supporting information).

We used the OTU table obtained with open-reference methods for downstream analyses. Two OTUs were removed because they were classified as chloroplasts and another two because they belonged to samples with <500 reads. The remaining 259 OTUs contained 231 706 sequences, representing 98.99% of all input sequences (Table S1, Supporting information).

At high taxonomic levels, the bacterial community found in the weevil's gut was quite stable, dominated by Alpha- and Gamma-Proteobacteria in 90% of the samples (Fig. 1). Firmicutes were abundant in Germany and some samples from Gotland (Sweden), but were absent from Spanish samples. Swedish samples harboured a higher bacterial diversity than samples from Germany or Spain, with low abundances of Actinobacteria and other taxa (Fig. 1). Most alpha-proteobacterial sequences were assigned to *Wolbachia*, which was

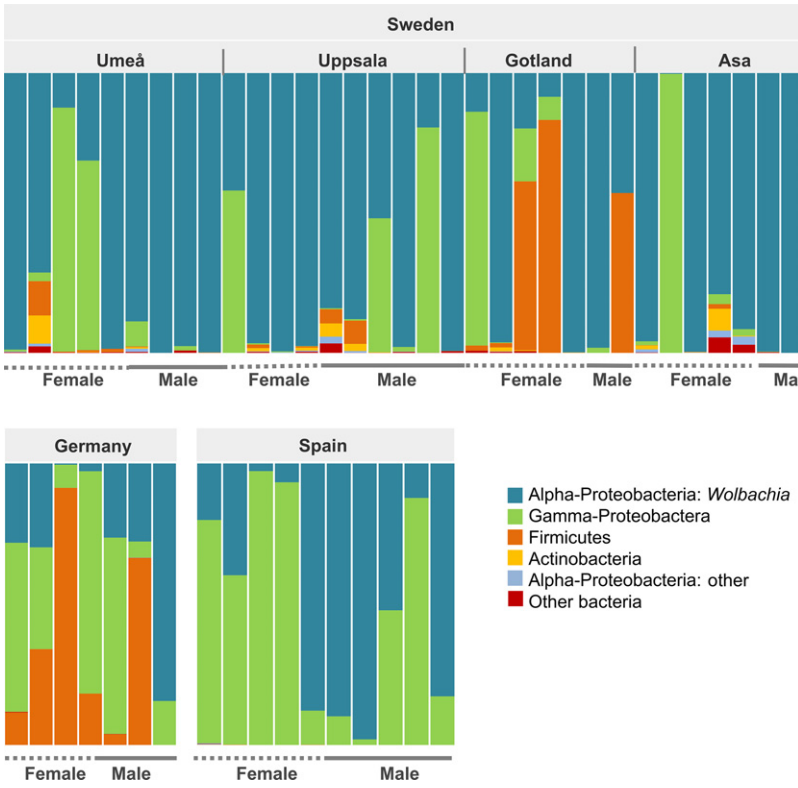

Fig. 1 Class-level gut bacterial community composition of *Hylobius abietis* across different geographical locations. Each bar corresponds to a pool of three to nine individuals of the same sex collected from the same trap.

present in all samples, ranging, in relative abundance, from 0.2% to 100% and comprising on average 73%, 28% and 49% of sequence reads in samples from Sweden, Germany and Spain, respectively. However, there was no statistical difference in the relative abundance of *Wolbachia* among countries, locations or sexes (Kruskal–Wallis test, d.f. = 2; d.f. = 5; d.f. = 1, $P = 0.4$; $P = 0.2$, and $P = 0.1$, respectively).

Due to its high abundance, *Wolbachia* overshadowed the remaining microbial community. Therefore, we eliminated *Wolbachia* sequences from the analysis to gain more detailed insights into other microbial associates. Following the removal of *Wolbachia*, 23 samples had <400 reads and were excluded from further analysis. To normalize the number of reads per sample, we rarefied the OTU table to a common depth of 481 reads per sample. Our final *Wolbachia*-free data set contained 13 468 reads that were binned to 162 OTUs distributed across 28 samples (Table S1, Supporting information).

Rarefaction analysis indicated that despite removing *Wolbachia* reads, our sampling of the underlying microbial community was still exhaustive (Fig. S4, Supporting information). Calculation of the Chao1 diversity index revealed considerable variability between samples, ranging from 6 to 48 OTUs, with the mean ± SD being 16 ± 8 OTUs (Fig. S4, Supporting information). However, there were no differences in richness between countries (Fig. S5, Supporting information).

After removing *Wolbachia* sequences, the most abundant and prevalent OTUs belonged to the families Enterobacteriaceae (Gamma-Proteobacteria) and Leuconostocaceae (Firmicutes) (Fig. 2). Within these two families, two patterns emerged. First, within the Enterobacteriaceae, *Erwinia* sp. was very abundant across Spanish and German samples, but virtually absent from all locations in Sweden. Second, within the Leuconostocaceae, *Weissella oryzae*, which was the most abundant taxon within the family, and *Lactococcus* sp., were present in Swedish and German samples, but completely absent from Spanish ones. Interestingly, some samples were dominated by Proteobacteria and others by Firmicutes, with no sample harbouring similar relative abundances of these two taxa (Fig. 2).

As expected based on the higher diversity, most OTUs (134) were present in Sweden, with 100 of those being exclusive, while only 51 and 38 were found in Germany and Spain, respectively (Fig. S6, Supporting information). Only 16 OTUs—13 Enterobacteriaceae and three Firmicutes—were shared between samples from all three countries. The analysis of the 'core' microbiota (i.e. those OTUs present in more than 50% of the samples) revealed just four OTUs, all of them belonging to the Enterobacteriaceae family, which were shared by all three countries. While genus-level classifications are generally difficult in the Enterobacteriaceae based on short 16S rRNA fragments, BLAST analyses of the 'core'

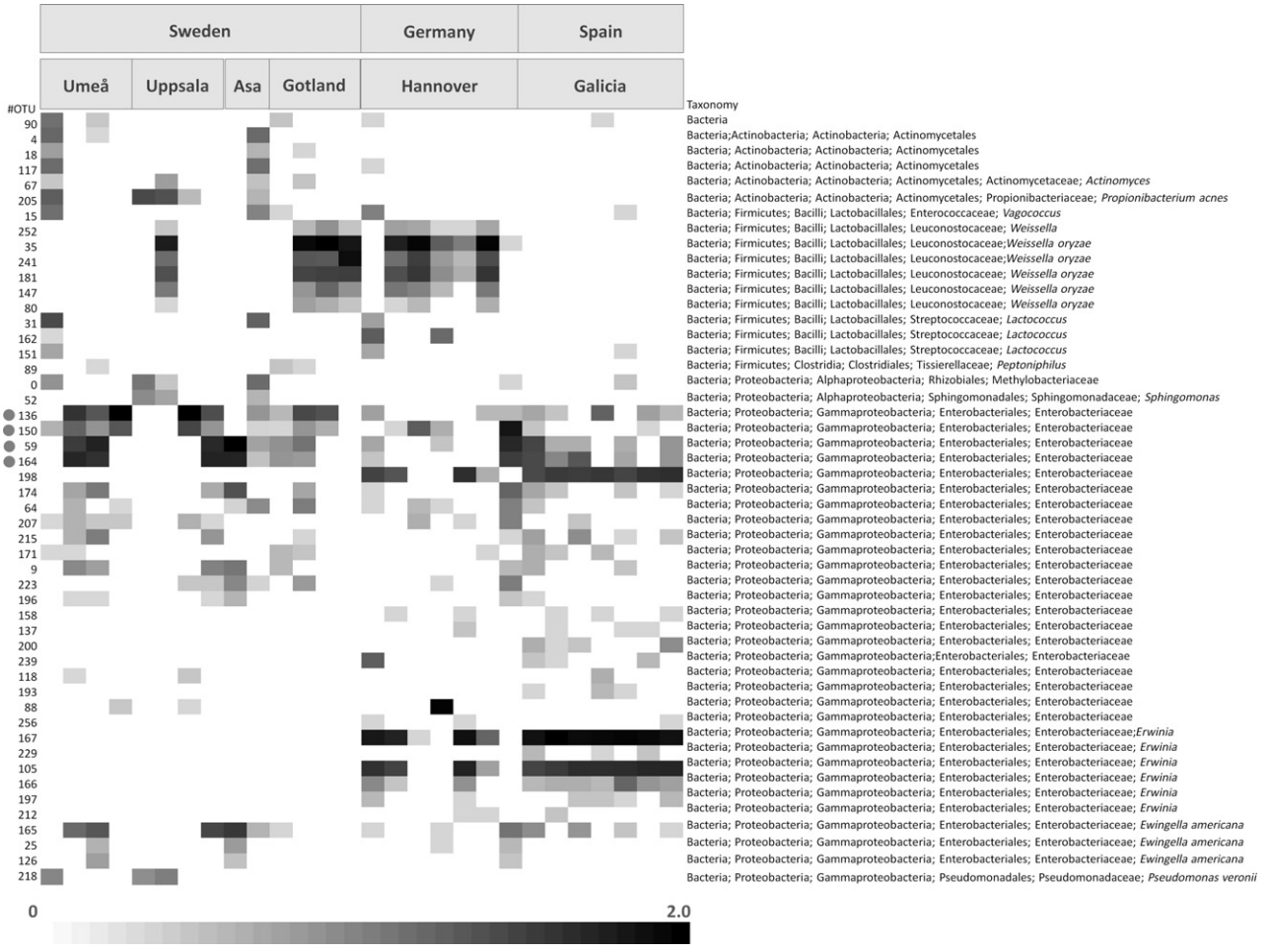

Fig. 2 Bacterial gut community composition of the pine weevil from different geographical locations after removal of *Wolbachia*-associated OTUs. Only the 50 most prevalent OTUs are shown. Relative abundances of bacterial taxa are displayed as a heat map on log scale, with dark colours representing high abundances and white indicating absence.

microbes suggested that their closest relatives are *Erwinia typographi*, *Rahnella* and *Serratia*.

From the 50 most prevalent OTUs (i.e. those that were found in most samples) that comprised 97.2% of the original sequences, 31 belonged to the Gamma-Proteobacteria and two to the Alpha-Proteobacteria. Within the Gamma-Proteobacteria, all OTUs except for one Pseudomonadales OTU belonged to the Enterobacteriaceae family. The remaining 17 OTUs comprised 12 Firmicutes and five Actinobacteria (Fig. 2).

Variation in microbial communities due to geography

We used several different distance metrics to assess differences in bacterial community profiles between sexes and geographical locations. PCoA based on an unweighted UniFrac distance matrix (Fig. 3) and DA revealed that the gut microbiota composition of weevils does not differ between sexes (ANOSIM $P = 0.623$; Adonis $P = 0,773$; Wilk's $\lambda = 0.88$ 1, $P = 0.546$). However, the composition significantly differed between the three countries tested (ANOSIM $P = 0.001$; Adonis $P = 0.006$; Wilk's $\lambda = 0.480$, $P = 0.028$). The microbial communities of the samples from Gotland tended to be intermediate between the other Swedish and the German samples, which agrees with the geography of the sampling localities. However, there were no statistically significant differences between locations in Sweden (ANOSIM $P = 0.267$; Adonis $P = 0.343$; Wilk's $\lambda = 0.295$, $P = 0.531$). These results were consistent with those obtained from all other distance matrices tested, that is weighted UniFrac, Sorensen, Bray–Curtis and Jaccard (Table S3, Supporting information).

Oligotyping

We used the oligotyping pipeline (Eren *et al.* 2013) to explore bacterial diversity within the four OTUs

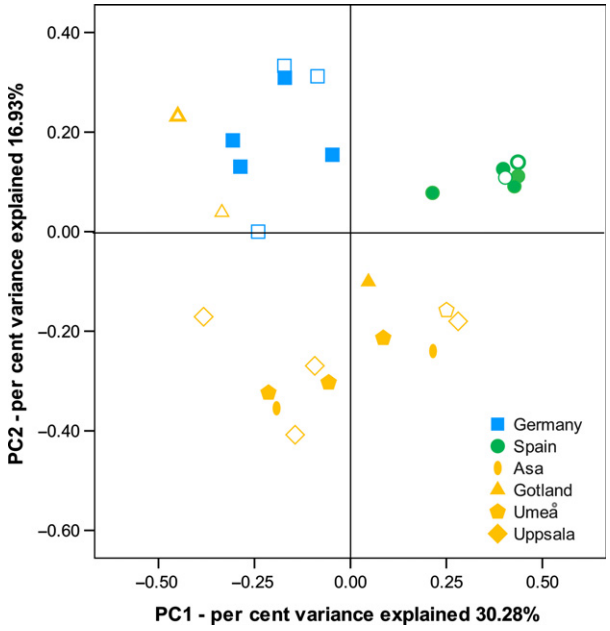

Fig. 3 PCoA plot based on an unweighted UniFrac distance matrix depicting differences in the composition of the gut microbiota from male and female weevils of different locations. Symbols represent community profiles of individual samples. Colours represent countries, shapes represent different locations; full symbols represent females and empty ones males. Thick lines indicate cases in which two communities (male and female) from the same location are identical.

comprising the 'core microbiota' as well as within the genus *Wolbachia*. The entropy analysis for *Wolbachia* revealed seven positions with high entropy values, all of which were located in homopolymer regions (Fig. S7, Supporting information). As the 454 technology is known to commonly produce errors in homopolymer regions, differences in these regions probably represent sequencing artifacts rather than true biological variation. Thus, we excluded hompolymer substitutions, which left only a single *Wolbachia* oligotype shared by all individuals regardless of their sex and geographical origin.

Entropy analysis of the core microbiota (OTUs 59, 136, 150 and 164) revealed 6, 4, 22 and 6 high entropy nucleotide positions, respectively (Table S5, Supporting information). Following oligotyping and quality filtering, we observed 15, 5, 6 and 10 oligotypes in the members of the core microbiota, respectively. We used a Bray–Curtis dissimilarity matrix to study the differences in oligotype composition between sexes and geographical locations. Statistical analysis showed that there was no difference in the oligotype composition between sexes for any of the core OTUs studied (Table S5, Supporting information). Likewise, we found no difference in composition across geographical locations for OTU 136 ($P = 0.1$) and OTU 150 ($P = 0.07$) (Fig. 4). However, the composition significantly differed between locations for OTU 59 ($P = 0.02$) and 164 ($P = 0.01$).

Functional inference

PICRUST predicted metagenome content to level 2 KOs based on the complete data set (i.e. including *Wolbachia*) revealed the putative presence of genes of possible symbiotic importance, such as amino acid, carbohydrate and vitamin metabolism genes (Fig. S8, Supporting information). The PICRUST analysis without *Wolbachia* showed the same gene functions with the addition of xenobiotic degradation and metabolism (Fig. 5). The NSTI values per sample ranged from 0.0089 to 0.03, showing that the 16S rRNA gene of microbes in the weevil's bacterial metagenome were on average more than 99% similar to those of sequenced genomes in the database.

Our independent prediction of the presence of diterpene degradation genes in the pine weevil bacterial metagenome suggested the putative occurrence of a complete *dit* gene cluster. The diterpene degradation cluster contains 20 annotated genes, all of which are likely to be present in the pine weevil's bacterial metagenome, based on the OTUs' closest fully sequenced relatives. Taxonomic classification of these genes showed that most of them were classified as belonging to the genera *Pseudomonas* (which has copies of all genes in the cluster), *Bacillus* (with all copies except dit E, F, G, H and K) and *Sphingomonas* (all genes present except ditN and ditP) (Table S6, Supporting information).

A second homologue search was performed against a CUSTOM database containing fully sequenced genomes of the closest relatives of the pine weevil's core microbiota (i.e. *Rahnella aquatilis, Serratia symbiotica, Serratia odorifera, Yersinia nurmii, Pantoea agglomerans* and *Erwinia typographi*). This analysis predicts the presence of all genes of the DDGC within these taxa (Table S7, Supporting information).

Comparison of Hylobius abietis' microbial community to those of other conifer-feeding beetles

To study whether *H. abietis* shares a microbiota consistent with that of other conifer-feeding beetles, and to gain some insights as to whether the stability of the pine weevil microbiota may have an ecological significance, we extended our data set with sequences from the gut microbiota of other beetles feeding either on conifers (*Dendroctonus ponderosae, D. frontalis, D. valens, D. rhizophagous* and *Ips pini*) or on other food sources (*Rhynchophorus ferrugineus, Otiorhynchus salicicola, O. rugostriatus, O. sulcatus* and *O. armadillo*) (Table S2, Supporting information). We added these sequences to

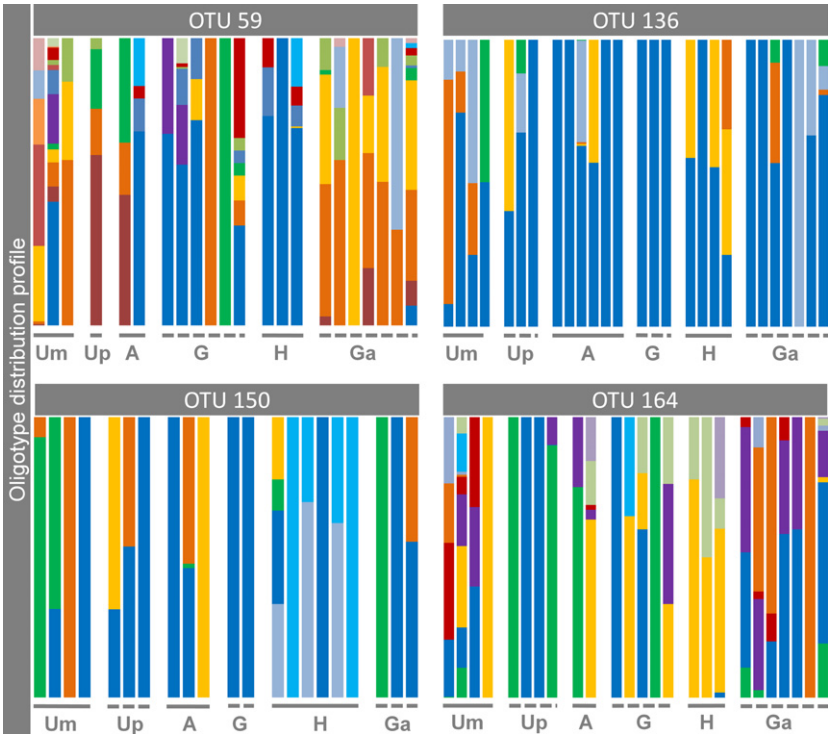

Fig. 4 Oligotype distribution profile of the four 'core' OTUs across geographical locations. Um, Umeå; Up, Uppsala; A, Asa; G, Gotland; H, Hannover; Ga, Galicia.

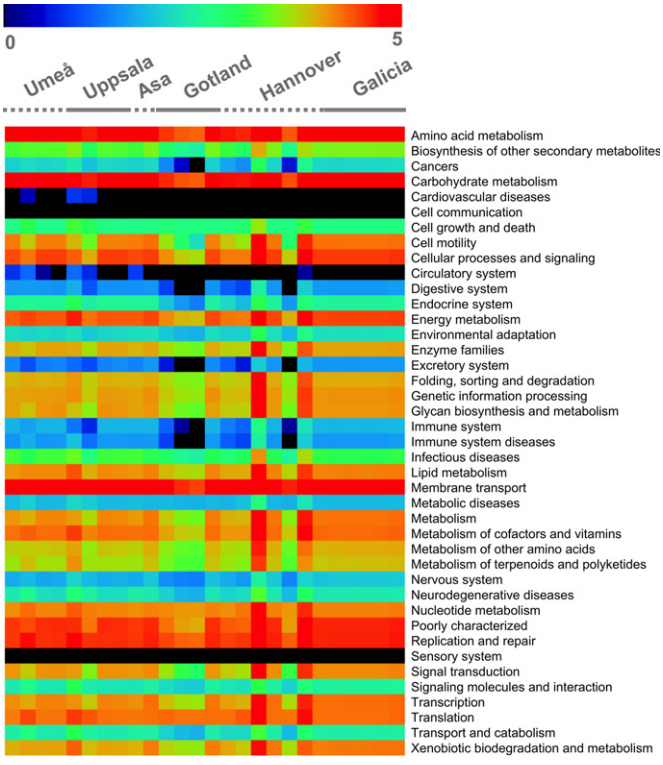

Fig. 5 Heat map in log scale depicting the PICRUSt-inferred gene abundance in the predicted bacterial metagenome of *Hylobius abietis* across different sampling locations in the absence of *Wolbachia*. Warm colours represent high abundances, cold colours represent low abundance and black indicates absence.

those of *H. abietis* and then reconstructed a phylogenetic tree, which was subsequently simplified by eliminating sequences that did not cluster with those of other beetle-associated bacteria.

Of the 444 sequences that were initially included in the phylogeny, 77 were removed because they clustered distantly from any of the *H. abietis*- or other Curculionidae-associated bacteria. Of the remaining 367

sequences, 123 belonged to bark beetle-associated bacteria and 44 were associated with other beetles, 82 were associated with *H. abietis* and the rest were obtained from the database and comprised bacteria from many different environments. Of the bark beetle-associated bacterial sequences that were included in the phylogeny, 110 (89.43%) clustered within the Gamma-Proteobacteria, the rest (10.56%) clustered as follows: two within the Alpha-Proteobacteria, four within the Beta-Proteobacteria, two within the Actinobacteria and five within the Firmicutes. Of the 44 bacterial sequences associated with other beetles, only 12 (27.27%) clustered within the Gamma-Proteobacteria, whereas the rest (72.72%) clustered within the Alpha-Proteobacteria (six sequences), Bacteroidetes (four sequences), Beta-Proteobacteria (10 sequences), Actinobacteria (three sequences), Firmicutes (eight sequences) and Chloroflexi (one sequence).

We observed different clustering patterns for the *H. abietis*-associated sequences in Enterobacteriaceae and Firmicutes, while no clustering at all occurred in any other phyla. The Enterobacteriaceae contained the vast majority of *H. abietis*-associated bacterial sequences, which clustered in two main groups (associated with the genera *Erwinia*, *Rahnella* and *Serratia*) (Fig. 6). These groups were large clusters mainly composed of *H. abietis*- plus bark beetle-associated bacteria, and they contained all four OTUs that constitute *H. abietis*' 'core' microbiota (Fig. 6). By contrast, within the Firmicutes and all other classes, most *H. abietis*-associated sequences occurred dispersed, with the exception of a cluster associated with the genus *Weissella*, which contained no other beetle-associated bacteria (Fig. 7).

Interestingly, some OTUs from *H. abietis* were closely related to those of bark beetles and other weevils, appearing as sister taxa. This occurred in eight cases, in four of which bark beetle associates within the Enterobacteriaceae were the closest relatives, while in four cases, weevil-associated bacteria, specifically from *R. ferrugineus*, were the pine weevil symbionts' sister taxa (twice within the Enterobacteriaceae and twice outside this family). Remarkably, within the Gamma-Proteobacteria, sister taxa occurred with *D. valens*, *R. ferrugineus* and *D. rhizophagous*, whereas outside that group, it occurred only with *R. ferrugineus*. Thus, while there were some *H. abietis*-associated sequences with high similarity to bacteria from other weevil species feeding on different diets, the most prevalent OTUs clustered with bark beetle-associated microbes in the Enterobacteriaceae.

Discussion

In the present study, we characterized the gut microbiota of different populations of the European pine weevil, an important pest of young conifer trees in Europe, and compared it to the microbiota of other beetles of the Curculionidae (including other weevils and bark beetles) feeding on the same and different food sources to place the bacterial community in a broader ecological context.

Analyses with both open- and closed-reference OTU picking strategies yielded very similar results. The most striking difference between both methods is the number of OTUs assigned to *Wolbachia*, 28 and 4 for open-reference and closed-reference methods, respectively. However, its abundance remains similar. Taxonomical resolution appears to be better using closed-reference methods. However, this does not affect the results, given that the difference between both approaches within the most abundant taxonomical groups in the pine weevil community is minimal.

We found *Wolbachia* to be present in all samples studied. The analysis of the microbiota with QIIME showed 28 OTUs binned to *Wolbachia*. However, oligotyping analysis revealed that all pine weevils harboured just one oligotype (a unique 16S rRNA sequence), indicating that all individuals share just one strain of *Wolbachia* regardless of sex or geographical location. This discordance on the number of *Wolbachia* strains is most likely due to the fact that the highly variable nucleotides found in the 16S rRNA sequence were located in homopolymer regions and thus are probably the result of sequencing errors (Quince *et al.* 2011). *Wolbachia* infects up to two-thirds of all known insects (Hilgenboecker *et al.* 2008), and it is most well known for its manipulative effects on host reproduction (Werren *et al.* 2008), but mutualistic associations for nutrition (Hosokawa *et al.* 2010) or defence (Hedges *et al.* 2008) of the host have also been described. In a previous study, 40% of sampled weevil species from central Europe were infected with *Wolbachia* (Lachowska *et al.* 2010). *Wolbachia* is most commonly found in insect reproductive tissues, but localization in other tissues including the gut has also been described (Dobson *et al.* 1999; Frost *et al.* 2014). In *H. abietis*, as has been speculated for *Acromyrmex* leaf-cutting ants (Andersen *et al.* 2012), the presence of *Wolbachia* in the gut may point towards a nutritional function, although reproductive manipulation or a commensal relationship cannot be ruled out without further experiments.

After removing *Wolbachia* sequences from the analysis, Proteobacteria and Firmicutes turned out to be the most abundant and prevalent phyla in the pine weevil microbiota, followed by Actinobacteria. Even though the composition of insect-associated microbial communities differs strongly among insect species, Proteobacteria and Firmicutes appear to be the most prevalent phyla (Colman *et al.* 2012). Both groups have also been

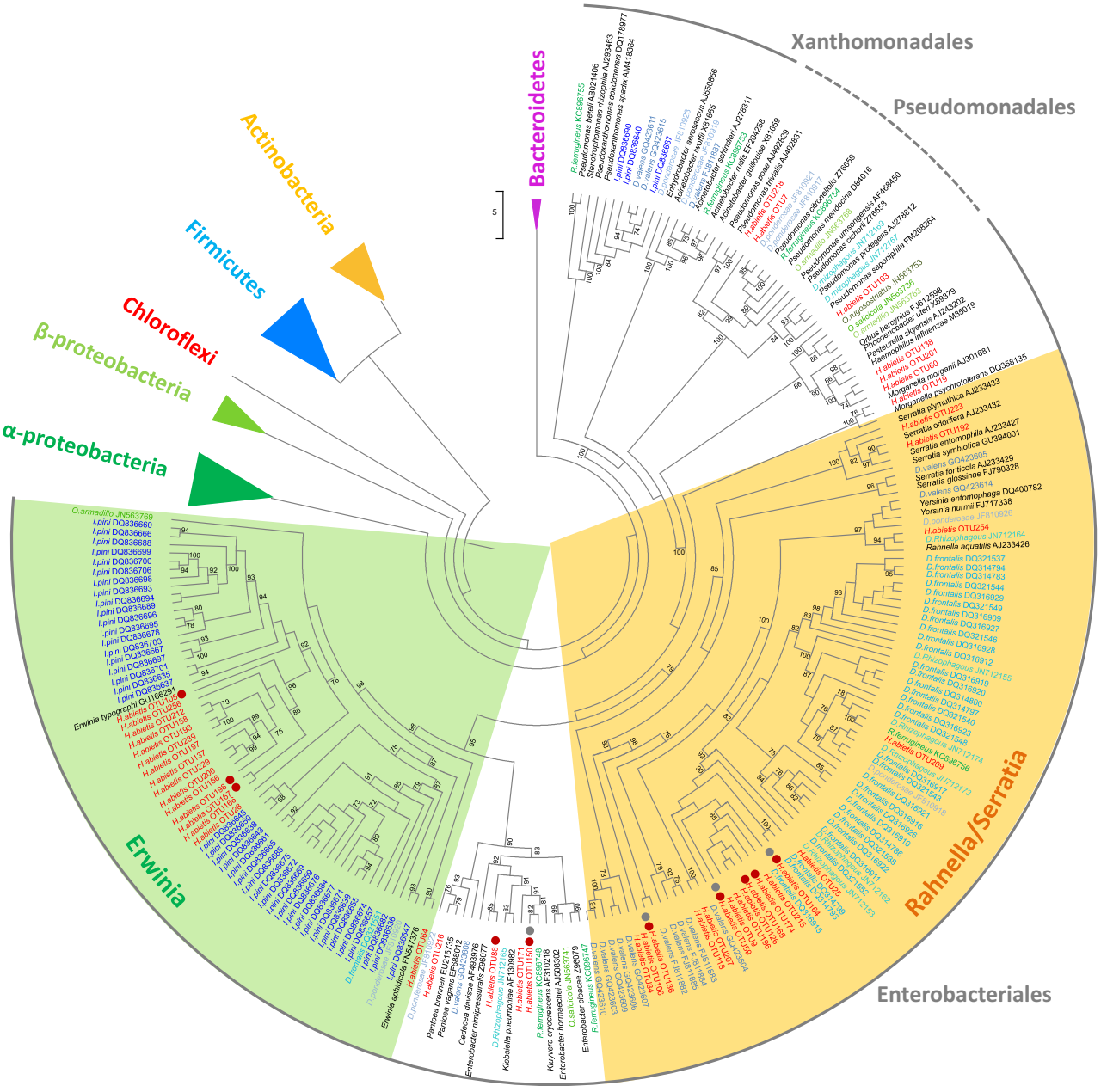

Fig. 6 Phylogenetic placement of the pine weevil gut microbiota (red taxa) in relation to that of other beetles feeding on conifers (blue taxa) or other food sources (green taxa). Only Gamma-Proteobacteria are depicted, all other groups are collapsed (see Fig. 7 for phylogenetic relationships within these groups). Red dots represent OTUs belonging *to Hylobius abietis* that are present in more than 1% abundance in total. Grey dots correspond to OTUs from the 'core microbiota'.

described to be dominant in the conifer-feeding bark beetles, *Dendroctonus valens* and *I. pini* (Delalibera *et al.* 2007; Morales-Jimenez *et al.* 2009). More specifically, we found Enterobacteriaceae to be the most dominant family harboured in the pine weevil gut. This family has also been reported in bark beetles such as *D. ponderosae* (Adams *et al.* 2013), *D. frontalis* (Vasanthakumar *et al.* 2006), *Dendroctonus rhizopagus* (Morales-Jimenez *et al.* 2012) and in *I. pini* (Delalibera *et al.* 2007).

While bacterial communities of insect guts can exhibit large variation in space and time (Toju & Fukatsu 2011; Zouache *et al.* 2011), a number of studies have nonetheless demonstrated that functionally relevant microbial communities can be remarkably stable, as exemplified in firebugs (Sudakaran *et al.* 2012), honey bees (Martinson *et al.* 2011) and termites (Hongoh *et al.* 2005). In bark beetles, the geographical stability of microbial communities varies across host taxa, being

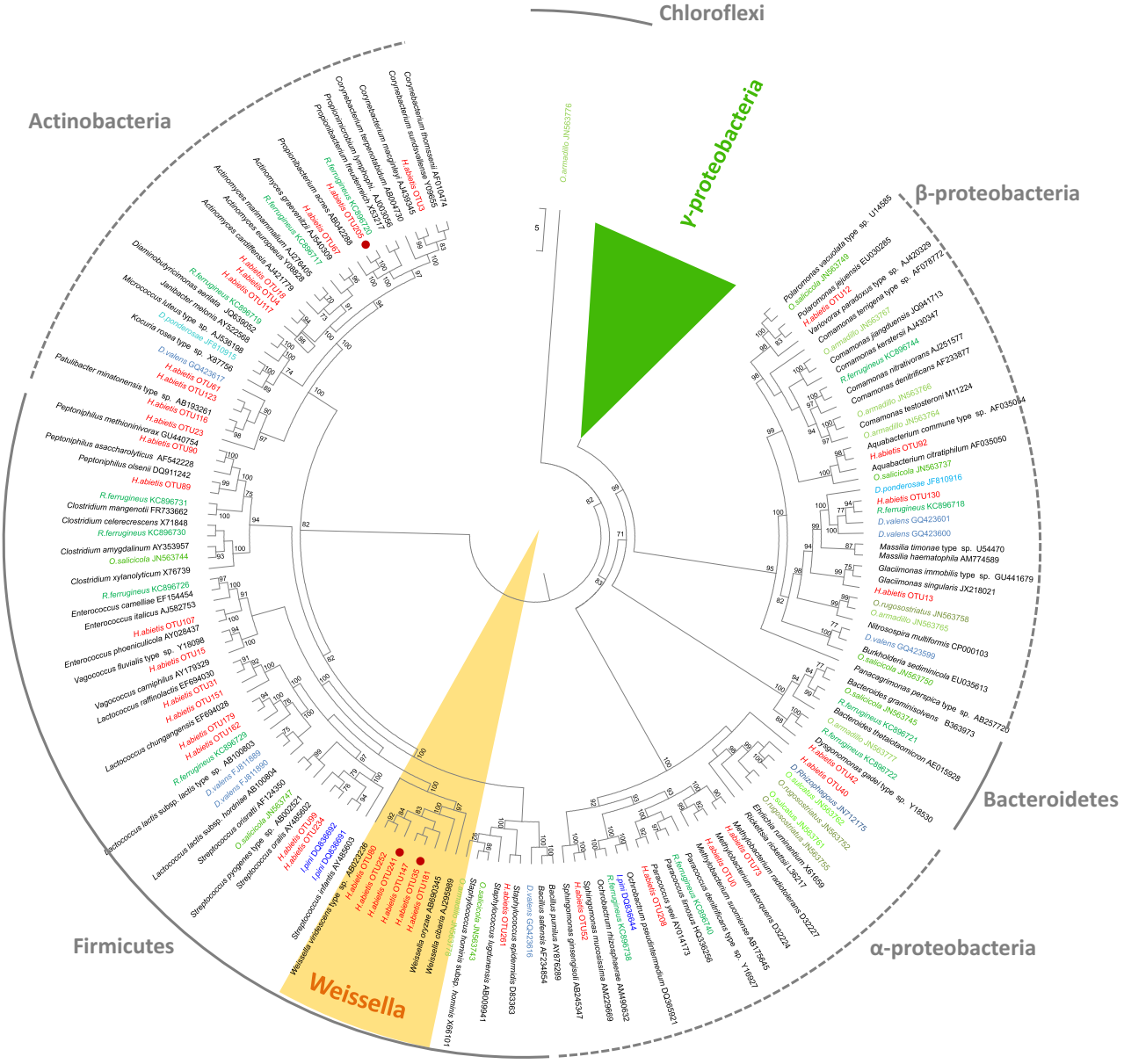

Fig. 7 Phylogenetic placement of the pine weevil gut microbiota (red taxa) in relation to that of other beetles feeding on conifers (blue taxa) or other food sources (green taxa). All bacterial groups except Gamma-Proteobacteria are depicted (see Fig. 6 for phylogenetic relationships within this group). Red dots represent OTUs belonging to *Hylobius abietis* that are present in more than 1% abundance in total.

high in *D. ponderosae* (Adams *et al.* 2013) and low in *D. valens* (Adams *et al.* 2010), although some microbial taxa were always present in the latter regardless of sampling location. Our data demonstrate a rather stable gut community in the pine weevil across different locations in Europe (Fig. 1). Around 50% of the most prevalent OTUs were present in every country, and members of the Enterobacteriaceae were present in every location. In particular, two oligotypes of this family were geographically stable and were present in all locations studied. However, differences between geographical locations were also detected (both at OTU and oligotype levels), resulting in the significant separation of sampling locations based on the beetles' microbial community profiles (Fig. 3). Interestingly, individuals that presented a high abundance of Proteobacteria harboured a low abundance of Firmicutes and vice versa (Fig. 2), which could suggest

mutual competitive exclusion of these two groups in the pine weevil's gut.

Colman and colleagues (2012) found that beetles feeding on bark and phloem of living trees harboured similar bacterial taxa and that their microbial communities generally exhibited low richness and phylogenetic diversity. We found the overall bacterial richness in guts of adult pine weevils (16 ± 8 OTUs/sample) to be slightly higher than the richness in guts of bark beetles (e.g. 5 and 3 OTUs/sample), based on Chao1 richness estimators after OTU clustering with 97% similarity thresholds (Vasanthakumar *et al.* 2006; Morales-Jimenez *et al.* 2009). Several factors may explain the higher diversity detected in our study as compared to previous reports on bark beetles. First, we screened a greater number of samples in our analysis compared to previous studies, which may have contributed to an increased OTU number. Second, we used 454 pyrosequencing as opposed to DGGE, which—due to its greater depth and sensitivity, but also the potential generation of sequencing artifacts—is known to yield more diverse community profiles (Gilles *et al.* 2011; Quince *et al.* 2011). Despite the higher microbial diversity in our study, we could confirm that conifer phloem-feeding beetles harbour species-poor communities compared to those of other insects, including xylophagous taxa (e.g. 89 ± 61 OTUs/sample in Isoptera) and detritivorous taxa (53.2 ± 33 OTUs/sample) (Colman *et al.* 2012). The low bacterial richness in conifer phloem-feeding beetles may reflect the antimicrobial properties of the toxic defensive chemicals encountered in conifer phloem, such as terpenoids (Trombetta *et al.* 2005; Adams *et al.* 2011). Bacteria from the Enterobacteriaceae family are known for their frequent association with insects as intra- as well as extracellular nutritional symbionts (Baumann 2005; Lauzon *et al.* 2009; Husnik *et al.* 2011; Nikoh *et al.* 2011). The recurrent presence of Enterobacteriaceae taxa in the pine weevil whose closest relatives are *Rahnella*, *Serratia*, *Pantoea* and *Erwinia*, the phylogenetic relatedness of these microbes to those harboured by different species of bark beetles and our functional prediction of the bacterial metagenome suggest that they are common associates of conifer phloem-feeding insects and might play an important role in overcoming the nutritional challenges that this diet poses. Specifically, the low concentration of nitrogen could be overcome by the association with nitrogen-fixing bacteria. *Enterobacter* spp. are known to fix atmospheric nitrogen in the apple maggot (Lauzon *et al.* 2000) as well as in termites (Potrikus & Breznak 1977), and they have been isolated from *Dendroctonus terebrans* (Bridges 1981). Additionally, *Pantoea, Rahnella aquatilis* and *Serratia* spp. are known to fix nitrogen in plant–microbe associations (Behar *et al.* 2005) and could potentially perform the same function in an insect host. Furthermore, *Serratia* spp., *Erwinia* sp. and *Enterobacter aerogenes* are facultatively anaerobic bacteria that have been proposed to generate micro-anaerobic sites in termite guts where nitrogen fixation as well as cellulose degradation can take place (Adams & Boopathy 2005).

Some members of the pine weevil microbial community could also be involved in the degradation of plant chemical defences. *Pseudomonas* spp. and close relatives of *Rahnella* spp. are known for their ability to degrade aromatic compounds (Sarma *et al.* 2004; Wongsa *et al.* 2004; Bicas *et al.* 2008). Moreover, the bacterial metagenome of *D. ponderosae* (Adams *et al.* 2013) contains a complete gene cluster involved in diterpene degradation. Adams *et al.* (2013) were able to match some of those genes to specific members of the bacterial community, that is *Pseudomonas*, *Rahnella*, *Stenotrophomonas*, *Serratia*, *Pantoea*, *Erwinia,* as well as *Burkholderia,* most of which are found in *H. abietis*' gut community in high abundances. Isolates from some of those taxa can degrade terpenes in vitro (Boone *et al.* 2013) and do so more efficiently at concentrations typical of constitutive rather than induced chemical defences (Raffa 2014). Our functional inference of the bacterial metagenome suggests as much, indicating that *Pseudomonas* sp., *Bacillus* sp. and *Sphingomonas* sp, as well as members of the genera *Rahnella*, *Serratia*, *Erwinia*, *Pantoea* among others, are likely to contain a *dit* gene cluster and hence could be involved in such detoxification. Interestingly, it seems that although there is some degree of metabolic redundancy, *D. ponderosae*'s gut community appears to be highly complementary from a metabolic standpoint, as distinct bacterial species have been found to degrade different terpenoids in vitro (Boone *et al.* 2013). Thus, given (i) the overlap in the microbial community between bark beetles and the pine weevil, (ii) the presence of a complete DDGC in the bacterial metagenome of the bark beetle *D. ponderosae*, (iii) the ability of some microbes that are closely related to *H. abietis*-associated bacteria to degrade diterpenes in vitro and (iv) the putative presence of diterpene degradation genes in the pine weevil's bacterial metagenome, it seems likely that the same taxa could be involved in the detoxification of terpenes in the pine weevil.

Besides diet, phylogenetic relatedness is one of the main factors shaping insect microbial communities (Colman *et al.* 2012; Ravussin *et al.* 2012). Indeed, all beetles compared in this study belong to the Curculionidae family and it is plausible that the shared microbiota found in these insects reflects their relatedness. However, outside this beetle family, a similar bacterial community has been described in a species of the Cerambycidae (*Monochamus galloprovincialis*) sampled in Portugal (Vicente *et al.* 2013), which also lives on

conifers (i.e. *Pinus pinaster*). The similarity of the pine weevil's microbiota to that of a cerambycid beetle and to bark beetles suggests that the ecological niche plays an important role in shaping the community of these insects. Distantly related insects exploiting similar ecological niches have previously been shown to harbour similar microbiotas or at least equivalent functional diversity (Muegge *et al.* 2011; Fan *et al.* 2012).

The taxonomic convergence of the microbiota of conifer-feeding beetles is remarkable given that all these insects have been sampled from distant locations spanning two continents. However, the functional homology of these bacterial taxa among different species of beetles remains to be confirmed by further metagenomic, transcriptomic and biochemical analyses. The similarity in the gut microbiota of different conifer-feeding beetles could be explained by two different hypotheses. The most likely scenario is that many of the microbial taxa found in this study are frequent residents of conifer tissue due to their ability to degrade specific conifer metabolites. Hence, they may be horizontally acquired by the insect and present only transiently in the gut. Alternatively, vertical transmission from mother to offspring may occur to ensure that a functionally relevant microbiota is passed from generation to generation. However, occasional horizontal acquisition of gut microbes would still be necessary to explain the geographical patterns observed for *H. abietis* in this study. Under both scenarios, the observed presence of conserved bacterial taxa in different species of conifer-feeding beetles from distant locations, and the putative occurrence of genes involved in terpenoid degradation, suggests that these microbes are of functional importance to their hosts.

Wood boring beetles such as the pine weevil and bark beetles represent a serious threat to conifer forests worldwide. In Europe, *H. abietis* and *Ips typography* are the most damaging conifer pests with a reported distribution spanning more than a dozen countries (Gregoire and Evans 2004, Langstrom and Day 2004). The pine weevil alone has caused very serious damage in more than 88 258 ha between 1980 and 2000, and 3 418 264 ha is considered to be under threat (Gregoire and Evans 2004). In North America, bark beetles also represent a serious ecological threat. Understanding the interaction between these insects, their symbionts and the conifer hosts at different levels (i.e. ecological, physiological and molecular) is key to understanding their ability to devastate forests and can potentially help in designing more efficient strategies to protect these ecosystems.

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A.B., M.K., A.S., J.G., A.K.B.K. and O.T. conceived the study; A.B. and K.A. collected weevils, K.A. performed dissections, O.T. performed DNA extraction and 454 sequencing preparation, and A.B. performed the analyses. A.B. and M.K. wrote the manuscript, which was edited and agreed for publication by all authors.

Data accessibility

Raw fasta files with complete set of bacterial 16S rRNA sequences, quality filtered fasta files, alignment files, phylogenetic tree files and mapping files are available on Dryad (doi: 10.5061/dryad.f3r67.2).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Map of Europe depicting the different sampling locations of *H. abietis*.

Fig. S2 Heat map in log scale depicting the gut bacterial community of *H. abietis* obtained with open-reference OTU picking methods.

Fig. S3 Heat map in log scale depicting the gut bacterial community of *H. abietis* obtained with closed-reference OTU picking methods.

Fig. S4 Rarefaction curves of bacterial 16S rRNA gene sequences from weevil gut samples from different geographical locations after eliminating *Wolbachia* sequences.

Fig. S5 Chao1 estimator in different countries after eliminating *Wolbachia* sequences.

Fig. S6 Venn diagram depicting the number of shared and exclusive bacterial OTUs in *H. abietis'* gut across countries and across locations in Sweden.

Fig. S7 Entropy analysis output showing the nucleotide positions with high entropy values present in homopolymer regions.

Fig. S8 Heat map in log scale depicting the PICRUST-inferred relative gene abundance in the bacterial metagenome of *H. abietis* of different sampling locations in the presence of *Wolbachia*.

Table S1 Statistics of 454 pyrosequencing *of Hylobius abietis* gut microbiota after open-reference OTU picking for downstream analyses.

Table S2 Sequences used for the meta-analysis of Curculionidae-associated bacteria.

Table S3 *P*-values of the different statistical analyses performed for assessing differences in microbial community profiles among countries, locations and sexes, based on different distance matrixes.

Table S4 Oligotyping statistics.

Table S5 *P*-values of the different statistical analyses performed for assessing differences in oligotype profiles in different OTUs among countries, locations and sexes, based on a Bray–Curtis similarity matrix.

Table S6 Taxonomic assignment of taxa predicted to harbor genes from the diterpene degradation gene cluster (DDGC).

Table S7 Summary of diterpene degrading genes predicted to occur in close relatives of the core microbes of the pine weevil.