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RESEARCH ARTICLE

Bacterial and fungal symbionts of parasitic *Dendroctonus* bark beetles

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One sentence summary: The parasitic bark beetles *Dendroctonus micans*, *D. punctatus* and *D. valens* house bacterial and fungal communities commonly associated with tree-inhabiting insects, and likely benefit from these symbionts in various ways.

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ABSTRACT

Bark beetles (Curculionidae: Scolytinae) are one of the most species-rich herbivorous insect groups with many shifts in ecology and host-plant use, which may be mediated by their bacterial and fungal symbionts. While symbionts are well studied in economically important, tree-killing species, little is known about parasitic species whose broods develop in living trees. Here, using culture-dependent and independent methods, we provide a comprehensive overview of the associated bacteria, yeasts and filamentous fungi of the parasitic *Dendroctonus micans*, *D. punctatus* and *D. valens*, and compare them to those of other tree-inhabiting insects. Despite inhabiting different geographical regions and/or host trees, the three species showed similar microbial communities. Enterobacteria were the most prevalent bacteria, in particular *Rahnella*, *Pantoea* and *Ewingella*, in addition to *Streptomyces*. Likewise, the yeasts *Candida/Cyberlindnera* were the most prominent fungi. All these microorganisms are widespread among tree-inhabiting insects with various ecologies, but their high prevalence overall might indicate a beneficial role such as detoxification of tree defenses, diet supplementation or protection against pathogens. As such, our results enable comparisons of symbiont communities of parasitic bark beetles with those of other beetles, and will contribute to our understanding of how microbial symbioses facilitate dietary shifts in insects.

Keywords: insect symbiosis; bacteria; fungi; *Dendroctonus*; parasitic bark beetles; ecology

INTRODUCTION

Herbivory by insects is evolutionary derived (Labandeira and Sepkoski 1993) and highly successful given the accelerated rates of speciation after switching to herbivorous diet (Mitter, Farrell and Wiegmann 1988; Farrell 1998). Microbial symbionts play a major role in this transition, because many plant materials are nutritionally imbalanced for herbivorous insects (Watanabe and Tokuda 2010). Symbiotic bacteria and fungi can provide their

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insect hosts with essential capabilities for synthesizing nutrients, overcoming plant defenses, digesting lignocellulosic plant tissue and detoxifying plant defensive chemicals (Douglas 2009; Gibson and Hunter 2010). In addition, microbial symbionts may also help herbivorous insects to protect themselves against microbial competitors, pathogens and/or predators (Flórez *et al.* 2015).

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are one of the most successful herbivorous insect groups with at least 6000 known species worldwide (Knížek and Beaver 2004). Most of these attack woody plant tissues and studied species harbor diverse bacterial and fungal symbionts. As for other insects, these symbionts offer different services to their hosts, which researchers are just beginning to understand (Six 2013; Hofstetter *et al.* 2015). The tremendous diversity of bark beetle feeding habits and ecologies as well as the repeated evolutionary switches between these make bark beetle–microbe symbioses a relevant model for studying the function and evolution of insect symbionts in relation to host ecology (Six and Klepzig 2004; Six 2012; Kirkendall, Biedermann and Jordal 2015).

The relatively small genus *Dendroctonus* (19 described species) is the best studied bark beetle group today, and fungal as well as bacterial symbionts have been studied in several species (Six and Klepzig 2004; Davis 2014; Hofstetter *et al.* 2015). They are fascinating from an evolutionary point of view, because of their diversity of host-use strategies which are expected to strongly shape symbiont communities (Six and Klepzig 2004; Six 2012). *Dendroctonus* species have been classified in three groups depending on their host use: (i) aggressive tree killers (henceforth termed *aggressive beetles*; e.g. *Dendroctonus frontalis, D. ponderosae*), (ii) parasites on living trees that do not kill their host (henceforth termed *parasitic beetles*; e.g. *D. micans, D. punctatus*) and (iii) early successional saprophages on dead or dying trees (henceforth termed *saprophagous beetles*; e.g. *D. approximatus*, although it has also been reported to attack healthy trees) (Lindgren and Raffa 2013; Six and Bracewell 2015). As already observed by Lindgren and Raffa (2013), *D. valens* is somehow unusual, with a flexible status. In its native range, it would usually rather qualify as an early saprophage on the stumps of freshly cut hosts, or on lightning-struck trees, but it can also be parasitic on living trees (Smith 1971). Quite contrastingly, the North American *D. valens* aggressively attacks and kills living trees in China, where it was introduced and became epidemic under climatic conditions stressful for the host trees (Sun *et al.* 2013). On the whole, this species should thus rather be seen as *near parasitic*.

Although all *Dendroctonus* spp. invariably attack conifer hosts, these aggressive, parasitic and saprophagous species are faced with completely different challenges according to the types of hosts they colonize, and are therefore expected to profit from symbionts in different ways (Six and Klepzig 2004; Lindgren and Raffa 2013). Aggressive beetles, for example, would profit from symbionts that help them to overcome the tree defenses and to deal with declining phloem quality (Six and Klepzig 2004; Bleiker and Six 2007). By contrast, parasitic beetles are not expected to associate with symbionts that seriously harm the host tree, but instead with ones that help them to detoxify constitutive and continuously induced chemical tree defenses. The fact that only symbionts of aggressive beetles are well studied, however, currently limits our ability to develop a theoretical framework describing how host-use impacts symbiont communities and their functional roles in *Dendroctonus* (Six and Bracewell 2015).

The filamentous fungi are the best studied symbionts of *Dendroctonus*. Many aggressive beetles are obligately dependent on fungal symbionts, which they transmit from their natal brood system to the new host in highly selective spore-carrying organs, called mycangia, on the exoskeleton (Six 2003). These mycangial fungi supplement the beetles' phloem diet with additional nitrogen and sterols (Ayres *et al.* 2000), and in some cases may help beetles to overcome tree defenses (Six and Klepzig 2004); although the latter role has been questioned recently (Six and Wingfield 2011). Many filamentous fungi can be isolated from bark beetles, but only those found with a high prevalence (notably in mycangia) can be regarded as obligate mutualists. The limited information available on parasitic bark beetles suggests that they do not have mycangia and do not engage in obligate mutualisms with filamentous fungi (Six and Bracewell 2015).

Yeasts are ubiquitous associates of bark beetles, but their functional role for the host is not well understood. In *Dendroctonus*, they are often isolated at high rates, but only few are host-specific (Rivera *et al.* 2009). They can play a beneficial role in beetle pheromone communication (Zhao *et al.* 2015) and some enhance the growth of fungal mutualists while suppressing fungal competitors and/or entomopathogens *in vitro* (Adams *et al.* 2008; Davis *et al.* 2013). Others negatively affect beetles by attracting natural enemies or by producing toxic chemicals (Boone *et al.* 2008). Again, in *Dendroctonus*, yeast communities are mostly described for the aggressive beetles.

Bacterial symbionts of *Dendroctonus* are ubiquitous in beetle guts, mycangia and galleries. Many taxa could provide nutritional benefits to the beetles by accessing sugars from complex polymers, recycling nitrogen from beetle excretions or fixing atmospheric nitrogen (Engel and Moran 2013; Morales-Jiménez *et al.* 2013). Other bacterial symbionts can detoxify host tree defensive compounds (Adams *et al.* 2013; Boone *et al.* 2013), defend their hosts against microbial competitors or pathogens (Cardoza, Klepzig and Raffa 2006; Scott *et al.* 2008) and/or facilitate the growth of the fungal mutualists (Adams *et al.* 2008). Generally, *Dendroctonus* species host relatively species-poor bacterial communities compared to other insects; this is likely due to the protected nature of the habitat and the toxicity of the phloem (Franceschi *et al.* 2005; Six and Bracewell 2015). Many symbionts of *Dendroctonus* are also known as symbionts of plants, likely because of their pre-adaptations to plant defenses. Whether indeed plant symbionts play a role in the beetles' success and what proportion of the insects' symbionts is picked up anew from the tree every generation is currently unknown (despite recent attempts, e.g. Mason, Hanshew and Raffa 2016).

Dendroctonus micans (Kugelann) and *D. punctatus* LeConte are two parasitic species with similar ecologies; *D. valens* LeConte seems to have a different ecology (see above). However, all three can complete their entire life cycle within living hosts: *D. micans* mainly on spruce in Europe and Asia, *D. punctatus* on spruce in Northern USA, Canada and Alaska, and *D. valens* on pines in Mexico, USA and Southern Canada (Smith 1971; Wood 1982; Grégoire 1988; Furniss 1995). A single female (*D. micans* and *D. punctatus*) or pair (*D. valens*) bores a gallery in the inner bark, often near ground level, and lays batches of eggs. Larvae feed gregariously in a communal chamber where frass is accumulated and pupation occurs. Emerging adults either mate with siblings or, in the case of *D. valens*, after dispersal. Microbial symbionts have been studied in several populations of *D. valens* (Six and Klepzig 2004; Rivera *et al.* 2009; Adams *et al.* 2010). Surveys of *D. micans*'s symbionts were limited to culturable bacteria in Turkish populations and ophiostomatoid fungi in one French population (Lieutier *et al.* 1992; Yilmaz *et al.* 2006). Non-culturable bacteria and other fungi have not been investigated in *D. micans*, and

the microbial communities of *D. punctatus* remain completely unknown.

Here we describe an extensive survey of bacterial and fungal symbionts of *D. valens*, *D. micans* and *D. punctatus* using a common set of culture-dependent and independent techniques (454 pyrosequencing). Our first objective is to characterize symbiont communities of three parasitic or near-parasitic *Dendroctonus* species. We further investigate whether these communities change during insect development or lab rearing, and if microorganisms could be taken up from the surrounding phloem. Second, we discuss how symbiont functional differences may be related to respective insect ecologies by comparing our microbial communities of parasitic bark beetles with published data on the communities of aggressive beetles.

MATERIALS AND METHODS

Beetle collection

Field insects

Mature adults and second and third instar larvae (L2-3) were sampled. *Dendroctonus micans* were collected on living trees in Commana, Merdrignac and Scrignac, Brittany, France (48°24′09.60″N 3°56′20.02″W; 48°27′51.71″N 3°38′14.97″W), in April–October 2012 and August 2013. Phloem samples were jointly taken 1 cm left or right from the edge of *D. micans* larval chambers in order to document the bacterial symbionts in the beetles' environment. *Dendroctonus punctatus* were collected on living trees west of Prince George, British Columbia, Canada (53°42′44″N 122°52′26″W; 53°43′04.57″N 122°53′07.01″W; 53°51′06″N 123°12′21″W) in July 2012 and July 2013. *Dendroctonus valens* were collected on fresh stumps and lightning-struck trees east of Redding, California, USA (40°30′28.80″N 121°51′52.77″W; 40°32′43″N 121°46′50″W; 40°43′03.78″N 121°59′27.04″W) in July 2012 and July 2013.

Laboratory insects

Only mature adults were sampled from the laboratory reared populations. *Dendroctonus punctatus* specimens were collected on a living tree north of Kamloops, British Columbia, Canada (51°04′29″N 120°19′52″W) in June 2011, and were reared until the second generation in the LUBIES quarantine room. *Dendroctonus valens* specimens were collected on fresh stumps in the province of Shanxi, China in 2007, and were reared until the 17th generation. As *D. micans* undergoes a reproductive diapause, laboratory adults could not be obtained for this species.

Culture-independent bacterial community analysis

DNA extraction, amplification and sequencing

Samples were stored in 70% ethanol at –80°C until analysis. Insect and phloem samples were taken out of the ethanol and dried under a sterile hood. Individual samples (whole insects or phloem) were flash frozen with liquid nitrogen and homogenized with a pestle. DNA extraction was conducted using the Epicentre MasterPure DNA Purification kit (Madison, Wisconsin, USA) in accordance with the manufacturer's instructions, including a treatment with lysozyme to break up Gram-positive bacterial cell walls. After DNA extraction, identical volumes of six individuals (except n = 4 for *D. micans* field larvae and n = 5 for *D. punctatus* field adults) or four phloem pieces (each 8 × 2 × 2 mm) were pooled per group for bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Pooled DNA samples were sent to 'Molecular Research Laboratory' (Shallowater, TX, USA) for bTEFAP with 16S rRNA primers Gray28F forward 5′-GAGTTTGATCNTGGCTCA-3′ and Gray519R reverse 5′-GTNTTACNGCGGCKGCTG-3′ (Sun, Wolcott and Dowd 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of HotStar and HotStar HiFidelity Taq polymerases (Qiagen, Hilden, Germany). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Molecular Research Laboratory (http://mrdnalab.com/).

454 sequencing data analysis

QIIME (i386 1.3.0-3) was used for analysis of the 454 sequencing data (Caporaso *et al.* 2010). All low-quality reads (minimum average quality cut-off = 25) were removed, as well as sequences shorter than 200 bp and longer than 600 bp. No errors in the barcode but one mismatch and one ambiguous base per sequence were allowed. USEARCH6.1 (QIIME) was used to identify and discard potential chimeras. The remaining reads were analyzed using a multistep operational taxonomic unit (OTU)-picking strategy with the cdhit (Li and Godzik 2006) algorithm set at 97% similarity cut-off. The representative sequences per OTU were determined by picking the most abundant sequence, and were aligned to the Greengenes core set (http://greengenes.lbl.gov/) with PyNast using a minimum sequence identity of 75%. Taxonomy was assigned using the uclust classifier and only sequences with more than 0.8 confidence were selected for further analyses. Finally, an OTU table was generated with the absolute and relative abundances of bacterial phylotypes within the samples, which is visualized in a heat map constructed with MultiExperiment Viewer 4.9.0 (Saeed *et al.* 2003). Chloroplast and mitochondria sequences were manually removed from the OTU table.

Alpha- and beta-diversity indices were calculated in QIIME based on the OTU table of absolute and relative abundances per sample. Rarefaction curves were generated by subsampling the OTU table with step increments of 10 sequences and 100 iterations to check for adequate sampling depth. Weighted and unweighted UniFrac distances were subjected to principal coordinates analyses (PCoA) to assess clustering of bacterial communities according to insect species, instar, phloem and laboratory-reared versus field samples. ANOSIM discriminant analyses between these groups were performed in QIIME using the unweighted and weighted UniFrac principal coordinates.

Phylogenetic placement of Dendroctonus *microbiota*

OTUs from the three sampled *Dendroctonus* spp. were compared with the bacterial communities of other bark beetle studies that used culture-independent methods. Our data set on *Dendroctonus*-associated bacteria was expanded by bacterial symbiont sequences of tree-inhabiting insect host species belonging to four genera (308 sequences from GenBank; Table S1, Supporting Information; see also Berasategui *et al.* 2016). All sequences were aligned using SINA 1.2.11 (Pruesse, Peplies and Glockner 2012), imported into ARB 5.5 (Ludwig *et al.* 2004) and then mapped onto the 16S SILVA rRNA database (which currently includes 597 607 curated bacterial sequences). Two to three OTUs, neighboring our OTUs, were then picked up from the SILVA database to construct a tree with our sequences and the quality checked bacterial sequences from SILVA (total 551 sequences; Table S1, Supporting Information). The quality of the alignment for each OTU sequence was manually checked and corrected. A total of 32 of the initial 182 OTUs that were imported in ARB did not fit among the 16S SILVA sequences and were excluded from our tree. The final tree was constructed with the remaining 150 OTUs in FastTree 2.1 using the GTR model (Price *et al.* 2010) and was edited in MEGA 6 (Tamura *et al.* 2011).

Culture-dependent microbiota analysis

Bacterial and fungal isolation

Live insects (n = 6) were individually surface-sterilized in 70% ethanol, rinsed in distilled water and crushed as a whole in 500 μL phosphate buffer solution with a Retsch MM301 grinder and beads (Haan, Germany). The supernatant was serially diluted 10-fold in 10 mM $MgSO_4$ (up to 10^{-8}). Dilutions were plated (n = 2) on potato dextrose agar (PDA), yeast extract malt agar (YEMA) and nitrogen-depleted medium described in Emtiazi, Pooyan and Shamalnasab (2007) to detect potential nitrogen-fixing bacteria. Plates were incubated at 30°C for optimal growth (1 to 14 days), and colony forming units (CFU) were counted based on plated dilutions. Pure cultures of all morphotypes were obtained and preserved in glycerol at −80°C. Yeasts were discriminated from bacteria microscopically.

DNA extraction, amplification and sequencing

Typical 2–3 mm diameter colonies of bacteria and yeasts were transferred to a cell lysis solution (67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 5 mM b-mercaptoethanol, 6.7 mM $MgCl_2.6H_2O$, 6.7 μM EDTA pH 8.0, 1.7 μM SDS), while filamentous fungi were grown on cellophane and ground in liquid nitrogen for DNA extraction using the MasterPure DNA Purification kit (Epicentre).

Bacterial 16S amplification

For bacterial isolates, the 16S rRNA gene was amplified with primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg *et al.* 1991) using the following PCR conditions: 94°C for 3 min; 35 cycles of 94°C for 40 s, 60°C for 1 min, 72°C for 1 min and a final extension of 72°C for 4 min. PCR was conducted with 25 U mL^{-1} TopTaq DNA polymerase and 1x TopTaq PCR buffer (Qiagen), 0.2 mM of each dNTP, 0.2 μM of each primer and typically 2.3-5 ng μL^{-1} of template DNA. PCR products were purified using 1.54 U μL^{-1} Exonuclease I (Epicentre) and 0.15 U μL^{-1} FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific, Erembodegem, Belgium), and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) with the same primers and additionally the primer R1087 (5′-CTCGTTGCGGCACTTAACCC-3′) for the 16S rDNA.

Fungal 18S and ITS amplification

For fungal isolates, two genetic markers were sequenced. First, the 18S rRNA gene was amplified with the primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTYCTTTAAG-3′) (White *et al.* 1990) using the following PCR conditions: 94°C for 30 s; 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min; and 72°C for 7 min. Second, the ITS region was amplified with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990) with PCR conditions as follows: 92°C for 2 min; 41 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 8 min (Six *et al.* 2009). PCR and sequencing mixtures were the same as for bacteria, except that primer couples NS1–NS4 and ITS1–ITS4 were used.

BLAST

Consensus sequences were assembled in Geneious Pro 4.8.5 (Biomatters, Auckland, New Zealand) and blasted against the GenBank database. Taxa were considered identified at the species level with a match level of at least 97% identity, at the genus level with a match of at least 95%, and were considered unidentified below 95%. Phylogenetic relationships among isolated strains and closest BLASTn matches were constructed using MOLE-BLAST with the neighbor-joining method.

RESULTS

Culture-independent bacterial community analysis

Our metagenomic analysis of *Dendroctonus micans*, *D. punctatus* and *D. valens* field adults, larvae, phloem and laboratory-reared adults yielded a total of 169 107 sequences (after quality trimming), which were clustered into 182 OTUs. OTUs were assigned to the level of 56 different genera, 57 families, 32 orders and 20 classes of bacteria. In the relative abundance heat map of the 50 most abundant OTUs (Fig. 1), OTUs 230 (*Luteibacter*), 124 (*Pseudomonas*), 251 (*Ralstonia*), 216 (*Erwinia*), 50 (other unidentified Xanthomonadaceae), 166 and in particular 52 (both other unidentified Enterobacteriaceae) were the most conserved among the three studied parasitic or near-parasitic bark beetles. Within *D. punctatus*, the prominent *Erwinia* (OTU 216) was almost absent from the second generation of lab-reared adults while an unidentified enterobacterium (OTU 52, close to *Rahnella* spp.) was 3.5 times as abundant as in field adults. Similarly, the rare enterobacterial OTU 123 (also close to *Rahnella* spp.) was more than 10 times as abundant and many less abundant OTUs were completely absent in the 17th generation lab-reared *D. valens*, in comparison with the field population.

Except *D. micans* field adults, whose community was predominated by Firmicutes (*Lactococcus*), all other samples mainly harbored Gammaproteobacteria (Fig. 2). Depending on the sample, these were mainly *Acinetobacter* (only in *D. micans* field larvae and nearby phloem) or *Erwinia* and other unidentified genera in all others. Betaproteobacteria (*Ralstonia* and other Comamonadaceae) were common only in *D. punctatus* and *D. micans* field larvae and nearby phloem. Rarefaction analyses demonstrated sufficient sampling depth for all our samples (Fig. S1, Supporting Information). Field-collected *D. micans* hosted less bacterial OTUs (25 ± 5 OTUs, mean ± SD) than *D. punctatus* (57 ± 23) and *D. valens* (46 ± 6) (Table 1). Field-collected adults and larvae were more species rich (45 ± 3) than those reared in the laboratory (25 ± 9).

The weighted PCoA components explained a total of 82.8% of the variance in the OTUs' relative abundance (Fig. S2, Supporting Information). All instars of *D. punctatus* and *D. valens* clustered together, except *D. punctatus* field larvae, which were a bit closer to another cluster composed of *D. micans* field larvae and nearby phloem. *Dendroctonus micans* field adults were distant from all other clusters. The unweighted PCoA components explained 45.7% of variance (Fig. S3, Supporting Information). There was no effect of species (ANOSIM $P = 0.71$) and origin ($P = 0.42$) on the bacterial communities based on unweighted UniFrac. However, a significant effect of stages ($P = 0.023$) on bacterial communities was found. This analysis needs to be interpreted with care since (i) abundance of OTUs lead to important differences between the weighted and unweighted PCoA plots (Figs S2 and S3, Supporting Information), and (ii) ANOSIM on weighted UniFrac values only detected a tendency for clustering for species ($P = 0.056$) but not for origin ($P = 0.39$) and stage ($P = 0.39$).

The phylogenetic position of the three sampled *Dendroctonus* species' OTUs is shown in comparison with the bacterial community of other bark- and wood-inhabiting insects, and bacteria isolated from the environment (Table S1; Figs. 3 and S4, S5, Supporting Information). The *Dendroctonus* OTUs were close

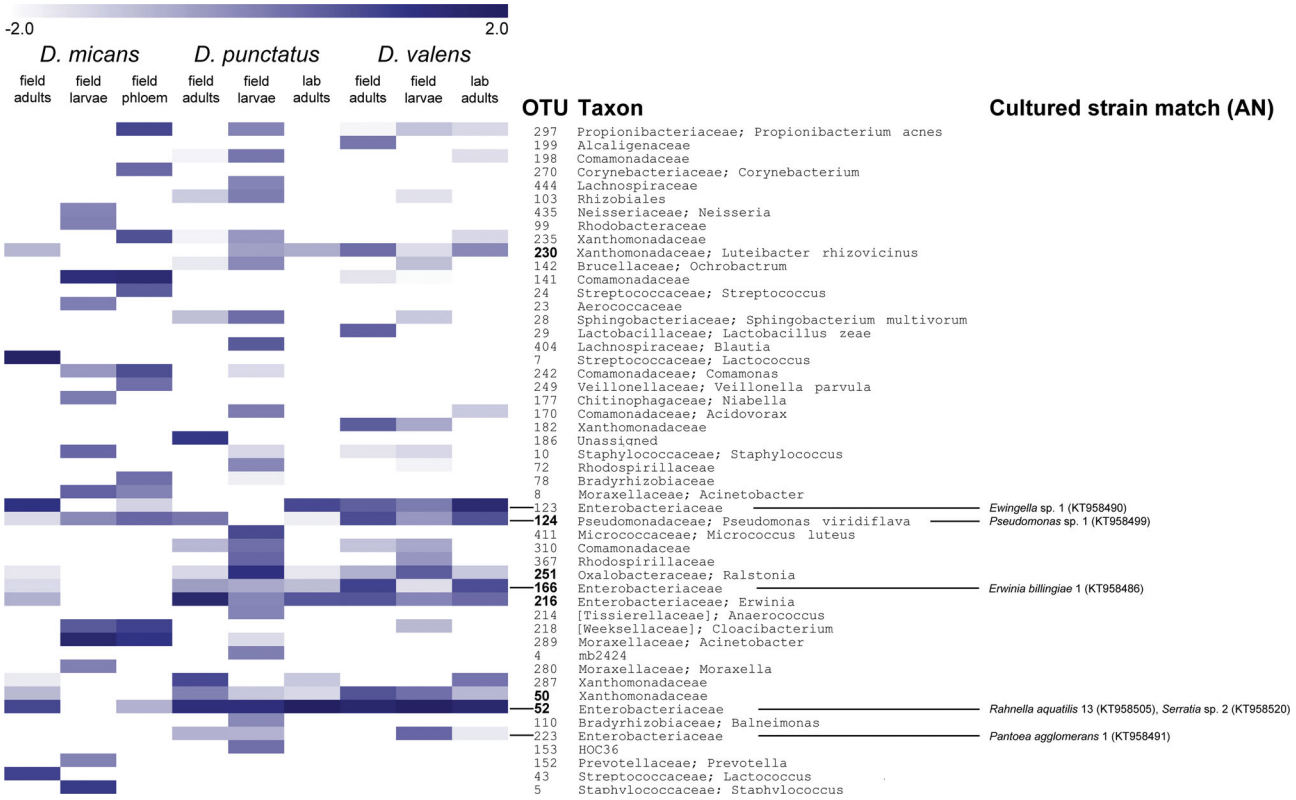

Figure 1. Relative abundance heat map (log-transformed, white = absence, dark blue = high) of the 50 most abundant pyrosequenced bacterial OTUs (based on the cumulative relative abundance among all groups) in *D. micans*, *D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem. Bold OTUs are the most conserved among all samples of the three beetle species, lines denote OTUs with corresponding isolates (i.e. identity > 99.3%) and accession numbers (AN) that we gained by culturing on PDA, YEMA and/or nitrogen-depleted media.

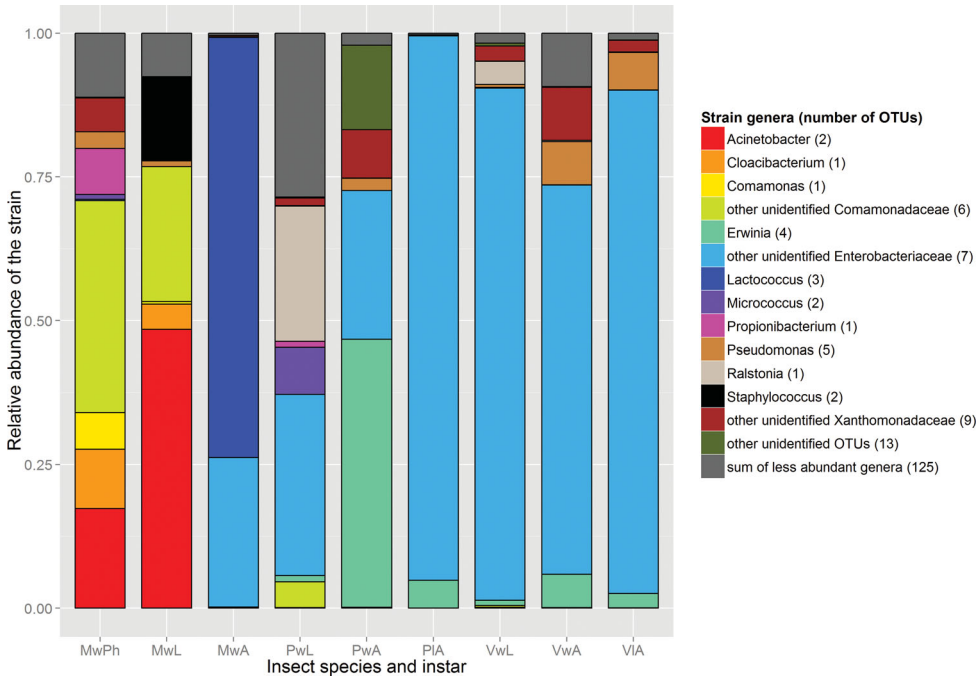

Figure 2. Relative abundance of pyrosequenced bacterial genera in *D. micans* (M), *D. punctatus* (P) and *D. valens* (V) field (w) and laboratory (l) larvae (L), adults (A) and distant phloem (Ph).

Table 1. Chao1 alpha-diversity indices of pyrosequenced bacterial OTUs (at equal sampling depth of 1762 sequences) in *D. micans*, *D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem.

Species	Origin	Instar	Total N of OTUs
Dendroctonus micans	Field	Adults	30
	Field	Larvae	20
	Field	Phloem	24
Dendroctonus punctatus	Field	Adults	42
	Field	Larvae	72
	Lab	Adults	15
Dendroctonus valens	Field	Adults	48
	Field	Larvae	45
	Lab	Adults	34

neighbors of bacteria from 16 different host insects belonging to 11 genera, in particular the bark beetles *Ips pini*, *D. frontalis* and to a lesser extend *D. rhizophagus*. There was an overlap of OTUs with previous studies on *D. valens*; for *D. micans* and *D. punctatus*, no culture-independent data on bacterial communities were available for comparison. Nevertheless, most OTUs were closely related to bacteria that were originally not isolated from insects, and were rather free-living bacteria.

Culture-dependent microbiota analysis

A total of 116 bacterial and 83 fungal isolates were cultured from *D. micans*, *D. punctatus* and *D. valens* field adults and larvae, including 37 and 27 identified strains, 16 and 18 species, and 9 and 7 genera, respectively (Table S2, Supporting Information).

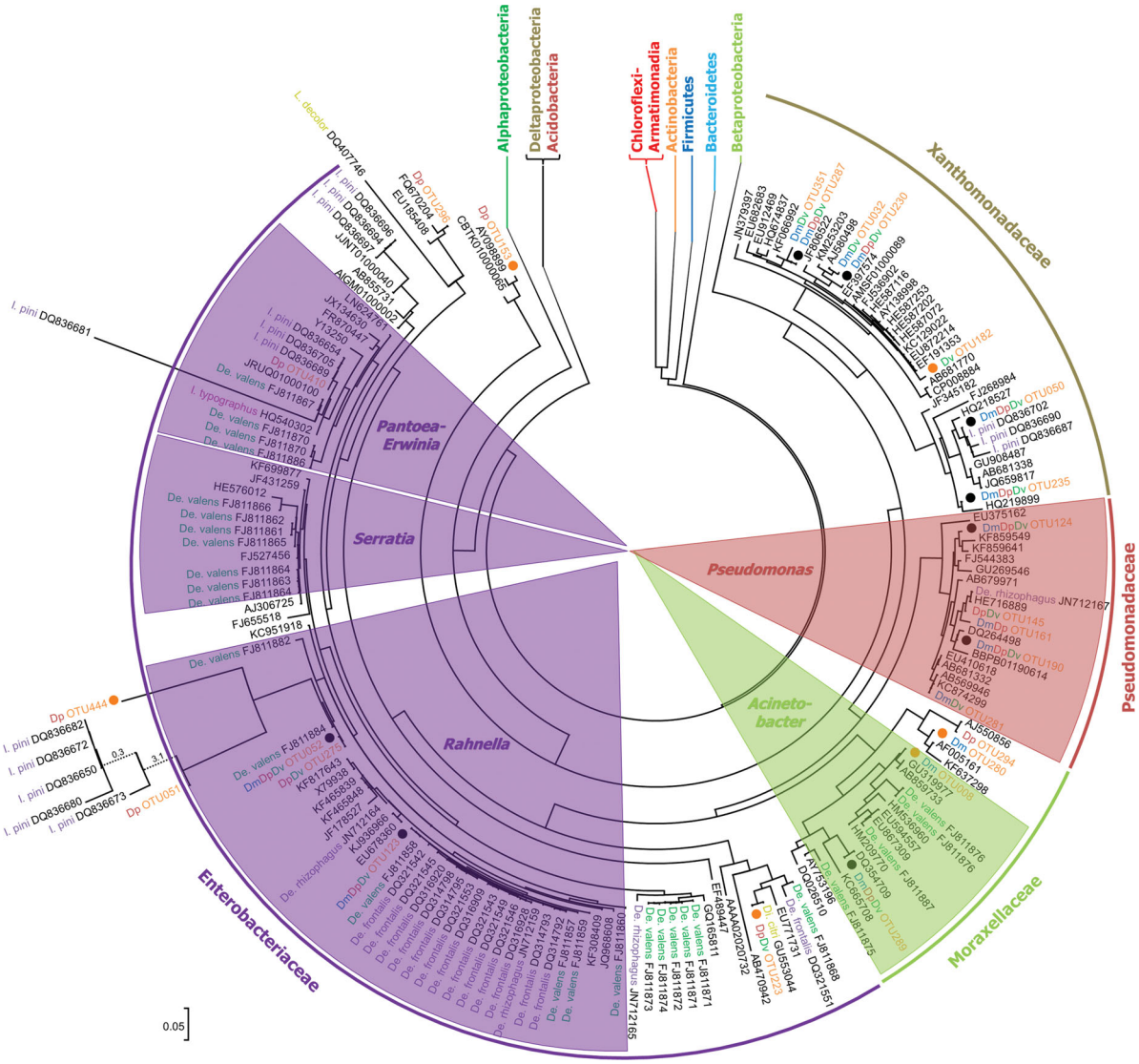

Figure 3. Phylogenetic tree of representative sequences of pyrosequenced bacterial OTUs associated with *D. micans*, *D. punctatus* and *D. valens* in relation to the symbionts of other insects and environmental bacteria. Only Gammaproteobacteria are shown, all other taxa are collapsed. Taxa from this study are highlighted in orange; the 50 most abundant OTUs are indicated by an orange dot; OTUs present in at least one instar of all three species are indicated by a black dot. Black accession numbers are provided for taxa from other studies. The insect host, when known, is attached to the taxa, where Dm = *D. micans* (in blue), Dp = *D. punctatus* (in red), Dv = *D. valens* (in green), A. = *Apis*, Ce. = *Cephalotes*, Cr. = *Cryptopone*, De. = *Dendroctonus*, Di. = *Diaphorina*, I. = *Ips*, L. = *Liposcelis*, M. = *Moechotypa*, R. = *Reticulitermes*, S. = *Saccharococcus* and X. = *Xylosandrus*. Spruce-feeding insects (except *D. micans* and *D. punctatus*) are in pink, pine-feeding insects (except *D. valens*) are in violet, and other insect hosts are in yellow. There is no mention of insect host for bacteria isolated from the environment. The length of plain branches represent the genetic distance according to the scale, dotted branches could not be plotted in full size and have their true genetic distance indicated.

Table 2. Identity and prevalence (% of sampled individuals) of bacterial and fungal isolates that we gained by culturing on PDA, YEMA and nitrogen-depleted media from *D. micans*, *D. punctatus* and *D. valens* field larvae and adults. Asterisk indicates isolates that were able to grow on nitrogen-depleted medium, = = denotes synonyms, / indicates assignments equally closely related.

Prevalence (%)	*Dendroctonus micans*		*Dendroctonus punctatus*		*Dendroctonus valens*	
Identified strains	Larvae	Adults	Larvae	Adults	Larvae	Adults
Bacteria						
Enterobacter sp.	0	0	0	0	0	33
Erwinia billingiae	0	0	0	0	0	17
Erwinia sp.	0	0	0	0	17	0
Erwinia typographi	0	0	0	33*	33*	0
Ewingella sp.	0	0	0	83*	33	0
Pantoea agglomerans	0	17*	0	0	0	67*
Pantoea cedenensis	0	0	67*	33*	50*	50*
Pseudomonas sp. 1	0	0	0	0	17*	33
Pseudomonas sp. 2	0	0	0	0	17*	0
Rahnella aquatilis	67*	100*	17*	0	83*	67
Rouxiella sp.	0	67*	0	0	0	0
Serratia liquefaciens	0	0	0	17	0	0
Serratia marcescens	0	0	0	0	17*	0
Serratia sp. 1	0	0	0	0	0	33*
Serratia sp. 2	0	17*	0	0	0	0
Streptomyces lienomycini	0	0	67	17	0	0
Fungi						
Acanthophysium cerussatum/Stereum gausapatum	17*	0	0	17*	0	0
Candida piceae	33	0	33	17	33	67
other unidentified *Candida* spp.	83*	33*	0	17*	0	67*
Candida sp. 1/*Candida* sp. 3	33*	17	0	0	0	17
Candida sp. 2/*C. fructus*	50*	67*	0	17*	0	0
Cyberlindnera americana/Candida sp. 3	33	17	0	0	33	17*
Hypocrea muroiana/Trichoderma viride	0	0	83*	0	0	0
Penicillium charlesii/P. pulvis	0	0	17*	0	0	17
Penicillium chrysogenum	0	17*	0	0	0	0
Penicillium corylophilum	0	17*	0	0	0	0
Penicillium decumbens	17	0	0	0	0	0
Penicillium purpurogenum = = *Talaromyces purpurogenus*	0	0	17	0	0	0
other unidentified *Penicillium* spp.	17*	0	17	17*	0	0
Penicillium sp. 1/*Talaromyces radicus*	0	0	17	0	0	0
Penicillium sp. 2/*Talaromyces variabilis*	17*	50*	0	0	0	17*
Pichia bispora/Yeast sp. 5	0	0	67	0	0	0
Fungus sp. 8	0	17	0	0	0	0
Yeast sp. 7	0	0	0	0	0	17

Bacterial community

The mean bacterial population was 1.7×10^7 CFUs/adult and 6.0×10^5 CFUs/larva (Figs S6 and S7, Supporting Information; Table 2). *Rahnella aquatilis* was the most widespread bacterium; it consistently inhabited all instars of *D. micans* and *D. valens* (≥ 67% of prevalence). Similarly, *Pantoea cedenensis* was present in at least 50% of *D. punctatus* larvae and all instars of *D. valens*. Other very prevalent taxa were *Ewingella* sp. in 83% of *D. punctatus* adults, *Streptomyces lienomycini* in 67% of *D. punctatus* larvae, *Pa. agglomerans* in 67% of *D. valens* adults and *Rouxiella* sp. in 67% of *D. micans* adults.

Fungal community

The mean fungal population was 4.9×10^7 CFUs/adult and 2.9×10^4 CFUs/larva. Overall, yeasts were the most abundant fungi (Figs 4, S8 and S9, Supporting Information; Table 2), especially *Candida/Cyberlindnera* spp. They were isolated from all three insect species and dominated particularly in *D. micans* and in *D. valens* field adults (≥67% of samples). These yeasts were less abundant in *D. punctatus* field larvae, where *Hypocrea muroiana/Trichoderma viride* (83%) and *Pichia bispora/Yeast* sp. 5 (67%) were found instead. *Penicillium/Talaromyces* spp. were the most abundant filamentous fungi and were only absent in *D. valens* field larvae. Despite being ubiquitous, these were inconsistent associates among the insects, except *Penicillium* sp. 2/*Talaromyces variabilis* in *D. micans* field adults (50%).

Complementarity of culture-dependent and independent analyses

Bacterial 16S sequences from cultured strains and pyrosequenced OTUs were aligned for potential matches between both methods. Seven OTUs (Fig. 1; Table S2, Supporting Information) corresponded with six different Enterobacteriaceae species (*Erwinia billingiae*, *E. typographi*, *Ewingella* sp. 1, *Pa. agglomerans*, *Ra. aquatilis*, *Serratia* sp. 2) and two Pseudomonadaceae species

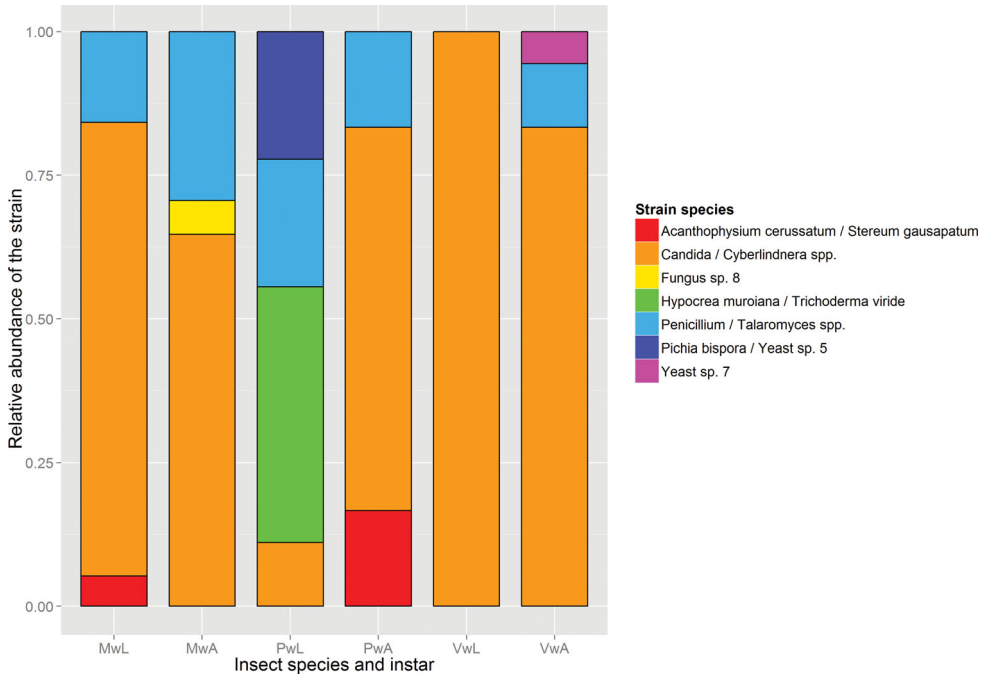

Figure 4. Relative abundance of cultured fungal taxa in *D. micans* (M), *D. punctatus* (P) and *D. valens* (V) field (w) larvae (L) and adults (A).

(*Pseudomonas* sp. 1 and 2), accounting for a total of 101 305 sequences (59.9% of sequences from all OTUs).

DISCUSSION

In this study, we characterized the bacterial and fungal communities associated with three bark beetles, *Dendroctonus micans*, *D. punctatus* and *D. valens*, which have a similar parasitic or near-parasitic ecology. Despite inhabiting distinct geographical regions and/or different host trees, these insects shared a large proportion of their microbial symbionts: (1) Enterobacteriaceae (Gammaproteobacteria) was the dominant bacterial family with the most prevalent species *Rahnella aquatilis*, *Pantoea cedenensis*, *Ewingella* sp., *Pa. agglomerans* and *Rouxiella* sp. (although this one is very close to *Ra. aquatilis*). Enterobacteria, especially the aforementioned genera and species, are often reported as symbionts of bark- and wood-boring insects (Morales-Jiménez *et al.* 2009, 2012; Hu *et al.* 2013; Aylward *et al.* 2014; Xu *et al.* 2015; Berasategui *et al.* 2016; Mason, Hanshew and Raffa 2016). (2) *Streptomyces lienomycini* was commonly isolated from *D. punctatus*. *Streptomyces* bacteria are frequent associates of North American bark beetles (Hulcr *et al.* 2011). (3) Yeasts were the most abundant fungi, especially *Candida/Cyberlindnera* spp. in *D. micans* and *D. valens*, whereas *D. punctatus* also commonly harbored *Pichia bispora*. Likewise, these yeasts are frequent symbionts of bark and ambrosia beetles (Rivera *et al.* 2009; Davis 2014; Lou, Lu and Sun 2014).

All these prevalent microorganisms are close relatives of common, free-living bacteria (Figs 3, S4 and S5, Supporting Information) and yeasts. *Rahnella aquatilis*, *Ewingella* spp., *Pa. agglomerans* as well as *Candida* spp. are regularly isolated from soil, water and also from plants as epiphytes and endophytes (Weinthal *et al.* 2007; Winder, Macey and Cortese 2010; Ribeiro and Cardoso 2012; Hu, Li and Chen 2015). The latter means that they likely occur in the vicinity of bark- and wood-boring beetles, which may explain why they are so frequently isolated from these insects (Colman, Toolson and Takacs-Vesbach 2012). As a striking illustration of endophyte intake, *D. micans* larvae housed bacteria very similar to nearby phloem, with prominent *Acinetobacter* and Comamonadaceae. Our phloem samples from just *D. micans*, however, have low explanatory power in this respect and it remains unknown which microorganisms are present in the phloem already before bark beetles establish their galleries there (Mason, Hanshew and Raffa 2016). Nevertheless, even if their associated microbes can occur in the environment, this does not exclude possible functional roles for the performance of *D. micans*, *D. punctatus* and *D. valens*. Facultative symbionts can make important contributions to host fitness, even when they are acquired from the environment (Kikuchi, Hosokawa and Fukatsu 2007; Engel and Moran 2013).

Overall, the bacterial community associated with (near-) parasitic bark beetles does not seem very constant. Unlike *D. punctatus*, the bacterial communities of *D. valens* and *D. micans* have already been studied before and greatly vary geographically (Yilmaz *et al.* 2006; Morales-Jiménez *et al.* 2009; Adams *et al.* 2010; Xu *et al.* 2015; this study). In *D. valens*, the bacterial community from the Californian population we sampled (where *D. valens* qualifies as a saprophagous-parasitic species) was much more distant to the one from the Northern USA (Adams *et al.* 2010) than to the ones from Mexico (Morales-Jiménez *et al.* 2009) or from invasive populations in China (although *D. valens* behaves aggressively there; Xu *et al.* 2015). Despite some geographical variation, *Rahnella* and *Pantoea* were consistent symbionts throughout all four studies. In the bacterial community of *D. micans*, there is only a small overlap (mainly *Serratia*) in the identified taxa from France (this study) and from Turkey (Yilmaz *et al.* 2006). By contrast to these parasitic beetles, the bacterial community of the aggressive *D. ponderosae* is much more stable (Adams *et al.* 2013). Variable bacterial communities could be related to rather opportunistic, loose symbiotic associations, with little effect for their host (Engel and Moran 2013). However, symbiont redundancy, where different microorganisms play similar roles (Six 2012), could also explain this pattern. Throughout

the developmental stages, the bacterial community of *D. valens* was very constant, but the ones of both *D. micans* and *D. punctatus* were quite distinct between field-collected adults and larvae. Several non-exclusive hypotheses could explain such community changes during the developmental stages: (i) successive molts disrupt the gut bacterial flora (Engel and Moran 2013); (ii) adults and larvae feed on different parts of the phloem, which may be of different quality (e.g. in terms of nutrition and defense compounds; Franceschi *et al.* 2005); (iii) larvae and adults select different symbionts according to their needs; (iv) temporal turnover of the symbionts (unlike *D. punctatus*, *D. micans* larvae and adults were collected in the same place but at different times).

The bacterial species richness was much lower in laboratory-reared adults in comparison with field insects. In addition, we observed differences in the relative abundance of several bacterial OTUs. These results are similar to those of Meeus *et al.* (2015) who observed that *Bombus terrestris* reared indoors had a subset of the bacterial community compared to field insects. It is possible that the differences in the bacterial communities result from the rearing conditions and could, in turn, affect the beetles' physiology. In many insects, the gut community is strongly influenced by the diet (Engel and Moran 2013). In our laboratory conditions, insects were reared on fresh logs and phloem sandwiches that are likely to differ from a standing, living tree (i.e. moisture content, toxic plant compounds and general food quality; Klepzig and Six 2004; Six and Klepzig 2004) and which different microorganisms may exploit. Alternatively, in the specific case of *D. valens*, the community change between field and lab adults may be related to the different collection places.

The fungal community of *D. micans*, *D. punctatus* and *D. valens* was dominated by widespread environmental yeasts and some ubiquitous filamentous fungi. Not a single ophiostomatoid fungus was isolated. Most aggressive *Dendroctonus* spp. are tightly associated with these fungi (Six and Bracewell 2015). In its native range, *D. valens* behaves like a saprophage and parasite, and is occasionally associated with ophiostomatoid fungi, although with high variability of prevalence (Six and Klepzig 2004, Sun *et al.* 2013). In China, where *D. valens* aggressively attacks living trees, it is associated with phytopathogenic strains of *Leptographium procerum* and other ophiostomatoid species, however, with a very low prevalence (Lu *et al.* 2009, 2010; Sun *et al.* 2013). These insect–fungus interactions are very complex: *D. valens* could benefit from symbiotic nutritional supplementation or help in overcoming the tree defenses (Sun *et al.* 2013), but feeding experiments demonstrated that *L. procerum*, and other ophiostomatoid associates of *D. valens*, compete with the insects for polysaccharides in the phloem and trigger an immune response in the beetles, which resulted in lower weight gain in larvae (Shi *et al.* 2012; Wang *et al.* 2013). Such a competition for the available resources might be one reason explaining why true parasitic beetles are not consistently associated with ophiostomatoid fungi specifically, or filamentous fungi in general, which in this case are regarded as opportunistic associates (Lieutier *et al.* 1992; Raffa, Phillips and Salom 1993; Six and Bracewell 2015). Another reason may be the secondary metabolites of living trees that are toxic to both insects and fungi (Raffa and Smalley 1995; Franceschi *et al.* 2005; Krokene 2015), but to which parasitic beetles evolved a high resistance (Everaerts, Grégoire and Merlin 1988). Last, it is also counteradaptive for parasitic beetles that derive large benefits from their life in living trees (i.e. a stable environment protecting them from generalist natural enemies and from competitors) to inoculate their galleries with fungi that have been shown (i) to trigger higher induced tree defenses, which harm the beetles (Raffa and Smalley 1995), or even (ii) kill the tree. Future surveys should focus on the prevalence and possible roles of ophiostomatoid associates of parasitic bark beetles, which could in turn improve our knowledge on those of aggressive bark beetles.

Parasitic bark beetles live in and feed on phloem that is loaded with constitutive and induced plant defensive compounds. Therefore, symbionts that are able to assist in detoxification should be very beneficial to the beetles, like *Ra. aquatilis* (Boone *et al.* 2013; Xu *et al.* 2015) and *Candida/Cyberlindnera* spp. (Rivera *et al.* 2009; Lou, Lu and Sun 2014) which we both isolated at high rates in our study. By-products of such microorganisms may also play a role in the intra- or interspecific chemical communication of the beetles (Boone *et al.* 2008; Zhao *et al.* 2015). Bark beetles could also benefit from symbionts by protection and improvement of their nutritional niche. Living phloem is lacking many nutrients, like nitrogen sources and sterols (Merrill and Cowling 1966; Bentz and Six 2006). In this context, associated microorganisms could provide a significant advantage to parasitic bark beetles. First, several prevalent bacteria were isolated on nitrogen-depleted medium in this study and are known as nitrogen fixing (diazotroph): *Ra. aquatilis* (Vasanthakumar *et al.* 2006; Morales-Jiménez *et al.* 2009) and *Pa. agglomerans* (Bridges 1981). Moreover, *NifH* genes were amplified from the microbial community of *D. micans*, *D. punctatus* and *D. valens* (Dohet and Biedermann, unpublished data). Likewise, *Ra. aquatilis* is also reported as nitrogen recycling (uricolytic; Morales-Jiménez *et al.* 2013). Second, cellulolytic microorganisms were detected in preliminary tests on Congo red agar (Dohet, unpublished data), which may enrich the beetles' phloem diet with additional free carbohydrates. Furthermore, microorganisms such as the isolated actinomycetes *Micrococcus luteus* or *Streptomyces* spp. could shape beetles' microbial communities by producing antibiotics as shown for strains of these taxa in *D. rufipennis* and *D. frontalis*, respectively (Cardoza, Klepzig and Raffa 2006; Scott *et al.* 2008).

Our study suggests that parasitic bark beetles lack consistent association with highly phytopathogenic fungi but could benefit, at the same time, from other symbionts. It also discloses striking differences between parasitic bark beetles, two of which (*D. micans* and *D. punctatus*) almost never kill their hosts and are inconsistently found associated with ophiostomatoid fungi (*D. micans*; Lieutier *et al.* 1992) or have never been studied so far in this respect (*D. punctatus*). On the other hand, some near-parasitic bark beetles, like *D. valens*, sometimes massively kill trees and harbor ophiostomatoid fungi with a low prevalence in this case (Lu *et al.* 2009). *Dendroctonus murrayanae*, which also qualifies as parasitic, has been observed killing trees (Wood 1982) and is consistently associated with ophiostomatoid fungi (Six *et al.* 2011). In summary, we believe that these important differences in the ecology of parasitic beetles call for a substantial revision of this whole group, distinguishing true parasites from near parasites.

This is the first comprehensive characterization of the bacterial and fungal symbionts of the (near-) parasitic bark beetles *D. micans*, *D. punctatus* and *D. valens*, using a combination of culture-dependent and independent methods. Many of the close relatives of the identified taxa have been previously characterized as free-living microorganisms, but others are known as symbionts of plants, bark beetles and other wood-boring insects. Parasitic and near-parasitic bark beetles could benefit from these symbionts in various ways, through detoxification of tree defenses, diet supplementation and/or protection against pathogens. Due to the high variability of associated bacteria and fungi, future studies should sample

all developmental stages of parasitic bark beetles more intensively from various populations and at different times to identify a potential core microbiota, spatiotemporal patterns and their concrete effects on their hosts. This sampling should also take the surrounding phloem and wood into account, which will help to clarify which microbes are present within the tree beforehand and are not inoculated by the beetles. Such studies will help to understand the roles of symbionts in shifts of ecologies and hosts in bark beetles, which are a great model for the development of a broad theoretical framework on the function and evolution of bacterial and fungal symbionts in insects as a whole.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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