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Bacterial Communities in Central European Bumblebees: Low Diversity and High Specificity

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Abstract Recent studies on the microbial flora of the honeybee gut have revealed an apparently highly specific community of resident bacteria that might play a role in immune defence and food preservation for their hosts. However, at present, very little is known about the diversity and ecology of bacteria occurring in non-domesticated bees like bumblebees, which are of similar importance as honeybees for the pollination of agricultural and wild flowers. To fill this gap in knowledge, we examined six of the most common bumblebee species in Central Europe from three locations in Germany and Switzerland for their bacterial communities. We used a culture-independent molecular approach based on sequencing the 16S rRNA gene from a selection of individuals and examining a larger number of samples by terminal restriction fragment length polymorphism profiles. The gut flora was dominated by very few and mostly undescribed groups of bacteria belonging to the Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria. This core set of bacteria was present in all of the examined bumblebee species. These bacteria are similar to, but distinct from, bacteria previously described from the honeybee gut. Significant differences were observed between the communities of bacteria in the different bumblebee species; the effect of sampling location was less strong. A novel group of Betaproteobacteria additionally shows evidence for host species-specific genotypes. The gut flora of bumblebees therefore is apparently composed of relatively few highly specialized bacteria, indicating a strong interaction and possibly important functions with their hosts.

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Introduction

Bacterial communities can be important for many vital functions of the host organism, such as digestive efficiency [20, 26, 33], for example in herbivorous insects [7, 38]; for the defence against major enemies [67]; or through interactions with infecting pathogens [13, 14]. We are, however, still lacking a good understanding of the diversity and distribution of bacterial communities in most naturally occurring organisms. For example, wild bees provide important pollination services and are known to host a number of potentially important bacteria in their gut. But so far, the existing studies have only been looking at a few individual hosts of few species [44, 51, 54] or limited the sampling to the genus *Bifidobacterium* [42, 43]. We here enlarge this database for a prominent group of large pollinators—the bumblebees, *Bombus* spp.

Bumblebees are a group of eusocial hymenoptera, most abundant in temperate and cold regions of the world [69]. Pollinating a variety of wild and agricultural flowering plants, they provide important ecosystem services [23]. They are also commercially bred for the pollination of greenhouse plants, most notably for the pollination of tomatoes [66]. Over recent years, a decline in the abundance and range of bumblebee species has been noted in several parts of the world [24, 70]. Whereas man-made changes of natural ecosystems are likely playing a major role in this decline, the spillover of new parasites into wild bumblebee populations caused by the international trade with commercially bred bumblebee colonies has been discussed as a further important factor as well [11, 24].

So far, the ecology and effect of eukaryotic bumblebee parasites, such as the microsporidian *Nosema bombi* and the trypanosomatid *Crithidia bombi*, have been studied intensively (e.g. 16, 55, 60]. In contrast, the diversity and ecology of prokaryotic organisms associated with bumblebees, such as bacteria, is much less known. As a yardstick, recent studies have shown a specialized community of bacteria inhabiting the honeybee gut [4, 12, 53]. This included lactic acid bacteria protecting the host against infections of the bacterium *Paenibacillus larvae*, the pathogenic agent of the American foulbrood [19]. Similar lactic acid bacteria and other members of the honeybee microbiota have been found in the bumblebee gut [44, 51, 54]: these might potentially play an important role in defence against parasites of bumblebees as well.

We here examined the gut bacterial community of six common bumblebee species from three locations in Central Europe. Since methods that depend on culturing the bacteria are likely to miss a considerable number of bacterial species in environmental samples [36], we used a culture-independent molecular approach that can be applied to field data. Specifically, we cloned and sequenced 16S rDNA, the most commonly used molecular marker for bacterial identification [63], for the gut bacteria of a sample of individuals. Terminal restriction fragment length polymorphism (T-RFLP) analysis [48] was then used for the characterization of a larger number of specimens. We identified the phylogenetic position of the most common bumblebee gut bacteria, especially in relation to the bacteria found in the honeybee gut. In addition, we examined the effects of host species and sampling location on the bacterial community composition.

Methods

Collection and Preparation of Samples

Bumblebee workers were collected in three regions [1]: (1) Northern Germany in June 2008 (location: Celle, 52° 38′0.96″ N, 10°3′9.71″ E; sample sizes: *Bombus terrestris*, *N*=39; *Bombus lapidaries*, *N*=10; *Bombus pascuorum*, *N*=20) [2]; (2) Northwestern Switzerland in the Jura range near Basel in August 2007 (location: Röschenz, 47°25′ 32.66″ N, 7°28′31.41″ E; sample sizes: *B. terrestris*, *N*= 18; *B. lapidaries*, *N*=22; *B. pascuorum*, *N*=24; *Bombus hortorum*, *N*=20) [3]; (3) Swiss Alps in the Swiss National Park in July 2007 (location: Stabelchod, 46°39′ 40.02″ N, 10°14′25.47″ E; sample sizes: *Bombus lucorum/ Bombus cryptorum*, *N*=24; *Bombus soroeensis*, *N*=22). Because of a lack of reliable traits to distinguish workers of *B. lucorum* from *B. cryptarum*, samples resembling these species from the Swiss Alps were not assigned to either of the two species and will be referred to as *B. lucorum*/*B. cryptarum* in the following.

Field-caught bumblebee workers were stored in pure ethanol at −20°C. Whole guts were dissected out by separating the abdomen from the thorax, cutting open the abdomen with a micro scissor along both sides, removing the ventral cuticula and transferring the gut to a 1.5-ml Eppendorf tube. All instruments used in the dissection process were flame-sterilized between each individual.

DNA Extraction and PCR

Whole guts were ground in a 1.5-ml Eppendorf tube in DNA lysis buffer (consisting of 20 mM Tris–Cl, 2 mM sodium EDTA and 1.2% Triton X-100) with a sterile plastic pestle until yielding a homogenous suspension. To digest cell walls of Gram-positive bacteria, 20 mg/ml lysozyme was added to the lysis buffer and the samples were incubated for 30 min at 37°C. DNA was then extracted with the Qiagen DNeasy kit for 96-well plates following the protocol for blood and tissue samples.

An approximately 1.5-kb-long fragment of the bacterial 16S rRNA gene was PCR-amplified with the universal eubacterial primers 27f (AGA GTT TGA TCM TGG CTC AG) and 1492r (ACG GYT ACC TTG TTA CGA CTT), annealing to *Escherichia coli* positions 8–27 and 1492–1512 [68]. These primers are amongst the most widely used to generate surveys of diverse microbial communities (e.g. [47, 50]). Primer 27f was FAM-labelled for samples used in the T-RFLP analysis. The PCR protocol consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C (30 s), 52°C (30 s) and 72°C (1.5 min) and a final extension step of 72°C for 5 min [47, 50]. The success of the PCR reaction was verified by running samples on a 1.5% agarose gel.

T-RFLP Analysis, Cloning and Sequencing

For the T-RFLP analysis, PCR products were purified with Sephadex™ G-50 (GE Healthcare, Glattbrugg, Switzerland) in 96-well filtration plates [37] and 10 µl of the purified PCR product was digested overnight at 37°C with the restriction enzyme *Hae*III, followed by a heat deactivation at 80°C for 20 min. Restriction digests were desalted by Sephadex™ G-50 purification, as described above, and resuspended in 10 µl ddH_2O. Of this preparation, 2 µl was mixed with 0.15 µl MegaBACE™ ET900-R size standard and 2.85 µl MegaBACE™ loading solution containing formamide, denatured for 2 min at 95°C, put on ice and subsequently run on a MegaBACE™ 1000 capillary sequencer (GE Healthcare; injection time, 45 s; voltage, 8 kV; run time, 200 min).

Based on the T-RFLP profiles, 14 bumblebee individuals were selected for cloning to cover the majority of the frequently observed T-RFLP peaks and to represent the different bumblebee species. PCR-amplified 16S rRNA gene fragments were generated for each of the selected bumblebees individually using the procedure described above. The products were purified with Wizard SV Gel and PCR Clean-Up columns and ligated into the pGEM-T Easy Vector (both Promega, Madison, WI, USA) following instructions by the manufacturer. *E. coli* cells of the electrocompetent strain DH5 alpha were transformed with the ligation product in an electroporator. Cells were allowed to recover in SOC medium for 1 h at 37°C and plated out on Luria–Bertani (LB) agar plates substituted with 100 μg/ml ampicillin. For a blue/white screening of successful transformants, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal were spread on the agar surface. The plates were incubated overnight at 37°C; for each sample, 48 clones were picked. The clones were grown overnight in 200 μl LB broth, and 10 μl of the overnight culture was diluted in 90 μl Milli-Q water (Millipore, Billerica, MA, USA), heated for 5 min at 95°C and used as PCR template. Inserts of all clones were PCR-amplified with the primer pair SP6 (CTA TTT AGG TGA CAC TAT AG) and T7 (TAA TAC GAC TCA CTA TAG GG). PCR products were run on a 1.5% agarose gel to check for inserts of the right size, and 3 μl of the PCR product of those matching the expected insert length was digested with *Hae*III for 2 h at 37°C. Digestion products were again run on a 1.5% agarose gel, and for each clone library, inserts with different restriction digest banding patterns were selected for subsequent sequencing. An incubation with exonuclease I and shrimp alkaline phosphatase removed unincorporated primers and dNTPs from the undigested PCR product of the selected clones. Cycle sequencing was conducted in a volume of 10 μl with 0.8 μl BigDye 3.1, 1.6 μl sequencing buffer (ABI, Foster City, CA, USA), 0.16 μl primer (10 μM), 4.94 μl ddH_2O and 2.5 μl PCR product. Sequencing primers used were SP6, T7 (see above), 790f (ATT AGA TAC CCT GGT AG) and 907r (CCG TCA ATT CCT TTR AGT TT). Products were run on an ABI 3130xl capillary sequencer (ABI).

Data Analysis

Sequence Data

Raw forward and reverse sequences from the four sequencing primers were aligned to create a consensus and edited in Sequencher 4.8 (Gene Codes, Ann Arbor, MI, USA); vector and primer sequences were removed. The sequences were checked for chimaeras using Bellerophon [35]; sequences of chimeric origin were removed from further analysis. The sequences were deposited in GenBank under accession numbers HM215010–HM215051. The curated 16S database of the Ribosomal Database Project (RDP) release 10.24 [10] was used to find both the most similar of all high-quality 16S sequences and the closest sequenced type strain for the different clades of bacteria presented in this study. A BLASTN search [2] was carried out to find additional related sequences in GenBank, especially from previous reports on bacteria associated with bees and other insects. These sequences were exported and incorporated into the analysis. The sequences from bacteria in the gut of *B. terrestris* from the study of Mohr and Tebbe [51] were not recovered among the closely matching sequences in the RDP and GenBank searches because of their limited length (approx. 370 bp). They were therefore checked separately and the matching sequences added to the analysis. The sequences were then aligned with ClustalW [62] (http://align.genome.jp) using standard settings for DNA (gap opening penalty 15, gap extension penalty 6.66). Using jModelTest 0.1 [56], an appropriate model of sequence evolution was determined, choosing the model with the lowest Akaike information criterion. A maximum likelihood phylogenetic tree was then calculated using a GTR+γ+inv model in PhyML [27], and branch support was assessed with 500 bootstrap replicates.

Individual-based rarefaction curves were computed in PAST v. 2.30 [28] for the 14 clone libraries to assess the success in obtaining a representative sample of the bacterial diversity in the gut by sampling 48 clones from each library. Clones were considered identical if they produced the same restriction pattern in the restriction digest described above; chimeric sequences were excluded.

T-RFLP Data

Fragment profile raw data were processed and sized in Fragment Profiler v. 1.2 (MegaBACE, GE Healthcare). The peaks were filtered from baseline noise and binned between the samples following the algorithms described in Abdo et al. [1]. The peak areas were then standardized by dividing the area of individual peaks by the total area of all peaks in a sample. To visualize the relationship between individual samples, a dissimilarity matrix from the proportioned data was produced using the Bray–Curtis coefficient [6] for an ordination with non-metric multidimensional scaling (NMDS) following the recommendations in Ramette [57] and Field et al. [18]. The NMDS analysis was carried out in SPSS 19 (IBM) with the PROXSCAL module. Goodness of fit of the NMDS solution was assessed with the help of a Shepard plot and Kruskal's $STRESS_1$ measure for two and three dimensions. The two-dimensional NMDS gave a relatively high $STRESS_1$ value (0.199) compared to a three-

dimensional solution (STRESS1=0.139). An examination of the three-dimensional coordinate space, however, showed a similar pattern in the distribution of the individual samples. Therefore, a two-dimensional NMDS was chosen to enable an easy visualization of the analysis whilst not affecting the interpretation. To test for significant differences between the community composition of different bumblebee species and the three sampling sites, a one-way analysis of similarity (ANOSIM) [9, 58] was carried out with 10,000 permutations on the Bray–Curtis dissimilarity matrix; p values of the pairwise comparisons between host species and sampling locations were corrected for multiple testing by a Bonferroni correction. ANOSIM compares the average rank similarity between samples within a group with the average rank similarity between samples between groups and computes an R value, which can range from −1 to 1 [9]. Positive R values indicate a higher similarity between samples within one group than between groups, and values around 0 indicate no difference in similarity between samples within and between groups. R values>0.75 are interpreted as indicating strong separation between groups, R>0.5 as separation with overlap and R<0.25 as barely separable [57]. Replicated permutation of group membership allows for testing of significant differences between groups [9]. The ANOSIM analysis was run in PAST v. 2.03 [28].

To identify the bacterial taxa behind the T-RFLP peaks, the terminal fragment of the 16S sequences obtained from the clone libraries was predicted by a virtual digest of the sequence with the enzyme *Hae*III in the programme EnzymeX 3 (Mekentosj, Aalsmeer, the Netherlands). The predicted fragment length was then compared to the observed peaks in the T-RFLP profile of the individual sample the 16S sequence originated from. The predicted T-RFLP peaks closely matched the observed peaks in the profiles (Table 1). The identity of the peaks in the T-RFLP profiles of the samples not subjected to cloning and sequencing of the 16S rRNA gene was then inferred from these identifications (Table 1).

Results

Phylogenetic Position of Bumblebee Bacteria

On average, 3.1 (SD=0.9) bacterial taxa per host individual were obtained from the 16S clone libraries of the 14 individual bumblebees, out of the 48 sampled clones each. Three of the nine bacterial taxa in total (Table 1) were exclusively observed in only one bumblebee individual. The individual-based rarefaction curves mostly reached an asymptote for the 48 sampled clones (Fig. 1), indicating a sufficient sampling of the low diversity in the gut. The phylogenetic placement of bacteria in the bumblebee gut relative to bacteria reported from the honeybee gut and the closest matches of type strains from the RDP [10] is

Table 1 SeqMatch results (RDP) of all groups of 16S sequences obtained in this study, similarity scores to closest match among all RDP sequences and type strains only, and corresponding T-RFLP peaks

Bacterium (clade)	Closest match[a]		Closest species[b]		T-RFLP peaks[c]		Presence (%)[d]
		Sim.		Sim.	Pred. (bp)	Obs. (bp)	
Gammaprot. (I)	AY370192 Gammaproteobacterium (*Apis mellifera*)	0.984	*Edwardsiella hoshinae*	0.924	202	202	93
Gammaprot. (II)	EF608541 Gammaproteobacterium (*Poecilus chalcites*)	0.973	*Pseudomonas nitroreducens*	0.936	39	39	60?
Betaprot. (III)	AY370189 Betaproteobacterium (*Apis mellifera*)	0.994	*Simonsiella muelleri*	0.963	223	223	92
Bacteroidetes (IV)	DQ837639 Bacteroidetes (*Apis mellifer*a)	0.983	*Empedobacter brevis*	0.903	39	39	60?
Firmicutes (V)	DQ837631 Firmicutes (*Apis mellifera*)	0.972	*Lactobacillus sharpeae*	0.906	322	320	43
Lactobacillus (VI)	DQ837634 *Lactobacillus* sp. (*Apis mellifera*)	0.978	*Lactobacillus acetotolerans*	0.953	247	244	40
Carnobacterium (VII)	AY573049 *Carnobacterium* sp.	1.000	*Carnobacterium maltaromaticum*	0.998	315	316	0.50
Fructobacillus (VIII)	AF360737 *Fructobacillus fructosus*	0.994	*Fructobacillus fructosus*	0.994	310	306	15
Bifidobacterium (IX)	FJ858733 *Bifidobacterium* sp. (*Bombus* sp.)	0.997	*Bombiscardovia coagulans*	0.992	257	259	65

[a] Closest hit with SeqMatch (RDP) for all good quality sequences >1,200 bp: accession number, taxonomic identity (host species) and similarity score (Sim.) = percent sequence identity over all pairwise comparable positions

[b] Closest hit with SeqMatch (RDP) for all types of strain sequences: taxonomic identity and similarity score (Sim.)

[c] Position of predicted (Pred.) and observed (Obs.) T-RFLP peaks in base pairs, prediction from virtual digest of 16S sequence with *Hae*III

[d] Percentage of host individuals with corresponding T-RFLP peak, clades II and IV uncertain because of identical restriction fragment length

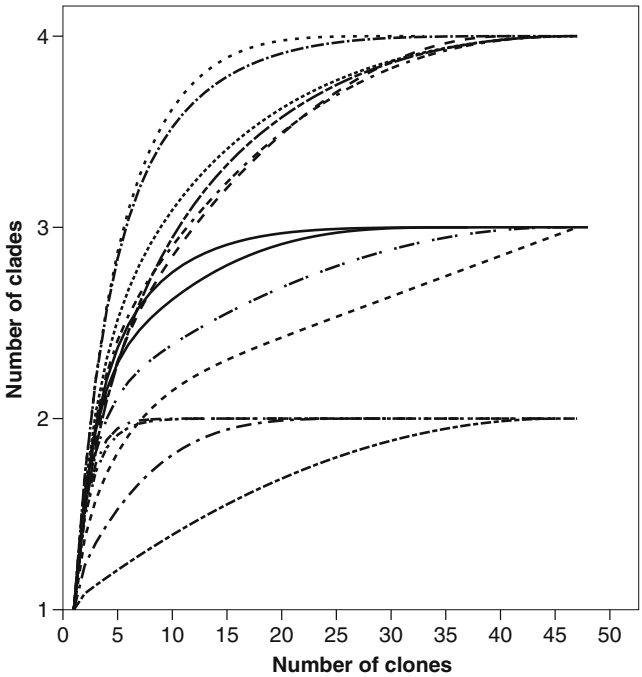

Figure 1 Individual-based rarefaction curves for the 16S clone libraries from guts of 14 different bumblebee individuals. *Individual lines* represent the diversity in the different host individuals

presented in Figs. 2 and 3 and Table 1. The bacteria can be grouped into nine major clades (I–IX). Figure 2 reveals four major clades of Gram-negative bacteria. In this tree, clade I and clade II are undescribed Gammaproteobacteria, clade III comprised Betaproteobacteria, and clade IV of Bacteroidetes. According to the entries in GenBank, the Gammaproteobacteria in clade I are most closely related to bacteria found in honeybees (*Apis mellifera*, *Apis cerana*), but similar bacteria have also been found in aphids (e.g. *Cinara* sp., *Stomaphis quercus*) and a ground beetle (*Poecilus chalcites*). Their closest matches amongst 16S sequences of the type strains in the RDP were *Orbus hercynius* and *Edwardsiella hoshinae*. The single sequence obtained for bacteria from clade II shows closest similarity to a bacterium from the honeybee gut and is also close to a bacterium found in the ground beetle *P. chalcites* (Fig. 2). Their closest described relative was found to be *Pseudomonas nitroreducens* (Table 1). The Betaproteobacteria (III) fall within the Neisseriaceae and are closest to *Stenoxybacter acetivorans* from the termite gut and *Simonsiella muelleri*, a human commensal (Fig. 2). They too have closely related representatives in the honeybee gut. The Bacteroidetes (IV) were found to be closest to *Empedobacter brevis*, but show a low similarity score (0.903, Table 1). Again, a closely related bacterium has previously been found in the honeybee gut (Fig. 2) [4].

The Gram-positive bacteria (Fig. 3) are represented by a novel group of Firmicutes (V), *Lactobacillus* (VI), *Carnobacterium* (VII), *Fructobacillus* (VIII) and *Bifidobacterium* (IX). Clade V is composed of Firmicutes highly divergent from any described bacterial species, but similar bacteria have also been reported from the honeybee gut before (Fig. 3). Furthermore, a species of *Lactobacillus* was found (VI), showing close affinities with lactobacilli previously described from the crop of honeybees [53]. A single sequence from the gut of *B. hortorum* was identical to *Carnobacterium maltaromaticum* (VII) and another one from *B. terrestris* almost identical to *Fructobacillus fructosus* (VIII). A group of Bifidobacteria (IX) from different *Bombus* species was also found in this study, similar to Bifidiobacteria previously isolated from bumblebees (Fig. 3) [43].

Grouping according to sampling location in the phylogenetic tree was not observed in those cases where 16S rRNA gene sequences for one clade of bacteria were obtained from several bumblebee individuals. However, the Betaproteobacteria (clade III) are separated into different well-supported clades according to their host species (Fig. 2).

Comparative Analysis of Gut Bacterial Communities of Different *Bombus* Species

The two-dimensional NMDS analysis ($STRESS_1$=0.199) of the T-RFLP profiles from six species and three localities reveals considerable overlap of the structure of the communities, both between and sampling locations and species (Figs. 4 and 5). Whilst this indicates a high degree of similarity of microbial communities in the sampled bumblebee individuals, significant differences between host species as well as localities were also observed. An ANOSIM revealed a pairwise significant difference between samples from the Swiss Alps to Northern Germany (Table 2 and Fig. 4), but not for the other pairwise comparisons between sampling locations. Most of the pairwise comparisons between species, however, indicated highly significant differences, but with mostly moderate to low R values (R<0.5, Table 3). The highest degree of separation was observed between *B. pascuorum* and *B. soroeensis* (R=0.64), and *B. pascuorum* and *B. terrestris* (R=0.53). *B. hortorum* and *B. pascuorum* had the most distinct microbiota, being significantly different from most other host species. *B. terrestris* in contrast showed less separation from the other species, with low R values especially for the German samples. Accordingly, the NMDS plot shows a high amount of scatter for *B. terrestris* individuals, whereas individuals of the other species tend to be more clustered in certain areas of the plot, especially *B. pascuorum* and *B. soroeensis* (Fig. 5).

No difference was observed within one host species when comparing the microbiota between sampling locations for *B. pascuorum* and *B. lapidaries*, with R values around 0

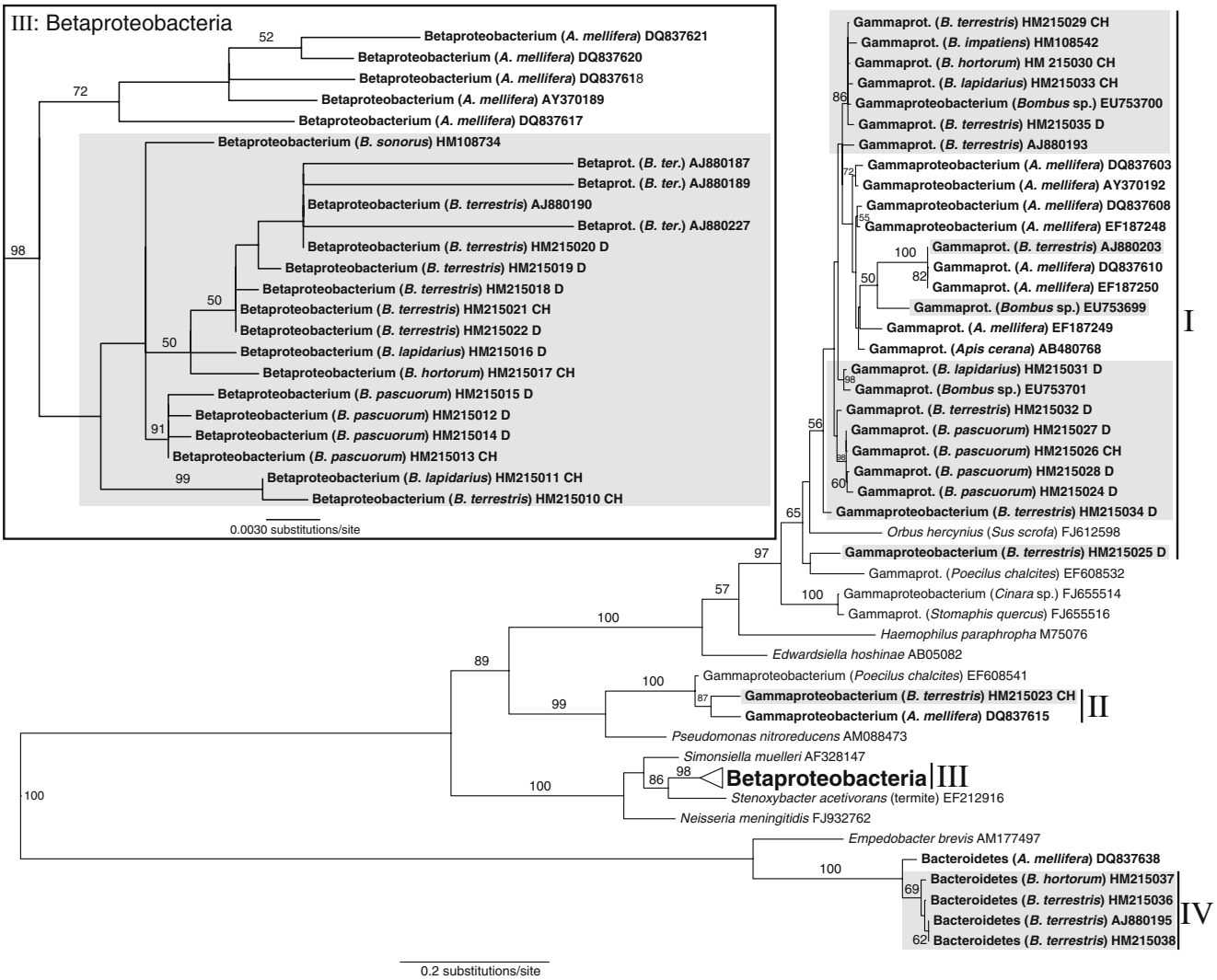

Figure 2 Maximum likelihood tree for 16S sequences of Proteobacteria and Bacteroidetes from this study (clades I to IV) and related sequences from GenBank. Clade III (Betaproteobacteria) is drawn to different scales in the *upper left corner*. *Figures on branches* are bootstrap support values (500 replicates); taxon labels denote bacterium (host species), GenBank accession numbers and sampling location (*CH*—Swiss lowlands, Jura of Basel; *D*—Northern Germany, Celle). Bacteria from honeybee and bumblebee guts in *bold font*, bacteria from the bumblebee gut additionally marked in *grey boxes*

(Table 3). A slight difference was, however, detected between *B. terrestris* from Switzerland and Germany.

Of the 49 T-RFLP peaks (the taxonomic units) recorded in total from all 199 bumblebee individuals, most were rare; only 11 peaks were detected in more than 20% of the sampled bumblebees. The rare peaks remained mostly unidentified because no corresponding 16S sequence could be obtained from the clone libraries, with the exception of a *Carnobacterium* (VII) found only in a single profile and a *Fructobacillus* found in only 15% of all individuals.

Assuming that the peaks at a certain position observed in the different profiles always correspond to the predicted peaks of the bacterial taxa identified in the clone libraries (Table 1 and Figs. 2 and 3), distributions of the most common bacteria in the bumblebee gut were as follows (Fig. 6). The most dominant bacteria in the bumblebee gut are one species from each of the following groups: Gammaproteobacteria (clade I, present in 92.5% of all individuals), Betaproteobacteria (III, 91.5%), and Firmicutes (V, 42.7%) and one species each of the genera *Lactobacillus* (VI, 40.2%) and *Bifidobacterium* (IX, 64.8%). In 60.3% of all samples, a peak, possibly corresponding to clade IV (Bacteroidetes), was observed. A single clone from one of the clone libraries also yielded a 16S sequence of an unknown Gammaproteobacterium (clade II) that produces a T-RFLP peak at the same position (39 bp, Table 1). An unequivocal identification of this peak is therefore not possible; however, the presence of the Bacteroidetes bacterium in several of the clone libraries might indicate this bacterium to be more widespread than

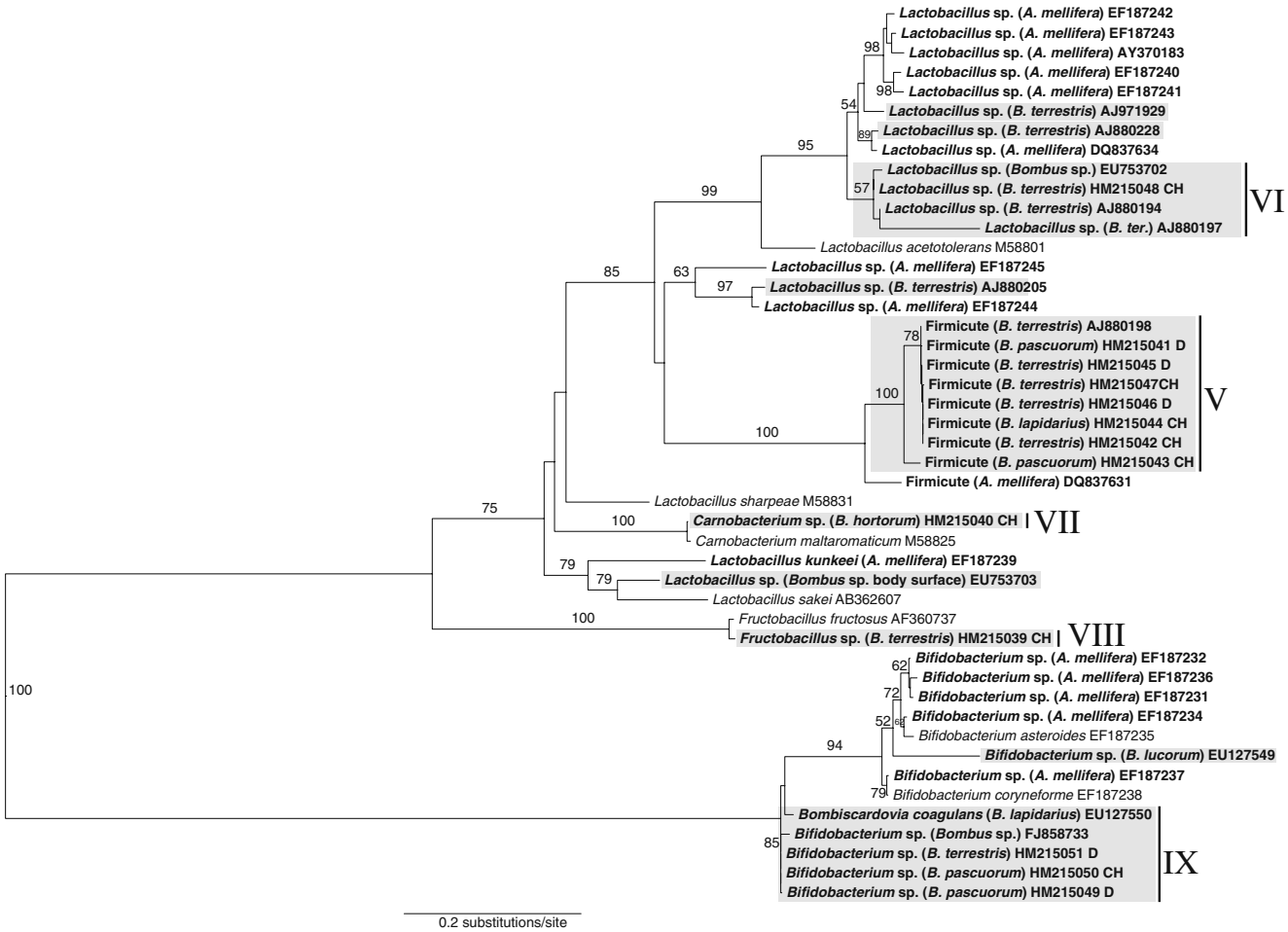

Figure 3 Maximum likelihood tree for 16S sequences of Firmicutes and Actinobacteria from this study (clades V–IX) and related sequences from GenBank; bootstrap support values (500 replicates) on *branches*. For further explanations, see Fig. 2

the Gammaproteobacterium (clade II). The average standardized peak signal intensity as a proxy for the relative abundance of the different bacteria within a host generally corresponds to the percentage of infected individuals (Fig. 6). For example, the two most common bacteria (clades I and III) are also the most abundant bacteria within infected hosts, whilst less common bacteria tend to be less abundant in infected hosts (e.g. clade VIII in *B. lucorum*/*cryptarum* and *B. soroeensis*; Fig. 6).

The aforementioned groups can generally be detected in all of the observed *Bombus* species at all localities (Fig. 6), with the exception of the Firmicutes bacterium (clade V) which was not found in *B. soroeensis* (Fig. 6). Marked differences in the frequency of some of the bacteria in different host species can be observed (Fig. 6). For example, *B. hortorum* relatively rarely harboured the otherwise much more common Gammaproteobacterium (I) and Betaproteobacterium (III), but was colonized more than expected by *Lactobacillus* (VI) and *Fructobacillus* (VIII). Certain patterns relating to the sampling location can also be found. For example, firmicute (V) is almost absent in the Swiss Alps, and *Fructobacillus* (VIII) seems to mostly occur in the Swiss lowlands (Fig. 6).

Discussion

The gut bacterial community of central European bumblebees appears to contain relatively few abundant species, which consistently appear in all examined *Bombus* species and localities. This low diversity is most likely not an artefact of limited sampling effort, as indicated by the rarefaction analyses of the clone libraries (Fig. 1). Bacteria in this abundant group belong to different phyla including the Gamma- and Betaproteobacteria, Bacteroidetes, Firmicutes and Actinobacteria. They generally are quite distant to any of the already described bacterial species. For most of the taxa and groups identified here, similar bacteria have been found in the honeybee gut [4, 12, 39, 44, 51]. The 16S sequences of gut bacteria from different bumblebee individuals and species generally form distinct clades with respect to similar bacteria from the honeybee gut (Figs. 2

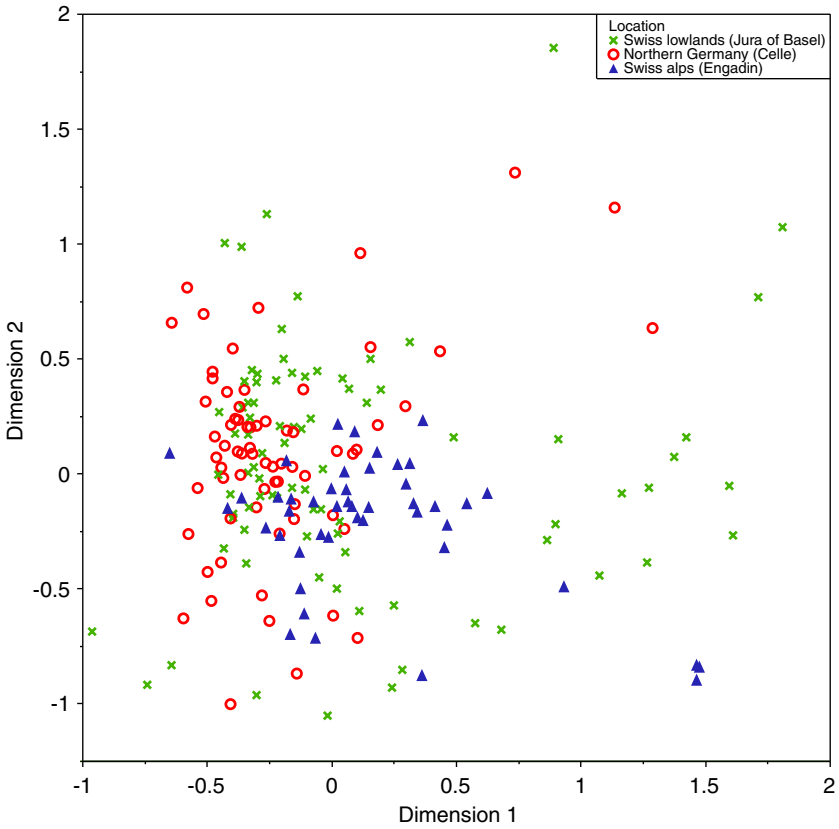

Figure 4 NMDS plot of 16S T-RFLP profiles for 199 bumblebee individuals. *Different symbols* indicate different sampling localities

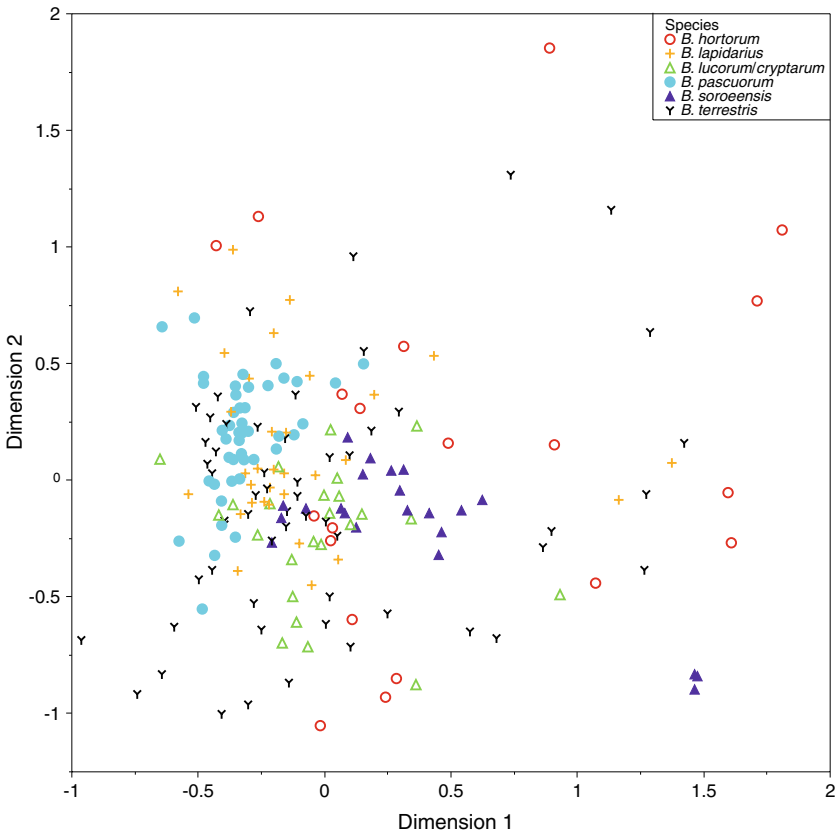

Figure 5 NMDS plot of 16S T-RFLP profiles for 199 bumblebee individuals. *Different symbols* indicate different host species

Table 2 Results of ANOSIM comparing T-RFLP profiles of all sampling locations

Location R	Swiss lowland	Northern Germany
Swiss lowland		
Northern Germany	0.03061	
Swiss Alps	0.01789	0.2299*

*$p<0.0001$ (R values, Bonferroni-corrected)

and 3). These bacteria may thus be specialized inhabitants of the bumblebee gut and similar but distinct from those observed in honeybees. This is in agreement with the findings of Martinson et al. [44] who recorded similar bacteria in two species of Northern American bumblebees and several species of honeybees, but not in a variety of solitary bees. This similarity between bacterial communities in honeybees and bumblebees is most likely not caused by the sampling method as a variety of extraction and PCR protocols have previously been used in the study of bacterial communities in bees [4, 12, 39, 44, 51] with similar results,

The observed sequence divergence of 2–5% between related bacteria in honeybees and bumblebees (Table 1) has to be judged against the background of a sequence divergence rate at the 16S rRNA locus of 1–2% per 50 million years [52] and an estimated split of the honeybee and bumblebee linage around 90 million years ago [25]. Hence, this fits well with a scenario where these bacteria became separated as these two host groups diverged. Additional sequence data for bacteria from the other tribes of corbiculate bees (Meliponini and Euglossini) could help expand on this hypothesis. Bumblebees originated only 25–40 million years ago, and most speciation events have occurred within the last 10 million years [31]. Therefore, as a note of caution, the 16S rRNA gene might be too highly conserved to yield a fine resolution of these bacteria in the recently diverged *Bombus* species. Using T-RFLP profiles furthermore reduces the resolution as several bacteria need to have different terminal restriction sites to be effectively differentiated. Hence, our results reflect robust differences, but will have to be refined by further studies.

The group of Betaproteobacteria found in this study not only appears to be differentiated between honeybees and bumblebees but also shows different clades perhaps specialized to different bumblebee host species regardless of their sampling location (Fig. 2). This result will have to be confirmed by examining a wider range of bumblebee species, but indications for co-divergence of the closely related genus *Simonsiella* with their mammalian host have been found previously [29]. Whilst co-speciation of bacterial symbionts with their hosts has mostly been observed in intracellular bacteria [38], extracellular insect gut bacteria have also been reported to have co-speciated with their plataspid bug hosts [34, 41].

With exception of sequences from related bacteria in the honeybee gut, for the majority of 16S sequences obtained in this study, no similar sequences were found among the more than two million 16S sequences stored in GenBank at the time of this study, many of which originate from environmental samples [5]. Therefore, these bacteria seem to constitute a specialized endogenous community in the bumblebee alimentary tract rather than bacteria accidentally taken up from the environment passing the gut. An exception to this may be the two strains with a high similarity to *C. maltaromaticum* and *F. fructosus*, which have been described from outside the bumblebee gut on decaying plant or animal matter [17, 45] and might therefore be unspecific bacteria taken up from the environment by the bees. Bacteria in bees could also come from another source—nectar and pollen. Yet, there is very

Table 3 Results of ANOSIM comparing T-RFLP profiles of all species pairs

	B. hortorum CHL	*B. lapidarius* CHL	*B. lapidarius* D	*B. luc./cryp.* CHA	*B. pascuorum* CHL	*B. pascuorum* D	*B. soroeensis* CHA	*B. terrestris* CHL
B. hortorum CHL								
B. lapidarius CHL	0.23***							
B. lapidarius D	0.12	−0.03						
B. luc./cryp. CHA	0.31***	0.14***	0.27					
B. pascuorum CHL	0.47***	0.26***	0.38**	0.43***				
B. pascuorum D	0.46***	0.20***	0.27	0.39***	0.06			
B. soroeensis CHA	0.29***	0.23***	0.30	0.23**	0.64***	0.57***		
B. terrestris CHL	0.23**	0.30***	0.27	0.36***	0.53***	0.43***	0.33***	
B. terrestris D	0.42***	0.09	0.04	0.12	0.09	0.00	0.31***	0.29*

Species with samples from more than one location were split by sampling locations (*CHL* Swiss lowlands, *CHA* Swiss Laps, *D* Northern Germany)

*$p<0.05$; **$p<0.01$; ***$p<0.0001$ (R values, Bonferroni-corrected)

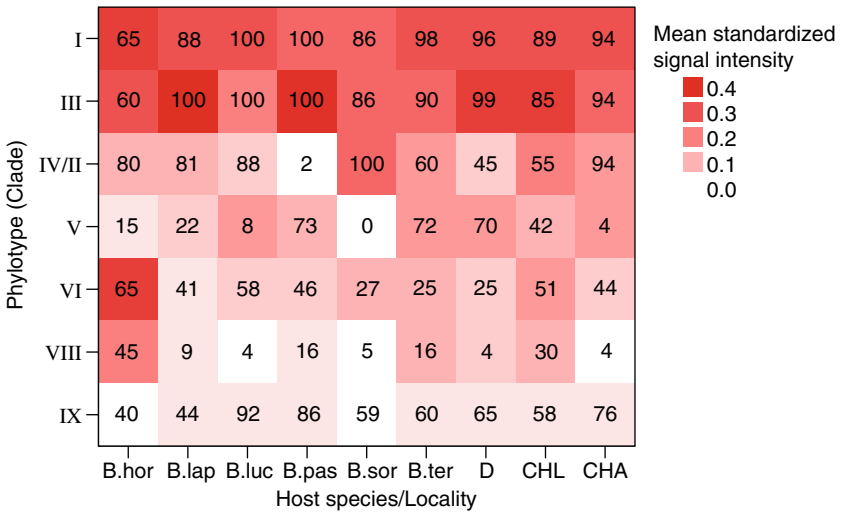

Figure 6 Heatmap of relative abundances and frequencies of different T-RFLP peaks in different bumblebee species and sampling localities. *Different shades* indicate average standardized signal intensity of individual T-RFLP peaks for the infected individuals, representing relative abundances of the different bacterial phylotypes. *Numbers in squares* indicate the percentage of individual hosts of each group in which the respective peak was detected. For the Roman numerals coding for the different bacterial phylotypes, see Table 1 and Figs. 2 and 3. Host species/locality: *B.hor B. hortorum* (n=20), *B.lap B. lapidarius* (n=32), *B.luc B. lucorum/cryptarum* (n=24), *B.pas B. pascuorum* (n=44), *B.sor B. soroeensis* (n=22), *B.ter B. terrestris* (n=57), *D* Northern Germany (n=69), *CHL* Swiss lowland (n=84), *CHA* Swiss Alps (n=46)

limited knowledge about bacteria living inside the pollen and nectar of flowers. Culture-independent studies of this potential microbial habitat are curiously absent, and one of the few culture-dependent studies found bacteria to be virtually absent in floral nectar [21]. This absence of bacteria has been attributed to plant-produced antimicrobial secondary compounds found in floral nectar [21]. However, recent studies have shown a surprising abundance of yeasts in nectar [30], transmitted by pollinating insects like bumblebees [8], pointing at the possibility of other microbial organisms inhabiting flowers. Flowers would represent a likely site of horizontal transmission for bacteria in bees. Such a transmission route has previously been demonstrated for the trypanosomatid *C. bombi*, an intestinal parasite of bumblebees [15], and RNA viruses in hymenopterans including bees [61]. This horizontal transmission route might result in a stable and consistent mutualistic association of the bee hosts and their bacteria, as has been found in symbionts of stinkbugs [40]. The apparent absence of these bacteria in solitary bees [44] points, however, towards a role of sociality in transmission. The life history of bumblebees with gynes staying in their mother colony several days after emergence and founding new colonies after hibernation in the next season [23] would facilitate vertical transmission. The higher probability of transmission of beneficial microbes within a colony and to the daughter colonies might thus represent an additional benefit of sociality in bumblebees and honeybees [44, 49]. This mechanism has also been suggested in termites [32].

Significant differences between the gut floras of different bumblebee species were found in this study (Fig. 5 and Table 3). Additionally, a comparison of the microbiota between sampling localities within one host species showed no difference for *B. pascuorum* and *B. lapidaries*, in contrast to a comparison with different host species at the same site (Table 3). This further strengthens the argument for the existence of species-specific bacterial gut communities in these hosts across geographical distances. These might relate to differences in the host ecology and physiology, thereby selecting different communities of bacteria in the gut. The sampled *Bombus* species have, for example, different preferred flower types, with the long-tongued *B. hortorum* visiting flowers with long corolla tubes, whilst the short-tongued *B. terrestris* is a generalist visiting a broad spectrum of flowers with short corolla tubes, but also robbing nectar from flowers with long corolla tubes [23]. Accordingly, the more specialized *B. hortorum* has very distinct bacterial communities (Table 3), whereas the generalist *B. terrestris* shows more variation and little distinction than the other examined *Bombus* species. The communities in *B. terrestris* from Northern Germany were also found to be significantly different from those in Switzerland, perhaps indicating a higher plasticity of the microbiota in this species. More extensive sampling of different bumblebee species is of course needed to substantiate these points. As discussed above, the significant differences among host species might also be driven by a predominantly vertical transmission of bacteria from a

mother colony to a daughter colony via the young queen whilst horizontal transmission would remain rare.

Our analysis could not test the functional role of the bacteria, nor is much known about these roles. However, the bacteria identified here are most likely non-pathogenic because, on one hand, they belong to the generally non-pathogenic lactic acid bacteria, Bifidobacteria or Bacteroidetes that are commonly found as part of the healthy gut flora of other organisms, including vertebrates and honeybees [22, 46, 53]. On the other hand, some of the identified bacteria occur in almost every bumblebee individual checked here (clades I and III, the Gamma- and Betaproteobacteria), which is indicative of their possible role as mutualists or commensals, but less likely so as pathogens.

Furthermore, the presence of an apparently highly conserved and specialized community in the bumblebee gut across different host species and geographic distances makes a functional relevance of these bacteria for their hosts seem likely. As in other insects [13], they could play a role in host immune defence. The main route of infection of insect pathogens is through ingestion and invasion of the gut, followed by the colonization of the haemocoel through the midgut wall [64]. Exclusion of potential pathogens from the gut is therefore an essential part of the insect immune system. In addition to direct control of pathogens by the insect immune system through, for example, the production of antimicrobial peptides and reactive oxygen species [64], the resident gut flora might play an important protective role as well [3, 13], either by producing antimicrobial substances themselves [19] or through competitive exclusion of newly invading bacteria [14]. Gilliam [22] speculated about a similar role of the gut microbiota of honeybees, and recently, the lactic acid bacteria of honeybees have been shown to produce antimicrobial substances and efficiently protect larvae against the foulbrood-causing bacterial pathogen *P. larvae* [19]. As the bacteria found in the bumblebee gut are highly similar to those in the honeybee gut, they may possess a protective role for their hosts as well.

Bacteria have furthermore been found to play a key role in the adaptation of herbivorous insects to novel food resources and subsequently in the diversification and ecological success of this group [38]. Even though the diet of bees consisting of pollen and nectar is highly nutritious [59], the highly resistant pollen wall has to be degraded first to make these nutrients available. Bee-specific symbiotic bacteria could aid in this process, either in the host gut or in stored bee bread [22, 65]. They could also help in preserving the stored provisions of honeybees and bumblebees by the production of antimicrobial substances [65].

In conclusion, we have provided the first detailed survey of bacteria in the bumblebee gut for different species from Central Europe. The resident gut bacteria are surprisingly well conserved and species poor, but apparently highly specialized to this group of organisms. Whilst this indicates a possibly strong interaction with their hosts as well as a functional role, further studies are needed to elucidate this.

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