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ORIGINAL ARTICLE

Microbial community types and signature-like soil bacterial patterns from fortified prehistoric hills of Thuringia (Germany)

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Abstract

16S rRNA profiling has been applied for the investigation of bacterial communities of surface soil samples from forest-covered areas of ten prehistorical ramparts from different parts of Thuringia. Besides the majority bacterial types that are present in all samples, there could be identified bacteria that are highly abundant in some places and absent or low abundant in others. These differences are mainly related to the acidity of substrate and distinguish the communities of lime stone hills from soils of sand/quartzite and basalt hills. Minority components of bacterial communities show partially large differences that cannot be explained by the pH of the soil or incidental effects, only. They reflect certain relations between the communities of different places and could be regarded as a kind of signature-like patterns. Such relations had also been found in a comparison of the data from ramparts with formerly studied *16S rRNA* profiling from an iron-age burial field. The observations are supporting the idea that a part of the components of bacterial communities from soil samples reflect their ecological history and can be understood as the "ecological memory" of a place. Probably such memory effects can date back to prehistoric times and might assist in future interpretations of archaeological findings on the prehistoric use of a place, on the one hand. On the other hand, the genetic profiling of soils of prehistoric places contributes to the evaluation of anthropogenic effects on the development of local soil bacterial diversity.

Keywords Soil · Bacteria · Diversity · 16S rRNA · Genetic profiling · Ecology · Ramparts · Archaeology

Abbreviations

DNA	Deoxyribonucleic acid
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymer chain reaction
ppm	Parts per million
rRNA	Ribosomal ribonucleic acid

Introduction

The composition of soil microbial communities is of crucial importance for all terrestrial habitats (Fierer and Jackson 2006). The high diversity of natural soils is mainly determined by the micro-organisms in their interactions with plants, animals, the mineral substrate, chemical ions and compounds dissolved in the soil water and dependent on physical conditions (Fierer and Lennon 2011). The variability of microbial communities is due to the qualitative and quantitative combination of microbial phyla, classes, orders, families, genera and species. The high number of different microbial species and strains generates the potential of a practically infinite size of combinatorial possibilities.

Besides the actual conditions of a place, its preceding development is important for the state of microbial communities, too. The appearance, growth and appearing of macro-organisms, changing weather and climate conditions as well as the varying availability of water and nutrients and the occasional impact of damaging substances affect the development of the different microbes in soil communities.

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These processes are modulating the different kinetics of the different interacting microbial strains (Aanderud et al. 2015).

Besides natural processes, the human impact contributes to the dynamics of the development of soil microbial communities, too. It is obvious that intensive use of surface by agriculture, the construction of industrial facilities and settlements as well as the release of industrial and urban waste have a strong impact on the microbial populations and vice versa, and microbial activities interfere with human impact on environment (Wu et al. 2015). Duration and intensity of human impacts affect the kinetics of microbial growth and the timescales for relaxation and adaptation of communities, too. Therefore, the composition of soil microbial communities reflects not only a recent ecological state of a place, but also its ecological history (Wegner and Liesack 2017). A strong effect is always observed in industrial mining areas, for example, and has also to be reconsidered for the temporal requirements for remediation of damaged areas (Haferburg and Kothe 2010).

Recent investigations argue for the fact that not only the environmental pollution and industrial damages during the last decades are reflected by the biological state of soil, but also earlier human impact is important for recent compositions of soil microbial communities. Also, there are indications for long-term effects of human activities on soil micro-organisms. They can be caused already by preindustrial changes in soil structure and contamination as well as by earlier influences on the distribution of microbial types and on the kinetics of developing microbial communities. Such early effects on recent microbial components in soils can be interpreted by a "soil microbial memory". Examples date back to local situations in the iron age (Margesin et al. 2017).

The investigation of microbial ancient DNA from human remains (Philips et al. 2017) was motivated by the recognition of pathogens (Rollo and Marota 1999; Moodley et al. 2009), on the one hand. On the other hand, profiling of soil microbial DNA was used for characterization of archaeological objects (Lenehan et al. 2017) and evaluation of degradation and preservation conditions (McNamara et al. 2005; Douterelo et al. 2010; Xu et al. 2017). The investigation of archaeological material by r-RNA profiling showed significant differences between the bacterial communities of excavated objects and the surrounding soil on lower taxonomical levels, but larger similarities on the phylum level (Kazarina et al. 2019). The determination of abundances of extremophiles could support specific insights into the interpretation of archaeological findings, for example by the enhanced presence of thermophilic bacteria (Chernysheva et al. 2017).

Here, it tries to distinguish between the influences of general natural conditions and specific local features on soil bacteria communities of some prehistorically used places by comparing data of *16S rRNA* profiling. Prehistorically fortified hills of Thuringia (Germany) in different geological situations have been chosen as an example for this investigation. In the following, the sequence data of soil samples from selected places will be discussed and the results are interpreted in comparison with each other and with recently obtained data from an archaeological excavation on an iron-age burial place (Köhler et al. 2018).

Experiments

Soil samples and sampling places

The soil samples were taken from open spots of the forest soil of areas of prehistoric ramparts. The majority of them are regarded to be fortified hill settlements. These settlements have been deserted in prehistoric times. In case of the hill of Hasenburg, the place was later used for constructing a mediaeval castle, which was deserted, too, about 800 years ago. The state of archaeological investigation of the ramparts is different. Detailed studies are reported from the multiphase hill settlement and Celtic oppidum Steinsburg near Römhild (Peschel 2005), from the pre-Roman iron-age settlements on Öchsen near Vacha (Donat 1966). Dating artefacts have also been found on Diesburg near Aschenhausen (Peschel 2004), Röderburg (near Dermbach), on Herrenberg near Theuern (Gall 1994) and from the late bronze/early iron-age rampart of Ole Burg near Sondershausen. The fortified multiphase hill settlements of Monraburg near Beichlingen (Simon 1984) and Hasenburg near Haynrode (Peschel 1986) are archaeologically studied, whereas the ramparts of Aaskehren (near Bleicherode) and the rectangular wall in the Lange Bahn forest (not identical with the deserted mediaeval settlement "Lange Bahn") near Suhl have not been archaeologically investigated in detail, up to now. The sample material was taken from vegetation-free spots on the surface. The sample material was filled into sterile 50-mL-sample tubes and stored and transported by them in the laboratory. The samples have been dried and stored at room temperature. The sampling locations (Fig. 1) are identified by GPS. The sample origin and locations are described in detail in Table 1.

DNA extraction, amplification, labelling and data processing

The DNA of soil samples was extracted by a Power Soil Isolation Kit (MO BIO, Carlsbad, USA) according to the manufacturer's protocol and processed as published (Köhler et al. 2018). An Edvocycler (Edvotek, Washington D.C.) was applied for DNA amplification by polymer chain reaction (PCR). Each PCR amplification step was verified by gel electrophoresis. Therefore, 1.2% agarose gels were applied.

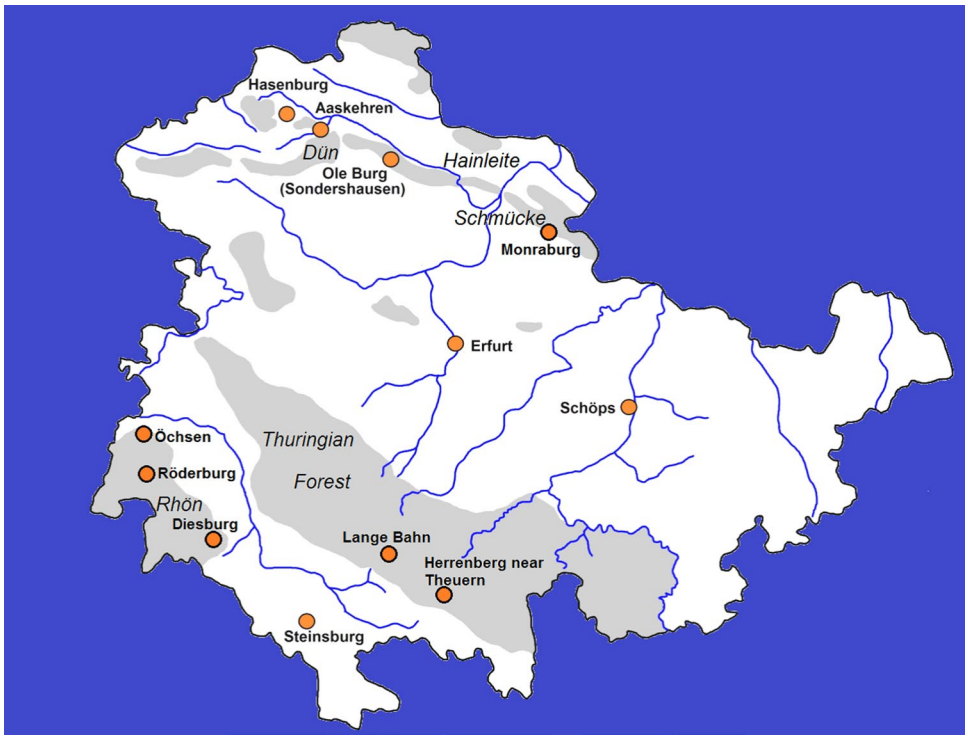

Fig. 1 Sampling locations in Thuringia/Germany

Single PCR products, as well as pooled DNA libraries, were purified with AMPure XP beads (Beckman Coulter, Brea, USA).

For amplicon PCR, adaptor primers A519F-Ad (5′ TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCAG CMGCCGCGGTAA 3′) and Bact_805R-Ad (5′-GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGACTACH-VGGGTATCTAATCC 3′) were used (100 pmol/µL), which were obtained from Eurofins Genomics (Ebersberg, Germany). The reaction mixtures with a total volume of 50 µl per reaction contained 1 µL of DNA isolation eluate, 2 mM $MgCl_2$, 200 µM PCR nucleotide mix, 1,25 Units GoTaq G2 Flexi DNA Polymerase, nuclease-free water (all reagents from Promega, Madison (USA)) and 1 µmol/l of each primer. Amplicon PCR programme settings were as follows: 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s; annealing at 50 °C for 30 s; extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The quality of the sequence data was checked by a sample of 16 of the data sets using the tool "Galaxy" (http://hannonlab.cshl.edu/fastx_toolkit/; A. Gordon 2010). All checked data sets show a high median quality score (examples in Supplementary 2). An average of 37.4 was found in the median quality scores (based on a scale from − 15 to 40). In total, 94.1% of the median quality scores are above 35. Only, a very low decay in the score values was observed (Graph in Supplementary 2).

The automatic software pipeline of the SILVAngs data analysis service (https://ngs.arb-silva.de/silvangs; Quast et al. 2013, Yilmaz et al. 2014, Klindworth et al. 2013) was used for the community analysis of the NGS data. All data files from GATC Biotech first had to be converted from fastq file format to fasta file format, because the fastq file format was not compatible to upload. The used fastq-to-fasta converter software was "phred33 conversion" from MR DNA Lab. All data sets were analysed using the preset parameters of the settings page and with the SILVAngs database release version 128 (Yilmaz et al. 2014).

Only a part of the assigned bacterial groups could be assigned down to the genus level. Other groups can only be identified with higher taxonomical levels as family, order, class or phylum. The lowest identified level was always referenced as "operational taxonomic unit" (OTU).

For similarity analyses, the absolute number of reads per place or per sample had been used, on the one hand. The absolute number n has mainly been applied for comparing samples by less abundant OTUs and by a search for rare patterns in the presence of soil bacteria. On the other hand, normalized frequency data had been calculated for the comparison of samples by higher abundant OTUs. Therefore, a relative abundance value r and a Shannon diversity-related h value were calculated for each OTU in each sample from the individual number of reads n, the total number of reads of each sample N and the total of r values from all samples R of each OTU.

$$r_{ij} = log\left[10^6 * \left(1 + n_{ij}\right)/N_j\right] \quad (1)$$

Table 1 Origin of samples

Location	Sample no.	Internal Laboratory sample No.	Gauss–Krüger coordinates	
			r	h
Römild, Steinsburg	1a	B11	4399747	5586335
Römild, Steinsburg	1b	B12	4399713	5587157
Römild, Steinsburg	1c	B13	4399713	5587164
Römild, Steinsburg	1d	B14	4399727	5587362
Römild, Steinsburg	1e	B15	4400109	5587413
Römild, Steinsburg	1f	B16	4400094	5587204
Römild, Steinsburg	1g	B17	4400238	5586986
Römild, Steinsburg	1h	B18	4400116	5586747
Aschenhausen, Diesburg	2a	T15-1	3586124	5606568
Aschenhausen, Diesburg	2b	T16-1	3586099	5606493
Aschenhausen, Diesburg	2c	T17-1	3586023	5606384
Aschenhausen, Diesburg	2d	T18-1	3586091	5606482
Aschenhausen, Diesburg	2e	T19-1	3586129	5606346
Vacha, Öchsen	3a	T61-1	3572501	5630023
Vacha, Öchsen	3b	T61-2	3572501	5630023
Vacha, Öchsen	3c	T62-1	357252	5630013
Vacha, Öchsen	3d	T62-2	357252	5630013
Vacha, Öchsen	3e	T63-1	3572523	563001
Vacha, Öchsen	3f	T63-2	3572523	563001
Vacha, Öchsen	3g	T64	3572854	5629539
Dermbach, Röderburg	4a	T31	3576057	5620666
Dermbach, Röderburg	4b	T32	3575996	5620664
Dermbach, Röderburg	4c	T33	357602	5620561
Dermbach, Röderburg	4d	T35	3576082	5620474
Haynrode, Hasenburg	5a	B1	3603574	5702945
Haynrode, Hasenburg	5b	B2	3603355	5702923
Haynrode, Hasenburg	5c	B3	3603337	5702946
Haynrode, Hasenburg	5d	B4	3603431	5702634
Haynrode, Hasenburg	5e	V1	3603342	5702981
Haynrode, Hasenburg	5f	V2-1	3603331	5702839
Haynrode, Hasenburg	5g	V2-2	3603331	5702839
Haynrode, Hasenburg	5h	V3-1	3603331	570282
Haynrode, Hasenburg	5i	V3-2	3603331	570282
Haynrode, Hasenburg	5j	V4-1	360336	360336
Haynrode, Hasenburg	5k	V4-2	360336	360336
Haynrode, Hasenburg	5l	V5	3603354	5702754
Beichlingen, Monraburg	6a	T72	4450469	5678312
Beichlingen, Monraburg	6b	T73	4450782	5678244
Beichlingen, Monraburg	6c	T74	4450792	5678243
Beichlingen, Monraburg	6d	T75	4450676	5678237
Beichlingen, Monraburg	6e	T76	4450687	5678271
Beichlingen, Monraburg	6f	T77	445071	5678328
Beichlingen, Monraburg	6g	T78	4450687	5678354
Beichlingen, Monraburg	6h	T79	4450577	5678428
Sondershausen, Ole Burg	7a	B43-1	4418982	5690304
Sondershausen, Ole Burg	7b	B45-1	4418897	5690399
Sondershausen, Ole Burg	7c	B46-1	4418862	5690465

Table 1 (continued)

Location	Sample no.	Internal Laboratory sample No.	Gauss–Krüger coordinates	
			r	h
Bleicherode, Aaskehren	8a	T1-1	4400216	5700617
Bleicherode, Aaskehren	8b	T2-1	4400548	5700676
Bleicherode, Aaskehren	8c	T3-1	4400731	570053
Bleicherode, Aaskehren	8d	T4-1	4400691	5700481
Theuern, Herrenberg	9a	T40-1	4430138	5589078
Theuern, Herrenberg	9b	T-40-2	4430138	5589078
Theuern, Herrenberg	9c	T41-1	4430187	5589051
Theuern, Herrenberg	9d	T41-2	4430187	5589051
Theuern, Herrenberg	9e	T42-1	4430152	5589113
Theuern, Herrenberg	9f	T42-2	4430152	5589113
Suhl, Forest Lange Bahn	10a	T51	4403701	5605545
Suhl, Forest Lange Bahn	10b	T52	4403665	5605577
Suhl, Forest Lange Bahn	10c	T53	4403641	5605553
Suhl, Forest Lange Bahn	10d	T54	4403608	5605573
Suhl, Forest Lange Bahn	10e	T55	4403607	5605573
Schöps*	HB11	urn	4471485	5633000
Schöps	HB12	reference	4471485	5633000
Schöps	HB13	urn	4471479	5633028
Schöps	HB14	reference	4471479	5633028
Schöps	HB15	urn	4471498	5633016
Schöps	HB16	reference	4471498	5633016
Schöps	HB17	urn	4471495	5633084
Schöps	HB18	reference	4471495	5633084
Schöps	HB19	urn	4471499	5633071
Schöps	HB20	reference	4471499	5633071

*The results on the investigations of the samples from Schöps are reported (Köhler et al. 2018): "urn" means a sample, which was taken from the soil material inside a urn, and "reference" means sample material, which was taken from the soil of the same spot, but outside the same urn

$$h_{ij} = (-1) * r_{ij}/R_j * \ln\left(r_{ij}/R_j\right) \quad (2)$$

The value r can be regarded as the log(ppm) value for the relative number of reads per sample + one. The "ppm" value means "part per million". The value h represents the contribution of each sample to the total Shannon diversity of the whole sample set. The total of these values h result in the Shannon diversity index H for each OTU in the investigated set of 72 samples with a H_{max} value of 4.2767 [=ln(72)]. This parameter is high if a micro-organism component is present in many samples in a similar concentration. It is low in case of the dominance of an OTU in a few samples, only.

Results and discussion

Community types of different types of sampling locations

The soil bacterial communities from the different sampling sites have been characterized by their taxonomic profiles. *Proteobacteria, Bacteriodetes, Actinobacteria* and *Acidobacteria* are the most abundant phyla (Fig. 2). Larger differences have been observed in the abundancies

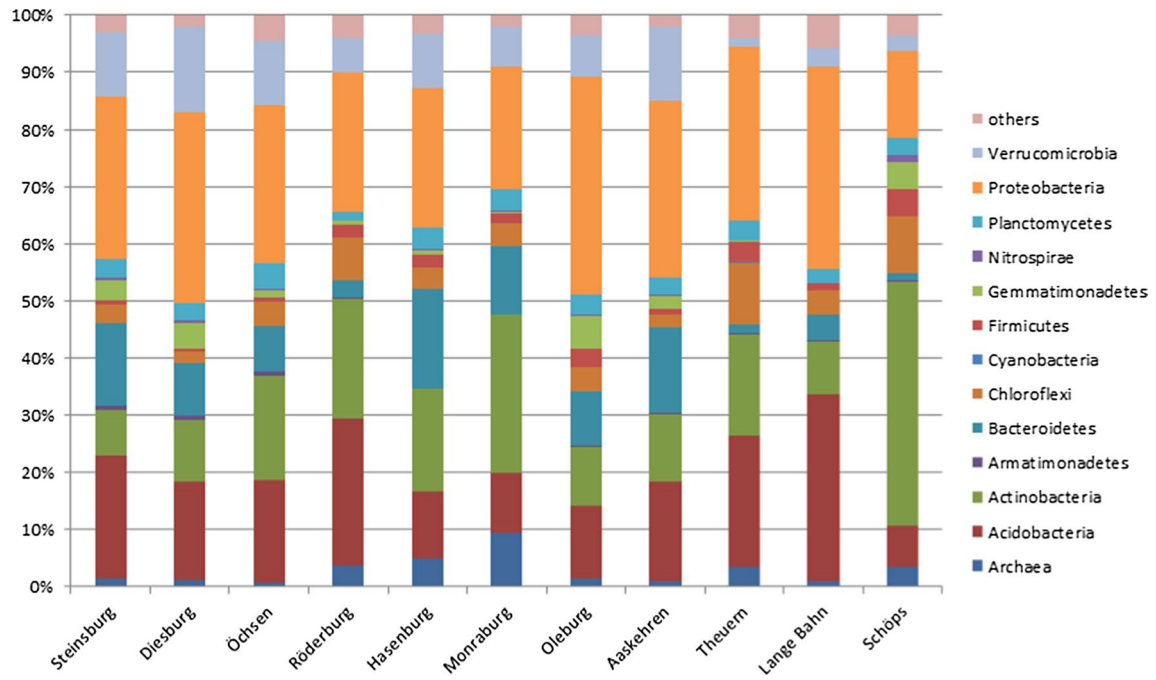

Fig. 2 Taxonomic profile (phylum level) of the investigated sampling places

of *Verrucomicrobia, Nitrospirae, Gemmatimonadetes, Firmicutes* and *Archaea*.

The more abundant OTUs can be distinguished by their diversity over the whole sample set. Figure 3 shows examples of correlations of pairs of arbitrarily selected OTUs from different ranges of H/H_{max} values (normalized Shannon diversity index). The upper part (Fig. 3a) shows the distribution of r values of *Acidimicrobiia IMCC26256* and *Pirellulaceae, uncult.*, which are marked by a narrow diversity corresponding to high H/H_{max} values of 0.96. They belong to a group of OTUs which are highly abundant in all places. The middle and the lower parts (Fig. 3b, c) show that the OTUs *Ilimobacteraceae, uncult.* and *Chloroflexi* TK10 ($H/H_{max} = 0.9$) have a higher distribution, and *Gemmatimonas* and *Acidobacteria, Subgroup 22* ($H/H_{max} = 0.76$) have a very high distribution of abundance in the whole set of investigated samples. Figure 3 illustrates the fact that groups of OTUs with different frequency distribution can be distinguished by their H values.

It was expected that the bacterial soil communities differ in dependence on the geological ground and the soil substrate. Therefore, it was investigated whether the groups of the different regions of Thuringia of different geological situations can be distinguished. A group of OTUs (supplementary Table 1) with higher abundance and moderate normalized diversity index H/H_{max} of ppm values has been chosen for a principal component analysis (PCA). OTUs with moderate values of H/H_{max} have been chosen to have a good representation of OTUs with high differences in their abundancies, on the one hand, and to avoid an over-representation of OTUs which are only present in a few samples, on the other hand. Comparable high values (near one) result from the logarithmic definition of r (Eq. 1). The application of the logarithmic values corresponds to the fact that the abundance of OTUs differs by orders of magnitudes. The application of logarithmic values helps to avoid an over-representation of very frequent OTUs and an under-estimation of OTUs with mediate abundance. The PC1/PC2 plot indicates the similarity of samples within each region and differences between the regions related to the selected OTUs (Fig. 4). The samples of the excavation of the burial place (Schöps, circles), the samples of all four Northern Thuringian hills (squares) and the samples from the investigated places of Thuringian Forest (triangles) are clustered and well separated from each other in the plane of the principal components 1 and 2 (Fig. 4a). A broader distribution was only observed in the case of the basalt hill samples (open diamonds). But the samples of this group can also mostly be distinguished from the quartz and sand-based soils (acidic mineral substrate) of the Thuringian Forest places by regarding the PC1/PC3 plot (third principal component, Fig. 4b). The samples of Hasenburg and Monraburg are particularly highly related. This is probably mainly caused by the similarities in soil substrate and vegetation, but it had also to be reconsidered that both places have important analogies in their ancient fate. Both places are multiphase hill settlements with artefacts from Neolithic

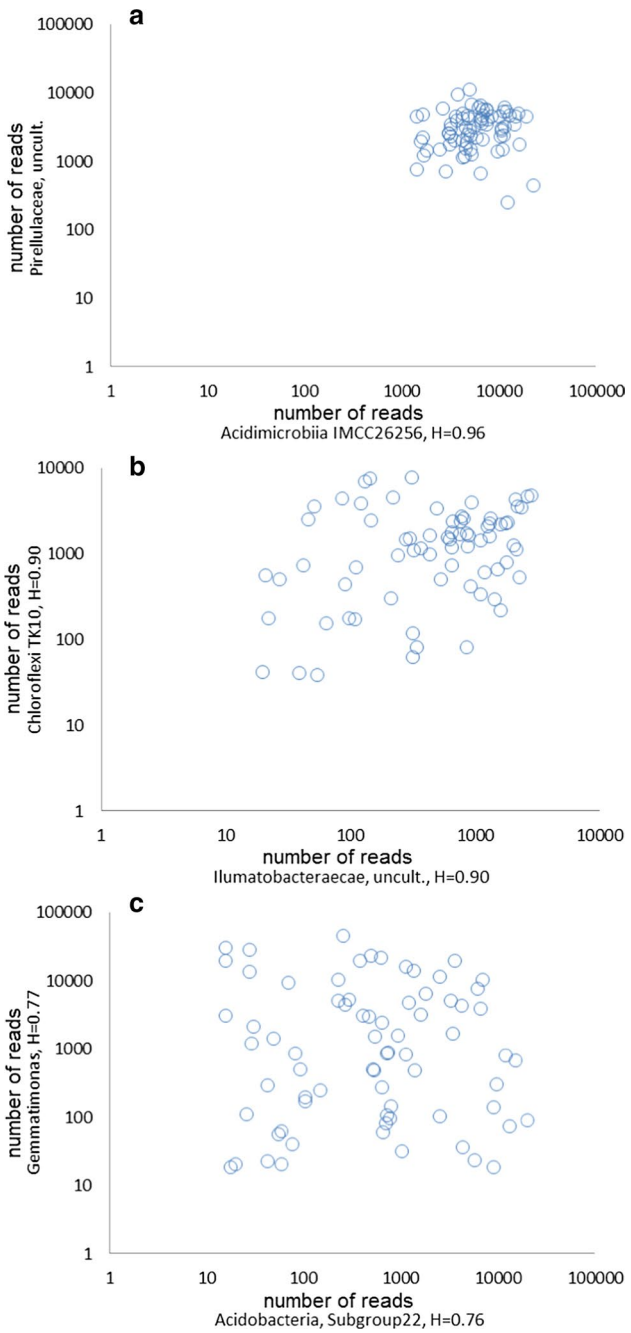

Fig. 3 Correlation of 3 pairs of selected OTUs (higher total abundance) of different Shannon diversity index: **a** $H/H_{max} = 0.96$; **b** $H/H_{max} = 0.90$; **c** $H/H_{max} = 0.76$

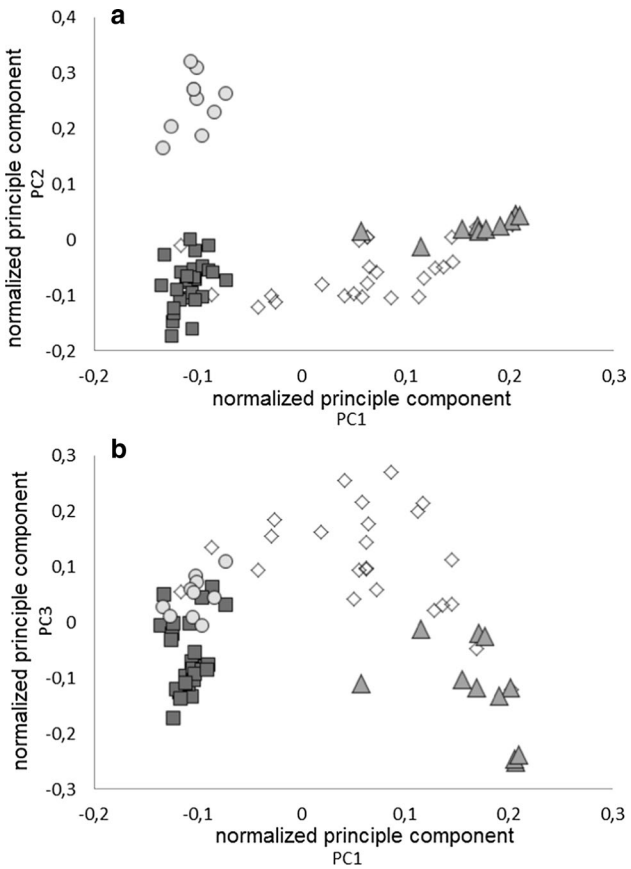

Fig. 4 Principle component analysis of all investigated samples from rampart hills and of the samples of the iron-age burial field of Schöps (Köhler et al. 2018) by 15 selected OTUs (list in Table 1 of supplementary material): symbols are related to soil types: circles: river valley sediments (Schöps), squares: lime stone hills, triangles: quartzite/sand soil of Thuringian Forest places, open diamonds: basalt hills (the PCA is based on the single contributions h of samples to the Shannon diversity index of each OTU with a H value between 0.18 and 0.84 of the group of the 200 most abundant OTUs in total over all samples)

Age over the late bronze age and the early iron age up to a mediaeval re-settlement and re-fortification (Peschel 1986; Simon 1984).

The clustering of regions is also visible by the abundant distribution of some selected single OTUs. Most samples of lime stone and basalt hills can be distinguished by the excavation samples of Schöps and the hills of Thuringian Forest by the abundance of *Ferruginibacter,* whereas the limestone samples and the samples from Schöps are mostly separated from the basalt samples and the Thuringian Forest samples by the abundance of *Rhodanobacter* (Fig. 5a). Ferruiginibacter was described as an isolate from freshwater sediment (Lim et al. 2009), the genus Rhodanobacter for a strain which is able to mineralize the insecticide Lindane (Nalin et al. 1999). In contrast to the basalt places, the three other sample groups are also well distinguishable by the abundances of *Adhaeribacter* and the *Actinobacter group MB-A2-108* (Fig. 5b). Like *Ferruginibacter, Adhaeribacter*, too, is related to freshwater and was isolated from a biofilm in a freshwater tank (Rickard et al. 2005). As it is visible, the basalt hill samples differ much more from each other, and some of these samples are closely related to the Thuringian Forest samples concerning the both last mentioned OTUs. The samples of Schöps have a high content of the

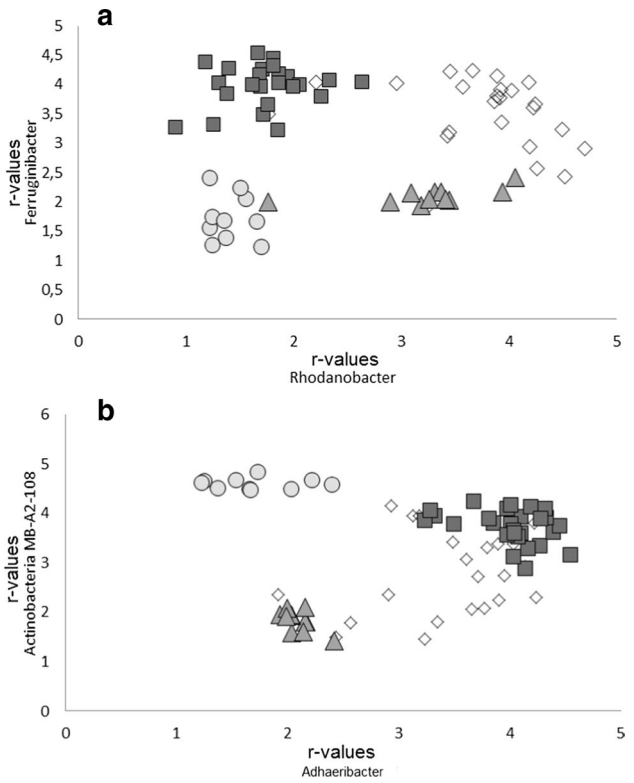

Fig. 5 Distinguishing soil type-related bacterial communities by the r values (log-ppm abundancies) of single pairs of OTUs: **a** *Ferruginibacter vs Rhodanbacter*, **b** *Actinobacter group MB-A2-108 vs Adhaeribacter* (symbols are related to soil types: circles: river valley sediments (Schöps), squares: lime stone hills, triangles: quartzite/sand soil of Thuringian Forest places, open diamonds: basalt hills)

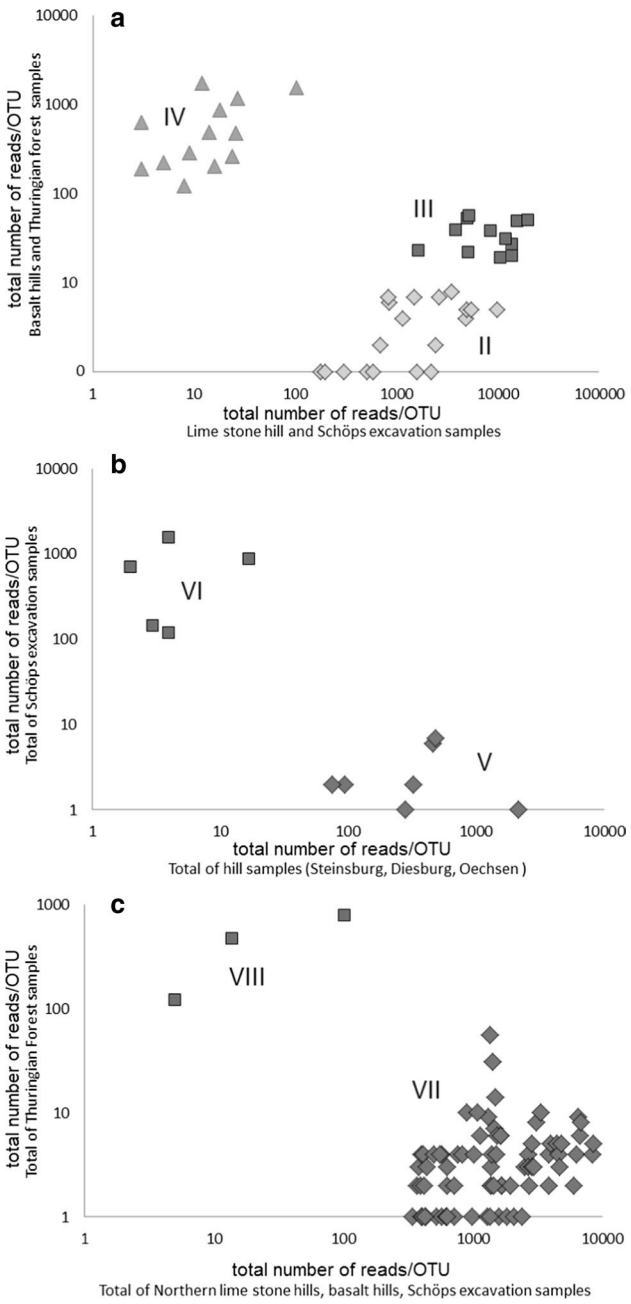

Fig. 6 Distinguishing sample groups by groups of OTUs (total number of reads per OTU for sample groups, Roman numbers characterize groups of OTUs): **a** Basalt hill and Thuringian Forest samples versus lime stone hill and Schöps samples, **b** Schöps samples versus three basalt hill samples, **c** Thuringian Forest samples versus all other samples

Actinobacter MB-A2-108 group, but no or low content of *Adhaeribacter*, whereas this last mentioned OTU is present in all limestone hill samples. The samples of both investigated Thuringian Forest places show low content of *Adhaeribacter* as well as of the *Actinobacter* group (Table 2). It seems to be plausible that the samples from the archaeological excavation (circles in Fig. 5a, b) are related to the low content of the freshwater-related OTUs because the concerned soil was covered by a soil top layer for about 20 centuries.

Besides the distinguishing of all four groups of places with different soil types, it is possible to distinguish sampling places by selected groups of OTUs (listed in supplementary 1). Thus, three groups of OTUs are well reflected by their abundancies on the Basalt hill and Thuringian Forest samples, on the one hand, and the lime stone hill and the Schöps excavation samples, on the other hand (Fig. 6). Two other groups of OTUs distinguish clearly between the Schöps excavation samples and samples of three of the basalt hills. It is remarkable that in this case, the fourth basalt hill place (Röderburg) does not match the other basalt hills. The Röderburg samples are marked by the absence or very low abundancies of organisms of group IV and group V, which corresponds to the features of the Thuringian Forest samples. These last mentioned places (Theuern and Lange Bahn) are distinguishable from all other types of samples (including the samples of

Table 2 Distinction of samples from different soil types by four selected OTUs

	Adhaeribacter	*Actinobacter MB-A2-108*	*Ferruginibacter*	*Rhodanobacter*
Schöps (river valley sediments)	Low	High	Low	Low
Northern Thuringia hill ramparts (lime stone soil)	High	High	High	Low
Thuringian Forest ramparts/settlements (quartzite/sand stone substrate)	Low	Low	Low	High
Southwest Thuringian hill settlements (basalt substrate)	Different	Different	High	High

Röderburg) by OTUs of two other groups (VII and VIII) as shown in Fig. 6c. The OTU groups II–VIII are listed in Table 2–4 of supplementary material 1.

Search for soil microbial signatures on rampart places

Besides bacterial types (OTUs) with higher abundance on several places ("majority components"), there are some "minority components" which are present at one rampart place only, but had not found in the profiling of any other place. The number of these "exclusively" found OTUs differ between the places (supplementary 1, Table 5). The number of reads per OTU is very low in most cases. It might be that some of these OTUs are typical for a place, but a considerable part of these OTUs was probably found accidentally.

Besides these types, there are other small groups of OTUs that are found in two or several samples from the same place. Also in these cases, some OTUs might be found accidentally, but some patterns seem to be characteristic of a group of samples from the same place (supplementary 1, Table 6). Similarities are observed in patterns of places with similar types of soil. This confirms the above-described significant relation between the bacterial communities of different places with the same geological situation. Thus, minority types that have been observed in samples from Steinsburg (basalt substrate) are partially found in other basalt hills, too. In the case of the set of samples from Hasenburg (limestone substrate), an enhanced number of reads of two minority OTUs (*Bacteriovoracaceae, uncultured* and *Rubinisphaeraceae, uncultured*) distinguishes these samples from the samples of other places. Even on the other limestone hills, only one of these both OTUs is enhanced in the samples (for data see also supplementary 1, Table 6; Hasenburg). An example of a signature-like pattern with stronger enhanced numbers of reads was detected of one sample of Ole Burg (place No. 7) and Aaskehren (place No. 8). The combined significant presence of a group of four OTUs (*Anaerosporomusa, Anaerolinea, Desulfuromonas, Gracilibacter*) was observed in these two samples, but in no other samples. It is remarkable that all four OTUs are described to thermophilic, partially, and are related to anaerobic conditions (Sekiguchi et al. 2003; Lee et al. 2006; Choi et al. 2016).

Despite the similarities between samples of the same place and samples with similar soil conditions, there are also small sets of minority OTUs which are found in a few places only. These OTUs or small sets of them can be regarded as connecting certain places (examples in Fig. 7). Thus, a set of four minority OTUs (*Desulf. MA-28-I98C, Ignavibacterium, Cand. Vogelbacteria, Vadin HA17*) connects the samples of Öchsen (basalt hill, Rhön) with samples of Ole Burg (limestone hill, Northern Thuringia). Another set of seven OTUs (*Thermoplasmata, uncult., Ktedenobacterales Bac C-u-018, Dermococcaceae, uncult., Acdiobacillus, Actinocatenispora, Methyloparacoccus, Methanobacterium*) connects the basalt hill of Röderburg (Rhön) with the rampart of Theuern in the Thuringian Forest (quartzite, sand soil). Four of these OTUs have been also found on the rampart of Lange Bahn in the Thuringian Forest, two of them on Öchsen. It is remarkable that a group of anaerobic and methanotrophic OTUs (Hoefman et al. 2014) is important for these relations between the places, too. The ramparts of Lange Bahn and Theuern supplied a group of four common OTUs (*Acidobacterium, Natroniella, Telmatobacter, Orenia*) which are known to be connected with extremeous pH, salt content and anaerobic conditions (Kishimoto et al. 1991; Zhilina et al. 1996; Mouné et al. 2000; Pankratov et al. 2012).

Interesting differences in the bacterial communities have been found between two samples of the interior of the rectangular rampart of Lange Bahn and two reference samples taken a few metres outside of this rampart. Inside the wall area, several OTUs had been found which are very low or not present outside (Table 7 of supplementary material 1). Among them are a conspicuous number of OTUs of *Ktedonobacteria*, which attract interest due to expectations to producing useful secondary metabolites (S. Yabe et al. 2017). Further, several OTUs of the family *Beijerinckiaceae* have been found inside the wall area, among them *Methylocapsa* and *Methylocella* two acidiophilic and methanotrophic bacteria (SN Dedysh et al. 2002, SN Dedysh et al. 2005). With *Streptacidiphilus* (S.B. Kim et al. 2003), *Catenulispora* (E. Busti et al. 2006) and *Actinospica* (L. Cavaletti et al. 2006) further

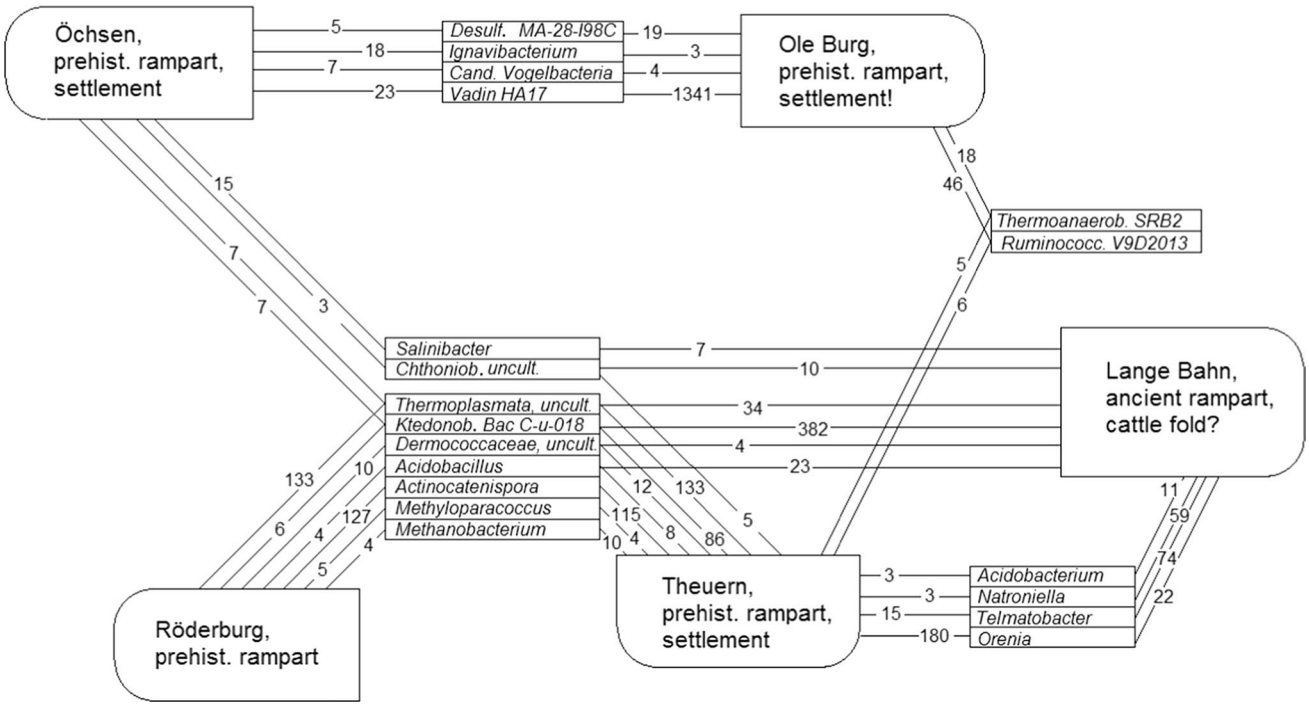

Fig. 7 Relations between samples of selected rampart hills by groups of minority OTUs found in the 16S-RNA profiling (the numbers give the total number of reads of the single OTUs for the related places)

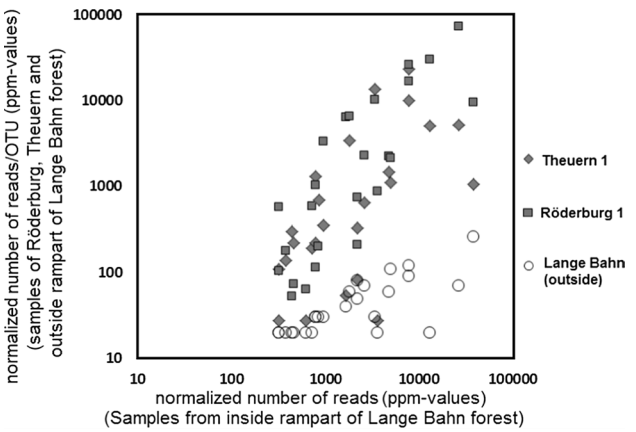

Fig. 8 Normalized numbers of reads (ppm values, 0 set to "20") for samples no. 4c (Röderburg, diamonds) and 9a (Theuern, squares) versus the reads of the inner part of Lange Bahn in comparison with the samples from outside Lange Bahn (open circles), selected OTUs corresponding to Table 7 of the supplementary material

acidophilic bacteria have been found. The observation of these differences could give a hint on the former use of the concerned wall rampart. It is supposed that this kind of ramparts (so-called Vieh-Halde) was mainly constructed for protecting cattle. The above-discussed asymmetries between inside and outside found OTUs would match to this purpose. Interestingly, the concerned area is a forest, recently, and the assumed construction and use of the rampart date back to centuries, at least, probably back to prehistoric times. Concerning the historical land use situation, the found features of minority components in the bacterial communities could be interpreted by a long-term ecological memory of the place. Two samples from the inner part of the ramparts of Theuern and Röderburg show a distribution pattern of the above-mentioned OTUs (Fig. 8) which is similar to the inner part of the Lange Bahn rampart and significantly different from the samples outside of Lange Bahn.

The observed differences in the compositions of soil bacterial communities in the different samples match the observation that archaeological residues can be distinguished by the analysis of the contained bacteria as it was found, e.g. in case of the investigation of Indigenous ochre from different sites of Australia (Lenehan et al. 2017). Besides the expectation to identify the provenance or archaeological samples, it has to be taken in mind that the mineral substrate and the local environment play a crucial role in the presence of many members of a recent bacterial community. Even though chemical conditions of soil mainly influence the composition of soil bacterial communities, sample-specific bacterial components seem to be present over long time in archaeological samples. This corresponds to the presence of DNA of bacterial human pathogens in ancient human remains and fits the finding that microbial communities are

sample specific and less dependent on geographical origin (Philips et al. 2017).

Comparison of lower abundant OTUs from samples of prehistoric hill settlements with RNA profiling data from the iron-age burial site near Schöps (Thuringia)

In an earlier study, NGS profiling of 10 soil samples of an iron-age burial field near Schöps (Germany) supplied characteristic features of the minority OTUs from the interior of urns and external reference soil samples (Köhler et al. 2018). In an analogy to these investigations, it is also possible to look for minority components of the soil bacterial communities that are present on the rampart places and the burial field of Schöps. In Fig. 9, such relations between the results from Schöps and some of the investigated rampart hills are shown. Besides groups of minority OTUs which are present on one rampart, mainly, other groups exist which have been found on two of these places. Thus, one group of OTUs (*Pseudoclavibacter, Tepidimicrobium, Renibacterium, Rhodobacteraceae, Rubellimicrobium*) connects Schöps with Hasenburg and Monraburg samples (both limestone substrate), another group (*Gordonia, Agreia, Yonghparkia*) with Hasenburg and Steinsburg (basalt substrate).

A significant set of minority OTUs relates the soil bacterial community of Schöps samples with the samples of sandy/quartzite substrate of the rampart of Theuern (*Caldibacillus, Caproiciproducens, Methanosarcina, Ureibacillus* and *Solobacterium*), a second set *(Desulfurispora, Desulfitibacter, Azospirales*, uncult. and *Methylophilaceae* MM2) to the samples of Ole Burg (limestone), a third (*Balneo*, uncult., *Muribaculaceae,* LD1-PA32) to Steinsburg and a forth (*Paenibacillaceae, Kurthia, Methanomassiliicoccales, Pigmentiphaga* and *Cryobacterium*) to Hasenburg. In these relations, anaerobic and methanogenic soil bacteria form an important part, too. The strong influence of the local environment on the composition of bacterial communities in the single samples corresponds to investigations on microbial communities inside and on the surface of ancient limestones of Maya buildings (McNamara et al. 2005) which showed significant differences in the epilithic and endolithic bacterial communities with high importance for the conservation of the archaeological residuals. Whereas the above-mentioned organisms reflect the general relations of the samples from Schöps, it is possible to analyse the specific OTUs from the internal material from the urns, too. In another earlier study of ours, significant differences between four of the samples from the urns and the reference material were detected (Köhler et al. 2018). The comparison of these data with the *16S rRNA* profiles from the ramparts

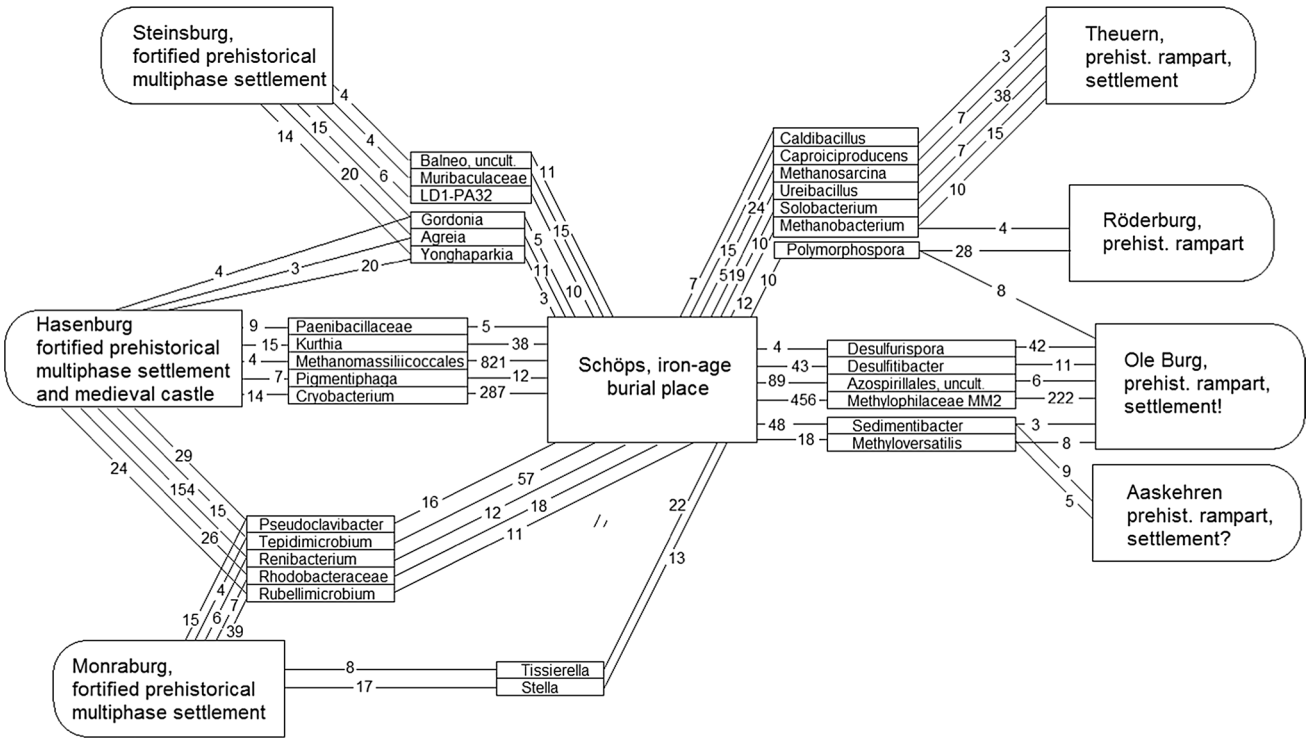

Fig. 9 Relations of samples of selected rampart hills to soil samples of the iron-age burial field of Schöps by groups of minority OTUs found in the 16S-RNA profiling (the numbers give the total number of reads of the single OTUs for the related places)

speaks of the existence of certain signature-like patterns. Thus, four OTUs (*Fodinicola, Sphingobacterales* NS11-12 marine group, *Sphingobacterales* S15-21 group and *Adhaeribacter)*, which had been found in several urns, but not in the reference samples from Schöps, are present significantly at three of the basalt hill ramparts and all limestone hills, but are only partially present in the samples of Röderburg, Theuern and Lange Bahn (data in Table 9 of supplementary material 1).

It is obvious that the observed pattern cannot be explained by the character of soil substrate, exclusively, but might have to do with the historical or even the prehistorical use of the places. Some of the OTUs found in one urn exclusively and seem also to be related to patterns of the rampart communities (data in Table 10 of supplementary material 1). The above-mentioned distribution pattern (significant presence on places number 1 (Steinsburg), 2 (Diesburg), 3 (Öchsen), 5 (Hasenburg), 6 (Monraburg), 7 (Ole Burg) and 8 (Aaskehren) and the absence or low presence on the three other places no. 4, 9 and 10) was also observed for two OTUs (*Aquaspirillum arcticum* group, *Segetibacter*) found only in urn sample HB11 and for four OTUs (*Polyangiaceae* strain, *Sulfuriferula, Rhodobacter, Solibacteraceae Elev-16S-1166*) found only in the urn sample HB13. A certain pattern (*Salinimicrobium, Marisediminicola, Alkanindiges*) could also be recognized in the samples of places number 2, 5 and 6) for three OTUs of urn sample HB17. In contrast, no hints of signature-like structures could be found for the exclusive OTUs of urn samples HB15 and HB19. Despite the possibility that a part of the observed "signature-like" relations between soil from urns and the investigated samples from hills might be caused accidentally, another part could hint to connections based on a former exchange of plants, animals or organic material. In the recent state of investigations, it is difficult to decide whether these patterns indicate, indeed, a relation between late pre-Roman iron-age soil samples from Schöps and the late Latène period settlements on hills as Steinsburg, Diesburg, Öchsen, Röderburg and Herrenberg near Theuern. In the future, more detailed soil genomic studies including larger metagenomic data sets and whole genome sequencings could allow for identifying such prehistorical connections.

Conclusions

The *16S rRNA* profiling of soil samples from 10 prehistoric ramparts of different regions of Thuringia shows that the bacterial communities of the places can clearly be distinguished by the geological substrate. On the one hand, soil bacteria that are represented in all samples by many reads (majority components) have been found. On the other hand, significant differences for other OTUs, which are highly present in some samples, but low presence or absent in others have been observed. In their presence, the sample origin is important and reflects differences in the composition of soil bacterial communities from a limestone, basalt or sand stone substrate. It was found that these community groups can also be distinguished by majority components from the bacterial communities identified in soil samples from an archaeologically investigated iron-age burial field in the Saale valley near Schöps.

The single samples show partially large differences in less abundant bacterial types (OTUs). These minority components of local soil bacterial communities form in some cases certain patterns, which could be regarded as "signature-like" features of a special soil. Such patterns are not limited to samples of the same soil type, but can also be found between samples of different places and between single samples of the studied ramparts and the interior material of iron-age urns from Schöps. The dominance and the presence or absence of certain OTUs are mainly connected with the acidity of soils. Despite the age of about two thousand years of the urns and continuous leaching by soil water, the cremated remains inside of urns might have contributed to similarities with the communities of limestone places. Other similarities should be regarded as a pH-independent ecological memory of soil. A pH-independent effect of usage past of a place seems to be also responsible for significant differences in the composition of soil communities inside and outside the rampart of Lange Bahn near Suhl. These soil samples are related to the same mineral substrate and are marked by the same pH. They could confirm the assumption of a former concentration of cattle inside the rampart.

The investigations have shown that 16S rRNA profiling of soil microbial communities is a valuable tool for the characterization of soils from prehistorically used areas. The results suggest a long-time ecological memory of soils that might be able to reflect earlier land use. It is to expected that more detailed metagenomics studies, the identification of special strains and metabolic potential of local soil microbial communities can supply a lot of information not only on the recent ecological situation but also on the ecological past of a place. Genetic profiling could help in the interpretation of archaeological findings of prehistoric constructions and their original use. Also, they can contribute to understanding the value of long-term anthropogenic effects on the diversity of local microbial communities.

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