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The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities

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Abstract

Verrucomicrobia are ubiquitous in soil, but members of this bacterial phylum are thought to be present at low frequency in soil, with few studies focusing specifically on verrucomicrobial abundance, diversity, and distribution. Here we used barcoded pyrosequencing to analyze verrucomicrobial communities in surface soils collected across a range of biomes in Antarctica, Europe, and the Americas (112 samples), as well as soils collected from pits dug in a montane coniferous forest (69 samples). Data collected from surface horizons indicate that *Verrucomicrobia* average 23% of bacterial sequences, making them far more abundant than had been estimated. We show that this underestimation is likely due to primer bias, as many of the commonly used PCR primers appear to exclude verrucomicrobial 16S rRNA genes during amplification. *Verrucomicrobia* were detected in 180 out of 181 soils examined, with members of the class *Spartobacteria* dominating verrucomicrobial communities in nearly all biomes and soil depths. The relative abundance of *Verrucomicrobia* was highest in grasslands and in subsurface soil horizons, where they were often the dominant bacterial phylum. Although their ecology remains poorly understood, *Verrucomicrobia* appear to be dominant in many soil bacterial communities across the globe, making additional research on their ecology clearly necessary.

Keywords

Verrucomicrobia; *Spartobacteria*; Bacteria; Relative abundance; Ecological dominance; Primer bias; Barcoded pyrosequencing; 16S rRNA genes; Oligotroph; Subsurface horizon

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1. Introduction

Bacteria belonging to the phylum *Verrucomicrobia* are nearly ubiquitous in soil (Zhang and Xu, 2008), but are usually considered to be one of the less frequent bacterial phyla. A recent meta-analysis of published clone library results indicated that, on average, only about 7% of soil bacterial sequences were verrucomicrobial (Janssen, 2006). *Verrucomicrobia* are also typically difficult to cultivate (Janssen et al., 1997, 2002; Janssen, 1998; Joseph et al., 2003; Davis et al., 2005). As a result, this phylum is currently represented by <100 isolates in public databases, such as Greengenes (<http://greengenes.lbl.gov/>) and the Ribosomal Database Project (<http://rdp.cme.msu.edu/>). To date, nearly all *Verrucomicrobia* cultured from soil have been free-living (Schlesner et al., 2006; Ward et al., 2006; Griffiths and Gupta, 2007) and most are mesophilic (Sangwan et al., 2004), facultatively or obligately anaerobic (Chin et al., 2001), saccharolytic (Janssen, 1998), and oligotrophic (da Rocha et al., 2010; Senechkin et al., 2010). Several studies have documented the distribution of *Verrucomicrobia* across specific soil gradients (O'Farrell and Janssen, 1999; Buckley and Schmidt, 2001; Sangwan et al., 2005), but relatively little information is available on the composition, diversity, and overall distribution of verrucomicrobial communities across a range of soil types.

In this study, we set out to obtain improved estimates of the relative abundance and distribution of *Verrucomicrobia* in soil, and identify the dominant taxa in this phylum across a range of soil types. In order to meet these objectives, we analyzed 181 soils from Antarctica, Europe, and the Americas, including samples from subsurface soil horizons in a montane coniferous forest. All soils were analyzed with a recently developed primer set that amplifies a phylogenetically informative region of the 16S rRNA gene with few selective biases against *Verrucomicrobia* or any other known bacterial taxa. A barcoded pyrosequencing protocol similar to that described previously (Fierer et al., 2008; Hamady et al., 2008) was used to sequence all PCR products. Estimates of verrucomicrobial abundance were then compared to those from previously published datasets. Patterns of diversity and distribution were examined in order to provide some insight into the ecology of soil *Verrucomicrobia*.

2. Methods

2.1. Sample collection

We collected 112 mineral soil samples from relatively undisturbed sites across North America, South America, Europe, and Antarctica. These represent a wide range of soil types and span a range of edaphic and environmental characteristics (e.g. latitudes between 68°N and 78°S, pH between 3.4 and 9.9, and total organic carbon concentrations between 0.12 and 24.94 mg C kg soil⁻¹). Sampling procedures followed the protocol outlined by Fierer and Jackson (2006). Briefly, samples were taken near the time of the peak growing season for vascular plants (except those from Antarctica where plants are absent). At each site, five to ten randomly selected samples were collected (to a depth of ~5 cm) within an area of about 100 m². These were combined into a single composite sample for each site, and then shipped at 4°C to the University of Colorado at Boulder. Upon receipt, samples were homogenized, sieved to 4 mm, and stored at -20°C until DNA extraction. To assess how verrucomicrobial abundance varied across soil depth profiles (from the surface down to 170 cm in depth), we also included an additional 69 samples from nine soil pits excavated in a montane coniferous forest in Colorado, USA (Pit 6 was in a meadow). These samples were processed following the procedure described above. Sampling locations and characteristics for all 181 soils included in this study are provided in Supplementary Table S1.

2.2. DNA extraction, PCR amplification and sequence analysis

DNA was extracted from soils using the MO BIO PowerSoil® DNA Isolation Kit, and following the method described in Lauber et al. (2008). Amplicons for bar-coded pyrosequencing were generated using the methods described in Lauber et al. (2009), except that triplicate PCR reactions were performed for each sample using the primer set 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3'). The 515F primer included the Roche 454-B pyrosequencing adapter and a GT linker, while 806R included the Roche 454-A sequencing adapter, a 12-bp barcode (unique to each sample), and a GG linker. The region amplified by this primer set is well suited for accurate phylogenetic placement of bacterial sequences (Liu et al., 2007) and, as explained below, the primer set should amplify nearly all bacterial taxa with few biases. Amplicons from the triplicate reactions were combined, cleaned and quantified to determine the volume needed to produce a single composite sample with equal representation of each individual sample. The composite sample was sent to Engencore at the University of South Carolina for sequencing on a Roche 454 automated sequencer.

Pyrosequencing data were processed using the QIIME pipeline (Caporaso et al. 2010). Briefly, sequences were assigned to specific samples based on their unique bar-codes and binned into phylotypes at 97% sequence similarity. A representative sequence of each phylotype was chosen and classified using the RDP II taxonomy (Cole et al., 2005). Relative verrucomicrobial abundances were calculated as the percentage of bacterial sequences in each sample assigned to the verrucomicrobial phylum.

Primer coverage predictions were based on the results from the Primer Prospector analysis pipeline (<http://pprospector.sourceforge.net/>). The Silva dataset (<http://www.arb-silva.de/>) was used in the *in silico* primer coverage prediction, and filtered by length and percent identity. Length was based on domain of life, with 1,450 bp as a minimum sequence length for Archaea and Bacteria, and 1,800 bp for Eukarya. Sequences were also filtered to 97% identity using Uclust (Edgar, 2010). Primers were scored by finding the best local alignment to Silva dataset sequences, and assessing a weighted score based on 3' and non-3' mismatches. Primer pair coverage (Figure S1) was generated by using the lowest primer score of the pair.

2.3 Meta-analysis

We reviewed studies that used “universal” bacterial primers for sequence-based surveys (clone library/Sanger sequencing or pyrosequencing) of 16S rRNA genes from soil samples in order to compare our results to previous estimates of relative verrucomicrobial abundances. Only those studies with at least 50 sequences per sample were used for meta-analysis. The ten studies included in the meta-analysis, and the primer sets used in each study are described in Tables S2 and S3, respectively.

3. Results

From the 181 soils included in this study, we obtained a total of 207,015 quality bacterial sequences, with an average read length of approximately 260 bp. Sequences assigned to the phylum *Verrucomicrobia* made up 48,802 of these sequences. *In silico* tests indicate that most of the commonly used primer sets considered to be “universal” for bacteria are biased against *Verrucomicrobia*, while the primer set used here (515F/806R) is relatively unbiased against this and other bacterial phyla (Tables S4 and S5). This primer bias has a clear effect on estimates of verrucomicrobial abundance, as the average relative abundance of verrucomicrobial sequences was 5.3% (0.0 – 20.0%) in our meta-analysis of 124 soils from previously published studies using other primer sets, but 23.5% (0.0 – 72.6%) in our direct

analysis of 181 soils (Figure 1a). In addition, for a subset of 55 soils that were analyzed using both 27F/338R (another commonly used “universal” primer set) and 515F/806R, verrucomicrobial abundances were only 0.9% with 27F/338R (Lauber et al., 2009), but they were 14.9% for these same soils analyzed using 515F/806R (Figure 1b).

The mean number of verrucomicrobial phylotypes per soil for all 181 soils was 49. Within the phylum, class *Spartobacteria* was dominant, with 92% of all verrucomicrobial sequences (44,847 sequences in total) assigned to this group (Table 1). Subdivision 3 and *Opitutae* were the second and third most abundant classes, constituting 5% and 1% of sequences, respectively. A few phylotypes were most closely related to “*Candidatus* Xiphinematobacter brevicolli,” an endosymbiont of the nematode *Xiphinema*, which inhabits the rhizosphere as a plant pathogen (Vandekerckhove et al., 2000, 2002). However, the majority of dominant phylotypes were most closely related to *Chthoniobacter flavus*, a free-living soil heterotroph (Sangwan et al., 2004) (Table 1).

Although sampling was unequal across biomes, soil from grasslands and prairies had the highest average relative abundances of *Verrucomicrobia*, averaging 35% of all bacterial sequences (Figure 2) in individual soils from these biomes. *Spartobacteria* were the most abundant class, but they dropped to only 36% in agricultural fields, while Subdivision 3 and *Opitutae* rose to 44% and 19%, respectively (Figure 3). We also examined verrucomicrobial distribution in nine pits from a montane coniferous forest, two of which were excavated below 160 cm. The relative abundances of *Verrucomicrobia* ranged from 2–20% near the surface (0–6 cm), but were as high as 52% at intermediate depths (approximately 10 to 50 cm). Class *Spartobacteria* was dominant in all horizons (Figure 4).

4. Discussion

It is likely that verrucomicrobial abundances have been underestimated in many studies, as a number of commonly used PCR primers targeting 16S rRNA are biased against members of the *Verrucomicrobia* (see Figure S1, and Tables S4 and S5; see also: Andersson et al., 2008; Hamady and Knight, 2009; Wang and Qian, 2009). Although the discrepancy in estimated verrucomicrobial abundances between the 515F/806R primer set used here and other primer sets (Figure 1a) could be due, in part, to differences among soils, or the taxonomic schemes used in the different studies, analyses conducted on the same 55 soils with identical methods, but different primers, indicate that verrucomicrobial abundances are likely many times higher than previously estimated (Figure 1b). Together with *in silico* tests (Tables S4 and S5), these results show that 515F/806R achieves good coverage with no significant bias against the bacterial phyla evaluated, including *Verrucomicrobia*, making it an effective “universal” primer set for bacterial analyses. *Verrucomicrobia* appear to be far more common in soil than previously thought, their average abundance in soil (23.5%) being comparable to that of other phyla considered to be dominant in soil, namely *Proteobacteria* (39%), *Acidobacteria* (20%), and *Actinobacteria* (13%) (Janssen, 2006).

We found that the dominant verrucomicrobial class in nearly all soils was *Spartobacteria* (Table 1), only one representative of which has been cultivated to date (Schlesner et al., 2006). However, other molecular studies have shown that *Spartobacteria* is the most common verrucomicrobial class in soil (Sangwan et al., 2004; Janssen, 2006).

The relative abundance of *Verrucomicrobia* was highly variable across soils, as confirmed in other studies (Buckley and Schmidt, 2001, 2002; Janssen et al., 2002; Jansen, 2006), with members of this phylum apparently most abundant in soils from grasslands and prairies (Figure 2). With so few soil *Verrucomicrobia* having been cultured, the ecological relevance of this finding remains to be determined. However, it is possible that *Verrucomicrobia* are

more abundant in grasslands due to an association with eukaryotic hosts. The most abundant verrucomicrobial class, *Spartobacteria*, contains free-living taxa, as well as a number of endosymbionts associated with nematode worms in the genus *Xiphinema* (Vanderkerckhove et al., 2000, 2002; Wagner and Horn, 2006). Because temperate grasslands typically harbor relatively high levels of nematode biomass (Fierer et al., 2009), this may, in part, explain why *Verrucomicrobia* appear to be so abundant in this biome (Janssen, 2006). However, further research is required to test this hypothesis.

In the soil profiles examined from a single, forested watershed, there was a striking increase in verrucomicrobial abundances at intermediate depths (Figure 3), with *Verrucomicrobia* dominating some of the bacterial communities in horizons 10 to 50 cm below the surface. Additional research is required to determine if this pattern is observed in profiles from other ecosystem types. Because C availability decreases as one moves down a soil profile (Fierer et al., 2003), *Verrucomicrobia* may be relatively abundant in subsurface horizons due to their oligotrophic life history strategy (da Rocha et al., 2010; Senechkin et al., 2010). Bacteria that are difficult to culture may be specialized for oligotrophy (Garland et al., 2001; Sigler et al., 2002), so the slow growth rate of *Verrucomicrobia* (Janssen et al., 1997; Janssen, 1998; Janssen et al., 2002) may allow them to exploit sparse resources in subsurface soils. Alternatively, if there are methanotrophic members of this phylum in soil, as there are in hot springs (Semrau et al., 2008; den Camp et al., 2009; Lee et al., 2009), then the subsurface peak in verrucomicrobial abundance may correspond to the peak in methane oxidation that occurs at similar depths in other forested soil profiles (Mohanty et al., 2006, 2007). *Verrucomicrobia* are likely to occupy very different ecological niches in both surface and subsurface horizons, but the environmental factors regulating their diversity and abundance within and across soil types remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- A total of 181 soils from Antarctica, Europe, and the Americas were analyzed via pyrosequencing.
- *Verrucomicrobia* was found to be a dominant phylum in soil, averaging 23% of bacterial 16S rRNA gene sequences.
- Relative abundance of *Verrucomicrobia* was highest in grasslands and some subsurface soils, where >50% of bacterial 16S rRNA gene sequences were verrucomicrobial.
- Abundance of *Verrucomicrobia* has been underestimated, probably due to primer bias.
- Class *Spartobacteria* was dominant within phylum *Verrucomicrobia*.

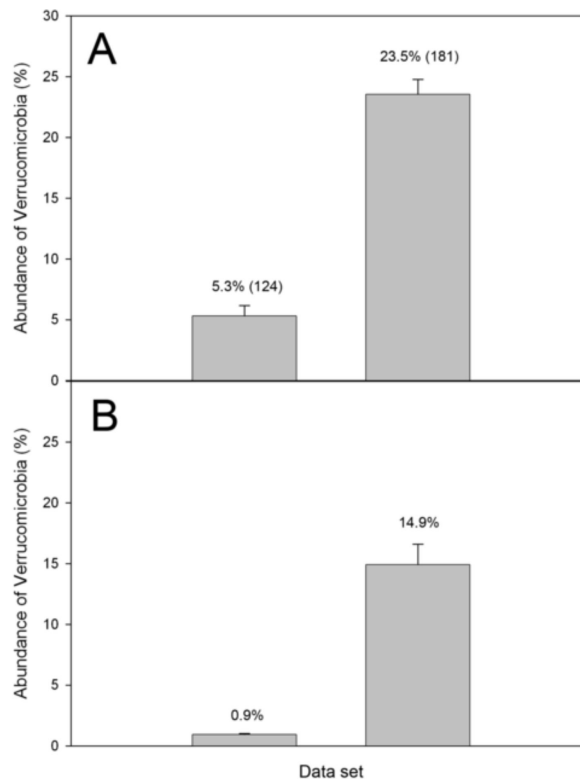


Figure 1. Relative abundance of *Verrucomicrobia* in soil. *A*) Percent abundance of *Verrucomicrobia* in previously published datasets (124 soils; left column) and this study (181 soils in all, including both surface and subsurface samples; right column). *B*) Percent abundance of *Verrucomicrobia* for a subset of 55 soils, analyzed using the 27F/338R primer set (Lauber et al., 2009; left column), then reanalyzed in the present study using the 515F/806R primer set (right column). Error bars represent standard error of the mean.

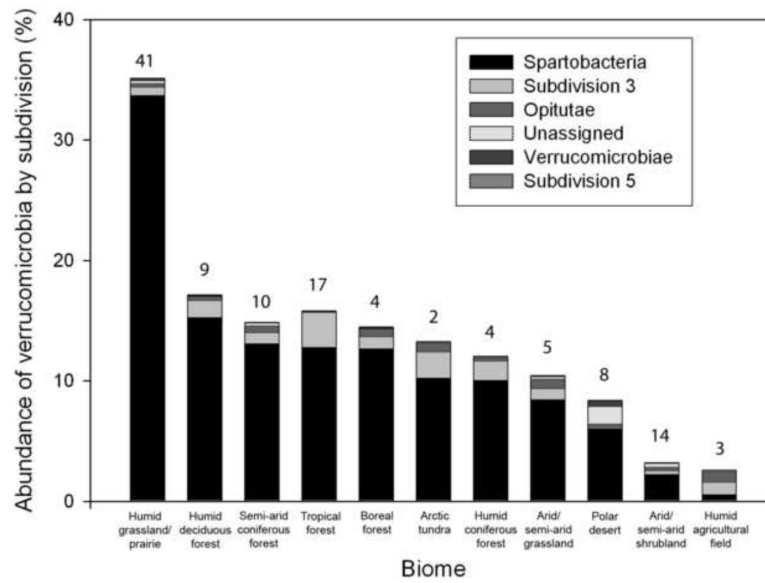


Figure 2. Abundance of *Verrucomicrobia* by biome. Numbers over each bar represent the number of soil samples for that biome. Total height of each bar represents the mean abundance of *Verrucomicrobia* (relative to all bacteria) in each biome.

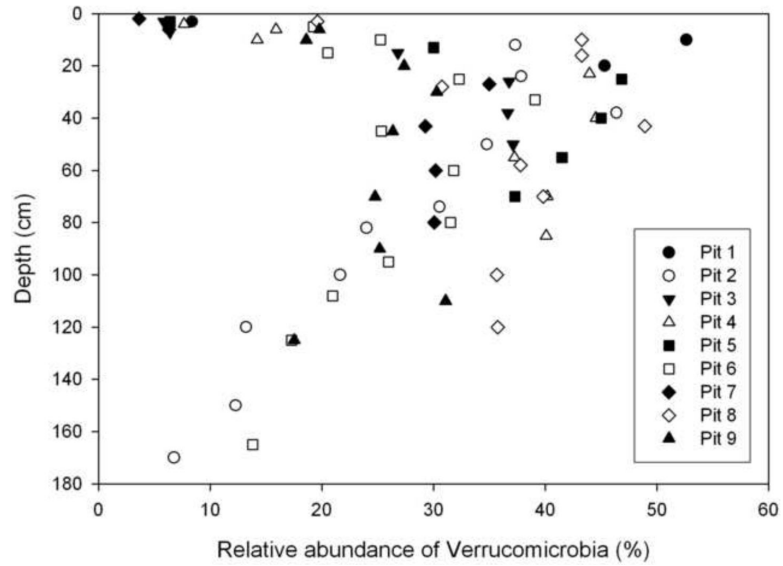


Figure 3. Relative abundance of *Verrucomicrobia* relative to other bacterial phyla in nine soil pits, ranging in maximum depth from 20 to 170 cm.

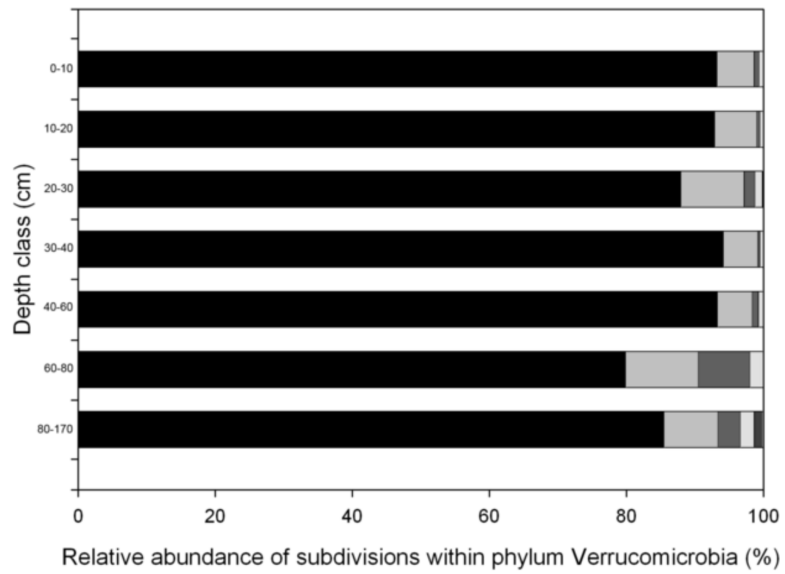


Figure 4. Abundance of classes within the phylum *Verrucomicrobia* in nine soil pits, ranging in maximum depth from 20 to 170 cm. Classes depicted on bar from left to right: *Spartobacteria*, Subdivision 3, *Opitutae*, Unassigned, and *Verrucomicrobiae*.

Table 1

Relative abundance of individual verrucomicrobial phylotypes across surface soils from different biome types.

Phylotype	% of sequences	Frequency	Closest matching isolate	GenBank #	% Identity	AT	ASAG	ASAS	BF	AF	CF	DF	GP	PD	SACF	TF
PT00001	30.01	89	<i>Chthoniobacter flavus</i>	AY388649	91	4	22	5	24	10	31	27	45	1	23	23
PT00002	19.18	77	<i>Chthoniobacter flavus</i>	AY388649	91	0	7	0	0	5	23	7	29	0	4	45
PT06005	6.69	73	<i>Chthoniobacter flavus</i>	AY388649	90	0	6	2	9	5	5	5	9	0	10	6
PT03824	6.08	81	<i>Chthoniobacter flavus</i>	AY388649	90	31	40	46	11	15	4	5	3	0	31	3
PT00683	4.50	89	<i>Chthoniobacter flavus</i>	AY388649	92	9	10	2	15	5	3	5	4	24	11	4
PT02737	2.36	72	<i>Chthoniobacter flavus</i>	AY388649	91	26	2	3	10	5	8	13	1	0	3	9
PT13121	1.96	40	" <i>Candidatus</i> Xiphinematobacter brevicollis"	AF217462	89	5	0	0	3	25	12	21	1	0	0	1
PT04198	1.76	101	<i>Chthoniobacter flavus</i>	AY388649	99	3	5	26	4	10	3	3	1	9	5	1
PT05991	1.69	62	<i>Chthoniobacter flavus</i>	AY388649	91	1	1	6	0	0	1	0	0	65	3	2
PT05512	1.35	55	<i>Chthoniobacter flavus</i>	AY388649	90	1	0	0	1	0	2	1	2	0	0	3
PT06508	1.02	59	<i>Chthoniobacter flavus</i>	AY388649	93	1	4	9	1	0	0	0	1	1	6	0
PT02553	0.98	52	<i>Chthoniobacter flavus</i>	AY388649	92	0	1	0	0	0	1	4	1	0	1	1
PT07240	0.97	47	" <i>Candidatus</i> Xiphinematobacter brevicollis"	AF217462	89	4	0	0	15	0	3	3	0	0	1	0
PT05715	0.93	40	<i>Chthoniobacter flavus</i>	AY388649	90	0	1	1	2	0	0	1	1	0	1	1
PT02535	0.77	58	" <i>Candidatus</i> Xiphinematobacter brevicollis"	AF217462	81	16	0	0	4	20	5	3	0	0	1	2

The biome categories are AT (arctic tundra; 134 total sequences), ASAG (arid/semi-arid grassland; 398 total sequences), ASAS (arid/semi-arid shrubland; 123 total sequences), BF (boreal forest; 508 total sequences), AF (agricultural field; 20 total sequences), CF (coniferous forest; 318 total sequences), DF (deciduous forest; 1,298 total sequences), GP (grassland/prairie; 13,460 total sequences), PD (polar desert; 418 total sequences), SACF (semi-arid coniferous forest; 1,979 total sequences), and TF (tropical forest; 1,327 total sequences). The 15 most abundant phylotypes (ID numbers given to the left, indicated by "PT") are depicted, and the table to the right gives the percent that each phylotype (by row) represents of all verrucomicrobial sequences (across all samples; n = 112), the frequency of a phylotype's occurrence (across all samples), and the closest matching cultured isolate. All phylotypes are known to be within the class *Spartobacteria*, except one (PT02535) for which the family-level classification could not be unequivocally determined. The origin of each sample is reported by biome in Supplementary Table S1.