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A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses

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Acidobacteria are ubiquitous and abundant members of soil bacterial communities. However, an ecological understanding of this important phylum has remained elusive because its members have been difficult to culture and few molecular investigations have focused exclusively on this group. We generated an unprecedented number of acidobacterial DNA sequence data using pyrosequencing and clone libraries (39 707 and 1787 sequences, respectively) to characterize the relative abundance, diversity and composition of acidobacterial communities across a range of soil types. To gain insight into the ecological characteristics of acidobacterial taxa, we investigated the large-scale biogeographic patterns exhibited by acidobacterial communities, and related soil and site characteristics to acidobacterial community assemblage patterns. The 87 soils analyzed by pyrosequencing contained more than 8600 unique acidobacterial phylotypes (at the 97% sequence similarity level). One phylotype belonging to Acidobacteria subgroup 1, but not closely related to any cultured representatives, was particularly abundant, accounting for 7.4% of bacterial sequences and 17.6% of acidobacterial sequences, on average, across the soils. The abundance of Acidobacteria relative to other bacterial taxa was highly variable across the soils examined, but correlated strongly with soil pH ($R = -0.80$, $P < 0.001$). Soil pH was also the best predictor of acidobacterial community composition, regardless of how the communities were characterized, and the relative abundances of the dominant Acidobacteria subgroups were readily predictable. Acidobacterial communities were more phylogenetically clustered as soil pH departed from neutrality, suggesting that pH is an effective habitat filter, restricting community membership to progressively more narrowly defined lineages as pH deviates from neutrality.
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Introduction

The founding member of the Acidobacteria, *Acidobacterium capsulatum*, was isolated from an acidic mineral environment in 1991 (Kishimoto *et al.*, 1991), and was shown to represent a unique bacterial phylum in 1995 (Hiraishi *et al.*, 1995). Molecular surveys of soil bacteria confirmed the existence of the Acidobacteria in soil and gave the first glimpse of the incredible phylogenetic diversity contained within this phylum (Kuske *et al.*, 1997).

Since then, further molecular surveys have revealed that Acidobacteria are ubiquitous and among the most abundant bacterial phyla in soil (Janssen, 2006). However, we know surprisingly little about the ecological characteristics of this dominant group of soil bacteria.

Cultured isolates can give some indication of the ecological characteristics of a lineage, but Acidobacteria have thus far been difficult to culture. Of 66 305 high-quality 16S rRNA gene sequences available from bacterial isolates (Ribosomal Database Project (RDP) II v10.4; http://rdp.cme.msu.edu/index.jsp), only 74 are classified as Acidobacteria. Culturing techniques that have contributed to the successful isolation of Acidobacteria include: media with lowered pH (Sait *et al.*, 2006); increased headspace CO_2 concentrations (Stevenson *et al.*,

2004); substrate amendments (Pankratov *et al.*, 2008); random selection from a wide variety of growth substrates (Joseph *et al.*, 2003); use of a diffusion chamber (Bollmann *et al.*, 2007) and extension of the incubation time (Davis *et al.*, 2005). Despite the successes of these culturing methods, the isolates poorly represent the known diversity of Acidobacteria; of the 74 acidobacterial isolates, nearly 75% (55/74) are in subgroup 1 (RDP v10.4). Nevertheless, the cultured isolates suggest that some Acidobacteria have slow growth rates and either require or tolerate specific environmental conditions.

The paucity of cultured isolates poses a significant limitation in understanding soil acidobacterial ecology. As an alternative approach, molecular techniques can be used to describe the biogeographical patterns exhibited by soil acidobacterial communities and such patterns can provide important insight into the ecology, physiology and life history characteristics of the group. Acidobacterial-specific 16S rRNA primers developed by Barns *et al.* (1999) have been used to quantify acidobacterial abundances in specific samples (Felske *et al.*, 2000; Fierer *et al.*, 2005; Fierer *et al.*, 2007a), to target acidobacterial genomic DNA (Quaiser *et al.*, 2003), to develop group-specific denaturing gradient gel electorphoresis (Boon *et al.*, 2002) and to generate clone libraries that specifically target acidobacterial diversity (Barns *et al.*, 2007; Bryant *et al.*, 2008). A recent comprehensive study used quantitative PCR to determine the relative abundances of six bacterial groups in 71 soil samples, and found that the relative abundance of Acidobacteria was negatively correlated with soil carbon availability (Fierer *et al.*, 2007a). Also, two recent studies of microbial succession in soil found the abundance of Acidobacteria to be low in young soils and high in older soils (Nemergut *et al.*, 2007; Tarlera *et al.*, 2008). Additional molecular work has found pH to influence acidobacterial abundances, with the highest abundances found in environments with the lowest pH (Fierer *et al.*, 2007a; Mannisto *et al.*, 2007; Lauber *et al.*, 2008). Taken together, these findings support the idea that Acidobacteria are often slow-growing oligotrophs and that their abundance within a community may be regulated by pH.

We examined acidobacterial communities in 87 diverse soils to gain some insight into the ecological characteristics of the Acidobacteria. More specifically, we wanted to determine which abiotic factors influence the abundance, diversity and composition of soil acidobacterial communities across a range of ecosystem types. We used two complementary methods, pyrosequencing and clone libraries, to generate sequence data and survey acidobacterial communities at an unprecedented breadth (surveying a large number of soils) and depth (detailed surveys of the acidobacterial communities in each of the individual soils).

Methods

Soil collection, characterization and DNA extraction
A total of 87 unique soils representing a diverse array of soil and site characteristics were collected from throughout North and South America. Each soil was collected near the peak of the plant-growing season at each location. We collected the upper 5 cm of mineral soil from each site, collecting soil from 5 to 10 locations within an area of $\sim 100\,m^2$ with the soils from each site combined into a single bulk sample. All samples were sieved to 4 mm and thoroughly homogenized before being stored at $-80\,^\circ$C. Additional details on the soil collection procedures and the measurements of the soil characteristics are provided elsewhere (Fierer and Jackson, 2006; Fierer *et al.*, 2007a). Supplementary Table 1 contains information on the sampling location and soil characteristics for each of the 87 soils included in this study.

DNA was isolated from each of the collected soil samples using the MoBio Power Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) with the following modifications. Soil samples of approximately 5 g were ground with a mortar and pestle in liquid nitrogen and three 0.5-g subsamples of this ground soil were placed into bead tubes for extraction. The bead tubes were heated to 65 °C for 10 min, then shaken horizontally for 2 min at maximum speed with the MoBio vortex adapter. The remaining steps were performed as directed by the manufacturer. DNA from the three replicate subsamples per site was combined, and samples were stored at $-20\,^\circ$C.

Pyrosequencing of bacterial 16S rRNA genes
Pyrosequencing of the DNA isolated from all 87 samples was conducted using a previously described method (Hamady *et al.*, 2008). Briefly, we amplified the 16S rRNA genes from each sample using a primer set identical to that described in Hamady *et al.*, but the reverse primer contained a slightly longer 12-bp error-correcting Golay barcode rather than the originally described 8-bp Hamming barcode. This primer set is 'universal' for bacterial 16S rRNA and has been found to be well suited for the phylogenetic analysis of pyrosequencing reads (Liu *et al.*, 2007, 2008). PCR reactions consisted of 0.25 μl (30 mM) of each forward and reverse primers, 3 μl template DNA and 22.5 μl Platinum PCR Super-Mix (Invitrogen, Carlsbad, CA, USA). Samples were initially denatured at 94 °C for 3 min, then amplified using 35 cycles of 94 °C for 45 s, 50 °C for 30 s and 72 °C for 90 s. A final extension of 10 min at 72 °C was added at the end of the program to ensure complete amplification of the target region. All samples were amplified in triplicate and no template controls were included in all steps of the process.

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A composite sample for pyrosequencing was prepared by pooling approximately equal amounts of PCR amplicons from each sample. The replicate PCR reactions for each sample were combined and cleaned with the Mobio UltraClean-htp PCR Cleanup kit (Mobio Laboratories) as directed by the manufacturer. Each sample was then quantified using PicoGreen dsDNA reagent (Invitrogen) and the appropriate volume of the cleaned PCR amplicons was combined in a sterile, 50 ml polypropylene tube and precipitated on ice with sterile, 5 M NaCl (0.2 M final concentration) and two volumes of ice cold, 100% ethanol for 45 min. The precipitated DNA was centrifuged at 7800 g for 40 min at 4 °C and the resulting pellet was washed with an equal volume of 70% ethanol and centrifuged again at 7800 g for 20 min at 4 °C. The supernatant was removed and the pellet was air dried for 7 min at room temperature, then resuspended in 100 µl DNA nuclease-free water. The sample was sent to the Environmental Genomics Core Facility at the University of South Carolina, Columbia, SC, USA, for pyrosequencing on a 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics, Indianapolis, IN, USA) machine. The average sequence length was 232 bp, and sequences obtained through pyrosequencing have been deposited in the GenBank short read archive.

Sequences were processed and analyzed following previously described procedures (Hamady *et al.*, 2008). Phylotypes were defined at the 97% sequence identity level, and a set of representative sequences selected from these phylotypes were aligned using NAST (DeSantis *et al.*, 2006) with a PH lanemask to screen out hypervariable regions of the sequence. Sequence taxonomy assignments were made using RDP 2 classifier (release 10.4), with a minimum support threshold of 60%. Of 128 477 classified sequences, 39 707 were classified as Acidobacteria. Sequences were further divided into 26 subgroups: subgroups 1–8 (Hugenholtz *et al.*, 1998); subgroups 9–11 (Zimmermann *et al.*, 2005) and subgroups 12–26 (Barns *et al.*, 2007).

Cloning and sequencing of acidobacterial 16S rRNA genes
We constructed clone libraries to survey acidobacterial communities in 22 of the 87 soils to provide more detailed phylogenetic information (that is, longer sequence reads) than that offered by pyrosequencing. The clone libraries were constructed by amplifying the DNA samples with the acidobacterial-specific forward primer 31f (5′-GATCCTGGCT CAGAATC -3′) developed by Barns *et al.* (1999) and the 'universal' reverse primer 1492r (5′-GGTTAC CTTGTTACGACTT-3′). Each 50-µl PCR reaction contained: $1 \times$ PCR buffer; 2.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of each primer; 0.5 U of *Taq* DNA polymerase (Invitrogen) and 2 µl of template DNA. Each DNA sample was amplified with three replicate PCR amplifications (30 cycles each,

annealing temperature of 52 °C) with the amplicons from each sample pooled prior to cloning. Amplicons were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and cloned using the TOPO TA for Sequencing kit (Invitrogen) following the manufacturer's instructions. Plates were grown overnight at 37 °C before plasmid amplification and sequencing. For each sample, a total of 96 clones were sequenced with single-pass reads at Agencourt Bioscience (Beverly, MA, USA).

DNA sequences from the clone libraries were aligned using the NAST aligner (DeSantis *et al.*, 2006). The aligned sequences were imported into ARB v06.04.26 (Ludwig *et al.*, 2004) and edited by hand. Sequences were exported using ARB's Acidobacteria Lane mask to exclude ambiguous base pair positions. A 16S rRNA gene sequence of *Escherichia coli* (accession no. AE016755) was included as an outgroup. After filtering out short sequences (those less than 590 bp), a total of 1787 Acidobacteria sequences remained for analysis (GenBank accession nos: FJ165555–FJ167342). Both ends of each DNA sequence were trimmed to minimize the number of ambiguous characters, and an additional 17 bp that could not be accurately aligned were removed. The alignment file is available upon request. Clone library DNA sequences were classified into subgroups using RDP 2 classifier (release 10.4). Acidobacteria have been divided into 26 subgroups: subgroups 1–8 (Hugenholtz *et al.*, 1998); subgroups 9–11 (Zimmermann *et al.*, 2005) and subgroups 12–26 (Barns *et al.*, 2007). We compared the relative abundances of Acidobacteria subgroups to environmental characteristics for all individual soils. Sequences were also used in community analyses (detailed below).

Community analyses—pyrosequencing data
We used the pyrosequencing data to estimate the relative abundance of Acidobacteria in each bacterial community by comparing the number of sequences classified as belonging to the Acidobacteria versus the number of classified bacterial sequences per sample. We used Spearman's rank correlation to compare this estimate of acidobacterial relative abundances to the following soil and site characteristics: pH, percent soil organic carbon, mean annual precipitation at location, mean annual temperature at location, soil C/N ratio, soil carbon mineralization rate, and percent silt and clay. We also used Spearman's rank correlations to compare the relative abundances of Acidobacteria subgroups across all of the individual soils, relating the relative abundance estimates for the individual subgroups to the soil and site characteristics. Spearman's rank correlations were performed using an online statistics calculator (Wessa, 2008). To estimate the pairwise similarity in acidobacterial communities, we generated Bray–Curtis dissimilarity matrices, as implemented in PRIMER-E v6 (PRIMER-E Ltd, Lutton,

UK), using the relative abundance of acidobacterial phylotypes (identified as described above) as an input. Three other Bray–Curtis dissimilarity matrices were constructed that excluded singletons (those phylotypes only observed once), singletons and doubletons, and phylotypes not observed at least 10 times. We used the Mantel test in PRIMER-E to compare dissimilarity matrices to pairwise distances in environmental characteristics as estimated using normalized Euclidean distances in the measured soil and site parameters, an approach described by Fierer and Jackson (2006).

Community analyses—clone library data
We compared levels of diversity in individual soils by determining the number of unique phylotypes at four different levels of sequence similarity: 90%, 93%, 95% and 97% using FastGroup II (Yu *et al.*, 2006) to identify unique phylotypes and EstimateS to calculate rarefaction curves (Colwell, 2005). To estimate the pairwise similarity in acidobacterial communities, we generated Bray–Curtis dissimilarity matrices, as implemented in PRIMER-E, using the relative abundance of acidobacterial phylotypes as an input.

In total, 100 bootstrapped maximum likelihood trees were constructed using RAxML v7.0.4 with the GTRGAMMA model without branch length constraints using the clone library sequence data (Stamatakis, 2006). Duplicate sequences were omitted, and a total of 1652 Acidobacteria 16S rRNA gene sequences were analyzed. We used the resultant trees in phylogenetic techniques that, unlike taxonomy-based or phylotype-based methods, incorporate evolutionary relatedness of community members into diversity descriptions and comparisons. Including evolutionary history into community comparisons is valuable because more closely related individuals are more likely to be ecologically similar to one another than distantly related individuals, and thus cannot be considered statistically independent (Felsenstein, 1985; Harvey and Pagel, 1991). We analyzed each bootstrapped tree ($n = 100$) independently in all phylogenetic analyses, because the true phylogeny is unknown and unknowable and it is important to incorporate phylogenetic uncertainty in phylogenetic analyses (Huelsenbeck *et al.*, 2000). Furthermore, when phylogenetic uncertainty is incorporated into microbial community comparisons, inferences are more conservative and robust (Jones and Martin, 2006). We used three phylogenetic metrics to further explore Acidobacteria diversity and community assembly patterns.

The phylogenetic metric, phylodiversity, was used to compare diversity across the acidobacterial communities. Phylodiversity was originally developed to prioritize conservation areas (Faith, 1992), but we use it here to describe the percentage of acidobacterial phylogenetic diversity contained in one soil sample relative to all acidobacterial diversity (that is, the sum of branch lengths in one community relative to the total branch lengths of all communities). Because sampling intensity is not equal among all soil samples and phylodiversity estimates will increase with comparatively higher sampling, we used a bootstrap approach to randomly assemble 100 communities with 65 taxa from each soil sample; each community bootstrap was evaluated with respect to each bootstrapped tree (that is, 100 communities $\times$ 100 phylogenetic trees = 10 000 data points). Phylodiversity values were determined using Phylocom v4.0.1 (Webb *et al.*, 2008).

We used the net relatedness index (NRI) to describe the phylogenetic evenness of Acidobacteria communities within individual soils, and compared phylogenetic evenness to environmental characteristics. The NRI (Webb, 2000, 2002) compares the pairwise distances (as determined by the total length of branches between two taxa) between all members of a community to pairwise distances of randomly selected taxa from the entire tree, and indicates whether a community's diversity is phylogenetically clumped (members of a community are more related to each other than by chance) or phylogenetically even (members of a community are less related to each other than by chance). Positive NRI values indicate phylogenetically clustered communities and negative values indicate phylogenetically even communities. Because NRI values are affected by sample size, we use the same community bootstrap approach detailed for phylodiversity measurements. NRI values were obtained using the 'comstruct' command in Phylocom v4.0.1 with 1000 randomizations and null model 1 (Webb *et al.*, 2008).

We used UniFrac to cluster soils based on the phylogenetic differentiation they contained (Lozupone and Knight, 2005; Lozupone *et al.*, 2006). UniFrac clusters microbial communities based on the fraction of unique branch lengths represented by a community in pairwise comparisons to all other communities. Communities separated by a small fraction of unique evolutionary diversity cluster close together and communities separated by large fractions of evolutionary diversity cluster farther apart. The underlying notion behind this approach is that longer unique branch lengths represent more evolutionary adaptation, which is necessary for survival in one community relative to the other (Lozupone and Knight, 2008). We used Mantel tests to test the significance of correlations between the pairwise UniFrac distances between soil acidobacterial communities and the normalized Euclidean distances in soil and site characteristics.

Results

We characterized the relative abundance, diversity and composition of soil acidobacterial communities

across a wide range of soil types using two sequencing approaches. Using a pyrosequencing approach with universal bacterial 16S rRNA gene primers, we generated 39 707 acidobacterial sequences from 87 soils. The clone library approach with Acidobacteria-specific 16S rRNA gene primers was used to generate 1787 sequences from 22 soils. The pyrosequencing yielded a more extensive survey of acidobacterial communities (more soils, more sequences per soil), but the clone library data allowed us to survey the communities at a higher level of phylogenetic resolution (owing to the longer lengths of the sequence reads). We used the two types of data to characterize Acidobacteria communities in three ways: (1) taxonomy-based, (2) phylotype-based and (3) phylogenetic. We compared community characterizations to soil environmental data (Supplementary Table 1) to gain a better understanding of the factors shaping Acidobacteria communities.

Overall abundance of Acidobacteria

We used the pyrosequencing data to estimate the phylum-level relative abundance of Acidobacteria in the 87 soils examined. Across all soils, Acidobacteria represented 30.9% of all classified bacterial sequences detected using pyrosequencing (39 707 sequences out of a total of 128 477 sequences). However, the relative abundance of acidobacterial sequences within an individual soil bacterial community varied from 2.4% to 78.5% (Figure 1). The relative abundance of soil Acidobacteria was strongly correlated with soil pH ($R = -0.80$, $P < 0.001$), with Acidobacteria representing a larger portion of the bacterial community in low pH soils (Figure 1). Other measured soil and site characteristics that were also significantly correlated included: mean annual precipitation ($R = 0.66$, $P < 0.001$), % soil organic carbon ($R = 0.48$, $P < 0.001$) and soil C/N ratio ($R = 0.45$, $P < 0.001$).
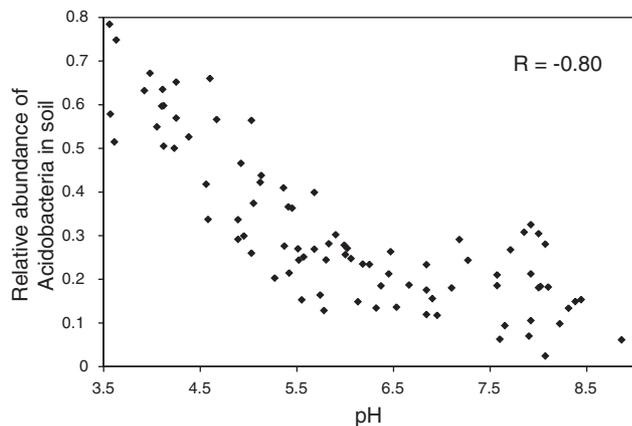

Figure 1 Relationship between pH and the relative abundance of Acidobacteria in 87 soil bacterial communities using pyrosequencing data. Abundance of Acidobacteria is strongly and negatively correlated with pH (Spearman's rank correlation: $R = -0.80$, $P < 0.001$).

However, it is important to note that these other environmental characteristics were more weakly correlated with acidobacterial abundances than soil pH. Also, these other environmental characteristics are, to varying degrees, also correlated with soil pH (Fierer and Jackson, 2006).

Relative abundance of Acidobacteria subgroups

We further classified Acidobacteria DNA sequences (from clone libraries and pyrosequencing) into acidobacterial subgroups using the RDP v10.4 classifier. We present the average relative abundances of Acidobacteria subgroups 1–26 for both the pyrosequencing and clone library data in Table 1; we also present the relative abundances of Acidobacteria subgroups 1–26 in individual soils for pyrosequencing data (Supplementary Table 2) and for clone library data (Supplementary Table 3). On the basis of the pyrosequencing results, we find that acidobacterial subgroups 1, 2, 3, 4 and 6 are the most abundant in soil: across all 87 soils, these subgroups accounted for 22.4%, 12.7%, 15.0%, 29.0% and 10.7% of the acidobacterial sequences, respectively. Subgroups 1, 3, 4 and 6 were also found to be

Table 1 Abundance of Acidobacteria subgroups detected using pyrosequencing and clone libraries

Subgroup	Pyrosequencing		Clone libraries	
	Average (%)	Range (%)	Average (%)	Range (%)
1	22.4	0.0–66.5	43.7	0.0–87.0
2	12.7	0.0–59.9	ND[a]	—
3	15.0	1.9–54.5	16.4	1.3–30.8
4	29.0	0.0–76.7	21.1	0.0–82.1
5	2.1	0.0–15.8	2.4	0.0–11.0
6	10.7	0.6–43.3	13.6	1.2–63.2
7	2.0	0.0–10.9	ND	—
8	0.1	0.0–3.4	ND	—
9	ND	—	ND	—
10	0.4	0.0–2.6	ND	—
11	0.3	0.0–4.9	0.3	0.0–6.1
12	<0.1	0.0–0.3	ND	—
13	0.4	0.0–5.2	0.5	0.0–3.2
14	ND	—	ND	—
15	0.2	0.0–1.1	0.2	0.0–2.6
16	2.3	0.0–15.9	ND	—
17	0.3	0.0–2.7	0.2	0.0–2.4
18	0.2	0.0–3.2	0.3	0.0–6.4
19	<0.1	0.0–0.3	ND	—
20	<0.1	0.0–0.3	ND	—
21	ND	—	ND	—
22	0.1	0.0–1.6	ND	—
23	<0.1	0.0–0.6	ND	—
24	ND	—	ND	—
25	0.3	0.0–4.5	ND	—
26	ND	—	ND	—
Unclassified	1.5	0.0–4.2	1.1	0.0–7.8

[a]ND indicates that the subgroup was not detected. DNA sequences were classified into 26 acidobacterial subgroups using the Ribosomal Database Project v10.4 classifier. The 26 subgroups are classified according to the following designations: subgroups 1–8 according to Hugenholtz *et al*. (1998); subgroups 9–11 according to Zimmermann *et al*. (2005) and subgroups 12–26 according to Barns *et al*. (2007).

abundant in the clone libraries, but the Acidobacteria-specific primers used to generate the clone libraries did not amplify sequences from a number of Acidobacteria subgroups (Table 2). Although some of these subgroups appear to be minor and ephemeral constituents of soil Acidobacteria communities, subgroups 2, 7 and 16 were often abundant and represented up to 59.9%, 10.9% and 15.9%, respectively, of Acidobacteria in individual soils as quantified by pyrosequencing (Figure 2). The relative abundance of most Acidobacteria subgroups was strongly correlated with the pH of the individual soil; groups 1, 2, 3, 12, 13 and 15 had negative correlations and groups 4, 6, 7, 10, 11, 16, 17, 18, 22 and 25 had positive correlations with pH (Figure 2; Supplementary Tables 4 and 5). Spearman's correlations between subgroup abundance and all environmental characteristics are also presented (Supplementary Tables 4 and 5).

Diversity of soil Acidobacteria
The Acidobacteria subgroups represent broad taxonomic groups and each group contains a tremendous amount of diversity. To examine the levels of diversity at finer levels of taxonomic resolution, we binned DNA sequences into phylotypes based on sequence similarity (90%, 93%, 95% and 97% for clone library data; 97% for pyrosequencing data). We constructed rarefaction curves for the clone library data (Supplementary Figure 1) and the absence of clear asymptotes suggests that we did not completely sample Acidobacteria diversity at the 93%, 95% or 97% phylotype designations. Of 1787 DNA sequences generated by the clone libraries, we found the following number of phylotypes across the 22 soils examined: 38 (90%), 102 (93%), 230 (95%) and 317 (97%). Of the 39 707 acidobacterial DNA sequences generated by pyrosequencing from the 87 soils, we identified 8643 unique phylotypes (97%). It is worth noting that the 97% phylotype designation is not necessarily equivalent between the Sanger sequences and pyrosequences because the region of 16S targeted by pyrosequencing is more variable than the region targeted by Sanger sequencing.

To explore the effects of the environment on acidobacterial diversity, we related phylodiversity to soil characteristics. Median phylodiversity values for individual soil communities ranged from 10.4% to 16.8%. However, phylodiversity did not correlate with any of the measured environmental characteristics ($P > 0.1$ in all cases), including pH (Supplementary Figure 2). This suggests that, across the wide range of soil types examined, the characteristics of these soils do not restrict or amplify the total amount of diversity contained within a given acidobacterial community.

We further explored environmental effects on phylogenetic diversity by comparing environmental characteristics to phylogenetic evenness. Soil pH strongly affected phylogenetic evenness; communities were the most even in soils with close to neutral pH and communities became phylogenetically clustered as pH departed from neutrality (Figure 3). In other words, the more the soil pH departed from neutrality, the more the communities were composed of closely related members. This result may seem at odds with the lack of correlation between phylodiversity and pH (Supplementary Figure 2). However, phylodiversity is a snapshot of diversity and is highly sensitive to the presence or absence of major lineages, whereas the NRI is based on pairwise comparisons between all taxa in a community and is more indicative of broad assemblage patterns.

Overall differentiation in Acidobacteria community composition
We used the relative abundances of each phylotype in individual samples to characterize differences in

Table 2 Mantel correlations between community structure and environmental variables

Phylotype definitions No. of phylotypes	Pyrosequencing	Clone library				
	97% 9822	97% 317	95% 230	93% 103	90% 38	UniFrac 1658
pH (log)	**0.734 (<0.001)**	**0.591 (<0.001)**	**0.611 (<0.001)**	**0.629 (<0.001)**	**0.638 (<0.001)**	**0.702 (<0.001)**
MAP[a] (mm)	**0.332 (<0.001)**	0.034 (0.336)	0.089 (0.195)	0.057 (0.261)	0.044 (0.274)	**0.229 (0.012)**
% C[b] (sqrt)	**0.228 (<0.001)**	0.103 (0.139)	0.057 (0.239)	0.090 (0.149)	0.088 (0.137)	**0.249 (0.005)**
C/N[c] (log)	**0.226 (<0.001)**	**0.244 (0.021)**	**0.300 (0.007)**	**0.279 (0.008)**	**0.241 (0.011)**	**0.164 (0.045)**
C min[d] (log)	**0.191 (<0.001)**	0.014 (0.417)	0.058 (0.263)	0.086 (0.186)	0.024 (0.354)	0.078 (0.203)
MAT[e] (°C)	**0.162 (<0.001)**	−0.045 (0.675)	0.007 (0.419)	−0.011 (0.483)	−0.054 (0.745)	0.040 (0.254)
% silt and clay (sqrt)	**0.093 (0.004)**	0.150 (0.066)	**0.191 (0.027)**	**0.226 (0.026)**	**0.194 (0.027)**	0.097 (0.138)

Bold values indicate a significance of <0.05. Significance values in parentheses.
[a]Mean annual precipitation.
[b]% organic carbon.
[c]Carbon to nitrogen ratio.
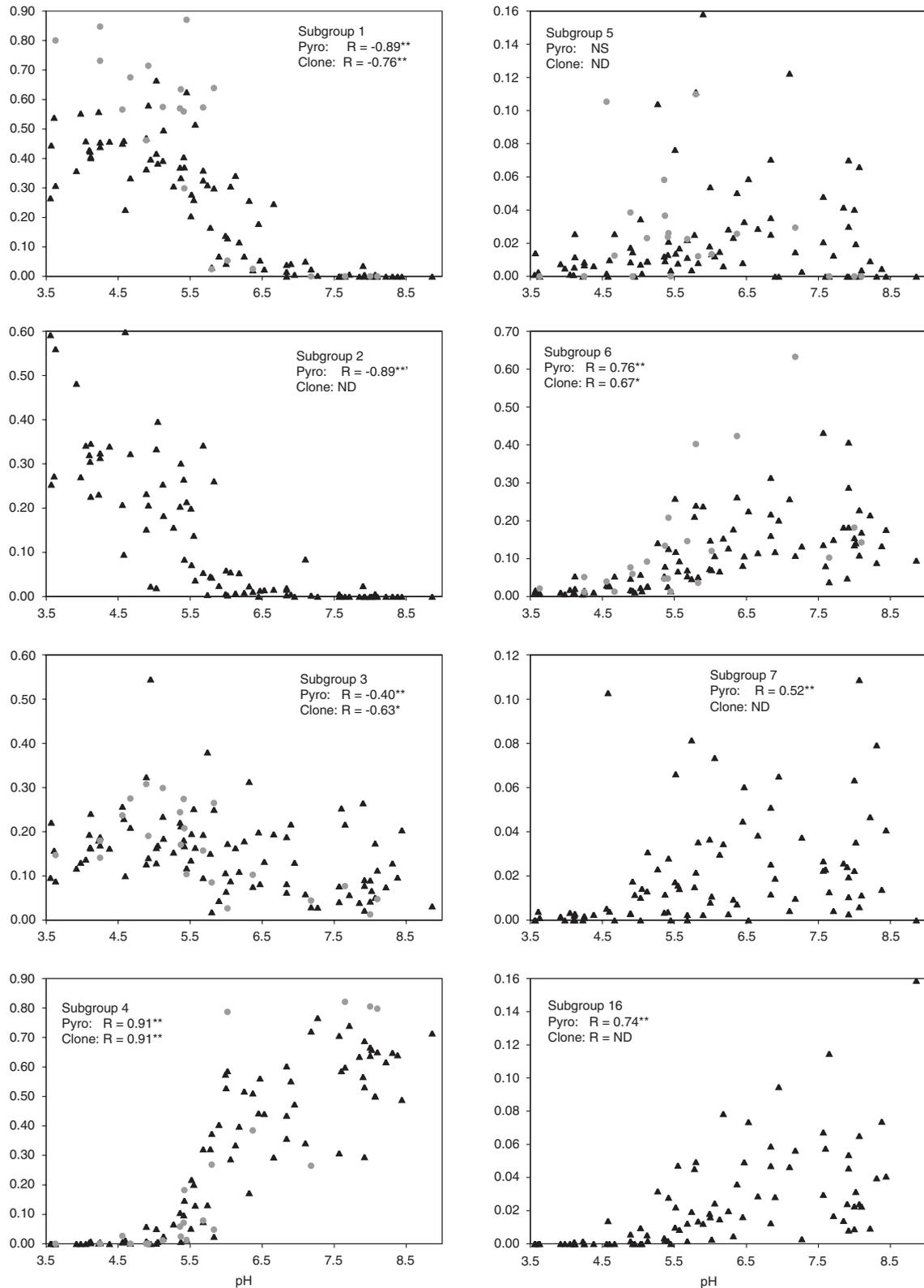[d]Carbon mineralization rate.
[e]Mean annual temperature.

Figure 2 Effect of pH on abundance of Acidobacteria subgroups (1–7 and 16) relative to all Acidobacteria using pyrosequencing and clone library data. Triangles (black) represent pyrosequencing data; circles (grey) represent clone library data. Spearman's rank correlations between subgroup abundance and pH are depicted. NS, not significant; ND, not detected. *$P < 0.05$; **$P < 0.001$.

acidobacterial community composition as determined by pyrosequencing. Mantel tests between Bray–Curtis-transformed community assemblage matrices and pairwise environmental matrices revealed significant correlations between community assembly patterns and pH and C/N ratios at all
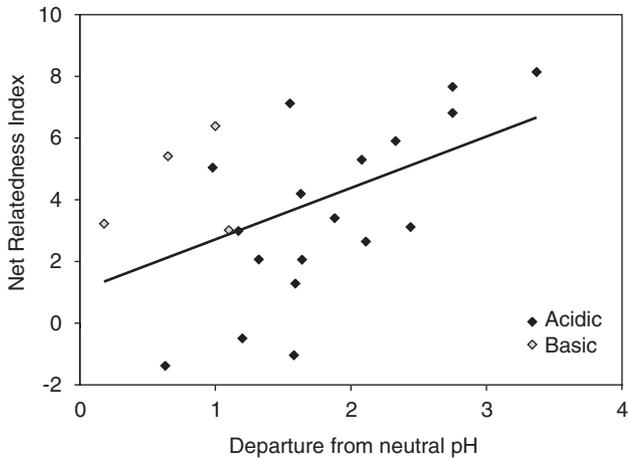
Figure 3 The net relatedness index (NRI) measures phylogenetic evenness. Positive NRI values indicate that communities are phylogenetically clustered and negative values indicate phylogenetic evenness. Here, phylogenetic clustering is positively correlated with departure from neutral pH ($R^2 = 0.229$, $P < 0.05$); the more the soil pH departs from neutrality, the more the communities are phylogenetically clustered. Median NRI values are plotted.
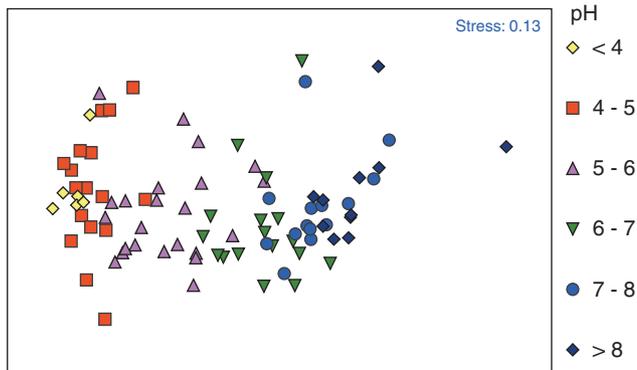

Figure 4 Non-metric multi-dimensional scaling plot of soil community assembly patterns (using Bray–Curtis-transformed pyrosequencing data) as related to soil pH. The first principal axis is dominated by soil pH effects, showing the key effect of pH on acidobacterial community assembly.

Figure 5 Relative abundances of the 20 most abundant phylotypes (97% OTU designation) in individual soil samples using clone library data. Subgroups are indicated over phylotypes. Soils are arranged from lowest pH (CF2) to highest pH (SA2). Phylotypes with asterisks share >97.0% sequence similarity with a cultured isolate. Percentages at bottom indicate the abundance of each phylotype relative to all detected acidobacterial sequences (values corrected for acidobacterial-specific primers not detecting certain subgroups; see text).

phylotype designations for both pyrosequencing and clone library data (Table 2). The correlation with pH was particularly strong and is clearly depicted in a non-metric multi-dimensional scaling plot of the pyrosequencing phylotype data (Figure 4). We see a similar pattern if we use phylogenetic approaches to compare Acidobacteria communities. We clustered Acidobacteria communities based on their unique phylogenetic diversity using the UniFrac algorithm with the clone library data. Mantel tests comparing UniFrac distances and environmental distances showed significant correlations between UniFrac distances and the following environmental parameters: pH, soil moisture, mean annual precipitation, % organic C and C/N ratio (Table 2). UniFrac distances showed more significant associations than phylotype-based approaches,
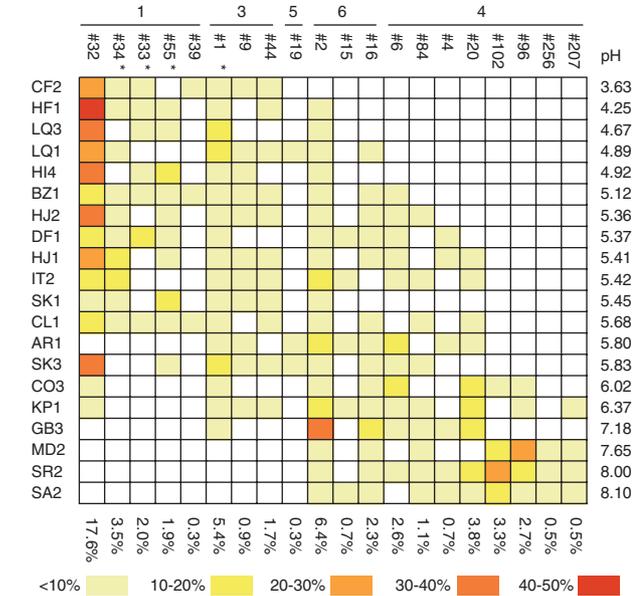
underscoring the importance of using a phylogenetic approach.

To further explore these trends, we quantified the abundances of the 20 most common phylotypes identified using the clone library approach in the 22 soils. These 20 phylotypes represented approximately 75% of all acidobacterial DNA sequences in the clone libraries. Again, we found a clear trend of phylotype presence correlated with pH (Figure 5). Importantly, only 4 of the top 20 most common phylotypes shared greater than 97% sequence identity with a cloned isolate (Figure 5). Phylotype no. 32 was particularly abundant: it accounts for 7.4% of all bacterial sequences in soil (17.6% of all Acidobacteria) and represents as much as 23.5% of bacterial sequences (41.3% of acidobacterial sequences) in individual soils. These estimated values were corrected for the clone libraries not detecting some subgroups (for example (abundance of phylotype no. 32 in clone libraries) × (proportion of subgroups 1, 3, 4, 5, 6, 11, 13, 15, 17 and 18 relative to all detected Acidobacteria using pyrosequencing data)).

Discussion

Our results show that soil pH strongly regulates the abundance of Acidobacteria (relative to all bacteria) in individual communities (Figure 1). Negative correlations between Acidobacteria phylum-level abundance and pH have also been seen in fine

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organic matter of streams and in Arctic soils (Fierer et al., 2007b; Mannisto et al., 2007). Our results differ, however, from a previous study that found that organic carbon availability was the best predictor of Acidobacteria abundance in soils (Fierer et al., 2007a). This disparity may be related to our finding (evident in Figure 2 and Table 1) that the primer set used by Fierer et al. (2007a), the same primer set used in the clone library analyses described here, excludes some common acidobacterial subgroups. We suggest that our pyrosequencing results (Figure 1) better represent Acidobacteria relative abundances in soil and that the strong correlation between Acidobacteria abundance and pH ($R = -0.80$) is more robust than the weaker correlation between Acidobacteria abundance and carbon availability ($R^2 = 0.26$) noted by Fierer et al. (2007a). Our results do not exclude the notion that Acidobacteria are often slow-growing oligotrophs, as suggested by a negative correlation between abundance and carbon availability (Fierer et al., 2007a), culturing studies (Davis et al., 2005) or studies of microbial succession (Nemergut et al., 2007; Tarlera et al., 2008). Nevertheless, based on our comprehensive survey of acidobacterial communities across a wide range of soil types, pH governs phylum-level abundance of Acidobacteria more than any other measured environmental characteristic. This is not unique to Acidobacteria; environmental pH has also been found to significantly correlate with the abundances of Alphaproteobacteria, Bacteroidetes, Flavobacteria and OP10 phyla in lakes (Percent et al., 2008).

Within individual Acidobacteria soil communities, composition is also strongly regulated by pH. This finding holds across all characterizations of Acidobacteria communities: taxonomy-based, phylotype-based and phylogenetic. The abundances of most Acidobacteria subgroups significantly correlate with pH: abundances of subgroups 1, 2, 3, 12 and 13 have a negative relationship; abundances of subgroups 4, 6, 7, 10, 11, 16, 17, 18, 22 and 25 have a positive relationship (Figure 2; Supplementary Tables 4 and 5). These results confirm the results of Eichorst et al. (2007) who found that the abundance of subgroup 1 was inversely related to pH in soil and other terrestrial environments and Barns et al. (1999) who found that some subgroups did not occur in low pH soils. Similarly, a higher proportion of Acidobacteria subgroup 1 colonies formed on acidic media versus neutral media (Sait et al., 2006). If we compare community assemblages using a range phylotype definitions (90%, 93%, 95% and 97% sequence similarity for clone library data; 97% sequence similarity for pyrosequencing data), we also find that pH strongly correlates with acidobacterial community composition, regardless of the data set or the phylotype definition (Table 2; Figure 4). As a final means to characterize Acidobacteria communities, we used UniFrac to cluster soils based on the unique fraction of phylodiversity contained in each soil based on pairwise comparisons between all samples (Lozupone et al., 2006; Lozupone and Knight, 2005). Again, we find that soil pH is a strong predictor of acidobacterial community composition (Table 2). These results are similar to other studies that have found pH to regulate fine-scale community composition, such as in the beta-subgroup of proteobacterial ammonia oxidizers (Stephen et al., 1998) and in the acI lineage of freshwater lake Actinobacteria (Newton et al., 2007). Broad-scale surveys comparing entire bacterial communities have also found that bacterial community composition is often well correlated with soil pH (Fierer and Jackson, 2006; Hogberg et al., 2007; Lauber et al., 2008; Singh et al., 2008). As soil pH can be considered a 'master variable' that integrates a number of other soil and site characteristics, we cannot determine whether pH is having a direct influence on acidobacterial communities or whether soil pH is correlated with some other, unmeasured, environmental parameter that, in turn, influences acidobacterial community composition. Regardless, our results confirm results from a range of other studies showing a strong influence of pH on microbial communities and suggest that pH is also a strong predictor of acidobacterial abundance and community composition in soil.

Not surprisingly, we found that the Acidobacteria phylum is phylogenetically diverse with even our relatively large libraries failing to capture the full extent of 'species'-level diversity (Supplementary Figure 1). This diversity, however, is not partitioned randomly across individual communities. We used the NRI metric to quantify the phylogenetic evenness of individual Acidobacteria communities and found that communities became more phylogenetically clustered as soil pH departed from neutrality (Figure 3). This suggests that pH acts as an effective habitat filter, restricting community membership to progressively more narrowly defined lineages as pH deviates from neutrality. Similar patterns were noted by Felske et al. (1998), who implicated low pH as a habitat filter for soil bacteria when many duplicate sequences were found in a peaty acidic soil. Intracellular pH of bacteria is close to neutral regardless of environmental pH, and bacterial cells persisting in acidic environments generally cope by having increased buffering capacities or less permeable cell membranes (Booth, 1985). Closely related Acidobacteria likely share similar, phylogenetically constrained, cellular mechanisms to compensate for low pH. For example, closely related Acidobacteria may share similar means of membrane lipid construction and these differences in acidobacterial membrane construction may explain the abundance of a novel group of branched glycerol dialkyl glycerol tetraether membrane lipids found to correlate negatively with soil pH (Weijers et al., 2007).

Although Acidobacteria are phylogenetically diverse, the 20 most common phylotypes (as defined

by 97% sequence similarity) accounted for >58% of community membership (Figure 5). These abundant Acidobacteria phylotypes should account for a disproportionate amount of biogeochemical cycling compared with the more rare phylotypes. However, the cultured Acidobacteria isolates are not representative of total Acidobacteria diversity or, more importantly, the most common Acidobacteria found in soil (Figure 5). Our results should help identify those specific environmental conditions best suited for targeted cultivation of important and abundant phylotypes of Acidobacteria. Of particular interest is one specific acidobacterial phylotype (phylotype no. 32, as defined by 97% sequence similarity), which was found to be particularly abundant in the clone libraries constructed from many soils (Figure 5). Phylotype no. 32 accounts for 7.4% of all bacterial sequences in soil (17.6% of all Acidobacteria) and represents as much as 23.5% of bacterial sequences (41.3% of acidobacterial sequences) in individual soils. Its abundance suggests that it plays an important role in soil biogeochemistry, and the abundance of phylotype no. 32 in soil may parallel that of the bacterioplankton phylotype SAR11 in oceans. SAR11 was first detected using molecular methods (Giovannoni *et al.*, 1990), and was found to be one of the most abundant bacteria in the oceans (Morris *et al.*, 2002). Over a decade passed after the molecular discovery of SAR11 before a member of the cluster was cultured (Rappe *et al.*, 2002). Culturing, particularly of abundant lineages, is important because it allows genomic surveys that provide more insight into the metabolic potential and biogeochemical function of a lineage (Giovannoni and Stingl, 2007). We suggest that Acidobacteria phylotype no. 32 be targeted for culturing to enable genomic analyses of this numerically dominant soil community member.

It is particularly noteworthy that our findings are robust across different data generation methods (clone libraries and pyrosequencing) and across a range of data analyses (taxonomy-based, phylotype-based and phylogenetic-based). There are important differences between results obtained using the two data generation methods. Regrettably, the Acidobacteria-specific primers (Barns *et al.*, 1999) failed to detect a number of Acidobacteria subgroups. Some of these subgroups are rather rare members of soil communities, but subgroup 2, 7 and 16 are often common members of Acidobacteria communities (Figure 2). Nevertheless, analyses of pyrosequencing and clone library data yielded similar results, demonstrating that the composition of soil acidobacterial communities are remarkably predictable across a wide range of soil and ecosystem types if we consider a single soil edaphic characteristic, namely soil pH. This is surprising considering the phylogenetic diversity contained within the soil Acidobacteria and it suggests that there is a lot of value in using these broad-scale biogeographic surveys for

revealing important insight into the natural history of bacterial lineages represented by few, if any, cultured isolates.

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